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HIV INFECTION AS AN INDEPENDENT RISK FACTOR FOR THE DEVELOPMENT OF LUNG NEOPLASMS

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Acronyms and Abbreviations

3TC – Lamivudine

8-OHdG - 8-hydroxy-2-deoxyguanosine

ADCs – AIDS-defining cancers

AIDSRE – AIDS-related events

AKT - Protein kinase B

ALK - Anaplastic Lymphoma Kinase

AM – Alveolar macrophages

ARE – Antioxidant Response Elements

ART – Antiretroviral therapy

AZT - Azidothymine

BAL - Bronchoalveolar lavage

CAT – Catalase

CD – Cluster of differentiation

CCR5 - CC-chemokine receptor-5

CI – Confidence Interval

COPD - Chronic Obstructive Pulmonary Disease

CSC - Cigarette Smoke Condensate

CYP - Cytochrome

CXCR4 - C-X-C chemokine receptor 4

EGFR - Epidermal Growth Factor Receptor

FoxP3 - Forkhead box P3

GPx – Glutathione peroxidase

GR – Glutathione redutase

GSH – Glutathione

GSSG – Oxidized Glutathione

HL – Hodgkin's Lymphoma

IFN-γ - Interferon-gamma

IL - Interleukin

KRAS - Kristen rat sarcoma

KS – Kaposi's sarcoma

LC – Lung Cancer

LPS - Lipopolysaccharide

MA - Microsatellite Alterations

MDSC - Myeloid Derived Suppressor Cells

MMP - Matrix Metalloproteases

NADCs – Non-AIDS defining cancers

Nef - Negative Regulatory Factor

NF-kB – Nuclear factor kappa B

NHL - Non-Hodgkin lymphomas

Nrf2 - Nuclear factor (erythroid-derived 2)-like 2

NNRTIs - Non-Nucleoside Reverse Transcriptase Inhibitors

NSCLC - Non-Small Cell Lung Cancer

OR – Odds Ratio

OS- Oxidative Stress

PD-1 - Programmed Death -1

PIs – Protease Inhibitors

PI3K - Phosphatidylinositol 3'-kinase

RANTES – Chemokine Ligand 5

ROS – Reactive Oxygen Species

RR – Relative risk

RT – Reverse Transcriptase

sCD14 – Soluble CD14

SIR - Standardized Incidence Ratio

SODs – Superoxide Dismutases

Tat – Trans-activator of transcription

TB – Tuberculosis

TGF-β1- Transforming growth factor beta-1

Th – T helpers

TNF-α - Tumor necrosis factor-alpha

TREC - T-cell receptor excision circle

Tregs – Regulatory T cells

VEGFR-2 - Vascular Endotelial Growth Factor Receptor-2

Vpr – Viral protein R

Abstract

With the introduction of antiretroviral (ART) regimens, the leading causes of morbimortality of HIV-infected individuals have shifted from AIDS-related events (AIDSRE) to other diseases that are also common in the general population. Infectious and non-infectious lung diseases are still a big burden, with Lung Cancer (LC) currently being the leading cause of death in this population. In this review we will discuss the impact that HIV infection may have on the pathogenesis of LC. HIV seems to be an independent LC risk factor. Although the etiology is certainly multifactorial, several specific mechanisms justify discussion. We will subdivide the HIV infection related risk factors in virus-related factors - effect of immunosuppression and immune dysfunction, HIV-associated lung infections and lung diseases, HIV-associated immunosenescence, Oxidative Stress (OS) and direct oncogenic potential of the virus - and in therapeutic-related factors: impact of ART exposure. Furthermore, we will also assess the effects of smoking on HIV-infected individuals, because other than being the dominant LC risk factor, it has a high prevalence in this population. Further studies must be addressed, because little is known about the exact physiopathological mechanisms that have a role on lung carcinogenesis in seropositive individuals.

KEYWORDS: HIV infection; Lung neoplasms; Immunologic deficiency syndromes; Oxidative Stress; Pulmonary Disease, Chronic Obstructive; Antiretroviral Therapy, Highly Active; Smoking.

Resumo

Com a introdução da terapêutica anti-retroviral, as principais causas de morbimortalidade dos indivíduos com infeção VIH deixaram de ser os eventos definidores de SIDA para passarem a ser outras doenças que também são comuns na população geral. Atualmente, o impacto das doenças pulmonares, infeciosas e não infeciosas, é cada vez maior, sendo o cancro do pulmão a principal causa de morte nesta população.

Nesta revisão vai ser discutido o impacto que a infeção VIH pode ter na patogénese do cancro do pulmão. A infeção VIH parece ser um fator de risco independente para o desenvolvimento destas neoplasias, mas, apesar da etiologia desta relação causal ser claramente multifatorial, há mecanismos específicos que devem ser discutidos. Os fatores de risco associados à infeção serão subdivididos em fatores relacionados com o vírus – efeito da imunossupressão e disfunção imune, doenças e infeções pulmonares associadas ao VIH, imunosenescência, *stress* oxidativo e potencial oncogénico direto do vírus – e fatores relacionados com a terapêutica: impacto da terapêutica anti-retroviral. Além disso, os efeitos do tabaco nos indivíduos infetados por VIH serão também mencionados, visto que, para além de ser o fator de risco dominante para o cancro do pulmão, a sua prevalência é também elevada nesta população. Os mecanismos fisiopatológicos que podem ter influência na carcinogénese pulmonar em indivíduos seropositivos são ainda pouco conhecidos, pelo que serão necessários mais estudos nesta área.

PALAVRAS-CHAVE: Infeção VIH; Neoplasias pulmonares; Síndromes de imunodeficiência; *Stress* Oxidativo; Doença Pulmonar Obstrutiva Crónica; Terapêutica Anti-retroviral; Tabaco.

Introduction

In the pre-ART era, the main causes of morbidity and mortality in HIV infected patients were AIDSRE, especially opportunistic infections and AIDS-defining cancers (ADC), such as Kaposi's sarcoma (KS) and Non-Hodgkin lymphomas (NHL)[1–5]. These patients had a life expectancy much smaller than the rest of the population, since there were no therapies available to limit viral replication and prevent the immunosuppression[6].

The introduction of ART changed the natural course of the disease by preventing the immunosuppression and, therefore, diminishing AIDSRE[1,3,7–12]. With the reduction of the morbimortality caused by these events, HIV infection has become a chronic disease, and the life expectancy for a newly diagnosed seropositive individual is now comparable to that of the general population[1,7]. Considering this, the impact of common causes of death in the general population, such as non-AIDS-defining cancers (NADCs) became greater[1,3–5,9–11,13]. NADCs are a heterogeneous group of malignancies, including lung, liver and anal cancer, Hodgkin's lymphoma (HL), and others.

Currently, LC is the third most common malignancy among HIV infected persons (preceded only by KS and NHL)[12,14,15] and the most common NADC[1,2,6,15–21], being the leading cause of mortality among HIV individuals[2,4,5,8,12,14–17,21]. Although LC is also a significant cause of mortality in general population, it assumes a particular importance in HIV-infected patients, since the incidence is this population can be up to three times greater when compared to non-infected individuals[2,5,22,23].

The reasons for this excess incidence of LC are not completely clarified. Beside the improved life expectancy and reduction of competing causes of death, the higher prevalence in the infected patients of traditional major risk factors for cancer, such as tobacco smoke,

could explain this higher incidence[2,4,5,19]. Nevertheless, even controlling for aging, smoking exposure, or other risk factors, some studies have shown that HIV infection is an independent risk factor[8,17], while others did not found a statistically significant difference[24]. When considering these studies as a whole, HIV infection appears to be an independent risk factor for LC in the HIV-positive individuals[5], but the HIV-associated biological factors that may have a crucial role in lung carcinogenesis are not fully understood. So far, the larger cohort study assessing the independence of HIV infection as a LC risk factor was performed by Sigel K. *et al.* (2012)[17]. In their study, HIV infection maintained an independent association with increased LC risk, even after adjusting for age, gender, race/ethnicity, smoking, baseline COPD (chronic obstructive pulmonary disease) and bacterial pneumonia (IRR 1.7, 95% CI: 1.5-1.9). However, they didn't had data for smoking intensity and duration adjustment.

This systematic review aims to give the current state of knowledge of the main physiopathologic mechanisms by which HIV infection *per se* can increase LC risk. The induced immunosuppression and immune dysfunction, the observed OS, direct oncogenic potential of the virus, the recurrent pulmonary infections that are so prevalent in this risk group or even the increased susceptibility to carcinogens, might explain this high incidence of LC.

Finally, we will also discuss the current knowledge gaps and future perspectives concerning the research of pathophysiological mechanisms of HIV-associated lung malignancies.

Methods

Research in PubMed database with the MeSH Terms: Lung neoplasms, HIV infection, HIV Infections/immunology, Immunologic deficiency syndromes, Oxidative Stress and smoking/adverse effects. The exclusion criteria were: articles published before 2012 and written in other language than English and Portuguese. Final research was made at 9/11/2017.

- ✓ Search nº1: (("2012"[PDAT] : "2017"[PDAT]) AND "lung neoplasms"[MeSH Terms]) AND "immunologic deficiency syndromes"[MeSH Terms] AND (English[lang] OR Portuguese[lang]). Of 136 results, 88 were excluded by the title, 14 excluded after reading the abstract and 32 were selected.
- ✓ Search nº2: (("2012"[PDAT] : "2017"[PDAT]) AND "hiv infections"[MeSH Terms]) AND "Oxidative Stress"[MeSH Terms] AND (English[lang] OR Portuguese[lang]). Of 89 results, 79 were excluded by the title and after reading the abstract were selected 10.
- ✓ Search nº3: "Smoking/adverse effects"[Mesh] AND "HIV Infections/immunology"[Mesh] AND (("2012/01/01"[PDAT] : "2017/12/31"[PDAT]) AND (English[lang] OR Portuguese[lang])). Of 16 results, 11 were excluded by the title and after reading the abstract 5 were select.

These three searches found a total of 47 articles. After excluding repeated results, 46 articles were selected. During the curse of this systematic review, studies that were referred in the analysed studies and were considered relevant and important to support evidences were also included.

HIV-related immunodeficiency

Contrarily to the ADCs, such as KS and NHL, where the risk of developing cancer is inversely related to the $CD4^+$ count, NADCs development can take place at any point of HIV infection[15]. Currently, there are no conclusive results whether immunodeficiency might influence the risk of LC.

Several studies have failed to demonstrate a relationship between the degree of immunodeficiency and $CD4^+$ T-cells count and the risk of developing cancer, but there are contradictory results (**see table 1**).

Favoring the hypothesis of immunosuppression being a causative factor of the excess LC incidence in HIV positive patients, we have the similarities in the rates of incidence with other immunosuppressed groups[16,23]. In fact, a meta-analysis of seven studies published between 2002 and 2006, including 1.297 LC cases, showed that the LC Standardized Incidence Ratio (SIR) among HIV infected patients was 2.72 (95% CI 1.91-3.87), while the SIR in immunosuppressed solid organ transplant recipients was 2.18 (95% CI 1.85-2.57)[15]. In the other hand, Hleyhel M. *et al.*[22] observed that the incidence of LC in patients with HIV-infection under ART and with $CD4^+$ counts over 500 cell/µl was similar to the incidence in general population **(see table 1)**. These evidences that LC risk diminishes after $CD4^+$ cell recovery can be explained by the reduction of pulmonary inflammatory markers at the lung, caused by the uncontrolled virus replication, and also by reduction of pneumonia episodes[22].

Table 1. Overview of the relationship between LC and immunosuppression

Author, year, reference	Design / population	LC cases in PLWH	Main findings	Other important findings
Frisch M. *et al.*, 2001[20]	Cohort study 302.834 HIV+ individuals	n= 808	RR of LC was the lowest in the distant pre-AIDS diagnosis period: 1.2. In the recent pre and post AIDS diagnosis periods the RR were 2.7 and 2.8.	LC was considered to have potential association with immunosuppression.
Guiguet M. *et al.*, 2009[25]	Cohort study 52.278 HIV+ participants	n=207	RR of LC was 2 times higher for individuals with $CD4^+$ counts of 350-499 cells/µl, and 8.5 for $CD4^+$ counts under 50, comparing to those with >500 cells/µl. The risk of LC increased as the $CD4^+$ count fell.	The association between immunosuppression and increased LC risk was still significant after adjusting for smoking.
Sigel K. et. al., 2012[17]	Cohort study 37.94 HIV+ patients, 75.750 HIV- patients	n=457	No significant differences between the median baseline $CD4^+$ count and median nadir $CD4^+$ of HIV+ patients with/without LC (p-values of 0.6 and 0.5, respectively). Previous diagnosis of bacterial pneumonia was associated with greater LC risk (IRR 1.5, 95% CI: 1.1-2.0).	Surprisingly, there was a significant difference in the baseline median HIV RNA, being lower in those diagnosed with LC (p=0.001).

Table 1. (cont.)

Author, year, reference	Design / population	LC cases in PLWH	Main findings	Other important findings
Clifford *et al.*, 2012[23]	Case-control study 68 LC cases and 337 non LC HIV+ controls	n=68	No proven association between HIV-related immunodeficiency and LC risk, including nadir $CD4^+$ cell count, median $CD4^+$ or $CD8^+$ counts or higher viral load, even after adjusting for smoking.	Borderline statistical significance between a $CD4^+/CD8^+$ ratio inferior of 0.25 within a year of LC diagnosis and LC risk, even after controlling for smoking (OR= 2.12 95%CI 0.94-4.77).
Petoumenos *et al.*, 2013[26]	Cohort study 2.181 HIV+ patients	n=6	Both low $CD4^+$ cell counts and prior AIDS diagnosis were associated with greater risk for all incident NADCs, but only a prior AIDS diagnosis was considered an independent predictor of NADCs.	LC was the second most frequent NADC, preceded by melanoma (n=10).
Hleyhel *et al.*, 2014[22]	Cohort study 84.504 HIV+ patients	n=763	Risk of LC in individuals with $CD4^+$ cell recover under ART (at least 500 $CD4^+$ cells/µl for two years) is close to the general population (SIR=0.9, 95% CI 0.6-1.3).	

Table 1. (cont.)

Author, year, reference	Design / population	LC cases in PLWH	Main findings	Other important findings
Hessol *et. al.*, 2015[24]	Two cohort studies: 2.549 HIV+ women (WIHS), 4.274 HIV+ men (MACS)	WHIS: n=31 MACS: n=15	In the WHIS study there wasn't an association between current $CD4^+$ and LC incidence. In the MACS study, there was a significant association between a higher incidence of LC and lower $CD4^+$ T-cell count (p=0.047 for <200cell/µl), higher peak HIV RNA levels and a prior diagnosis of AIDS.	A prior diagnosis of AIDS pneumonia was an independent risk factor both for WHIS and MACS study (in the ART era (1995-2011) IRR=3.51, 95% 1.61-7.67).
Bruyand *et al.*, 2015[27]	Case-control study 1.447 HIV+ patients	n=382	Association between a low $CD4^+$ cell count nadir and higher risk of LC.	
Sigel K *et al.*, 2017[21]	Cohort study 21.666 HIV-positive patients	n=277	Increased LC risk in individuals with higher cumulative episodes of bacterial pneumonia and for $CD4^+/CD8^+$ ratios below 0.4 or 0.4-1.0, when compared to >1.0 (p=0.001). These two factors were considered to be the most robust predictors of LC risk.	Higher HIV RNA levels and cumulative exposures of $CD4^+$ counts of 100-199 and 200-500, when compared to ≥500 cell/µl were associated with a greater risk (p=0.001).

LC: Lung cancer; PLWH: people living with HIV; RR: relative risk; CI: confidence interval; IRR: incidence rate ratio; OD: odds ratio; SIR: standardized incidence ratio; WHIS: Women's Interagency HIV Study; MACS: Multicenter AIDS Cohort Study.

HIV-associated lung infections

Lung infections, such as bacterial, tuberculosis (TB) and *Pneumocystis* pneumonias, are particularly important in HIV-infected individuals, because, in addition to being more frequent in this population[8,28], the inflammatory response to serious lung infections can be a stimulus for LC[4,5,19]. Indeed, the outcomes of lung infections in HIV-positive individuals are thought to have a more deleterious effect due to an aberrant inflammatory response with tissue damage[4,9], as it will be discussed later.

HIV-infected population is particularly prone to bacterial pneumonias[7,21,29]. The community-acquired pneumonia correlates inversely with $CD4^+$ cell counts[28], and the risk of developing it is estimated to be between two- to five-fold in HIV-positive patients[3]. Even in HIV-positive individuals with over 500 cells/µl, the incidence of pneumonias is six-times higher than in non-infected controls[5].

TB has been considered the most important opportunistic infection in HIV-patients in sub-Saharan Africa and other areas of the developing world[3], and has been previously considered a risk factor for LC[4,9]. The treatment of TB itself may be implicated in the pathogenesis of LC, as it can induce severe pulmonary inflammation, pulmonary fibrosis and augmented production of tumor necrosis factor-alpha (TNF-α), due to its long time of treatment[9]. However, a recent study[17] revealed that, in HIV-infected patients, there wasn't an epidemiological difference in a previous TB diagnosis between individuals with and without LC.

Pneumocystis pneumonias are also one of the most frequent opportunistic infections in HIV-positive patients, typically appearing when $CD4^+$ counts are lower than 200 cell/µl[8]. The lung damage that advents from this infection is more associated to the degree of

pulmonary inflammation than the direct effect of *Pneumocystis*[8]. It was found that HIV-infected individuals have higher rates of colonization of *Pneumocystis jirovecii*[3] and that patients with LC have even higher rates; in fact, there are reports of a colonization rate of 100% in individuals with small-cell lung cancer[3]. This chronic colonization with *P. jirovecii* has also been implicated in the development of HIV-related COPD, which could mean a potential link between lung infections and a LC precursor state[4]. Although Sigel K. *et al.* (2012)[17] found that baseline diagnosis of *Pneumocystis* pneumonia didn't differ statistically between patients with HIV infection that developed LC *vs.* the ones that didn't, there are some case-control studies that have reported an association between *Pneumocystis jirovecii* pneumonia and higher LC risk[9].

In short, despite the fact that HIV-positive patients are under ART and with low levels of immunosuppression, they are still particularly susceptible to lung infections. The mechanism that renders these individuals vulnerable is still unclear, but one possibility is the increased OS within the alveolar space[30], explored later.

According to the results of their most recent study, Sigel K. *et al.* (2017)[21] supposed that the immunosuppression may be linked to a higher risk of LC through the risk of developing bacterial pneumonia, as in their mutually adjusted model, bacterial pneumonia remained a significant risk factor ($p=0.004$), and $CD4^+$ didn't ($p=0.10$)[21]. As a matter of fact, in another study by Sigel K. *et al.* (2012)[17], they also noticed that bacterial pneumonia was more prevalent in the infected individuals and that the HIV positive patients with LC were more likely to have had a previous diagnosis of a bacterial pneumonia than those HIV-positive but without LC ($p=0.01$)[17]. Furthermore, in an analysis combining the WHIS and MACS participants[24], a prior AIDS pneumonia diagnosis was found to be an independent

risk factor for a higher LC incidence. The authors considered that two-thirds of the effect of HIV infection were explained by a diagnosis of a prior-AIDS pneumonia, corroborating previous results which found that, even after adjusting for age, race, gender, HIV transmission mode, $CD4^+$ count and AIDS diagnosis year, HIV-positive patients with recurrent pneumonias have a significantly higher LC risk than those without this history[24].

Other previous studies have also found a contribution of prior pulmonary infections to LC risk in general population[1].

Immune dysfunction and pulmonary chronic inflammation

HIV enters the cells via the CD4 receptor, CC-chemokine receptor-5 (CCR5) – found in microglia, T-lymphocytes, macrophages and dendritic cells – or via C-X-C chemokine receptor -4 (CXCR4), used as a co-receptor to enter preferentially T-cell lines[7].

The early stages of the infection are characterized by a substantial depletion of $CD4^+$ cells and CCR5-expressing memory T-cell population of the gut[29], with T-helper (Th) 17 cells being particularly susceptible[3].

The immunity of the lung seems to be intact in the early curse of the infection[9], mainly due to the resilience to HIV of airway Th1 and Th17 cells and to the high quantity of large alveolar macrophages (AM), instead of small macrophages that are preferentially infected by HIV[3,7]. Beside the resistance of alveolar cells to the virus, there is also an inferior quantity of secondary lymphoid tissue susceptible to the HIV than in the gut and the pulmonary immune mechanisms are also more effective: high prevalence of mucosal HIV-specific

polyfunctional $CD4^+$ cells and an efficient response by HIV-specific $CD8^+$ cytotoxic T-cells[3,9].

Nonetheless, at some point of the infection, the immune defenses progressively deteriorate and start to fail to control the evasion of HIV[3,9]. In the cytosol of $CD4^+$ cells occurs an accumulation of incomplete HIV reverse transcripts that triggers a powerful inflammatory response that culminates in pyroptosis, a cell-death mediated by intense inflammation[3]. Also, the prolonged exposure of $CD4^+$ T-cells to HIV-proteins Vpr (Viral protein R) and Tat (trans-activator of transcription) could lead to apoptosis, mediated by several mechanisms, including programmed death (PD)-1[3]; in fact, the HIV-specific T-cells of the lung can remain elevated, comparing to other anatomic regions, but display a dysfunctional phenotype associated with increased expression of PD-1[3]. On top of this, there is also a massive destruction of HIV-infected $CD4^+$ T-cells by the HIV-antigen specific cytotoxic T-cells, which translates in an intense-pulmonary alveolitis[3]. All of this contributes for a low $CD4^+/CD8^+$ ratio. The impaired function of alveolar $CD4^+$ cells translates in inferior antigenic specific $CD4^+$ T-cells against *Streptococcus pneumonia* and *Mycobacterium tuberculosis*[28], rendering these subjects susceptible to infections as stated before. The follicular T-helper cells that are found in the mediastinal pulmonary lymph nodes are also extremely susceptible to the virus; their permissiveness guarantees a major reservoir for the virus, while altering the production of specific antibodies[3].

If HIV downregulates the receptors of the host, it may evade immune surveillance and hide in the cells; the HIV protein Nef (negative regulatory factor) has an important role in this pathogenesis, by downregulating the major histocompatibility complex-1 (MHC-1) and CD4 receptors[7]. This CD4 downregulation can also enhance viral replication in T-cells[7].

The lung itself can function as an anatomic reservoir for HIV, mainly due to their high content in macrophages[7,9]. Macrophages and dendritic cells are long-lived myeloid cells and, together with memory T-lymphocytes, are potential cellular sources of HIV, as they can remain transcriptionally silent for long periods of time, while having integrated copies of the HIV genome[7,31]. It is thought that the alveolar lymphocytes are more susceptible to the virus than macrophages[7,32]. The reservoir is reseeded when these cells are activated, as they start to produce new infectious particles, which guarantees the perpetuation of viral replication[7].

During the setting of the reservoirs, HIV acts as a silent pathogen in the lung. The simple presence of HIV virions and/or proteins compromises the innate immunity of the lung[15], with diminished bronchoalveolar T-cells and macrophage immune responses[7]. Analyses of bonchoalveolar lavage (BAL) fluid of infected individuals confirmed decreased innate and adaptative immune responses, such as lymphocytes, activation of complement and humoral responses[7].

It is important to refer that these reservoirs are not affected by classical antiretroviral drug regimens, which explains the reappearance of viral particles shortly after interrupting ART, even after an apparent clearance of the virus in the blood-stream[7,9,31]. Indeed, a study evaluated the bronchoalveolar cells of HIV-positive patients, and found levels of HIV proviral DNA 7,6 times higher than those of the peripheral blood cells[9].

Although the hallmark of HIV immunosuppression is considered to be the destruction of $CD4^+$ T-cells, the virus has many other implications in immune system cells.

The infection of the macrophages originates a massive release of inflammatory mediators in the alveolar space[9], including pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-18, TNF-α and interferon-gamma (IFN-γ)-inducing protein 10[33]. TNF-α

and IL-18 induce viral replication, even after prolonged ART[34]. Beside this, some of these inflammatory markers could also have a role on LC risk. Apart from being found elevated prior to the LC diagnosis[19], a study with more than 5.000 HIV-positive participants has observed an association between increased levels of IL-6 and LC incidence[4,7]. AM also have impaired bacterial phagocytosis and decreased antifungal activity, predisposing the individual to co-infections[7]. Lysozyme, immunoglobulins and chemokine ligand 5 (RANTES) are increased as well in BAL fluid[7,15].

During the evolution of the infection there is also an augmented production, which is thought to be ROS-mediated[33], of transforming growth factor beta-1 (TGF-β1), an immunosuppressive cytokine[3,9]. This cytokine is intended to oppose the chronic immune activation, but ends contributing to the HIV-related immunosuppression through the induction of Forkhead box P3 (FoxP3)[3]. FoxP3 is a transcription factor that in addition to inducing the differentiation of $CD4^+$ T-cells into $FoxP3^+CD25^+$ immunosuppressive regulatory T-cells (Tregs)[33], promotes the deposition of collagen and fibrosis in the secondary lymphoid tissues, causing a disruption in T-cell colonization and function[3,9]. Surprisingly, even in individuals receiving ART it's possible to detect elevated levels of TGF-β1, which reflects the presence of ongoing chronic immune activation[3,9], which cannot be fully controlled so far. This immunosuppression mediated by TGF-β1 is particularly important, as this cytokine was previously implicated in the pathogenesis of LC and other types of cancer[3,9].

The importance of $FoxP3^+$ Tregs and $CD8^+$ lymphocytes in tumor growth was studied by Ganesan *et al.*[35]. They analysed the immune phenotype of Non-Small Cell Lung Cancers (NSCLCs) and found a clear predominance of lymphocyte infiltration, including $CD4^+$Tregs

and cytotoxic $CD8^+$ T-cells. These latter cells infiltrate lung tumor tissue and are critical to limit its growth; in turn, they are inhibited by $CD4^+FoxP3^+$ Tregs. So, whereas a higher Tregs predominance is associated to a poor prognosis, $CD8^+$ has shown an improved outcome. An inhibition of $CD4^+FoxP3^+CD25^+$ Tregs by an anti-CD25 monoclonal antibody was proven to reduce significantly the tumor burden, due to a high infiltration of $CD8^+$ cells. These findings prove the role of $CD8^+$ cells in limiting tumor progression and Tregs in inhibiting the anti-tumor activity of $CD8^+$ T-cells[35].

Another important process that happens during the acute phase of HIV primary infection is the microbial translocation. It consists on the disruption of gut integrity, with passage of pro-inflammatory bacterial products, such as DNA and bacterial Lipopolysaccharide (LPS) through the gut to extra-intestinal sites, increasing the already ongoing immune activation/inflammation[7,33,36]. Both monocytes and neutrophils are exposed to chronic inflammation processes, mostly due to the virus itself and its proteins, but also by this process of microbial translocation[3]. Indeed, plasma soluble CD14 (sCD14) is a marker of microbial translocation because traduces the LPS-induced monocyte activation[36]. Monocytes from HIV infected individuals also produce higher levels of hydrogen peroxide, which can promote the production of pro-inflammatory cytokines[33], and subsequently contribute to T-cell apoptosis and dysfunction[3].

Furthermore, it was shown that the HIV protein Gp120 stimulates the immunosuppressive $CD33^+CD14^+$ Myeloid Derived Suppressor Cells (MDSC), which can downregulate the production of INF-γ by activated T-cells[33]. This process is ROS-dependent, which means it can be restored once the OS is also controlled[33]. Another study by Vollbrecht *et al.*[37] analyzed both phenotypes of MDSC (CD14 positive and negative) in the curse of HIV

infection and, despite not showing a significant association between HIV-infection and the levels of MDSC $CD14^+$, found some interesting results relatively to the $CD14^-$ lineage. Comparing the levels of these cells in HIV-infected patients to healthy controls and to individuals with NSCLCs, they discovered that the HIV-infected individuals ART-naïve had significantly higher levels of $CD33^+CD14^-$ MDSC than healthy controls. Beside this, there was a negative correlation between the levels of MDSC $CD14^-$ and $CD4^+$ counts, with individuals with $CD4^+$ counts higher that 500 cell/µl having lower levels of MDSC that those with $CD4^+$<200 cell/µl. There was also observed a positive correlation of MDSC $CD14^-$ levels with the viral load and the immune activation, measured by the CD38 expression on both bulk and HIV-specific $CD8^+$ T-cells. All of this was proven to be controlled by ART, with individuals under ART having lower MDSC than individuals ART naïve. Of note, these MDSC are thought to have a role on the impaired T-cell responses[37].

Nef protein can also activate the transcription nuclear factor kappa B (NF-kB) and induce the release of pro-inflammatory cytokines[12], contributing to the altered immune function during the course of the infection.

In summary, chronic inflammation is caused by HIV itself, and exacerbated by lung infections. Markers of inflammation have been previously linked to LC[5,7,13,16]. It is thought that this continuous immunosuppression, chronic immune activation and inflammation can cumulatively promote carcinogenesis[2] and uncontrolled tumor growth[5].

The low ratio of CD4/CD8 count is also thought to be intrinsically associated with the immune dysfunction and chronic inflammation observed in the HIV-positive patients, and was considered a robust predictor of LC risk, as we've seen before[21]. Finally, CXCR4 and its natural ligand are associated with malignancies, including NSCLC; however, it's still

necessary further studies to confirm if the interaction of HIV with CXCR4 is, indeed, linked to development of LC[7].

HIV-associated immunosenescence

Aging, or organismal senescence, can be defined by a reduced ability to respond to stress and maintain a homeostatic balance, allied to an increased risk of developing age-related diseases[38]. HIV-infection is linked to several of these age-related diseases that, other than being more prevalent in infected individuals, appear at all ages, which suggests that HIV is associated with accelerated aging[38].

Several studies concerning the HIV-associated immunosenescence have proven that the aging phenotype of immune markers of non-infected individuals is similar to the one found in much younger HIV-infected individuals[39].

It is known that age and HIV infection reduce the number of naïve B-lymphocytes and naïve T-cells ($CD4^+$ and $CD8^+$)[29]. Although HIV can reduce the levels of T-lymphocytes to those typically found in non-infected individuals 20-30 years older, the difference between the quantities of naïve $CD8^+$ T-cells between HIV-positive older adults and non-infected age-matched individuals is not statistically significant. Furthermore, in aging uninfected individuals, the number of $CD8^+$ cytotoxic lymphocytes decreases, but increases in infected persons, both in young and old adults. Also, the repeated activation of these cells leads to a loss of cell surface markers CD28 and CD27 that indicate replicative senescence. The senescent phenotype - verified both in HIV-infected and older individuals - consists on low CD28 expression, short telomeres, low proliferative capacity and induction of inflammatory

cytokines in $CD8^+$ lymphocytes, associated with early mortality or faster progression to AIDS in infected individuals[29].

Rickabaugh *et al.*[40] compared the two subsets of naïve $CD4^+$ T-cells –T-cell receptor excision circle (TREC) high ($CD31^+$) and TREC low ($CD31^-$) - between HIV-infected and uninfected individuals; both seropositive/seronegative groups included a younger and an older subset of participants. They found that aging and HIV-infection were associated with a depletion of $CD31^+CD4^+$ T-cells, and, surprisingly, the levels of these cells in young HIV-positive individuals, with 20-32 years, were very close to those found in older HIV-negative participants, with 47-60 years, indicating a cumulative effect of HIV on the aging process. Another interesting finding was the depletion of $CD31^-CD4^+$ T-cells that was verified only in the HIV-infected individuals: young seropositive individuals had 2.9 times less $CD31^-CD4^+$ T-cells than young seronegative individuals ($p=0.007$). This depletion was shown to be independent of age: no significant difference between young and older groups of infected participants ($p=0.9455$). Also, young and older groups of seronegative participants presented normal levels of these cells, proving that the effects of HIV-infection on these subset of cells are distinct from the ones provoked by physiological aging. Furthermore, both HIV-infection and aging were associated with telomere shortening in the two subsets of $CD4^+$ cells, for young and older individuals of both seropositive and seronegative groups. In none of the naïve subsets of $CD4^+$ cells was found an interaction between age and HIV-infection ($p=0.9564$ for $CD31^+$ and $p=0.5493$ for $CD31^-$), which proves that these effects are additive, not synergic. This study also evaluated the effect of ART on $CD4^+$ naïve T-cells. No difference was found between the $CD31^+CD4^+$ cells in infected individuals after 2 years of ART and non-infected controls ($p=0.2670$), which means that ART can successfully

reconstitute these cells to normal levels. However, ART didn't have any impact on $CD31^-$ $CD4^+$ cells: even after two years of ART, infected and non-infected controls continued to show a significant difference in the biodisponibility of $CD31^-CD4^+$ cells ($p=0.0022$). Importantly, the homeostasis of naïve $CD4^+$ T-cells is maintained by the proliferation of $CD31^-CD4^+$ naïve cells, and not by $CD31^+CD4^+$ naïve T-cells (recent thymic emigrants). If HIV-infected individuals have a depletion of this subset of cells, that cannot be reconstituted with ART, this has implications on the quantity of naïve $CD4^+$ cells available to be recruited to the effector/memory pool and on the response to neoantigens. Other than that, telomere shortening is associated with decreased proliferative capacity, which means that T-cells have deficient immune responses to infections; beside the normal aging process, this is aggravated by HIV-infection[40].

In short, all of this findings indicate that HIV-positive patients experience an accelerated immunologic aging, putting them at an increased risk for cancer[39]. This is congruent with the age of diagnosis of LC in HIV-positive individuals. Compared to the expected rate for the age-adjusted general population, the curve of LC incidence in HIV-positive patients is left-shifted[39], which means it its diagnosed earlier than general population[2,4,7–9,12,15,16,18,19,22].

Data review of 19 studies which covered periods between 1983 and 2010 point out that the average age of LC diagnosis is 70 years in general population and between 38 and 57 years for patients with HIV infection[5]. However, other studies refer a slight inferior difference in the age of diagnosis: Mena. A. *et al.*[9] observed mean ages of diagnosis in seropositive patients between 45 and 50 years, compared to the 62 years old of general population. Staitieh and Guidot[6] describe a mean age at diagnosis of approximately 48 years

for HIV-positive patients, and approximately 60 years for the HIV-negative patients. Furthermore, Palacios R. *et al.*[14] found a mean age of 48 years at the time of diagnosis of LC for infected participants and 66 years for non-infected individuals. There are even reports of smaller differences: 50 years for infected and 54 for uninfected patients[15]. In summary, the early age of LC diagnosis in HIV-positive patients is consistently observed.

It is consensual that advanced age is an established independent risk factor for LC in the general population and also in HIV-infected patients[4,5,12,17,21,26,39]. A study observed a 28-fold increase in LC incidence in infected individuals with ages>60 years, compared to adults under 30 years[5]. As the overall survival of individuals with HIV-infection is increasing, part of the excess incidence of LC in this population can be explained by the normal aging process, but it is necessary to take in account the additive effects of HIV-associated immunosenescence, which accelerates the aging process.

HIV-associated non-infectious diseases

Chronic lung diseases, such as COPD and asthma are associated with lung inflammation and can contribute to the LC risk[9]. COPD consists in a preventable and irreversible limitation of expiratory airflow, and currently is the fourth leading cause of death worldwide[7,12]. Emphysema is included in the COPD spectrum and it is characterized by apoptosis of epithelial and alveolar cells, with destruction of lung parenchyma and with several degrees of inflammation[7,28]. Asthma is characterized by an airway inflammation with a reversible/inducible airflow limitation[6,28].

Although most studies have shown that COPD is more prevalent in HIV-infected individuals [7,9,11–13,41] (including a study with almost 100.000 participants, infected and non-infected)[11], findings of Sigel K. *et al.*(2012)[17] in their study with over 113.000 participants (both HIV-infected and non-infected) observed that baseline COPD prevalence did not differ by HIV status (p=0.15). Furthermore, since the major risk factor for developing COPD is tobacco exposure[6,7,12,28,42], the high prevalence of smoking in this population can explain part of the excess burden[12].

George MP. *et al.*[43] found that the obstructive pattern on spirometry low FEV1/FVC ratio, and subsequent airflow obstruction, was independently associated with the pack-year smoking history (p<0.001), but also with bacterial pneumonias (p=0.007) and, surprisingly, to ART use (p=0.04). Gingo MR. *et al.* (2010)[44] also found ART to be an independent risk factor for irreversible airway obstruction, together with pack-year (tobacco exposure) and use of intravenous drugs. Other studies have also observed abnormal spirometry results, including airflows declines in both smokers and non-smoker under ART[6]. However, some studies have shown that undetectable viral loads and use of ART are protective factors[13], while others have observed that, with the introduction of ART, there was no change in the severity of COPD in these patients[12]. The mechanism by which ART can cause airway obstruction is still unknown[12].

There are also evidences that low $CD4^+$ counts and *Pneumocystis jirovecii* colonization are risk factors for airflow limitation[12,13]; the latter has been implicated in the pathogenesis of COPD in both infected and uninfected individuals[8,12]. We've seen that HIV-positive patients have higher rates of *Pneumocystis* pneumonias and colonization[3,28]. Associated to this, patients with COPD have higher colonization rates with *P. jirovecii*[3].

Studies in animal models have shown that this colonization may increase the lung susceptibility to the development of emphysema[6]; in fact, both pneumonia and colonization by *Pneumocystis* have been associated to an accelerated development of emphysema[8,13]. In terms of the contribution of immunosuppression, Risso K. *et al.*[41] found that low $CD4^+$ cell counts and low nadir $CD4^+$ counts were significantly associated with COPD (p=0.001 and p=0.007, respectively), but, in a multivariable analysis, only $CD4^+$ counts remained an independent risk factor for COPD (p<0.001). Of note, they did not find any correlation between ART exposure and COPD[41].

High viral loads also seem to have an impact[6,12] and were even independently associated to the development of COPD[3]. Individuals with high viral loads and low $CD4^+$ counts present a faster decline in FEV1 values, when compared to patients without advanced HIV disease or non-infected[42]. Infected individuals with over 200.000 copies/mL have a 3.4 fold increase of developing COPD compared to non-infected individuals[28].

The pulmonary immune dysfunction of the infected patients is another factor that deserves attention. Pro-inflammatory markers of monocyte activation, such as sCD14, are higher in infected individuals with COPD, when compared to non-infected COPD patients[4]. Beside this, macrophages of these individuals seem to produce higher levels of Matrix Metalloproteases (MMP) that can degrade the extracellular matrix, damaging lung tissues and contribute to the development of emphysematous areas[6]. The upregulation of MMP is even higher in HIV-positive smokers, comparing to HIV-positive non-smokers[7], showing a cumulative effect of smoking and HIV infection in the development of emphysema. Barjaktarevic *et al.*[45] have studied the impact of IL-23 in the upregulation of metalloproteinases. IL-23 is a Th17 related cytokine, which is augmented in HIV-positive

individuals, independently of smoking status; *in vitro* studies have shown that infected AM have an expression of IL-23 10-times higher than uninfected AM. Exposing AM or lymphocytes to IL-23 showed an upregulation of MMP-9 ($p<0.01$), promoted by an interaction between AM and T-lymphocytes. Other cytokines, such as IL-1β, TNF-α and IL-6 – which we've seen to be elevated in HIV-positive patients – are also inducers of MMP-9[45]. This relationship between emphysema and MMPs was also observed by Yearsley *et al.* (2005)[46], which noticed that the expression of MMP-9 was more prominent in the emphysematous areas of lung tissue of HIV-positive individuals.

Finally, the cytotoxic T-lymphocytes can also contribute to COPD pathogenesis[3]. In animal models it was observed that $CD8^+$ lymphocytes – which are elevated during the alveolitis – produce INF-γ and may promote the development of emphysema[8]. Analyses of BAL fluid of HIV-positive individuals showed higher levels of cytotoxic lymphocytes in those with emphysema, compared to the patients without emphysema[12,46]. In short, individuals with COPD have an abnormal accumulation of $CD8^+$ T-cells in the lung[7,12,13,19] and its severity is directly correlated to the degree of CD8 infiltration in the lungs[4]. *Pneumocystis* infection may contribute to the development of emphysema/COPD due to the strong cytotoxic T-lymphocyte response that incites, or also due to the induction of macrophage activation and formation of ROS provoked by their cell-wall component β-glucans[12].

All of these factors intrinsically related to the HIV-infection and immune dysfunction support the evidence that HIV infection is independent-risk factor for COPD[3,11,13,28,42]. This relationship remains significant even controlling for smoking[7,41], and a worse control of the infection is associated to a higher risk[11,13,28]. Indeed, a longer duration of HIV infection has

a high association with airflow obstruction[11]. HIV-positive patients appear to have an accelerated decline in lung function[11] and this HIV impact remains evident in non-smokers[7,8,12,13,28]. Actually, in one study, HIV non-smokers presented a higher obstructive pattern in spirometry than controls non-HIV smokers[8].

It is still uncertain whether the impact of HIV on COPD is mediated by HIV associated events, such as colonization of infection by pathogens or by a direct effect of the virus[28]. Supporting this last hypothesis, it was found that the emphysematous regions of the lung were precisely the areas with the highest HIV viral loads; this finding, allied with the absence of HIV-positive cells in normal lung areas, may indicate a direct pathogenic role of the virus or its proteins in emphysema[46]. However, it is still unclear whether HIV infection increases the susceptibility to COPD or accelerates the course of the disease[3] .

Popescu *et al.*[42] compared the immune markers of 27 HIV-infected individuals with and without COPD, and also with 7 HIV-negative patients with COPD. They found that HIV-positive participants with COPD had a marked lung mucosal $CD4^+$ cell depletion and impaired HIV-specific $CD4^+$, but not $CD8^+$, T-cell immunity. It was also demonstrated that this $CD4^+$ depletion was mediated through the Fas death receptor, which was elevated; the pro-apoptotic marker programmed cell death protein (PD)-1 levels were also augmented. Surprisingly, between HIV-positive patients with and without COPD there wasn't any statistical difference between viral loads levels, $CD4^+$ counts or ART exposure, opposing to the results of some other studies. Finally, also a decreased CD4/CD8 ratio was associated with a faster FEV1 decline, despite the preservation of $CD8^+$ levels in the participants with HIV-infection and COPD[42].

COPD is an independent LC risk factor in non-infected individuals and it also appears to be for HIV-positive persons[4,17,19]. In fact, a large cohort study with over 37.000 HIV-positive patients has found that COPD diagnosis was associated with an increased LC risk (IRR 1.9, 95% CI 1.5-2.3)[17]. However, they didn't found any difference in LC risk according to baseline COPD status[17]. Sigel K. *et al.*(2017)[21] also found that patients with LC were more likely to have been diagnosed with COPD than those who didn't developed LC. Currently, there aren't studies assessing the relationship between COPD severity and LC risk in HIV-infected individuals[19]. It is also important to refer that although HIV-infected patients have higher rates of *P. jirovecii* colonization and that this is implicated in the COPD pathogenesis, baseline diagnosis of *Pneumocystis* pneumonia was not different between HIV-infected patients with and without LC[17].

Even though the relationship between HIV and emphysema is clearer than with asthma[6], there are still some reports of higher rates of asthma incidence in HIV-infected individuals than in the general population[6], with some patients developing asthma in adulthood, often only after becoming seropositive[3,28]. Chemokine RANTES is elevated in HIV-infection, as we've seen before, and has also been implicated in asthma[6,28].

HIV-associated asthma is important for the carcinogenesis hypothesis, since it has been implicated to have a role in the development of LC. In the WHIS cohort with 2.549 HIV-positive women[24], history of asthma was independently associated with LC incidence (p=0.01). There are also more evidences that seropositive patients with bronchial asthma are at high-risk of developing LC[5,16], but the reports that link HIV-associated asthma and LC are very scarce.

Oxidative Stress

1) What is OS and which are the major endogenous redox systems?

The OS can be defined by the imbalance between the oxidant and antioxidant pathways, with accumulation of Reactive Oxygen Species (ROS) that cannot be neutralized[47,48].

The main endogenous antioxidant agents are vitamins A, C and E and glutathione (GSH) and ROS-detoxifying enzymes like superoxide dismutases (SODs), catalase (CAT), glutathione s-transferase (GST) and glutathione peroxidase (GPx)[31,33,47]. The nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor that recognizes Antioxidant Response Elements (ARE) in the promoters of ROS-converting enzymes genes, responsible for controlling a wide set of antioxidant enzymes[31].

It is important to refer that GSH is considered the primary antioxidant in the alveolar space, and that GSH deficiency has been associated with reduced survival in HIV-infected individuals[30]. GSH is crucial for many immunologic functions, including T and B-cell differentiation and activation of cytotoxic T-cells[30].

2) Which redox alterations can we observe in HIV positive patients?

Most studies analyzing the OS in HIV infected individuals, comparing to non-infected patients, showed:

- ✓ Decreased plasma GSH, SOD, CAT and GPx[31];
- ✓ Increased levels of lipid oxidation products[15,31] and oxidized nucleic bases[31] in plasma;

- ✓ Decreased GSH and glutathione/oxidized glutathione (GSH/GSSG) ratio in epithelial lung fluid[31];
- ✓ Increased levels of alkanes in the breath output[15,31];
- ✓ Increased ROS production in monocytes[31,33] and in other HIV-infected cells[31].

Also, there is evidence that individuals with AIDS have a higher urinary excretion of vitamin A comparing to HIV-infected non-AIDS individuals or to healthy controls[48].

On the contrary, Cribbs *et al.*[30] didn't observe any statistical difference between GSH BAL fluid levels of HIV-positive patients and HIV-negative controls.

3) How can HIV alter the redox status of the host?

It is well established that HIV infection triggers OS in the host cell, but the mechanism by which it occurs is not fully explained. Most studies showed that this unbalance is caused both by the over-production of reactive oxygen species (ROS) and reduction of the endogenous antioxidant capacity[31]. The induction of ROS production is due to HIV-mediated inflammation[33] and directly by HIV-associated proteins, including the reverse transcriptase (RT)[31], envelope protein Gp120, Tat [31,33], Nef protein and Vpr[31,33].

Tat protein can impair the mitochondrial function[31], decrease the glutathione levels[33,47,48] - by downregulating glutathione synthase (GSS) - and also alter GPx and gluthatione reductase (GR) activities[31]. These latter three enzymes are also suppressed by Gp120[31]. The impairment of these enzymes decreases both glutathione and GSH/GSSG ratio[47]. Vpr induces directly the production of ROS in mitochondria and can also cause mitochondrial dysfunction. The interaction of Vpr with adenine nucleotide translocator, a component of the permeability pores of the mitochondrial membrane that is responsible for

the influx of Ca^{2+}, can reduce glutathione levels, but by an indirect mechanism, because the biosynthesis of GSH requires two molecules of ATP[31].

Gp120 can generate ROS by upregulating cytochrome CYP2E1[31] and modulate the T-cell function, as mentioned before, but through a ROS-dependent pathway. Nef protein can stimulate the release of superoxide anions from macrophages[33] and trigger ROS production by interacting with NADPH oxidases, responsible for the production of superoxide anions[31]. Furthermore, the mechanism by which RT can trigger the formation of ROS is still unknown[31].

Finally, Tat and Gp120 viral proteins also seem to downregulate the antioxidant Nrf2/ARE pathway in the alveolar epithelium[48]. By altering this pathway, the antioxidant defenses and barrier function are impaired, augmenting the lung susceptibility to OS and to subsequent harmful outcomes[7,48].

4) Why is OS benefic to HIV?

The importance of OS for the HIV infection lies on the fact that ROS play a key role in cell signaling[33] and are important components of the innate and adaptive immune response[48]. They can exacerbate the inflammatory status of the cell by inducing the production of pro-inflammatory cytokines, such as IL-1β, IL-6, interferons and TNF-α, which will ultimately contribute to further ROS production[33].

It is also important to acknowledge that HIV infection enhances the OS of the host, and the OS itself may augment the rate of viral replication of HIV, creating a vicious cycle[33,38,47–49]. On one hand, ROS can activate the translocation of the NF-kB to the nucleus,

promoting the transcription of the virus genome[33,48,49], but, on the other hand, GSH inhibits the expression of the viral protein p24, required to form the viral coat[47].

Other important consequences of OS are the induction of PD-1[33] and DNA irreversible damage[48] that lead to apoptosis of $CD4^+$ T-cells, exacerbating the depletion of these cells during the HIV-infection. Indeed, the severity of OS during HIV infection is inversely-correlated to the $CD4^+$ T cell count: higher $CD4^+$ counts correlate to the total levels of glutathione and at low counts of $CD4^+$ T cells corresponds a more severe OS[31]. This could be explained by the host cell exhaustion of antioxidant mechanisms facing the virus-induced OS[31].

All of this is corroborated by the fact that the administration of antioxidants reverses the plasmatic lipid peroxidation, reduces the viral load and increases the number of $CD4^+$ T-cells[48].

5) How does OS correlates with an increased risk of lung pathologies?

The over-production of ROS results in a deleterious process that can have many cellular implications. It can alter redox-depending metabolic pathways[47], and reduce the amount and activity of macrophages, CD4+ and CD8+ cytotoxic T-lymphocytes[47]. Of note, the free radicals that are generated in this process have high affinity for specific biological components, such as phospholipids, polysaccharides, proteins and, most importantly for the HIV-tumorigenesis hypothesis, DNA[33,38,47,48], inducing profound alterations in cell functioning[48]. In fact, the hydroxyl radical, $HO^{\cdot}$, is the most reactive ROS and can oxidize almost every molecule that is nearby[31]. H_2O_2, in turn, has a low reaction potential, but can be converted to the hydroxyl radical[31].

It was hypothesized that accumulation of damaged/oxidized products might contribute to the aging process[38]. Protein oxidation was implicated in the development of age related diseases, such as cancer, and DNA oxidation can also contribute to aging, malignant tumors or other degenerative diseases[38]. The oxidation of DNA can induce genomic instability[31], possibly explaining the HIV-associated malignancies[47,48]. On top of this, it was demonstrated that high levels of ROS and a pro-inflammatory status may facilitate the development of tumor cells[48].

Kolgiri *et al.*[38] studied the relationship between protein oxidation and DNA damage in 300 HIV-infected individuals under ART or ART naïve and 300 HIV-negative controls. The protein oxidation marker chosen was carbonyl content, and DNA damage marker was 8-OHdG (8-hydroxy-2-deoxyguanosine), a product of oxidation of DNA base guanine, which is known to be mutagenic. They found that protein carbonyl levels were positively associated to DNA damage levels, and both were higher in HIV-positive patients compared to uninfected controls. These biological markers were also increased in the older group (40-60 years), compared to the younger group (20-40 years), which indicates a cumulative effect of HIV infection and aging on OS and DNA oxidation/damage, promoting mutations and carcinogenesis[38].

The other excess lung pathologies observed in HIV positive individuals could be explained, in part, by the overproduction of ROS. In fact, OS seems to contribute to the development of COPD: the redox unbalance can lead to the inactivation of anti-proteases and consequent lung damage, inhibiting also the repair mechanism[12]. Although studies of HIV individuals with COPD have found low levels of pulmonary and serum GSH[7], a study by Diaz *et al.*[50] comparing the BAL regional distribution of GSH levels in HIV-positive patients

with emphysema found an interesting result. In their study, emphysema scores were higher in the upper lobes, where GSH levels were also higher, comparing to the middle lobes; these results were stated both in smokers and non-smokers. The authors have hypothesized that increased GSH values are suggestive of an adaptive/compensatory response to the high OS in the upper lobes, as data of both *in vitro* and *in vivo* studies have shown that chronic OS can induce GSH synthesis[50]. Corroborating the effect of OS on COPD pathogenesis, antioxidants were proven to reduce COPD exacerbations[13].

Furthermore, it was observed that the OS in the lung diminished the expression of tight junction proteins, causing a disruption in the lung epithelium and boosting the susceptibility to infections[7,31]. Lung infections are a risk factor for LC, as we saw before. Beside this, the presence of ROS creates a favorable environment to the HIV replication and also other viruses[47], which could be a contributing factor for the high prevalence of viral co-infections seen in these individuals. The impact of other viral co-infections is discussed later.

Direct oncogenic potential of the virus

The pathogenesis of NADCs is somewhat complex, but a viral contribute is verified in many of these cancers[15,18,39]. In fact, viral co-infections of HIV-positive individuals have been previously associated with increased cancer risk. For example, the co-infection of HIV and hepatitis B and C virus puts the individual at a higher risk of developing hepatocellular carcinoma, while the co-infection with human papilloma virus (HPV) is associated with a wide variety of HPV-associated tumors, such as cervix and anal cancer[4,39,51]. Currently, LC is the only NADC that doesn't have a viral co-factor to explain the excess risk of LC in HIV-

infected individuals[4,18,19], although it was hypothesized the effect of HPV on lung carcinogenesis, especially in non-smokers[1,4]. Until now, these data are still inconclusive, but the excess burden may be explained by the direct oncogenic potential of HIV itself.

It was observed in previous studies that several HIV proteins could enhance the cancer risk in HIV positive individuals. These proteins include Tat, matrix protein Gag p17 and envelope protein Gp120[4,52]. The Nef protein was detected in a third of head and neck squamous cell carcinomas in HIV-positive patients, but there are no other findings that could explain the importance of this discovery[2].

Compared to the other mentioned viral proteins, the oncogenic role of HIV-1 Tat protein is the best described. *In vitro*, it has been shown that Tat modulates the expression of some proto-oncogenes and downregulates the tumor suppressor gene TP53 in bronchoalveolar carcinoma cells lines[15,18]. This protein can also disrupt tight junction proteins[7] and bind to VEGFR-2 (vascular endothelial growth factor receptor-2) - expressed in several cell-types -, inducing the production of growth factors and, so, can act as an oncogenic and angiogenic factor. Tat can also induce the expression of Platelet Derived Growth Factor (PDGF) and proliferation of smooth muscle cells on the lung[7]; these endothelial abnormalities seen during HIV-infection may permit the tumor growth[39]. Some studies have shown that Tat protein and cocaine can have additive effects on the endothelium[7,13], particularly important to intravenous drug users. It was also observed that, both *in vitro* and in nude mice, the downregulation of HIV-*tat* interacting protein (TIP30) promotes metastasis of LC[15].

Gp120 protein can induce apoptosis of lung endothelial cells[7,13], and Nef protein is able to induce OS in pulmonary artery endothelial cells[28].

The direct effect of viral proteins Tat and GP120 on endothelial cells of the pulmonary vasculature is particularly relevant, because these cells don't express the CD4 receptor, neither CXCR4 or CCR5, which means that they are resistant to HIV[7]. Nevertheless, they are still affected by HIV infection by the exposure to viral proteins, or due to the related inflammation, as IL-6 also appears to upregulate VEGFR-2[12].

Kawabata *et al.*[52] tested the impact of HIV on lung tumorigenesis in an animal model. This study used a HIV-transgenic mouse model, where the mice are immunocompetent and, thus, don't have the abnormal augmentation in inflammatory markers seen in HIV positive individuals (IL-6 levels are normal), but express the viral proteins Tat, p17, gp120 and Nef. The hypothesis tested was that the induction of LC is due to the HIV proteins *per se*, independently of immunosuppression or inflammation. They used saline injections for the control group and tobacco carcinogens injections - that are known to induce LC - for the experimental group. The control mice didn't develop lung tumors, while two thirds of the mice treated with tobacco carcinogens, as expected, did. This showed that, in mice that express HIV proteins with known oncogenic activity, the increased LC risk can only be validated if exposed to tobacco, and not by the viral proteins *per se*, at least in a host with full immunocompetence and without immune dysfunction/continuous inflammation[52]. Although these results are important for a better understanding of the pathogenesis of HIV-mediated LC, they don't exclude the possibility that HIV proteins may have an impact in lung carcinogenesis in humans.

Mutations in Lung Cancers of HIV-positive patients

After discovering specific Epidermal Growth Factor Receptor (EGFR) mutations and Anaplastic Lymphoma Kinase (ALK) rearrangements in NSCLCs of individuals without extensive smoking histories[2], other studies evaluated the presence of clinically relevant oncogenic mutations in the LCs of both HIV-infected and uninfected patients. However, the prevalence of EGFR mutations[53] and KRAS (Kristen rat sarcoma) and ALK rearrangements in NSCLCs were proven not to differ by HIV status[4,19,53], which means that there are still not specific mutations that we can link to HIV-related LC.

Moreover, according to Wistuba *et al.*[54], LCs in HIV-positive patients exhibit microsatellite alterations (MA), which traduces genomic instability. These MA are 6-fold higher than the ones found in LC of non HIV-positive individuals. However, no viral sequences were found in the neoplastic cells from the microdissected tumor samples, which supposes an indirect -instead of direct- mechanism of HIV in the pathogenesis of LC, which is still unknown. Furthermore, the absence of MA in non-malignant tissues, does not favor the hypothesis of a general somatic effect of HIV on the mutation repair system[54]. Okuma *et al.* also found that EGFR mutations are not associated with genetic instabilities[53].

ART exposure

The impact of ART exposure in the cancer risk is debated. On one hand, it is expected to have a protective role, since it was proven to reduce the incidence of ADCs[5,26] – absolute decrease in the incidence of KS up to 80%[51] - and because the first-line treatment of these ADCs also includes ART[9]. On the other hand, some studies evaluating LC risk in pre and

ART era showed a higher incidence of LC in the ART era[1,3,12,18,19,55] while others observed a flat trend[2,15,39]. Hleyhel *et al.*[22], conversely, found that the RR of LC of HIV-positive patients fell during the study period (1997-2009), but the risk remained three-fold elevated, compared to the general population. Of course that this increased incidence described in some studies can be considered an artefact of the higher life expectancy of HIV infected individuals and the inability to fully adjust for the exponential increase in LC that naturally advents from ageing[23].

Nevertheless, in the ART era, LCs are still more prominent in HIV-infected subjects, which reflects the importance of assessing the possibility of ART being a causative factor of LC. In reality, the majority of studies performed in the ART era include a much higher number of patients under therapy than the ones untreated, which could explain the high risk of LC in HIV-positive patients if proven to be a risk factor.

Some authors have suggested that ART may have oncogenic potential[1]. It was hypothesized that nucleoside analogs may cause host cell DNA damage and potentially mutations that lead to carcinogenesis[2]. In animal models was observed that the nucleoside reverse transcriptase inhibitors azidothymine (AZT) and lamivudine (3TC) had mutagenic effects[4]. Furthermore, *in vitro*, AZT and didanosine have shown to elicit mutagenic responses[1]; also *in vitro* models have also proven that PIs, especially ritonavir, may have a pathogenic role on the development of some tumors, such as LC[27]. Finally, AZT and tenofovir were discovered to be genotoxic to neonates exposed to these drugs *in utero*[4].

The results of human studies evaluating the linkage between ART exposure and cancer risk are inconsistent. Some studies have found an association between non-nucleoside

reverse transcriptase inhibitors (NNRTIs) and HL and between protease inhibitors (PIs) and anal cancer[9].

It was hypothesized that PI-mediated inhibition of cytochrome (CYP) P450 could potentiate the effects of tobacco carcinogenesis, as CYP450 is responsible for the metabolism of polyaromatic hydrocarbons and nitrosamines that are found in the tobacco smoke[4,9,27,51]. In fact, several polymorphisms in CYP450 have been associated with LC[51]. Fortunately, a recent study with 383 LC cases[27] that addressed this particular hypothesis observed that exposure to PIs did not increase cancer risk in HIV-infected patients with smoking history (OD=0.83, 95% CI 0.61-1.12); the results did not differ in sensitivity analyses considering only HIV-positive smokers or ritonavir alone. Another interesting fact discovered by Bruyand *et al.*[27] was that the patients that were under PIs treatment, including ritonavir, for more than 5 years might have a lower LC risk (OD=0.39, 95% CI 0.19-0.79 for ritonavir exposure ≥5 years). This finding is congruous with the results of other two epidemiologic studies of HIV-associated cancers; for HL and anal cancer it was demonstrated a decreased risk with 5 or more years of treatment with ART[51].

It is needed to state that these latter findings could be related to the biologic effects of PIs. In addition to the inhibition of HIV aspartate proteases, these drugs have some off-target effects, such as the protein kinase B (AKT) signaling, that can have some role on lung carcinogenesis[51]. The phosphatidylinositol 3'-kinase (PI3K)-AKT pathway promotes reactive changes that could lead to LC and, so, the off-target AKT inhibition could possibly lead to a decreased LC risk. Currently, nelfinavir has been considered an anti-neoplastic drug, as its effect on AKT inhibition of peripheral blood mononuclear cells was proven *in vivo*[51].

Similarly, most of the recent studies didn't found any significant difference in cancer risk between ART exposed and non-exposed groups. The Swiss HIV Cohort Study[23] didn't reveal any statistical difference between the history of ART use and LC risk (OD for ever *vs* never=0.67, 95% CI:0.29-1.52). Likewise, Sigel K. *et al.* (2012)[17] and Hessol *et.al* [24] observed that ART exposure did not impact the risk of developing LC (p=0.08 and p=0.60, respectively).

Alongside the hypothesized oncogenic effect previously referred, there are some evidences that ART can contribute to the OS observed in HIV-positive patients, although a study presented contrary results. Mandas A. *et al.*[56] in their study with 116 HIV-infected and 46 non-infected participants revealed that the individuals treated with antiretrovirals, compared to the untreated (both infected and non-infected) had higher levels of serum ROS, and, comparing to the HIV positive untreated group, lower levels of plasma antioxidants. These findings revealed that, surprisingly, patients with poor adherence to ART had better antioxidant levels, suggesting a synergistic effect of ART in OS with HIV-infection. Also, different ART regimens don't seem to alter the oxidative status[56]. It was also observed that HIV positive patients under ART presented higher levels of serum oxidation products and lower levels of GSH, when compared to their own levels before the treatment and to healthy controls[31]. Kolgiri *et al.*[38] found that HIV-positive patients of both age groups (20-40 and 40-60 years) under ART had higher levels of carbonyl content and DNA damage markers, compared to infected individuals ART naïve or uninfected controls.

On the contrary, Cribbs S. *et al.*[30] in their study with 22 HIV-positive and 21 HIV-negative non-smokers observed that the OS – reflected by low BAL GSH levels - was more

prominent in HIV patients that weren't receiving ART, when comparing with patients on ART or uninfected persons.

Smoke exposure in HIV-positive patients

Smoking is currently the dominant risk factor for LC[5,15,19,28,55] and is still the largest preventable cause of death[3]. Because the HIV-positive individuals are more likely to be smokers – and less likely to be never smokers – than the non-infected persons[17,21], the tobacco exposure acts as a major confounder in studies assessing the independent association between HIV infection and LC risk. Epidemiological studies have shown that 20% of the uninfected individuals smoke, compared to the 40 to 70% of HIV-infected individuals[2]; this latter percentage could be even bigger in high-resource countries[18] or among intravenous drug users[3,5,15]. Adding to this, infected individuals have a tendency to start to smoke earlier and continue during more years[2].

In a cohort study with over 37.000 infected participants, it was observed that HIV-infected individuals that developed LC were more likely to be current smokers, and less likely to be never smokers, than infected individuals without LC ($p<0.001$)[17]. The Swiss HIV Cohort Study[23] confirmed that smoking was a very strong risk factor for LC in HIV-infected individuals (OR *vs* never= 14.4, 95% CI: 3.36-62.1), and that there is a small augmentation of the OR for smokers with more than 30 pack-years: 11.5 fold increase for exposures less than 30 pack years and nearly a 16-fold increase for exposures ≥30 pack-years. Hessol *et al.*[24] concluded that 10 or more pack-years exposure was an independent risk factor for higher LC incidence. In summary, the majority of studies demonstrate the impact of smoking on LC

risk and, most importantly, demonstrate that most of patients with HIV infection and LC are smokers[2,7,14–16,53,55,57].

The histological types of LC more closely related to tobacco exposure are squamous cell and small cell LCs[2], which curiously, aren't the most frequent histologic types of LC in HIV-positive patients. Most studies have shown that adenocarcinoma is the preponderant LC histological type in the HIV-infected individuals[14,17,18,23,24,27], which is the most common type in non-smokers, non-infected individuals[1].

These epidemiologic data are particularly important in infected patients, as it is thought that the tobacco-related harm in the setting of HIV infection is underestimated, due to reactive changes that seem different, and more deleterious, than those in non-infected individuals[10]. Indeed, Palacios R. *et al.*[14] observed that the HIV-negative group developed LC at a greater pack year consumption than HIV-positive individuals. Furthermore, although confirming the independence of HIV infection as a LC risk factor, Sigel K *et al.* (2012)[17] found that smoking conveys a much greater magnitude of risk for LC than HIV infection. Currently, smoking accounts for more life years lost than HIV-associated mortality[3,10].

Impact of smoking on immune system

Smoking seems to have an immunosuppressive and pro-inflammatory effect similar to HIV infection[3,9]. It can alter immune functions, impacting both innate and adaptive host immunity by several mechanisms[36]. Chronic smokers present defective T-cell responses and higher levels of pro-inflammatory cytokines[36]. Although it is known that HIV-infected smokers have decreased immune responses, poorer control under ART and a higher risk of

virological rebound than HIV-infected non-smokers[3,10,32], the mechanism by which it occurs is yet to be fully comprehended.

Nicotine mimics acetylcholine, a neurotransmitter[10]. Its receptors are expressed on the surface of neural cells and also in immune cells, and its cholinergic immunomodulatory effects on macrophages, T-lymphocytes and B-lymphocytes can alter pathways that are involved in the regulation of the inflammatory response[10].

Also the OS that is induced by cigarette smoke can contribute to the inflammation and lung damage[10]. Lymphocytes exposed to cigarette smoke have induced formation of superoxide anion and downregulation of antioxidant enzymes, which could lead to a DNA damage and subsequent fragmentation[32]. Ande A. *et al.* (2015)[34] found that HIV-negative smokers have a 2-fold induction of Nrf2 comparing to HIV-negative non-smokers; however, in HIV-positive smokers there was no such induction, reflecting the failure of this defense mechanism to compensate the smoking-induced oxidative stress in these patients. On the other hand, Mandas *et al.*[56] observed that the OS, in non-infected individuals, was more prominent in smokers than non-smokers. Furthermore, the OS markers in HIV-positive patients were higher than controls, and patients under ART also had higher levels than the untreated infected patients or healthy controls, independently of smoking status. These latter findings suggesting a more important role of HIV infection and ART on OS than smoking[56].

Importantly, the immunologic effects associated to tobacco exposure in HIV-infected people can be different than those among general population[10].

Several CYP enzymes are responsible for the metabolism of cigarette smoking compounds and the subsequent toxicity has been implicated in various types of cancer[32]. Liver CYP2A6 and lung-specific CYP2A13 metabolize nicotine to cotinine and other

metabolites[32,34], and activate nitrosamines into reactive compounds that cause liver/lung damage[32]. CYP1A1 and CYP1B1 also generate reactive compounds during the activation of polycyclic aromatic hydrocarbons, others constituents normally found in cigarette smoke[32,49].

CYP2A6 is one of the most abundant CYP enzymes expressed in U937 monocytic cell line and it was recently proven that nicotine induces OS in monocytes through the activation of this cytochrome[32,34,49]. The exposure of monocytes/macrophages to cigarette smoke downregulates the expression of antioxidant genes and alters the redox status[49]. The higher viral replication seen in HIV-infected smokers[34] is thought to be subsequent to the OS[32]; in fact, it was demonstrated *in vitro* that nicotine exposure stimulates the replication of HIV in the AM, microglia and also in T-cells[32,34].

Rao *et.al* (2016)[49] studied the impact of cigarette smoke condensate (CSC) – which holds the majority of cigarette smoke components – on HIV-infected and non-infected monocytic cells. Exposure of HIV non-infected U937-monocytic cells to CSC resulted in a time-dependent increase in ROS production and enhancement of caspase-3 activity, and also in a significant increase in apoptosis (mediated by caspase-3). Authors believe that the activity of this enzyme is boosted by OS, as it was significantly reduced with a concomitant antioxidant treatment. Furthermore, CSC exposure of U1 cells (HIV-infected U937 cells) also enhanced ROS production and resulted in a higher HIV-protein p24 expression, suggesting a significantly elevated HIV replication due to smoking components[49], consistent with the results of another of their studies from 2015[34], where they found a higher expression of p24 in CSC treated HIV-infected macrophages. In addition to these findings, they[49] also found that, after being treated with CSC, both U937 and U1 monocytic cells exhibit an induction of transcription of CYP1A1 (increase in mRNA production), but not in the

expression of the enzyme itself in HIV-infected macrophages. Nonetheless, both the expression of mRNA and protein of CYP1B1 were significantly augmented in HIV-infected primary macrophages treated with CSC. In summary, they concluded that cigarette constituents stimulate directly ROS production, activate CYP1 enzymes that aggravate redox unbalance, induce apoptosis and cellular toxicity and enhance the viral replication in monocytes[49]. Finally, another study refers that this activation of CYP1A1 can stimulate the production of cytokines, which will activate T-lymphocytes and perpetuate the chronic inflammation[32].

Adding to the findings of Kolgiri *et al.*[38] that reported increased levels of protein carbonyl and 8-OHdG in older individuals and in HIV-infected persons, Ande A. *et al.* (2015)[34] found higher levels of 8-OHdG in HIV-infected individuals and in smokers, proving a cumulative effect of aging, smoking and HIV infection in DNA damage.

Although there are reports of increased $CD4^+$ counts in smokers[34], other studies have shown that smoking is associated with an accelerated depletion of $CD4^+$ T-cells during the course of the HIV infection[3,10] and that this effect could be reversible after smoking cessation[10]. Beside the reduction in quantity, there is an increased activation of these cells and also $CD8^+$ lymphocytes, both in general population and in HIV-positive patients[36] . Upon activation, T-cells produce several cytokines, including IL-1β, IL-6, IL-8, IL-17 and TNF-α; higher levels of these cytokines were found in smokers of both HIV-positive and negative individuals, when compared to their corresponding non-smoking group. In addition, the immune markers of $CD4^+$ and $CD8^+$ cell exhaustion, such as PD-1, are higher in HIV+ patients, as observed before, but are also greater in infected and non-infected smokers. In conclusion, smoking and HIV-infection influence independently the T-cell immune

activation and function and together they present the worst immune profile[36]. It also appears to exist a relationship between smoking and leucocyte telomere shortening, contributing to the already existing HIV-associated immunosenescence[10].

It seems that nicotine can decrease the immune response of lymphocytes by altering the production of cytokines and chemokines[32]. Another effect that tobacco smoke appears to have in blood lymphocytes is the induction of CYP1A; the levels of this nicotine-metabolizing enzyme are usually extremely low in these cells, but a component of cigarette smoking can induce this enzyme by 20-fold[32]. Another residual enzyme of lymphocytes is CYP2A6. A study revealed that there is a higher expression of this enzyme in the blood lymphocytes of individuals with LC, but there aren't studies performed about the expression of these nicotine-metabolizing enzymes in the lymphocytes of HIV infected smokers and non-smokers[32]. The importance of the induction of these CYP enzymes by cigarette smoke lies on the fact that they generate an redox unbalance and augment DNA oxidation[32,49], as it was stated before.

Another important consequence of smoking exposure is the increment in plasma levels of sCD14 and bacterial LPS, two markers of microbial translocation[36] . Indeed, a recent study observed that these levels were significantly higher in HIV-negative smokers compared to the uninfected non-smoker group. This proves the effect of smoking on microbial translocation regardless of HIV infection. They also found higher microbial translocation markers in HIV-positive patients[36], confirming the HIV-mediated microbial translocation previously described. It is important to refer that both smoking and HIV infection cause microbial translocation, but it is thought that smoking exposure leads to an increased alveolar epithelial permeability, while HIV affects predominantly the intestinal epithelium[3].

Another immunosuppressive effect of smoke relates to its ability to increase the production of TGF-β1[3], which we saw previously that promotes Tregs. All of these smoking-induced immunosuppressive effects lead to a higher probability of developing lung infections. In fact, smoking is a clear risk factor for bacterial, TB or *Pneumocystis* pneumonia[3], and could additionally impact LC risk through pulmonary infections.

Discussion

Currently it is well established that HIV-infected individuals have higher rates of co-morbidities and NADCs than the general population. However, there are specific guidelines for the follow-up of cardiovascular, hepatic, renal, neurocognitive/psychiatric and metabolic disorders in these individuals, but no specific recommendations on primary prevention of chronic pulmonary diseases are yet available[13,41]. It is, thus, essential to fully comprehend the impact of HIV on lung diseases to decide if screening of these pathologies should be implemented.

Since the $CD4^+$ T-cell count depletion is considered the hallmark of HIV-infection, understanding of the impact of low $CD4^+$ counts on LC risk is crucial to recognize the impact of HIV-infection *per se*. Most of the original articles analysed - and also systematic reviews – refer that there is no relationship between low $CD4^+$ counts and increased LC risk. However, a large longitudinal cohort study has proven that cumulative exposures of low $CD4^+$ counts were associated with a greater risk. It is important to refer that this conflicting data can be due to several confounders. On one hand, patients that have a bigger degree of immunosuppression often have opportunistic infections, which leads to more investigation

procedures and may increase the probability of a surveillance bias[23]. On the other hand, low $CD4^+$ counts are related to other events with high mortality, which could explain the absence of association between CD4+ counts and cancer risk, due to death-competing risks[26]. Before validating this causal relationship between immunosuppression and LC risk, these confounders should be properly assessed. This same longitudinal cohort[21] observed that low $CD4^+/CD8^+$ ratios and episodes of bacterial pneumonia were the most robust predictors of LC risk, corroborating the findings previously described relatively to the immune dysfunction and bacterial pneumonias. The chronic inflammation and immune dysfunction are considered important risk factors for LC, mostly due to abnormal immune responses to pathogens.

It is consistently observed that people with HIV infection have different immune phenotypes than the general population. Furthermore, levels of immune markers of HIV-positive individuals are comparable to those found in older non-infected individuals. It is also hypothesized that this accelerated aging can augment the cancer risk, as it is well established that with increased age comes a higher risk of developing cancer.

The prevalence of several lung diseases, such as COPD and asthma, are higher in HIV-positive patients compared to HIV-negative individuals[3,7,28]. It is still unclear whether the higher incidence of COPD in infected patients is due to a direct effect of the virus –and its proteins - or to the effects of HIV sequelae, such as lung infections[28]. The findings of Yearsley *et al.*[46] supported the hypothesis of a direct viral role on the development COPD, as high quantities of HIV-infected cells were identified in the emphysematous areas, and, in normal lung areas, no infected cells were found. However, we previously observed that persistent viral replication can have further implications on the immune dysfunction and also

that low $CD4^+/CD8^+$ ratio is associated with a faster FEV1 decline[42]. Other HIV-associated risk factors can explain the high COPD prevalence, such as OS[12,50] and *Pneumocystis jirovecii* colonization[3], but the mechanism by which HIV can mediate the development of COPD is yet fully known. COPD was proven to be an independent risk factor for LC in general population and in HIV-infected individuals[19].

Individuals with HIV infection have an unbalanced redox status, with overproduction of ROS and decrease of antioxidant enzymes. This puts this population at a high risk for oxidization of cellular components, including proteins, measured by protein carbonyl, and DNA damage, directly related to 8-OHdG. The presence of 8-OHdG is also a marker for mutagenesis, possibly contributing to the development of malignancies.

The direct oncogenic potential of HIV is a challenging matter to address. Although some *in vivo* studies have shown that, in HIV-infected individuals, there is a higher expression of proto-oncogenes and downregulation of tumor suppressive genes, such as TP53, studies in animal models indicate that there is not a relationship between LC and exposure to viral products and proteins; however, these latter results cannot be extrapolated to humans. Moreover, the absence of viral sequences in tumor samples suggests that the HIV-mediated tumorigenesis is not mediated by the virus itself, but by some other indirect mechanisms.

The effect of ART on lung immunology and lung carcinogenesis is still not fully known. Studies present contradictory results, but the most recent studies don't seem to found a relationship between ART exposure and cancer risk. However, recently ART has been linked to OS, with several studies founding an increase of OS on patients who are more adherent to therapy[38,56], while other studies have found contrary results[30]. This inconsistency

of results can be due to the characteristics of participants; for example, in the study performed by Mandas *et al.*[56], nearly 66% of the ART exposure individuals were smokers, where only 46 and 43% of HIV-positive non-treated, and negative controls, respectively, were smokers. This augmented OS in the treated group could be influenced by tobacco exposure. On the other hand, Cribbs *et al.*[30] had very strict exclusion criteria and individuals were otherwise healthy, with a mean age of 40 years. This sample may not correspond to the actual HIV-infected population. Finally, the differences in the biomarkers evaluated (OS markers of serum *vs*. alveolar space) and the different regimens used should also be taken in account. There are evidences that patients treated with NNRTIs have lower peroxide concentrations than those treated with PIs[31]. It is still necessary to address further studies in this matter to comprehend the exact pathways that lead to OS in ART-treated individuals and the impact that this redox imbalance can have on viral replication and progression of the infection.

Finally, relatively to the smoking habits of seropositive patients, it is necessary to perform more studies relatively to the real impact of tobacco carcinogens on lung immunity. In fact, it is still not clear whether the effects of smoking on HIV-infected patients are cumulative or synergic with those of the HIV infection[10]. Currently, in most studies with HIV-positive patients, the majority of the LC cases are smokers. If tobacco smoke exposure is proven to have more harmful effects on seropositive patients than the ones that provokes in general population, it is possible that some studies that have adjusted their results for smoking could have had underestimated the impact of smoking.

Conclusions and future perspectives

The relationship between HIV-associated immunosenescence and increased LC risk although hypothesized, is not established. This means that it is essential to address further studies comparing the immune phenotype of HIV-patients that develop LC with those that don't develop LC to verify if exists a causal relationship. If studies confirmed this association, the LC incidence rates shouldn't be adjusted for age-matched control individuals, but to older individuals of general population. In fact, it is possible that rates of incidence of LC are similar to those observed in older seronegative individuals, which means that the mean age for LC diagnosis in HIV-positive patients is not diminished, but, in fact, congruent with their immunological age.

Relatively to the HIV-associated lung diseases, it is important to have a better understanding of the impact of the virus on the development of COPD and asthma. Some of the proposed HIV-associated COPD risk factors are contradictory: both low $CD4^+$ counts and high viral loads, and ART treatment – that would increase $CD4^+$ counts and decrease viral replication - have been associated with worse lung function, proving that this matter should be further investigated. As we know, these two lung diseases were previously considered risk factors for LC. If the exact mechanisms by which the viral infection can induce or accelerate the development of COPD and asthma are understood, targeting strategies could be developed to prevent the evolution of these lung diseases and subsequent LC.

The OS is mediated by HIV-infection itself, aggravated by tobacco exposure and possibly by ART. There aren't studies assessing the direct impact of OS in LC risk. In fact, the redox status – measured by ROS and anti-oxidants levels – of individuals that were diagnosed with LC should be compared to those who didn't develop LC, to investigate if

there is, indeed, a relationship between them. Currently, the hypothesis of oxidization of DNA by ROS and its consequences in genomic instability and lung tumorigenesis is not confirmed in any study.

The knowledge of the physiopathological mechanisms that are responsible for the high incidences of LC in HIV-positive patients is germane to better adjust the therapeutic strategies needed to these patients. For example, if proven that the OS is, in fact, a risk factor for LC, anti-oxidant treatments should be included in therapeutic protocols for infected patients. On the other hand, if proven that HIV has a direct oncogenic potential, since is not yet possible to address this problem directly, HIV-infected individuals should be considered a high-risk group to develop LC, and some screening tests should be implemented during their follow-up.

Finally, while HIV-associated risk factors for development of LC are still unclear, prevention strategies can help to reduce the incidence of this neoplasm. Since smoking is still the dominant risk factor and the most effective strategy to reduce LC incidence, encouraging and assisting HIV-infected smokers to quit and sustain cessation of smoking is imperative.

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