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Ciclo XXVII

Study of Immune Senescence and Premature Aging in Two Populations at Higher Risk of Cancer: Elderly People and Perinatally Human Immunodeficiency Virus (HIV)-Infected Children

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Sommario

L'immunosenescenza e l'invecchiamento prematuro rappresentano importanti fattori di rischio per l'insorgenza di tumore; il deterioramento delle funzioni immunitarie e l'accumulo di danni al DNA con l'avanzare dell'età determinano un generale peggioramento dello stato di salute e predispongono alla malattia neoplastica. Come risultato della maggiore aspettativa di vita, è previsto che il numero di tumori nella popolazione anziana aumenterà significativamente nei prossimi anni. E' sempre più importante, pertanto, trovare dei marcatori prognostici per l'insorgenza di cancro e/o di risposta alla terapia, anche al fine di migliorare il trattamento di questa popolazione più vulnerabile. Va inoltre sottolineato che certe condizioni, come l'infezione da HIV, sembrano accelerare il processo di invecchiamento e di immunosenescenza; l'attivazione cronica del sistema immunitario potrebbe giocare un ruolo critico in queste disfunzioni, che, oltre all'immunodepressione, potrebbero costituire un importante fattore di rischio per l'insorgenza dei tumori associati ad HIV. L'alta incidenza di tumore fra gli individui HIV-infetti, rispetto alla popolazione generale di pari età, anche dopo l'introduzione della terapia antiretrovirale (ART) che riduce i livelli di HIV e parzialmente ripristina il sistema immunitario, va a sostegno dell'ipotesi che altre condizioni, oltre all'immunodepressione, possano giocare un ruolo nel processo di cancerogenesi nelle persone HIV-infette. Questo è di particolare rilievo nei bambini con infezione perinatale da HIV, nei quali il sistema immunitario co-evolve dalla nascita con il virus. Dopo l'introduzione dell'ART, la maggior parte dei bambini HIV-infetti diventa adolescente ed entra nell'età adulta; nonostante la terapia, questi rimangono ad alto rischio di tumore.

Il mio progetto di Dottorato si è focalizzato sullo studio dell'immunosenescenza e dell'invecchiamento prematuro in due popolazioni ad alto rischio di cancro: i soggetti anziani e i bambini con infezione perinatale da HIV.

1. Immunosenescenza e cancro negli anziani

In questo studio sono stati analizzati l'output timico, il profilo immunofenotipico dei linfociti $CD4^+$ e $CD8^+$ e la lunghezza del telomero (TL) nel sangue periferico di pazienti con cancro di età superiore ai 70 anni. Sono stati arruolati 52 soggetti anziani con cancro e 39 controlli di pari età senza storia di cancro. Le percentuali di cellule $C8^+$ naive e $CD8^+$ di recente uscita timica e i livelli di TREC ("*T-cell receptor excision circles*", marker molecolare per valutare l'output timico) erano significativamente più bassi nei pazienti con cancro che nei controlli (16.7% [9.3-25.2] vs 24.6% [14.7-33.5], p=0.003; 34.2% [24.7-46.4] vs 44.9% [36.0-50.7], p=0.004; 16.0 [7.7-31.5] vs 25.0 [14.0-56.0] TREC copies/10^5 PBMC, p=0.031; rispettivamente). La TL nelle cellule mononucleari di sangue periferico (PBMC) era significativamente più corta nei pazienti con cancro che nei controlli (p=0.046) e correlava con l'età solo in quest'ultimi (r=-0.354, p=0.031). Il profilo telomeri corti(≤mediana)/bassi livelli di TREC (≤mediana) era associato con un alto rischio di cancro (OR=3.68 [95%CI 1.22-11.11]; p=0.021). E' in corso un sottostudio longitudinale al fine di valutare l'impatto di questi marcatori sulla risposta alla chemioterapia e/o sul decorso della malattia. Dati preliminari hanno mostrato, che dopo il trattamento chemioterapico, vi è un aumento delle cellule $CD8^+$ differenziate allo stadio terminale (p=0.031), e che l'accorciamento del telomero durante il follow-up tende ad essere maggiore nei pazienti con malattia di stadio III rispetto a quelli di stadio II. Inoltre, l'analisi del danno al DNA nei PBMC, prima e dopo l'esposizione ad una sorgente di raggi gamma, ha suggerito che i pazienti con cancro hanno una ridotta efficienza nei processi di riparo del DNA. In generale, questi risultati indicano che l'immunosenescenza è significativamente più grave nei pazienti anziani con cancro rispetto ai controlli di pari età. Il basso output timico e i telomeri più corti nelle cellule di sangue periferico

potrebbero riflettere una condizione pre-esistente, che facilita l'insorgenza di tumore nelle persone anziane e che potrebbe influenzare la risposta alla chemioterapia.

2. Invecchiamento prematuro nei bambini con infezione perinatale da HIV

Sono stati studiati 71 bambini HIV-infetti (HIV+) nati da madre HIV-infetta, dai 0 ai 5 anni di età, 65 bambini esposti non infetti (HEU) nati da madre HIV-infetta e 56 non esposti non infetti (HUU) di pari età. Il 42% dei bambini HIV+ non era in ART. La TL era significativamente più corta nei bambini HIV+ rispetto ai bambini HEU e HUU ($p<0.0001$, dopo aggiustamento per età); inoltre, i bambini HIV+ ART-naive mostravano una TL più corta rispetto ai bambini in ART (mediana 2.11 [range interquartilico (IQR) 1.75-2.37 vs 2.46 [2.07-2.68]; $p=0.0029$, dopo aggiustamento per età). Le cellule $CD8^+$ di recente uscita timica ($CD45RA^+CD31^+$) e i livelli di TREC erano significativamente più bassi nei bambini HIV+ rispetto ai bambini HEU e HUU ($p=0.005$ e $p=0.0249$, rispettivamente), mentre le percentuali delle cellule $CD8^+$ di memoria effettrici ($CD45RA^-CD27^-$) e differenziate allo stadio terminale ($CD45RA^+CD27^-$) erano maggiori nei primi ($p=0.033$ e $p<0.001$, rispettivamente). Le cellule $CD8^+$ senescenti ($CD28^-CD57^+$) erano maggiori negli HIV+ che nei bambini HEU e HUU (25.8% [12.4-43.2] vs 8.5% [6.8-16.7] vs 9.7% [3.3-27.3]; $p=0.004$), così come le cellule $CD8^+$ attivate ($CD38^+HLA\text{-}DR^+$) (7.0% [5.2-12.2] vs 3.5% [3.9-7.6] vs 3.7 [2.4-6.7]; $p<0.001$) e le cellule $CD8^+PD\text{-}1^+$ (7.1% [5.0-12.4] vs 3.5% [2.1-5.9] vs 3.7% [2.4-5.3]; $p<0.001$). All'interno della sottopopolazione di cellule $CD4^+$, la percentuale delle cellule senescenti non era significativamente diversa fra HIV+ e controlli, sebbene l'espressione di PD-1 tendesse ad essere maggiore negli HIV+ ($p=0.050$). Nell'insieme, questi dati suggeriscono che i bambini HIV-infetti mostrano un invecchiamento prematuro e uno

stato di immunosenescenza accelerato che colpisce per lo più la sottopopolazione delle cellule $CD8^+$. L'infezione da HIV per sé sembra influenzare il processo di invecchiamento, piuttosto che l'esposizione all'ART per profilassi o trattamento. I meccanismi attraverso i quali HIV potrebbe influenzare queste funzioni rimangono ancora da chiarire; comunque, l'attivazione cronica del sistema immunitario dovuta alla persistenza degli antigeni virali potrebbe promuovere la rapida proliferazione delle cellule $CD8^+$ causando l'erosione dei telomeri e determinando il fenotipo immunosenescente.

Conclusioni

Nel complesso questi studi suggeriscono che la lunghezza dei telomeri, l'output timico e l'analisi immunofenotipica rappresentano dei marcatori utili per monitorare l'invecchiamento prematuro e l'immunosenescenza. Nei pazienti anziani, il basso output timico e i telomeri corti sono significativamente associati al tumore e potrebbero influenzare la risposta alla chemioterapia. Pertanto, questi marcatori potrebbero essere utili nella popolazione anziana per predire l'insorgenza di neoplasia e pianificare strategie terapeutiche. Nel contesto dell'infezione pediatrica da HIV, il nostro studio indica l'importanza di analizzare, in aggiunta agli esami correnti, come la plasmaviremia HIV e il profilo delle cellule $CD4^+$, la lunghezza del telomero e le sottopopolazioni delle cellule $CD8^+$, al fine di valutare lo stato di invecchiamento prematuro e l'immunosenescenza in questa popolazione ad alto rischio di cancro.

Summary

Immune senescence and premature aging are important tumor risk factors. Decline in immune functions and accumulation of DNA damages influence overall health and predispose to malignancies. As a result of longer life expectancy, the number of cancers in the elderly population is expected to increase significantly over the next years. Considering the effect of the growing geriatric population on cancer care, the need to develop prognostic markers for cancer onset and/or chemotherapy response for improved treatment in this frail population is becoming greater. Furthermore, several conditions, such as HIV infection, also appear to accelerate aging and immune senescence; chronic immune activation may play a critical role in these dysfunctions which, besides immune depression, may constitute important risk factors for the onset of HIV-associated tumors. The persistence of a higher incidence of malignancies in HIV-infected individuals compared with the age-matched general population, even after the introduction of antiretroviral therapy (ART), which can reduce HIV levels and partially restore the immune functions, indicates that other conditions than immune depression play a role in the cancerogenesis occurring in HIV-infected patients. This is of particular importance in the perinatally HIV-infected children in whom the immune system co-evolves with HIV from birth. After the introduction of ART, most perinatally HIV-infected children are entering into adolescence and young adulthood and, despite ART, they remain at high risk of malignancies.

My PhD project focuses on the study of immune senescence and premature aging in two populations at higher risk of cancer: elderly people and perinatally HIV-infected children.

1. Immune senescence and cancer in elderly people

This study analyzed thymic output, immunophenotypic profile of $CD4^+$ and $CD8^+$ lymphocytes and peripheral blood telomere length (TL) in cancer patients 70≥ years old. Fifty-two elderly cancer patients and 39 age-matched controls without personal history of cancer were enrolled. The percentages of $CD8^+$ naïve and $CD8^+$ recent thymic emigrant cells (RTE) cells and levels of TREC (T-cell receptor excision circles, a molecular marker to evaluate thymic output) were significantly lower in cancer patients than in controls (16.7% [9.3-25.2] vs 24.6% [14.7-33.5], p=0.003; 34.2% [24.7-46.4] vs 44.9% [36.0-50.7], p=0.004; 16.0 [7.7-31.5] vs 25.0 [14.0-56.0] TREC copies/10^5 PBMC, p=0.031; respectively). TLs in peripheral blood mononuclear cells (PBMC) were significantly shorter in cancer patients than they did in controls (p=0.046) and did not correlate with age in patients, whereas it did in controls (r=-0.354, p=0.031). Short telomere(≤median)/low TREC(≤median) profile was associated with a higher risk of cancer (OR=3.68 [95%CI 1.22-11.11]; p=0.021). A longitudinal substudy is in progress to evaluate the effect of these markers on chemotherapy response and/or disease outcome. Preliminary data from this longitudinal substudy showed that, after chemotherapy treatment, there was an increase in $CD8^+$ terminally differentiated cells (p=0.031), and telomere shortening at follow-up tended to be greater in stage III patients than in those at stage II. In addition, analysis of DNA-damage in PBMC, before and after exposure to a source of y-radiation, suggested that the DNA repair process is less efficient in cancer patients than in controls. Taken together, these findings indicate that immune senescence is significantly worse in elderly cancer patients than in age-matched controls. The low thymic output and the short telomeres in peripheral blood cells of cancer patients may reflect a pre-existing condition which facilitates the onset of malignancies in elderly people and may affect the response to chemotherapy and disease outcome.

2. Premature aging in perinatally HIV-infected children

Seventy-one HIV-infected (HIV+) children born to HIV-infected mothers, aged from 0-5 years, 65 HIV-exposed-uninfected (HEU) born to HIV-infected mothers and 56 HIV-unexposed-uninfected (HUU) age-matched children were studied. 42% of the HIV+ children were not on ART. TLs were significantly shorter in HIV+ than in HEU and HUU children (overall $p<0.0001$, adjusted for age); in addition, HIV+ ART-naïve children had shorter TLs than children on ART (median 2.11 [interquartile range (IQR) 1.75-2.37] vs 2.46 [2.07-2.68]; $p=0.0029$ adjusted for age). $CD8^+$ RTE ($CD45RA^+CD31^+$) and TREC levels were significantly lower in the HIV+ group than in the HEU and HUU groups (overall, $p=0.005$ and $p=0.0249$, respectively), whereas percentages of $CD8^+$ effector memory (CD45RA-CD27-) and terminally differentiated cells ($CD45RA^+CD27^-$) were higher in the former (overall, $p=0.033$, and $p<0.001$, respectively). $CD8^+$ senescent cells ($CD28^-CD57^+$) were higher in HIV+ than in HEU and HUU children (25.8% [12.4-43.2] vs 8.5% [6.8-16.7] vs 9.7% [3.3-27.3]; $p=0.004$), as were $CD8^+$ activated cells ($CD38^+$HLA-DR^+) (7.0% [5.2-12.2] vs 3.5% [3.9-7.6] vs 3.7% [2.4-6.7]; $p<0.001$) and $CD8^+PD\text{-}1^+$ cells (7.1% [5.0-12.4] vs 3.5% [2.1-5.9] vs 3.7% [2.4-5.3]; $p<0.001$). Within the $CD4^+$ cell subset, percentages of senescent cells did not differ between HIV+ and controls, although PD-1 expression tended to be up-regulated in HIV+ children (overall, $p=0.050$). Overall, these data suggest that HIV-infected children exhibit premature aging and accelerated immune senescence, which particularly affects the $CD8^+$ cell subset. HIV infection *per se* seems to influence the aging process, rather than exposure to ART for prophylaxis or treatment. The mechanism(s) by means of which HIV may affect these functions remain to be investigated; however, chronic immune activation due to viral antigen persistence may promote rapid proliferation of $CD8^+$ cells, resulting in erosion of telomeres and immunosenescent phenotype.

Conclusions

Taken together these studies indicate that TL, TREC and immunophenotypic analysis are useful markers in monitoring premature aging and immune senescence. In elderly patients, low thymic output and short telomeres are significantly associated with tumor and may affect the response to chemotherapy. Thus, these markers may be useful in the elderly population to predict tumor onset and to plan therapeutic strategies. In the context of pediatric HIV infection, in addition to current assays, *i.e.* HIV plasmaviremia and $CD4^+$ cell profile, our study indicates the need to analyze TL and $CD8^+$ cell subsets to assess the status of premature aging and immune senescence in this population at high risk of cancer.

Chapter 1

Introduction

1. INTRODUCTION

1.1 Aging and Cancer

1.1.1 Aging and elderly people

Aging represents the accumulation of changes in a person over time and coincides with a gradual decline in the functional reserve of multiple organ system, thus leading to impaired function and increased vulnerability to death. The progressive loss of physiological integrity that occurs with aging is the primary risk factor for major human chronic diseases, including cancer.

Advances in medicine and socioeconomic development have substantially reduced mortality and morbidity rates due to infectious conditions and, to some extent, non-communicable diseases. As a result of both longer life expectancy and declining fertility rates, the proportion of people aged over 60 years is growing faster than any other age group in almost every country. Between 2000 and 2050, the proportion of the world's population over 60 years will double from about 11% to 22% [United Nations, 2013]. The absolute number of people aged 60 years and over is expected to increase from 605 million to 2 billion over the same period, and the number of people aged 80 years and over will have almost quadrupled between 2000 and 2050 (Figure 1). During the next 5 years, for the first time in the world’s history, people aged 65 years and older will outnumber children aged younger than 5 years [United Nations, 2013].

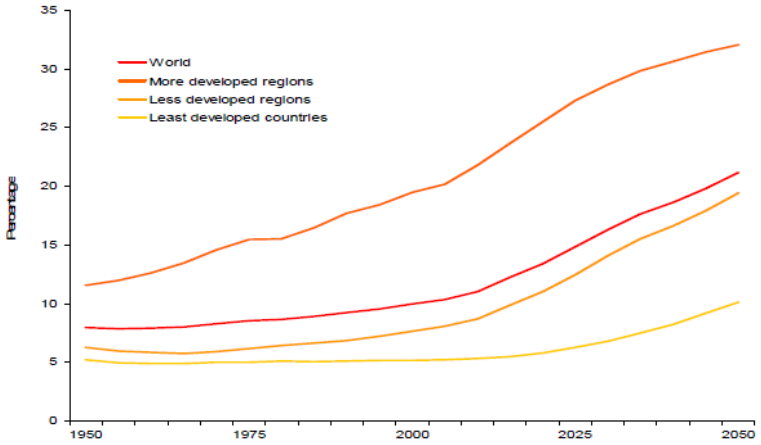

Figure 1. Proportion of population aged 60 years and over between 1950-2050. [From United Nations, Department of Economic and Social Affairs 2013].

These demographic and epidemiological changes are accompanied by an increase of age-associated diseases, where cardiovascular diseases and cancers (15.1%) are the leading contributors to disease burden in older people. In particular, the burden of malignancies in elderly people is forecasted to increase by 69% to 2030 [Prince MJ, Lancet 2014] (Figure 2).

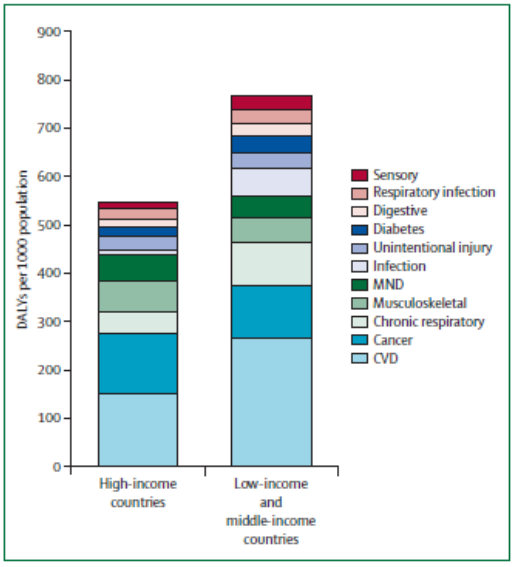

Figure 2. Leading contributors to burden of disease in people aged 60 years and older [From Prince MJ et al, Lancet 2014].

1.1.2 Biological aging and cellular senescence

The aging process is an open question. However, the improvement of knowledge allowed to identify a set of distinctive characteristics of aging, and nine hallmarks have been recently advanced: genomic instability, telomere attrition, epigenetic alterations, mitochondrial dysfunction, loss of protein homeostasis, deregulated nutrient sensing, cellular senescence, stem cell exhaustion and altered intercellular communication [López-Otín C et al, Cell 2013] (Figure 3).

Figure 3. The hallmarks of aging. Nine common denominators of aging characterizing the mammalian aging: genomic instability, telomere attrition, epigenetic alterations, loss of protein homeostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication. [From López-Otín C et al, Cell 2013].

The genomic damages include point mutations, translocations, chromosomal alterations and gene copy number variations [Moskalev AA et al, Ageing Res Rev 2013]. These all can be generated by exposure to genotoxic agents and lack of efficiency of DNA repair mechanisms [Neri S et al, J Gerontol A Biol Sci Med Sci 2005; Engels WR et al, Cell Cycle 2007; St Laurent G et al, Mech Ageing Dev 2010].

In addition to the genetic lesions, also the epigenetic alterations (*i.e.* variation in DNA methylation or acetylation patterns) can contribute to the aging process, as these changes may influence chromatin remodeling and DNA stability [Fraga MF et al, Proc Natl Acad Sci USA 2005; Calvanese V et al, Ageing Res Rev 2009; Esteller M et al, N Engl J Med 2008]. Despite the accumulation of DNA-damage occurs randomly in the genome, telomeres are the region more susceptible to age-related damage [Blasco MA, Nat Rev Genet 2005]. Since they are progressively shortened during each cell division, due to end-replication problems of DNA polymerase, they represent a mitotic counter that determines the proliferative capacity of any normal somatic cell. Defects in telomere length have been implicated in the pathogenesis of several age-related diseases, including cancer (see section 1.1.3) [Blasco MA, Nat Rev Genet 2005]. Mitochondrial DNA represents another target more susceptible to injury, so that mitochondrial dysfunction has been hypothesized be a central mechanism driving mammalian aging [Kujoth GC et al, Science 2005] and capable to modulate risk of several age-associated diseases [Kenney MC et al, BMC Med Genet 2013; Lezi E & Swerdlow RH, Adv Exp Med Biol 2012; Ashar FN et al, J Mol Med 2014].

Cellular senescence plays a key role in the aging process. It was originally described more than 40 years ago as a process that limited the proliferation (growth) of human fibroblast after a cultivation period [Hayflick L & Moorhead PS, Exp Cell Res 1961]. Cellular senescence defines a condition of permanent cell-cycle arrest, in response to various stress beyond telomere shortening (replicative senescence), that are oncogenic *RAS* activation (oncogene-induced senescence), non-telomeric DNA-damage or perturbation to chromatin organization [Campisi J and d'Adda di Fagagna F, Nat Rev Mol Cell Biol 2007]. The cellular senescent phenotype includes a permanent arrest of cell proliferation, resistance to apoptosis (in some cells), and an altered pattern of genes'

expression. Senescent cells accumulate in aged tissues [Dimri GP et al, Proc Natl Acad Sci USA 1995; Price JS et al, Aging Cell 2002; Sis B et al, Kidney Int 2007; Takayama K et al, J Orthop Res 2014].

Along with the accumulation of senescent cells, the regenerative potential of tissue is a distinctive marker of aging. Indeed, it has been demonstrated that, with age, the bone regeneration capacity [Gruber R et al, Exp Gerontol 2006] as well as T-cell lymphopoiesis and B-cell lymphopoiesis are compromised [Linton PJ & Dorshkind K, Nat Immunol 2004; Miller JP & Allman D, Semin Immunol 2005; Wang J et al, Curr Opin Immunol 2011]. Adult stem cells experience many stressful insults in the course of a lifetime of tissue repair, and thus the decrease of their function with aging may play an important role in the development of aging-related disease [Stenderup K et al, Bone 2003; Wagner W et al, PLoS One 2009; Mansilla E et al, Stem Cells Int 2011; Chakkalakal JV et al, Nature 2012].

The altered intercellular communication represents another mechanism that contributes to the aging process and to the determination of aging phenotype. As described above, senescent cells acquire widespread modifications in gene expression including changes in cell-cycle inhibitors or activators, and also in genes encoding secreted proteins, such as proinflammatory cytokines and growth factors that alter the neighboring (non senescent) cells and the tissue microenvironment [Parrinello S et al, J Cell Sci 2005; Bavik et al C, Cancer Res 2006]. This phenomenon of chronic, low-grade inflammation is defined as “inflammaging” [Franceschi C & Campisi J, J Gerontol A Biol Sci Med Sci 2014]. Senescent cells display a unique phenotype, which has been termed "senescence-associated secretory phenotype" (SASP) [Erusalimsky JD & Kurz DJ, Exp Gerontol 2005; Acosta JC et al, Cell 2008; Coppé JP et al, PLoS One 2010; Salminen A et al, Eur J Neurosci 2011]. Senescent cells have been shown to disrupt normal tissue structures and

differentiated functions in complex cell culture models [Tsai KK et al, Cancer Res 2005], thus resulting in an important driver of age-related diseases [Gorenne I et al, Cardiovasc Res 2006; Burton DG et al, Exp Gerontol 2010; Salminen A et al, Eur J Neurosci 2011; Kumar M et al, Am J Respir Cell Mol Biol 2014].

Finally, the loss of protein homeostasis and the deregulation of nutrient sensing are another two common features of aging, since remarkable examples of genetic manipulation of the signaling pathways that regulate proteolytic system and metabolic system showed that these can modulate the aging process and contribute to diseases [Hipp MS et al, Trends Cell Biol 2014; Fontana L et al, Pro Natl Acad Sci USA 2004; Colman RJ et al, Nat Commun 2014].

1.1.3 Cellular senescence and cancer

The link between cell senescence and cancer has been deeply investigated to understand the parallel and opposite forces that drive aging and cancer. On the one hand, cancer is characterized by an aberrant gain of cellular fitness and aging by a loss of fitness; on the other hand, both cancer and aging are different manifestations of the accumulation of cellular damage.

As described before, cell senescence is not simply a halt to cell proliferation, but coincides also with a senescent phenotype (SASP), frequently characterized by secretion of degradative enzymes, cytokines and growth factors that may have deleterious effect on function and/or homeostasis of neighboring tissues (Figure 4).

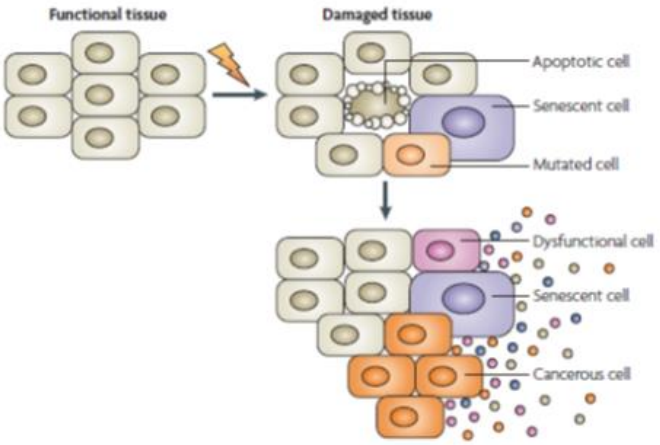

Figure 4. Tissue damage responses and potential deleterious effect of senescent cells. [From Campisi J & d'Adda di Fadagna F, Nat Rev Mol Cell Biol 2007].

As briefly depicted before, the cellular proliferative span is determined by telomere shortening. Telomeres are the repetitive DNA sequences, bound by a complex of proteins (the shelterin complex) at the end of linear chromosomes [Blackburn EH et al, Nat Med 2006], that progressively shorten at each division due to the end-replication problem in human somatic cells that do not express telomerase. This enzyme is a telomere-specific

ribonucleoprotein reverse transcriptase that adds single-stranded telomeric repeats to the chromosomal 3' end, preventing telomere shortening in germ-line and in many cancer cells. When telomeres become critically short, they are no longer protected by the shelterin complex and are recognized as DNA double strand breaks [d'Adda di Fagagna F et al, Nature 2003]. Telomeric DNA damage causes persistent DNA-damage response (DDR) activation [Fumagalli M et al, Nat Cell Biol 2012], that leads to replicative cellular senescence and/or apoptosis [Fumagalli M et al, PLoS One 2014]. p53 and p16-retinoblastoma (pRB) proteins are involved in the principal pathways engaged in the DDR response after senescent-inducing signals and also in the major tumor suppressor pathways. Dysfunctional telomeres, recognized as double strand breaks, activates p53, a crucial upstream inducer of the apoptotic pathway. Mutations that dampen the p53 or p16/pRB pathways cause the loss of their tumor suppressor activity, conferring resistance to senescence or apoptosis. Progressive shortening of telomeres increases genetic instability and risk of cancer [Popov N & Gil J, Epigenetics 2010; Muller PA & Vousden KH, Nat Cell Biol 2013].

Key components of the DDR, such as (Ataxia Telangiectasia Mutated) *ATM*, (Nijmegen Breakage Syndrome) *NBS1* and (Checkpoint Kinase-2) *CHK2*, that are the first proteins to initiate and maintain the response to DNA-damage, drive the secretion of potent inflammatory cytokines, as IL-6 and IL-8, that can enforce senescence but also play a tumor-promoting role by favoring tumor growth, cancer cell invasiveness and angiogenesis [Coppé JP et al, PLoS Biol 2008; Rodier F, Nat Cell Biol 2009].

1.2 Human Immunodeficiency Virus (HIV) Infection and Aging

1.2.1 Pediatric HIV Infection

Today, approximately 35 million people are living with HIV worldwide; more than three million are children [UNAIDS, Global Report 2013]. The number of new infections in children has decreased in the last years [UNAIDS, 2013 progress report], due to increased access to antiretroviral treatment (ART) to prevent mother-to-child transmission (MTCT). However, 240000 new pediatric HIV infections still occurred worldwide in 2013, because treatment coverage remains suboptimal in many countries [WHO, UNAIDS, UNICEF 2013] (Table 1).

Table 1. Global summary of the AIDS epidemic in 2013

Number of people living with HIV in 2013	Total	35.0 [33.1-37.2] million
	Adults	31.8 [30.1-33.7] million
	Children (<15 years)	3.2 [2.9-3.5] million
People newly infected with HIV in 2013	Total	2.1 [1.9-2.4] million
	Adults	1.9 [1.7-2.1] million
	Children (<15 years)	240000 [210000-280000]
AIDS deaths in 2013		
	Total	1.5 [1.4-1.7] million
	Adults	1.3 [1.2-1.5] million
	Children (<15 years)	190000 [210000-280000]

[From WHO, UNAIDS, UNICEF 2013]

MTCT is the main source of pediatric HIV-infection and, without prevention of MTCT, the overall risk of transmission is up to 40%. Most of MTCT occurs around the time of delivery, but it may occur also *in utero*; breastfeeding is an additional mode of MTCT [The Working Group on MTCT of HIV, J Acquir Immune Defic Syndr Hum Retrovirol

1995]. MTCT is a multifactorial event in which high maternal plasma HIV RNA level represents the major risk factor; mode of delivery and gestational age are also important factors [The European Collaborative Study, AIDS 1999]. Moreover, a body of evidence has established that also host genetic factors are important determinants of MTCT and HIV infection outcome [Ometto L et al, J Infect Dis 2001; Ricci E et al, J Acquir Immune Defic Syndr 2009; Ricci E et al, J Transl Med 2010; Freguja R et al, New Microbiol 2012; Gianesin K et al, PLoS One 2012].

The course of disease progression in infants markedly differs from that in adults. The median time to AIDS and death in ART-untreated adults is approximately 10 years from infection, but in children the course of infection is faster [Collaborative Group on AIDS Incubation and HIV Survival, Lancet 2000; Blanche S et al, New Engl J Med 1994; Barnhart HX et al, Pediatrics 1996] (Figure 5). In ART-untreated children, progression of HIV infection follows two patterns: about 15-20% develop rapid progression to AIDS, whereas the others progress more slowly [Blanche S et al, Am J Dis Child 1990]. In a recent meta-analysis conducted in 12112 infants born from HIV-infected mothers, it was estimated that survival from acquisition of infection postnatally in breast-feeding children was higher than in those with infection acquired around delivery: 1-year mortality was 26% among children infected through breast milk compared to 52% for peri-partum infected children [Becquet R et al, PLoS One 2012]. Furthermore, in a study that distinguished *in utero*, intrapartum and postnatal infections, it was estimated that median time from infection to death was 208, 380, and >500 days, respectively [Marinda E et al, Pediatr Infect Dis J 2007]. These differences in survival time are partly due to the immunological immaturity at the time of infection [Chakraborty R, Curr HIV Res 2005].

The levels of plasma HIV RNA are generally higher in vertically infected children than in adults, persist at high levels and decline slowly with age [De Rossi A et al, J Clin Invest

1996; McIntosh K et al, Pediatr Infect Dis J 1996] (Figure 5). Conversely to adults, the relationship between $CD4^+$ cell count and plasma HIV RNA is quite complex in children. High levels of plasmaviremia may be observed despite normal $CD4^+$ lymphocyte counts, since $CD4^+$ cell counts physiologically fall with age and plasma HIV RNA declines non-linearly up to 5 years of life [Mofenson LM et al, J Infect Dis 1997; PENTA, AIDS 1998].

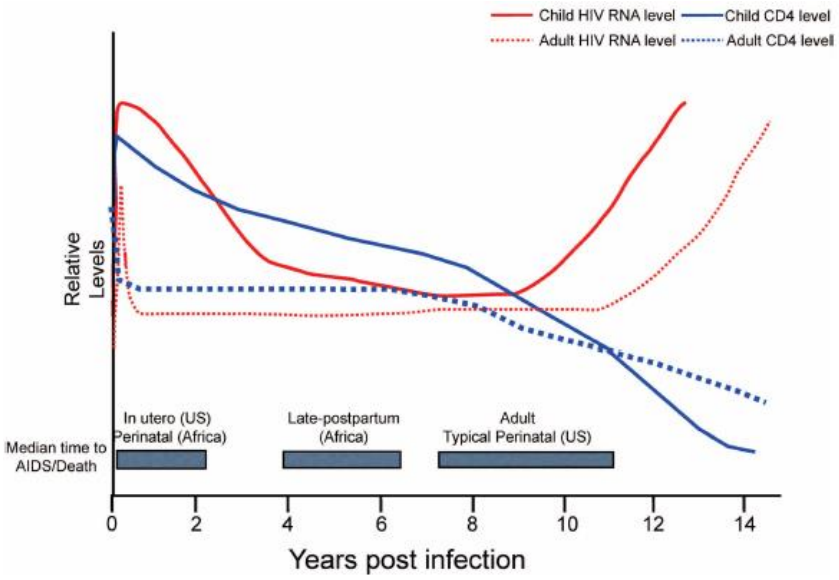

Figure 5. Dynamics of HIV RNA and $CD4^+$ levels in adults and children. Relative levels of HIV RNA (red) and $CD4^+$ cells (blue) in adults (dotted lines) and children (solid lines) in the years following acquisition of HIV. Gray boxes highlight the median time to AIDS/Death for infants by region and route of infection in comparison to adults. (US: United States). [From Tobin NH & Aldrovandi GM, Immunol Rev 2013].

1.2.2 Pediatric HIV infection and antiretroviral therapy

The introduction of the ART has changed the natural history of pediatric HIV-1 infection, reducing the rate of MTCT of HIV to less than 2% [Cooper ER et al, J Acquir Immune Defic Syndr 2002] and resulting in a substantial decline in mortality rates and in an improved quality of life in HIV-infected children [Gibb DM et al, BMJ 2003; Judd A et al, Clin Infect Dis 2007]. ART has transformed pediatric HIV infection into a chronic disease and now infected infants and children often survive to adolescence and adulthood [Deeks SG et al, Lancet 2013; de Martino M et al, JAMA 2000; Chiappini E et al, AIDS 2007]. Early ART leads to a sustained viral suppression and allows the recovery of thymic function, which in children is a pivotal event in immune reconstitution, and normalization of immunologic responses to non-HIV antigens [Luzuriaga K et al, J Virol 2000; Chiappini E et al, AIDS 2006; Zanchetta M et al, Antivir Ther 2008; Goetghebuer T et al, AIDS 2009]. However, despite early ART treatment controls HIV replication and reduces viral load, it does not significantly prevent the establishment of a reservoir of latently infected cells that precludes virus eradication [Zanchetta M et al, Antivir Ther 2008]. Therefore, perinatal infected children require life-long ART with the risk of important complications, including drug toxicities and adverse clinical events, consistent with those experienced by an aging population, including malignancies.

1.2.3 Cancer risk in children with perinatal HIV-infection

HIV-infected children and adults have an higher cancer risk compared to the general population; in particular, Kaposi sarcoma (KS), non-Hodgkin lymphoma (NHL), and invasive cervical cancer (ICC) represent the cancers with the higher incidence rates among people with HIV/AIDS and are defined as AIDS-defining malignancies (ADM) [CDC; Morb Mortal Wkly Rep 1992]. The long-term HIV-induced immunodeficiency together with co-infection with oncogenic viruses (KS-associated herpes virus (KSHV), Epstein-Barr virus (EBV) and Human Papilloma Virus (HPV), etiological agents of KS, NHL and ICC, respectively) represent the explanation for the increased risk of ADM [Grulich AE et al, Lancet 2007].

The impact of ART on cancer incidence in adulthood has been well described [Engels EA et al, AIDS 2006; Biggar RJ et al, J Natl Cancer Inst 2007; Grulich AE et al, Lancet 2007; Crum-Cianflone N et al, AIDS 2009; Simard EP et al, Cancer 2011], whereas it has been evaluated only by few studies in childhood [Kest H et al, Pediatr Infect Dis J 2005; Chiappini E et al, J Clin Oncol 2007; Alvaro-Meca A et al, Pediatr Infect Dis J 2011; Simard EP et al, Cancer Epidemiol Biomarkers Prev 2012]. Overall, epidemiological data suggest that with the widespread of ART, the incidence of ADM significantly decreased in both adults and children, due likely to partial immune restoration associated with an improved immune surveillance against oncogenic viruses [Biggar RJ et al, J Natl Cancer Inst 2007; Simard EP et al, Cancer Epidemiol Biomarkers Prev 2012]. In an Italian pediatric population of 1190 perinatal HIV-infected children, the ADM incidence decreased significantly from pre-ART to ART era [Chiappini E et al, J Clin Oncol 2007] (Table 2). The same trend was observed in an US study that analyzed cancer incidence between the pre-ART (1980-1995) and ART eras (1996-2007) in people diagnosed with AIDS during childhood (0-14 years): KS and NHL declined by 87% and 60%,

respectively in the ART era [Simard EP et al, Cancer Epidemiol Biomarkers Prev 2012]. Furthermore, a Spanish study that evaluated epidemiological trends of cancer diagnosed through 3 calendar periods (early-period ART: 1997-1999, middle-period ART: 2000-2002, and late-period ART: 2003-2008), found that the rate of ADM diagnoses decreased significantly (from 9.1 to 3.6 to 1.0 per 1000 children/year) during the study period [Alvaro-Meca A et al, Pediatr Infect Dis J 2011] (Table 2). However, despite ART reduced ADM, the overall incidence of malignancies in HIV-infected children do not significantly differ between pre-ART and ART-era [Chiappini E et al, J Clin Oncol 2007; Alvaro-Meca A et al, Pediatr Infect Dis J 2011; Simard EP et al, Cancer Epidemiol Biomarkers Prev 2012] (Table 2). The improvement in long-term life expectancy has led to change the spectrum of cancer among people living with HIV, shifting from ADM to non-ADM. In the Spanish study cited before, the rate of overall non-ADM increased from 0.6 to 5.0 to 8.7 cases of cancer per 1000 children/year during the early, middle and late ART eras [Alvaro-Meca A et al, Pediatr Infect Dis J 2011]. Malignant neoplasm of bone and articular cartilage, Hodgkin lymphoma (HD) and leiomyosarcoma were the cancers with the highest incidence [Simard EP et al, Cancer Epidemiol Biomarkers Prev 2012].

The pathogenesis for the increased non-ADM incidence remains to be fully elucidated. Immunodepression was long considered to play pivotal role in the etiology of cancer; however, given the ART-associated immune recovery, the immunodeficiency is not sufficient to explain this excess risk. The dysfunction of immune system due to persistent immune activation, premature aging and immune senescence may be the drivers of non-ADM [Deeks SG et al, Curr Opin Immunol 2012; Phillips AN et al, AIDS 2008].

Table 2. Epidemiologic data on cancer rate in HIV-infected children over time

Study population	Study period	Incidence of cancer*		
		All cancer	ADM	non-ADM
5840	1980-1995	5.59	4.70	0.65
(United States)[1]	1996-2007	2.13	1.50	0.63
1190	1985-1995	4.49	2.97	1.69
(Italy)[2]	1996-1999	4.09	2.60	1.48
	2000-2004	0.76	0.76	-
1307	1997-1999	9.70	9.10	0.60
(Spain)[3]	2000-2002	8.70	3.60	5.00
	2003-2008	9.70	1.00	8.70

*Incidence rates are per 1000 children/year

From: [1] Simard EP et al, Cancer Epidemiol Biomarkers Prev 2012
[2] Chiappini E et al, J Clin Oncol 2007
[3] Alvaro-Meca A et al, Pediatr Infect Dis J 2011

1.2.4 Premature aging and immune senescence in HIV-infection

Accumulating evidence suggest that HIV-infected people undergoing long-term ART tend to show an accelerated aging phenotype, that involves the immune system and leads to the increase in the frequency of non-AIDS-related complications (*i.e.* cardiovascular, liver and kidney diseases, non-ADM) [Deeks SG, Annu Rev Med 2011; Chiappini E et al, Cancer Lett 2014].

The immunological alterations that characterized immune senescence both in physiological aging and in HIV infection have several common features, and in both conditions immune activation/inflammation play a crucial role in promoting the gradual decline of the functionality of the immune system [Desai S and Landay A, Curr HIV/AIDS Rep 2010]. These processes affect all aspects of immunity, but the T-cell compartment seems to be the most damaged. These changes are characterized by a reduction of number and function of hematopoietic stem cells, thymic involution, loss of naïve cells, progressive enrichment of terminally differentiated T-cells, shrinkage of the T-cell repertoire, reversed ratio of $CD4^+$ to $CD8^+$ cells and increased levels of pro-inflammatory cytokines [Deeks SG, Annu Rev Med 2011].

In the context of HIV infection, the virus replicates and releases virions into circulation. A low-grade, chronic and systemic replication occurs despite ART and causes a continuous stimulation of immune system and inflammatory response. In addition, the injury to the immune component of gastrointestinal mucosal surface, along with damage to the intestinal epithelial microenvironment with its antimicrobial functions, may affect systemic immune activation during the chronic phase of HIV infection through the release of microbial products into blood circulation [Brenchley JM et al, Nat Med 2006], a phenomenon defined as microbial translocation. The strong pressure on the immune system, exerted by viral antigens, leads to the clonal expansion of activated cells,

resulting in differentiation and accumulation of senescent cells with loss of effector functions and proliferative capacity [Desai S & Landay A, Curr HIV/AIDS Rep 2010; Khaitan A & Unumatz D, Curr HIV/AIDS Rep 2011] (Figure 6). Finally, the accumulation of senescent cells secreting proinflammatory factors results in a chronic inflammation status that leads to the development of age-related pathologies [Ovadya Y & Krizhanovsky V, Biogerontology 2014]. Indeed, the progressive decline of immune function may result in an impaired immune surveillance against tumor antigen favoring cancer onset. The chronic immune activation may also induce B-cell stimulation leading to an expansion of EBV-infected cells, thus increasing the risk of developing EBV-associated malignancies [Petrara MR et al, Front Microbiol 2013]; both leiomyosarcoma and HD, two of the non-AIDS-defining cancers with higher incidence in the ART-era, are EBV-associated cancers [Bhatia K et al, Curr Opin Oncol 2012].

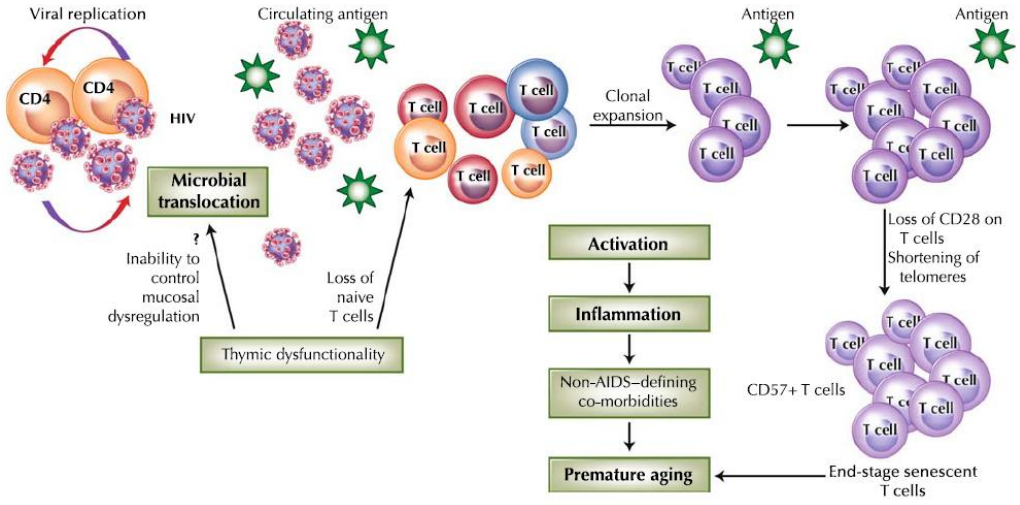

Figure 6. Accelerated aging model in HIV infection. [From Desai S & Landay A. Curr HIV/AIDS Rep 2010]

Since telomeres have been postulated as a universal biological clock that shorten in parallel with aging of cells and whose attrition reflects cellular replication history and cellular senescence, shortening telomere length is considered the best marker of biological aging. Telomere length in peripheral blood mononuclear cells (PBMC) is representative of that of many tissues; indeed, intra-individual correlation between telomere lengths in different tissue is high [Daniali L et al, Nat Commun 2013]. Short telomere length has been associated with older age [Barrett EL and Richardson DS, Aging Cell 2011], age-related diseases and low survival in the general population [Brouilette SW et al, Lancet 2007; Cawthon RM et al, Lancet 2003; Heidinger BJ et al, Proc Natl Acad Sci U S A 2012]. Recently, short telomere length has been associated with HIV infection [Côté HC et al, PLoS One 2012; Pathai S et al, AIDS 2013]. Moreover, considering the capability of nucleoside reverse transcriptase inhibitors (NRTIs) to repress not only HIV reverse transcriptase but also human telomerase, it has been suggested that ART may be a potential factor contributing to HIV-associated accelerated shortening of telomere and premature aging [Liu X et al, Nucleic Acids Res 2007; Hukezalie KR et al, PLoS One 2012; Leeansyah E et al, J Infect Dis 2013].

Chapter 2

Immune Senescence and Cancer in Elderly Patients: Results from an Exploratory Study and Preliminary Results of Long-term Follow-up

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The results of the case-control study have been published in :

"Falci C, Gianesin K, Sergi G, Giunco S, De Ronch I, Valpione S, Soldà C, Fiduccia P, Lonardi S, Zanchetta M, Keppel S, Brunello A, Zafferri V, Manzato E, De Rossi A, Zagonel V. Immune senescence and cancer in elderly patients: results from an exploratory study. Exp Gerontol 2013; 48:1436-42"

The longitudinal substudy is in progress.

ABSTRACT

Background. The challenge of immune senescence has never been addressed in elderly cancer patients. This study compares the thymic output and peripheral blood telomere length in 70≥ years old cancer patients. A longitudinal substudy is in progress to evaluate the impact of these markers on chemotherapy response and disease outcome.

Patients and Methods. Fifty-two elderly cancer patients and 39 age-matched controls without personal history of cancer were enrolled. All patients underwent a Comprehensive Geriatric Assessment. Peripheral blood samples were studied for naïve and recent thymic emigrant (RTE) $CD4^+$ and $CD8^+$ cells by flow cytometry. T-cell receptor rearrangement excision circle (TREC) levels, telomere length and telomerase activity in peripheral blood cells were quantified by real-time PCR. Patients with colorectal cancer has been enrolled in the longitudinal substudy.

Results. The percentages of $CD8^+$ naïve and $CD8^+$ RTE cells and TREC levels were significantly lower in cancer patients than in controls (p=0.003, p=0.004, p=0.031, respectively). Telomere lengths in peripheral blood cells were significantly shorter in cancer patients than in controls (p=0.046) and did not correlate with age in patients, whereas it did in controls (r=-0.354, p=0.031). Short telomere(≤median)/low TREC(≤median) profile was associated with higher risk of cancer (OR=3.68 [95%CI 1.22-11.11]; p=0.021). Preliminary data from the longitudinal substudy suggested that after chemotherapy there was an increase of $CD8^+$ terminally differentiated cells (p=0.031), and shortening of telomeres tended to higher in patients with stage III than in those with stage II.

Conclusions. Immune senescence is significantly worse in elderly cancer patients than in age-matched controls. The low thymic output and the shorter telomeres in peripheral blood cells of cancer patients may reflect a pre-existing condition which facilitates the onset of malignancies in elderly people and may impact the response to chemotherapy and the disease outcome.

1. INTRODUCTION

People over 65 years old are the fastest-growing age bracket in the population and will account for an estimated 20% of Americans and 25% of Europeans by the year 2030 [Fries JF, Ann Intern Med 2003]. The incidence of malignancies increases with age, so the number of cancers in the elderly is expected to increase significantly in years to come [American Cancer Society, 2012]. Several studies have shown that elderly patients are less likely to be treated according to guidelines, and their under-treatment may be detrimental to both survival and quality of life [Sargent DJ et al, N Engl J Med 2001; Bouchardy C et al, J Clin Oncol 2003; Dale DC, J Support Oncol 2003; Ng R et al, Clin Lung Cancer 2005]. Elderly cancer patients may benefit from chemotherapy just as much as younger adults, but at a higher risk of hematological toxicity [Muss HB et al, JAMA 2005; Hurria A et al, J Natl Compr Canc Netw 2012; Muss HB et al, J Clin Oncol 2007]. Better understanding of the physiological and functional changes that occur with aging will enable to improve strategies for treating elderly cancer patients. Since the aging process coincides with a gradual decline in the functional reserve of multiple organ systems [Balducci L, J Support Oncol 2003] the search for laboratory markers of biological aging and organ reserve should be a priority of clinical research in the field of geriatric oncology.

Over a lifetime, the immune system undergoes profound remodeling process with major impact on health and survival [Grubeck-Loebenstein B et al, Aging Clin Exp Res 2009; Fulop T et al, Ann N Y Acad Sci 2010]. Thymic involution and diminished output of T lymphocytes are thought to be among the major factors contributing to the loss of immune function with age [Berzins SP et al, J Exp Med 1998]. T-cell output begins to decline exponentially from early in life, and at 75 years of age the immune repertoire

appears to be severely impaired [Douek DC et al, Nature 1998; Naylor K et al, J Immunol 2005]. However, recent data suggest that the thymus may remain active even late in life, supplying functional T cells to the periphery [Nasi M et al, Aging Cell 2006; Mitchell WA et al, Clin Exp Immunol 2010].

Measuring T-cell receptor rearrangement excision circle (TREC) levels in peripheral blood lymphocytes has been suggested as a method for quantifying thymic output [Douek DC et al, Nature 1998; Zhang L et al, J Exp Med 1999; Ometto L et al, AIDS 2002; De Rossi A et al, J Infect Dis 2002]. TRECs are generated by T-cell receptor gene rearrangement and maintained in thymic emigrant cells as DNA episomes. Because TRECs are not duplicated during mitosis, their concentration is diluted out with each cell division. The frequency of recent thymic emigrant (RTE) cells in peripheral blood, identified by the marker $CD31^+$ among the $CD45RA^+$ naïve T cells [Kimmig S et al, J Exp Med 2002], decreases with aging and correlates well with the decline in TREC levels [Kohler et al, Eur J Immunol 2005; Junge S et al, Eur J Immunol 2007]. Very little is known about the relationship between TRECs and cancer, especially in elderly patients. One study on head and neck cancer patients, including just a few ≥70 years old, showed that the age-associated decrease of TREC numbers and naïve T lymphocytes was significantly greater in cancer patients than in controls, suggesting altered lymphocyte homeostasis in the former [Kuss I et al, Clin Immunol 2005].

The immune system function depends largely on its capacity for extensive cell division and clonal lymphocyte expansion. Telomere length, and its regulation by telomerase have attracted considerable attention, due to their potential roles in controlling cell replication [Blackburn E et al, Nat Med 2006]. Telomeres are capping end structures of eukaryotic

chromosomes essential for protecting chromosome integrity [Blasco MA, Nat Rev Genet 2005]. Telomeres are progressively shortened during each cell division due to end-replication problems of DNA polymerase; when a critical length is reached, the cell undergoes cycle arrest and apoptosis [Blasco MA, Nat Rev Genet 2005]. Permanent cell growth relies on telomere maintenance, and certain human cell subsets, as well as most cancer cells, have telomerase activity which enables telomere elongation [Dolcetti R & De Rossi A, Med Res Rev 2012]. Despite their telomerase activity, most tumor cells have shorter telomeres than the corresponding normal tissues, and there is a relationship between short telomeres and genetic instability [Garcia-Aranda C et al, Cancer 2006; Rampazzo E et al, Br J Cancer 2010]. Since telomere shortening reflects cell turnover and exposure to oxidative and inflammatory damage, which are crucial processes of biological aging, it has been suggested that telomere length serves as an indicator of aging process [Wong JMY & Collins K, Lancet 2003; Aviv A, J Gerontol A Biol Sci Med Sci 2006; Baird DM, Exp Gerontol 2006]. Telomere shortening in peripheral blood cells has been associated with a number of chronic diseases, such as coronary heart disease, hypertension, dementia, obesity, insulin resistance, and osteoporosis. However, two clinical trials failed to confirm any relationship between telomere length and frailty syndrome in elderly non-cancer patients [Woo J et al, Mech Ageing Dev 2008; Collerton J et al, Mech Ageing Dev 2012].

Several studies have investigated the relationship between telomere length in peripheral blood cells and cancer risk and outcome. Although few manuscript report that longer telomere length is a risk factor [Lan Q et al, PLoS One 2013; Svenson U et al, Cancer Res 2008], most of the studies indicate that short telomere lengths are associated with a cancer risk and/or worse prognosis [Bojesen SE et al, Nat Genet 2013; Martinez-Delgado B et al,

J Med Genet 2012; Riegert-Johnson DL et al, Int J Biol Markers 2012; Shao L et al, J Urol 2007; Wu X et al, J Natl Cancer Inst 2003; Qu F et al, Mol Oncol 2014; Chen Y et al, Ann Oncol 2014]. To date, however, telomere length in peripheral blood cells has been measured in elderly non-cancer patients and younger cancer patients, but no data are available for elderly cancer patients.

In this study, we present the results of a prospective observational study providing the first description of immune senescence markers and frailty scores in elderly cancer patients and age-matched controls. Furthermore, we report the preliminary results of the ongoing longitudinal substudy planned to evaluate the impact of these markers on chemotherapy response and/or risk of tumor relapse.

2. MATERIAL and METHODS

2.1 Study design and study population

Patients enrolled in this prospective observational study were aged ≥70 years, with stage I-III breast or colorectal cancer, diagnosed during the previous 2 months and radically resected, consecutively admitted to the Medical Oncology Units from September 2010 to March 2012. Controls included patients ≥70 years old with no personal history of cancer, consecutively admitted to the Geriatric Clinic. For both groups, the exclusion criteria were: any hematological disorders, chronic diseases requiring immunosuppressive treatment, prior immunodeficiency, blood transfusion ≤4 weeks before blood sampling, active infectious diseases, extremely severe comorbidities suggesting a life expectancy <6 months, or severe cognitive impairment hampering communication with the physician.

A subgroup of patients with colorectal cancer have been enrolled in the longitudinal substudy.

The study was approved by the institutional Ethics Committee and conducted in accordance with the Helsinki Declaration and Good Clinical Practice guidelines. Written informed consent was obtained from all patients.

2.2 Clinical assessment

Complete demographic and clinical details were collected at baseline for each patient (Table 1). At the first visit, both cases and controls underwent traditional comprehensive geriatric assessment (CGA) administered by a multidisciplinary team, including medical oncologist, geriatrician and psychologist. As reported elsewhere [Basso U & Monfardini S, Eur J Cancer Care 2004], the CGA included Activities of Daily Living (ADL), Instrumental Activities of Daily Living (IADL), Short Portable Mental Status Questionnaire (SPMSQ), and the Mini Mental Status Examination (MMSE). Comorbidities and their severity were studied according to the Cumulative Illness Rating Scale-Comorbidity Index (CIRS-CI) and Cumulative Illness Rating Scale-Severity Index (CIRS-SI) [Conwell Y et al, J Am Geriatric Soc 1993]. Affective status was assessed with the Geriatric Depression Scale (GDS) [Yesavage JA et al, J Psychiatr Res 1983]. Nutritional status was explored with the Mini Nutritional Assessment (MNA) [Guigoz Y & Vellas B, Nutr Workshop Ser Clin Perform Programme 1999]. Social aspects included household composition, institutionalization, amount of assistance provided by a caregiver. The number of drugs taken by patients for concomitant diseases was also recorded. The CGA results were interpreted as previously reported by Balducci [Balducci L, J Support Oncol 2003], classifying patients as fit, vulnerable or frail. A multidimensional prognostic index (MPI) score was also calculated based on findings for the ADL, IADL, SPMSQ, CIRS-CI, CIRS-SI, MNA, number of drugs and social conditions, assuming a low,

moderate and high mortality risk for MPI ≤0.33, 0.34-0.66, >0.66, respectively [Pilotto A et al, Rejuvenation Res 2008; Giantin V et al, J Geriatric Oncol 2013].

2.3 Biomarker analyses

Peripheral blood samples were collected at the time of enrollment, prior to any oncological medical treatment (endocrine therapy, chemotherapy, radiotherapy or immune therapy). In the longitudinal substudy, samples were collected at the end of adjuvant chemotherapy treatment and/or at tumor relapse; the median period of follow-up was 22 [20-28] months. Samples were analyzed for the standard blood parameters (Table 1S, Supplementary data), for Cytomegalovirus (CMV) and for the following tests.

2.4 Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by centrifugation on a Ficoll-Paque gradient [Freguja R et al, Clin Exp Immunol 2011]. Cells were stained with the following labeled monoclonal antibodies (mAbs): anti-CD3 (FITC), anti-CD4 (PerCP), anti-CD8 (PerCP), anti-CD31 (PE) and anti-CD45RA (APC). Appropriate isotypic controls (mouse IgG1-PE and mouse IgG2b-APC) were used to assess non-specific staining. All samples were analysed by four-color flow cytometry on a fluorescence-activated cell sorter (FACS)Calibur (Becton-Dickinson) [Freguja R et al, Clin Exp Immunol 2011]. Data were processed with CellQuest Pro Software (Becton-Dickinson) and analysed using Kaluza® Analysis Software v.1.2 (Beckman Coulter). Anti-CD45RA and anti-CD27 were used to identify, within $CD3^+CD4^+$ and $CD3^+CD8^+$ gates, naïve ($CD45RA^+CD27^+$), central memory ($CD45RA^-CD27^+$), effector memory ($CD45RA^-CD27^-$) and terminally differentiated memory ($CD45RA^+CD27^-$) cells. Moreover, anti-CD45RA and anti-CD31 were used to identify recent thymic emigrant

(RTE) ($CD45RA^+CD31^+$), and anti-CD28 and anti-CD57 to identify senescent cells ($CD28^-CD57^+$).

2.5 TREC quantification

Thymic output in PBMC was studied by measuring TREC levels by real-time polymerase chain reaction (PCR), exactly as described previously [Ometto L et al, AIDS 2002; De Rossi A et al, J Infect Dis 2002]. TREC levels were expressed as the number of TREC copies per 10^5 PBMC [De Rossi A et al, J Infect Dis 2002; Anselmi A et al, Clin Exp Immunol 2007].

2.6 Telomere length measurement by quantitative real-time PCR

Telomere length in PBMC was determined by real-time PCR exactly as described elsewhere [Rampazzo E et al, Br J Cancer 2010; Rampazzo E et al, Haematologica 2012], and values were expressed as relative telomere/single-copy gene (T/S) ratio [Rampazzo E et al, Br J Cancer 2010].

2.7 Telomerase activity quantification

Telomerase activity was quantified by real-time PCR, as described elsewhere [Rampazzo E et al, Haematologica 2012] and expressed in relative units (RU).

2.8 Irradiation of PBMC and detection of DNA-damage by flow cytometry

After isolation of PBMC by Ficoll-Paque gradient, cells (~$8*10^6$) were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS, Life Technologies), 10 ng/ml IL-2 (Roche), 2mM L-glutamine (Gibco) and 50 ug/ml Gentamycin (Sigma) (standard medium) at 37°C in 5% CO_2, in 6-well plate for 12 hours before y-irradiation. Following the removal of ~10^6 PBMC (untreated control), the remaining cells were irradiated to a

dose of 2.5 Gy using y-radiation from a ^{137}Cesium source (CisBIO International, France) at room temperature and were immediately incubated in 12-well plate in standard medium at 37°C in 5% CO_2; aliquots of ~10^6 PBMC cells were taken 1, 5 and 24 h post-irradiation. Cells were fixed in 4% paraformaldehyde (Sigma) for 10 min at 37°C, chilled on ice for 1 min, washed in PBS, permeabilized with 90% methanol in 0.5% bovine serum albumin (BSA; Sigma)-PBS, and kept at -20°C for 1 h. Cells were washed three times with 0.5% BSA-PBS and incubated in blocking buffer [PBS supplemented with 8% FCS, 0.1 g/L RNAseA (Life Technologies), 10 mM NaF (Sigma) and 1 mM $NaVO_3$ (Sigma), 0.25 g/L salmon sperm DNA (Sigma), 0.2% Triton X-100 (Roche)] for 1 h prior to y-H2AX staining with the AlexaFluor-488 mouse anti-H2AX (pS139) antibody (BD Pharmigen) diluted in blocking buffer for 1 h at room temperature. Cells were washed twice in 0.5% BSA-PBS and resuspended in PBS. Isotype antibody was included as a background reference. A minimum of 100000 cells were acquired using a FACSCalibur (Becton-Dickinson). Cellular debris were excluded from the analysis by gating a forward scatter versus side scatter plot. Analysis of flow cytometry data was conducted using the CellQuest Pro Software (Becton-Dickinson). Percentage of y-H2AX positive cells and median fluorescence intensity were calculated at each time point.

2.9 Statistical analysis

Differences in geriatric parameters between groups were examined with the Mann-Whitney test for non-parametric data and Pearson's χ^2 test with odds ratios (OR) and 95% confidence intervals (CI) for nominal data (CGA, MPI, ADL, IADL). The Mann-Whitney test was used to compare subsets of lymphocytes, TREC levels and telomerase activity. Student's t-test was used to compare the telomere lengths. Correlations between age and TREC levels, or telomere length in both groups were analysed with Pearson's χ^2 test.

Spearman's rank correlation was used to analyse associations between geriatric parameters and TREC levels, telomere lengths or telomerase activity. TREC levels and telomere length were analysed also as dichotomous variables (cut-off: ≤median) and OR were estimated using logistic regression model. All statistical analyses were performed using SPSS software, version 19 (SPSS Inc.). All p-values were two-tailed, and were considered significant when lower than 0.05.

3. RESULTS

3.1 Characteristics of study population

Ninety-one patients, 52 with cancer (26 breast and 26 colorectal cancer) and 39 controls, were enrolled in the study. Their demographic and clinical characteristics are listed in Table 1. There were more females in the control group (82.1%) than among the cancer patients (63.5%); the latter had better Karnofsky performance status than controls. As established by the study protocol, before enrollment, cancer patients underwent a thorough radiological assessment to rule out metastases. According to the TNM staging system, 16 patients (30.8%) had stage I, 33 (63.5%) had stage II, and 3 (5.7%) had stage III disease. Thirty-one patients were prescribed the same adjuvant therapy as for younger adults, according to good clinical practice, 18 patients received an adapted treatment, due to problems detected at the CGA, and 3 were given no adjuvant therapy, due to frailty detected at the CGA and were only followed up.

3.2 Comprehensive geriatric assessment

All cancer patients and controls underwent the full CGA; 17.3% of the former and 43.6% of the latter were classified as frail (OR=0.29, 95% CI 0.10-0.82), while the proportion of vulnerable patients in the two groups did not differ significantly (Table 2). 5.8% of cancer

patients and 23.1% of controls had a moderate MPI (OR=0.20, 95% CI 0.05-0.79), and only one control had a severe MPI score. When MPI was calculated as a continuous variable, the median score was significantly lower for cancer patients than for controls (Table 2). In ADL and IADL, cancer patients tended to have preserved greater physical autonomy than controls, although the difference was not statistically significant (Table 2). Controls had better cognitive status in both the MMSE and SPMSQ, but their burden of associated diseases (as shown by CIRS-CI and CIRS-SI) was higher, as was their use of drugs for these comorbidities (Table 2).

3.3 Standard hematological and biochemical parameters

The hematological parameters of cancer patients and controls differed only in their platelet count (Table 1S, Supplementary data). Controls had significantly lower levels of nutritional markers, such as total plasma proteins, triglycerides, and LDL-cholesterol; CRP and IL-6 were significantly higher in controls than in cancer patients (Table 1S, Supplementary data). The overall prevalence of CMV-IgG seropositive individuals was 96.7%. Antibodies against CMV were found in 49 (94.2%) of cancer patients and 39 (100%) of controls. No statistical difference was found between CMV antibody titers between the groups (p=0.498) (Table 1S, Supplementary data).

3.4 Immune senescence markers

Cancer patients and controls had comparable percentages of total $CD4^+$ lymphocytes, naïve $CD4^+CD45RA^+$ and RTE $CD4^+CD45RA^+CD31^+$ cells (Table 3). By contrast, the median (interquartile) percentages of naïve $CD8^+CD45RA^+CD27^+$ and RTE $CD8^+CD45RA^+CD31^+$ cells were significantly lower in cancer patients than in controls (16.7% [9.3-25.2] *versus* 24.6% [14.7-33.5]; p=0.003) and (34.2% [24.7-46.4] *versus*

44.9% [36.0-50.7]; p=0.004), respectively (Table 3). Notably, the lower percentage of $CD8^+$ naïve cells in cancer patients was compensated by a higher expansion of $CD8^+$ memory cells; thus, cancer patients and controls did not differ in terms of their percentages of total $CD8^+$ cells (Table 3). In particular, among the $CD8^+$ cell subsets, the $CD45RA^-CD27^-$ (effector memory) cells were found to be significantly higher in cancer patients than in controls (21.7% [12.7-31.7] *versus* 16.3% [10.4-25.0]; p=0.042). The imbalance in favor of the $CD8^+$ memory cell subset in cancer patients was evident even when the absolute cell count was considered (not shown). For a few subjects with available frozen samples (11 cancer patients and 8 controls), markers of immune senescence ($CD28^-CD57^+$) were investigated. Cancer patients and controls exhibited percentages of $CD4^+CD28^-CD57^+$, but $CD8^+$ cells with senescent phenotype tended to be higher in the former (p=0.082) (Table 3S, Supplementary data).

TREC levels in PBMC were significantly higher in controls than in cancer patients (25.0 [14.0-56.0] *versus* 16.0 [7.7-31.5] TREC copies/10^5 PBMC; p=0.031) (Table 3). TREC levels decreased significantly with increasing age in cancer patients (r=-0.478; p<0.001), while did not correlate with age in controls (r=0.071; p=0.677) (Figure 1A).

The mean telomere length in PBMC was significantly lower in cancer patients than in controls; the former had a mean (± standard deviation (SD)) telomere length of 1.09±0.31 T/S *versus* 1.22±0.30 T/S in controls, p=0.046 (Table 3). Telomere length correlated inversely with age in controls (r=-0.354, p=0.031), but not in cancer patients (r=-0.011, p=0.938) (Figure 1B). Telomerase activity was significantly higher in cancer patients, being detected in 38/52 patients (73.1%; median value 14.6 RU) and 20/38 controls (52.6%; median value 1.4 RU); p=0.003. Telomerase activity was not influenced by age in either group.

TREC was defined as high and low, and telomere length as long or short according to their value above and below the median, respectively. Subjects with TREC low/telomere short profile were at higher risk of cancer than subjects with only low TREC or short telomere (Table 4).

3.5 Relationship between TREC levels, telomere length and geriatric characteristics

In the control group, the CIRS-SI was the only tool which revealed a significant positive association with TREC level (r=0.45, p=0.01) and telomere length (r=0.35, p=0.03), whereas it had a significant negative correlation with the number of drugs taken for concomitant diseases (r=-0.39, p=0.02) (Table 2S, Supplementary data). No relationship emerged between telomere length, thymic output and geriatric features of cancer patients (Table 2S, Supplementary data). Neither a classification of unfitness at CGA (not shown) nor the MPI score (Table 2S, Supplementary data) correlated significantly with the thymic output or telomere length in either group. Among the controls, TREC levels correlated positively with IL-6 (r=0.34, p=0.04), and negatively with total plasma protein levels (r=-0.38, p=0.02). Neither of these correlations emerged in the cancer patients (data not shown).

3.6 Preliminary results from the longitudinal substudy

Twenty-two patients have been already enrolled in this substudy. Eight patients received capecitabine plus oxaliplatin, 1 capecitabine plus bevacizumab, 8 capecitabine alone for 6 months while 5, all with stage II disease, received no adjuvant chemotherapy. Four patients suspended therapy before the deadline due to poor tolerability and/or onset of cardiovascular complications. Three patients had a relapse: one with stage II disease (no

adjuvant chemotherapy) and two with stage III (both with a complete chemotherapy treatment).

Overall, telomere length tended to decrease during the follow-up period ($T/S_{baseline}$ 1.23±0.28 *versus* $T/S_{follow\text{-}up}$ 1.14±0.25; p=0.201), even if at not significant level. At baseline, telomere length was not associated with tumor staging, being T/S of 1.20±0.26 and 1.27±0.32 for stage II and III respectively (p=0.300), but their shortening at follow-up tended to be higher in patients with stage III than in those with stage II ($\Delta_{baseline\text{-}follow\text{-}up}$ T/S 0.13 *versus* 0.07). The ΔT/S of 3 patients with tumor relapse were: 0.15, 0.05 and -0.14, respectively; the low number and the heterogeneity (stage disease and treatment) did not allow to draw any conclusions. TREC levels tended to be lower in samples at follow-up compared to baseline (6 [3-18] *versus* 10 [6-22] TREC copies/10^5 PBMC; p=0.161). These results are in agreement with phenotypic analysis of peripheral blood cells. Frequencies of $CD4^+$ and $CD8^+$ naïve T cell did not change during follow-up period. Conversely, within the $CD8^+$ memory cell subset, we found a shift of cells from effector ($CD45RA^-CD27^-$) to terminally differentiated ($CD45RA^+CD27^-$) phenotype (Figure 2). Moreover, senescent cells tended to be more expanded at follow-up than at baseline (63.0% [57.9-73.5] *versus* 58.5% [46.5-64.0]; p=0.099). The higher frequencies of these subsets may explain the tendency of shorter telomere length in samples at follow-up.

The higher levels of memory cells in elderly cancer patients, both before and after treatment, is important. It is known that DNA-damage recognition and repair efficiency varies between memory and naïve cells [Scarpaci S et al, Mech Ageing Dev 2003; Cossarizza A et al, Mech Ageing Dev 1996]. In a preliminary experiment, we analyzed the DNA-damage repair in PBMC exposed to a dose of 2.5 Gy using y-radiation from a ^{137}Cesium source. By flow cytometric assay, we found that the level of y-H2AX, a robust

marker of cellular double strand breaks [d'Adda di Fagagna F et al, Nature 2003], was higher in untreated PBMC from cancer patients than in those from controls, suggesting a more unstable genome in the former. Soon after irradiation, the level of y-H2AX increased in both groups, but at 24 h it remained higher in cancer patients than in controls (Figure 3), suggesting a lower efficiency in DNA repair process in the former [Banáth JP et al, BMC Cancer 2010].

4. DISCUSSION

This is the first study aiming to shed light on two essential aspects of the aging process (thymic output and telomere length in peripheral blood cells) in elderly cancer patients. We found that 70- to 92-year-old cancer patients had significantly lower TREC levels, lower percentages of naïve and RTE $CD8^+$ T lymphocytes, and a more expanded $CD8^+$ memory cell subset than age-matched controls. These results are partially consistent with those of a small trial in which head and neck cancer patients (most of them under 70-years-old) revealed lower TREC levels than controls [Kuss I et al, Clin Immunol 2005]. The expansion of memory $CD8^+$ cells, particularly those with effector phenotype, is also consistent with the findings of two recent studies conducted on breast cancer patients [Hueman MT et al, Cancer Immunol Immunother 2007; Poschke I et al, Int J Cancer 2012]. The shift from naïve T cells to memory cells was probably due to greater stimulation by tumor antigens; as $CD8^+$ T cells are the key components of tumor immune surveillance, the tumor-induced dysfunction may be more evident in the CD8-cell subset than in the $CD4^+$ T-cell compartment [Klebanoff CA et al, Immunol Rev 2006; Williams MA & Bevan MJ, Annu Rev Immunol 2007].

Our findings confirm reports by other authors that thymic activity does not stop completely beyond 70 years of age [Nasi M et al, Aging Cell 2006; Mitchell WA et al, Clin Exp Immunol 2010]. Notably, while TREC levels in cancer patients dropped significantly with increasing age, they remained relatively constant in controls. Thymic output may compensate for the loss of peripheral blood lymphocytes in elderly patients, but this homeostatic phenomenon seems to disappear in cancer patients. In our study, the control group included a higher proportion of frail patients, with higher average MPI scores and more chronic diseases and comorbidities than the cancer patients. Levels of pro-inflammatory cytokine IL-6 were also significantly higher in controls than in cancer patients. Systemic inflammation and loss of peripheral blood cells may stimulate thymic output, which plays an important role in immunological homeostasis. In fact, TREC levels did correlate significantly with CIRS-CI in controls group, but not in cancer patients. The imbalance between the two groups in terms of their geriatric conditions reinforces the magnitude of the difference observed for TREC levels between cancer patients and controls. While the hypothesis of immune homeostasis may justify a higher thymic output in controls, the lower age-related TREC levels seen in cancer patients may point to a pre-existing condition, favoring immune escape and the onset of malignant disease.

In agreement with the lower percentage of $CD8^+$ naïve and RTE cells in cancer patients than in controls, the former tended to have a higher percentage of $CD8^+$ immune senescent cells. Several data indicate that latent CMV infection leads to significant changes in the $CD8^+$ repertoire, and it may be an important driver of immune senescence [Almanzar G et al, J Virol 2005; Solana R et al, Immun Ageing 2012]. The above results

are unlikely to be due to CMV infection, because all except three cancer patients were CMV-positive and with similar titers of anti-CMV antibodies.

As regards telomere length, there are many data available on the relationship between telomere length and aging-associated changes and chronic [Wong JMY & CollinsK , Lancet 2003; Aviv A, J Gerontol A Biol Sci Med Sci 2006; Baird DM, Exp Gerontol 2006], but data are lacking for elderly cancer patients. Our study showed that cancer patients' telomeres in PBMC are significantly shorter than those seen in controls. In addition, while control patients' telomeres become shorter with age, as expected according to previous studies [Blasco MA, Nat Rev Genet 2005; Der G et al, PLoS One 2012; Steenstrup T et al, Eur J Epidemiol 2013;, no such relationship between age and telomere length was seen in our cancer patients. This difference cannot be explained by lower telomerase activity, because it was detected in the PBMC of most cancer patients. The fact that controls scored worse on the severity and comorbidity indexes than cancer patients clearly highlights the magnitude of the difference in telomere length between the two groups.

Several studies have found shorter telomeres in tumor cells than in surrounding non-cancer cells [Rampazzo E et al, Br J Cancer 2010; Bisoffi M et al, Int J Cancer 2006], but the intriguing finding of shorter telomeres in PBMC cannot be explained by the presence of cancer cells in peripheral blood. Our cancer patients had radically-resected early breast or colorectal cancer, and had undergone through radiological assessment to exclude both persistent local disease and distant metastases. As reported elsewhere [Franken B et al, Breast Cancer Res 2012; Shimada R et al, Oncol Rep 2012], the number of epithelial cells detectable in the peripheral blood on patients with early breast or colorectal cancer is so small that it cannot influence telomere length or telomerase activity. We surmised that our control patients' longer telomeres are related to their higher proportion of naïve $CD8^+$

cells. To explore this issue, we estimated telomere length separately in $CD8^+$ naïve and $CD8^+$ memory cells from 3 cancer patients: the two subsets had a similar telomere length, indicating that the shorter telomeres cannot be explained by an excessive replication/expansion of the memory cell subset (not shown). We therefore suggest that telomere length in peripheral blood cells reflects the more advanced biological aging of the immune system in cancer patients than in controls, a pre-existing condition which facilitates the onset of cancer in the former. In addition, the analysis of y-H2AX expression in PBMC, before and after exposure to a source of y-radiation, revealed that cancer patients, have a higher baseline level of DNA-damage and a lower capacity to repair it compared to controls. Because genomic instability is a driver of tumorigenic process, subjects with higher genomic instability are at a greater risk for developing cancer and relapse.

In summary, from the exploratory study emerged that elderly cancer patients have significantly lower thymic output and shorter telomeres in their peripheral blood cells than age-matched non-cancer patients; these results indicate that immune senescence may hamper the ability of the immune system to prevent cancer initiation and/or control tumor progression. Moreover, considering that the effective immune responses are dependent upon optimal lymphocyte division and clonal expansion, and that immune senescence may contribute to chemotherapy response and outcome of disease, the search of these markers may be useful to predict tumor onset and treatment response.

Table 1. Demographic and clinical characteristics of cancer patients and controls

	All patients, *n* (%)	**Cancer patients,** *n* (%)	**Controls,** *n* (%)
Age (yrs)			
n.	91 (100)	52 (57.2)	39 (42.8)
Median, range	81, 70-92	81, 72-92	80, 70-91
Gender			
Male	26 (28.6)	19 (36.5)	7 (17.9)
Female	65 (71.4)	33 (63.5)	32 (82.1)
Performance status (ECOG)			
0-1	83 (91.2)	51 (98.1)	32 (82.1)
≥2	8 (8.8)	1 (1.9)	7 (17.9)
Social Condition			
Home	90 (98.9)	51 (98.1)	39 (100)
Nursing home	1 (1.1)	1 (1.9)	0 (0.0)
Type of Assistance		9 (17.3)	14 (35.9)
Alone	23 (25.3)	38 (73.1)	21 (53.8)
Family	59 (64.8)	5 (9.6)	4 (10.3)
Others	9 (9.9)		
Caregiver Assistance (hours/day)			
Median time, range	24, 2-24	24, 2-24	24, 3-24
Tumor Stage (TNM)			
I	-	16 (30.8)	-
II	-	33 (63.5)	-
III	-	3 (5.7)	-
Modality of Diagnosis			
Symptoms/Self examination	-	40 (76.9)	-
Screening	-	9 (17.3)	-
Incidental diagnosis	-	3 (5.8)	-
Therapeutical Choice			
Adjuvant therapy as for younger adults	-	31 (59.6)	-
Adapted treatment	-	18 (34.6)	-
No adjuvant therapy	-	3 (5.8)	-
Agreement to Proposed Therapy			
Ready	-	51 (98.1)	-
Patient refusal	-	1 (1.9)	-
Cause of Admission to hospital/ Outpatient Services			
Cardiovascular disease	-	-	18 (46.2)
Peripheral deep venous thrombosis	-	-	4 (10.2)
Gastrointestinal inflammatory disorders or bleeding	-	-	5 (12.8)
Screening of osteoporosis with no active comorbidity	-	-	12 (30.8)

Table 2. Scores of geriatric tools in cancer patients and controls

Geriatric Tool	All patients	Cancer patients	Controls	OR (95% CI)	*p*-value
CGA, *n* (%)					
Fit	37 (40.7)	24 (46.2)	13 (33.3)	1	
Vulnerable	28 (30.7)	19 (36.5)	9 (23.1)	1.14 (0.40-3.24)	0.801 [a]
Frail	26 (28.6)	9 (17.3)	17 (43.6)	0.29 (0.10-0.82)	0.020 [a]
MPI, *n* (%)					
Low	78 (85.7)	49 (94.2)	29 (74.3)	1	
Moderate	12 (13.2)	3 (5.8)	9 (23.1)	0.20 (0.05-0.79)	0.022 [a]
Severe	1 (1.1)	0 (0.0)	1 (2.6)	<0.01	ns [a]
MPI					
Median, range	0.25, 0.00-0.69	0.19, 0.00-0.44	0.25, 0.06-0.69	-	0.001 [a]
Dependencies in ADL, *n* (%)					
No	71 (78.0)	44 (84.6)	27 (69.2)	1	
≥1	20 (22.0)	8 (15.4)	12 (30.8)	0.41 (0.15-1.14)	0.790 [a]
Dependencies in IADL, *n* (%)					
No	57 (62.6)	36 (69.2)	21 (53.8)	1	
≥1	34 (37.4)	16 (30.8)	18 (46.2)	0.52 (0.22-1.23)	0.133 [a]
MMSE					
Median [IQR]	27.1 [24.4-28.4]	26.2 [24.4-27.3]	28.3 [26.4-29.3]	-	0.001 [b]
SPMSQ					
Median [IQR]	1.0 [0.0-2.0]	1.0 [0.0-2.0]	0.0 [0.0-2.0]	-	0.013 [b]
CIRS-CI					
Median [IQR]	3.0 [1.0-4.0]	2.0 [1.0-3.0]	3.0 [2.0-4.0]	-	0.032 [b]
CIRS-SI					
Median [IQR]	1.5 [1.3-1.6]	1.4 [1.2-1.5]	1.6 [1.4-1.8]	-	<0.001 [b]
GDS					
Median [IQR]	3.0 [1.0-6.0]	3.0 [1.2-6.0]	3.0 [1.0-6.0]	-	0.888 [b]
BMI					
Median [IQR]	25.7 [24.0-27.4]	25.8 [24.2-26.9]	25.0 [23.0-28.6]	-	0.911 [b]
Drugs for concomitant diseases					
Median [IQR]	5 [3-6]	4 [2-5]	5 [4-8]	-	0.003 [b]

[a] Pearson's χ^2 or Fisher test; [b] Mann-Whitney *U* test

Table 3. Thymic output and telomere length in cancer patients and controls

Parameter	All patients Median [IQR]	Cancer patients Median [IQR]	Controls Median [IQR]	*p*-value
% $CD3^+$	54.3 [40.8-65.2]	53.6 [37.8-65.5]	56.3 [41.8-65.2]	0.560 [a]
% $CD4^+$	35.5 [25.6-49.1]	35.0 [21.1-46.7]	35.6 [26.6-51.8]	0.389 [a]
% $CD8^+$	15.7 [10.1-22.4]	15.4 [9.7-22.6]	16.4 [10.5-22.8]	0.717 [a]
% naïve, $CD4^+CD45RA^+CD27^+$	34.0 [22.5-48.5]	29.6 [17.9-48.3]	38.0 [27.0-49.8]	0.066 [a]
% naïve, $CD8^+CD45RA^+CD27^+$	20.5 [10.9-29.5]	16.7 [9.3-25.2]	24.6 [14.7-33.5]	0.003 [a]
% RTE, $CD4^+CD45RA^+CD31^+$	21.3 [14.2-33.4]	20.3 [10.4-32.0]	23.3 [16.8-33.5]	0.176 [a]
% RTE, $CD8^+CD45RA^+CD31^+$	37.6 [29.1-48.9]	34.2 [24.7-46.4]	44.9 [36.0-50.7]	0.004 [a]
TREC copy number/10^5 PBMC	19.0 [10.1-37.0]	16.0 [7.7-31.5]	25.0 [14.0-56.0]	0.031 [a]
Telomere length (T/S ratio)[c]	1.15 ± 0.30	1.09 ± 0.31	1.22 ± 0.30	0.046 [b]
Telomerase activity				
n positive/*n* tested	58/90	38/52	20/38	
Relative Units	5.6 [0.0-26.0]	14.6 [0.0-36.8]	1.4 [0.0-6.4]	0.003 [a]

[a] Mann-Whitney *U* test
[b] Student *t*-test
[c] Data are expressed as mean ± SD

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FIGURE LEGEND

Figure 1. Correlation between age and (A) TREC levels and (B) telomere lengths in cancer patients and controls. Panel (A): TREC levels decline with age in the peripheral blood cells of cancer patients ($r=-0.478$, $p<0.001$), but not in controls ($r=0.071$, $p=0.677$). Panel (B): telomere lengths decrease with age in peripheral blood cells of controls ($r=-0.354$, $p=0.031$), but not in cancer patients ($r=-0.011$, $p=0.938$).

Figure 2. $CD8^+$ cell subsets distribution at baseline (A) and at follow-up (B). Pie charts show that frequencies of naïve and central memory cells did not significantly differ between baseline and follow-up ($p=0.309$ and $p=0.137$, respectively); conversely, the proportion of effector memory cells significantly declined ($p=0.017$), while terminally differentiated cells increased ($p=0.031$).

Figure 3. Flow cytometry analysis of y-H2AX induction in controls and cancer patients. y-H2AX expression in untreated PBMC and after exposure to a dose radiation of 2.5 Gy at 24 h, in control (n=2) and cancer patients (n=3). Error bars represent standard deviation of the median fluorescence levels.

Figure 1.

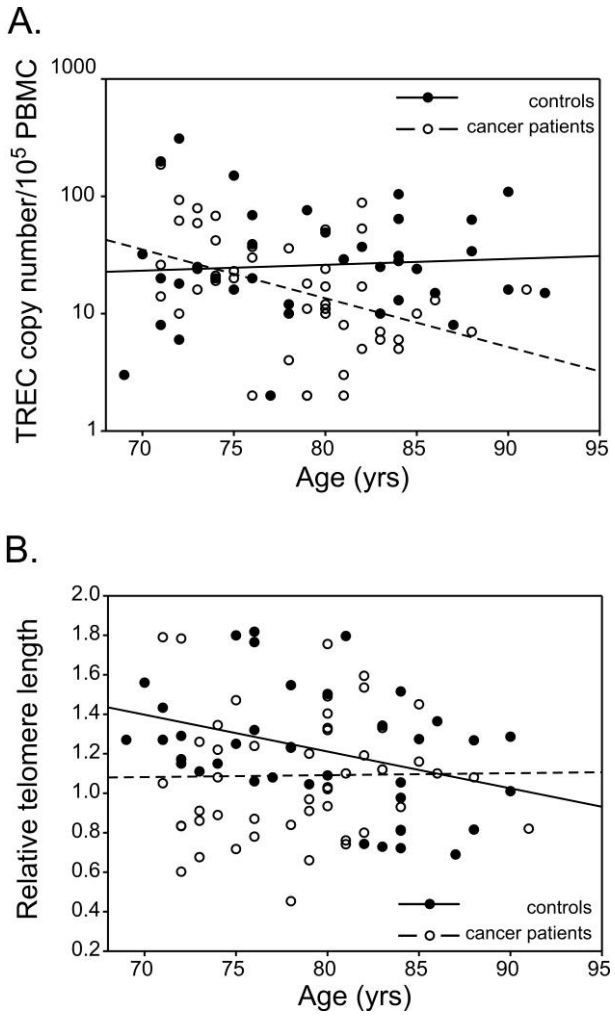
A.
TREC copy number/10^5 PBMC
1000
100
10
1
70
75
80
85
90
95
Age (yrs)
controls
cancer patients
B.
Relative telomere length
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
70
75
80
85
90
95
Age (yrs)
controls
cancer patients

Figure 2.

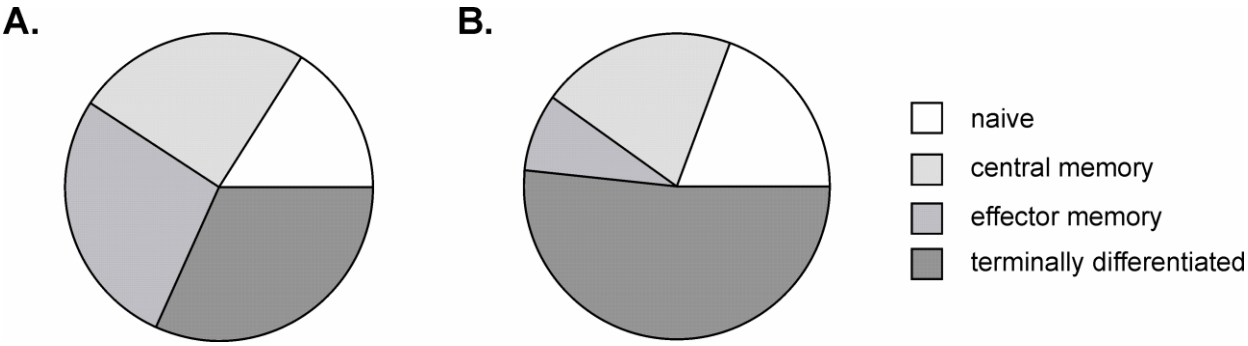
A.
B.
naive
central memory
effector memory
terminally differentiated

Figure 3.

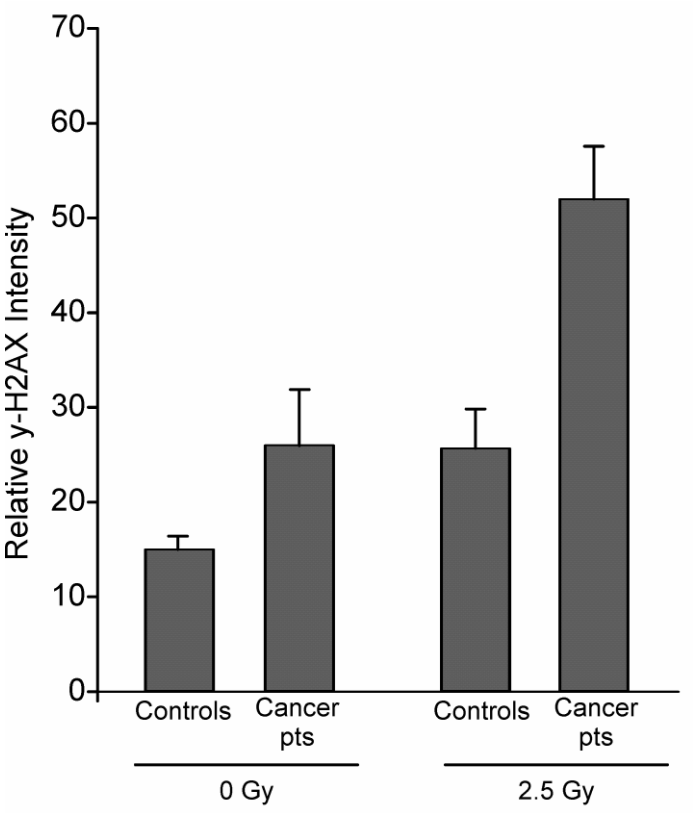
Relative y-H2AX Intensity
70
60
50
40
30
20
10
0
Controls
Cancer pts
Controls
Cancer pts
0 Gy
2.5 Gy

Table 1S. Standard blood parameters: comparison between cancer patients and controls

Parameter	All patients Median [IQR]	Cancer patients Median [IQR]	Controls Median [IQR]	*p*-value [a]
Leucocytes (x10^9/l)	6.6 [5.3-7.9]	6.7 [5.5-7.9]	5.9 [5.0-7.5]	0.110
Lymphocytes (x10^9/l)	1.6 [1.3-1.9]	1.7 [1.3-1.9]	1.5 [1.2-1.9]	0.165
Neutrophils (x10^9/l)	3.8 [3.0-4.9]	3.9 [3.2-4.9]	3.6 [2.7-4.9]	0.141
Hemoglobin (g/l)	127.0 [114.0-140.0]	128.0 [111.0-142.0]	126.0 [116.0-138.0]	0.665
Platelets (x10^9/l)	236.0 [204.0-291.0]	253.5 [212.5-317.0]	225.0 [183.0-260.0]	0.011
Total plasmatic proteins (g/l)	66.8 [63.5-71.1]	68.4 [66.1-74.3]	64.0 [60.7-68.7]	<0.001
Albumin (%)	57.0 [54.0-59.6]	57.9 [54.7-60.6]	55.9 [53.9-58.2]	0.530
Gamma globulins (%)	15.2 [13.2-17.3]	14.9 [13.3-17.1]	15.8 [13.0-18.0]	0.549
Triglycerides (mmol/l)	1.1 [0.8-1.4]	1.2 [0.8-1.7]	0.9 [0.7-1.2]	0.009
Total cholesterol (mmol/l)	4.9 [3.9-5.8]	5.1 [4.3-5.7]	4.5 [3.7-5.8]	0.229
LDL-cholesterol (mmol/l)	2.8 [2.0-3.7]	3.3 [2.7-3.8]	2.4 [1.7-3.6]	0.006
HDL-cholesterol (mmol/l)	1.4 [1.1-1.9]	1.4 [1.1-1.7]	1.6 [1.1-1.9]	0.194
Creatinine (umol/l)	76.0 [68.0-93.0]	77.5 [68.2-95.0]	75.0 [68.0-93.0]	0.642
IgG (g/l)	10.5 [8.5-12.2]	10.3 [8.9-11.6]	11.0 [7.7-12.6]	0.934
IgA (g/l)	2.3 [1.8-2.9]	2.6 [1.8-3.4]	2.2 [1.6-2.7]	0.104
IgM (g/l)	0.9 [0.5-1.3]	0.8 [0.5-1.3]	0. 9 [0.5-1.4]	0.853
IL-6 (ng/l)	3.0 [1.9-5.3]	2.6 [1.9-4.3]	3.7 [2.1-6.3]	0.033
CRP (mmol/l)	3.5 [2.9-6.6]	3.5 [2.9-5.5]	3.5 [3.4-10.8]	0.044
CMV [b]				
***n* positive/*n* tested (%)**	88/91 (96.7%)	49/52 (94.2%)	100/100 (100%)	
IgG (UI/ml)	15000 [8200-21000]	15000 [8125-19750]	15000 [9300-24000]	0.498

[a] Mann-Whitney *U* test; [b] CMV serology: Serum CMV antibody (IgG) determined by ELISA; antibodies titers>230 were considered to be positive.

Table 2S. Relationship between markers of immune senescence and geriatric scores in cancer patients and controls

	TREC level			Telomere length			Telomerase activity		
	All patients	**Cancer patients**	**Controls**	**All patients**	**Cancer patients**	**Controls**	**All patients**	**Cancer patients**	**Controls**
MPI	-0.03, 0.77	-0.04, 0.78	-0.24, 0.16	-0.04, 0.74	0.01, 0.95	-0.26, 0.13	-0.03, 0.81	0.16, 0.25	0.00, 0.98
MMSE	-0.1, 0.35	-0.28, 0.05*	-0.14, 0.42	0.06, 0.61	0.15, 0.28	-0.31, 0.06	-0.14, 0.19	-0.17, 0.22	0.19, 0.25
CIRS-CI	-0.04, 0.71	0.04, 0.79	-0.31, 0.07	0.04, 0.70	0.05, 0.72	-0.05, 0.76	-0.12, 0.26	0.01, 0.94	-0.18, 0.27
CIRS-SI	0.26, 0.02*	-0.07, 0.61	0.45, 0.01**	0.16, 0.13	-0.11, 0.42	0.35, 0.03*	-0.06, 0.60	0.01, 0.95	0.18, 0.27
GDS	-0.03, 0.79	-0.19, 0.19	0.22, 0.18	-0.10, 0.34	-0.09, 0.54	-0.05, 0.75	-0.24, 0.02*	-0.20, 0.15	-0.26, 0.12
BMI	-0.03, 0.79	0.01, 0.96	-0.10, 0.57	0.02, 0.83	0.14, 0.31	-0.11, 0.53	0.27, 0.01**	0.29, 0.04*	0.14, 0.39
Concomitant drugs	-0.01, 0.91	0.11, 0.43	-0.39, 0.02*	-0.13, 0.24	-0.18, 0.21	-0.24,0.15	-0.02, 0.89	0.13, 0.36	0.08, 0.65

Data are expressed as Spearman's rho coefficient, *p*-value.
* Correlation is significant at the level 0.05 (two-tailed)
** Correlation is significant at the level 0.01 (two-tailed)

Table S3. Flow cytometry markers of replicative senescence in cancer patients and controls

Parameter[a]	All patients Median [IQR]	Cancer patients Median [IQR]	Controls Median [IQR]	*p*-value[b]
% $CD4^+CD28^-CD57^+$	6.6 [2.6-23.9]	8.1 [1.9-33.3]	5.8 [3.1-18.5]	0.700
% $CD8^+CD28^-CD57^+$	55.7 [49.9-62.4]	58.5 [55.4-73.7]	50.7 [38.5-58.9]	0.092

[a] Flow cytometry was performed on frozen samples. Cells were stained with the following labeled mAbs: anti- CD3 (FITC), anti-CD4 (PerCP), anti-CD8 (PerCP), anti-CD57 (PE) and anti-CD28 (APC). Appropriate isotypic controls (mouse IgG1-PE and mouse IgG2b-APC) were used to assess non-specific staining. All samples were analysed by four-color flow cytometry on a fluorescence-activated cell sorter (FACS)Calibur (Becton-Dickinson). Data were processed with CellQuest Pro Software (Becton-Dickinson) and analysed using Kaluza® Analysis Software v.1.2 (Beckman Coulter). The percentage of $CD28^-CD27^+$ was calculated within the $CD3^+CD4^+$ or $CD3^+CD8^+$ gate. $CD28^-CD27^+$ cells were defined as replicative senescent cells.

[b] Mann-Whitney *U* test

Chapter 3

Premature Aging and Immune Senescence in HIV-Infected Children

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Manuscript in preparation

ABSTRACT

Background. Several data indicate that HIV-infected adults undergo premature aging and immune senescence; chronic immune activation may play a critical role in these dysfunctions which, besides immune depression, may constitute important risk factors for the onset of AIDS-associated and non-AIDS associated malignancies in HIV-infected individuals. Limited data are available for HIV-infected children, in whom immune activation and senescence are likely to be more deleterious, since their immune system co-evolves from birth with HIV.

Patients and Methods. 71 HIV-infected (HIV+) children, aged from 0-5 years, 65 HIV-exposed-uninfected (HEU) and 56 HIV-1-unexposed-uninfected (HUU) age-matched children were studied. 42% of the HIV+ children were not on antiretroviral therapy (ART). Telomere length (TL) and T-cell receptor rearrangement excision circle (TREC) levels were quantified in peripheral blood cells by real-time PCR. Subgroups of 24 HIV+, 21 HEU and 18 HUU children were studied for $CD4^+$ and $CD8^+$ cell differentiation (CD45RA, CD27, CD31), senescence (CD28, CD57) and activation/exhaustion (CD38, HLA-DR, PD-1) markers by flow cytometry. Statistical analyses were performed with SPSS v22.

Results. TL were significantly shorter in HIV+ than in HEU and HUU children (overall $p<0.001$, adjusted for age); moreover, HIV+ ART-naïve children had shorter TL compared with children on ART (median 2.11 [interquartile range (IQR) 1.75-2.37] vs 2.46 [2.07-2.68]; $p=0.0029$ adjusted for age). $CD8^+$ recent thymic emigrant cells ($CD45RA^+CD31^+$) and TREC levels were significantly lower in HIV+ than in HEU and HUU groups (overall, $p=0.005$ and

p=0.0249, respectively), while percentages of CD8 effector memory ($CD45RA^{-}CD27^{-}$) and terminally differentiated cells ($CD45RA^{+}CD27^{-}$) were higher in the former (overall, p=0.033, and p<0.001, respectively). CD8 senescent cells ($CD28^{-}CD57^{+}$) were higher in HIV+ than in HEU and HUU children (25.8% [12.4-43.2] vs 8.5% [6.8-16.7] vs 9.7% [3.3-27.3]; p=0.004), as were $CD8^{+}$ activated cells ($CD38^{+}HLA\text{-}DR^{+}$) (7.0% [5.2-12.2] vs 3.5% [3.9-7.6] vs 3.7% [2.4-6.7]; p<0.001) and $CD8^{+}PD\text{-}1^{+}$ cells (7.1% [5.0-12.4] vs 3.5% [2.1-5.9] vs 3.7% [2.4-5.3]; p<0.001). Within $CD4^{+}$ cell subset, percentages of senescent cells did not differ between HIV+ and controls, although $PD\text{-}1^{+}$ expression tended to be up-regulated in HIV+ children (overall, p=0.050).

Conclusions. HIV-infected children exhibit a premature biological aging with accelerated immune senescence which affects in particular the $CD8^{+}$ cell subset. HIV infection *per se* seems to influence the aging process, rather than exposure to ART for prophylaxis or treatment.

1. INTRODUCTION

The introduction of Antiretroviral Therapy (ART) has changed the natural history of pediatric HIV infection; indeed, ART-based prophylaxis regimens have reduced mother-to-child transmission (MTCT) of HIV from 15-20% to under 2% in high-income countries and have also given rise to substantial improvements in terms of survival and quality of life in HIV-infected children [Gibb DM et al, BMJ 2003; Judd A et al, Clin Infect Dis 2007]. In view of this increased survival, HIV infection is now considered a chronic disease that persists for many decades [Deeks SG et al, Lancet 2013]. However, despite its improvement of the immune function and reduction of AIDS-related complications, including AIDS-associated malignancies (ADM), ART does not restore full health. Indeed, many studies have evidenced that ART-treated HIV-infected adults have a higher risk of non-AIDS-related overall morbidity and mortality, compared to age-matched HIV-uninfected individuals. This increased risk is mainly due to a range of non-AIDS-defining illnesses that are associated with aging, including malignancies [Guaraldi G et al, Clin Infect Dis 2011], and it has been advanced that the increase of non-AIDS-defining diseases, including non-AIDS-defining malignancies (non-ADM) among HIV-infected patients is due to a premature aging [Effros RB, Clin Infect Dis 2008]. The pathogenic mechanism underlying this increased risk is still poorly understood. Chronic immune activation due to the persistence of circulating HIV virions may play a key role for the senescent pathway. Activated cells undergo clonal expansion in response to viral persistence, resulting in differentiation and accumulation of nonfunctional senescent cells [Desai S & Landay A, Curr HIV/AIDS Rep 2010]. It has been also advanced that premature and accelerated aging in HIV patients can be caused by adverse effects of antiretroviral drugs. Nucleoside reverse transcriptase inhibitors (NRTIs) have been

shown to inhibit telomerase activity in replicating cell lines *in vitro*, leading to accelerated shortening of telomere length, a marker of biological aging [Liu X et al, Nucleic Acids Res 2007; Hukezalie KR et al, PloS One 2012].

Clinical complications of HIV infection and ART treatment in children are more serious than in adults. The course of HIV infection in the former is characterized by faster disease progression and shorter time to AIDS, compared to adults. After infection, HIV plasma RNA levels are higher in vertically infected children than in adults. In addition they persist at high levels and decline slowly with age [De Rossi A et al, J Clin Invest 1996; McIntosh K et al, Pediatr Infect Dis J 1996], whereas in adults a control of viral load is reached within few weeks after infection [Henrard DR et al, JAMA 1995]. This gradual control of viral replication has been associated with increasing maturation and development of the immune system. The innate immunity, in children, is of particular relevance and plays a critical role in HIV pathogenesis [Ricci E et al, J Acquir Immune Defic Syndr 2009; Freguja R et al, New Microbiol 2012; Gianesin K et al, Plos One 2012] given that the adaptive immune system is still under development [Prendergast AJ et al, Nat Rev Immunol 2012]. Since in children the immune system co-evolves from birth with HIV and ART is required for decades, the effects of chronic immune activation and immune senescence together with ART toxicity in the course of their immune system's maturation might be more deleterious.

To date only little data are available on premature aging in HIV-infected children [Mansoor N et al, Clin Immunol 2009; Côté HC et al, PLoS One 2012; Diaz L et al, J Acquir Immune Defic Syndr 2012; Méndez-Lagares G et al, Clin Microbiol Infect 2013]. In this study we evaluated the status of biological aging and the degree of immune senescence in vertically HIV-infected children.

2. PATIENTS & METHODS

2.1 Study Population

A total of 71 perinatally HIV-infected (HIV+) children, aged 0-5 years, 65 exposed-uninfected (HEU) and 56 unexposed-uninfected (HUU) age-matched children were included in the study. All HIV+ and HEU children attended to the Department of Mother and Child Health, University of Padova (Padova, Italy) or to the Infectious Diseases Unit, Pediatrics Department, Hospital Saint Joan de Déu, Universitat de Barcelona, (Barcelona, Spain). For each HIV+ and HEU child, the first cryopreserved available sample after birth has been chosen for the study. No HIV+ or HEU child was breastfed. HUU children were recruited at the Pediatric Emergency Department of Azienda Ospedaliera Padova or Hospital Saint Joan de Déu, Universitat de Barcelona. Exclusion criteria were: malignancies, chronic infections, sarcoidosis, diabetes mellitus type-1, rheumatoid arthritis and systemic lupus erythematosus. The study was approved by the Ethics Committee of Azienda Ospedaliera Padova (Prot. n.#2921P); informed consent was obtained for patients and controls from their parents/guardians.

2.2 Sample preparation

Peripheral blood mononuclear cells (PBMC) were isolated from EDTA-treated peripheral blood by centrifugation on a Ficoll-Paque gradient (Pharmacia Biotech, Uppsala, Sweden). PBMC and plasma samples were cryopreserved and stored in liquid nitrogen until use. Two million PBMC were lysed as previously described [Righetti E et al, AIDS 2002].

2.3 Telomere length measurement by quantitative real-time PCR

Relative telomere length (TL) was determined by monochrome quantitative multiplex PCR assay [Cawthon RM, Nucleic Acids Res 2009] with minor modifications. Each PCR reaction was performed in a final volume of 25 µl containing 5 µl sample (~2 ng DNA/µl) and 20 µl reaction mix containing 0.75 x SYBR Green I (Invitrogen), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM each dNTP (Applied Biosystems), 1 mM DTT, 0.625 U AmpliTaq Gold DNA polymerase, 1% DMSO (Sigma) and 900 nM of each of the primers. The primer pair employed for telomere amplification were TELG 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3', and TELC 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3', and the primer pair for the amplification of the single-copy gene albumin were ALBU 5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG-3' and ALBD 5'-GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT-3'. The single-copy gene albumin was amplified simultaneously with the telomere template in the same well and was used as a reference to adjust for different amounts of DNA in different samples. The PCR reactions were performed on a LightCycler®480 real-time PCR detection system (Roche Applied Science). The thermal cycling profile was 15 min at 95ºC, 2 cycles of 15 s at 94ºC, 15 s at 49ºC, followed by 40 cycles of 15 s at 94ºC, 10 s at 62ºC, 15 s at 74ºC, 10 s at 84ºC, 15 s at 88ºC, with signal acquisition at the end of both the 74°C and 88°C steps. A standard curve was generated at each PCR run, consisting of DNA from the RAJI cell line serially diluted from 100 to 0.41 ng/µl. All DNA samples and reference samples were run in triplicate. LightCycler raw text files were converted to grid format using LC480Conversion free software developed by the Heart Failure Research Center (HFRC),

Amsterdam,Netherlands(http://www.hartfaalcentrum.nl/index.php?main=files&fileName=LC480Conversion.zip&description=LC480%20Conversion&sub=LC480Conversion). The converted data were analyzed using LinRegPCR free software developed by Ruitjer et al [Ruitjer JM et al, Nucleic Acids Res 2009]. Mean Ct values were used to calculate the relative telomere length (TL) using the telomere/single-copy gene ratio according to the formula: $\Delta Ct_{sample} = Ct_{telomere} - Ct_{albumin}$, $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference\ curve}$ (where $\Delta Ct_{reference\ curve} = Ct_{telomere\_RAJI} - Ct_{control\ RAJI}$) and then $T/S = 2^{-\Delta\Delta Ct}$ [Cawthon RM, Nucleic Acids Res 2009]. Intra and inter-assay reproducibility of both telomere and albumin PCR results was evaluated using dilutions of the reference curve. The standard deviation of Ct values was ≤0.19 (% coefficient of variation ≤0.94) in six replicates of samples amplified in the same PCR run, and ≤0.28 (% coefficient of variation ≤1.31) among mean values of triplicates in different PCR runs.

2.4 T-cell receptor rearrangement excision circle (TREC) levels quantification

Thymic output in PBMC was studied by measuring TREC levels by real-time PCR, as described previously [Ometto L et al, AIDS 2002]. TREC levels were expressed as the number of TREC copies per 10^5 PBMC.

2.5 Viral load quantification

Plasma HIV RNA levels were determined in all HIV-infected children using the COBAS *Taq*man HIV-1 test (Roche, Branchburg, NJ, USA). The lower limit of detection was 50 HIV RNA copies/ml. HIV DNA levels in PBMC were measured by real-time PCR, as described previously [Ometto L et al, AIDS 2002]. The HIV copy number was normalized against the

TERT copy number, and the final results were expressed as HIV DNA copies/10^6 cells. Samples for HIV DNA quantification were available for 44 children.

2.6 Flow cytometry analysis

T-cell phenotyping was performed on cryopreserved PBMC. Cells were thawed, washed, stained for 20 min in the dark with Live/Dead Fixable Near-IR Dead Cell Stain Kit (Life Technologies), and with fluorescent-conjugated mononuclear antibodies: CD3-FITC, CD31-PE, CD38-PE, CD57-PE, CD45RA-APC and PD-1-PECy7 (Becton-Dickinson), CD27-PECy7 (Beckman Coulter) and CD4-VioBlue, CD8-VioGreen, HLA-DR-APC and CD28-APC (Miltenyi Biotec). Appropriate isotypic controls (mouse IgG1-PE, mouse IgG2b-APC and mouse IgG1k-PECy7) were used to evaluate non-specific staining. Cells were then washed with Automacs Buffer (Miltenyi BioteC) and resuspended in PBS supplemented with 1% paraformaldehyde. All samples were analysed using LSRII Flow cytometer (Becton-Dickinson Biosciences). A total of 100000 events were collected in the lymphocyte gate using morphological parameters (forward- and side-scatter). Data were processed using FACSDiva™ Software (Becton-Dickinson) and analysed using Kaluza® Analyzing Software v1.2 (Beckman Coulter). Figure 1 showed the gating strategies in identifying the $CD4^+$ and $CD8^+$ T-cell subsets. Samples for flow-cytometry analysis were available for 24 HIV+ (15 with undetectable plasmaviremia (<50 copies/ml) and 9 with detectable plasmaviremia (median 3.64 [2.75-5.16] $\log_{10}$ copies/ml)), 21 HEU and 18 HUU children. Two HIV+ children were in therapy interruption at sample collection.

2.7 Statistical analysis

Comparisons between groups were made using nonparametric tests, the Mann-Whitney *U*-test or Kruskal-Wallis test, where appropriate. Correlations were explored using Spearman's rho test. Linear regression was used to model the relationship between TL and age. Samples with undetectable plasma HIV RNA were assigned a value of 20 copies/ml to include them in the statistical analyses. All statistical analyses were performed using SPSS software version 22 (SPSS, Inc., Chicago, IL, USA). All *P*-values were two-tailed, and were considered significant when lower than 0·05.

3. RESULTS

3.1 Characteristics of the study population

Characteristics of the 71 HIV+, 65 HEU and 56 HUU children are reported in Table 1. The median age of HIV+ children was 3.11 [interquartile range (IQR) 1.41-4.48 years], 1.73 [0.99-3.20] years for HEU and 1.85 [0.85-3.46] years for HUU children. 30/71 (42%) HIV+ children were ART-naïve, while the remaining were in ART (median time of 18 [11.0-36.5] months). ART-naïve HIV+ children were younger than those under therapy (1.70 [0.69-3.59] and 3.62 [2.17-4.81] years, respectively; p=0.005). 61/65 (93.8%) HEU children were exposed to prophylaxis during gestational period (median 34 [17-38] weeks) and received postnatal prophylaxis (6 weeks of zidovudine (AZT) monotherapy). Regarding HUU children, 38 of them were hospitalized for acute infections (pneumonia, influence, gastroenteritis) and 18 were healthy and referred for minor surgery.

3.2 Telomere length is shorter in HIV-infected children

The median TL in PBMC was significantly lower in HIV+ children than in HEU and HUU children, being the median TL of 2.21 [1.94-2.58], 2.63 [2.25-3.21], 2.88 [2.49-3.1], respectively (overall, $p<0.0001$ adjusted for age). TL inversely correlated with age in HEU and HUU groups (regression coefficient (β)=-0.01024, p=0.0079 and β=-0.01001, p=0.0112, respectively), but not in HIV+ children (β=-0.00175, p=0.5870) (Figure 2A). Of note, among HIV+ group, ART-naïve children had shorter TL compared with children on ART (2.11 [1.75-2.37] *versus* 2.46 [2.07-2.68]; p=0.0029 adjusted for age). Neither for ART-naïve children nor for those on ART, TL was associated with age (β=-0.00540, p=0.2269; β=-0.00455, p=0.2579, respectively) (Figure 2B).

3.3 Thymic output is lower in HIV-infected children

Given that an effective immune response depends on thymic function, thymic output was quantified by measuring TREC levels. HEU and HUU children had higher levels of TREC than HIV+ children (5409 [3411-6712], 5370 [2380-8101], 3498 [2051-6780] TREC copies/10^5 PBMC; overall p=0.0249 adjusted for age). TREC levels decreased significantly with increasing age in HEU and HUU groups (β=-61, p=0.0088 and β=-86, p=0.0003, respectively), but not in HIV+ children (β=-17, p=0.3526) (Figure 2C). No significant differences in TREC levels/10^5 PBMC were found between ART-treated children and ART-naive children (p=0.2995) (Figure 2D).

3.4 Phenotypic T-cell alterations occur early in HIV-infected children

There were no differences in the frequencies of $CD3^+$ T cells between the three groups (overall, p=0.590). Percentage of total $CD4^+$ T cells were lower in the HIV+ group than in the other groups (overall, p=0.001). Within $CD4^+$ T cells, HIV+ children and control groups did not significantly differ in percentages of naïve ($CD45RA^+CD27^+$), central memory ($CD45RA^-CD27^+$), effector memory ($CD45RA^-CD27^-$) and terminally differentiated ($CD45RA^+$CD27-) cell subsets (Table 2). However, when central and effector memory cell subsets, the major cellular reservoirs for HIV [Chomont N et al, Nat Med 2009] where considered together, they tended to be lower in HIV+ children than in HEU and HUU children [21.9% [15.3-38.3] versus 29.8% [18.6-39.4] and 34.3% [24.1-42.6]; p=0.158). Percentage of senescent cells ($CD4^+CD28^-CD57^+$) were similar between the three groups (p=0.568), while activated $CD38^+$HLA-DR^+ and exhausted PD-1^+ were more expanded in HIV+ children than in controls. In particular, $CD4^+CD38^+$HLA-DR^+ and $CD4^+$PD-1^+ were higher in children with detectable viral load than in aviremic children (p=0.056 and p=0.037, respectively).

The median of $CD8^+$ cell percentage tended to be higher in HIV+ children than in control groups (overall, p=0.063). Notably, significant differences emerged among $CD8^+$ cell subsets. HIV+ children showed a lower percentage of naïve cells ($CD45RA^+CD27^+$) than HEU and HUU children (46.2% [37.6-75.1], 77.1% [55.9-84.7], 71.0% [46.7-86.1]; overall p=0.019). In particular, HIV+ children had a lower frequency of $CD8^+$ recent thymic emigrant (RTE) (overall, p=0.005) and a higher percentage of peripheral expanded cells (PEC; $CD45RA^+CD31^-$) than control groups (overall, p=0.040), suggesting a strong peripheral proliferation. Moreover, the amount of $CD8^+$ RTE cells decreases with age in

HEU and in HUU children, but not in HIV+ children (Figure 3A-C). Interestingly, $CD8^+$ RTE cells were lower in children with detectable viral load than in aviremic children (41.8% [22.6-64.4] versus 55.9% [53.6-76.7], p=0.039), and tended to inversely correlate with HIV RNA (r=-0.363, p=0.080) (Figure 3D). Taken together, these findings suggest that, likely because the pressure exerted by HIV, RTE cells are induced to rapidly proliferate in order to mount an effective response against viral antigens.

The alteration of thymic function in HIV+ children is also evidenced by the absence of correlation between $CD4^+$ RTE and $CD8^+$ RTE, as shown in Figure 4A, conversely from what is observed in HEU and HUU children (Figure 4B and C).

Among memory cell subsets, frequency of $CD8^+$ central memory did not differ between HIV+, HEU and HUU children (overall p=0.278); however in the HIV+ group, this subset was more expanded in children with detectable viremia than in those with undetectable HIV RNA (p=0.027) (not shown). Both effector memory ($CD45RA^-CD27^-$) and terminally differentiated cells ($CD45RA^+CD27^-$) were more expanded in HIV+ children than in control groups (Table 2). Furthermore, in HIV+ children the proportion of $CD8^+$ cells with a senescent phenotype ($CD28^-CD57^+$) was higher than in HEU and HUU groups (25.8% [12.4-43.2] versus 8.5% [6.8-16.7] and 9.7% [3.3-27.3]; p=0.004). This expansion was observed only in children with detectable plasmaviremia, suggesting that the presence of viral load stimulates the production of a senescent phenotype (41.6% [18.5-45.8] versus 4.4% [2.1-13.4]; p=0.002). Additionally, the activation of $CD8^+$ ($CD38^+HLA\text{-}DR^+$) cells was significantly higher in HIV+ children than controls, being the median 6.9% [4.4-12.2] versus 4.5% [3.7-7.6] in HEU and 3.5% [2.9-7.6] in HUU children (overall p=0.015), and increased significantly with HIV RNA levels (r=0.672; p<0.001). In all groups, the percentage of

activated $CD8^+$ cells correlated with the percentage of senescent cells (HIV+: r=0.505, p=0.016; HEU: r=0.731, p<0.001; HUU: r=0.564, p=0.027). Surprisingly, the frequency of $CD8^+$ activated cells tended to be higher in HEU children than in HUU (4.7% [3.9-7.6] versus 3.4% [2.8-6.7] cells/ul; p=0.083). The percentage of PD-1 expressing $CD8^+$ cells were significantly higher in HIV+ children than in HEU and HUU groups (overall p=0.003 and p<0.001, respectively). In particular, PD-1 expression in viremic subjects was significantly higher than in those with undetectable plasmaviremia (p=0.002), and correlated with HIV RNA levels (r=0.471, p=0.021) and immune activated cells ($CD8^+CD38^+HLA\text{-}DR^+$) markers (r=0.528, p=009).

As shown in Figure 5, telomere length was inversely correlated with percentages of $CD8^+$ senescent (Figure 5A), activated (Figure 5D) and exhausted cells (Figure 5G) in HIV+ children, but not in HEU (Figure 5B, E, H) and HUU children (Figure 5C, F, I).

4. DISCUSSION

This is the first study describing biological aging and immune senescence in HIV-infected children (aged 0-5 years), compared with age-matched HIV-exposed-uninfected and unexposed-uninfected children.

Overall, the results demonstrated that HIV-infected children exhibit a premature biological aging with an accelerated immune senescence that affects in particular the $CD8^+$ T-cell subset. Conversely, from the CARMA cohort study of Côté *et al.* [Côté HC et al, PLoS One 2012], in which no difference in telomere length was found between HIV+ children and HIV- controls, in our study telomere length was significantly shorter in HIV-infected children than in HEU and HUU children. This discordant result could be due to the different

age of the two cohorts: children enrolled in our study are younger (aged 0-5 years, median 3.1) compared to those of Côté's study (aged 0-19 years, median 13.3). As telomere shortening in PBMC is very rapid during the first 3 years of life [Zeichner SL et al, Blood 1999], it is possible that in our cohort the difference between HIV+ and controls has better emerged. Moreover, the two cohorts differed for the duration of ART exposure. The longer exposure among children of CARMA cohort (median 338 weeks), resulting in a better control of plasmaviremia and in a reduction of immune activation, may lead to a loss of association that conversely was found in our study population, consisting of ART-naïve or recently under ART (median 48 weeks) children. Among HIV+ group, the shorter telomere length in PBMC of ART-naïve compared to ART-treated children suggests that HIV infection *per se*, rather than exposure to therapy, influences the aging process. The mechanism behind shorter telomere length in HIV-infected individuals is unknown. Short telomere lengths could be due to an excessive cellular replication consequent to chronic immune activation, but virus in itself may play an active role. Interestingly, our results could be partially explained by the evidence that telomerase activity is severely impaired in uninfected $CD34^+$ haematopoietic progenitor cells isolated from HIV-infected patients [Vignoli M et al, AIDS 1998]. Moreover, HIV-infection and HIV-Tat protein have been shown to down-modulate telomerase expression and activity in lymphoblastoid cells and in peripheral blood lymphocytes [Ballon G et al, J Infect Dis 2001; Reynoso R et al, AIDS Res Hum Retroviruses 2006; Comandini A et al, Mol Immunol. 2013].

Despite $CD4^+$ T-cell subset is the target of HIV-infection, analysis of T-cell phenotype showed that also $CD8^+$ T-cell compartment is largely impaired in the HIV-infected children. HIV+ children had a lower frequency of $CD8^+$ naïve cells, whose reduction does not

correlate with age as it occurs in the control groups. In particular, the production of new $CD8^+$ cells, identified by the expression of CD31 marker, decreases with parallel increasing level of HIV plasmaviremia, suggesting that HIV induces $CD8^+$ peripheral expansion and differentiation into effector cells, which play a central role in immunity against viruses by producing cytokines and by killing infected target cells [Northfield JW et al, J Virol 2007]. As already described in cohorts of adults and older children [Brenchley JM et al, Blood 2003; Montesano C, New Microbiol 2010; Diaz L, J Acquir Immune Defic Syndr 2012], the decrease of naïve cell subset is associated with a skewed maturation of $CD8^+$ T-cell towards an effector phenotype that, without an adequate replenishment of new $CD8^+$ naïve cells, that induces an accumulation of cells with a senescent phenotype. The evidence that immune senescence is more serious in children with detectable HIV viremia focuses the need to control HIV replication and chronic immune activation. In particular, this aspect must be taken into consideration when a treatment interruption has been planned [Klein N et al, PLoS One 2013]. Notably, the importance to control the levels of immune activation is also shown by studies during infancy and in older HEU children that revealed immunological alterations from HUU [Clerici M et al, Blood 2000; Vigano A et al, AIDS 2007; Afran L et al, Clin Exp Immunol 2014].

In conclusion, HIV-infected children exhibit a premature biological aging with an accelerated immune senescence that affects in particular the $CD8^+$ T-cell subset. The lower telomere length and the higher percentages of senescent cells in HIV+ children, compared to children on ART or HEU, suggest that HIV infection in itself, rather than exposure to ART for prophylaxis or treatment, influences the aging process. These data support the importance of maintaining undetectable viral load to avoid premature immune senescence and dysfunction

of $CD8^+$ cells, compromising their tumor immune surveillance function and increasing the risk of malignancies. Although ART suppresses viral load and partially restores immune functions, immunological alterations persist in ART-treated children making them more vulnerable to cancer development compared to controls.

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Table 1. Demographic and clinical characteristics of HIV+, HEU and HUU children

	HIV+ (n=71)	HEU (n=65)	HUU (n=56)
Age, median [IQR] yrs	3.11 [1.40-4.48]	1.74 [0.99-3.31]	1.85 [0.84-3.46]
Gender, n (%)			
M/F	39 (55%)/32 (45%)	34 (52%)/31 (48%)	29 (52%)/27 (48%)
Exposed to prophylaxis, n (%)	5 (7%)	61 (93.8%)	-
Exposed to ART, n (%)	41 (58%)	-	-
Duration of ART exposure, median [IQR] weeks	48 [72-144]	-	-
Percentage of lifetime on ART	57.5 [42.6-84.5]	-	-
Detectable plasmaviremia, n (%)	55 (77.5%)	-	-
Plasmaviremia at sample collection (log_{10} copies/ml)		-	-
ART-naive	5.25 [4.75-5.60]	-	-
on ART	1.70 [2.54-4.28]	-	-

Table 2. Frequencies of $CD4^+$ and $CD8^+$ subsets

		HIV+ (n=24)	HEU (n=21)	HUU (n=18)	overall *p*-value
$CD3^+$					
	%	64.4 [58.8-69.2]	61.8 [49.8-68.4]	60.2 [54.6-66.4]	0.590
$CD4^+$					
	%	39.6 [35.1-45.3]	53.4 [43.5-64.7]	52.5 [38.0-60.6]	**0.001**
naive					
	%	76.8 [59.9-84.4]	70.1 [59.9-81.0]	64.7 [56.9-75.3]	0.423
central memory					
	%	18.1 [13.2-31.1]	27.3 [17.4-32.6]	27.9 [20.7-35.1]	0.206
effector memory					
	%	3.4 [1.2-5.8]	2.5 [1.8-6.3]	4.4 [2.9-7.8]	0.220
t. differentiated					
	%	0.5 [0.2-1.0]	0.3 [0.1-0.6]	0.4 [0.3-0.1]	0.330
RTE					
	%	63.6 [54.9-72.4]	58.3 [46.0-68.2]	54.2 [49.3-61.7]	0.181
PEC					
	%	12.4 [5.3-16.4]	11.7 [8.2-16.1]	10.4 [8.1-15.2]	0.738
senescent					
	%	0.4 [0.1-0.7]	0.2 [0.1-0.5]	0.2 [0.1-1.3]	0.568
activated					
	%	3.3 [2.2-5.9]	2.1 [1.3-3.5]	2.8 [1.6-3.8]	**0.041**
exhausted					
	%	4.1 [3.4-6.6]	3.5 [1.9-5.2]	3.2 [2.7-5.1]	**0.050**
$CD8^+$					
	%	31.2 [25.8-39.6]	28.9 [21.2-35.0]	25.9 [22.8-29.0]	**0.063**
naive					
	%	46.2 [37.6-75.1]	77.1 [55.9-84.7]	71.0 [46.7-86.1]	**0.019**
central memory					
	%	11.0 [6.9-23.2]	16.3 [10.9-26.5]	12.7 [9.8-18.9]	0.278
effector memory					
	%	7.1 [2.3-13.1]	2.1 [1.1-4.9]	4.3 [1.3-11.4]	**0.033**
t. differentiated					
	%	16.3 [4.5-36.4]	2.5 [1.0-8.6]	4.2 [2.1-16.2]	**0.001**
RTE					
	%	55.3 [41.4-71.8]	69.8 [60.4-80.1]	68.1 [59.5-79.3]	**0.005**
PEC					
	%	17.1 [6.5-29.2]	5.9 [3.8-15.3]	9.8 [4.6-15.2]	**0.040**
senescent					
	%	25.8 [12.4-43.2]	8.5 [6.8-16.7]	9.7 [3.3-27.3]	**0.004**
activated					
	%	7.0 [5.2-12.2]	4.7 [3.9-7.6]	3.4 [2.8-6.7]	**<0.001**
exhausted					
	%	7.1 [5.0-12.4]	3.5 [2.1-5.9]	3.7 [2.4-5.3]	**<0.001**
Ratio $CD4^+/CD8^+$					
		1.23 [0.97-1.87]	1.78 [1.20-2.43]	1.81 [1.59-2.11]	**0.026**

FIGURE LEGENDS

Figure 1. Example of flow cytometry gating strategy to identify $CD4^+$ and $CD8^+$ cell subsets. Lymphocyte population was identified using FSC versus SSC plot (A), to select live cells (B). Sequential gating was used to select the total $CD3^+$ cell population (C) and the $CD3^+CD4^+$ and $CD3^+CD8^+$ cell subsets (D). Within $CD3^+CD4^+$ or CD3+CD8+ gate, CD45RA and CD27 markers were used to identify naïve ($CD45RA^+CD27^+$), central memory ($CD45RA^-CD27^+$), effector memory ($CD45RA^-CD27^-$) and terminally differentiated cells ($CD45RA^+CD27^-$) (E); CD45RA and CD31 markers were used to identify recent thymic emigrant (RTE, $CD45RA^+CD31^+$) and peripherally expanded cells (PEC, $CD45RA^+CD31^-$) (F); CD57 and CD28 markers were used to select senescent cells ($CD28^-CD57^+$) (G); CD38 and HLA-DR were used to identify activated cells ($CD38^+$HLA-DR^+) (H); finally, exhausted cells were identify by the expression of PD-1 (I).

Figure 2. Correlation between age and telomere length (A-B) and TREC levels (C-D). (A) Telomere length as function of age in HIV+ (in black), in HEU (in grey) and HUU (in white) children. (B) Telomere length as function of age in HIV+, subdivided in ART-naïve (in grey) and ART-treated children (in black). (C) TREC levels as function of age in HIV+ (in black), in HEU (in grey) and HUU (in white) children. (D) TREC levels as function of age in HIV+, subdivided in ART-naïve (in black) and in ART-treated children (in grey).

Figure 3. Correlation between frequency of $CD8^+$ recent thymic emigrants cells and age (A-C) and viral load (D). Scatter plots showing correlation between age and %$CD8^+$ RTE cells in HUU (A), HEU (B) and HIV+ (C) children. (D) Relationship between HIV-1 viral load and %$CD8^+$ RTE cells in HIV+ children.

Figure 4. Correlation between $CD4^+$ and $CD8^+$ recent thymic emigrants cells. Scatter plots showing absence of correlation between frequencies of naïve $CD4^+$ and $CD8^+$ RTE cells in HIV+ children (A); conversely, a strong relationship was found in HEU (B) and HUU (C) children.

Figure 5. Correlation between telomere length and percentages of senescent (A-C), activated (D-F) and exhausted cells (G-I). Telomere length correlates with senescent cells in HIV+ (A) children but not in HEU (B) and HUU (C) children. In addition, in HIV+ children, telomere length was inversely correlated with activated (D) and exhausted (G) cells; conversely, these relationships were not found in HEU (E, H) and HUU (F, I) children.

Figure 1.

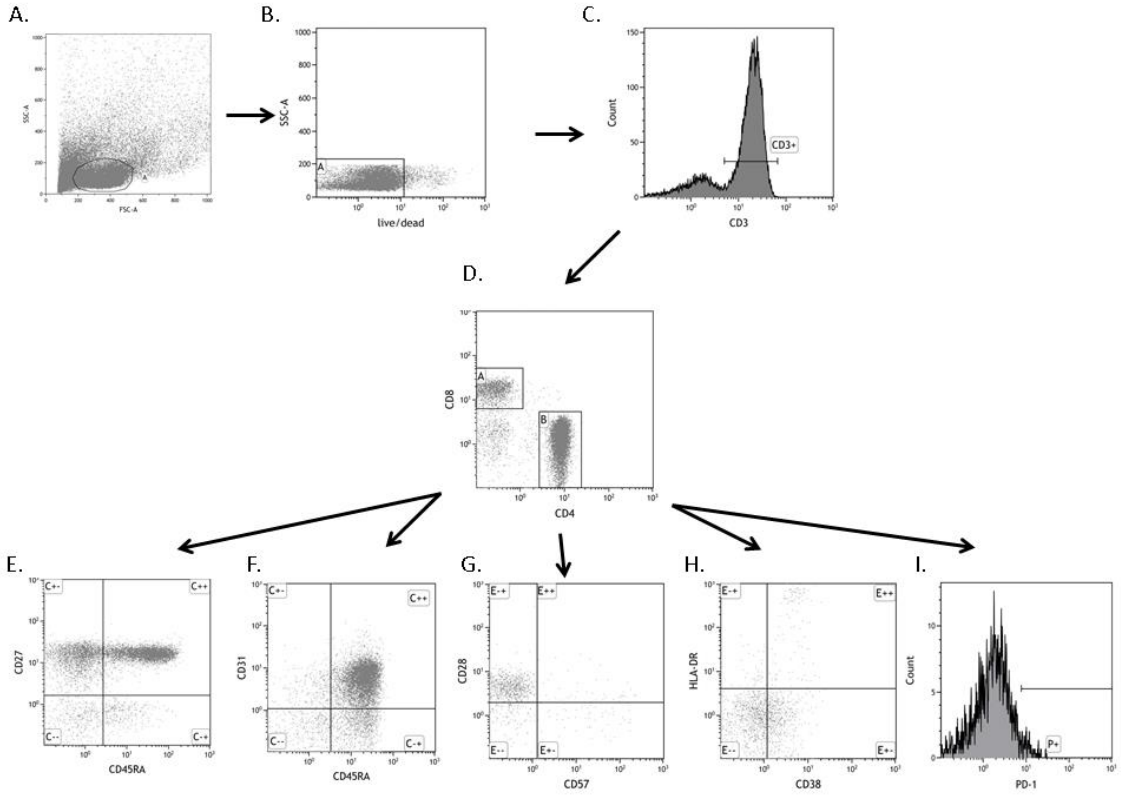

A.
B.
C.
SSC-A
FSC-A
live/dead
Count
CD3+
CD3
D.
CD8
CD4
E.
F.
G.
H.
I.
CD27
CD45RA
CD31
CD45RA
CD28
CD57
HLA-DR
CD38
Count
PD-1

Figure 2.

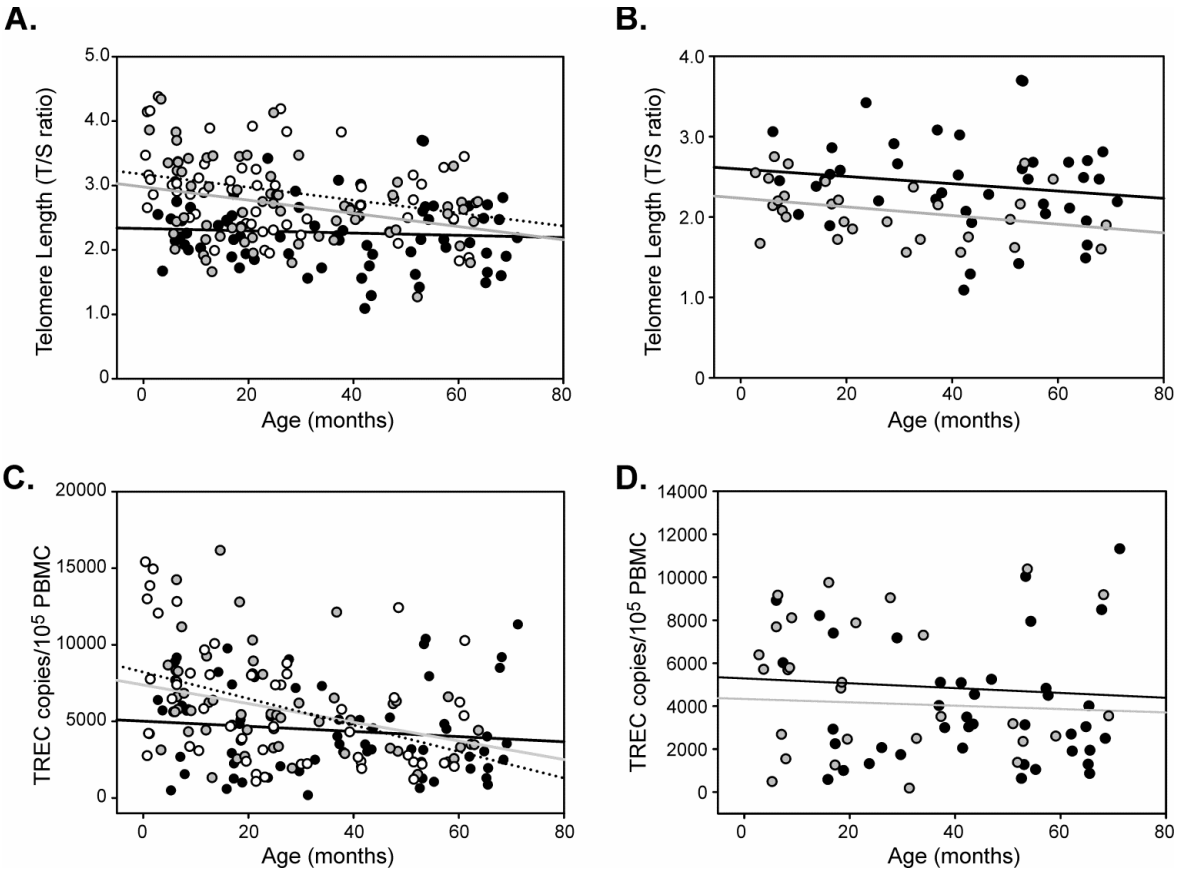

A.
Telomere Length (T/S ratio)
Age (months)
B.
Telomere Length (T/S ratio)
Age (months)
C.
TREC copies/10^5 PBMC
Age (months)
D.
TREC copies/10^5 PBMC
Age (months)

Figure 3.

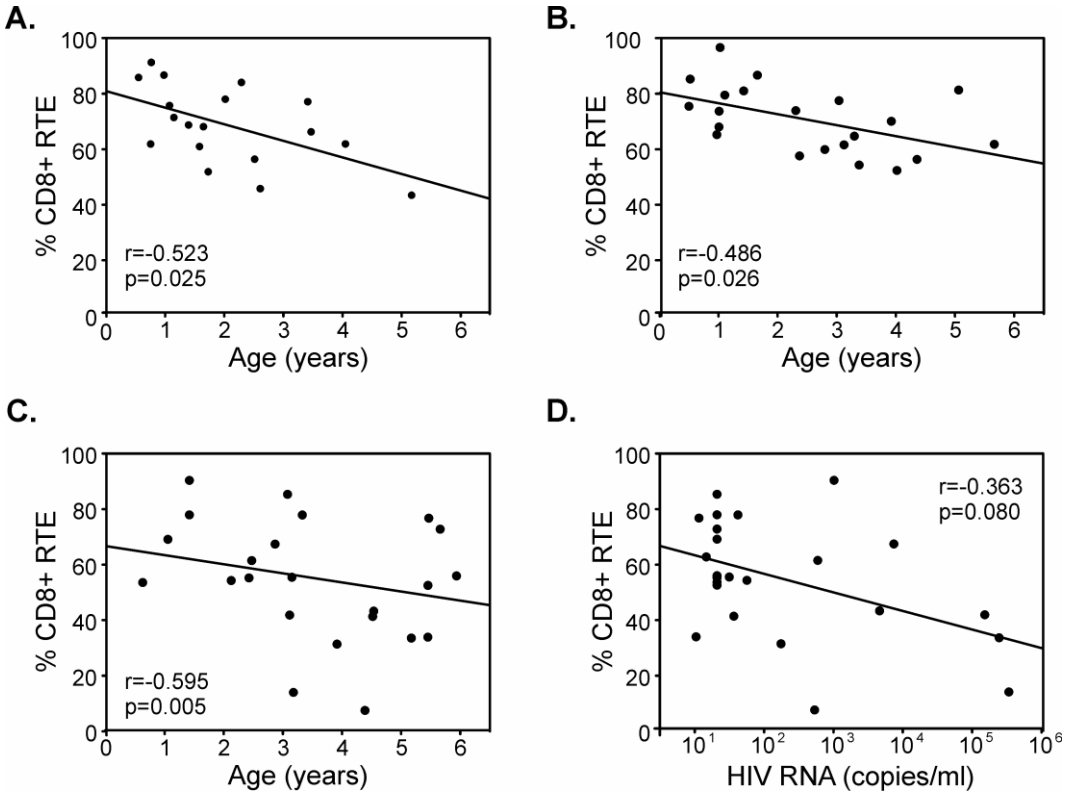

A.
% CD8+ RTE
Age (years)
r=-0.523
p=0.025
B.
% CD8+ RTE
Age (years)
r=-0.486
p=0.026
C.
% CD8+ RTE
Age (years)
r=-0.595
p=0.005
D.
% CD8+ RTE
HIV RNA (copies/ml)
r=-0.363
p=0.080

Figure 4.

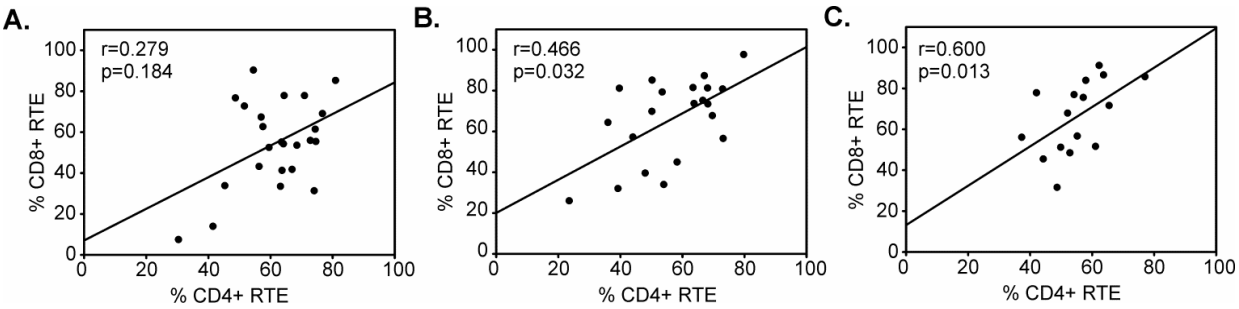
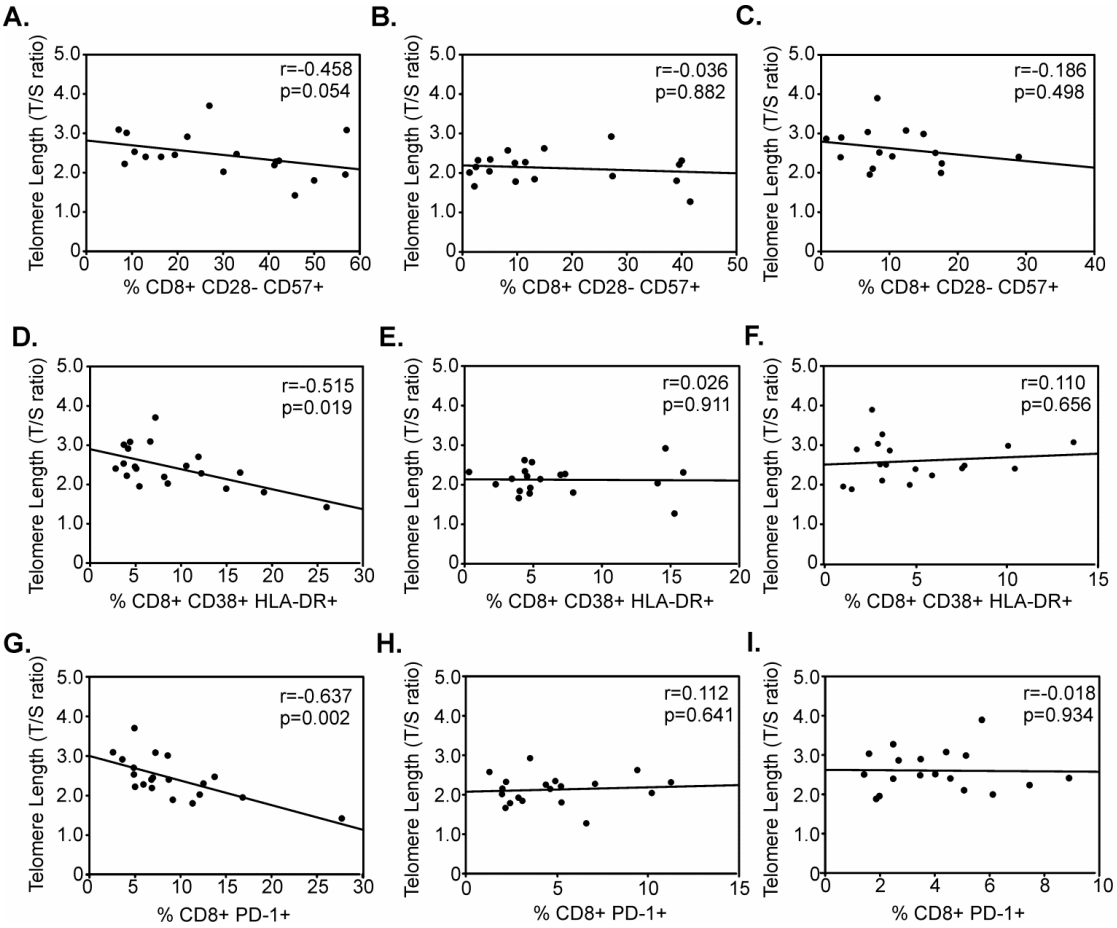

A.
r=0.279
p=0.184
% CD8+ RTE
% CD4+ RTE
B.
r=0.466
p=0.032
% CD8+ RTE
% CD4+ RTE
C.
r=0.600
p=0.013
% CD8+ RTE
% CD4+ RTE

Figure 5.


A.
r=-0.458
p=0.054
B.
r=-0.036
p=0.882
C.
r=-0.186
p=0.498
D.
r=-0.515
p=0.019
E.
r=0.026
p=0.911
F.
r=0.110
p=0.656
G.
r=-0.637
p=0.002
H.
r=0.112
p=0.641
I.
r=-0.018
p=0.934
Telomere Length (T/S ratio)
% CD8+ CD28- CD57+
% CD8+ CD38+ HLA-DR+
% CD8+ PD-1+

Chapter 4

4. GENERAL CONCLUSIONS

This research PhD program has been conducted in the frame of exploratory studies, aimed at evaluating the immune senescence and biological aging in two populations at higher risk of cancer.

1. The world's population is aging and, since cancer incidence increases exponentially with advancing age, diagnosis of new cancer cases among elderly people is expected to increase significantly in the coming years, leading to important issues for their treatment. Aging is a multidimensional process difficult to define well; elderly cancer patients differ each other in their biologic age, metabolism, comorbid conditions, life expectancy, performance status, social status, and in their treatment preferences (side-effects profile and convenience). In this light, the need to better define this more vulnerable population is growing. Our study represents the first attempt to investigate cellular parameters associated with immune aging and senescence in elderly cancer patients, alongside a thorough cognitive and frailty assessment. We found that in elderly cancer patients immune senescence is significantly worse compared to age-matched controls. Indeed, cancer patients have lower thymic output, lower percentages of naïve $CD8^+$ and recent thymic emigrant $CD8^+$ cells, but an expanded $CD8^+$ memory pool. Moreover, they have lower telomere length in peripheral blood cells. The fact that control patients present a more severe burden of associated diseases enforces the magnitude of the difference observed for these immune senescence and biological aging markers between the two groups, and it makes the possibility of a pre-existing condition that would facilitate the onset of malignancies. In addition, preliminary data available from the follow-up substudy seem to indicate that adjuvant chemotherapy aggravates the aging process. These findings are in agreement with other studies [Unryn BM et al, Clin Cancer

Res 2006; Sanoff HK et al, J Natl Cancer Inst 2014] conducted in younger cohorts. These observations could have important implications for the clinical treatment in elderly cancer patients. Indeed, the chemotherapy-related DNA-damage may have a synergistic effect on relapse risk, since it occurs in a setting of age-related reduction of DNA-damage repair ability [Scarpaci S et al, Ageing Dev 2003]. Our study highlights the value of aging biomarkers in addressing adjuvant chemotherapy to those patients who may better benefit without late side-effects risk. However, the little number of subjects included in the study undoubtedly represents a limitation to the study. In order to consolidate these findings, the enrolment of other patients is in progress.

2. With the advent of ART and the consequent substantial improvement in life expectancy, the proportion of perinatally HIV-infected children that become adolescents and young adults is expanding. The aim of our study was to assess parameters of biological aging and immune senescence in the context of HIV infection, given the increasing data about the excessive risk of age-related morbidity, particularly in malignancies. Our study demonstrated that HIV-infected children had a lower telomere length compared to age- and gender- matched controls, suggesting an accelerated biological aging. The phenotypic analysis of peripheral blood cells revealed that immune senescence is worse in $CD8^+$ cell compartment than in $CD4^+$, and it is mainly due to the accelerated replication of $CD8^+$ cells in their attempt to control the viral infection. Indeed, in HIV-infected children but not in control groups, telomere length was inversely correlated with frequency of senescent, activated and exhausted cells. Moreover, age-adjusted telomere length is shorter in ART-untreated than in ART-treated children, thus suggesting that HIV itself, rather than exposure to antiretroviral drugs, influences the aging process. It is known that NRTIs are able to inhibit telomerase

activity *in vitro*, leading to accelerated shortening of telomere length [Liu X et al, Nucleic Acids Res 2007; Hukezalie KR et al, PLoS One 2012], but no study has proved that NRTIs inhibited telomerase activity *in vivo*. The inverse association between telomere length and duration of NRTI-containing ART has been recently demonstrated in a cohort of adults [Leeansyah E et al, J Infect Dis 2013], but the small sample size and lack of an optimal control of ART-naïve patients preclude definitive conclusions. The shortening of telomeres is a slow process, and it is possible that, in this study, we were not able to find any association between telomere length and ART, because the exposure was not long enough (median time of ART exposure 48 [72-144] weeks).

Overall, these findings, together with the evidence that a worse immune senescence may predispose to cancer onset, highlight the importance to control immune activation and the need to maintain a persistent viral suppression, in order to delay the aging process that seems already compromised at birth. In addition to current assays, *i.e.* HIV plasmaviremia and $CD4^+$ cells profile, our study indicates the relevance to analyze telomere length and $CD8^+$ cell subsets to assess the status of premature aging and immune senescence in this population at high risk of cancer.

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