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Lung function and airway inflammation monitoring after hematopoietic stem cell transplantation

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Thesis submitted to fulfill the requirements for the degree of doctor in medical science 2013

« Dans les sciences, le chemin est plus important que le but. Les sciences n'ont pas de fin. »

De Erwin Chargaff

REMERCIEMENTS

Je souhaite tout d'abord remercier mon promoteur, le Professeur Renaud Louis, pour m'avoir accueillie au sein de son laboratoire et pour toute la confiance dont il a fait preuve à mon égard en me laissant mener à bien mon projet. Je le remercie également chaleureusement pour sa patience ainsi que pour sa gentillesse. Je lui suis reconnaissante pour sa disponibilité, ses conseils et ses grandes qualités scientifiques et humaines.

Je remercie également mon co-promoteur, le Professeur Yves Beguin d'avoir suivi ce projet et d'avoir pris le temps de relire mes écrits.

J'exprime toute ma reconnaissance à l'ensemble de mon jury de thèse, le Professeur Michel Moutschen, président de mon comité, ainsi qu'aux Professeurs Jean-Louis Corhay, Didier Cataldo et Frédéric Baron pour leurs conseils et le temps consacré à l'évaluation de mon travail.

Je voudrais également remercier chaleureusement le Professeur Geert Verleden de l'Université catholique de Louvain ainsi que le Professeur Ibrahim Yacoub-Agha du CHUR de Lille, membres extérieurs du jury, pour leur aimable participation.

Merci à tout le service de pneumologie du CHU de Liège: à Cédric Graas pour son aide précieuse, au Dr Florence Schleich, à Jocelyne Sele, au Dr Raluca Asandei, au Dr Thais Ribera Jorba, à Clarissa Hilzendeger, à Els Rubens (merci Els pour ton aide !), à Virginie Paulus, à Claude Fouyn, à toute l'équipe infirmière, au Dr Jane Da Silva et au Dr Delphine Nguynen Dang.
Sans oublier notre technicienne: Monique Henket. Monique, merci à toi pour ton soutien, tes compétences, ton humour, ta bonne humeur et pour tous ces moments partagés. Travailler avec toi a été un réel plaisir et j'espère pouvoir continuer notre collaboration encore de longues années.

Merci également aux secrétaires de pneumologie et spécialement à Mme Mady Moor pour sa gentillesse et son aide inestimable.

Mes pensées et ma reconnaissance vont particulièrement à l'ensemble des patients qui ont accepté généreusement de participer à cette étude et sans qui ce travail n'aurait pas été possible.

Un grand merci à tous mes collègues et amis qui m'ont supportée toutes ces années et avec qui j'ai partagé beaucoup de moments inoubliables: Julien, Nassim, Zheshen, Cédric, Sophie, Edith, Stéphanie, Bibba, Florence, Amélie, Céline, Julie, Laurence, Pakito et tous les autres.

Merci ensuite au laboratoire de Physiologie cellulaire et moléculaire, notamment à Fabrice, Christophe, Catherine, Coraline, Claire, Laurence, Dimitri et Marie.

Ce travail n'aurait pu être mené à bien sans le soutien financier du Télévie (FNRS), du PAI et de la fondation Léon Frédéricq.

Enfin, merci à ma famille chérie et à toi Christophe pour tout le soutien et la confiance que vous m'avez témoignés.

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LIST OF ABBREVIATIONS

aGVHD	Acute Graft-Versus-Host-disease
APC	Antigen presenting cell
ATG	Anti-thymocyte globulin
BAL	Bronchoalveolar lavage
BD	Bronchodilation
BMI	Body mass index
BO	Bronchiolitis Obliterans
BOOP	Bronchiolitis obliterans organizing pneumonia
BOS	Bronchiolitis Obliterans Syndrome
cGVHD	Chronic Graft-Versus-Host-disease
CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disease
CT	Chest high-resolution computer tomography
DAH	Diffuse alveolar hemorrhage
DLCO	Diffusing capacity
DLI	Donor lymphocyte infusion
EBC	Exhaled breath condensate
FeNO	Fraction of exhaled nitric oxide
FEV1	Forced expiratory volume in 1 s
FVC	Forced vital capacity
GVT	Graft versus tumor
Gy	Gray
HLA	Human Leucocyte Antigen
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes simplex virus
HZV	Herpes zoster virus
ICS	Inhaled corticosteroids
IFN	Interferon
IL	Interleukin
IPS	Idiopathic pneumonia syndrome
IS	Induced sputum
LPS	Lipopolysaccharide
LT	Lung transplantation
MA	Myeloablative
mHA	Minor histocompatibility antigen
MMF	Mycophenolate mofetil
MSCs	Mesenchymal stem cells
MTX	Methotrexate

NIH	National Institutes of Health
NK	Natural killer
NMA	Nonmyeloablative
NO	Nitric oxide
NRM	Nonrelapse mortality
OS	Overall survival
PBSCs	Peripheral blood stem cells
PFT	Pulmonary function test
PPB	Parts per billion
ROS	Reactive oxygen species
RV	Residual volume
SNPs	Single nucleotide polymorphisms
TBI	Total body irradiation
TLC	Total lung capacity
TLI	Total lymphoid irradiation
TNF	Tumor necrosis factor
UCB	Umbilical cord blood

SUMMARY

Allogeneic hematopoietic stem cell transplantation (HSCT) has been proposed to treat haematological malignancies since the 1960s. Unfortunately, pulmonary complications are frequent after this type of transplantation and are a major cause of post-transplant mortality.
Induced sputum (IS) and measurement of exhaled nitric oxide (FeNO) are non-invasive methods to investigate airway inflammation, which have been extensively used to assess airways inflammatory diseases. However, these procedures have not been studied in the context of HSCT.

In the first part of this work, in an attempt to detect modifications at airway level, we monitored lung function and airway inflammation in 182 patients who underwent HSCT. We prospectively measured FEV1, FVC, DLCO, KCO, TLC, RV, FeNO as well as sputum cell counts before and 3, 6, 12, 24 and 36 months after HSCT. We observed that there was a progressive loss in lung function after HSCT featuring a mild restrictive pattern while bronchiolitis obliterans rarely occurred (3.5%). Moreover, the patients undergoing a HSCT exhibited a high sputum neutrophil count, which was sustained over all the period of observation. By contrast, the FeNO value remained in the accepted normal range throughout the post-transplant course.
The myeloablative (MA) conditioning was associated with early rise of sputum neutrophils and greater alteration in lung function over the first year. Overall survival at 1 year after HSCT were 71% and none of the baseline functional and airway inflammation features had a prognostic value for survival at one year.

In a second part of our work, we measured Th1, Th2 and Th17 cytokines in the IS supernatant in order to better characterize the airway inflammatory process in patients with HSCT. Then, we compared the results with those from healthy subjects matched for age and tobacco habits and with patients suffering from chronic obstructive pulmonary disease (COPD), a chronic airway inflammatory disease also marked by a airway neutrophilic inflammation. We observed that that patients undergoing HSCT display raised sputum levels of IL-6 and IL-8 compared to healthy subjects though the levels remained lower than those found in COPD.

In the third part of our work, we assessed the impact of mesenchymal stem cell (MSC) co-infusion on lung function after HSCT. Indeed, MSC are used to promote engraftment and prevent graft-versus-host-disease in the context of HSCT but were shown to have opposite effects on lungs after systemic administration in animals. Their effects have never been studied in human after HSCT. Thus, we monitored lung functions, FeNO values, the occurrence of pulmonary infections and cytomegalovirus (CMV) reactivation in 30 patients who received an allogeneic unrelated HSCT after non-myeloabaltive (NMA)

conditioning with MSC co-infusion. They were compared with 28 patients undergoing the same type of transplantation and conditioning but without MSC infusion. The impact of MSC on pulmonary infections and CMV reactivation was analysed by uni and multivariate Cox models adjusted for competing risks. The principal observations were that MSC were safe at lung level after HSCT and did not lead to lung function changes 1 year post transplantation. However, MSC could promote pulmonary infections involving the need for a close monitoring of their occurrence.

Conclusion

There was a progressive loss in lung function after HSCT, featuring a restrictive pattern. Myeloablative conditioning was associated with early rise of sputum neutrophils and greater alteration in lung function over the first year. Moreover, patients undergoing HSCT exhibited a neutrophilic airway inflammation associated with raised sputum levels of IL-6 and IL-8, a picture similar but developed to a lesser extent of what is seen in COPD.
Finally, MSC co-infusion did not induce pulmonary deterioration 1 year after HSCT with NMA conditioning. MSC appeared to be safe for the lung but close monitoring of pulmonary infections remains essential.

RESUME

La greffe allogénique de cellules souches hématopoiétiques est utilisée pour traiter les hémopathies malignes depuis les années 60. Malheureusement, les complications pulmonaires sont fréquentes après ce type de greffe et représentent une cause majeure de mortalité.
L'expectoration induite et la mesure de l'oxyde nitrique exhalé (FeNO) sont des méthodes non-invasives permettant d'investiguer l'inflammation des voies aériennes qui ont été largement utilisées pour évaluer les maladies inflammatoires des voies respiratoires. Cependant, ces procédures n'ont jamais été étudiées dans le contexte de la greffe de cellules souches hématopoiétiques.

Dans la première partie de ce travail, dans le but de détecter les modifications au niveau des voies aériennes, nous avons suivi la fonction pulmonaire et l'inflammation des voies aériennes chez 182 patients ayant subi une greffe. Nous avons mesuré de manière prospective le VEMS, la CVF, la DLCO, le KCO, la CPT, le VR et le FeNO ainsi que la cytologie de l'expectoration induite avant et 3, 6, 12, 24 et 36 mois après la greffe. Nous avons observé une chute progressive de la fonction pulmonaire après greffe figurant un léger syndrôme restrictif tandis que l'apparition d'une bronchiolite oblitérante était rare (3.5% des patients). De plus, les patients ayant subi une greffe de cellules souches hématopoiétiques présentaient un taux élevé de neutrophiles dans les expectorations qui persistait pendant toute la période d'observation. A l'opposé, la valeur du FeNO est restée dans les valeurs normales pendant tout le suivi. Le conditionnement myéloablatif (MA) était associé à une élévation précoce du taux de neutrophiles dans les expectorations et une altération plus importante de la fonction pulmonaire pendant la première année après greffe. La survie globale à 1 an était de 71% et aucune caractéristique fonctionnelle ou inflammatoire avant greffe n'est apparue avoir une valeur pronostique de la survie à 1 an.

Dans la seconde partie de ce travail, nous avons mesuré des cytokines Th1, Th2 et Th17 dans le surnageant d'expectoration dans le but de mieux caractériser l'inflammation des voies respiratoires chez les patients greffés. Nous avons ensuite comparé les résultats avec ceux provenant de volontaires sains matchés pour l'âge et le statut tabagique et également avec des patients atteints de bronchopneumonie obstructive chronique (BPCO), une maladie chronique des voies respiratoires également caractérisée par une inflammation neutrophilique. Nous avons observé que les patients subissant une greffe de cellules souches hématopoiétiques présentaient une augmentation du taux d'IL-6 et d'IL-8 dans le surnageant d'expectoration comparés à des sujets sains bien que ces taux demeuraient plus faibles que ceux observés chez des patients BPCO.

Dans la troisième partie de ce travail, nous avons évalué l'impact de la co-infusion de cellules souches mésenchymateuses (MSC) sur la fonction pulmonaire après greffe. En effet, les MSC sont utilisées pour promouvoir la prise de greffe et prévenir la réaction du greffon contre l'hôte lors de greffe de cellules souches hématopoiétiques mais il a été démontré qu'elles pouvaient avoir des effets contradictoires sur le poumon après administration par voie générale chez l'animal. Leur effet n'a jamais été étudié à ce niveau après greffe chez l'humain. Ainsi, nous avons surveillé la fonction pulmonaire, la valeur du FeNO, l'apparition d'infections pulmonaires et la réactivation du cytomégalovirus (CMV) chez 30 patients ayant reçu une greffe allogénique non-familiale après un conditionnement non-myeloablatif (NMA) et une co-infusion de MSC. Ces patients ont été comparés avec 28 patients ayant reçu le même type de transplantation et de conditionnement mais sans co-infusion de MSC. L'impact des MSC sur l'apparition d'infections pulmonaires et sur la réactivation du CMV a été analysé par un modèle de Cox uni et multivarié ajusté pour les risques compétitifs. Les principales observations ont été que la co-infusion de MSC était sans danger au niveau pulmonaire et n'a pas entrainé de changement de fonction respiratoire 1 an après la greffe. Cependant, la co-infusion de MSC pourrait promouvoir les infections pulmonaires, nécessitant un suivi étroit de leur apparition.

Conclusion

Nous avons observé une chute progressive de la fonction respiratoire après greffe de cellules souches hématopoiétiques figurant un syndrôme restrictif. Le conditionnement myéloablatif était lié à une augmentation précoce du taux de neutrophiles dans les expectorations et une plus grande altération de la fonction pulmonaire dans la première année après greffe. De plus, les patients subissant une greffe de cellules souches hématopoiétiques présentaient une inflammation neutrophilique des voies respiratoires associée à une augmentation des taux d'IL-6 et d'IL-8 dans les expectorations, une image similaire mais moins prononcée que celle observée lors de BPCO. Finalement, la co-infusion de MSC n'a pas induit de détérioration au niveau pulmonaire 1 an après greffe associée à un conditionnement non-myeloablatif. La co-infusion de MSC apparait sans danger au niveau pulmonaire, cependant, un suivi étroit des infections pulmonaires demeure essentiel.

SAMENVATTING

Allogene hematopoïetische stamceltransplantatie (HSCT) wordt gebruikt voor de behandeling van hematologische maligniteiten sinds de jaren zestig. Helaas komen na dit type transplantaties vaak pulmonale complicaties voor die de grootste oorzaak van post-transplantatie mortaliteit zijn. Geïnduceerd sputum (IS) en metingen van uitgeademde Stikstof Monoxide (FeNO) zijn niet-invasieve methoden voor het onderzoeken van luchtweginflammatie die uitgebreid gebruikt worden om inflammatoire longziektes te evalueren. Deze methodes zijn echter nog niet onderzocht in het kader van HSCT.

In het eerste deel van dit werk, in een poging om veranderingen ter hoogte van de luchtwegen op te sporen, werden bij 182 patiënten die een transplantatie ondergingen de longfunctie en de luchtweginflammatie gecontroleerd. Prospectief werden de longfunctiewaarden FEV1, FVC, DLCO, KCO, TLC, RV, FeNO alsook de hoeveelheid cellen in het sputum gecontroleerd vóór de transplantatie en 3, 6, 12, 24 en 36 maanden erna.
Na de transplantatie werd een progressieve daling van de longfunctie vastgesteld in combinatie met een mild restrictief syndroom terwijl bronchiolitis obliterans zelden voorkwam (3.5%). Bovendien vertoonden de patiënten die een HSCT ondergingen een hoog aantal neutrofielen in hun sputum en dit gedurende de hele observatieperiode. De FeNO waarden daarentegen bleven binnen de normaalwaarden gedurende de gehele periode. De myeloablatieve (MA) conditionering ging gepaard met een vroege stijging van het aantal neutrofielen in het sputum en een grotere verandering van de longfunctie tijdens het eerste jaar na de transplantatie. De globale overleving 1 jaar na transplantatie was 71% en geen van de functionele of inflammatoire kenmerken vóór de transplantatie bleken een prognostische waarde te hebben voor de overlevingskansen na 1 jaar.

In het tweede deel van het onderzoek werden Th1, Th2 en Th17 cytokines bepaald in het supernatans van het geïnduceerd sputum om een beter inzicht te krijgen in het ontstekingsproces van de luchtwegen bij patiënten met HSCT. Vervolgens werden de resultaten vergeleken met deze van gezonde vrijwilligers, gematcht voor leeftijd en rookgewoontes, alsook met deze van patiënten die leden aan COPD (chronisch obstructief longlijden), een chronische inflammatoire longziekte ook gekenmerkt door een neutrofiele inflammatie. Bij patiënten die een HSCT ondergingen, werd een verhoging van de IL-6 en IL-8 waarden in het sputum vastgesteld in vergelijking met gezonde personen. Toch bleven de waarden onder deze vastgesteld bij patiënten met COPD.
In het derde deel van ons onderzoek werd de impact van co-infusie met mesenchymale stamcellen (MSC) op de longfunctie na transplantatie geëvalueerd. MSC worden immers gebruikt om het aanslaan te bevorderen en

om graft-versus-host ziekte te voorkomen bij HSCT. Er werd echter aangetoond dat deze een tegenovergesteld effect kunnen hebben op de longen na systemische toediening bij dieren. Hun effect werd nooit bestudeerd in mensen na HSCT. Dus werden de longfuncties, de FeNO waarde, de aanwezigheid van pulmonaire infecties en CMV (cytomegalovirus) reactivatie gecontroleerd in 30 patiënten die een allogene niet-gerelateerde HSCT ondergingen na een non-myeloablatieve (NMA) conditionering met een co-infusie van MSC. Zij werden vergeleken met 28 patiënten die eenzelfde type transplantatie en conditionering ondergingen, maar zonder co-infusie van MSC. De impact van MSC op pulmonaire infecties en CMV reactivatie werd geanalyseerd met een uni- en multivariant Cox model aangepast aan concurrerende risico's. De voornaamste observaties waren dat de co-infusie van MSC veilig was na HSCT met betrekking tot de longen en geen veranderingen van de longfunctie 1 jaar na transplantatie veroorzaakte. De co-infusie van MSC zou echter wel pulmonaire infecties kunnen bevorderen, met de noodzaak deze nauwlettend op te volgen.

Conclusie

Na HSCT werd een progressieve daling van de longfunctie vastgesteld in combinatie met een mild restrictief syndroom. De myeloablatieve (MA) conditionering ging gepaard met een vroege stijging van het aantal neutrofielen in het sputum en een grotere verandering van de longfunctie tijdens het eerste jaar na transplantatie. Bovendien vertoonden de patiënten die een HSCT ondergingen een neutrofiele luchtweginflammatie in associatie met verhoogde IL-6 en IL-8 waarden in het sputum, een beeld vergelijkbaar met dat bij COPD patiënten, maar minder uitgesproken.
Co-infusie van MSC gaf geen aanleiding tot een verslechtering van de longfunctie 1 jaar na HSCT met NMA conditionering. MSC zou blijkbaar veilig zijn voor de longen, maar een nauwlettende opvolging van pulmonaire infecties blijft essentieel.

INTRODUCTION

1. Hematopoietic stem cell transplantation

1.1. Definition

Allogeneic hematopoietic stem cell transplantation (HSCT) is defined as the transplantation of pluripotent hematopoietic stem cells from a healthy donor to a recipient. These cells are able to regenerate all hematopoietic tissues leading to replacement of the bone marrow of the recipient by the donor cells. HSCT is performed in patients who are suffering from hematological malignancies and other immuno-hematopoietic disorders. An association of a conditioning and an immunological conflict, linked to the donor T lymphocytes, mediates the eradication of malignant cells[1-3]. The graft is composed of stem cells, but contains also immune cells such as T cells, NK and monocytes. Stem cells are responsible for the hematological and long-term immune reconstitution (> 6 months) and T cells are linked to early immune recovery. Historically, the use of HSCT began in the 50‘s when it was shown that the transplantation of bone marrow cells to irradiated rodents could prevent death from marrow failure[4-8]. The first attempt in humans was performed by Thomas et al[9] in 1957 and his team received the Nobel Prize in Medicine in 1990 for his further work and discoveries on bone marrow transplantation[10]. After many years of improvements, concerning Human Leucocyte Antigen (HLA) compatibility and immunosuppressive management, HSCT is currently a routine procedure for patients with a variety of hematological disorders and nowadays, more than 50 000 transplantations are performed yearly worldwide (www.bmdw.org).

1.2. Indications

Allogeneic HSCT has been used to treat hematological diseases, solid tumors and selected nonmalignant disorders. Hematological malignancies can be classified as acute or chronic and as deriving from the lymphoid or myeloid lineages. They can develop in the bone marrow (leukemia) or in lymph nodes (lymphoma). They include therefore chronic or acute myeloid leukemia, chronic or acute lymphoid leukemia, myelodysplastic or myeloproliferative syndromes, non-Hodgkin’s lymphoma, Hodgkin’s lymphoma and multiple myeloma (Table 1). Allogeneic transplantation for hematologic cancers offers the best chance for cure in case of first remission in patients with a poor prognosis with conventional chemotherapy. It is also used after relapse in patients with a favorable prognosis with chemotherapy.
The eligibility for HSCT depends on the type and stage of the underlying malignancy, patient age and health status.

Table 1: indications of allogeneic transplant procedures for adults, current practice in Europe 2009

Disease	Disease status	Sibling donor	Well matched unrelated donor	mm unrelated > 1 Ag mm related
Leukemia				
AML	CR1 (low risk)	CO	D	GNR
	CR1 (intermediate)	S	CO	D
	CR1 (high reisk)	S	S	CO
	CR2	S	S	CO
	CR3, incipient relapse	S	CO	D
	M3 molecular persistence M3	S	CO	GNR
	molecular CR2	S	CO	GNR
	Relapse or refractory	CO	D	D
ALL	CR1 (standart/intermediate)	D	GNR	GNR
	CR1 (high risk)	S	S	CO
	CR2, incipient relapse	S	S	CO
	Relapse or refractory	CO	D	D
CML	First chronic phase (CP), faling imatinib	S	S	CO
	Accelerated phase or > first CP	S	S	CO
	Blast crisis	CO	CO	CO
Myelofibrosis	Primary or secondary with an intermediate or high Lille score	S	S	D
Myelodysplastic syndrome	RA, RAEB	S	S	CO
	RAEBt, sAML in CR1 or CR2	S	S	CO
	More advanced stages	S	CO	D
CLL	Poor-risk disease	S	S	D
Lymphomas				
Diffuse large B-cell lymphoma	CR1 (intermediate/high IPI at dx)	GNR	GNR	GNR
	Chemosensitive relapse; ≥ CR2	CO	CO	GNR
	Refractory	D	D	GNR
Mantle cell lymphoma	CR1	CO	D	GNR
	Chemosensitive relapse; ≥ CR2	CO	D	GNR
	Refractory	D	D	GNR
Lymphoblastic lymphoma and Burkitt's lymphoma	CR1	CO	CO	GNR
	Chemosensitive relapse; ≥ CR2	CO	CO	GNR
	Refractory	D	D	GNR
Follicular B-cell NHL	CR1 (intermediate/high IPI at dx)	GNR	GNR	GNR
	Chemosensitive relapse; ≥ CR2	CO	CO	D
	Refractory	CO	CO	D
T-cell NHL	CR1	CO	D	GNR
	Chemosensitive relapse; ≥ CR2	CO	CO	GNR
	Refractory	D	D	GNR
Hodgkin's lymphoma	CR1	GNR	GNR	GNR
	Chemosensitive relapse; ≥ CR2	CO	CO	CO
	Refractory	D	D	GNR
Lymphocyte predominant	CR1	GNR	GNR	GNR
	Chemosensitive relapse; ≥ CR2	GNR	GNR	GNR
	Refractory	GNR	GNR	GNR

nodular HL				
Other diseases		CO	CO	GNR
Myeloma		CO	CO	GNR
Amyloidosis	Newly diagnosed	S	CO	CO
Severe aplastic anemia	Relapsed/refractory	S	S	CO
PNH		S	CO	CO
Breast cancer		GNR	GNR	GNR
Breast cancer		D	D	GNR
Germ cell tumours		GNR	GNR	GNR
Germ cell tumours				
Ovarian cancer	Third-line refractory	GNR	GNR	GNR
Ovarian cancer	CR/PR	GNR	GNR	GNR
Medulloblastoma	Platinum-sensitive relapse	D	GNR	GNR
Small-cell lung cancer	Post-srugery	GNR	GNR	GNR
Renal cell carcinoma	Limited	GNR	GNR	GNR
Soft cell sarcoma including	Metastatic, cytokine-refractory	CO	CO	GNR
Immune cytopenias	Metastatic, responding	D	GNR	GNR
Systemic sclerosis		CO	D	D
Rheumatoid arthritis		D	GNR	GNR
Multiple sclerosis		GNR	GNR	GNR
SLE		D	GNR	GNR
Crohn's disease		D	GNR	GNR
CIDP		GNR	GNR	GNR
		GNR	GNR	GNR

Adapted from Ljungman, Bone Marrow Transplantation 2010[11]

CIDP= chronic inflammatory demyelinating polyradiculoneuropathy; CO= clinical option; can be carried after assessment of risks and benefits; CR1, 2, 3= first, second, third complete remission; D= developmental; further trials are needed; GNR= generally not recommended; IPI= international prognostic index; mm= mismatched; MRD= minimal residual disease; PNH= paroxysmal nocturnal hemoplobinuria; RA= refractory anaemia; RAEB= refractory anaemia with excess blasts, S= standart of care; generally indicated in suitable patients; sAML= secondary acute myeloid leukaemia; SLE= systemic lupus erythematosus. Low-intermediate-high categories are based mainly on number of white blood cells, cytogenetics at diagnosis and molecular markers, and time to achieve remission according to international trials.

This classification does not cover patients for whom a syngeneic donor is available

1.3. Conditioning

To allow the donor cell engraftment, the immune system of the recipient is previously destroyed by a conditioning based on irradiation and/or chemotherapy. Another important role of this conditioning is the eradication of tumor cells. Since the 90s, an association of total body irradiation (TBI) and cyclophosphamide has been the standard regimen[12,13].
However, because of high toxicity of the conditioning, patients over 55 years were considered as non-eligible [14]. The fact that these hematological diseases develop mainly between 60 and 70 years leaded to the development of reduced intensity conditioning in the 90's[15-17]. Non-myeloablative conditioning uses lower doses of radiation and/ or chemotherapeutic agents and can be performed in case of elderly patients or patients with comorbidities, i.e. considered ineligible for high dose conventional conditioning[18].
After non-myeloablative conditioning, eradication of cancer cells is performed by the Graft versus Tumor reaction instead of high dose cytotoxic agents, and this effect is mediated by the donor T cells. It initially allows the establishment of a mixed hemopoietic chimerism defined as the co-existence of donor and recipient hematopoietic cells that should evolve into full donor chimerism.
This type of regimen induces less organ damages (on gastro-intestinal tract, kidneys, liver, lungs)[19,20] than myeloablative conditioning. In addition, the neutropenia phase is shorter[21] leading to less bacterial infections early after HSCT[22]. The need for red blood cell transfusions is also decreased[23] and the patient follow-up can be done in an ambulatory care setting.
The incidence of grade II-IV acute Graft-Versus-Host-Disease (aGVHD)[24] is lower after this kind of conditioning compared to myeloablative conditioning but the rate of chronic GVHD (cGVHD) remains similar. The mortality is mainly due to relapse or GVHD. A common example of non-myeloablative conditioning combines a purine analog (Fludarabine) and total body irradiation (TBI, 2 grays (Gy))[25]. This combination was set up by Storb et al and is use in association with post-transplant immunosuppression consisting in ciclosporine and mycophenolate mofetil to prevent GVHD.

1.4. Type of transplant

Hematopoietic stem cells may be derived from the bone marrow, the peripheral blood or the umbilical cord blood. To collect peripheral blood stem cells or PBSCs, the donor is injected for 5-6 days with granulocyte colony-stimulating factor (G-CSF) that induces the mobilization of high stem cell numbers from the bone marrow to the peripheral blood. These cells are then harvested by apheresis. Compared to bone marrow, PBSCs contain an increased number of nucleated cells, natural killer (NK) and CD34-positive (surface marker of hematopoietic cells) and CD3-positive (T-cell marker) cells. Although PBSCs compared to bone marrow allow faster engraftment, the cGHVD incidence

appears higher. The aGVHD incidence seemed to be similar and the overall survival similar or even increased[26-28].
As all patients do not have an available sibling or unrelated donor, especially for patients from racial or ethnic minorities, alternative stem cell sources were needed.
Since the 80s, the umbilical cord blood (UCB) is also used as a source of transplantable cells[29]. It contains sufficient numbers of hematopoietic stem cells and low numbers of mature T cells, allowing some degree of HLA mismatch. Advantages also include a rapid availability and a reduced risk of GHVD[30]. Nevertheless, the graft failure rate is increased and the immune reconstitution is delayed, leading to a higher infection rate[31]. Furthermore, cord blood units contain a limited number of hematopoietic stem cells, so their use in adult patients is limited due to the recommended cell dose but results continue to improve. Today, more than 500 000 cord blood units are available in cord blood banks around the world (www.bmdw.org).

1.5. Type of donor

The transplanted stem cells may come from the recipient himself in case of autologous transplantation or are allogeneic when the cells come from another person than the recipient himself. The donor can be related or unrelated to the recipient. The HLA mismatch level between the donor and the recipient is linked to the occurrence of graft rejection as well as GVHD. The importance of a complete (10/10) matching for HLA A, B, C, DRB1 and DQB1 has been established[32]. The ideal situation is the identification of a sibling with the same HLA haplotypes. A twin donor, although being the most suitable donor in immunological terms, is associated with higher relapse risk because of the lack of GVT effects[33]. Partially HLA-identical family members can also be used with reasonable success rates.
Only around 30% of recipients have a suitable family donor, therefore the use of HLA-matched unrelated donors has increased during the last decades and results appear similar to what is observed with identical sibling donors when a 10/10 matched donor is used[34,35]. Nowadays, large registries of volunteer donors are established and around 20 million donors are available worldwide. Nevertheless, this type of transplantation is associated with delayed immune reconstitution leading to higher infection incidence[36].

1.6. Graft-Versus-Tumor effect

The graft-versus-tumor (GVT) effect was discovered in the 70-80s[37,38] although already suggested by Barnes et al. in the 50s[39]. It is linked to donor cells present in the graft such as CD4 and CD8 T cells, macrophages, dendritic cells and NK cells. These cells are responsible for the destruction of malignant cells and

decrease the relapse risk, although T cells also increase the risk of GVHD. The GVT reaction depends mainly on the T cells as T-cells depletion of the graft reduces it strongly[40,41].
It seems that T cells are directed against minor histocompatibility antigens (mHLA) recognized on the normal (leading to GVHD) and tumor recipient cells[42-44]. In this context, donor lymphocyte infusions (DLI) have been used to treat patients relapsing after tranplantation, especially in those with chronic myeloid leukemia[45,46]. However, the major complications of DLI include GVHD and marrow aplasia.

1.7. Graft-Versus-Host Disease

Graft-versus-Host disease (GVHD) remains an important limitation of the allogeneic HSCT, which disrupts the quality of life of the patient. This reaction is due to the donor cells recognizing the recipient cells as foreign and mounting an immunological attack[47]. The underlying mechanism is based on the recognition of the recipient major (HLA system) or minor histocompatibility molecules by T cells of the donor[48].
GVHD can be classified as acute or chronic depending on the time when it appears after the HSCT.

- Acute GVHD (aGVHD) occurs within 100 days after HSCT, often within 2 to 6 weeks. The major risk factors include HLA-mismatches, unrelated donor, CMV infection and high dose conditioning[49]. It occurs in up to 50% of HLA-matched and 70% of HLA-mismatched recipients[50]. AGVHD may involve the skin, liver, digestive tractus and lead to rash, jaundice, diarrhea or intestinal bleeding respectively[51]. This is due to apoptosis of epithelial cells of these organs. AGVH is responsible for 10 to 30 % of deaths observed after HSCT.
AGVHD is classified according to the injury severity from grade 0 (mild) to IV (very severe)[52]. The physiopathology involves, first, host tissue injuries linked to pre-transplant conditioning. The epithelial and endothelial injuries induce the production of pro-inflammatory chemokines and cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-alpha. At gastrointestinal level, damages due to conditioning induce a translocation of bacterial lipopolysaccharides (LPS). The release of these cytokines and LPS is responsible for an over-expression of adhesion and major and minor histocompatibility molecules on recipient cells. The recognition of these molecules is then facilitated. The second phase corresponds to the implementation of an immune response and depends on the interactions between antigen-presenting cells (APCs) and donor T lymphocytes. This phenomenon leads to the production of Th1 pro-inflammatory cytokines such as IL-2, IL-15 and IFN-gamma. The latter activates NK cells and macrophages. Inflammatory mediators are released such as TNF-alpha, IFN-gamma, nitric

oxide (N0) as well as IL-1, which amplify the tissue injury. Others cytokines were also shown to play a role in the development of aGVHD including Th2 cytokines (IL-4, IL-5, IL-6, and IL-10)[53]. The last phase is based on apoptosis of target cells by T lymphocytes and NK cells. AGVD progression can thus be summarized in three phases: 1) activation of APCs, 2) donor T cell activation, proliferation, differentiation and migration and 3) target tissue destruction (Figure 1). T reg, which are implicated in immune tolerance, may mitigate GVHD[54]. This observation led to newer approaches for the treatment of GVHD.

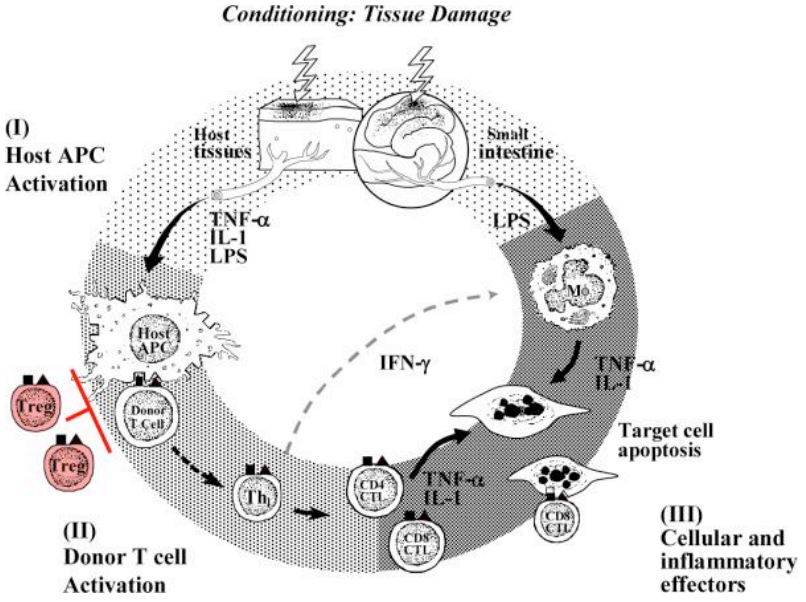


Figure 1: pathophysiology of acute GVHD (from Ferrara LM, Lancet, 2009)[55].

- Chronic GVHD (cGHVD) appears usually after 100 days post-transplantation in around 50 % of transplanted patients. It remains a major cause of transplant morbidity and mortality, which is attributable to immunodeficiency, infectious complications and organ failure. The histological picture is characterized by fibrosis that can lead to the organ failure. The most frequently injured organs include the skin, mucous membrane, eye, liver, lung and muscles that leads to lichenoid and sclerodermal skin changes, mucositis, keratoconjunctivitis, cholestatic hepatic dysfunction, bronchiolitis obliterans and myositis, respectively. CGHVD may occur in three different forms: as part of a continuous spectrum from aGVHD (=progressive), after a free interval (=quiescent) or without previous aGVHD (= *de novo*). In 1980, a staging system was defined and cGVHD was classified as limited or extensive[56]. Recently, the National Institutes of Health (NIH) proposed new consensus criteria for cGVHD diagnosis and staging (mild, moderate, and severe)[57].
Although underlying mechanisms have not been elucidated, some factors have been shown to increase cGHVD risk, such as: graft from PBSCs, previous aGVHD, advanced age, HLA disparities, use of DLI and female donor to male recipient. The latter is due to minor histocompatibility complex antigens, which are encoded by genes on the Y chromosome[49].
Moreover, chronic GVHD can be linked to an autoimmune syndrome. Indeed, auto-antibodies are generated, possibly due to the alteration of thymic function by previous aGVHD, previous conditioning or advanced age. The autoimmune T lymphocytes are not eliminated and together with alloreactive T cells cause tissue injuries via Th2 cytokine release such as IL-4, IL-6, and IL-10. But Th1 cytokines are also involved (IFN-gamma and TNF-alpha). They also promote B-cell activation leading to the production of auto-antibodies.
Current studies continue to analyze the involvement of donor/recipient genotype in transplantation outcome including in term of GVHD. For example, polymorphism of some single nucleotide (SNPs) or microsatellites of cytokine genes[58-61], or other genes[62], was shown to have an impact on the pathophysiology of GVHD.

The primary treatment for GVHD consists of ciclosporine A (or tacrolimus) and steroids (local therapy if GVHD is classified as mild, systemic therapy if classified as moderate or severe); however, these immunosuppressive treatments are responsible for a high infection level in patients with GVHD. Others strategies include extra-corporeal photopheresis or the use of antibodies directed against TNF-alpha and IL-2. Others are currently under investigation, such as use of mesenchymal stem cells and B-cell suppressive agents agents such as rituximab.
GVHD prevention based on T-cell depletion is to avoid because it induces an increased risk of graft rejection, infection and disease relapse[41,63,64]. Common immunosuppressive drugs used to prevent GVHD are methotrexate (MTX) combined with cyclosporine A or Tacrolimus[65-68], or a combination of a purine

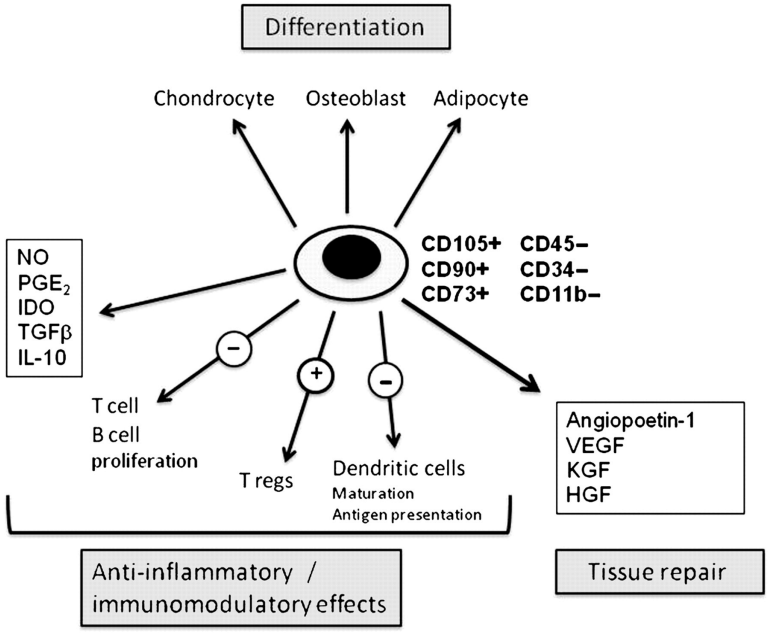
synthesis inhibitor (mycophenolate mofetill, MMF), with cyclosporine A[69] or Tacrolimus. Anti-thymocyte globulin (ATG) infusion producing T cell depletion is used in the context of mismatched transplant, reduced-intensity conditioning or when PBSC are used.
The important observation that patients who are suffering from GVHD have lower relapse rates indicates that these processes are closely linked[37,38]. Thereby, the best survival is observed for an adjusted balance between GHVD and GVT reaction.

1.8. Mesenchymal stem cells

Mesenchymal stem cells (MSC) are multipotent non-hematopoietic stem cells derived from the bone marrow and several other tissues. In vitro, they have the ability to differentiate into mesenchymal lineage including osteoblasts, adipocytes and chondrocytes[70]. They express surface markers such as CD73, CD90 and CD105 but not hematopoietic markers as CD14, CD34 and CD45[71]. In vitro, they show immunosuppresive properties; they are able to inhibit T- and B-cell proliferation and dendritic cell maturation and could promote regulatory T cells. They possess anti-inflammatory properties and release anti-inflammatory mediators such as IL-10. Indeed, they have the ability to secrete paracrine growth factors and cytokines able to down-regulate inflammation. Moreover, the ability of MSC to differentiate into functional cells and produce epithelial growth factors may be a key in promoting adequate organ repair[72]. Thus, the beneficial effects of MSCs may result from the secretion of soluble factors rather than long-term engraftment inside the damaged tissues. Indeed, MSCs become rapidly undetectable after systemic administration. However, MSC may also act as antigen presenting cells (APCs) and pro-inflammatory cells. The functional polarization of MSCs seems to be dependent of the administration timing, the priming origin and the concentration and duration of the stimulus[73].

In the context of HSCT, MSC have stimulated a high level of enthusiasm for their potential therapeutic use due to their immunosuppressive activity. Moreover, due to their lack of major histocompatibility complex (MHC) molecule expression, MSC are weakly immunogenic in humans, allowing administration to patients without HLA matching[74]. Some studies have demonstrated that MSC infusion was safe in human[75,76] and that MSCs could have a role in engraftment promotion and graft-versus-host disease (GVHD) prevention[76-78].
In recent years, there have been small studies investigating the effect of MSC treatment for aGHVD and cGHVD. However, Phase II and III studies evaluating the use of a commercial MSC product termed Prochymal®

(produced by Osiris Therapeutics®, Inc., Columbia, MD) in combination with corticosteroids to treat aGVHD gave conflicting results[79,80].
Other authors have recently shown that MSC could be successfully used for the treatment of severe steroid-resistant aGVHD[81-83]. In addition, some studies analyzed the usefulness of MSC as treatment for cGVHD and found that MSC could be an effective salvage therapy for some patients with severe cGHVD[84,85]. The real advantage of MSCs therapy to prevent or to treat GVHD remains, however, to be confirmed in randomized clinical studies. Moreover, further studies evaluating the most efficient culture condition, dose and schedule of administration are needed.



Rankin S., Thorax 2012

2. Pulmonary complications after HSCT

Pulmonary complications, that are potentially lethal, occur in 40%-60% of patients who receive an allogeneic HSCT and account for 50% of transplant-related deaths. Reports on intensive care outcome showed a strong association between mechanical ventilation and mortality after HSCT[86]. Complications can be divided into infectious and non-infectious and are classified as early or late depending on whether they occur before or after 100 days post-transplantation[87]. They arise as a consequence of the immunosuppressed status of the recipient, of the chemotherapy and radiotherapy conditioning regimens that precede HSCT, and of allo-immune reactions as host-versus-graft and graft-versus-host responses[88].
With the development of potent antibiotics and antifungal agents as effective prophylaxis in recent years, the spectrum of pulmonary complications has shifted from infectious to non-infectious etiologies.

Pulmonary complications after HSCT are known to follow a predictable time pattern, which facilitate the diagnosis (Figure 2).

2.1.Early complications

In the immediate phase after HSCT, there is a period of profound neutropenia for 2-3 weeks causing mainly fungal and bacterial infections, but non-infectious complications are also common.

Non infectious

Pulmonary edema occurs typically in the second or third week after HSCT. Patients present weight gain, dyspnea and hypoxemia and the diagnosis is based on chest radiographic pictures. It may be due to increased pulmonary capillary permeability or increased pressure in these capillaries linked to the conditioning regimen.

Engraftment syndrome develops within few days of recovery from neutropenia and is reported mainly after autologous transplantation. It is characterized by a "cytokine storm " and clinically by fever, skin rash, diarrhea, renal impairment and diffuse pulmonary infiltrates. The treatment is supportive and death occurs in around 25% of patients.

Diffuse alveolar hemorrhage (DAH) occurs around day 12 with a frequency average of 5% after HSCT[89]. It represents a common cause of acute respiratory failure after HSCT and often leads to hospitalization and mechanical

ventilation. The risk factors include intensive chemotherapy or irradiation before transplantation as well as older age and renal insufficiency. The pathogenesis involves injuries to endothelial cells of small blood vessels and thrombotic microangiopathy. Clinical signs include dyspnea, cough, fever and hypoxemia. The best tool for diagnosis is the bronchoalveolar lavage (BAL) fluid, which shows a progressively bloodier return, but infection must be excluded[90]. The treatment is based on empiric high doses of steroids and the prognosis is poor.

Idiopathic pneumonia syndrome (IPS) is a diffuse lung injury with no etiology identified. The frequency may be up to 10% of patients[91]. It appears in the first two months following HSCT. The risk factors include older age, intensive conditioning regimen, high-grade aGVHD and positive donor status for CMV[92]. Clinical manifestations include fever, dyspnea, bilateral pulmonary infiltrates and hypoxemia. The diagnosis is based on chest imaging and BAL with infection exclusion. High doses of corticosteroids are used as supportive treatment but the mortality rate is high (70-90%)[93].

Bronchiolitis obliterans organizing pneumonia or BOOP (also called cryptogeneic organizing pneumonia or COP) is a restrictive lung disease seen in 1.4% of patients. It may be related to GVHD and the pathological features observed are the presence of intraluminal granulation in the small airways, alveolar ducts and alveoli[94]. It usually develops within 1-3 months after HSCT and patients present dyspnea, cough and fever. The diagnosis is based on chest imaging and lung biopsy. Patients with BOOP have a good response to corticosteroid therapy.

Drug toxicity
This is associated with some chemotherapeutic agents such as bleomycin, methotrexate and busulfan, and is increased when chemotherapy is associated with radiotherapy. It can induce acute pneumonitis and pulmonary fibrosis at a later stage.

Infectious

Due to the impaired immunity resulting from conditioning or corticosteroid therapy for GVHD, patients following HSCT are susceptible to develop severe pulmonary complications from bacterial, viral or fungal origin.

Bacterial infections remain the main cause of complications after HSCT and are correlated with the period of granulocytopenia before engraftment. The main risk factors are the neutropenia, mucositis, skin damage, gastrointestinal failure associated with GVHD and intravenous catheters. Gram-negative

bacteria as Pseudomonas or Klebsiella are common in the neutropenia phase. Gram-positive pathogens include Staphylococcus and Streptococcus.
Pneumocystis carinii pneumonia (PCP) can occur from the first month but its frequency diminishes due to better prophylaxis based on trimethoprim/sulfamethoxazole.

Viral infections

Cytomegalovirus (CMV) infection occurs in 70% of patients and CMV pneumonitis usually occurs in 10 to 40% of patients 6 to 12 weeks after HSCT. This period corresponds to the gradual recovery of neutrophils. The diagnosis is based on antigen detection by polymerase chain reaction (PCR), performed weekly in routine, and allows diagnosing CMV viremia before disease develops. The most frequent causes of infection are the reactivation of latent virus in a CMV seropositive patient or the transmission from a CMV seropositive donor to seronegative recipient. Other risk factors implicate older age, irradiation, ATG use, neutropenia and aGVHD. Pneumonia is the most severe manifestation of this infection. The associated symptoms are cough, fever, dyspnea and hypoxemia. Routine prophylaxis using ganciclovir in reactivated patients is started within the first 100 days after HSCT. Treatment involves ganciclovir as well, and leads to CMV control in most cases.

Other respiratory viruses include respiratory syncytial virus pneumonia, which has a high mortality rate once pneumonia develops. Among Herpes virus, Herpes zoster virus (HZV) and Herpes simplex virus (HSV) may lead to severe infections.

Fungal infections

Candida species represent a commonly isolated fungal agent after HSCT. Routine prophylaxis uses fluconazole during the neutrophilic phase. Treatment uses fluconazole or amphotericin B.
Aspergillus infection is the most common fungus leading to severe pneumonia. The onset of aspergillus infection is observed to occur according to a bimodal distribution. The first peak is at a median of 16 days and the second peak at a median of 96 days after HSCT. Risk factors include older age, male sex, transplantation outside of laminar flow room, aGVHD and corticosteroid therapy. Clinical features include fever, dyspnea, dry cough, wheezing, chest pain and sometimes hemoptysis.
Diagnosis is suspected by chest imaging showing nodular or cavitary infiltrates and is confirmed by BAL or lung biopsy. The treatment of choice is amphotericin B or voriconazole but the mortality rate is high.

2.2.Late complications

The immune system of the patient usually recovers to normal by 1 year leading to predominantly non-infectious complications thereafter. The exception is in case of GVHD because of then delayed immune system recovery and the immuno-suppressive therapy predisposing patients to infections (mainly CMV, encapsulated bacteria and P. carinii).
The most dangerous late-onset non-infectious pulmonary complication is bronchiolitis obliterans, detailed in the next paragraph.

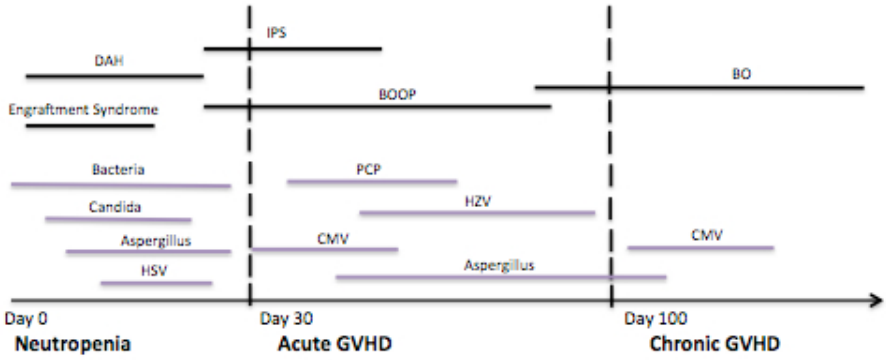


Figure 2: Pulmonary complications according to time course after HSCT. (Adapted from Soubani AO, Crit Care Med 2006)[95] DAH: Diffuse alveolar hemorrhage; IPS: Idiopathic pneumonia syndrome; BOOP: Bronchiolitis obliterans organizing pneumonia; PCP: Pneumocystis carinii pneumonia; BO: bronchiolitis Obliterans; CMV: cytomegalovirus; HZV: Herpes zoster virus; HSV: Herpes simplex virus.

2.3. Bronchiolitis Obliterans

First described in 1978[96], bronchiolitis obliterans (BO), also known as constrictive bronchiolitis, occurs between 6 months and 1 year in less than 10% of patients after allogeneic HSCT[97-101] and was proven to be associated with chronic GVHD[102,103]. However, an incidence range of 2 to 26% has been reported and is explained by varying diagnostic criteria and BO definition. BO represents a non-specific inflammatory injury affecting primarily the small airways but its pathogenesis remains unclear. The strong association with cGVHD suggests that the host bronchiolar epithelial cells serve as targets for donor cytotoxic T cells. Risk factors that have been associated with BO development include busulfan-based conditioning regimens, intensity of the conditioning, use of methotrexate, older age, aGVHD, long duration from leukemia diagnosis to HSCT, respiratory infection during the first 100 days (especially viral and mycoplasma infections), aspiration of oral material, HLA mismatch, female donor to male recipient sex match, cigarette smoking, PBSCs rather than bone marrow transplantation, airflow limitation prior to transplantation and hypogammaglobulinaemia[101,102,104,105]. The observation of heterogeneous histological findings and clinical courses implies that the most likely explanation is that BO has a multifactorial origin, based on allo-immune and non allo-immune mechanisms; it may result from events linked to infection, injuries (related to drugs, irradiation and ischemia) and an inappropriate immunological response (GHVD).

The molecular underlying mechanism involve injury to airway epithelial cells (due to the conditioning, allo-reaction by donor T cells and respiratory infections) leading to the release of pro-inflammatory cytokines, chemokines and growth factors implicated in inflammatory events and chronic fibroproliferation. The local recruitment of inflammatory cells such as lymphocytes, neutrophils, and macrophages leads in turn to inflammatory and fibrotic process amplification. This inflammatory reaction is characterized by an increase in cytokines such as IL-1, IL-6, IL-8, IFN-gamma and TNF-alpha[106].

BO is clinically recognized by the occurrence of a non-reversible airflow limitation within a few months that is linked to fibrosis affecting the small airway wall and thereby reducing airway lumen. Pulmonary function tests show an obstructive pattern defined by a fall of forced expiratory volume in 1 s (FEV1) > 20% from the pretranplantation value and a FEV1/forced vital capacity (FVC) ratio <0.7. Chien et al have introduced new spirometric criteria for BO diagnostic and suggested that annualized rate of predicted FEV1 decline > 5% per year and a lowest FEV1/FVC ratio < 0.8 was a better definition of airflow obstruction[104]. Diffusing capacity (DLCO) is usually reduced and the residual volume often increased (> 120 % of predicted value). Due to the lack of standardized criteria for its diagnosis, the NIH consensus included criteria for the BO diagnostic[57] (see table 2).

The classic respiratory symptoms include dry cough, absence of fever, dyspnoea and wheezing but BO may also develop as an asymptomatic deterioration in pulmonary function tests in 20% of cases[107]. The diagnosis is based on the exclusion of all the causes leading to an obstructive pulmonary syndrome. It requires negative microbiological tests from BAL, showing neutrophilic or lymphocytic inflammation in case of BO, but this finding is non-specific[108]. Chest radiography is usually normal. Other chest high-resolution computer tomography (CT) findings include signs of hyperinflation with decreased lung attenuation, segmental bronchial dilatation and proximal bronchiectasis. The hallmark is an evidence of air trapping during expiration on the chest scan[109] responsible for a mosaic picture (Figure 3). The confirmation by surgical lung biopsy is rarely indicated. Histologically, the principal findings are granulation tissue and scarring obliterating the small airways (Figure 3). The inflammatory infiltrates are peribronchiolar and consist of neutrophils and lymphocytes.

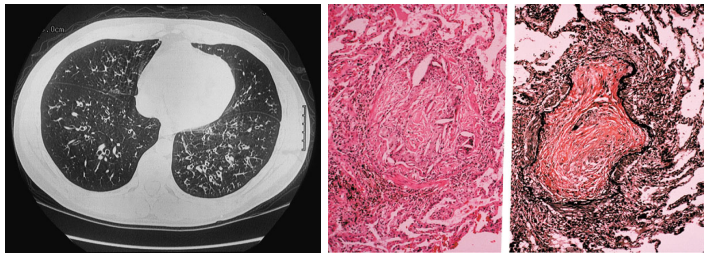


Figure 3: left side: A computed tomography of the chest of a patient with Bronchiolitis obliterans showing air trapping. Right side: Lung pathology in Bronchiolitis obliterans showing bronchiolar inflammation and luminal obliteration associated with excess fibrous connective tissue. (From Afessa B., Bone Marrow transplantation 2001)[107]

BO has a very poor prognosis with an overall survival rate of 13% at 5 years[99]. Chien et al noted that the rate of airflow decline between day 100 and 1 year was an indicator of mortality risk[110].

Due to poor understanding of its pathogenesis, the treatment is empiric and based on high-dose systemic corticosteroids (1 mg/kg/d) and augmentation of immunosuppressive therapy (cyclosporine A, mycophenolate mofetil or tacrolimus), but only a minority of patients shows clinical improvement. Other treatment options include inhaled corticosteroids (as fluticasone), bronchodilators, anti-CD20 monoclonal antibodies that targets B cells (Rituximab), Thalidomide, ATG, anti-TNF alpha (infliximab, etanercept), macrolides (azithromycin), antifibrotic agent such as imatinib, leukotriene receptor antagonist (montelukast), total lymphoid irradiation (TLI) and extracorporeal photodynamic, but their potential benefits are still under

consideration and need reliable results[111-114]. Finally, lung transplantation may represent an option for some patients.
Supportive treatment is essential in the management of patients with BO. Patients should be maintained on prophylactic measures against bacterial, fungal and viral infections. Moreover, appropriate vaccinations (mainly against influenza, pneumococcus) are recommended. Patients with advanced disease may require oxygen therapy and may benefit from pulmonary rehabilitation.

In conclusion, further trials evaluating the efficacy and safety of these treatments are urgently needed to prevent the high morbidity and mortality associated with BO.
Moreover, the onset of BO is insidious and the treatment appears ineffective once the fibro proliferative tissue process is initiated. By spirometry, the diagnosis of expiratory airflow obstruction indicating BO is possible only when the disease is widespread and advanced. Therefore there is a crucial need for early diagnostic biomarkers of BO, which could identify the early signs of the disease at a time when the patients are still clinically asymptomatic and could alter the course of this fatal disease.

Table 2: diagnostic criteria proposed by NIH consensus in 2005 for BOS after HSCT

Absence of infection
Another cGVHD manifestation in another organ
FEV1 < 75% predicted or > 10% decline from baseline
Signs of obstruction: FEV1/FVC ratio < 0.7 or RV > 120% predicted or RV/TLC > 120%
CT or lung biopsy with evidence of air trapping

Bronchiolitis obliterans occurs also and is more common after lung transplantation (LT). This procedure is a well-established treatment for patients with end-stage pulmonary diseases and around 50-60 % of patients who survive over 5 years after LT develop BO[115] which is responsible for the majority of deaths. The diagnosis is made latter in case of LT compared to HSCT (16-20 months post-transplant). The International Society of Heart and Lung Transplantation proposed a clinical description of BO termed BO syndrome (BOS) and defined it by pulmonary function changes (fall of FEV1) rather than histology[116]. This classification was revised later due to the inclusion of a decrease of FEF_{25-75} suggested to be a more sensitive early marker of obstruction[117]. The term BOS is also in use in the context of HSCT.

After LT, BO is regarded as the manifestation of chronic allograft rejection. Many risk factors are incriminated in the context of BO after LT as the number and severity of acute rejection episodes, respiratory infection (maily CMV pneumonitis), HLA disparities, lymphocytic bronchitis/bronchiolitis, gastroesophageal reflux and airway ischemia during the LT procedure[115]. Furthermore, genetic polymorphism may increase susceptibility to BO development.

At molecular level, BO is a sequel of the host T cells (CD4 and CD8), which trigger adaptive immune responses against allogeneic donor lung epitopes (MHC class I and II). Thus, the immunopathogenesis of BO after LT is a "mirror" picture of the abnormalities among HSCT patients[118]. The allo-immune dependent and independent mechanisms lead to a similar histological and clinical outcome. Damage to the airway epithelium plays a key role in the pathogenesis and epithelial cells are responsible for the release of IL-8, which is a chemoattractant for neutrophils. These neutrophils produce reactive oxygen species (ROS) and proteases (elastase and matrix metalloproteinases) leading to excessive oxidative stress and damage to lung tissue. Alveolar macrophages produce profibrotic cytokines that induce fibroblast proliferation and extracellular matrix deposition leading to obliteration of the small airways.

The clinical and radiological findings are similar after HSCT or LT[119]. However, the transbronchial biopsy is useful and allows detecting acute rejection. The management is also similar as well as the high mortality rate. Nevertheless, in this setting, compared to BOS after HSCT, azithromycin has been reported to stop the progression of BOS and even improve lung function[120], at least in patients with neutrophilic reversible allograft dysfunction[121].

3. Non-invasive airway monitoring methods

A close pulmonary monitoring of patients who underwent HSCT is crucial to diagnose complications, prevent progression of abnormalities and may guide therapy.
Clinically, patients can be followed using physiological parameters assessed by pulmonary function tests. From biological point of view, bronchial biopsy and BAL are the reference methodologies to assess airway inflammation. Nevertheless, less invasive methods have been introduced along the years in order to assess biomarkers in induced sputum or exhaled air.

3.1.Pulmonary function tests (PFT)

In order to early detect pulmonary functions alterations, it is recommended to monitor the lung function tests before HSCT and at regular interval in the months and year following the transplantation. Patients showing abnormalities must be investigated for possible pulmonary infection, GVHD or BO.
The lung function assessment prior to transplantation usually serves as baseline reference to evaluate the changes in pulmonary function after HSCT[122]. Lung function can be assessed using a body box plethysmography allowing to measure flow rates, lung volumes and diffusion capacity according to ATS/ERS standard criteria[123-126].
Spirometry measures the rate of exhaled or inhaled air volume as a function of time. Thereby, the measurements include the forced expiratory volume in 1 second (FEV_1) and the maximum volume of expired air after a maximum inhalation (forced vital capacity: FVC). The spirometry tests are performed before and after 400 µg inhaled salbutamol MDI administered through a Volumatic.
The total lung capacity (TLC) is defined as the volume of air in the lungs after a maximal inspiration and the residual volume (RV) as the volume of air remaining in the lungs after a maximal exhalation.
Diffusion for carbon monoxide (DLCO) is a measure of the ability to absorb alveolar gases into the capillary blood flow. It is depending on the alveolar membrane thickness, the hematocrite level, the cardiac output and the disparity between the ventilation and perfusion. DLCO is measured by the single breath wash-out technique and is corrected for the hemoglobin content. The correction for hemoglobin content is particularly important in HSCT patients as their hemoglobin levels may vary significantly over time. The lung function parameters can be expressed as absolute value or percentage of predicted value, which is calculated according to age, size, weight and gender.
These tests allow defining three main categories of lung function abnormalities. Obstructive lung disease occurs when there is airway narrowing leading to diminished FEV1 with a preserved FVC value resulting in a decreased FEV1/FVC ratio (commonly < 70%). Restriction is defined by a reduction in

TLC (commonly < 80% predicted) with a preserved FEV1/FVC ratio. The final defect concerns the DLCO, which decreases in case of thickening of the alveolo-capillary membrane as it occurs in several restrictive pulmonary diseases. It may also be altered in case of peripheral ventilation/perfusion mismatch or neuromuscular weakness impeding the gas to travel through the conducting airways. Therefore altered diffusing capacity may be observed in both obstructive and restrictive disorders.

The lung function results before the transplantation commonly showed decreased DLCO values to 70-80% predicted[127,128]. This is possibly due to interstitial infiltration by the underlying malignancy, vascular changes secondary to the chemotherapy, irradiation[129,130] or infections.
Some studies have assessed pre-transplantation pulmonary function parameters as predictive factor of the outcome following HSCT. A study conducted by Clark et al showed a correlation between the pretransplant and 1 year FEV1/FVC ratio[131]. Others found a doubled risk of post-transplant airflow decline in case of either pretransplant FEV1/FVC ratio < 0.8 or the same ratio < 0.8 at 3 months following HSCT[104,110]. In a large study including 1,297 patients, an association was observed between abnormal DLCO and alveolar-arterial oxygen gradient before HSCT and the use of mechanical ventilation and death after HSCT[128]. FEV1 < 80% of predicted value was associated with the occurrence of acute severe pulmonary complications following HSCT[132]. Low values of both FEV1 and DLCO pre-transplantation were previously identified to be associated with poor outcome after transplantation[133]. These two parameters were used by Parimon et al in the context of HSCT to establish a risk score for mortality[134]. However, this score was not validated in a recent study with patients receiving non-myeloablative conditioning. The authors found that lower pre-transplantation TLC showed a predictive value for the occurrence of pulmonary complications, nonrelapse mortality (NRM) and inversely related to overall survival (OS), while low FVC showed a correlation with pulmonary complications and lower OS[127]. A similar finding associating a pulmonary restriction prior HSCT and an increased risk for early respiratory failure and NRM after HSCT was reported in another study[135].
Overall, the current data are conflicting and did not show indisputable association between baseline pulmonary function parameters and early post-transplantation outcomes[136,137]. Nevertheless, it is important to specify that most of these studies concern relatively small cohorts allowing insufficient power to detect associations.

The pulmonary function parameters obtained early after the transplantation, were also linked to the occurrence of late and non-infectious pulmonary complications[138]. In an interesting study, restrictive defect at three months after the transplant was associated with a greater risk of death[139]. Others found a link between airflow decline by day 100 and an increase risk of airway obstruction 1

year after HSCT and an increased mortality risk associated with a fast rate of decline between 100 days and 1 year post-transplant[110]. A more recent analysis highlighted the fact that early decline in FEV1 and DLCO were linked to a higher risk of non relapse mortality at 5 years and that low FEV1 was associated with the occurrence of cGHVD within the first year after HSCT[140].
It is noted that the predictive value of pulmonary function parameters is different when assessed before or after transplantation due to the selection bias. Indeed, not all patients are able to tolerate the transplant and survive until the next PFT measure, others may have severe complications and the PFT may be not performed, explaining the different results observed.

Pulmonary function abnormalities after HSCT were assessed in a systemic review of 20 studies published between 1983 and 1999. What came out from this analysis is a diminished DLCO in 83%, a restriction in 35% and an obstruction in 23% of patients[141]. Many factors could contribute to the fall in DLCO and to the restriction in this population: thoracic irradiation, cytotoxic chemotherapy, pulmonary edema, muscle weakness, GVHD, infectious or idiopathic interstitial pneumonia and BOOP. Obstruction was linked to several factors as GVHD, TBI, infection, HLA mismatch, leukemia, busulfan conditioning and methotrexate use. However, in a more recent cohort, the same research team found a lower obstruction rate reaching only 5%, possibly due to the introduction of cyclosporine[142].
Few studies have analyzed long term lung function variation following HSCT. One study described the outcome of children with leukemia or lymphoma who underwent transplantation conditioned with cyclophosphamide associated with TBI. The authors found an immediate decrease in DLCO and lung volumes, which recovered in the following years. Nevertheless the patients still exhibited subclinical pulmonary restrictive pattern at a median of 8 years after the graft[143]. In adult patients, a 5-year monitoring was performed in two studies and showed a reduction in lung volumes and DLCO with subsequent recovery over time[142,144]. Likewise, another study in 111 patients followed over 2 years showed an initial decline in lung volumes and DLCO post-transplant. Factors associated with a delayed lung function recovery were GHVD, pulmonary infections and high irradiation[145].
Discrepancies in lung function evolution after HSCT in the studies published so far may be related to different conditioning, different immunosuppressive treatments, allogeneic vs autologous transplantation and different underlying diseases.

3.2.Exhaled nitric oxide

Nitric oxide (NO) is a small molecule synthesized within many cells in the respiratory tract by NO synthases from L-arguinine. Increased NO production

may play antiviral/antibacterial role. The regulation of its production has been implicated in the pathophysiology of airway diseases. Fraction of exhaled nitric oxide (FeNO) is a non-invasive marker of airway eosinophilic inflammation[146], and is correlated to the level of NO formed in airway epithelium[147].
The procedure to measure FeNO was shown to be simple and reproducible, allowing measurement in children and patients with severe disease. FeNO is measured using a chemoluminescence analyser at a constant flow rate of 50 ml/sec (as FeNO is highly flow-dependent), in accordance with the recommendation of the ATS/ERS task force[148]. Following a full inspiration, seated patients (wearing a nose clip) are asked to exhale slowly from total lung capacity. Patients maintain a mouth pressure of 5-20 cm H_2O by using a feedback visual display; this pressure allows closing the nasal route and avoids the contamination by nasal NO. Because of the influence of spirometric manoeuvre on FeNO values, this measurement is performed before starting the lung function tests. The result is expressed in parts per billion (ppb) and is the average value of at least two measurements with less than 10% of variability. Travers et al proposed reference ranges of normal values[149]. However, concerning FeNO measurement, special caution must be paid to demographic and environmental cofounding factors that are known to modify the value like gender, atopy, cigarette smoke exposure and acute viral airway infections.

FeNO values are often raised in stable asthmatics. Its clinical utility was mainly demonstrated in asthma to make diagnosis[150,151] and predict response to inhaled corticosteroids (ICS) treatment[152]. Its role as monitoring tool to adjust ICS treatment is more controversial[153,154].

FeNO was also investigated in chronic obstructive pulmonary disease (COPD), a chronic inflammatory disease linked mainly to cigarette smoke. Some authors reported an increased FeNO level in COPD patients[155], and some others found a negative correlation between FeNO level and FEV1 in COPD patients[156]. An association was also observed between FeNO values and sputum neutrophilia and IL-8[157]. However, FeNO seems to be less useful in COPD as it is in asthma as the levels often lie in the normal range in stable state and only elevate in case of exacerbations[158,159].

In the setting of LT, it is likely that the nitric oxide pathway plays a role in the inflammatory changes leading to BO. There is an increase level of inducible nitric oxide synthetase (iNOS) mRNA activity in the epithelial cells in a rat model of BO after LT[160], iNOS being correlated with FeNO level. Similarly, patients with BO exhibit higher FeNO value compared to stable patients or healthy subjects[161]. The measure of FeNO after LT could be also helpful to assess the treatment response of BOS patients. Indeed, a decrease in FeNO level was reported after switching the immunosuppressive agents from cyclosporin A to tacrolimus and was linked to the stabilization of FEV1 and a

reduction of airway inflammation[162]. Others described an increase in FeNO only in early stage of the disease followed by a decrease when the inflammatory phase is replaced by fibrosis[163]. Moreover, due to its good reproductibility and repeatability, FeNO has been used to monitor lung transplantation rejection[164] and was able to detect BOS several months (263 ± 169 days) before the diagnosis based on the fall of FEV1. Others found that elevated FeNO levels were observed in case of unstable BOS patient resulting in a further decline of lung function[165]. This result was corroborated and extended by Neurohr and al who reported also a higher FeNO level in case of unstable non-BOS patients with future deterioration. Moreover, serial measurements of FeNO may provide a tool for risk stratification in these patients due to the excellent negative predictive value to identify patients with stable course[166]. Finally, FeNO appeared to be linked with BAL neutrophilia in stable and in those developing BOS after LT, confirming the finding of Gabbay et al[167,168].

FeNO has been poorly assessed after HSCT in adult patients. In the context of HSCT, a report showed an increased FeNO value in some patients suffering from BOOP[169] which is suggested to be linked to GVHD as GVHD was associated with raised serum nitrite/nitrate levels previously[170,171]. A recent paper analysed FeNO in 68 patients following HSCT and showed a slight increase of FeNO 3 and 6 months after HSCT, associated with risk of death[172] but this association needs to be validated in a larger cohort. Patients of a more recent study had values within the normal range over time after HSCT but in patients with BO, they specify that others factors unrelated to BO such as atopy or conditioning-related lung injury could affect the FeNO value and make any relationship hard to detect[173]. In contrast, new data reported by Ditschkowski et al showed a decrease of FeNO in BO patients compared to patients without BO after HSCT and healthy subjects[174]. In this study, a threshold level of 15 ppb was able to differentiate BO from non-BO patients. In children who underwent HSCT, FeNO showed a predictive value for early pulmonary complications[175] after transplantation.

3.3.Induced sputum

Induced sputum (IS) is recognized as a safe and non-invasive technique to collect cells from airways and to assess the cellular composition as well as the inflammatory biomarkers in airway diseases such as asthma[176] and COPD[177]. Induced sputum cell count and mediator measurements have been well validated[178,179]. Additionally, IS allows to perform a large spectrum of analysis such as flow cytometry[180], proteomics[181] and genomics[182,183].

The sputum is induced by inhalation of hypertonic (4.5%) or isotonic (0.9%) saline solution according to the post-bronchodilation FEV_1 value, as previously described[184]. It is not clear how the inhalation of saline produces airway secretions. Proposed mechanisms involve an increase of the mucociliary clearance and/or of the airway lining fluid osmolarity, which raises the vascular permeability in the bronchial mucosa inducing mucus production.
Briefly, the inhalation is performed through an ultrasonic nebuliser (output 1-3ml/min) after a bronchodilation using 200-400 µg inhaled salbutamol to avoid bronchospasm. For this purpose, additional salbutamol is also added in the cup of the nebulizer. The duration of the inhalation is critical as it influences the cell and biochemical content of the sputum sample[185]. Therefore, the duration of inhalation is standardized. The patients are asked to inhale for 3-4 periods of 5 min and then to cough and expectorate in a plastic container at each time interval. Patients are also asked to rinse the mouth before expectoration to minimise the salivary contamination. The lung function is monitored throughout the induction which should be stopped in case of a decrease of FEV1 > 20% from baseline.
After collection, the sample is placed on ice until lab processing, usually within 2 hours, as recommended[178]. There are two options to process the induced sputum: the whole sample can be taken up or only the viscous part (plug) can be selected. Both methods are validated and reproducible but may display different results regarding the measure of mediators[178]. The use of a mucolytic agent such as dithiothreitol (DTT) or dithioerythritol (DTE) facilities the homogenization of the sample (by breaking the disulphide bonds in mucus molecules) and thereby the reading of the cytospin[186]. However, it may interfere with biochemical analyses[179]. To assess the cell composition, a cytospin is performed from the cell content and the supernatant is collected for further biochemical soluble markers analyses (as mediators, cytokines/chemokines). The cytology results must report the total non-squamous cell count expressed per ml or g of sputum. The cell viability is assessed by trypan blue exclusion and the differential leukocyte count performed on cytospins stained with May-Grünwald-Giemsa on 500 non-squamous cells using haemocytometer. The differential leukocyte count includes the proportion of macrophages, neutrophils, eosinophils, lymphocytes and columnar epithelial cells. Comparing to BAL, the sputum cell count display a greater number of granulocytes and a lower number of macrophages and lymphocytes due to a more central sampling of the airways[187].

Normal values in healthy adults for total cell count and differential cell count and cut-off points were reported[188]. A sputum eosinophil count greater than 2-3% is considered as abnormal. Age of the patients was proven to have an impact on the sputum neutrophil count and this highlight the importance of age matching in comparative studies[189].

Failure to get sputum cell count can be explained either by unsuccessful sputum induction or by poor quality sample (squamous cell fraction greater than 80%, cell viability < 50%). The success rate of sputum induction even in healthy subjects is usually ≥ 80%[190,191].

In asthma, sputum eosinophil count was proposed as a diagnostic marker and may help in deciding about treatment options because the presence of eosinophils (≥3%) in the airways predicts a good short-term response to inhaled corticosteroids. Furthermore, it is a critical tool to define asthma inflammatory phenotype[192].

In COPD, the IS is characterized by high number of neutrophils, which is correlated to disease severity and increase even more in case of exacerbation[193]. Mediators were also evaluated in the sputum supernatant of patients suffering from asthma and COPD and confirm a predominance of Th2 cytokines in asthma compared to Th1 profile in COPD.

In the context of LT, BAL neutrophilia and IL-8 may predate the spirometric criteria for BOS[194] (approximately 131 days before the BOS diagnosis based on FEV1 decrease) and increased concentrations of various cytokines in the BAL were demonstrated in BOS patients[195,196]. However, induced sputum was assumed to be the most relevant compartment to study the BO pathophysiology[197]. Indeed, IS was shown to be useful in the assessment of chronic lung rejection after lung transplantation[198]. In this study, stable LT patients displayed higher total sputum cell count and percentage of neutrophils compared to healthy subjects. Moreover, in patients with chronic rejection, there was an increase in sputum neutrophil percentage compared to stable patients. Another paper reported a negative correlation between the sputum neutrophil count and the percentage of change in FEV1 from baseline in double lung transplant recipients who developed BOS. The total cell count and the percentage of neutrophils were also correlated with the disease severity[199]. Others confirmed this result but found also a higher proportion of eosinophils and chemoattractant RANTES in IS compared to non-BOS patients. In contrast to BAL, the IL-8 level in IS was not a useful predictive marker of BOS development[200]. This highlights the fact that BAL and IS provide different and complementary results, each technique investigating distinct airway compartments.

IS has never been studied to assess the airway inflammation in patients undergoing HSCT. In this context, previous studies used rather BAL or bronchial biopsy analysis to assess the airway inflammation. A previous study has shown that BAL neutrophil and lymphocyte counts increased during the first 6 months after HSCT in patients without overt pulmonary complications[201]. Another group measured cytokines in BAL of transplanted

patients and showed an increased in TNF-α and IL-10 expression in BAL cells of patients with pulmonary complications after HSCT[202]. A high level of TNF-α expression was also correlated with poor post-tranplant outcome. However, the BAL procedure, although well tolerated in general, has to performed with caution in case of fragile patients with advanced disease, because it may precipitate an acute airflow obstruction or pneumothorax[106]. Moreover, trained personnel and emergency equipment are needed for BAL collection. Finally, it remains an invasive procedure that cannot be performed as often as necessary. IS may be performed more frequently with lower risks and costs.

PURPOSES OF THE STUDY

In the context of HSCT, few studies have analysed the usefulness of non-invasive techniques assessing airway inflammation such as FeNO value and none have investigated the potential role of induced sputum in airway inflammation monitoring of patients who underwent this type of transplantation.
The aim of this work was to characterize both the lung function status and the airway inflammatory profile of patients after HSCT using FeNO and induced sputum. In an attempt to better understand the underlying molecular mechanisms linked to cellular inflammation, we also measured soluble inflammatory mediators in the sputum supernatant from patients undergoing HSCT and compared them with patients suffering from COPD and healthy subjects.
In order to prevent GVHD in patients undergoing HSCT, some authors advocate mesenchymal stem cell co-infusion. However, some data from animal models show deleterious lung effects after systemic administration of mesenchymal stem cells. Therefore, another aim of our work was to investigate the functionnal impact and the infection risk in patients who underwent HSCT with mesenchymal stem cell co-infusion.

In the first part of this project, we have conducted a prospective observational study looking at pulmonary function indices and airway inflammation using sputum analysis and FeNO measurement in patients who underwent allogeneic HSCT.

We performed pulmonary function tests, measured FeNO value, and induced sputum to look at airway cell composition prior to HSCT and, 3 and 6 months, 1, 2 and 3 years after transplantation in 182 patients.

In the second part of the project, we have measured cytokines in sputum supernatants in patients who underwent HSCT and compared the results with those found in COPD patients and healthy volunteers.

Fourty patients undergoing HSCT were followed for one year and a set of cytokines reflecting Th1, Th2 and Th17 patterns were measured in sputum supernatant before HSCT and after 3, 6 and 12 months. These results were then compared with those from 40 COPD patients and 54 healthy subjects matched for age.

In the third part, we have investigated the impact of MSC co-infusion on pulmonary function after HSCT.

In this study, we monitored the evolution of lung function indices, FeNO and the occurrence of pulmonary infections and CMV reactivation in patients who underwent allogeneic unrelated HSCT with MSC co-infusion after non-myeloablative conditioning. These patients were compared with patients matched for conditioning regimen and the type of donor but who did not receive MSC co-infusion.

Part I

PUBLICATION

Lung function and airway inflammation monitoring after hematopoietic stem cell transplantation.

Moermans C, Poulet C, Henket M, Bonnet C, Willems E, Baron F, Beguin Y, Louis R.

Respir Med. 2013 Oct 16.

Respiratory Medicine (2013) 107, 2071–2080

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/rmed

Lung function and airway inflammation monitoring after hematopoietic stem cell transplantation

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Received 24 June 2013; accepted 8 October 2013
Available online 16 October 2013

KEYWORDS
Hematopoietic stem cell transplantation;
Lung function;
Sputum;
Exhaled nitric oxide

Summary
Background: Induced sputum is a non-invasive method to investigate airway inflammation, which has been used to assess pulmonary inflammatory diseases. However, this procedure has not been studied in the context of hematopoietic stem cell transplantation (HSCT).
Methods: We monitored lung function in 182 patients who underwent HSCT and measured airway inflammation by sputum induction in 80 of them. We prospectively measured FEV1, FVC, DLCO, KCO, TLC, RV, exhaled nitric oxide (FeNO) as well as sputum cell counts before and 3, 6, 12, 24 and 36 months after HSCT.
Results: For the whole cohort there was a progressive decrease in TLC, which was significant after 3 years ($p < 0.01$). By contrast, there was no change in other lung functions parameters or in FeNO. Baseline sputum analysis revealed increased neutrophil counts in patients {Median (IQR): 63% (38–79)} compared to healthy subjects matched for age {Median (IQR): 49% (17–67), $p < 0.001$} but there was no significant change in any type of sputum cell counts over the three years. When comparing myeloablative (MA) vs non-myeloablative (NMA) conditioning, falls in FEV1, FVC and DLCO, and rise in RV and sputum neutrophils were more pronounced over the first year of observation in those receiving MA.
Conclusions: There was a progressive loss in lung function after HSCT, featuring a restrictive pattern. Myeloablative conditioning was associated with early rise of sputum neutrophils

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0954-6111/$ - see front matter
http://dx.doi.org/10.1016/j.rmed.2013.10.013

and greater alteration in lung function over the first year.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) has been proposed to treat haematological malignancies since the 1960s. This type of transplantation requires the administration of high doses of chemotherapy and/or total body irradiation (TBI) with the aim of eradicating malignant cells [1]. In the late 1970, it has been recognized that the allograft itself confers immune-mediated antileukaemic effect termed graft-versus-tumour effects. This observation led to the development of allogeneic HSCT following non-myeloablative conditioning, in which the burden for tumour eradication has been shifted from high-dose radio/chemotherapy towards graft-versus-tumour effects [2].

Unfortunately, pulmonary complications are frequent after HSCT and are a major cause of post-transplant mortality [3]. They can be classified as infectious or non-infectious and as early or late depending on whether they occur before or after 100 days post-transplantation. Among late and non-infectious complications, bronchiolitis obliterans (BO) is characterized by a rapid and progressive decrease in expiratory flow rates and a rise in residual volume. It is admitted that less than 10% of patients develop BO after HSCT [4—7]. Follow-up of pulmonary function tests is critical in recognizing its early stages. The lung function assessment prior to transplantation usually serves as baseline reference to evaluate the changes in pulmonary function after HSCT [8].

Induced sputum is a safe method for recovering bronchial inflammatory cells. It was shown to be useful in the assessment of chronic lung rejection after lung transplantation [9] but it has never been studied in patients undergoing HSCT. A previous study has shown that bronchoalveolar lavage neutrophil counts increased during the first 6 months after HSCT in patients without overt pulmonary complications [10]. Similarly, fraction of exhaled nitric oxide (FeNO), a non-invasive marker of airway inflammation, has been used to monitor lung transplantation rejection [11] but its utility has been poorly assessed after HSCT.

In an attempt to detect modifications at airway level in patients who underwent allogeneic HSCT, we performed pulmonary function tests (PFTs), measured FeNO value, and induced sputum to look at airway cell composition prior to HSCT as well as 3 and 6 months, 1, 2 and 3 years after transplantation.

Material and methods

Subjects

We assessed 182 patients who underwent HSCT for haematological diseases at the University Hospital Center of Liege between January 2006 and October 2011, and who were reassessed 3 and 6 months, 1, 2 and 3 years later for their PFTs and FeNO measurement (Fig. 1). Over 1 year post-transplant, patients were essentially assessed if they had signs of Graft-Versus-Host-Disease (GVHD). The characteristics of these patients are presented in Table 1.

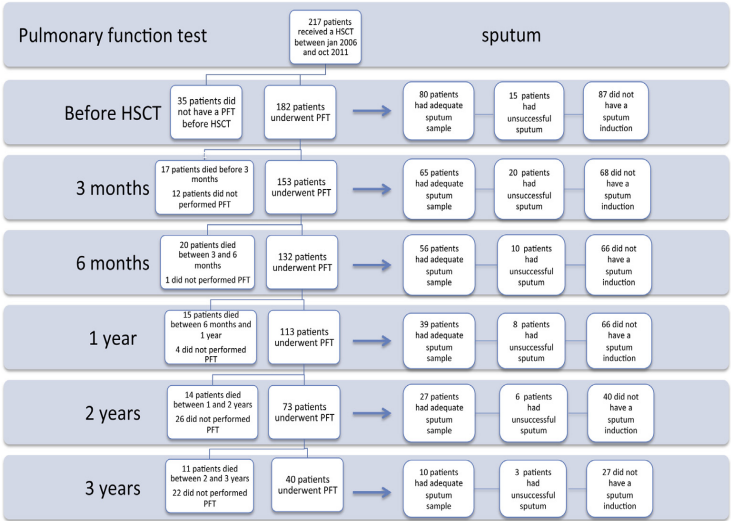

Figure 1 Flow chart of the study population PFT: pulmonary function test, HSCT: hematopoietic stem cell transplantation; unsuccessful sputum means too little material quantity or poor quality sample (squamous cell fraction > 80%) or sputum induction failure. PFT and Sputum were not performed in case of technical staff unavailability or patient poor health status.

Table 1 Demographic characteristics of the patients.

Age (years)	52 ± 13
BMI	25 ± 4
Gender (M/F)	108/74
Tobacco habits (n/ex/cs)	79/65/38
MA/NMA conditioning	49/133
Underlying malignancy	
AA	3
AIHA	1
AML	60
MPD	4
CML	3
MDS	20
ALL	9
HL	6
NHL	35
MM	29
CLL	10
PLL	2
Disease risk: low/standard/high	43/92/47
Donor: Unrelated/related	125/57
Patient/donor compatibility	
10/10 HLA-identical (allelic level)	80
Other	102
Comorbidities (HSCT-CI score)	2 (0—9)
No. of cells transplanted ($\times 10^6$/kg)	
$CD34^+$ cells	5.13 (0.04—15.88)
$CD3^+$ cells	318 (0—1216)

Results are expressed as mean ± SD except for HSCT-CI score and number of $CD3^+$ and $CD34^+$ transplanted expressed as Median (range); BMI = body mass index, *n* = non-smoker, ex = ex-smoker, cs = current smoker, MA = myeloablative conditioning, NMA = non-myeloablative conditioning, AA = aplastic anaemia, AIHA = autoimmune hemolytic anaemia, AML = acute myeloid leukaemia, MPD = myeloproliferative disease, CML = chronic myeloid leukaemia, MDS = myelodysplastic syndrome, ALL = acute lymphoblastic leukaemia, HL = Hodgkin's lymphoma, NHL = non-Hodgkin's lymphoma, MM = multiple myeloma,, CLL = chronic lymphocytic leukaemia, PLL = prolymphocytic leukaemia.

Forty-nine patients underwent a myeloablative-conditioning regimen (MA), with either 8 gray (Gy) single dose or 12 Gy fractionated TBI and high-dose chemotherapy ($n = 43$), or high-dose chemotherapy alone ($n = 6$). GVHD prevention consisted in cyclosporine A or tacrolimus with ($n = 28$) or without ($n = 21$) short methotrexate (15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6 and 11), with additional anti-thymocyte globulin (ATG; 45 mg/kg) in 22 of them. One hundred thirty-three patients underwent non-myeloablative conditioning (NMA). Two patients did not receive TBI but a chemotherapy associating fludarabine (90 mg/m^2) and cyclophosphamide (3000 mg/m^2). The others received a conditioning consisting in low-dose TBI (2 Gy) with ($n = 107$) or without ($n = 24$) fludarabine (90 mg/m^2). Their immunosuppressive regimen associated tacrolimus and mycophenolate mofetil (45 mg/kg from day 0 to day 28 in case of HLA-identical sibling donor or day 42 in case of alternative donor).

Healthy controls ($n = 116$) were recruited by local advertisement in the hospital. None of them exhibited respiratory symptoms and all had normal spirometric results (FEV1 > 80% predicted value) and none had airways hyperresponsiveness (provocative concentration of methacholine causing a fall in FEV1 of 20% > 16 mg/ml). They were well matched with patients undergoing HSCT according to age (53 ± 8 years) and tobacco habits (non-smokers: $n = 60$; ex-smokers: $n = 38$ and current smokers: $n = 18$).

This study was approved by the Ethics Committee of the Faculty of Medicine at the University of Liege and all subjects gave written informed consent for participation.

Methods

Lung function tests

Each subject underwent a global lung function assessment using a body box plethysmography (Sensormedics, Vmax series 22, Viasyhealthcare, Yorba Linda, California, USA) allowing to measure flow rates, lung volumes and diffusion capacity according to ATS/ERS standard criteria [12—14]. Spirometry (measure of Forced Expiratory Volume in 1 s: FEV_1 and Forced Vital Capacity: FVC) was performed before and after 400 μg inhaled salbutamol MDI administered through a Volumatic. Diffusion for carbon monoxide was measured by the single breath wash-out technique and corrected for the blood haemoglobin value. FeNO was measured using a chemoluminescence analyser (NIOX, Aerocrine, Stockholm, Sweden) at a flow rate of 50 ml/s, in accordance with the recommendation of the ATS/ERS task force [15].

Bronchiolitis obliterans syndrome

The international Society for Heart and Lung Transplantation (ISHLT) proposed a clinical description of BO termed *bronchiolitis obliterans syndrome (BOS)* and defined it by pulmonary function changes (fall of FEV1) rather than by histology [16]. In our series we assessed the proportion of patients who satisfied the criteria of BOS stage > 1 according to this classification (fall of FEV1 > 20% from baseline).

Sputum induction and processing

The sputum was induced on the same day after completion of lung function tests by inhalation of hypertonic (4.5%) or isotonic (0.9%) saline solution according to the post-bronchodilation FEV_1 value, as previously described [17]. Ninety-five patients had a sputum induction. Only 80 patients out of 95 produced an adequate sample suitable for cell count analysis (Fig. 1). Failure to get sputum cell count was explained either by unsuccessful sputum induction or by poor quality sample (squamous cell fraction greater than 80%). Cell viability was assessed by trypan blue exclusion and the differential leucocyte count performed on cytospins stained with May-Grünwald-Giemsa on 500 non-squamous cells.

Statistical analysis

Survival analyses were made using the Kaplan–Meier method. The assessment of the distribution normality was made with the Kolmogorov–Smirnov test. Lung function parameters were normally distributed, expressed as mean ± SD (tables) or ± SEM (figures) and compared to baseline using "*t*" tests while sputum cell counts and FeNO, which were not normally distributed, were expressed as median (IQR) and compared to baseline using Wilcoxon rank test or Mann–Whitney test for paired or unpaired data respectively. Bonferroni correction was applied to take into account multiple comparisons, so that only p value < 0.01 at each time point was considered as significant vs baseline. Statistical analyses were performed with Graph Pad Prism 5.0.

Results

Survival analysis

Overall survival at 1, 2 and 3 years after HSCT were 71%, 63% and 56% respectively (Fig. 2).

BMI

The body mass index (BMI) decreased significantly at day 100 compared to before HSCT (25 ± 4; 22 ± 4; $p < 0.0001$) and then begun to increase progressively to return to values close to baseline at 3 years (24 ± 5). Overall, the BMI remained within the normal range at each time point.

Lung function

Unpaired comparisons on the whole cohort shows that, compared to baseline, TLC decreased significantly after 3 years ($p < 0.01$) (Table 2). In contrast, there was no significant change in FEV1, FVC, FEV1/FVC, DLCO, KCO and FeNO over time compared to baseline, this latter remaining in accepted normal range [18]. For patients who provided paired data TLC and RV decreased over time (3 years vs baseline $p < 0.01$ for both) while there was no change in FEV1, FVC, FEV1/FVC, DLCO, KCO and FeNO (Fig. 3). Patients who underwent MA conditioning generally exhibited greater falls in FEV1, FVC and corrected DLCO compared to those receiving NMA conditioning (Fig. 3) (NMA vs MA: FEV1-D100, $p < 0.01$; FVC-D100, $p < 0.01$; D200: $p < 0.01$; DLCO: $p < 0.01$ for D100, D200 and 1 year; RV-D100, $p < 0.01$). Among the one hundred thirteen patients who were followed up for at least one year, only 4 developed BOS based on functional criteria (3.5%). Among them, only 3 had a consistent and persistent fall in lung function indices (2.6%) (Fig. 4).

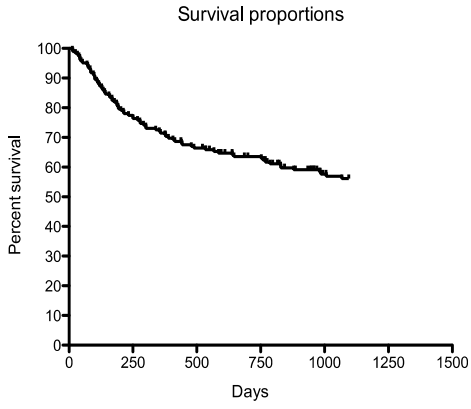

Figure 2 Survival analysis over 3 years of the 182 patients who underwent HSCT.

Sputum cell counts

Baseline sputum cells counts showed a dominant neutrophilic inflammation, which was greater than that seen in healthy subjects matched for age (Fig. 5, $p < 0.001$). Based on unpaired comparisons on the whole cohort, there was no change in total cell counts or in the percentages of different cell types over time (Table 3). Likewise, for patients who provided paired data, there was no significant change in total or in differential cell counts (Fig. 6). However, when examining the variations according to the conditioning regimen, those receiving MA conditioning showed a sharp and early rise in the percentage of neutrophils at 3 months compared to baseline ($P < 0.01$) an observation not found in those receiving NMA conditioning (Fig. 6). In addition, the increase in sputum neutrophils at 3 months was significantly greater after MA compared to NMA ($p < 0.001$) and mirrored by a greater fall in macrophages ($p < 0.01$).

There was no significant correlation between the change in lung function indices and the rise in sputum neutrophil count over the first 12 months even if there was a trend for an inverse correlation between the change in DLCO and the change in sputum neutrophil count at 3 months ($r = -0.31$; $p = 0.052$).

Predictive factors for survival at one year

There was no significant difference in baseline demographic and functional features between those who survive at one year and those who died in the first year of observation (Table 4). Likewise there was no difference with respect to sputum cells counts.

Discussion

The originality of our study is the combination of lung function assessment, including FeNO, with airway inflammation by measuring sputum cell counts in patients who underwent HSCT. Our main findings are the intense neutrophilic airway inflammation at baseline and the progressive appearance of a restrictive lung function defect associated with a persistent airway neutrophilic inflammation after HSCT. By contrast, occurrence of BOS was rare and only found in 3.5% of patients in our series.

Table 2 Lung function test results over time.

	Before HSCT $N = 182$	3 months $N = 153$	6 months $N = 132$	12 months $N = 113$	24 months $N = 73$	36 months $N = 40$
FEV1 pre-BD (% predicted)	89 ± 18	90 ± 18	91 ± 18	89 ± 20	87 ± 17	82 ± 21
FEV1 post-BD (% predicted)	88 ± 17	91 ± 18	91 ± 17	90 ± 22	86 ± 17	83 ± 19
FVC pre-BD (% predicted)	96 ± 17	96 ± 15	98 ± 16	96 ± 19	96 ± 15	91 ± 18
FVC post-BD (% predicted)	96 ± 15	96 ± 16	98 ± 16	97 ± 18	94 ± 17	91 ± 18
FEV1/FVC (%)	76 ± 10	77 ± 10	76 ± 11	76 ± 10	75 ± 10	74 ± 13
FEV1/FVC pots BD (%)	76 ± 10	79 ± 11	77 ± 12	76 ± 12	76 ± 10	76 ± 12
Corrected DLCO (% predicted)	74 ± 25	72 ± 27	72 ± 21	69 ± 19	67 ± 18	65 ± 17
Corrected KCO (% predicted)	90 ± 33	88 ± 28	91 ± 30	88 ± 27	88 ± 24	93 ± 29
TLC (% predicted)	96 ± 14	97 ± 15	96 ± 17	96 ± 16	94 ± 18	87 ± 20*
RV (% predicted)	104 ± 38	108 ± 44	103 ± 41	104 ± 41	100 ± 43	89 ± 35
FE_{NO} (ppb)	18 (12—29)	16 (12—27)	20 (15—35)	20 (12—28)	17 (11—22)	14 (11—23)

Results are expressed as mean ± SD except for FeNO value expressed as median (IQR). FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; DLCO: diffusing capacity of the lung for carbon monoxide; KCO: gas transfer coefficient; TLC: total lung capacity; RV: residual volume; BD: bronchodilation; FeNO: exhaled nitric oxide; * <0.01 vs before HSCT; unpaired comparisons.

At baseline our patients had flow rates, lung volumes and FeNO values within the normal range. By contrast, they showed a slight impairment of diffusing capacity that could reflect peripheral mismatch between ventilation and perfusion of the lung as a consequence of past chemotherapy and radiotherapy. As far as sputum cells are concerned, patients were characterised at baseline by a neutrophilic inflammation compared to figures usually seen in healthy subjects matched for age, although it was to a lesser extent than that seen in severe COPD [19—21]. Neutrophils are cells that easily and readily migrate into the lung in response to chemoattractants released upon airway irritation or injury [22,23]. In our patients, high sputum neutrophil counts at baseline are likely to reflect the impact of heavy treatment before HSCT. The persistence of intense neutrophilic airway inflammation after HSCT is remarkable and could indicate recurrent airways micro-injuries or bacterial colonisation in fragile patients because of their immune suppressive treatment. It also might be a sign of a repair process following aggressive therapy prior to the transplantation.

Our finding of a restrictive pattern that appears in the months and years following HSCT is in line with what most authors reported so far as highlighted in a review article [24]. However, we have to recognise that, in our study, the changes remained quite limited in their magnitude. The restrictive pattern was characterised by a reduced lung volume including TLC with preserved FEV1/FVC ratio. The overall decline in lung volume was progressive and maximal at 3 years. Those receiving NMA conditioning did not show an early decline in the first year, but displayed a similar loss in lung volumes at 3 years compared to those receiving MA conditioning. However, the diffusion capacity was minimally altered after NMA conditioning (5% fall) even after 3 years when compared to MA conditioning where the fall in DLCO was quite marked (exceeding 25% at this time point).

The reason for the appearance of the restrictive pattern in our study is unclear. The fact that most of our patients followed beyond the first year had a GVHD is likely to contribute to this process as GVHD was shown to favour lung restriction after HSCT though the underlying mechanism remains unclear [25]. As BMI remained quite stable we cannot incriminate a cachectic status to explain the loss of lung volumes. One could potentially think of skeletal muscle weakness or sequel of recurrent lung injuries likely to occur in these immuno-compromised patients. On the other hand thoracic irradiation and cytotoxic chemotherapeutic agents are recognized factors contributing to the occurrence of lung fibrosis that may lead to restriction [24]. This probably explains the marked alteration in flow rates and diffusing capacity observed in the first year after HSCT in those patients receiving myeloablative conditioning.

Only less than 5% of patients developed clinically significant BOS, which is in line with a large review on more than 6000 patients [26], but contrasts with what is generally observed after lung transplantation where it can occur in up to 50% of patients surviving beyond 6 months [27].

FeNO has been initially validated as a marker of airway eosinophilic inflammation in asthma [28,29] with thresholds values of 40—50 ppb being indicative of sputum eosinophilia >3%. By contrast, it has been recently shown that FeNO values remained within the normal range in non-eosinophilic asthma including neutrophilic asthma [30]. This exhaled biomarker has also been shown to rise early after lung transplantation complicated by BO [31] but the value of FeNO after HSCT has been poorly investigated so far. In our subjects FeNO values, which were within the normal range [18] at baseline (median 18 ppb), did not change significantly over time. This is not surprising as the airway inflammation in patients undergoing HSCT is rather neutrophilic, a feature that amplifies after transplantation. Our finding contradicts what was recently reported by Enocson A et al. [32] who followed 68 patients for 6 months after HSCT. However, in the latter study the baseline values of FeNO were similar to ours (median 15 ppb) and the change over time, although statistically significant, was of small magnitude with a median only reaching 20 ppb at 6 months. On the other hand, Lahzami et al. did not find any change in FeNO over a one-year

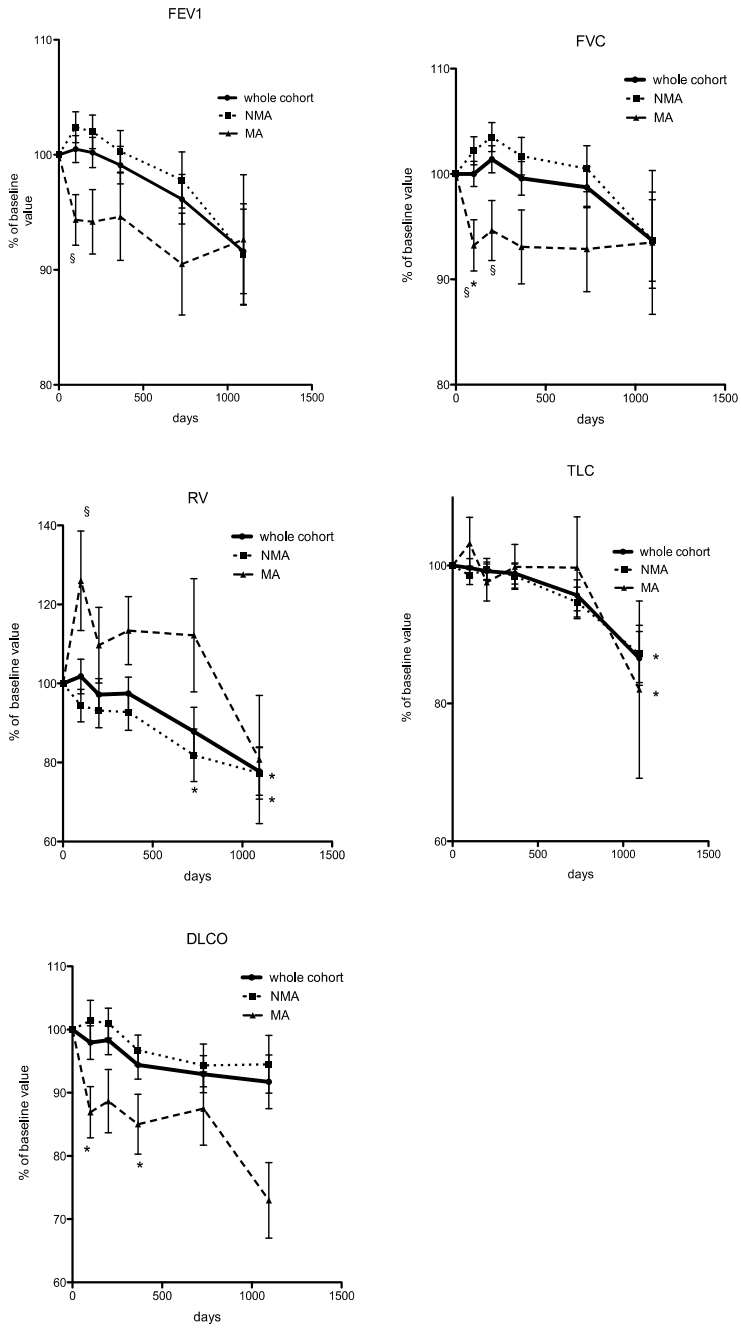

Figure 3 Changes of lung function indices over time in patients who provided paired data. Results are presented as % of baseline value ± SEM; 100% represent the baseline value. The number of patients at each time point is: 152, 133, 113, 70 and 40 for 3, 6, 12, 24 and 36 months, respectively. For NMA conditioning, the number of patients at each time point is 117, 101, 87, 54 and 32 for 3, 6, 12, 24 and 36 months, respectively. For MA conditioning, the number of patients at each time point is 35, 32, 26, 16 and 8 for 3, 6, 12, 24 and 36 months respectively. FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; TLC: total lung capacity; RV: residual volume; DLCO: diffusing capacity of the lung for carbon monoxide; NMA: non-myeloablative conditioning; MA: myeloablative conditioning. * < 0.01 vs before HSCT; paired comparisons. § < 0.01 vs NMA conditioning; unpaired comparisons.

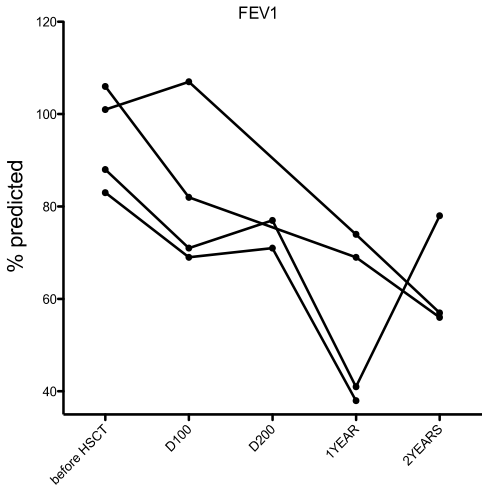

Figure 4 Individual changes in FEV1 over time in the 4 patients who had bronchiolitis obliterans.

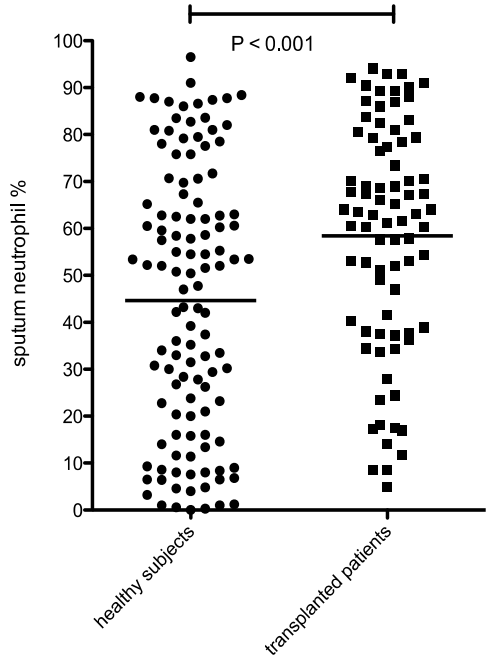

Figure 5 Sputum neutrophil percentage comparisons between patients before HSCT (n = 80) and healthy subjects (n = 116) matched for age and tobacco status. Unpaired comparisons.

follow-up after HSCT even if the patients showed signs of small airway dysfunction [33].

High sputum neutrophil counts have been linked to an irreversible airway obstruction in chronic obstructive pulmonary disease (COPD) [19] and BO following lung transplantation [34]. Neutrophilic airway inflammation is also seen in diseases with intense airway bacterial load such as bronchiectasis [35] and cystic fibrosis [36]. On the other hand, neutrophils in induced sputum were reported to be increased in lung transplant recipients even without BO [9]. To the best of our knowledge, we are the first to report on sputum cell count after HSCT. Even if baseline values of sputum neutrophil counts were already elevated prior to HSCT, we found a further rise in sputum neutrophils after HSCT in those receiving myeloablative conditioning. Recruitment of neutrophils in the airways is a major event in case of activation of innate immunity in response to bacterial and viral infections [37,38] and toxic or pollutant exposure [39]. The rise in neutrophils during the first year after MA conditioning may be related to the greater injury of respiratory mucosal system caused by the intense irradiation and chemotherapy. Even if not significant because of the limited number of subjects assessed at three years in our study, there was in our study, a trend for a new wave in sputum neutrophil increase by this time, whose underlying mechanism may differ from the first wave. Whether the rise in sputum neutrophils contributes to loss of lung function indices remains uncertain but neutrophils are a potent source of proteases endowed with remodelling capabilities. Our finding at the late time point needs certainly to be confirmed in a larger cohort as our patient number analysed at 3 years is limited.

None of the baseline functional and airway inflammation features had a prognostic value for survival at one year. This may seem discrepant from previous findings that

Table 3 Total and differential sputum cell counts over time.

	Before HSCT N = 80	3 months N = 65	6 months N = 56	12 months N = 39	24 months N = 27	36 months N = 10
Total cells (10^6/g)	0.73 (0.34—1.57)	0.94 (0.37—1.97)	0.55 (0.34—1.98)	0.73 (0.25—3.02)	1.17 (0.30—4.01)	2.91 (0.66—7.10)
Cell viability (%)	70 (58—80)	69 (57—84)	70 (53—80)	77 (66—86)	64 (54—83)	74 (65—87)
Macrophages (%)	27 (14—43)	27 (14—46)	27 (14—44)	23 (13—42)	16 (9—39)	15 (11—34)
Eosinophils (%)	0.0 (0.0—0.8)	0.0 (0.0—0.8)	0.0 (0.0—0.9)	0.0 (0.0—0.7)	0.4 (0.0—2.0)	0.0 (0.0—0.4)
Neutrophils (%)	63 (38—79)	67 (45—81)	65 (48—78)	65 (51—78)	72 (36—84)	74 (55—78)
Lymphocytes (%)	1.8 (0.6—4.4)	1.4 (0.2—4.0)	1.0 (0.5—3.0)	1.5 (0.2—5.0)	1.0 (0.5—2.2)	0.9 (0.4—2.0)
Epithelial cells (%)	2.3 (1.0—5.2)	2.7 (0.6—6.0)	2.0 (1.0—4.8)	1.8 (0.8—4.0)	2.7 (0.7—7.0)	2.7 (0.7—16.3)

Results are expressed as median (interquartile range); unpaired comparisons.

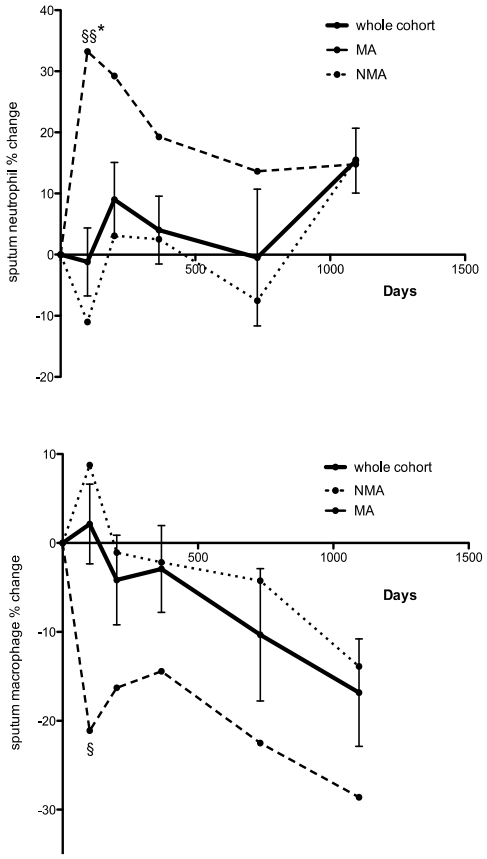

Figure 6 Sputum neutrophil and macrophage changes over time in patients who provided paired data. Results are presented as mean of % sputum neutrophils or macrophages changes ±SEM. For clarity reason, SEM were removed for NMA and MA conditioning lines; the number of patients at each time point is: 45, 35, 24, 12 and 5 for 3, 6, 12, 24 and 36 months, respectively. For NMA conditioning, the number of patients at each time point is 35, 25, 20, 8 and 4 for 3, 6, 12, 24 and 36 months, respectively. For MA conditioning, the number of patients at each time point is 10, 10, 4, 4 and 1 for 3, 6, 12, 24 and 36 months, respectively; NMA: non-myeloablative conditioning; MA: myeloablative conditioning. * < 0.01 vs before HSCT; paired comparisons. § < 0.01, §§ < 0.001 vs NMA conditioning; unpaired comparisons.

reported that low FEV1 and DLCO were associated with poorer prognosis [40,41]. However, we recognise that lung function impairment at baseline was rather mild in our cohort. On the other hand, survival after HSCT has dramatically improved over the last ten years [42] and the potential disadvantage of small lung function impairment in our series may have been compensated by the marked improvement in the management of the post-transplant period.

Table 4 Difference in baseline demographic and functional features between patients who survived at one year and those who died in the first year of observation.

Baseline parameter	Patients who died before 1 year; $N = 52$	Patients who did not die before 1 year; $N = 130$	P value
Age	54 ± 13	52 ± 13	0.30
BMI	24 ± 3	25 ± 5	0.52
NMA/MA conditioning	34/18	99/31	0.14
Gender: F/M	22/30	52/78	0.87
Tobacco habits (n/ex/cs)	22/20/10	57/45/28	0.87
FEV1 (% predicted)	87 ± 18	90 ± 17	0.19
FVC (% predicted)	94 ± 16	97 ± 16	0.33
FEV1/FVC	75 ± 10	76 ± 9	0.30
TLC (% predicted)	95 ± 16	97 ± 13	0.44
RV (% predicted)	103 ± 39	104 ± 37	0.94
DLCO (% predicted)	77 ± 28	72 ± 24	0.24
KCO (% predicted)	94 ± 35	88 ± 33	0.29
FeNO (ppb)	18 (14–35)	18 (12–25)	0.47
Total sputum cell number (10^6/g)	0.7 (0.4–1.4)	0.7 (0.3–1.5)	0.92
Neutrophils (%)	63 (38–82)	63 (38–75)	0.47
Macrophages (%)	26 (14–46)	28 (16–41)	0.56
Lymphocytes (%)	2.7 (1.0–4.8)	1.5 (0.5–3.8)	0.20
Eosinophils (%)	0.0 (0.0–0.6)	0.2 (0.0–1.3)	0.08
Epithelial cells (%)	2.5 (0.8–6.7)	2.2 (1.0–5.2)	0.99

Results are expressed as mean ± SD except for FeNO value, sputum cell count and cell percentages, which are expressed as median (IQR). BMI: body mass index, NMA: non-myeloablative conditioning, MA: myeloablative conditioning, n = non-smoker, ex = ex-smoker, cs = current smoker, FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; TLC: total lung capacity; RV: residual volume; DLCO: diffusing capacity of the lung for carbon monoxide; KCO: gas transfer coefficient; FeNO: exhaled nitric oxide.

Our study shows that patients who undergo HSCT display mild progressive loss of total lung capacity associated with sustained airway neutrophilic inflammation. By contrast bronchiolitis obliterans syndrome rarely occurs after HSCT.

Conflict of interest statement

All authors have declared the absence of potential conflict of interest related to this manuscript.

Acknowledgements

The authors would like to thank the lung function department of the CHU of Liege for their help in the collection of the data. The study was financially supported by TELEVIE (Grant F.N.R.S. 7.4.642.09.F) associated with the Fonds National de la Recherche Scientifique (FNRS) Belgium, PAI 6/35 and Fonds Leon Fredericq.

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Part II

PUBLICATION

Sputum cytokines levels in patients undergoing hematopoietic stem cell transplantation (HSCT): comparison with COPD.
Moermans C, Bonnet C, Willems E, Baron F, Nguyen M, Henket M, Sele J, Corhay JL, Beguin Y, Louis R.
Submitted in BMC Pulmonary Medicine

Abstract

Background: Patients undergoing hematopoietic stem cell transplantation (HSCT) display an airway neutrophilic inflammation before the transplantation that persists over the years. In this study, we have investigated the cytokine profile in sputum supernatant of patients who underwent HSCT over a period of one year.

Methods: We have measured sputum supernatant levels of TNF-α, TGF-β1, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, and IFN-γ in 40 HSCT patients by the multiplex technology and compared the results with those found in 40 COPD and 54 healthy subjects matched for age.

Results: Compared to healthy subjects, before the transplantation, HSCT patients exhibited raised levels of IL-6 ($p<0.01$) and IL-8 ($p<0.05$) while the other cytokines were generally poorly detectable. This picture was rather similar to what is seen in COPD even if cytokine levels were much greater in the latter with IL-8 being significantly greater in COPD than in HSCT patients ($p<0.0001$). In the 12 months following the transplantation, sputum IL-6 and IL-8 did not differ any longer compared to healthy subjects. However TGF-β1, although overall poorly detectable, was more often detected one year after HSCT (27%) than in healthy subjects (1%) and COPD (0%). Overall in HSCT patients, sputum IL-8 and IL-6 correlated with sputum neutrophil counts ($r=0.36$; $p<0.0001$, $r=0.31$; $p<0.01$, respectively). Baseline sputum levels of IL-6 and IL-8 were not different in those who died over the first year (5/40) compared to those who survived.

Conclusion: IL-6 and IL-8 may play a role in neutrophilic airway inflammation seen in patients undergoing HSCT.

Key words: HSCT- sputum-neutrophil- cytokines-IL-6-IL-8

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is now widely used to treat a lot of hematologic malignancies. Unfortunately, pulmonary complications are frequent after this type of transplantation and are a major cause of post-transplant mortality[88].
In a previous paper, we described the lung function status and the airway inflammatory profile of patients after HSCT using FeNO and induced sputum[203]. The principal observation was a progressive appearance of a restrictive lung function defect associated with a persistent airway neutrophilic inflammation after HSCT.
While high sputum neutrophil counts have been linked to an irreversible airway obstruction such as that in chronic obstructive pulmonary disease[204] (COPD), no airway obstruction was seen in patients who underwent HSCT.
In an attempt to better understand the underlying molecular mechanisms linked to cellular inflammation, we measured soluble inflammatory mediators in the sputum supernatant from patients undergoing HSCT and compared the results with those obtained in COPD and healthy subjects.
Our previous study based on the sputum and blood cell culture model highlighted the Th1 profile in the airway and blood compartment of COPD patients with raised levels of IFN-γ[205].
In this study, we undertook a comprehensive sputum cytokine analysis looking at Th1, Th2 and Th17 cytokines in sputum supernatant. Additionnally, we also measured pro-inflammatory (TNF-α, IL-6) and anti-inflammatory (IL-10) cytokines together with TGF-β1, a recognized factor involved in airway and lung remodeling.

Material and methods

Patients

Between January 2006 and October 2011, we assessed 40 patients who underwent HSCT for h63aematological diseases at the University Hospital center of Liege and who were reassessed 3, 6 and 12 months later for their PFT and FeNO measurement. The characteristics of these patients are presented in table 1. The diagnoses were: Hodgkin's lymphoma (n=3), non-Hodgkin's lymphoma (n=11), acute lymphoblastic leukaemia (n=1), chronic lymphocytic leukaemia (n=4), acute myeloid leukaemia (n=12), chronic myeloid leukaemia (n=1), myelodysplastic syndrome (n=3), multiple myeloma (n=5). The stem cells were obtained from a related donor in 11 patients and from an unrelated donor in 29. The diagnosis of acute GVHD was based on clinical findings according to commonly accepted diagnostic criteria[206]. It was confirmed by

biopsy and was categorized as present (grade 2-4) or absent (grade 0-1), as previously described[24,207]. Eleven patients developed grade 2-4 acute GVHD and were treated with methylprednisolone. CMV infection was monitored weekly and if CMV PCR tests became positive (n=17), patients were treated with ganciclovir.

Eight patients underwent a myeloablative conditioning regimen with either 8 Gy single dose or 12 Gy fractionated TBI and high-dose chemotherapy. GVHD prevention consisted in cyclosporine A or tacrolimus with (n=4) or without (n=4) short methotrexate (15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6 and 11. Thirty-two patients underwent nonmyeloablative conditioning consisting in low-dose TBI (2 Gy) with (n=26) or without (n=6) fludarabine (90 mg/m^2). Their immunosuppressive regimen associated tacrolimus and mycophenolate mofetil (45 mg/kg from day 0 to day 28 in case of HLA-identical sibling donor or day 42 in case of alternative donor).

Healthy controls (n=54) were recruited by local advertisement in the hospital. None of them exhibited respiratory symptoms and all had normal spirometric results (FEV1 > 80% predicted value) and none had airways hyperresponsiveness (provocative concentration of methacholine causing a fall in FEV1 of 20 % > 16 mg/ml). They were well matched with patients undergoing HSCT according to age, gender and tobacco habits.

Fourty COPD patients were recruited through the outpatient clinic and pulmonary rehabilitation center (University Hospital Center, Sart-Tilman, Liege). All the COPD patients fulfilled the criteria proposed by the Global Initiative for Chronic Obstructive Lung Disease (GOLD)[208] : FEV1/FVC ratio less than 0.7 measured 20 minutes after the inhalation of 400 µg of salbutamol. COPD patients were in stable condition at the time of sputum collection and no patients were studied within 8 weeks of having an upper tract infection or any exacerbations requiring change in maintenance treatment or oral steroid and antibiotic prescription. To avoid confounding factors influencing systemic inflammation, patients with significant comorbidities (such as diabetes mellitus or cancer) were excluded. Patients were matched to age and gender with patients undergoing HSCT.

This study was approved by the Ethics Committee of the Faculty of medicine at Liege University and all subjects gave written informed consent for participation.

Sputum induction and processing

The sputum was induced on the same day after completion of lung function tests by inhalation of hypertonic (4.5%) or isotonic (0.9%) saline solution according to the post-bronchodilation FEV_1 value, as previously described[184]. The whole sputum was collected in a plastic container, weighed and homogenized by adding three volumes of PBS, vortexed for 30 sec and centrifuged at 2000 rpm for 10 min at 4°C. Supernatant was separated from cell pellet and stored at -80°C. The cells were resuspended in a solution containing 5 mM dithiothreitol (DTT) without Ca^{++} and Mg^{++} and gently shaked for 20 min at room temperature. The cell suspension was then centrifuged again at 1600 rpm at 4°C for 10 min and the cell count was performed using a manual haemocytometer. Cell viability was assessed by trypan blue exclusion and the differential leukocyte count performed on cytospins stained with May-Grünwald-Giemsa on 500 non-squamous cells.

Cytokines

The concentrations of TNF-α, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17 and IFN-γ were assessed by ELISA multiplex using Fluorokine® Multianalyte Profiling (MAP) Kits (R and D systems, Minneapolis, USA) according to the manufacturer's instructions. The concentration of TGF-β1 was measured separately by ELISA (DuoSet, R and D systems, Minneapolis, USA). Before assay, TGF-β1 was activated by acidification of the samples as recommended by the manufacturer. Detection limits were 3.0, 10.0, 4.0, 1.0, 5.0, 1.0, 2.0, 1.5, 5.0 and 2.0 pg/ml for TNF-α, TGF-β1, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, and IFN-γ respectively. Spiking experiments of cytokines in sputum supernatants showed that recovery were 123%, 126%, 100%, 119%, 81%, 145%, 94%, 105%, 110% and 143% for TNF-α, TGF-β1, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, and IFN-γ respectively.

Statistical analysis

Results are expressed as mean ± SD or median (interquartile range) according to the distribution of the data. Intergroup comparisons were made using Kruskal Wallis test followed by Mann Withney test in case of significance. Comparisons between values before and after HSCT were performed using Wilcoxon matched-pairs signed rank test for sputum cell counts and cytokines levels. Correlations between variables were assessed using the Spearman rank test. Differences were considered statistically significant when a two-sided p-value was < 0.05.

Results

a) Baseline comparison between HSCT, healthy and COPD

As expected, COPD patients were characterized by greater total sputum cell counts and greater proportion of neutrophils and eosinophils compared to both healthy subjects and transplanted patients while patients who underwent HSCT were intermediate between healthy subjects and COPD (Table 2). Interestingly, both percentage and absolute epithelial cell counts were lower in patients undergoing HSCT compared to healthy subjects.

Results regarding cytokines are given in table 3. Baseline levels of IL-6 in the sputum supernatant of patients undergoing HSCT and COPD patients were greater compared to healthy subjects ($p<0.01$ and $p<0.0001$ respectively) (Figure 1). Baseline levels of IL-8 were higher in COPD compared to healthy subjects and HSCT patients ($p<0.0001$ for both) and higher in patients with HSCT compared to healthy subjects ($p<0.05$) (Figure 2). The levels of IL-4, IL-10, IL-13, IL-17, TNF-a and IFN-γ were undetectable in the large majority of patients and no difference was seen between the groups. TGF-β1 was more oten detected in the group of HSCT at one year than in healthy subjects ($p<0.01$) or COPD ($p<0.001$). IL-5 was more often detectable in COPD compared to healthy ($p<0.01$)(Table 3).

b) Longitudinal assessment of cytokines in HSCT

Among the 40 HSCT patients recruited at baseline for cytokines measurement, only fifteen produced adequate sputum samples at each visit until 1 year. This was due to the patient's death or unsuccessful sputum in case of insufficient quantity or poor quality of the sample (squamous cell fraction > 80%) or sputum induction failure. Moreover, sputum induction was not performed in case of technical staff unavailability or patient poor health status.

There was no significant change in sputum cell counts over the 1-year follow-up with persistence of a high sputum neutrophil count (data not shown, in agreement with the results our whole cohort[203]).

With respect to cytokines, we noted a significant decrease of IL-6 levels 3 months after HSCT compared to baseline {n=36; from 50 pg/ml (25-89) to 32 pg/ml (10-61); $p<0.01$)} but no difference was noted at 6 {n=17; from 57 pg/ml (31-65) to 28 pg/ml (6-68); $p=0.55$) and 12 months {n=15; from 41 pg/ml (13-71) to 44 pg/ml (13-102); $p=0.84$} (Figure 3). There was no significant change in IL-8 levels over time even if there was a trend for a reduced level at 3 months compared to baseline.

c) Relationship between sputum cytokines and sputum cell counts and lung functions.

When pooling all time points, IL-8 was positively correlated with the sputum neutrophil (n=122, r=0.36, p<0.0001) (Figure 4) and eosinophil absolute numbers (n= 122, r=0.35, p<0.0001) and negatively with FEV1 % predicted (n= 122, r=-0.32, p< 0.001) and FEV1/FVC ratio (n= 122, r=-0.35, p<0.0001) and FeNO (n=95, r=-0.34, p<0.01). As for IL-6, the cytokine was correlated with absolute macrophage (n=122, r=0.29, p<0.01) and neutrophil numbers (n=122, r= 0.31, p<0.01) and negatively with FeNO (n= 95, r=-0.32, p< 0.01).

d) Prognostic values of baseline sputum cytokines

Five patients out of 40 died over the 1-year period. The level of IL-6 was 57 pg/ml (27-279) in these patients vs 50 pg/ml (21-96) in the survivors (p=0.44). The level of IL-8 was 7124 pg/ml (1853-14278) in those who died vs 2039 pg/ml (942-3657) in the survivors (p=0.14).

Discussion

Our work shows, for the first time, that patients undergoing HSCT display raised sputum levels of IL-6 and IL-8 compared to healthy subjects matched for age though the levels remained lower than those found in COPD. The other cytokines tested including pro-inflammatory or pro-fibrotic cytokines as well as Th1 and Th2 remained undetectable in the majority of patients.

To the best of our knowledge, cytokines have not been measured in sputum from patients undergoing HSCT so far. IL-6 is a complex cytokine involved in many immunological pathways. It is released in acute inflammation but plays also a role in chronic inflammatory process[209]. The cytokine is mainly produced by mononuclear phagocytic cells and the correlation we found between sputum IL-6 and sputum macrophages is in keeping with this cell source being important in patients undergoing HSCT. The irradiation and the chemotherapy prior to HSCT may be involved in the release of IL-6 into the airways of patients as it was demonstrated in animal models[210]. In our study, the increase in IL-6 is slightly but not significantly lower than that seen in COPD, another neutrophilic airway disease, which was previously found to exhibit raised sputum IL-6[211-213]. IL-6 is thought to be pivotal in inducing IL-17 mediated neutrophilic inflammation[214]. In our series IL-17 was poorly detectable in all our group of patients despite satisfactory recovery in spiking experiments. Of interest, however, is the relationship we found between sputum IL-6 and sputum neutrophil counts which may confirm the link between IL-6 and neutrophilic inflammation. Our paired analysis of samples collected over one year also suggest an early decrease in sputum IL-6 levels 3 months after HSCT though this is not confirmed at further time points. The reduction in the cytokine level might be linked to starting immunosuppressive treatment like cyclosporine and tacrolimus, which can downregulate IL-6 expression[215]. Why

this decrease is not confirmed at 6 months and 1 year is unclear but this might be related to a loss of statistical power as a result of a lower number samples as the study went by. On the other hand, the patients for whom we obtained samples at 3 months but not at 6 and 12 were those who showed the sharpest drop in IL-6 at three months (n=19, from 54 (23-117) to 24 pg/ml (10-56) $p<0.01$).

IL-8 is a key chemoattractant for neutrophils and its sputum level was repeatedly shown to be increased in a series of neutrophilic airway diseases such as COPD, cystic fibrosis and bronchiectasis[213,216,217]. Our study extends this series by showing raised sputum IL-8 in patients undergoing HSCT although the levels remained significantly lower than those found in COPD. The role of IL-8 in recruiting sputum neutrophils in HSCT patients was further supported by the clear correlation found between sputum neutrophil counts and IL-8 levels over the 1 year period of observation.

Bronchial epithelial cells are known to contribute to local release of IL-6 and IL-8 upon activation. In our series of HSCT patients, the bronchial epithelial cell count appeared to be lower than that seen in healthy subjects. We can speculate that irradiation and chemotherapy are likely to cause injury and structural alteration of the epithelial layer thereby promoting release of cytokines involved in inflammatory cell trafficking in the airways.

Of note is the fact that, although higher before the transplantation, sputum levels of IL-6 and IL-8 in the the 12 months following HSCT did not strikingly differ from those seen in healthy subjects, an observation that might relate to the strong immunosuppressive treatment received by the patients.

We were wondering whether the baseline cytokine levels could have any prognosis value for the survival. The death rate at one year was 12.5% (5 out 40) and actually rather low in the series of patients studied here compared to the whole cohort in which mortality at one year was 29%. Consequently, it was unlikely to find any statistical difference in cytokine levels at baseline between those who died vs those who survived even if there was trend for higher IL-8 in those who died.

Other cytokines tested, including IL-4, IL-5, IL-10, IL-13, TNF-a, TGF-β1 and IFN-γ were poorly detectable. As aforementioned for IL-17, the poor detection cannot be accounted for by the use of a mucolytic agent that would have disturbed the immunoassay as our samples were only homogenised by diluting them in phosphate buffered saline. Furthermore, our spiking experiments show adequate recovery of all cytokines. One explanation for the poor detection may be linked to the fact that we used whole sputum instead of the plug thereby diluting somewhat the mediator concentration found in the supernatant[218,219].

Although generally poorly detectable, TGF-β1 was more frequently detected at one year, but not at baseline, in HSCT patients (27%) compared to healthy (2%) and COPD (0%). This finding could be of pathophysiological interest for airway tissue remodelling that may appear after transplantation but should be confirmed in a larger population. Similarly, IL-5 was more often detectable in

COPD (15%) than in healthy subjects (0%), a finding in agreement with the observation that some COPD may display airway eosinophilia[220,221].
There are other models looking at cytokines in the airway by using sputum sample. We previously showed that measuring cytokine after 24h sputum cell culture could provide biological signals distinguishing asthmatics[222] and COPD[205] from healthy subjects. In this model, it is noteworthy that COPD were shown to release more IFN-γ but less IL-6 than healthy subjects. Therefore, the results with IL-6 in COPD are highly discrepant depending on the model chosen. The difference could be explained by the fact that cellular sources that contribute to the supernatant content are more varied than those contributing to supernatant from the sputum cell culture model. Supernatant content not only depends on the secretion from airway cells lying within the lumen but also from resident structural cells and in particular from the epithelial layer, which is not strongly represented in the sputum cell culture. This highlights the complexity of the system and the caution needed before drawing definitive pathophysiological conclusion from cytokine measurement in the airway.
We can conclude that patients undergoing HST exhibit a neutrophilic airway inflammation associated with raised sputum levels of IL-6 and IL-8, a picture similar but developed to a lesser extent of what is seen in COPD.

Table 1: Demographic and functional characteristics of patients and healthy subjects

Parameters	**HSCT patients N= 40**	**Healthy subjects N= 54**	**COPD patients N= 40**
Age (years)	51 ± 12	51 ± 11	60 ± 10
BMI	24.6 ± 3.9	25.3 ± 5.9	26.0 ± 5.2
Gender (F/M)	16/24	25/29	12/28
Smokers/ Ex smokers/Non-smokers	10/15/15	9/15/30	8/32/0
FEV_1 pre-BD (% pred)	91 ± 15	104 ± 15	46 ± 16
FVC pre-BD (% pred)	99 ± 15	109 ± 17	72 ± 25
FEV_1/FVC pre-BD (%)	76 ± 8	79 ± 4	52 ± 14
FeNO (ppb)	16 (13-28)	18 (14-24)	24 (15-32)
Corrected DLCO (% pred)	74 ± 25	ND	ND
Corrected KCO (% pred)	88 ± 36	ND	ND
TLC (% pred)	97 ± 12	ND	ND
RV (% pred)	104 ± 30	ND	ND

Results are expressed as mean±SD except for exhaled nitric oxide (FeNO) expressed as median (IQR); BMI: body mass index; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; DLCO: the carbon monoxide diffusing capacity; KCO: the carbon monoxide transfer coefficient; TLC: total lung capacity; RV: residual volume; pred: predicted.

Table 2: Sputum total and differential cell counts in patients and healthy subjects

Cells	Pre-HSCT N= 40	D100 N=39	D200 N=28	1 YEAR N=18	Healthy subjects N=54	COPD patients N=40
Total cells (10^6/g)	0.82$^{\$\$\$}$ (0.30-1.59)	0.78 (0.42-2.21)	0.70 (0.38-1.84)	0.91 (0.42-2.14)	0.71 (0.39-1.51)	*** 2.73 (1.23-9.19)
Cell viability (%)	70 (58-82)	71 (60-85)	68 (50-87)	78 (64-84)	65 (55-81)	79 (56-87)
Macrophages (%)	30$^{\$\$\$}$ (21-43)	20 (11-51)	28 (20-44)	23 (13-37)	35 (20-57)	*** 12 (5-26)
Eosinophils (%)	0.0 (0.0-0.6)	0.0 (0.0-0.7)	0.1 (0.0-1.3)	0.0 (0.0-0.1)	0.0 (0.0-0.4)	** 0.4 (0.0-2.7)
Neutrophils (%)	61$^{\$}$ (37-71)	73 (41-84)	63 (46-74)	65 (54-78)	52 (19-68)	*** 77 (55-93)
Lymphocytes (%)	1.3 (0.4-3.8)	1.2 (0.2-2.3)	1.7 (0.1-3.7)	1.5 (0.1-2.3)	1.5 (0.5-2.8)	0.7 (0.0-1.9)
Epithelial cells (%)	1.9* (0.9-5.4)	1.5 (0.4-4.2)	2.2 (1.0-6.2)	2.0 (0.9-4.8)	5.5 (2.7-13.5)	2.4 (0.4-8.9)
Macrophages 10^3 /g	178 (78-491)	281 (96-547)	221 (111-399)	185 (99-459)	221 (85-472)	252 (102-749)
Eosinophils 10^3/g	0$^{\$\$}$ (0-0)	0 (0-1)	0 (0-0)	0 (0-0)	0 (0-3)	** 10 (0-194)
Neutrophils 10^3/g	300$^{\$\$\$}$ (197-815)	466 (230-1393)	484 (156-892)	606 (179-1504)	276 (78-679)	*** 2346 (646-8268)
Lymphocytes 10^3/g	11 (2-39)	6 (1-48)	11 (1-34)	8 (1-25)	8 (2-29)	15 (0-30)
Epithelial cells 10^3/g	15$^{*\$}$ (3-43)	18 (4-55)	17 (9-50)	16 (8-58)	29 (14-108)	59 (17-180)

Unpaired comparisons, results are expressed as median (IQR).
* < 0.05; ** < 0.01; *** < 0.001 vs healthy subjects
$ < 0.05; $$ < 0.01; $$$ < 0.0001 vs COPD patients

Table 3: Sputum cytokine concentrations in patients and healthy subjects.

Cytokine	Pre- HSCT N=40	D100 N=39	D200 N=28	1 YEAR N=15	Healthy subjects N=54	COPD patients N=40
IL-4 (pg/ml)	0 (0-0) D= 0/40	0 (0-0) D= 0/39	0 (0-0) D= 1/28	0 (0-0) D= 0/15	0 (0-0) D=0/54	0 (0-0) D= 0/40
IL-5 (pg/ml)	0 (0-0) D= 2/40	(0-0) D= 1/39	0 (0-0) D= 2/28	0 (0-0) D= 0/15	0 (0-0) D=0/54	0 (0-0) D= 6/40**
IL-6 (pg/ml)	** 54 (23-110)	35 (10-63)	30 (11-94)	39 (17-87)	25 (13-40)	*** 90 (38-168)
IL-8 (pg/ml)	*$$$ 2117 (962-6360)	2048 (840-4235)	1353 (720-2461)	2048 (900-8249)	1496 (806-2150)	*** 10361 (3133-18629)
IL-10 (pg/ml)	0 (0-0) D= 3/40	0 (0-0) D= 2/39	0 (0-0) D= 0/28	0 (0-0) D= 0/15	0 (0-0) D= 3/54	3 (0-5) D= 7/40
IL-13 (pg/ml)	0 (0-0) D= 0/40	0 (0-0) D= 1/39	0 (0-0) D= 0/28	0 (0-0) D= 0/15	0 (0-0) D= 0/54	0 (0-0) D= 1/40
IL-17 (pg/ml)	0 (0-0) D= 2/40	0 (0-0) D= 0/39	0 (0-0) D= 0/28	0 (0-0) D= 0/15	0 (0-0) D= 1/54	0 (0-0) D= 1/40
IFN-γ (pg/ml)	0 (0-0) D= 3/40	0 (0-0) D= 0/39	0 (0-0) D= 0/28	0 (0-0) D= 0/15	0 (0-0) D= 0/54	0 (0-0) D= 1/40
TNF-α (pg/ml)	0 (0-6) D= 11/40	0 (0-0) D= 7/39	0 (0-0) D= 2/28	0 (0-0) D= 1/15	0 (0-0) D= 10/54	0 (0-19) D= 12/40
TGF-β1 (pg/ml)	0 (0-0) D= 1/40	0 (0-0) D= 1/39	0 (0-0) D= 1/28	0 (0-34) D= 4/15**$$$	0 (0-0) D= 1/54	0 (0-0) D= 0/40

Unpaired comparisons, results are expressed as median (IQR). D: detectable
Kruskal-Wallis test for IL-6 gave a p value of 0.0001 and Kruskal-Wallis test for IL-8 gave a p value < 0.0001. Then, Mann Withney tests were performed.
* < 0.05; ** < 0.01; *** < 0.001 vs healthy subjects
$ < 0.05; $$ < 0.01; $$$ < 0.0001 vs COPD patients

Figure 1: Sputum IL-6 concentrations comparison between HSCT, healthy and COPD

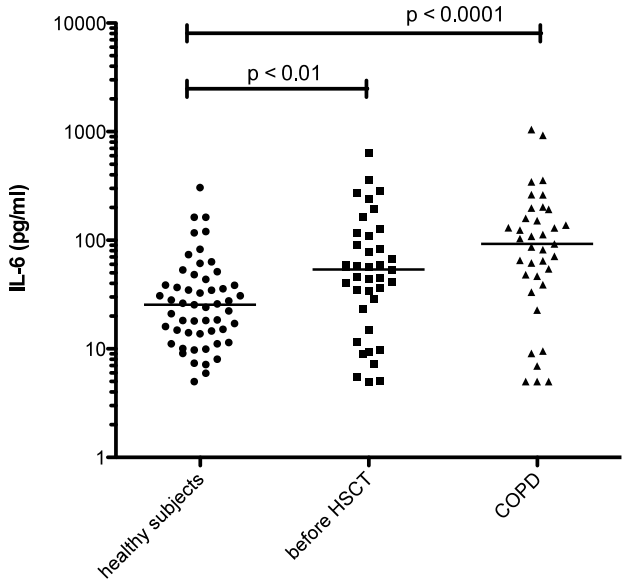


Figure 2: Sputum IL-8 concentrations comparison between HSCT, healthy and COPD.

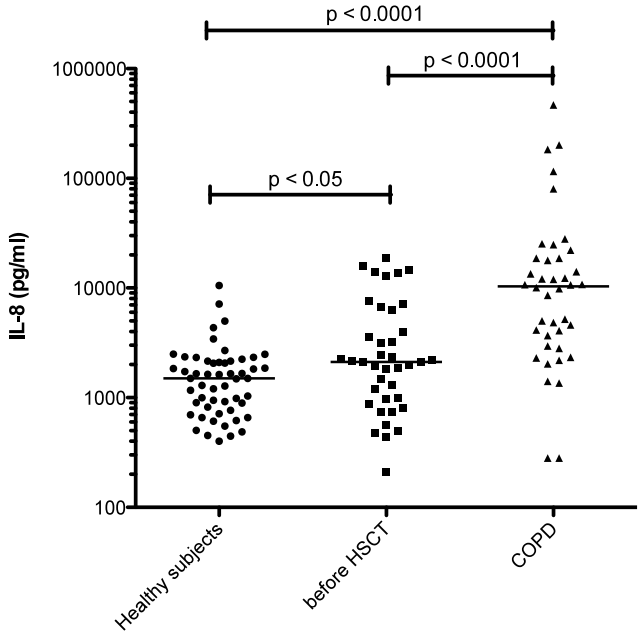


Figure 3: Sputum IL-6 concentration evolution in tranplanted patients.

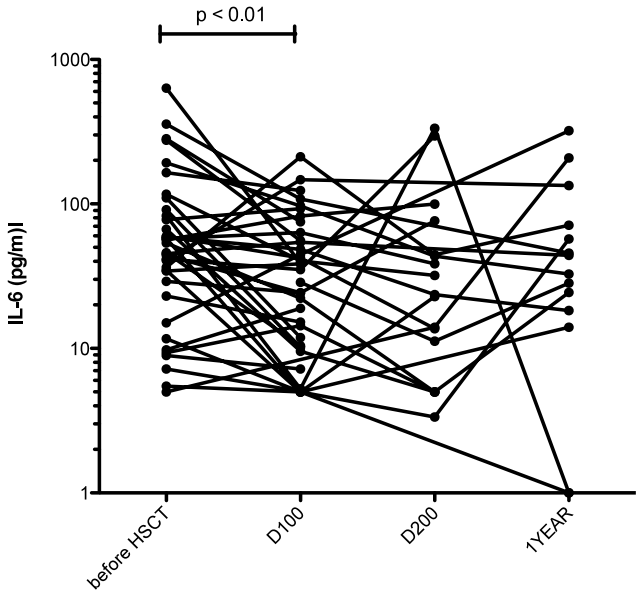
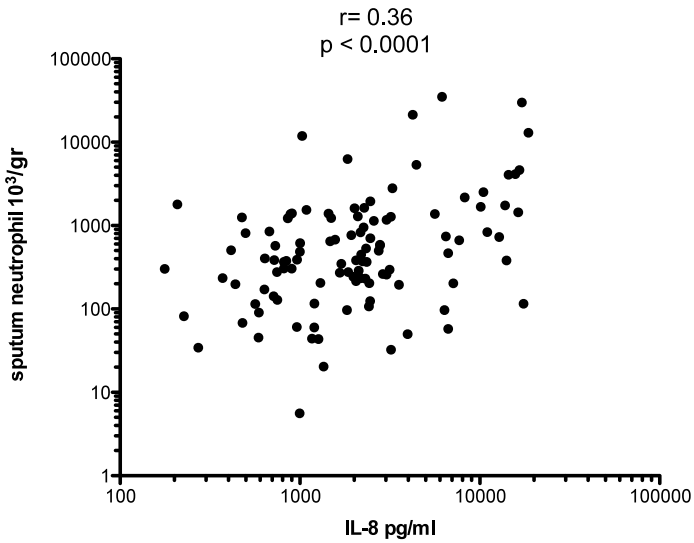


Figure 4: Correlation between sputum IL-8 concentration and sputum neutrophil counts in transplanted patients. The graph includes all sputum samples collected over the one-year period (n=122).



Part III

PUBLICATION

Impact of co-transplantation of mesenchymal stem cells on lung function after unrelated allogeneic hematopoietic stem cell transplantation following non-myeloablative conditioning.

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Transplantation, submitted October 2013.
Allowed to submit a revised manuscript (dec. 2013)

Footnotes : **Footnote to *The Title*** : author's contributions

Moermans Catherine [1]: wrote the manuscript, performed the research and participated in data analysis; was supported by: Grant F.N.R.S. 7.4.642.09.F) associated with the Fonds National de la Recherche Scientifique (FNRS) Belgium, PAI 6/35 and Fonds Léon Frédéricq

Lechanteur Chantal [2]: participated in the procurement of bone marrow from MSC donors, supervised the preparation of MSC products and ensured quality management of MSC production.

Baudoux Etienne [2]: collected bone marrow from MSC donors and participated in the preparation of MSC products and ensured quality management of MSC production.

Giet Olivier [2]: participated in the preparation of MSC products and ensured quality management of MSC production.

Henket Monique [1]: participated in data analysis

Seidel Laurence [3]: performed statistical analysis

Lejeune Marie [4]: participated in patient care and collected clinical data.

Baron Frederic [4]: participated in patient care, collected clinical data and participated in the writing of the manuscript.

Willems Evelyne [4]: participated in patient care, collected clinical data and participated in the writing of the manuscript.

Louis Renaud [1]: participated in the writing of the manuscript.

Beguin Yves [2,4]: designed the study, participated in patient care and co-wrote the manuscript.

All co-authors have approved the work and have declared the absence of potential conflict of interest related to this manuscript.

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Abbreviations

aGVHD	Acute Graft-Versus-Host-Disease
cGVHD	Chronic Graft-Versus-Host-Disease
CMV	Cytomegalovirus
DLCO	Diffusion for carbon monoxide
FeNO	Exhaled nitric oxide
FEV1	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
GVHD	Graft-Versus-Host-Disease
HSCT	Hematopoietic stem cell transplantation
KCO	Transfer coefficient
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
MSC	Mesenchymal stem cells
NMA	Non-myeloablative conditioning
PBSC	Peripheral blood stem cells
PFT	Pulmonary function tests
RV	Residual volume
TBI	Total body irradiation
TLC	Total lung capacity

Abstract

Background: In the context of hematopoietic stem cell transplantation (HSCT), mesenchymal stem cells (MSC) have been used to promote engraftment and prevent graft-versus-host-disease. However, in animal models, MSC were shown to cause pulmonary alterations after systemic administration. The impact of MSC infusion on lung function has not been studied in humans. The objective of the study was to investigate the impact of MSC co-infusion on lung function and airway inflammation as well as on the incidence of pulmonary infections and cytomegalovirus (CMV) reactivation after HSCT.

Methods: We have prospectively followed 30 patients who underwent unrelated HSCT with MSC co-infusion after non-myeloablative conditioning (NMA). Each patient underwent detailed lung function testing (FEV_1, FVC, FEV_1/FVC, RV, TLC, DLCO and KCO) and measurement of exhaled nitric oxide before HSCT and 3, 6 and 12 months posttransplant. The incidence of pulmonary infections and CMV reactivation were also monitored. This group was compared with another group of 28 patients who underwent the same type of transplantation but without MSC co-infusion.

Results: Lung function tests did not show important modifications over time and did not differ between the MSC and control groups. There was a higher 1-year incidence of infection, particularly of fungal infections, in patients having received a MSC co-infusion. There was no difference between groups regarding the 1-year incidence of CMV reactivation.

Conclusions: MSC co-infusion does not induce pulmonary deterioration 1 year after HSCT with NMA conditioning. MSC appear to be safe for the lung but close monitoring of pulmonary infections remains essential.

Introduction

Mesenchymal stem cells (MSC) are pluripotent cells derived from the bone marrow or other sources, which have stimulated a high level of enthusiasm in recent years for their potential therapeutic use.

In the context of allogeneic hematopoietic stem cell transplantation (HSCT), MSC co-transplantation appears to be safe (1, 2). These cells could have a role in engraftment promotion (3) and graft-versus-host disease (GVHD) prevention (4) through their immunosuppressive activity. Due to their lack of major histocompatibility complex (MHC) molecule expression, MSC are weakly immunogenic in humans, allowing administration to patients without HLA matching (5).

However, in some animal models, MSC infusion has been shown to cause pulmonary alterations. Indeed, after IV injection in mice, the cells are trapped within the pulmonary capillaries, thereby causing embolism (6). Moreover, mouse MSC could differentiate into tumor cells in lungs after systemic administration (7), and could promote tumor development (8). Another study in mice disclosed differentiation of MSC in the lung after irradiation, depending on the infusion timing (9): MSC differentiated into functional lung cells if injected at an early stage or into cells involved in fibrosis if injected once chronic inflammation and fibrosis had started. Furthermore, Salazar K et al showed evidence of a mitogenic potential of human and mouse MSC on lung fibroblasts in vitro (10). In contrast, other papers have shown that, in response to injury caused by endotoxin or bleomycin, MSC migrated to the lung, decreased tissue damage and improved lung repair (11, 12). In addition, in rats with chronic obstructive lung disease, intra-tracheal MSC administration restored lung function (13). These observations may result from the ability of MSC to secrete paracrine growth factors and cytokines able to down-regulate inflammation. Moreover, the ability of MSC to differentiate into functional cells may be a key in promoting adequate lung repair. MSC are also suggested

to be able to attenuate oxidative stress in inflammatory lung diseases induced by previous irradiation or by subclinical pathogen colonization in a context of immunosuppression (14).

The impact on the lung of MSC co-infusion after HSCT has not been studied so far in humans. In this study, we monitored the evolution of lung function, the value of exhaled nitric oxide (FeNO) and the occurrence of pulmonary infections and cytomegalovirus (CMV) reactivation in patients who underwent HSCT with MSC co-infusion. We also investigated the impact of MSC co-infusion on CMV reactivation and pulmonary infections in univariate and multivariate Cox models adjusted for competing risks.

It is widely accepted that performing pulmonary function tests (PFT) before and after transplantation is crucial to detect early signs of pulmonary complications. Lung function assessment prior to transplantation usually serves as baseline reference to evaluate changes after HSCT (15). We followed 30 patients who received HSCT for hematological malignancies from unrelated donors and a co-infusion of MSC after non-myeloablative conditioning (NMA). The results were compared with those of 28 patients who received the same type of transplant but without MSC. Pulmonary function parameters, including airway flow rates, lung volumes and diffusing capacity (DLCO), as well as FeNO value, were measured before HSCT as well as 3, 6 and 12 months post-transplantation. FeNO is a non-invasive marker of airway inflammation used to monitor rejection after lung transplantation (16) but its utility has not been really assessed after HSCT.

Results

At baseline, all patients displayed normal spirometric and lung volume values but a slight impairment of diffusing capacity. The median FeNO value was within the accepted normal range for both patient groups (17).

MSC co-infusion had no detrimental effect on lung function indices and was even associated with a slight improvement in FEV1 and FVC at some time points, which contrasted with the decrease in DLCO seen at 1 year in the group without MSC co-infusion ($p<0.05$) (see Supplemental Digital Content, Table 1).

The other lung parameters (FEV1/FVC, KCO, TLC, RV and FeNO) were not significantly changed after 3, 6 or 12 months in any of the patient groups and did not show differences between groups.

The one-year cumulative incidence of pulmonary infection appeared higher in the MSC group compared to the control group ($p <0.01$; Table 1 and Supplemental Digital Content, Figure 1A). Infection etiologies showed a trend for a higher rate of fungal infections in the MSC group (6 versus 1; $p=0.06$). In contrast, the cumulative incidence of CMV reactivation did not show any difference between groups (Supplemental Digital Content, Figure 1B).

In univariate Cox analysis of the whole cohort (n=58), MSC co-infusion showed a significant association with the occurrence of pulmonary infections ((HR=2.96 (1.15-7.60), $p<0.05$)) (Table 2). After multivariate analysis adjusting for aspergillosis before HSCT, female donor-to-male recipient and MSC co-infusion, there was only a trend for an association between MSC and pulmonary infections ($p=0.09$; Table 2). The only significant risk factor was aspergillosis before HSCT ((HR: 3.19 (1.08-9.43), $p<0.05$)).

For CMV reactivation, MSC-co infusion did not come out significantly in univariate analysis. In multivariate analysis, the only parameters that appeared significant were the donor and recipient CMV positive status ((HR=2.54 (1.10-5.84), $p=0.03$ and HR=7.56 (2.18-26.23), $p=0.001$, respectively)) (Table 2).

Discussion

In this study, we investigated the impact of MSC co-infusion on several clinical and laboratory outcomes of patients who underwent HSCT. In order to investigate the effect of MSC on the lung, we focused our analysis on pulmonary function monitoring and pulmonary infections.

At 1 year, even if all patients in the MSC group and none in the control group received a graft from HLA-mismatched donors, we did not observe any difference between groups for the incidence of acute GVHD (aGVHD). Indeed, transplantation from HLA-mismatched donors is known to give less favorable outcome because of a higher incidence of GVHD. In addition, the higher proportion of the female-to-male combination, usually associated with a higher risk of chronic GVHD (cGHVD) (18), was not correlated with a greater incidence of cGVHD in the MSC group. These observations might attest for a protective effect of MSC against GVHD but this remains to be studied in prospective randomized trials.

The original finding of our study is the fact that patients receiving MSC exhibited no deterioration in lung function indices over the first year of observation. There was even a slight improvement in airway flow rates and vital capacity and no change in lung diffusing capacity in the MSC group, while the group without MSC exhibited a significant decrease in diffusion lung capacity reaching on average 10% after one year. Although some animal data drew attention to the potential fibrotic effect of MSC (10), our observation attests that infused MSC did not result in excessive airway or lung remodeling that could potentially alter lung function.

By contrast, the incidence of pulmonary infection appeared to be higher after MSC co-infusion. This observation could theoretically be linked to the immunosuppressive effects of MSC, GVHD or CMV reactivation (19). However, these factors were not predictive of lung infections neither in univariate nor in multivariate analyses. Likewise, neutropenia,

aspergillosis prior to HSCT and tobacco status, all recognized risk factors for pulmonary infections in general (20-22) were evaluated but only a previous episode of aspergillosis before HSCT was significantly predictive. It should be emphasized that these analyses investigated not only the impact of MSC alone but also the combined effect of HLA mismatch and MSC co-infusion, as the two factors were systematically associated. HLA mismatching could have caused a higher incidence of severe GHVD in the MSC group and hence a greater risk of fungal infections (23). However, we did not observe any difference between the groups for the incidence of aGVHD and cGVHD, indicating that MSC might have mitigated this increased risk.

Although MSC have been shown to possess antimicrobial properties, this ability has been essentially observed *in vitro* or in animal models and in *ex vivo* models of human lung tissues (24). Moreover this property was mainly described against bacterial, viral and parasitic infections (25-27). Interestingly, we encountered a higher incidence of fungal infections after MSC co-infusion. Forslow et al also found an association between MSC co-infusion and pneumonia-related death (28). The majority of patients had mold-related pneumonia, but authors were not able to prove the relation with MSC co-infusion due to low patient numbers in this study. However, in a further paper, the use of MSC was associated with a higher incidence of invasive fungal infections in case of severe aGVHD (29), which is in line with our results.

Contrary to pulmonary infections, MSC co-infusion did not have an impact on CMV reactivation and this was attested by the fact that the cumulative incidence of CMV reactivation did not differ from the control group. A similar finding was observed in a recent paper by Lucchini et al (30), which demonstrated that MSC did not interfere with antiviral responses *in vivo*. The only parameters found to be strongly predictive of CMV reactivation in

this study were donor and recipient CMV positive status, which are widely recognized as risk factors for such reactivation (31).

In conclusion, the main finding of our study is the fact that patients who underwent HSCT with MSC co-infusion after NMA showed no deterioration of lung function over a period of one year after transplantation. However, the pulmonary infection rate (mainly the occurrence of fungal infections) appeared to be increased. This indicates the need for prolonged antifungal prophylaxis and close monitoring of pulmonary infections in patients following HSCT and MSC co-transplantation.

Material and Methods

Subjects

Patients who underwent HSCT after NMA conditioning in the University Hospital Center of Liege between December 2006 and December 2012 were screened for the following inclusion criteria: (1) conditioning with total body irradiation (TBI) 2 Gy and fludarabine; (2) immunosuppression with mycophenolate mofetil (MMF) and tacrolimus; (3) transplantation with peripheral blood stem cells (PBSC); (4) unrelated donor; (5) minimal follow-up of 100 days. Thirty-eight patients met the inclusion criteria in the MSC group. Among them, 8 were excluded because they did not have lung function assessment 3 months after HSCT. Indeed, three patients died before day 100 (1 died of graft rejection and sepsis, 1 of relapse and 1 of Graft-Versus-Host Disease and sepsis associated with organ failure). The other 5 patients were not able to perform lung function tests at day 100 (2 had invasive lung aspergillosis, complicated by renal failure in 1; 2 had a relapse associated with severe encephalopathy in 1; 1 had severe GVHD and organ failure). Consequently, 30 patients remained eligible in the MSC group. They took all part in one of two consecutive clinical trials investigating the safety and efficacy of MSC co-infusion at time of HSCT from mismatched unrelated donors

after NMA conditioning (1). In the non-MSC group, thirty-five patients also undergoing HSCT after NMA conditioning were included. However, 3 died before 100 days (2 of relapse, 1 of GVHD associated with sepsis and organ failure), and 4 did not perform lung function tests because of poor health status (2 had a relapse combined with sepsis, 1 had severe GVHD associated with organ failure and 1 had GVHD combined with encephalopathy). Therefore, 28 patients remained eligible in the control group. Characteristics of the patients are presented in Table 3. Patients with HLA mismatches (1 allele up to 2 antigens) were eligible for the NMA MSC trial, while all controls were 10/10 HLA-matched at allelic level.

MSC were cultured as described previously (1, 32). The conditioning regimen consisted in Fludarabine 90 mg/m^2, followed by a single dose of 2 Gy TBI administered on day 0 before infusion of cells. MSC were infused first, followed by PBSC infused at least 60-120 minutes later. MMF was administered orally from day 0 through day 42 at the dose of 15 mg/kg three times a day. Tacrolimus was given orally at the dose of 0.06 mg/kg twice a day starting on day -3 until day 180 and then progressively tapered to be definitely discontinued by day 365 in the absence of GVHD. The conditioning regimen and postgrafting immunosuppression used were identical in the MSC and the control groups.

The diagnosis and clinical grading of aGVHD were performed according to standard criteria (33, 34). Diagnosis and grading of cGVHD were made using the National Institute of Health consensus criteria (35).

All subjects gave written informed consent for participation as well as for collection and analyses of post-transplant data. The MSC clinical trials were approved by the Ethics Committee of the University of Liege.

Pulmonary function assessment

At each time point, subjects underwent a global lung function assessment using a body box plethysmography (Sensormedics, Vmax series 22, Viasyhealthcare, Yorba Linda, California, USA) allowing to measure flow rates, lung volumes and diffusion capacity according to ATS/ERS standard criteria (36-38). Spirometry (measure of Forced Expiratory Volume in 1 second: FEV_1 and Forced Vital Capacity: FVC) was performed before and after 400 µg inhaled salbutamol MDI administered through a Volumatic®. Diffusion for carbon monoxide (DLCO) was measured by the single breath wash-out technique and corrected for the hemoglobin content. FeNO was measured using a chemoluminescence analyser (NIOX, Aerocrine, Stockholm, Sweden) at a flow rate of 50 ml/sec, in accordance with the recommendation of the ATS/ERS task force (39).

Pulmonary infections and CMV reactivation

Standard prophylaxis against infections was used (40), and disease evaluation was routinely carried out on days 40, 100, 180 and 365. Pulmonary infections were diagnosed based on respiratory symptoms, microbial analysis of bronchoalveolar lavage, and chest radiography and/or CT scan. Bronchitis leading to hospitalization and pneumonia were recorded. The day of the first CMV reactivation episode, defined as the first viral load > 1000 copies/ml by PCR, was also recorded and positive patients were treated preemptively with ganciclovir. Secondary neutropenia was defined as an episode of at least two weeks with absolute neutrophil count < 500 cells/µl occurring at least 1 month after HSCT.

Statistical analysis

Categorical parameters were compared using the Chi-square test. Comparisons between PFT values before and after HSCT were performed using paired t tests or Wilcoxon matched-pair

signed rank tests for the FeNO values. Intergroup comparisons were made using unpaired t tests or Mann-Whitney tests as appropriate. Cumulative incidences of aGVHD, cGHVD, neutropenia, CMV reactivation and pulmonary infection were calculated as previously described (41), taking death as competing event. The impact of MSC co-infusion (and correlatively of HLA mismatch because of the linkage between the two characteristics) on CMV reactivation and pulmonary infections was assessed in univariate and multivariate Cox models. Factors analyzed in univariate analysis were as follows: patient age, gender, tobacco habits, acute leukemia vs other diagnoses, number of CD34+ cells transplanted, aspergillosis before HSCT, secondary neutropenia, grade II-IV aGVHD, female donor to male recipient vs other sex combinations, and MSC co-infusion. CMV reactivation was also assessed as risk factor for pulmonary infection, and pulmonary infection, donor and recipient CMV serostatus were evaluated as risk factors for CMV reactivation. Factors with a P value < 0.10 were then introduced into multivariate analysis. Differences were considered statistically significant when a two-sided p-value was < 0.05. Statistical analyses were carried out with Graphpad Prism (Graphpad Software San Diego, CA) and SAS version 9.3 (SAS institute, Cary, NC).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

The authors would like to thank all participants of the study and thank the lung function laboratory of the University Hospital Center of Liege for their help in the collection of the data. The study was financially supported by TELEVIE (Grant F.N.R.S. 7.4.642.09.F) associated with the Fonds National de la Recherche Scientifique (FNRS) Belgium, PAI 6/35 and Fonds Léon Frédéricq.

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Table 1: Cumulative incidence of aGVHD, cGVHD, CMV reactivation, secondary neutropenia and pulmonary infection (including etiology).

	MSC group n=30	Controls n=28	P value
1- year Cumulative incidence of Grade II-IV acute GVHD (%)	30	36	0.6
1- year Cumulative incidence of Chronic GVHD (%)	67	52	0.14
Moderate-severe GVHD (%)	46	33	0.3
1-year cumulative incidence of CMV reactivation (%)	40	43	0.76
1-year cumulative incidence of neutropenia (%)	14	4	0.17
1-year cumulative incidence of pulmonary infection (%)	48	15	0.0074
Unknown origin (n=)	9	3	0.12
Fungal (n=)	6	1	0.06
Viral (n=)	1	0	0.35
Bacterial (n=)	3	2	0.71
Total (n=)	19	6	0.02

GVHD: graft versus host disease; CMV: cytomegalovirus.

Table 2: Univariate and multivariate Cox analysis of factors affecting the rate of pulmonary infections (left) and CMV reactivation (right) in the MSC and control groups combined (n=58)

Pulmonary infections			CMV reactivation		
Factor	**HR (95% CI)**	**P value**	**Factor**	**HR (95% CI)**	**P value**
Univariate anlalysis			***Univariate anlalysis***		
Age	1.00 (0.97-1.04)	0.85	Age	1.04 (1.00-1.08)	0.07
Gender	0.72 (0.30-1.73)	0.47	Gender	1.26 (0.58-2.75)	0.57
Tobacco habits	1.15 (0.48-2.76)	0.76	Tobacco habits	0.93 (0.40-2.15)	0.87
Acute leukemia vs other diagnoses	0.61 (0.25-1.51)	0.29	Acute leukemia vs other diagnoses	1.00 (0.45-2.21)	1.00
Aspergillosis prior to HSCT	2.73 (1.00-7.44)	0.05	Aspergillosis prior to HSCT	0.50 (0.12-2.11)	0.35
Female donor to male recipient	2.32 (0.89-5.98)	0.08	Female donor to male recipient	2.12 (0.89-5.06)	0.09
Number of CD34 cells transplanted	0.89 (0.76-1.04)	0.14	Number of CD34 cells transplanted	1.08 (0.95-1.23)	0.23
HLA mismatch and MSC	2.96 (1.15-7.60)	0.02	HLA mismatch and MSC	0.96 (0.44-2.09)	0.92
Secondary neutropenia	1.35 (0.40-4.56)	0.63	Secondary neutropenia	0.28 (0.04-2.04)	0.21
aGVHD	1.04 (0.42-2.58)	0.92	aGVHD	1.74 (0.79-3.83)	0.17
CMV reactivation	1.12 (0.47-2.66)	0.79	Pulmonary infection	0.98 (0.43-2.21)	0.96
			Donor CMV+ serostatus	2.28 (1.03-5.03)	0.04
			Recipient CMV + serostatus	7.78 (2.31-26.3)	0.0009
Multivariate analysis			***Multivariate analysis***		
Aspergillosis prior to HSCT	3.19 (1.08-9.43)	0.04	Age	1.01 (0.97-1.06)	0.53
Female donor to male recipient	2.29 (0.80-6.56)	0.13	Female donor to male recipient	2.25 (0.87-5.81)	0.09
HLA mismatch and MSC	2.35 (0.88-6.29)	0.09	Donor CMV+ serostatus	2.54 (1.10-5.84)	0.03
			Recipient CMV+ serostatus	7.56 (2.18-26.23)	0.001

GVHD: graft versus host disease; CMV: cytomegalovirus; HR: hazard ratio

Table 3: Patient characteristics.

	MSC group n=30	Controls n=28	P value
Age (years)	54 ± 13	57 ± 11	0.40
BMI	25 ± 4	26 ± 4	0.54
Gender (M/F)	21/9	12/16	0.04
Tobacco habits (n/ex/cs)	12/8/10	8/13/7	0.29
Comorbidities (HSCT-CI score)	2 (0-9)	3 (0-7)	0.16
Underlying malignancy			
HL	2	2	0.83
NHL	6	4	
CLL	2	3	
ALL	1	2	
CML	0	1	
AML	11	10	
MPD	0	1	
MM	7	5	
PLL	1	0	
Disease risk (42): low/standard/high	7/17/6	7/16/5	0.98
No. of cells transplanted (x 10^6/kg)			
$CD34^+$ cells	4.3 (1.0-11.7)	6.1 (2.6-14.5)	0.05
$CD3^+$ cells	271 (92-540)	320 (140-598)	0.24
Patient/donor compatibility			
10/10 HLA identical (allelic level)	0	28	<0.0001
Other	30	0	
Female donor to male recipient	9	2	0.03
Other sex combinations	21	26	
Aspergillosis before HSCT	5	3	0.51
CMV serologic status, recipient-donor			
Negative-negative	5	9	0.19
Negative-positive	3	6	
Positive-negative	12	6	
Positive-positive	8	5	

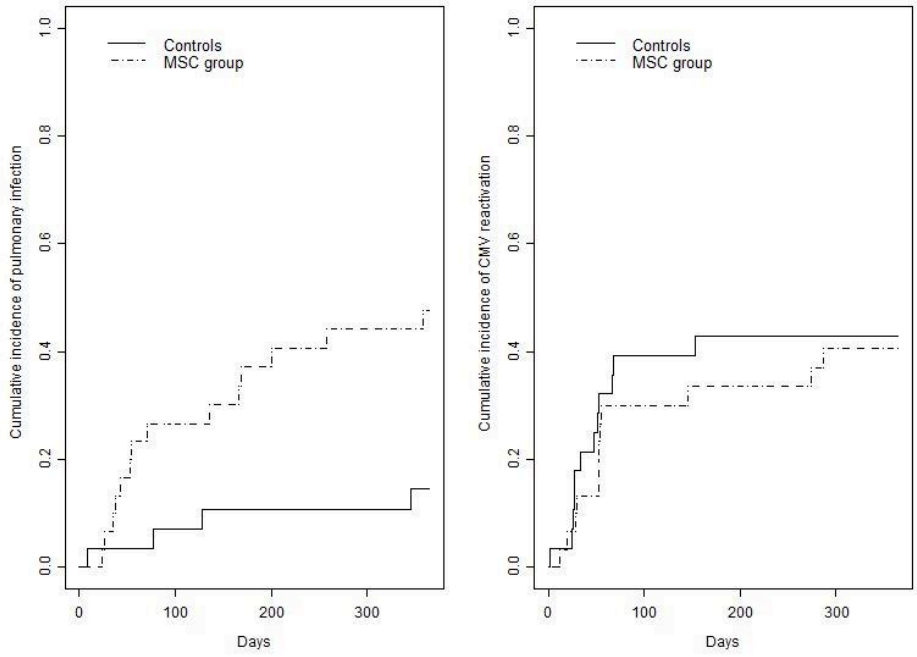
Results are expressed as mean ± SD except for HCT-CI score and number of $CD3^+$ and $CD34^+$ cells transplanted expressed as median (range); BMI= body mass index, n= non-smoker, ex= ex-smoker, cs=current smoker, HL= Hodgkin's lymphoma, NHL= non-Hodgkin's lymphoma, CLL= chronic lymphocytic leukaemia, ALL= acute lymphoblastic leukaemia, CML= chronic myeloid leukaemia, AML= acute myeloid leukemia, MPD= myeloproliferative disorder, MM= multiple myeloma, PLL= prolymphocytic leukaemia, HSCT: hematopoietic stem cell transplantation; CMV: cytomegalovirus.

SDC Table 1: Changes in PFT 3, 6 and 12 months after HSCT in the MSC and control groups.

		Before HSCT	3 months	6 months	12 months
FEV1 pre-BD (L)	*MSC*	2.7 ± 0.9	2.9 ± 0.9 *	2.8 ± 0.8	2.7 ± 0.8
	No MSC	2.5 ± 0.7	2.4 ± 0.7 §	2.6 ± 0.7	2.5 ± 0.8
FEV1 pre-BD (% pred)	*MSC*	89 ± 23	95 ± 22	93 ± 22	91 ± 19
	No MSC	89 ± 17	87 ± 17	90 ± 15	89 ± 21
FEV_1 post-BD (L)	*MSC*	2.9 ± 0.9	2.9 ± 0.9	2.6 ± 0.8	2.8 ± 0.8
	No MSC	2.5 ± 0.6	2.5 ± 0.7	2.6 ± 0.6	2.5 ± 0.9
FEV_1 post-BD (% pred)	*MSC*	88 ± 23	94 ± 22	92 ± 23	90 ± 20
	No MSC	87 ± 13	87 ± 15	92 ± 16	89 ± 24
FVC pre-BD (L)	*MSC*	3.6 ± 1.1	3.8 ± 1.1	3.8 ± 1.1 *	3.6 ± 1.0
	No MSC	3.3 ± 0.9	3.3 ± 0.9	3.3 ± 0.8	3.3 ± 0.8
FVC pre-BD (% pred)	*MSC*	94 ± 19	99 ± 18	101 ± 17	98 ± 15
	No MSC	97 ± 17	96 ± 16	97 ± 15	99 ± 20
FVC post-BD (L)	*MSC*	3.8 ± 1.1	3.8 ± 1.2	3.5 ± 1.0	3.7 ± 1.0
	No MSC	3.3 ± 0.6	3.3 ± 0.9	3.4 ± 0.7	3.4 ± 0.9
FVC post-BD (% pred)	*MSC*	95 ± 18	99 ± 21	100 ± 18	96 ± 13
	No MSC	95 ± 12	94 ± 13	100 ± 14	99 ± 21
FEV1/FVC (%)	*MSC*	76 ± 11	77 ± 10	75 ± 10	74 ± 7
	No MSC	76 ± 7	74 ± 7	75 ± 7	74 ± 9
FEV1/FVC post BD (%)	*MSC*	74 ± 11	79 ± 12	77 ± 15	75 ± 10
	No MSC	76 ± 8	77 ± 8	76 ± 8	74 ± 12
Corrected DLCO (mmol/KPa .min)	*MSC*	6.6 ± 2.4	6.2 ± 1.8	6.6 ± 2.2	6.6 ± 2.2
	No MSC	6.3 ± 1.8	6.4 ± 1.7	6.3 ± 1.3	5.8 ± 1.1 *
Corrected DLCO (% pred)	*MSC*	73 ± 25	69 ± 21	75 ± 24	75 ± 23
	No MSC	74 ± 22	75 ± 16	74 ± 17	70 ± 15
Corrected KCO (DLCO/L)	*MSC*	1.4 ± 0.6	1.4 ± 0.7	1.4 ± 0.5	1.4 ± 0.6
	No MSC	1.4 ± 0.4	1.5 ± 0.4	1.4 ± 0.4	1.3 ± 0.4
Corrected KCO (% pred)	*MSC*	86 ± 27	89 ± 39	95 ± 34	98 ± 37
	No MSC	97 ± 33	101 ± 34	98 ± 32	89 ± 25
TLC (L)	*MSC*	5.9 ± 1.2	5.6 ± 1.1	6.0 ± 1.6	5.6 ± 1.4
	No MSC	5.5 ± 1.5	5.4 ± 1.2	5.4 ± 1.2	5.3 ± 1.3
TLC (% pred)	*MSC*	94 ± 14	91 ± 13	94 ± 15	96 ± 12
	No MSC	94 ±19	92 ± 13	92 ± 16	94 ± 22
RV (L)	*MSC*	2.0 ± 0.9	1.9 ± 0.8	2.2 ± 1.1	2.0 ± 1.0
	No MSC	2.1 ± 1.1	2.1 ± 0.8	2.0 ± 0.9	2.0 ± 1.0
RV (% pred)	*MSC*	93 ± 36	104 ± 45	97 ± 39	99 ± 46
	No MSC	99 ± 46	99 ± 34	96 ± 41	99 ± 48
FE_{NO} (ppb)	*MSC*	21 (13-29)	16 (10-22)	26 (17-39)	21 (13-26)
	No MSC	15 (11-23)	13 (12-19)	16 (13-30)	19 (13-35)

Results are expressed as mean ± SD except for FeNO value expressed as median (interquartile range). % pred: predicted percentage; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; DLCO: diffusing capacity of the lung for carbon monoxide; KCO: gas transfer coefficient; TLC: total lung capacity; RV: residual volume; BD: bronchodilation. ; FeNO: exhaled nitric oxide; * < 0.05, ** < 0.01, *** < 0.001 vs before HSCT; § < 0.05, §§ < 0.01, §§§ < 0.001 vs MSC group.

Figure 1: Cumulative incidence of pulmonary infection (A, $p < 0.01$) and CMV reactivation (B, NS) in the MSC and control groups.



DISCUSSION

It is well recognized that patients undergoing HSCT are at high risk of pulmonary complications over the months and year following the transplantation. The current work aimed to monitor the lung function and the airway inflammation in patients undergoing HSCT. To this end we applied the non-invasive technique of induced sputum to characterize the cellular and molecular inflammation prevailing in the airway of patients.

In the first part of this project, we have described the modifications at airway level in a cohort of 182 patients who underwent HSCT. We assessed PFT, FeNO and sputum cell counts before and 3, 6, 12, 24 and 36 months after transplantation. Our main finding was the occurrence of a progressive restrictive pattern 3 years after HSCT associated with a sustained airway neutrophilic inflammation.

Baseline functional status of our patients showed normal flow rates and lung volume but a slight impairement of difusing lung capacity even after correction for hb level. Over the follow-up, there was appearance of a progressive restrictive syndrome, which was confirmatory of large data base report[141]. The reasons for the restrictive pattern remain unclear but the change in lung function might be related to several factors such as GVHD, skeletal muscle weakness or sequel of recurrent lung injuries likely to occur in these immuno-compromised patients. On the other hand, thoracic irradiation and cytotoxic chemotherapeutic agents are recognized factors contributing to lung fibrosis that may lead to restriction. MA conditioning was associated with greater functional restriction and greater impairement in total diffusing capacity compared to NMA. However, this does fit a pure restrictive change as residual volume increased in the first two years thereby maintaining a normal total lung capacity. Remarkably the ratio FEV1/FVC remained stable over the 3 years for the whole cohort and only a very limited number of patients exhibited BOS (3.5%).

To the best of our knowledge, we are the first to report on sputum cell counts after HSCT. We observed an intense neutrophilic airway inflammation at baseline in these patients, which persisted in the months and years after the transplantation. This could indicate ongoing innate immune system activation as a result of recurrent airways micro-injuries due to irradiation and chemotherapy or bacterial colonisation in fragile patients because of their immune suppressive treatment. Indeed, the clear rise in neutrophils during the first year after MA conditioning may lend support to this hypothesis and might be related to the greater injury of respiratory mucosal system caused by the intense irradiation and chemotherapy. It also might be a sign of a repair process following aggressive therapy prior to the transplantation. It is noted that subclinical pulmonary infections cannot be definitely ruled out. However, none of subjects had overt pneumonia when assessed and the microbiological

analysis of sputum, when performed, did not bring out others germs than commensal germs.
The neutrophils are thought to play a critical role in several lung diseases. They are able to release several proteases and ROS and could be therefore involved in lung injuries and remodeling seen in neutrophilic diseases as COPD, cystic fibrosis or bronchiolitis obliterans after lung transplantion. Whether neutrophils are involved in the change in lung function status in our patients is unclear but we did not see convincing relationship between the changes in sputum neutrophil count and the change in lung function parameters. Whether neutrophils could be considered as a therapeutic target to decrease airway inflammation in severe airway diseases is still debated because of the role of neutrophils in immune defence[223]. There has been, however, several recent attempts to combat neutrophilic inflammation in bronchiolitis obliterans by non-immunosuppressive drugs like cystéinyl-leukotrienes receptor antagonists (LTRA), phosphodiesterase inhibitors and azithromycine could be a strategy of choice. This strategy was shown successful to reverse neutrophilic bronchiolitis obliterans after lung transplantation[121].
Due to the persistent high sputum neutrophil percentage in HSCT patients compared to healthy subjects, great caution should be taken when interpreting the sputum composition from patients with lung complications. In order to avoid drawing wrong conclusions, these data should be compared with those obtained from a control group of HSCT patients without lung complications and not from healthy volunteers.
In contrast, the value of FeNO remained in the normal range and did not change over time in our cohort of patients. This is not surprising as FeNO has rather been shown to reflect airway eosinophilic inflammation while inflammation in patients with HSCT is clearly neutrophilic.
Another thing coming out from our study is the demonstration of the feasibility of repeated sputum in a fragile population. When pooling together all our sputum inductions attemps in HSCT patients, we obtained a success rate of 73%, which is close to the figures reported in other diseases in which the technique has been validated such as asthma, COPD and even heathy subjects. Furthermore the procedure was well tolerated with no serious adverses effects noticed over almost 300 procedures of induction. Consequently induced sputum proved to be a valuable method to evaluate aiway inflammation after HSCT. It can be used as a complementary method with clinical, imaging and functional parameters when bronchoscopy is contraindicated or when tissue samples are not available.
Thus, induced sputum is a promising method for clinical and experimental applications in patients who underwent HSCT.

In the second part of this project we have assessed the cytokine profile seen in 40 patients undergoing HSCT by measuring a range of cytokines using the multiplex ELISA technology**.**

Our data indicated that, compared to age matched healthy subjects IL-6 and IL-8 were increased in sputum supernatant of patients before the HSCT and were associated with sputum neutrophilic inflammation. The levels of these cytokines remained however lower than those seen in COPD and it was especially the case for IL-8. IL-8 and IL-6 are key cytokines involved in neutrophilic inflammation either by direct chemotactic effect as for IL-8 or by promoting IL-17 pathway as for IL-6. The high levels of these cytokines in patients prior to transplantation are likely to result from an activation of innate immunity as a consequence of previous chemotherapy and irradiation. The cell source of these cytokine could be the epithelial layer activated by previous traitements.
Baseline levels of IL-6 and IL-8 were not significantly different between survivors and non-survivors at one year although this finding has to be cautiously interpreted because the number of patients who died was very limited in this series (5/40). Indeed the levels of IL-8 tended to be higher in those who will die over the first year.
When HSCT patients were followed up over one year (sputum samples assayed: 39 at 3 months, 28 at 6 months and 15 at one year), sputum levels of IL-6 and IL-8 tended to return to normal values. Interestingly, some patients with HSCT (27%) exhibited raised levels of TGF-β 1 one year after the transplantation whereas the cytokine remained undetectable in more than 95% of healthy subjects, COPD and HSCT patients prior to transplantation. Our finding needs caution in the interpretation as the number of sputum samples assayed at this time was rather low which decreases the stastical power and may cause a biais of selection.

In the third part, we assessed the impact of mesenchymal cells (MSC) co-infusion on lung function after HSCT. Indeed, conflicting results exist in the literature concerning MSC administration and their impact on the lungs.
We evaluated the impact of MSC co-infusion on lung after unrelated allogeneic HSCT with NMA conditioning. We monitored the lung function, FeNO, pulmonary infection and CMV reactivation until 1 year in 30 patients who underwent HSCT with MSC co-infusion. They were compared with 28 patients who underwent the same type of transplantation but without MSC.
We observed that MSC did not have deleterious effect on lung function 1 year after HSCT. However, the patients receiving MSC exhibited higher severe pulmonary infection incidence compared to controls. By multivariate Cox model analysis, we found that MSC could be linked to this increased occurrence of infections, particularly to fungal infection.
In contrast, MSC did not favour CMV reactivation. However, it is noted that the patients who received MSC were all HLA-mismatch transplant cases, then their immune recovery could have been slower than in the control group, which could have caused increased pulmonary fungal infections.

Limitation of our work

The limitations of these studies are plurial; they were limited by the heterogeneity of the patient group and underlying diseases and they were performed in a single institution limiting the patient number.
The original part of our work is to investigate the airway inflammatory status of patients undergoing HSCT by using the technique of induced sputum. Although the technique is relatively non invasive, it has, however, not been simple to systematically obtain paired sputum samples of sufficient quality over the follow-up period for several reasons including death, poor health status of the patients and/or sometimes poor material collected. This has certainly limited the statistical power of our cell and biochemical analyses and the assessment of their predictive values.

PERSPECTIVES AND CONCLUSIONS

Our current work has clearly shown that HSCT exhibit a neutrophilic airway inflammation associated with raised sputum IL-6 and IL-8 levels. Other cytokines are difficult to detect in sputum supernatants. Applying the model of sputum cell culture may provide additional information on the nature of inflammatory process operating in the airways. This model requires however a great number of cells recovered and is time consuming precluding its broad clinical application in the future. On the other hand, work focusing on transcriptomic should also allow giving more insight in the complex cytokine and growth factor network prevailing in HSCT patients.
Our data do not support a key role in FeNO in following the HSCT as the values remain in the normal ranges and do not predict poor outcome.
In addition to sputum and FeNO, other potential new markers have stimulated great interest and are under investigation in many pulmonary inflammatory disorders incuding exhaled gases or condensates[224] and sophisticated lung function indices. Among exhaled gases, exhaled carbon monoxide[225], volatile organic compounds[226], electronic nose[227] and breathprint would worth being investigated as predictive markers of infections or poor outcome. In exhaled breath condensate, PH has been shown to be the most robust parameter so far[228]. Among lung function indices, the slope of the alveolar plateau for nitrogen or helium, multibreath nitrogen washout could spot the patients at risk of deleterious lung function evolution.
All these potential biomarkers will have to be investigated and validated in the follow-up of HSCT patients in order to predict occurrence of severe infectious pulmonary complications, BO and death.

In conclusion, airway monitoring is of vital importance after HSCT to evaluate the clinical course of the patients. It requires the use of multiple techniques that assess different aspects. Overall, in our series, the changes in lung function remain rather limited in the follow-up of HSCT with a small restriction appearing at 3 years. We reported the faisability and interest of induced sputum method after HSCT and highlighted the central role of neutrophils in airway inflammation before and after the transplantation, particularly in case of myeloablative conditioning. This neutrophilic inflammation appeared linked to IL-6 and IL-8 sputum level and remained less intense than in COPD patients. Although sputum was never collected during an overt phase of severe pulmonary infection, it is worth noting that cumulative incidence of significant pulmonary infection approaches 50% over the first year of follow-up. The use of MSC co-infusion to prevent GVHD did not interfere on lung function 1 year after HSCT but could promote opportunistic pulmonary infections.

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ANNEXE

PUBLICATION

Local and systemic cellular inflammation and cytokine release in chronic obstructive pulmonary disease.
Moermans C, Heinen V, Nguyen M, Henket M, Sele J, Manise M, Corhay JL, Louis R.,
Cytokine. 2011 Nov;56(2):298-304.

Cytokine 56 (2011) 298–304

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

Local and systemic cellular inflammation and cytokine release in chronic obstructive pulmonary disease

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ARTICLE INFO

Article history:
Received 26 January 2011
Received in revised form 23 June 2011
Accepted 11 July 2011
Available online 30 August 2011

Keywords:
COPD
Sputum
Blood
Cell count
Cytokine release

ABSTRACT

Background: Chronic obstructive pulmonary disease (COPD) is a chronic airway inflammatory disease caused by repeated exposure to noxious gases or particles. It is now recognized that the disease also features systemic inflammation. The purpose of our study was to compare airway and systemic inflammation in COPD to that seen in healthy subjects and to relate the inflammation with the disease severity.
Methods: Ninety-five COPD patients, encompassing the whole severity spectrum of the disease, were recruited from our outpatient clinic and rehabilitation center and compared to 33 healthy subjects. Induced sputum and blood samples were obtained for measurement of inflammatory cell count. Interleukin (IL)-4, IL-6, IL-10, TNF-α and IFN-γ produced by 24 h sputum and blood cell cultures were measured.
Results: Compared to healthy subjects, COPD exhibited a prominent airway neutrophilic inflammation associated with a marked IL-10, IL-6 and TNF-α release deficiency that contrasted with a raised IFN-γ production. Neutrophilic inflammation was also prominent at blood level together with raised production of IFN-γ, IL-10 and TNF-α. Furthermore, sputum neutrophilia correlated with disease severity assessed by GOLD stages. Likewise the extent of TNF-α release from blood cells also positively correlated with the disease severity but negatively with that of sputum cell culture. Blood release of TNF-α and IL-6 negatively correlated with body mass index. Altogether, our results showed a significant relationship between cellular marker in blood and sputum but poor relationship between local and systemic release of cytokines.
Conclusions: COPD is characterized by prominent neutrophilic inflammation and raised IFN-γ production at both bronchial and systemic level. Overproduction of TNF-α at systemic level correlates with disease severity and inversely with body mass index.

1. Introduction

Chronic obstructive pulmonary disease (COPD) causes a significant health burden worldwide, in terms of morbidity and mortality. COPD is characterized by progressive and not fully reversible airflow limitation. It is a chronic airway inflammatory disease with a systemic component related to repeated inhalation of noxious gas and particles, in particular tobacco smoke [28].

The principal abnormalities in airways are the presence of a persistent inflammatory response as well as a structural remodelling that thickens the airway wall. A destruction of alveoli is also present and leads to the occurrence of emphysema [7]. The epithelial cells are damaged by the inhalation of noxious particles and there is an activation of innate (mainly neutrophils and macrophages) and adaptative immune cells (mainly CD8 cells). These cells are responsible for the release of proteases, cytokines/chemokines and mediators, which lead to inflammation and remodelling. COPD has been seen as a Th1 disease but some data suggest that Th2 cytokine may also play a role [1,2]. On the other hand some data have suggested that COPD may be favoured by a lack of anti-inflammatory cytokine such as IL-10 [38].

COPD is not only associated with an abnormal inflammatory response in the lung but also with systemic inflammation, including systemic oxidative stress, activation of circulating inflammatory cells and increased levels of circulating inflammatory cytokines [16].

Induced sputum is a recognized non-invasive technique to assess the cellular composition in chronic airway disease including COPD [29]. In addition, induced sputum cell culture has been shown to be a valid model to investigate cytokine production from airway cells in asthma [3,22,27,32].

The purpose of our study was twofold. First, to compare airway and systemic inflammation between COPD and healthy subject and secondly, to assess how this inflammation relates to disease severity in COPD. Here, we evaluated the sputum and blood cell

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doi:10.1016/j.cyto.2011.07.010

composition as well as the sputum and blood cell cytokine production in 95 COPD and 33 healthy subjects. As for cytokine, we decided to analyse the level of interleukin-4 (IL-4) and interferon-γ (IFN-γ) as markers of the Th2/Th1 balance, TNF-α and IL-10 as pro- and anti-inflammatory cytokines, respectively, and IL-6 as a cytokine playing a role in the transition from innate toward adaptive immunity [18].

2. Materials and methods

2.1. Study design and subject characteristics

The demographic and functional characteristics of patients are given in Table 1. COPD patients ($n = 95$) were recruited from our outpatient clinic and rehabilitation centre CHU-Sart Tilman. Diagnosis of COPD was made according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (post-bronchilator-400 μg salbutamol-ratio FEV1/FVC < 70%). We divided COPD patients in three groups according to GOLD classification of severity (GOLD II: FEV1 < 80% and ⩾50%; GOLD III: FEV1 < 50% and ⩾30% and GOLD IV: FEV1 < 30%. All COPD patients were in stable condition at the time of blood and sputum collection and no patients were studied within 8 weeks of having an upper tract infection or any exacerbations requiring change in maintenance treatment or oral steroid and antibiotic prescription. Healthy subjects ($n = 33$) were recruited by advertisement among the hospital. None of them exhibited respiratory symptoms and all had normal spirometric results and airways responsiveness (provocative concentration of metacholine causing a fall in FEV1 of 20% > 16 mg/ml). Atopy was not formally studied but no healthy subject was reporting overt allergy symptoms by the time of sampling. According to the medical file, the large majority of COPD patients (87 out of 95) had not reported clinical history of allergy in the past nor were they taking regular anti-allergic drugs such as H1-antagonists. All participants gave informed consent and the study was approved by the local Ethic's Committee.

2.2. Peripheral blood sampling and cell count

Peripheral blood samples were collected in apyrogenic, heparinised tubes (Venosafe; TERUMO®, Leuven, Belgium). The total and differential blood cell counts were obtained with an Advia 120 automatic counter (Siemens, Erlangen, Germany). Counting and cell typing were based on flow cytometry with bidimensional volume distribution, peroxydase concentration, and lobularity of leucocytes as parameters.

Table 1
Demographic and functional characteristics.

	Healthy $N = 33$	COPD $N = 95$	p-value
Age (years)	40 ± 12	62 ± 12	<0.0001
Sex (m/f)	24/10	73/21	0.55
Tobacco status (ns/es/cs)	22/4/8	0/64/30	<0.0001
BMI (kg/m^2)	24.1 ± 3.3	26.0 ± 5.59	0.07
Inhaled CS	0	64	
Oral CS	0	9	
LABA	0	59	
SABA	0	52	
LAMA	0	30	
SAA	0	39	
FEV1 (L)	3.93 ± 1.23	1.22 ± 0.44	<0.0001
FEV1 (% predicted)	110 ± 16	43 ± 15	<0.0001
FVC (L)	4.69 ± 1.50	2.47 ± 1.86	<0.0001
FVC (% predicted)	110 ± 18	61 ± 17	<0.0001
FEV1/FVC (%)	87 ± 13	55 ± 17	<0.0001
GOLD II	0	24	
GOLD III	0	51	
GOLD IV	0	19	

Results are expressed as mean ± SD. FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; BMI, body mass index; CS, corticosteroids; LABA, long acting beta 2 agonist; SABA, short acting beta 2 agonist, LAA, long acting muscarinic antagonist; SAA, short acting anticholinergic.

2.3. Sputum induction and processing

After premedication with 400 μg inhaled salbutamol, sputum was induced by inhalation of hypertonic (NaCl 5%) or isotonic (NaCl 0.9%) saline according to the FEV1 value (> or < than 65% predicted). Saline was combined with additional salbutamol delivered by an ultrasonic nebulizer (Ultra-Neb 2000; Devilbiss, Somerset, PA, USA) with an output set at 0.9 ml/min as previously described [9]. Each subject inhaled the aerosol for three consecutive periods of 5 min for a total of 15 min. For safety reasons, FEV1 was monitored throughout the induction and this one was stopped if FEV1 fell by more than 20% from baseline.

The whole sputum was collected in a plastic container, weighed, and homogenized by adding three volumes of phosphate-buffered saline (PBS), vortexed for 30 s, and centrifuged at 800g for 10 min at 4 °C. Supernatant was separated from cell pellet, which was washed twice in Roswell park memorial institute medium (RPMI) 1640 supplemented with 100 U penicillin/ml, 100 μg streptomycin/ml at 400g for 10 min at 4 °C. Squamous cells, total cell counts and cell viability checked by trypan blue exclusion were performed with a manual hemocytometer. The differential leukocyte count was performed on cytospins stained with May–Grünwald–Giemsa on 500 cells. A determined volume of RPMI+ antibiotics was then added to the cell suspension to obtain a concentration of 2×10^6 nonsquamous cell/ml.

2.4. Blood and sputum cell culture and cytokine assay

Cytokines (IL-4, IL-6, IL-10, TNF-α and IFN-γ) were measured by immunotrapping assay which is based on early capturing of cytokines secreted during cell culture. The antibodies and standards were purchased from Biosource (Cytosets; Biosource, Invitrogen, Merelbeke, Belgium). Fifty microliters from standards or whole blood (diluted twice) or sputum cell suspension (2×10^6 cells/ml) was incubated at 37 °C with 200 μl RPMI 1640 supplemented with 100 U penicillin/ml, 100 μg streptomycin/ml (Cambrex, Verviers, Belgium), and 2% of inactivated foetal calf serum (Cambrex) in apyrogen microwells, which were coated previously with specific antibodies directed toward the chosen cytokines. After 24 h, the wells were washed, and 150 μl of a solution containing biotinylated detection antibodies specific to the cytokines was added for 2 h at room temperature. The wells were washed again and filled with a solution containing streptavidin horse-radish peroxidise for 45 min at room temperature. Then, 100 μl tetramethylbenzidine chromogen solution was added for 10–20 min in the dark. The reaction was stopped by adding 50 μl H2S04 1 M. The amount of substrate converted to products was thereafter detected as optical densities at 450 nm in an enzyme-linked immunosorbent assay (ELISA) reader (Multiscan Ascent; Thermo Labsystems, Helsinki, Finland). The sensitivity of the ELISAs was determined by running a set of ten blanks and was calculated as the mean response plus 2 standard deviations. The sensitivities of our assays were 6 pg/ml for IL-4, 6 pg/ml for IL-6, 4 pg/ml for IL-10, 6 pg/ml for TNF-α, and 7 pg/ml for IFN-γ.

2.5. Statistical analysis

The demographic and functional characteristics were expressed as mean ± SD and comparisons between groups were performed by

unpaired student "*t*" test for continuous variables and Chi-square test for categorical analyses.

Blood and sputum cell counts as well as cytokine levels were expressed as median (IQR).

Comparisons between groups were performed by a Mann–Whitney test and correlations were analysed with a Spearman rank test. Regarding production according to the age, differences between tertiles were analysed by a Kruskal–Wallis test. A p-value < 0.05 was considered as statistically significant.

3. Theory

COPD prevalence and morbidity continue to increase throughout the world and it represents a significant burden on the healthcare system. It is the fourth leading cause of death in most industrialized countries and it is projected to be the third worldwide by 2020.

Therefore, the identification of biomarkers involved in the immunopathogenesis of COPD is crucial and may provide myriad opportunities for possible intervention. Indeed, such biomarkers may be useful for monitoring disease progression and evaluating the effects of therapeutics or providing targets for therapies.

In addition, the lack in the understanding of the relationships between plasma and airway secretions in COPD highlights the importance of studying immune cells and mediators regulation at local and systemic level.

4. Results

4.1. Patient characteristics

Patients in both groups consisted mostly of men and COPD patients were all current or ex-smokers. Other demographic and treatment characteristics are given in Table 1. The pulmonary function parameters of COPD patients were significantly reduced compared to the healthy volunteers.

4.2. Sputum and blood cell counts

Detailed cell counts are given in Table 2. As for sputum COPD were characterized by a raised total sputum cell number ($p < 0.01$). The proportion of neutrophils ($p < 0.0001$) and, to a lesser extent, that of eosinophils ($p < 0.01$) were increased compared to healthy subjects while the proportions of macrophages, lymphocytes and epithelial cells were decreased in COPD. When expressed as absolute cell counts per gram of sputum, both neutrophils and eosinophils were clearly raised in COPD but no difference was detected with respect to other cell types.

At blood level, COPD displayed raised total leucocyte counts ($p < 0.0001$) as well as a raised proportion of neutrophils ($p < 0.01$) whereas we observed a lower proportion of lymphocytes ($p < 0.01$) and monocytes ($p < 0.05$) as compared to healthy subjects. Absolute neutrophil number per blood volume was twice as great in COPD as in healthy subjects ($p < 0.0001$). Likewise, absolute lymphocyte and monocyte numbers were slightly greater in COPD ($p < 0.05$ and $p < 0.01$ respectively).

4.3. Cytokine release from sputum and blood cell culture

Sputum cytokine levels are shown in Table 3. Sputum cells from COPD strikingly released less IL-10 ($p < 0.001$), TNF-α ($p < 0.0001$) and to a lesser extent IL-6 ($p < 0.01$) as compared to those of healthy subjects. IL-6 was detectable in sputum cell culture of 76% of healthy subject while it was only detectable in 52% of COPD. By contrast, IFN-γ Fig. 1a) was more often detectable in COPD (29% of subjects) than in healthy subjects (6%) ($p < 0.05$). IL-4 was rarely detectable in both COPD and healthy subjects (less than 10% of subjects).

Blood leukocytes from COPD released more IFN-γ Fig. 1b) and TNF-α as compared to those of healthy subjects ($p < 0.01$ for both). IFN-γ was detectable in 61% of COPD and only 27% of healthy subjects. Although undetectable in the majority of the subjects, IL-10 was more frequently detected in COPD (46%) than in healthy subjects (7%) ($p < 0.01$). No difference was observed for IL-4 and IL-6

Table 2
Sputum and blood cell counts.

	Healthy $N = 33$	COPD $N = 95$	p-value
Sputum			
Squamous (%)	18 (12–33)	22 (8–36)	0.99
Total non squamous (10^6/g)	0.53 (0.33–1.33)	1.23 (0.49–5.22)	0.001
Viability (%)	61 (54–74)	62 (50–75)	0.99
Macrophages (%)	42.8 (29.8–61.3)	16 (6.7–34.6)	<0.0001
Macrophages (10^3/g)	245 (100–600)	220 (100–520)	0.83
Neutrophils (%)	32.6 (14.6–53.9)	72.6 (39.2–83.3)	<0.0001
Neutrophils (10^3/g)	195 (35–485)	615 (245–4035)	<0.0001
Lymphocytes (%)	2 (0.9–3.7)	1.2 (0.5–2.2)	<0.05
Lymphocytes (10^3/g)	10 (0–30)	30 (10–310)	0.74
Eosinophils (%)	0 (0–0.5)	0.6 (0–2.5)	<0.01
Eosinophils (10^3/g)	0 (0–5)	35 (10–1030)	<0.001
Epithelial cells (%)	9.6 (2.7–19.8)	3.6 (0.8–13)	<0.05
Epithelial cells (10^3/g)	45 (15–125)	40 (15–95)	0.80
Blood			
Leucocytes (1/μl)	6700 (5405–7650)	9150 (7420–11,080)	<0.0001
Neutrophils (%)	54.5 (49–61.6)	62.9 (56.1–69.5)	<0.01
Neutrophils (1/μl)	3665 (2695–4535)	5910 (4405–6865)	<0.0001
Lymphocytes (%)	33.7 (26.6–37.5)	25.7 (20.3–31.6)	<0.01
Lymphocytes (1/μl)	1875 (1445–2510)	2305 (1760–2885)	<0.05
Monocytes (%)	6.1 (5.3–7.8)	5.8 (4.9–6.8)	<0.05
Monocytes (1/μl)	415 (335–495)	500 (415–645)	<0.01
Eosinophils (%)	1.8 (1.2–3.2)	2 (0.9–3.4)	0.94
Eosinophils (1/μl)	130 (85–185)	160 (100–325)	0.12
Basophils (%)	0.7 (0.4–0.8)	0.5 (0.3–0.7)	0.06
Basophils (1/μl)	40 (30–65)	50 (30–60)	0.84

Results are expressed as median (IQR). Differences were calculated by a Mann–Whitney test.

Table 3
Sputum and blood cytokine release.

	Healthy $N = 33$	COPD $N = 95$	p-value
Sputum (10^5 cells/well) standardized cell concentration			
IL-4 (pg/ml)	0 (0–0)	0 (0–0)	0.31
IL-6 (pg/ml)	29 (5–207)	7 (0–49)	<0.01
IL-10 (pg/ml)	50 (34–77)	22 (8–57)	<0.001
IFN-γ (pg/ml)	0 (0–0)	0 (0–11)	<0.05
TNF-α (pg/ml)	2248 (1501–3923)	1116 (544–2042)	<0.0001
Blood (50 μl diluted twice) Standardized blood volume			
IL-4 (pg/ml)	0 (0–7)	0 (0–0)	0.72
IL-6 (pg/ml)	0 (0–10)	0 (0–23)	0.12
IL-10 (pg/ml)	0 (0–0)	4 (0–14)	<0.01
IFN-γ (pg/ml)	0 (0–7)	11 (0–36)	<0.01
TNF-α (pg/ml)	31 (0–86)	146 (22–585)	<0.01

Results are expressed as median (IQR). Differences were calculated by a Mann–Whitney test.

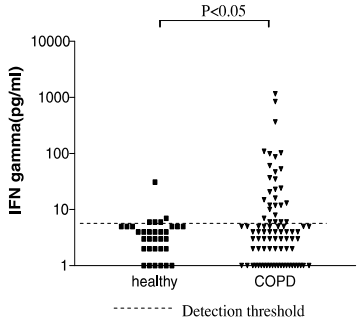

Fig. 1a. IFN-γ released from sputum cells of COPD vs healthy subjects.

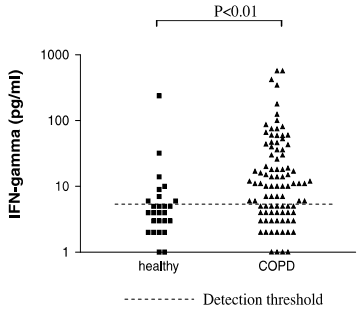

Fig. 1b. IFN-γ released in blood of COPD vs healthy patients.

which were undetectable in the majority of both COPD (80% and 57% respectively) and healthy subjects (70% for both cytokines). The differences for TNF-α, IL-10 and IFN-γ remained significant when cytokine levels were normalized according to blood cell count (data not showed).

4.4. Relationship between sputum and blood cells and cytokines released in sputum and blood

The percentage of sputum neutrophils, lymphocytes and eosinophils were weakly but significantly correlated with their blood counterparts in COPD patients ($p < 0.0001$ and $r = 0.44$; $p < 0.01$ and $r = 0.33$; $p < 0.0001$ and $r = 0.43$ respectively). As for cytokines, we found a weak negative correlation between the TNF-α released from sputum and blood cells in the COPD group ($p < 0.05$ and $r = -0.27$). For other cytokines, no correlation was found between cytokine released at sputum and blood levels in any group of subjects.

4.5. Relationship between cells, cytokine release and disease severity

In COPD, the% predicted post-bronchodilator FEV1 was inversely related to the total sputum cell number ($r = -0.28$; $p < 0.01$) and the proportion of neutrophils ($r = -0.33$, $p < 0.01$) but not to the proportion of eosinophils ($r = -0.08$, $p > 0.05$). Accordingly there was a significant increase in sputum neutrophil proportion with the GOLD stages (Fig. 2). Similarly, we found a relationship between blood cell TNF-α production and disease severity as reflected by the GOLD stages (Fig. 3). Indeed, GOLD stage IV patients exhibited a greater level of TNF-α than GOLD III (542 pg/ml (104–486) versus 172 pg/ml (28–414), $p < 0.05$) and tended to have a greater level than GOLD II (542 pg/ml (104–486) versus 42 pg/ml (13–913), $p = 0.07$).

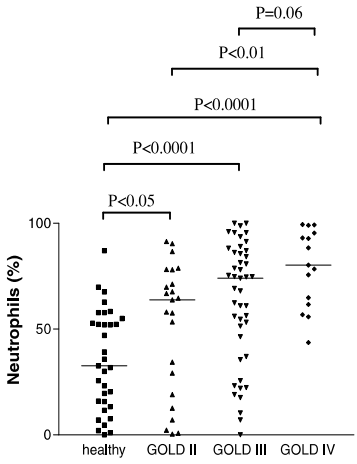

Fig. 2. Sputum neutrophil count according to the disease severity (—: median value).

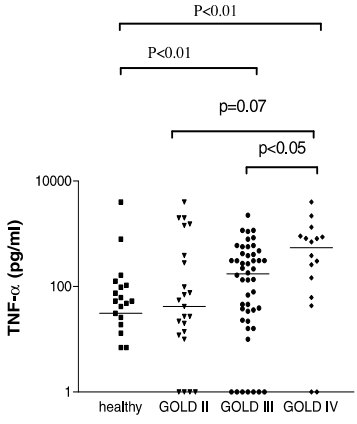

Fig. 3. Blood cell TNF-α production according to disease severity (—: median value).

Table 4
Cytokine release according to age in healthy and COPD patients.

	Tertile 1 Healthy subjects: $N = 11$ COPD patients: $N = 32$	Tertile 2 Healthy subjects: $N = 11$ COPD patients: $N = 32$	Tertile 3 Healthy subjects: $N = 11$ COPD patients: $N = 31$	p-value
Age (years)	Healthy: 26 (21–32) COPD: 51 (37–59)	Healthy: 41 (34–44) COPD: 62 (59–67)	Healthy: 52 (45–63) COPD: 73 (67–82)	<0.0001 <0.0001
Sputum (10e5 cells/well)				
IL-4 (pg/ml) Healthy	0 (0; 0)	0 (0; 0)	0 (0; 0)	0.64
IL-4 (pg/ml) COPD	0 (0; 0)	0 (0; 0)	0 (0; 22)	<0.05
IL-6 (pg/ml) Healthy	44 (0; 479)	31 (23; 461)	22 (0; 216)	0.84
IL-6 (pg/ml) COPD	0 (0; 54)	0 (0; 28)	24 (0; 91)	<0.05
IL-10 (pg/ml) Healthy	47(22; 63)	85 (48; 167)	44 (37; 52)	0.13
IL-10 (pg/ml) COPD	22 (8; 53)	11 (7; 30)	51(8; 80)	<0.05
IFN-γ (pg/ml) Healthy	0 (0; 0)	0 (0; 0)	0 (0; 0)	0.16
IFN-γ (pg/ml) COPD	0 (0; 0)	0 (0; 4)	0 (0; 36)	<0.05
TNF-α (pg/ml) Healthy	2238 (1303; 3633)	2942 (1091; 4084)	2221 (1574; 4033)	0.75
TNF-α (pg/ml) COPD	1132 (577; 2576)	1106 (567; 1769)	1088 (362; 2124)	0.94
Blood (50 μl diluted twice)				
IL-4 (pg/ml) healthy	0 (0; 90)	0 (0; 4)	0 (0; 4)	0.56
IL-4 (pg/ml) COPD	0 (0; 0)	0 (0; 9)	0 (0; 0)	0.31
IL-6 (pg/ml) healthy	0 (0; 17)	0 (0; 14)	0 (0; 10)	0.7
IL-6 (pg/ml) COPD	0 (0; 23)	3 (0; 22)	0 (0; 31)	0.99
IL-10 (pg/ml) healthy	0 (0; 3)	0 (0; 0)	0 (0; 0)	0.51
IL-10 (pg/ml) COPD	5 (0; 14)	2 (0; 20)	2 (0; 14)	0.85
IFN-γ (pg/ml) healthy	0 (0; 0)	0 (0; 20)	0 (0; 9)	0.14
IFN-γ (pg/ml) COPD	12 (0; 45)	3(0; 19)	11 (0; 43)	0.27
TNF-α (pg/ml) healthy	0 (0; 130)	42 (6; 427)	42 (7; 90)	0.47
TNF-α (pg/ml) COPD	347 (17; 745)	135 (27; 606)	113 (22; 386)	0.75

Patients were divided in tertiles according to the age. Age is expressed as mean (range) and other results are expressed as median (IQR). Differences were calculated by a Kruskal–Wallis test.

4.6. Relationship between cytokine release and demographic and treatment characteristics

To take into account the impact of age, we divided our two groups in tertiles (Table 4). In healthy subjects, there was no correlation between age and cytokine release either from sputum or blood cells. In COPD, the production of IL-4, IL-6, IL-10 and IFN-γ was slightly greater in the oldest tertile but there was no impact of age on cytokine production from blood cells. In the COPD group, the body mass index (BMI) appeared to be negatively correlated with TNF-α and IL-6 released from blood cells ($r = -0.3$ and $r = -0.27$ respectively; $p < 0.01$ for both). BMI did not correlate with any cytokine produced from sputum cells. In order to exclude the influence of current smoking on cytokine release, we compared the results in ex or non smokers healthy subjects ($n = 26$) with those seen in ex smokers COPD ($n = 64$). The differences in cytokine release persisted as for the whole groups of COPD and healthy subjects (Table 5). In COPD, TNF-α release from sputum cells was lower in current smokers ($n = 30$) than in ex-smokers ($n = 64$) (678 (483–1119) pg/ml vs 1434 (874–2202) pg/ml respectively; $p < 0.01$).

In COPD no difference in cytokine released either from sputum or blood cells was observed between those receiving inhaled corticosteroids (ICS) and those who were not treated by ICS.

Table 5
Cytokine released in non or ex-smokers healthy subjects versus ex-smokers COPD patients.

	Healthy $N = 26$	COPD $N = 64$	p-value
Sputum (10e5 cells/well)			
IL-4 (pg/ml)	0 (0–0)	0 (0–0)	NS
IL-6 (pg/ml)	60 (14–500)	7 (0–53)	<0.01
IL-10 (pg/ml)	50 (32–74)	25 (8–64)	<0.05
IFN-γ (pg/ml)	0 (0–0)	0 (0–12)	0.07
TNF-α (pg/ml)	2644 (1818–4000)	1328 (788–2202)	<0.001
Blood (50 μl diluted twice)			
IL-4 (pg/ml)	0 (0–8)	0 (0–0)	NS
IL-6 (pg/ml)	0 (0–10)	0 (0–23)	NS
IL-10 (pg/ml)	0 (0–0)	0 (0–14)	<0.01
IFN-γ (pg/ml)	0 (0–0)	10 (0–41)	0.0001
TNF-α (pg/ml)	25 (0–101)	155 (24–650)	<0.01

Results are expressed as median (IQR). Differences were calculated by a Mann–Whitney test.

5. Discussion

COPD exhibited a raised airway neutrophilic inflammation associated with a marked IL-10, IL-6 and TNF-α release deficiency that contrasted with a raised IFN-γ production. Neutrophilic inflammation was also prominent at blood level together with raised production of IFN-γ, IL-10 and TNF-α. Furthermore, we found a significant correlation between the sputum neutrophil count and the disease severity reflected by the GOLD stages. At the systemic level, production of TNF-α also correlated to GOLD stages and inversely to BMI.

Here we used here an original model of cell culture previously validated [13,26]. Our study showed a raised production of IFN-γ from sputum cells of COPD while there was no evidence of overproduction of IL-4, which remained rarely detectable in both groups. Our finding highlights the Th1 profile that prevails in the airway inflammation of COPD. The reason for this IFN-γ production is not clear at present but is likely to reflect ongoing immune response towards microbial agents although arguments also exist to support auto-immune mechanisms [7]. Our results are in keeping with those of Di Stefano et al. who reported greater IFN-γ expression in bronchial biopsies of COPD patients [11].

In contrast to what was found for IFN-γ, the production of TNF-α, IL-6 and IL-10 from sputum cells were reduced in COPD patients as compared to healthy subjects. The reduced production of TNF-α confirmed our previous results [10] and those of reduced IL-6 and IL-10 are novel. We postulate that the lower release of TNF-α might impair local immune defence and could make the subjects more prone to chronic infection while the deficient IL-6 release might make them unable to properly resolve acute airway inflammation following infectious assault [18]. On the other hand, re-

duced IL-10 production extends the finding of a previous study looking at sputum supernatant [38] and may be seen as a deficient anti-inflammatory mechanism.

There may be, however, some discrepancy between the results found in sputum supernatant and those reported here from sputum cell culture. Indeed, there are some, but not all [10,40] reports showing a significant increase of TNF-α in sputum supernatant [19] or BAL fluid [36]. Likewise, some studies reported greater IL-6 levels in sputum supernatant from COPD [4,14] while their sputum failed to release more IL-6 in our study. As for IFN-γ it was reported to be normal in sputum supernatant of COPD compared to healthy subjects [14,42]. It highlights the fact that sputum supernatant and sputum cell culture supernatant are not the same milieu. The difference could be explained by the fact that cellular sources that contribute to the supernatant content are more varied than those contributing in the sputum cell culture model. Supernatant content not only depends on the secretion from airway cells lying within the lumen but also from resident structural cells and in particular from the epithelial layer, which is not strongly represented in the sputum cell culture. In addition, plasma exudation may also contribute to a rise in mediator/cytokine concentration found in the supernatant whereas it does not influence the level measured in sputum cell culture [34]. Finally, another factor that has to be taken into account in the interpretation of our results is the fact that COPD patients display a greater number of cells per gram of sputum which may counterbalance in vivo the reduced production of cytokines per standardized cell number.

Regarding cytokine release from blood cells, there was a clear overproduction of IFN-γ, IL-10 and TNF-α in COPD. The enhanced release of IFN-γ extents the Th1 concept at the systemic level. The majority of COPD patients (61%) produced INF-γ from their blood cells, which was not the case in healthy subjects. Interestingly, we observed that 75% of COPD patients producing IFN-γ from their sputum cells also released this cytokine from their blood cells. Neither the sputum nor the blood production of IFN-γ appeared to be related to disease severity reflected by the GOLD stages. The IFN-γ producers did not distinguish from their counterparts according to their demographic and treatment features. The raised IL-10 and TNF-α release at the blood level contrasts with what was found in sputum. Our result of raised TNF-α production in COPD is in keeping with results from Schols et al. [33]. These cytokine over-productions seen in COPD persisted even after normalization per cell number, which reinforces the finding.

Because we found a relationship between TNF-α release and GOLD stages as well as an inverse relationship between TNF-α and the BMI, our data support an important role for systemic TNF-α in the disease progression and the cachexia often observed in the advanced stages of the disease. It is line with studies showing relationship between losing weight and serum TNF-α in COPD [8,12,15,37]. Alteration of FEV1 values might not be strictly related to change in airway calibre but may also depend on pulmonary elastolysis and reduced respiratory skeletal muscle forces, both processes likely to be influenced by TNF-α [6,15]. Surprisingly, the production of IL-6, which is the most important inducer of acute-phase protein synthesis, was not different from that in healthy volunteers. This might be explained by the fact that our patients were in stable conditions when sampled as there are reports of increased blood IL-6 levels during exacerbations [41].

By contrast to smoking, taking inhaled corticosteroids (ICS) did not seem to change the cytokine release from sputum or blood cell culture as there was no difference between those who were or were not on ICS.

The main limitation of our study is the age difference between our COPD and our healthy controls. Although age may influence the neutrophil cell counts [39], our results showed that the reduced release of IL-6 and IL-10 from sputum cells in COPD can not be accounted for by the age as the youngest were those in whom this reduction was the most apparent. However, we can not rule out a role of age in raised IFN-γ production from sputum cells in COPD even if it was not observed in healthy subjects. Furthermore, no relationship between age and cytokine release from blood cells was found in any of the two groups. This makes us confident that, overall, differences in cytokine release between COPD and healthy subjects are unlikely to be accounted for by the age difference between the two groups.

Another limitation of the study is the mismatch in tobacco habits between healthy subjects and COPD. However, it clearly appeared from our data that ex smokers COPD still markedly distinguished from non or ex-smokers healthy subjects. This highlights the fact that COPD by itself, irrespective of the current smoking status, is characterized by a disturbed cytokine production both at systemic and local level.

As for the cell component of inflammation, our results showed a raise in total sputum cell number linked to higher proportion of neutrophils in COPD patients which is keeping with previous studies [25,30,31]. In addition, we confirmed that the% predicted post bronchodilator FEV1 was inversely correlated to the proportion of neutrophils [23,25,35]. It suggests that neutrophilic inflammation is an important factor in the pathogenesis of irreversible airflow limitation in COPD [5]. On the other hand, high sputum neutrophil count may reflect constant activation of local innate immunity in response to bacterial colonization developing in remodelled and altered airways [20]. Interestingly, although sputum eosinophil count was also increased in COPD but not related to the magnitude of airway obstruction. In previous studies, eosinophilic COPD was found to be associated with asthmatic features such as greater reversibility to β2 agonist, raised exhaled NO [24] as well as sign of mast cell activation [21]. Raised circulating number of leucocytes and mainly of neutrophils was also seen in blood of COPD patients. This observation is in line with previous studies and supports the concept of systemic inflammation [17]. In our study, COPD patients also show raised circulating number of lymphocytes and monocytes per blood volume even if this was less conspicuous than for neutrophils. The raised number of monocytes and lymphocytes is likely to partly contribute to the enhanced cytokine release from whole blood sample of COPD.

6. Conclusions

Our study confirms the neutrophilic inflammation in COPD both at the local and the systemic level. It shows raised local and systemic IFN-γ production highlighting the pertinence of Th1 concept in COPD. A systemic enhanced release of TNF-α is also an important feature of COPD which is related to disease severity and BMI.

Acknowledgements

The authors would like to thank all participants of the study and thank the lung function department of the CHU of Liege for their help in the collection of the data. The study was financially supported by the National Fund for Scientific Research (FNRS, Belgium) and TELEVIE (Grant 7.4.642.09.F).

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