Universidade do Minho
Escola de Medicina

Rita Ribeiro da Silva

Immune recovery of HIV infected patients and thymus function

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I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

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Recuperação imunológica de doentes com infeção por HIV e função tímica

Resumo

A maioria dos doentes infetados pelo vírus da imunodeficiência humana (HIV, do inglês *human immunodeficiency virus*) sob terapêutica antirretrovírica (ART) apresenta um aumento das contagens de células T CD4+ em comparação com os valores antes do início da ART, refletindo a recuperação do sistema imunológico associada à supressão da replicação do vírus. No entanto, uma proporção significativa de doentes mantém contagens anormalmente baixas apesar de supressão da replicação vírica, apresentando um alto risco de morbilidade e mortalidade. Vários termos e critérios são utilizados para identificar estes doentes. Esta heterogeneidade dificulta a comparação de dados de diferentes origens e a expansão do conhecimento sobre esta condição, à qual nos iremos referir como "não-resposta imunológica" (INR, do inglês *immunological non-response*).

Com vista a determinar a diversidade e a frequência dos termos e critérios utilizados na definição de INR, foi feita uma revisão sistemática usando-se um conjunto predefinido de palavras associadas a esta condição. Dos 1360 artigos inicialmente identificados na PubMed, 103 foram incluídos no estudo. Vinte e dois termos e 73 critérios diferentes foram encontrados, sendo o termo mais frequente "não-resposta imunológica", e o critério mais frequente, "contagens de células T CD4+ < 350 células/μL após ≥ 24 meses de supressão virológica". Informação detalhada sobre os termos e os critérios foi analisada nesta revisão. Este trabalho representa um contributo para a discussão e estabelecimento futuro de uma definição consensual de INR entre a comunidade de investigadores em HIV.

Para investigar o papel do timo na recuperação imunológica dos doentes com HIV durante a ART, 33 indivíduos de uma coorte prospectiva foram seleccionados por apresentarem linfopenia grave (< 200 CD4+ T cells/μL) no início da ART e um tempo de seguimento ≥ 36 meses. Utilizando análise de clusters baseada nas trajectórias das contagens de células T CD4+, esses doentes foram classificados como tendo "resposta imunológica pobre" ou "resposta imunológica adequada" (PIR ou AIR, respectivamente). Diferentes parâmetros imunológicos foram analisados ao longo do tempo, verificando-se que várias estimativas da função tímica se encontravam aumentadas nos doentes com AIR em relação aos doentes com PIR. Modelos preditivos de uma resposta imunológica pobre ou adequada aos 36 meses de ART foram construídos com base em observações feitas nos primeiros 2 a 6 meses de tratamento, e esses modelos identificaram correctamente 77-87 % dos casos. Este estudo destaca a importância da função tímica na recuperação imunológica de doentes com linfopenia grave.

O trabalho apresentado nesta tese contribui para o avanço do conhecimento sobre a recuperação imunológica durante o tratamento da infeção por HIV e aponta para um subgrupo de doentes que poderão beneficiar de estratégias de reforço da função tímica em combinação com a ART.

Palavras-chave: Células T CD4+, HIV, Modelos preditivos, Recuperação imunológica, Timo

Abstract

Most patients infected by the human immunodeficiency virus (HIV) on antiretroviral therapy (ART) present an increase of $CD4^+$ T cell counts compared to pre-ART values, reflecting the recovery of the immune system associated with the suppression of viral replication. However, a significant proportion of patients maintain abnormally low $CD4^+$ T cell counts despite full virological suppression, exhibiting a relatively high risk of morbidity and mortality. Different terms and criteria are used to identify these patients. This heterogeneity hampers the comparison of data from different sources and the expansion of knowledge of this condition, which we will refer to as "immunological non-response" (INR).

To determine the diversity and relative frequency of terms and criteria used to define INR, we performed a systematic review using a predefined set of words related to this condition. From the 1360 retrieved PubMed papers, 103 met the inclusion criteria. Twenty-two terms and 73 distinct criteria were found. The most frequent term was "immunological non-responders", and the most frequent criterion was "a $CD4^+$ T-cell count < 350 cells/μL after ≥ 24 months of virologic suppression". Detailed information on terms and criteria was collected, analyzed and discussed. This work may foster the discussion and future establishment of a consensual INR definition among the community of HIV researchers.

To investigate the role of thymic function in the process of immune recovery of HIV-infected patients on ART, we selected, from a prospective cohort study, 33 individuals with severe lymphopenia (< 200 $CD4^+$ T cells/μL) at ART onset and follow-up ≥ 36 months after ART initiation. Using cluster analysis grounded on their $CD4^+$ T cell count trajectories, these patients were classified as "poor" or "adequate" immunological responders (PIR or AIR, respectively). A variety of immunological parameters were evaluated over time, with AIR presenting higher values of several thymic function surrogates compared to PIR. Predictive models of PIR/AIR outcome after 36 months of ART built based on observations made until 2-6 months, were able to correctly predict 77-87 % of cases. This study highlights the importance of thymic function in the immune recovery of severely lymphopenic patients.

The work presented in this dissertation contributes to advance the body of knowledge regarding the immunological response during the treatment of HIV infection, and pinpoints a subset of patients that may benefit from new strategies to boost thymic function in combination with ART.

Keywords: $CD4^+$ T cells, HIV, Predictive models, Immune recovery, Thymus

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Abbreviation list

ABC	Abacavir
ABT	Albuvirtide
ADD	AIDS-defining disease
AICD	Activation-induced cell death
AIDS	Acquired immune deficiency syndrome
AIR	Adequate immunological responders
APC	Antigen presenting cell
ART	Antiretroviral therapy
ARV	AIDS-associated retrovirus
ATV	Atazanavir
AZT	Azidothymidine
BIC	Bictegravir
cART	Combination antiretroviral therapy
CBG	Cabotegravir
CDC	Centers for Disease Control and Prevention
CDR	Complementarity-determining region
CLP	Common lymphoid progenitor
CT	Computed tomography
CTL	Cytotoxic T-lymphocytes
CMV	Cytomegalovirus
d4T	Stavudine
DCs	Dendritic cells
ddC	Zalcitabine
DDHS	Department of Health and Human Services
ddI	Didanosine
DGS	Direção-Geral de Saúde
DIR	Discordant immunological response
DLV	Delavirdine
DNA	Deoxyribonucleic acid
DOI	Digital object identifier
DOR	Doravirine
DP	Double positive
DRV	Darunavir
DTG	Dolutegravir
EACS	European AIDS Clinical Society
EFV	Efavirenz
EIA	Enzyme immunoassay
EPLM	Early progenitors with lymphoid and myeloid potential
erc	Epithelioreticular cell
ETR	Etravirine
EVG	Elvitegravir
FCT	Foundation for Science and Technology
FPV	Fosamprenavir
FTC	Emtricitabine
FTV	Fostemsavir
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B Virus
HF-TOC	Human fetal-thymus organ culture
HIV	Human immunodeficiency virus

HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HTLV	Human T-cell Lymphotropic Virus
IAS	International AIDS Society
IBA	Idalizumab
ICVS	Life and Health Sciences Research Institute
IDV	Indinavir
IL	Interleukin
INN	Enfuvirtide
INR	Immunological non-response
INSTI	Integrase strand transfer inhibitor
IRIS	Immune reconstitution inflammatory syndrome
LAV	Lymphadenopathy-associated virus
LCs	Langerhans cells
LPV	Lopinavir
MESH	Medical subject heading
MHC	Major histocompatibility complex
MOOSE	Meta-analyses of Observational Studies in Epidemiology
MVC	Maraviroc
NFV	Nelfinavir
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleos(t)ide reverse transcriptase inhibitor
NVP	Nevirapine
PIR	Poor immunological responders
PCR	Polymerase chain reaction
PEP	Post exposure prophylaxis
PBMC	Peripheral blood mononuclear cell
pmcID	PubMed Central identifier
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analyses
RAL	Raltegravir
RNA	Ribonucleic acid
RPV	Rilpivirine
RTV	Ritonavir
SCID	Severe combined immunodeficiency
SCR	Suboptimal $CD4^+$ T cell recovery
SIV	Simian immunodeficiency virus
SP	Single positive
SQV	Saquinavir
STD	Sexually transmitted disease
STI	Sexually transmitted infections
T20	Enfuvirtide
TAF	Tenofovir alafenamide fumarate
TCR	T cell receptor
TDF	Tenofovir disoproxil fumarate
TEC	Thymic epithelial cells
TPV	Tipranavir
Treg	Regulatory T cells
UNAIDS	Joint United Nations Programme on HIV/AIDS
USA	United States of America
WHO	World Health Organization
ZDV	Zidovudine
3TC	Lamivudine
/c	cobicistat-booster
/r	ritonavir-booster

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Context, Aims and Outline of the Thesis

The number of people living with human immunodeficiency virus (HIV) is progressively increasing, year by year, having reached almost 37 million in 2017. This shows us that HIV infection is still a public health problem, although the challenges are now different from those at the beginning of the epidemic. While in the 1980s, all patients infected by HIV died from AIDS-related complications in a relatively short period, nowadays HIV infection is considered a manageable chronic disease.

The outstanding improvement of HIV patients' prognosis is mainly due to the development of effective and safe antiretroviral therapy (ART) regimens, which can revert the $CD4^+$ T cell depletion, a major hallmark of HIV infection. Most patients on ART show suppression of the viral replication and an adequate recovery of the $CD4^+$ T cell counts. However, a significant proportion of patients maintain low $CD4^+$ T cell counts despite full virologic suppression. The mechanisms responsible for this are not fully understood. One hypothesis is that the immunological recovery, which relies on peripheral cell proliferation and on the activity of thymus (the organ responsible for the production of new T cells), is compromised by an impairment of thymic function in some of these patients. This hypothesis has been explored over the years, but results are still inconclusive for many reasons, such as the inexistence of a direct method to assess thymic function and the difficulty to discriminate the incorporation of new T cells in the periphery from proliferation of existing cells.

In this work, we focused on the following general question:

What is the role of thymic function in the immunological recovery of HIV-infected patients on ART with persistently low $CD4^+$ T cell counts despite full virologic suppression?

This question is particularly important for two main reasons: 1) patients on ART whose $CD4^+$ T cell counts do not normalize despite full virologic suppression present higher morbidity and mortality than those whose $CD4^+$ T cell counts normalize, and there is a

urgent need to find strategies to improve their outcome; 2) a better understanding of the role of thymic function in the immunological recovery during ART, would allow to identify the patients that could benefit the most from associating ART with strategies to boost thymic function.

The specific aims defined for this work were:

AIM 1: To determine the different terminologies and criteria applied to patients on ART who fail to achieve satisfactory $CD4^+$ T cell counts despite virologic suppression, and their relative frequency, based on a set of predefined terms related to the condition.

AIM 2: To build predictive models of the long-term immunological recovery of HIV-infected patients on ART using thymic function surrogates evaluated during the first months of ART.

To achieve these aims, we followed two different approaches.

For **AIM 1**, we performed a systematic review on the definition of patients on ART who fail to achieve satisfactory $CD4^+$ T cell counts despite full virologic suppression (presented in **Chapter 3**; paper submitted). Briefly, PubMed database was searched for all records containing a set of predefined terms related to the condition, published in English between January 2009 and September 2018. The search retrieved 1360 studies, of which 103 were eligible for analysis, based on the title and abstract screening. These 103 studies used 22 different terms and 73 distinct criteria for the definition, demonstrating high heterogeneity in the literature. The most frequent term was "immunological non-responders", followed by "discordant immunological response" and "suboptimal $CD4^+$ T-cell recovery". Of the 73 criteria, only 9 were used by more than one team. The most frequent criterion was "$CD4^+$ T cell count < 350 cells/μL after ≥ 24 months of virologic

suppression". We discuss the terms and criteria and suggest the combination of two definitions that could potentially reach a consensus.

For **AIM 2**, we studied a cohort of 100 HIV-infected patients from Centro Hospitalar do Porto (Porto, Portugal), followed since 2009. Clinical evaluations and blood sampling were performed at regular time points from the date of ART initiation to up to 60 months of follow-up. Blood samples were analyzed by flow cytometry at our laboratory. A subset of patients was submitted to chest CT scans to determine the volume and aspect of the thymus. For 37 of these patients, quantification of T cell receptor excision circles in peripheral mononuclear blood cells, an indirect method to estimate the thymic activity, was performed at Institut Cochin (Paris, France). The results of this part of the work, presented in **Chapter 4** of this dissertation, are separated into two subchapters:

Subchapter 4.1 includes the article "Thymic Function as a Predictor of Immune Recovery in Chronically HIV-Infected Patients Initiating Antiretroviral Therapy" (published in Frontiers in Immunology, in March 2019). In this study, we selected 33 HIV patients from our cohort who presented severe lymphopenia at ART initiation and had a follow-up of at least 36 months, and separated them into two groups with different immunological recoveries ("poor immunological responders" *versus* "adequate immunological responders") using cluster analysis grounded on their $CD4^+$ T cell count trajectories. Thymic function surrogates and immunological parameters were investigated and predictive models for the immune recovery were built.

Subchapter 4.2 includes the results obtained through the application of mathematical tools and R programming in the analysis of the data from the cohort, comprising two topics: 1) the thymic score, a combination of two different thymic function surrogates (thymic volume and thymic index); and 2) a mathematical model for the thymic output/peripheral proliferation ratio.

Overall, the work presented herein sets the basis for a standardization of "immunological non-response" definition, highlights the importance of thymus in the immune recovery of severely lymphopenic patients on ART, and may help to select those that will benefit from closer follow-up or from combination of ART with strategies to boost thymic function.

PART I | General Introduction

CHAPTER 1.

Infection by the Human Immunodeficiency Virus

1. Introduction

In the beginning of 1980s, several cases of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma were diagnosed in homosexual men living in the United States of America (USA) [1]. These diagnoses, uncommon in immunocompetent individuals, led to the discovery of a new syndrome characterized by a defect in cell mediated immunity: the acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV) infection. The virus was described for the first time in 1983, by Françoise Barré-Sinoussi and Luc Montagnier in France, and a few months later by the American investigator Robert Gallo [2-4]. At that time, the research groups led by these investigators did not agree on the morphologic and immunological characteristics of HIV, and called it lymphadenopathy-associated virus (LAV) and human T-lymphotropic virus III (HTLV-III), respectively. Another American investigator, Jay Levy, working contemporaneously with the Montagnier and Gallo groups, independently described another HIV strain, which he named AIDS associated retrovirus (ARV) [3]. In 1985, HTLV-III, LAV and ARV were recognized as variants of the same virus, and in 1986 a subcommittee of the International Committee for the Taxonomy of Viruses recommended the term "HIV" to designate the causal agent of AIDS [5, 6].

In 1986, a second type of HIV was discovered in West Africa. Since then, HIV is classified in two types: HIV-1 and HIV-2 [7]. Both have the same modes of transmission and are associated with similar opportunistic infections and AIDS, but they present significant genetic differences, distinct clinical evolutions and geographic patterns of incidence and prevalence. HIV-1 is responsible for most of the global AIDS pandemic, it is more infectious and progresses faster than HIV-2, presenting a higher mortality rate [8]. This thesis focuses on HIV-1, to which we will refer to as HIV from now-on.

Almost four decades after the first diagnoses of HIV infection, there is still no acceptable curative strategy, and vaccines to protect against the disease are still being tested [9]. Fortunately, the development of effective and potent, safe, tolerable, and easy-to-take therapeutic schemes, and the regular guideline updates resulted in great improvement of patients' prognosis, who now have much higher life expectancy and life quality. Despite those great achievements and the vast knowledge that has been acquired about this infection, research efforts are still needed to find new ways to stop HIV transmission, prevent the disease progression, and improve the treatment and immunological recovery of these patients.

2. Epidemiology

The HIV infection is a global public health problem, with the number of people living with HIV increasing every year (Figure 1) and cases reported from virtually every country [10]. In 2017, 36.9 million people were living with HIV, 1.8 million new cases were diagnosed and 940.000 people died from HIV-related causes, making it the fourth leading cause of mortality in low-income countries (UNAIDS/WHO estimates). Two-thirds of people living with HIV were in sub-Saharan Africa; more than half of cases were women and about 6 % of cases were children (aged 0–14 years) [11].

The HIV epidemic has occurred in "waves" in distinct regions of the world. Demographics and timing are the two main variables responsible for the different characteristics of each wave. In Portugal, about 58.000 people were diagnosed with HIV infection since the beginning of the epidemic. In 2017, approximately 40.000 people were living with HIV, and 1064 new cases of HIV infection were reported, with two times more cases in men compared to women (Figure 2) [12]. The comparative study The Nation's Health 1990–2016, concluded that the disability caused by HIV/AIDS in Portugal increased 794.3%, ranking as the 10th leading cause of disability in 2016 [13]. However, it should be noted that in 1990 the prevalence of HIV/AIDS in Portugal was still very low.

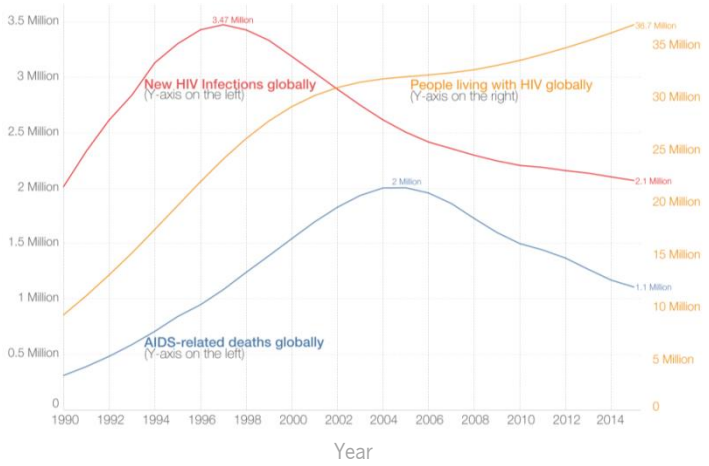

Figure 1 – Global number of AIDS-related deaths, new HIV infections, and people living with HIV (1990-2015). Source: UNAIDS (via http://www.aidsinfoonline.org).

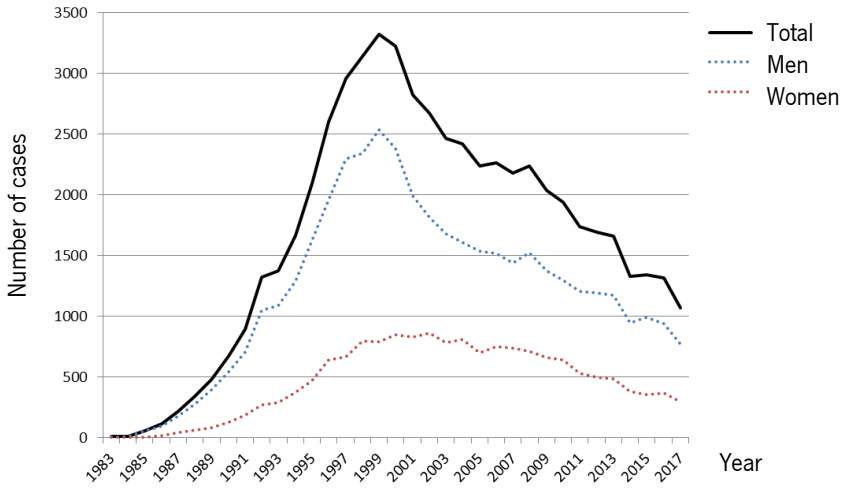

Figure 2 – New cases of HIV infection in Portugal by year and by sex.
Source: Instituto Nacional de Saúde (Portugal), 2019.

An international commitment is needed to stop HIV transmission and ensure that everyone living with HIV has access to HIV treatment. In 2014, UNAIDS proposed new and ambitious aims: that 90% of all people living with HIV will know their HIV status; 90% of all people with diagnosed HIV infection will receive sustained antiretroviral therapy; and 90% of all people receiving antiretroviral therapy will have viral suppression, by 2020 (90-90-90 targets). This strategy underpins the Sustainable Development Goals which aim for zero new infections and zero AIDS related deaths by 2030 [14].

3. Acute phase, chronic phase and acquired immunodeficiency syndrome

The natural course of the HIV infection is classically described by three phases: an initial acute retroviral syndrome, followed by an asymptomatic chronic phase that, without any treatment, evolves to AIDS after seven to ten years on average (Figure 3) [10].

The acute phase occurs in a short period of weeks just after exposure/transmission and is characterized by depletion of $CD4^+$ T cells and massive viral replication. Symptoms present in this phase (fever, headache, malaise, pharyngitis, and lymphadenopathy) are often too vague or nonspecific to lead to a diagnosis [15]. The chronic phase can endure for long periods of time, even decades, before the onset of symptoms, and it is characterized by immune hyperactivation and slow depletion of $CD4^+$ T cells. Finally, without adequate treatment, the disease progresses to its most advanced phase, AIDS. To be diagnosed with AIDS, a person with HIV must have a $CD4^+$ T cell count < 200 cells/mm^3 or an AIDS-defining condition (Table 1). In this phase, patients present very low $CD4^+$ T cell counts, high plasma viral loads and increased susceptibility to opportunistic infections, certain cancers and other disorders, ultimately leading to death [16].

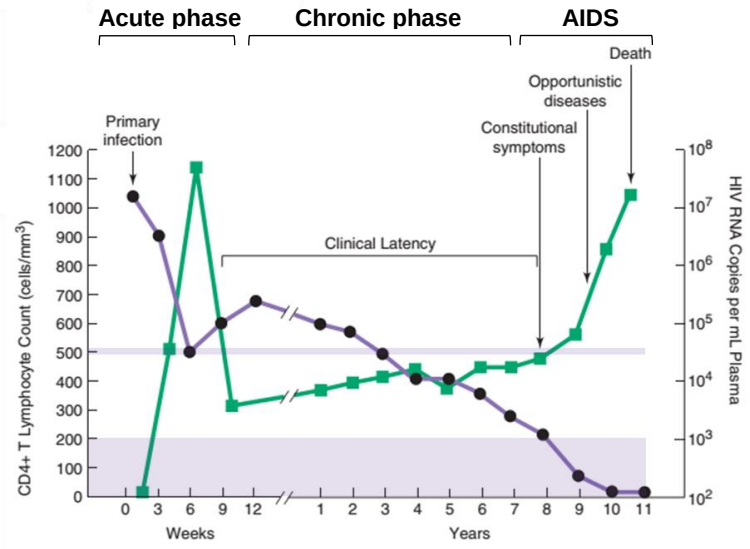

Figure 3 – Natural course of HIV disease.
Schematic evolution of the $CD4^+$ T cell counts (in purple) and plasma HIV viral load (in green). Adapted from [10].

Table 1 – AIDS-defining conditions according to the CDC Morbidity and Mortality Weekly Report Recommendations and Reports 2014.

AIDS-defining opportunistic illnesses
Bacterial infections, multiple or recurrent*
Candidiasis of bronchi, trachea, or lungs
Candidiasis of esophagus
Cervical cancer, invasive†
Coccidioidomycosis, disseminated or extrapulmonary
Cryptococcosis, extrapulmonary
Cryptosporidiosis, chronic intestinal (>1 month's duration)
Cytomegalovirus disease (other than liver, spleen, or nodes), onset at age >1 month
Cytomegalovirus retinitis (with loss of vision)
Encephalopathy attributed to HIV§
Herpes simplex: chronic ulcers (>1 month's duration) or bronchitis, pneumonitis, or esophagitis (onset at age >1 month)
Histoplasmosis, disseminated or extrapulmonary
Isosporiasis, chronic intestinal (>1 month's duration)
Kaposi sarcoma
Lymphoma, Burkitt (or equivalent term)
Lymphoma, immunoblastic (or equivalent term)
Lymphoma, primary, of brain
Mycobacterium avium complex or Mycobacterium kansasii, disseminated or extrapulmonary
Mycobacterium tuberculosis of any site, pulmonary†, disseminated, or extrapulmonary
Mycobacterium, other species or unidentified species, disseminated or extrapulmonary
Pneumocystis jirovecii (previously known as "Pneumocystis carinii") pneumonia
Pneumonia, recurrent†
Progressive multifocal leukoencephalopathy
Salmonella septicemia, recurrent
Toxoplasmosis of brain, onset at age >1 month
Wasting syndrome attributed to HIV§

Adapted from [17].

* Only among children aged <6 years.

† Only among adults, adolescents, and children aged ≥6 years.

§ Suggested diagnostic criteria for these illnesses, which might be particularly important for HIV encephalopathy and HIV wasting syndrome, are described in:

- CDC. 1994 Revised classification system for human immunodeficiency virus infection in children less than 13 years of age. MMWR 1994;43(No. RR-12).
- CDC. 1993 Revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. MMWR 1992;41(No. RR-17).

Besides the three described phases, HIV infection may be further classified into six distinct laboratory stages ("Fiebig stages") based on the progression of HIV markers during the primary HIV infection (Figure 4) [18]:

- stage I: only viral RNA is detected by PCR;
- stage II: viral RNA and p24 antigen tests positive, HIV-specific antibody enzyme immunoassay (EIA) non-reactive;
- stage III: viral RNA, HIV antigen and HIV IgM-sensitive EIA reactive, but Western blot without HIV-specific bands;
- stage IV: as stage III, but in addition indeterminate Western blot pattern;
- stage V: as stage IV, but reactive Western blot pattern, except lacking p31 reactivity;
- stage VI: as stage V, but full Western blot reactivity including a p31 band.

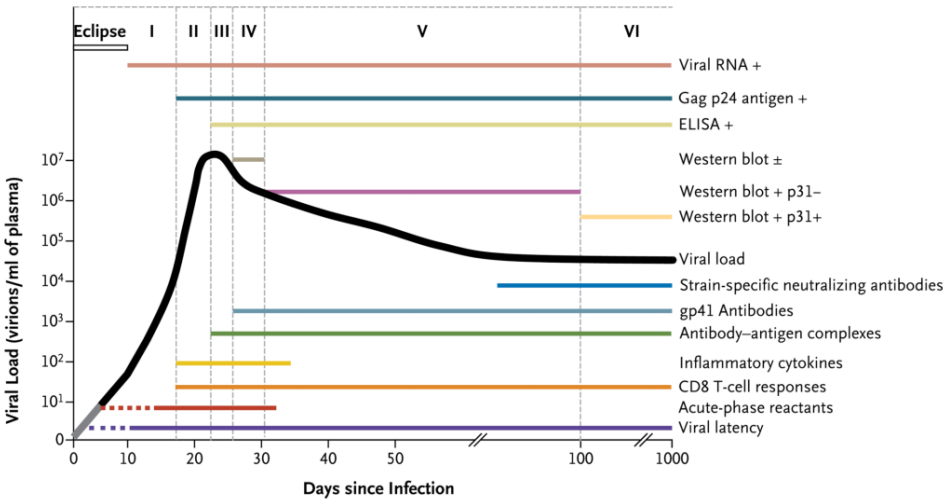

Figure 4 – "Fiebig stages" of HIV infection, based on clinical laboratory tests.
The six stages (indicated by Roman numerals) are defined according to the sequential appearance in plasma of HIV viral RNA; the gag p24 protein antigen; antibodies specific for recombinant HIV proteins; and antibodies that bind to fixed viral proteins, including p31. +, positive test result; -, negative test result; ±, borderline-positive result. The lines below the viral load curve show the timing of key events and immune responses. Extracted from [19].

The pattern of emergence of laboratory markers is highly consistent, allowing to distinguish:

- the *eclipse period,* the initial interval after infection with HIV when no laboratory markers are detectable (from day 0 to day 11);
- the *seroconversion window period*, the interval between infection with HIV and the first detection of antibodies (from day 0 to day 21, maximum day 42);
- *primary HIV infection*, defined as the 6 to 12 weeks between HIV exposure (day 0) and the appearance of anti-HIV-antibodies;
- *acute HIV infection*, between the appearance of detectable HIV RNA and the first detection of antibodies (from day 11 to day 21, maximum day 42);
- *established HIV infection*, characterized by a fully developed IgG antibody response sufficient to meet the interpretive criteria for a positive Western blot (from 1-month post-infection).

Without ART, most HIV-infected patients present the typical clinical evolution, becoming symptomatic about ten years after seroconversion, but a minority of patients remains asymptomatic for longer periods. These patients are called *elite controllers*, if they are able to maintain undetectable plasma viral RNA levels without ART (less than 1% of the total of HIV infected patients), or *long term non progressors*, if they have detectable plasma HIV RNA levels [20, 21].

Based on randomized trials demonstrating clinical and public health benefits of starting ART as soon as possible after HIV diagnosis [22, 23], World Health Organization (WHO) guidelines recommend, since 2015, to provide ART to all people living with HIV, irrespective of $CD4^+$ T cell counts [24].

4. Mechanisms of CD4+ T cell dysfunction and depletion

The HIV immunopathogenesis is an extremely complex phenomenon, in which CD4+ T cells play a major role. The gradual loss of CD4+ T cells is a hallmark of HIV infection. Dysfunction and depletion of these cells are responsible for the immunodeficiency state and, consequently, for the morbidity and mortality associated to this disease [10].

Considering the mucosal transmission, HIV infection begins with migration of free viruses, infected cells, or virions attached to dendritic cells or Langerhans cells across a mucosal barrier to the lamina propria [25]. Then, the viruses infect susceptible targets, *i.e.* cells expressing the molecule CD4, as this is the primary cellular receptor for HIV [26, 27]. A co-receptor must also be present together with CD4 for efficient binding, fusion, and entry of the virus in the cells: CCR5 (CCR5-tropic, R5-tropic or non-syncytium-inducing viruses) or CXCR4 (CXCR4-tropic, X4-tropic or syncytium-inducing viruses) [28]. The majority of HIV-infected individuals harbor CCR5-tropic viruses. CXCR4 is the co-receptor used in some cases of advanced infection. Initially, the principal targets are partially activated CD4+ T cells dispersed in the lamina propria, where viral replication occurs at low-level. At this point, strong innate and adaptive immune responses occur, but they provide incomplete viral replication control [29]. Then, activated CD4+ T cells replicate and propagate at high-levels [16]. Dissemination of HIV occurs first from mucosa-associated lymphoid tissue (MALT), in lamina propria, to the draining lymph nodes, and then to other lymphoid tissue, resulting in the establishment of viral reservoirs [30-32].

Mechanisms responsible for the imbalance in CD4+ T cell homeostasis are not fully understood, but include increased cell death, decreased cell life-span, decreased cell production, cell redistribution, as well as immune exhaustion due to aberrant immune activation [16, 33-36].

Death of HIV-infected CD4+ T cells results from both HIV direct cytotoxicity and a bystander effect of viral replication. HIV promotes caspase-3-mediated apoptosis [37, 38] and formation of short-lived syncytia [39]. Infected cells are more susceptible to death due to

loss of plasma membrane integrity caused by viral budding [40]; accumulation of unintegrated viral DNA [41]; activation of DNA-dependent protein kinase during viral integration into the host genome [42]; mitochondrial dysfunction [43]; and interference with the cellular RNA processing [40]. Furthermore, HIV-infected $CD4^+$ T cells are also eliminated by the virus-specific $CD8^+$ T cell response, which contributes to the initial decline of viremia during acute infection [44-47].

The vast $CD4^+$ T cell depletion cannot be justified only by the destruction of infected cells, which correspond to a disproportionally small percentage of all $CD4^+$ T cells. In fact, most dying cells are uninfected bystander cells, eradicated by indirect effects of viral replication, including: activation-induced cell death (AICD) mediated by Fas/Fas ligand; apoptosis stimulated by HIV proteins released from infected cells; or caspase-1-mediated pyroptosis [48-50].

The contribution of decreased production of $CD4^+$ T cell in the thymus to HIV pathogenesis is controversial [10]. While some data suggest that impaired thymic function significantly contributes to the $CD4^+$ T cell depletion [51-54], other studies suggest that thymus plays a minor role in that process [55-57]. This topic will be further explored in the next chapter.

Chronic HIV viremia and stimulation with HIV antigens lead to increased cell turnover and changes in cellular phenotype and function that are consistent with immune hyperactivation and exhaustion. Analysis of T cell turnover in humans with HIV infection suggests that the fraction of dividing $CD4^+$ T cells in untreated HIV disease can be elevated two- to threefold [58-60]. The increase in cell turnover is seen in $CD4^+$ and $CD8^+$ T cells, as well as B cells, natural killer cells and monocytes [61-64]. Up-regulation of lymphocyte homing receptors leads to increased cell migration from bloodstream to lymphoid tissues, such as lymph nodes and bone marrow [65].

Sustained inflammation and immune hyper-activation are reflected not only by increased cell turnover and up-regulated lymphocyte homing receptors, but also by enhanced expression of activation markers (*e.g.* CD38 and HLA-DR) and increased pro-inflammatory

cytokine levels [66-70]. The precise stimulus that triggers these phenomena is not clear, and probably they are driven by multiple factors (Table 2) [71-73].

Table 2 – Possible causes of immune activation during HIV infection.

Potential immune activation triggers
• Innate and adaptive immune response to HIV and its antigens; • Stimulation by the viral gene products Nef, Tat, Vpr, and Vpu; • Damage of the mucosal barrier and increased translocation of microbial products; • Concomitant infections, either opportunistic or non-opportunistic; • Increased levels of pro-inflammatory and/or pro-apoptotic cytokines resulting in activation of bystander (*i.e.*, non-HIV specific) T cells; • Depletion and/or dysfunction of regulatory $CD4^+$ T cells.

Adapted from [73].

On one hand, sustained inflammation and immune dysfunction contribute to HIV persistence by generating new target cells, altering the migration patterns, increasing proliferation of infected cells, promoting viral replication and hampering HIV-specific clearance mechanisms from function. On the other hand, by depleting $CD4^+$ T cells, viral replication also leads to immune dysfunction and, consequently, to persistent inflammation, creating a vicious cycle [71]. Several studies have demonstrated that the level of immune activation in HIV-infected subjects is a better predictor of disease progression than the HIV RNA plasma load, and that inflammation and immune activation are associated with a higher risk of non-AIDS-related complications, such as cardiovascular disease, osteoporosis, renal disease and non-AIDS-defining malignancies [67, 74, 75].

5. Antiretroviral therapy

Antiretroviral therapy (ART) completely changed the burden of HIV infection, by improving patient prognosis, quality of life and life expectancy [19, 20]. As of end-2017, 21.7 million people were receiving ART worldwide [11].

The first antiretroviral drug (zidovudine or azidothymidine, AZT) was developed in 1986. Since then, more than one hundred agents have been approved to treat patients with HIV infection/AIDS. Combination ART (cART) or highly active ART (HAART) were introduced in 1996 and became the cornerstone of management of patients with HIV infection [76]. ART consists in the use of multiple antiretroviral drugs targeting different stages in HIV-life cycle. Currently licensed drugs fall into five categories: (1) those that interfere with viral entry (fusion inhibitors; CCR5 antagonists; anti-CD4 monoclonal antibodies; anti-gp120; anti-gp41); (2) those that inhibit the viral reverse transcriptase enzyme (nucleoside, nucleotide or nonnucleoside reverse transcriptase inhibitors); (3) those that inhibit the viral integrase enzyme; (4) those that inhibit the viral protease enzyme; and (5) pharmacokinetic enhancers (Table 3).

Usually HAART in naïve patients combines two nucleos(t)ide reverse transcriptase inhibitors (NRTIs), as the 'backbone', along with a third drug that might be an integrase inhibitor, a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (Table 4). The choice of a specific regimen depends on several factors, including potential side effects, drug interactions, presence of drug resistance and cost [77]. Recently, complete dual drug regimens have been tested, and their use may be considered in specific cases (*e.g.* dolutegravir and lamivudine, darunavir/ritonavir and raltegravir). With the availability of potent and safe drugs, it is probable that ART schemes significantly change in the future.

ART have two main goals: primarily, preventing HIV-associated morbidity and mortality; and secondarily, reducing the risk of HIV transmission. Both goals are best accomplished when plasma HIV RNA (viral load) is maintained below the limits of quantification of the

commercially available assays (undetectable viral load) [78]. As adherence to ART is essential to maintain undetectable viral load, adherence barriers must be promptly identified and solved through the adequate interventions [79].

Table 3 – Antiretroviral drugs with FDA approval or in Phase III clinical trials.

Class	Drug name	FDA approval
Entry inhibitors	Enfuvirtide (T20 or INN)	March 2003
	Maraviroc (MVC)	August 2007
	Ibalizumab (IBA)	March 2018
	Fostemsavir (FTV)	Phase III
	Albuvirtide (ABT)	Phase III
Reverse transcriptase inhibitors		
• Nucleoside analogues	Zidovudine (AZT)	March 1987
	Didanosine (ddI)	October 1991
	Zalcitabine (ddC)	June 1992
	Stavudine (d4T)	June 1994
	Lamivudine (3TC)	November 1995
	Abacavir (ABC)	December 1998
	Emtricitabine (FTC)	February 2003
• Nucleotide analogue	Tenofovir disoproxil (TDF)	October 2001
	Tenofovir alafenamide (TAF)*	November 2015
• Non-nucleoside reverse-transcriptase inhibitors	Delavirdine (DLV)	April 1997
	Nevirapine (NVP)	September 1998
	Efavirenz (EFV)	September 1998
	Etravirine (ETR)	January 2008
	Rilpivirine (RPV)	May 2011
	Doravirine (DOR)	August 2018
Integrase inhibitors	Raltegravir (RAL)	October 2007
	Dolutegravir (DTG)	August 2013
	Elvitegravir (EVG)*	September 2014
	Bictegravir (BIC)	February 2018
	Cabotegravir (CBG)	Phase III
Protease inhibitors	Saquinavir (SQV)	December 1995
	Indinavir (IDV)	March 1996
	Nelfinavir (NFV)	March 1997
	Lopinavir (LPV)*	September 2000
	Fosamprenavir (FPV)	October 2003
	Tripanavir (TPV)	June 2005
	Atazanavir (ATV)	June 2006
	Darunavir (DRV)	June 2006
Pharmacokinetic enhancers	Ritonavir (RTV or /r)**	March 1996
	Cobicistat (COBI or /c)	March 2018

* Available only in combination for the treatment of HIV infection.
** Ritonavir was approved as a protease inhibitor, but it is now used as a pharmacokinetic enhancer.
Sources: Medscape® and AIDSinfo | UNAIDS.

Table 4 – International and national antiretroviral therapy guidelines.

Initial Combination Regimen for ART-naïve Adult HIV-positive Persons			
	EACS guidelines 2018	**IAS guidelines 2018**	**DHHS guidelines 2018**
	<u>Recommended regimens:</u> **2 NRTIs + INSTI:** - ABC/3TC/DTG* - TAF/FTC or TDF/FTC + DTG - TAF/FTC/BIC - TAF/FTC or TDF/FTC + RAL **2 NRTIs + NNRTI:** - TAF/FTC/RPV or TDF/FTC/RVP **2 NRTIs+ PI/r or PI/c:** - TAF/FTC or TDF/FTC + DRV/c or DRV/r <u>Alternative regimens:</u> **2 NRTIs + INSTI:** - ABC/3TC + RAL* - TAF/FTC/EVG/c or TDF/FTC/EVG/c **2 NRTIs + NNRTI:** - TDF/FTC/EFV - ABC/3TC + EFV **2 NRTIs+ PI/r or PI/c:** - ABC/3TC + ATV/c or ATV/r - ABC/3TC + DRV/c or DRV/r - TAF/FTC or TDF/FTC + ATV/c or ATV/r **Other combinations:** - DTG + 3TC - RAL + DRV/c or DRV/r	<u>Generally recommended:</u> **2 NRTIs + INSTI**: ABC/3TC/DTG* **[AI]** TAF/FTC + DTG **[AI]** TAF/FTC/BIC **[AI]** <u>Recommended when previous regimens are not available or not an option:</u> **2 NRTIs + INSTI:** - TDF/FTC/EVG/c **[AI]** - TDF/FTC or TAF/FTC + RAL **[AI]** **2 NRTIs + NNRTI:** - TDF/FTC/EFV **[AI]** - TDF/FTC/RPV or TAF/FTC/RVP **[AI]** **2 NRTIs + PI/r or PI/c:** - TDF/FTC or TAF/FTC + DRV/c **[AI]** - TDF/FTC or TAF/FTC + DRV/r <u>For the rare situation when individuals cannot take abacavir, TAF, or TDF:</u> **2-drug regimens:** - DTG + 3TC - RAL + DRV/r - 3TC + DRV/r	<u>For most people with HIV:</u> **2 NRTIs + INSTI:** - ABC/3TC/DTG* **[AI]** - TAF/FTC or TDF/FTC + DTG **[AI]** - TAF/FTC/BIC **[AI]** - TDF/FTC or TAF/FTC + RAL **[BI]** or **[BII]** <u>In certain clinical situations:</u> **2 NRTIs + INSTI:** - TDF/FTC/EVG/c or TAF/FTC/EVG/c **[BI]** - ABC/3TC + RAL* **[CII]** **2 NRTIs + NNRTI:** - TDF/FTC or TAF/FTC + EFV **[BI or BII]** - TDF/FTC/RPV or TAF/FTC/RVP **[BI]** - TDF/3TC/DOR **[BI]** or TAF/FTC + DOR **[BIII]** **2 NRTIs + PI/r or PI/c:** - TDF/FTC or TAF/FTC + (DRV/c or DRV/r) **[AI]** - TDF/FTC or TAF/FTC + (ATV/r or ATV/c) **[BI]** - ABC/3TC + (DRV/c or DRV/r)* **[BII]** **Other combinations:** - DTG + 3TC **[BI]** - RAL + DRV/r **[CI]** - 3TC + DRV/r **[CI]**
	Portuguese guidelines 2016		
	<u>Preferential regimens:</u> **2 NRTIs + INSTI:** - TDF/FTC + DTG - TDF/FTC + RAL - ABC/3TC/DTG* - ABC/3TC + RAL* - TDF/FTC/EVG/c **2 NRTIs + NNRTI:** - TDF/FTC/RVP - TDF/FTC/EFV - ABC/3TC + RVP - ABC/3TC + EFV - TDF/FTC or ABC/3TC + DRV/r or ATV/r		

[], Rating scheme for recommendations, according to the strength of recommendations (A, Strong; B, Moderate; C, Optional) and the quality of evidence (I, Data from randomized controlled trials; II, Data from well-designed nonrandomized trials or observational cohort studies with long-term clinical outcomes; III, Expert opinion).
Components separated with a slash (/) indicate that they are available as coformulations.
* Only for patients who are HLA-B*5701 negative.
ABC, abacavir; ATV, atazanavir; DRV, darunavir; DTG, dolutegravir; EFV, efavirenz; EVG, elvitegravir; FTC, emtricitabine; INSTI, integrase strand transfer inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside (or nucleotide) analogue reverse transcriptase inhibitors; PI, protease inhibitor; RAL, raltegravir; RPV, Rilpivirine; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; /c, cobicistat-booster; /r, ritonavir-booster.

Adapted from the European AIDS Clinical Society (EACS), the International AIDS Society (IAS), the U.S. Department of Health and Human Services (DDHS) and the Portuguese Direção-Geral de Saúde (DGS) guidelines [77, 78, 80, 81].

Over the last decades, the ideal moment for starting ART has been debated among clinicians, researchers and policy makers. During the late 1990s, the recommendations were to start ART only in symptomatic individuals with severe lymphopenia and AIDS, in which the benefits of therapy clearly outweighed the risks. At that time, treatment was expensive; drug regimens were complex, involving multiple drugs with a variety of administration schedules; drugs were significantly more toxic and the risk of drug resistance due to mutations was relatively high. Following improvement of the efficacy of antiretroviral drugs and limitation of its possible side effects and multi-drug interaction, the WHO recommendations for ART initiation changed from starting therapy in patients with < 200 $CD4^+$ T cells/μL, in 2002; to < 350 $CD4^+$ T cells/μL, in 2010; to < 500 $CD4^+$ T cells/μL, in 2013 [82]. The results from two important studies (INSIGHT START and SMART) showed a benefit of starting ART as soon as HIV infection is diagnosed, irrespective of $CD4^+$ T cell counts, and maintaining the therapy without interruptions. The most recent guidelines recommend that all patients start ART as soon as possible, to avoid the progressive damage to the immune system and to prevent HIV transmission [22, 77, 83].

Although ART has played an incredible role in changing a deathly disease into a chronic condition, it does not offer a cure: patients need to take ART drugs daily for the rest of their lives, which brings compliance problems, toxicity, and untenable costs. All ART interruption attempts led to viremia rebound, with only two exceptions in the entire world: the "Berlin patient", reported in 2009, and the "London patient", reported in March 2019. Both these patients suffered from a malignant hematological disease (respectively acute myeloid leukemia and Hodgkin lymphoma) and were submitted to CCR5Δ32/Δ32 haematopoietic stem-cell transplantation (HSCT) [84, 85]. Homozygous CCR5Δ32/Δ32 donor cells did not express CCR5, rendering these cells resistant to infection with HIV variants using the CCR5 co-receptor [86, 87]. Both patients were infected by CCR5-tropic HIV before the transplant, and interrupted ART after this procedure. The "Berlin patient" remained with undetectable

levels of HIV in their plasma and $CD4^+$ T cells for 12 years, and the "London patient", for 18 months, demonstrating that remission of HIV infection can be achieved [84, 85].

A definite cure for HIV infection implies complete eradication of the latent viral reservoirs. These reservoirs correspond to cells in which proviral DNA is integrated in the host's genome but it does not actively replicate, being "invisible" to the host immune system and unaffected by existing antiviral drugs [88]. It is not clear if a CCR5Δ32/Δ32 HSCT leads to a definite cure or just a "functional cure", given that positive low-signals in ultra-sensitive HIV-1 LTR Droplet Digital polymerase chain reaction (ddPCR) were observed in both "Berlin patient" and "London patient" [85, 89]. These signals may reflect a false ddPCR signal, potential contamination, or very low levels of persistence of HIV infected cells. Nevertheless, a HSCT is not an option to HIV-infected patients without cancer, because of the high morbidity and mortality risks, the difficulty in finding CCR5 Δ 32/ Δ 32 HLA-matched donors and the costs of this procedure [90]. While curative options for HIV infection are not available, many on-going studies aim to find a strategy to allow patients to interrupt ART, at least temporary [9]. Scheduled interruptions could improve patient motivation and compliance to ART, reduce the risk of toxicity and the high costs of continuous therapy.

6. Immunological recovery during antiretroviral therapy

The response to ART is usually evaluated from the virologic perspective (*"Is viral replication suppressed?"*) and from the immunological perspective (*"Is the immune system recovering from the damage cause by the infection?"*). Considering patients with good adherence to ART and without resistance-associated mutations, it is expected that plasma viral load drops to undetectable levels and, consequently, that $CD4^+$ T cell count increases to normal ranges [10] (Figure 5).

The human immune system is highly variable between individuals, due to heritable influences, exposure to different symbiotic and pathogenic microorganisms, among other factors [91]. In the same way, the immune recovery during ART varies significantly from patient to patient [92]. However, the rise in $CD4^+$ T cell counts typically follows a tri-phasic pattern [93]. In the first phase, that lasts 3 to 6 months, there is a rapid $CD4^+$ T cell count increase (20-30 cells/μL per month). The second phase is characterized by a slower increase (about 5 to 10 cells/μL per month) and lasts until the end of the second year of therapy. After that, in the third phase, the increase of $CD4^+$ T cells is even slower (about 2 to 5 cells/μL per month) [94, 95] (Figure 5). Some authors describe a $CD4^+$ T cell count plateau, while others observe increases even after several years of therapy, depending on different factors such as age, nadir $CD4^+$ T cell count and pre-ART viral load [96-99].

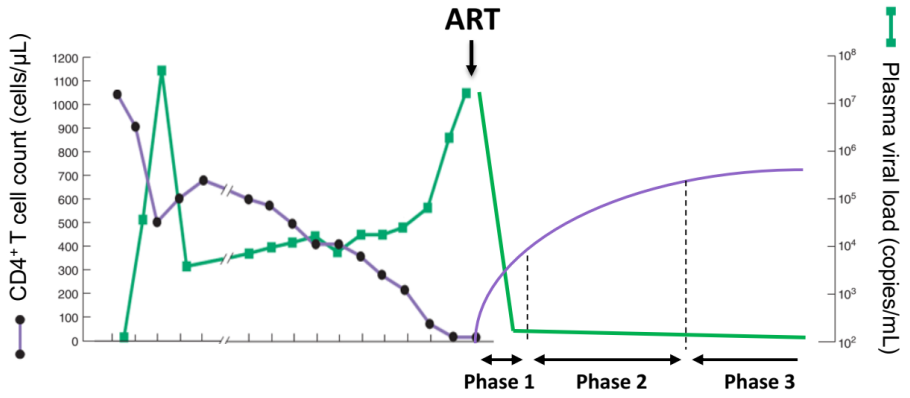

Figure 5 – Evolution of HIV plasma load and $CD4^+$ T cell counts before and after ART onset.
ART, Antiretroviral therapy. Adapted from [10] and [100].

Several mechanisms are responsible for the $CD4^+$ T cell recovery. In the first phase, the main cause is migration of memory $CD4^+$ T cells from the lymphoid tissues to the bloodstream (cell redistribution) due to down-regulation of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) [101]. In the second and third phases, other mechanisms become more relevant, including thymic output of naïve $CD4^+$ T cells; peripheral proliferation, extension of cell half-life and decrease of cell death [94, 102]. Although all these mechanisms contribute to the increase of $CD4^+$ T cells, some authors suggest that the thymic activity plays a predominant role [103, 104].

A significant proportion of patients on ART maintain unexpected low $CD4^+$ T cell counts despite full suppression of viral replication [105]. Different criteria are used in the literature to identify and classify these patients, known as "immunological non-responders", "discordant immune responders", "poor or suboptimal immune reconstituters", among other terms. The heterogeneity of these criteria makes prevalence estimates difficult. The percentage of patients who present immunological non-response (INR) varies between 9 % and 45 %, depending on the cohorts and criteria used [106, 107]. Notwithstanding, it is consensual that these patients present higher morbidity and mortality rates [108]. Many factors have been associated with INR, such as older age, male gender, long pre-ART period, presence of co-infections, low nadir $CD4^+$ T cell counts, increased lymphoid tissues fibrosis, enhanced immune activation, higher immunosenescence and inflammation, altered Treg/Th17 ratio, persistent latent viral burden, increased T cell death and impaired thymic function [109-113]. In addition, based on a prospective cohort of HIV patients, our group showed that levels of regulatory T cells (Treg, $CD3^+CD4^+CD127^-CD25^{high}FOXP3^+$ cells) correlate negatively with $CD4^+$ T cell counts during ART, and that alterations in Treg subpopulations persist, at least until 24 months of ART, in patients with severe lymphopenia before therapy (< 200 $CD4^+$ T cells/μL) [114, 115]. Patients with severe lymphopenia have higher risk of presenting INR, as $CD4^+$ T cell count at baseline is the most important factor predicting the $CD4^+$ T cell recovery during ART [116]. A better

understanding of the pathophysiological mechanisms behind INR will contribute to the identification of interventions to reduce the mortality and morbidity in this vulnerable group of patients.

Other problem related to the immune restoration during ART is that it may lead to an acute inflammatory response, known as immune reconstitution inflammatory syndrome (IRIS). Patients with IRIS experience clinical deterioration from a few days to 6 months after ART initiation, despite efficient control of HIV viral replication and no apparent drug toxicity, due to a concomitant autoimmune disorder, an immune-mediated inflammatory condition or, most frequently, a co-infection [117]. Common infectious agents associated with IRIS are *Mycobacterium tuberculosis*, Kaposi's sarcoma-associated herpesvirus or human herpes virus 8 (HHV-8), *Cytomegalovirus* and *Cryptococcus neoformans* [118]. IRIS is classified as unmasking or paradoxical, depending on the infection being previously subclinical/undiagnosed or diagnosed and treated, respectively [119]. IRIS presentation is heterogeneous, from slight to exuberant clinical manifestations, depending on the trigger and on the host immune response. IRIS treatment may include antibiotics, antivirals or antifungals to reduce the antigen load; supportive measures; and, in some cases, immunosuppression with corticosteroids [117].

It is important to highlight that $CD4^+$ T cell depletion represents only one of the many alterations occurring during HIV infection. The immune system damage is also reflected by inversion of CD4/CD8 ratio, increased levels of pro-inflammatory cytokines, enhanced expression of cell activation markers, etc. A recent study showed that the increases in $CD4^+$ T cell counts poorly correlate with those of percentage of $CD4^+$ T cells and CD4/CD8 ratios [120]. ART is not able to reverse all the alterations caused by HIV infection and to restore the balance between innate and adaptive immunity. Additionally, ART may be responsible for a wide range of toxicities.

In conclusion, the immunological recovery of HIV-infected patients on ART is a multifactorial and heterogeneous process. Typically, the increase of $CD4^+$ T cell count

follows a tri-phasic pattern, reaching the range of $CD4^+$ T cell counts in healthy individuals. In some cases, immunological recovery may be erratic, with the occurrence of IRIS or INR. Even in patients with adequate immunological response to ART, the immune recovery is not perfect, and inflammation, immune hyperactivation and microbial translocation persist [121].

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CHAPTER 2.
The Thymus

1. Thymus development and morphology

The thymus is the primary lymphoid organ responsible for the production of T cells, for the establishment of a broad peripheral T cell receptor (TCR) repertoire, as well as for the maintenance of a diverse T cell pool in the periphery. It is located in the superior mediastinum, anterior to the heart and great vessels. It develops bilaterally from the third or both the third and fourth branchial pouches [1, 2]. During the 4th and 5th weeks of gestation, the epithelium invaginates and a thymic rudiment grows caudally as a tubular projection of the endodermal epithelium, into the mediastinum [3]. The advancing tip proliferates and ultimately becomes disconnected from the branchial epithelium, forming a rudimentary thymus, called thymic anlage [4]. The thymic anlage then attracts cells of hematopoietic origin, the common lymphoid progenitor (CLP) cells, which invade it during the 8th and 9th weeks of gestation, and occupy spaces between the epithelial cells to give rise to a large number of thymocytes [3]. In addition to CLP cells, other cells migrate from the bone marrow to be part of the thymic stroma. These bone marrow-derived cells include macrophages, dendritic cells, B cells, NK cells, eosinophils and basophils [5].

Macroscopically, thymus is a pinkish-gray, soft and bilobed organ. It is enclosed in a fibrous capsule which invaginates forming the septa that divides the two lobes into many pseudolobuli [6]. Each pseudolobule comprises four main regions: the medulla, the cortico-medullary junction, the cortex and the subcapsular zone. CLP cells start their differentiation pathway at outermost subcapsular region; the cortex contains the developing thymocytes; and the inner area, the medulla, is where the final steps of the T cell differentiation occur before the mature T cells exit to the periphery at the cortico-medullary region [7, 8]. The microenvironment is provided mainly by an extracellular matrix and by stromal cells [six types of thymic epithelial cells (TECs), also known as

epithelioreticular cells (*erc*), macrophages, dendritic cells, fibroblasts, and other cells in small number], which influence the developing thymocytes via cell-surface and secreted molecules. For example, type VI epithelioreticular cells, which form concentrically arranged structures (Hassal's corpuscules) present in the thymic medulla(Figure 6), are a potent source of the cytokine thymic stromal lymphopoietin (TSLP), interfering with the thymocytes differentiation [9]. Actually, the survival, proliferation and differentiation of thymocytes are finely regulated by not only by the microenvironment [that provide interleukin (IL)-7, IL-4, IL-15 and stem cell factor (SCF)], but also by the thymic architecture and several molecular factors, such as Wnt molecules, Notch1 and Hedgehog [10-13].

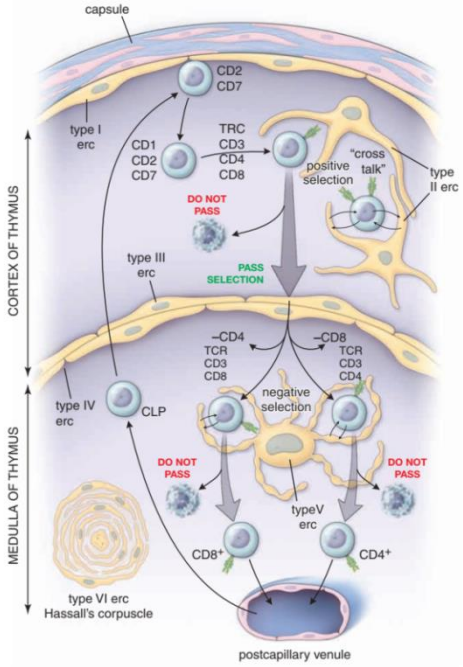

Figure 6 – Different epithelioreticular cells present in the thymic cortex and medulla. CLP, common lymphoid progenitor cells; erc, epithelioreticular cells; TCR, T cell receptor. Adapted from [5]

Based on the stimuli to which thymocytes are exposed, they differentiate in one of two major T cell subpopulations, that distinguished based on the type of TCR present in the cells: αβ T cells (90-99 %) and γδ T cells (1-10 %) [14]. Among αβ T cells, two main cell subsets are identified through the co-receptor expression: $CD4^+$ T cells and $CD8^+$ T cells. Each of these cell subsets plays a different role on the adaptive immunity by recognizing

different peptides in the context of distinct Major Histocompatibility Complex (MHC). $CD4^+$ T cells recognize antigens presented by MHC class II, while $CD8^+$ T cells recognize antigens presented by MHC class I [5, 7].

The thymus is fully formed and functional at birth. It reaches its maximum size (approximately 25 cm^3) within the first 12 months of life, and thereafter undergoes gradual involution, such that the volume of thymic tissue reduces progressively throughout life, being replaced by adipose tissue (Figure 7). Thymic involution starts as early as one year of age and continues at a rate of approximately 3 % per year until middle age, when it slows down to less than 1 % per year [15, 16]. Some authors suggest that reduction of thymic tissue accelerates at puberty due to increases in sex steroid and declines in growth hormone production [17].

Thymic atrophy is not solely associated with aging. Alterations in thymocyte numbers and thymic size have been described in different physiological and pathological states including puberty and pregnancy, inflammation, bacterial and viral infections, stress and other psychological conditions, environmental exposures, malnutrition, glucocorticoid therapy, chemotherapy and radiotherapy. Many of these processes are transient and reversible [18-27]. In general, thymi from persons over 90 years have lost more than 98 % of the functional thymic tissue [15]. However, thymopoieseis still occur in elderly and the organ can be re-stimulated under conditions that demand rapid T cell proliferation [28, 29].

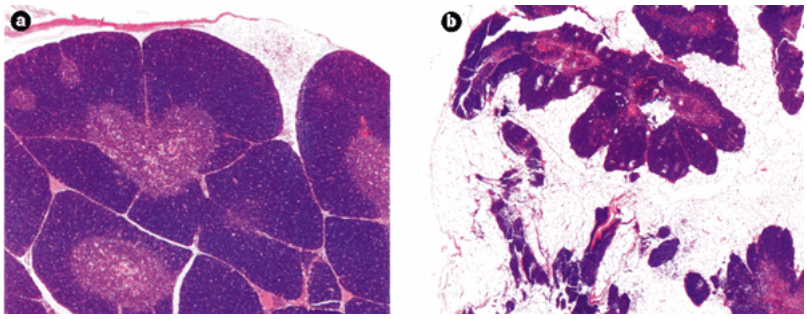

Figure 7 – Effect of aging on thymic morphology.
Two examples of thymic morphology: (a) thymus from a child, with multiple pseudolobuli, each one with evident central medulla and peripheral cortex; and (b) thymus from an old adult, massively infiltrated by adipose tissue, but still harbouring several islands of lymphoid tissue.
Reproduced from [151].

2. T cell differentiation

T cells are produced in the thymus through a differentiation process, highly regulated by the complex thymic microenvironment, in which CLP cells become immunocompetent cells of the adaptive immune system. As mentioned, this process depends on NOTCH, Wnt molecules, and Hedgehog, amongst others. NOTCH genes encode transmembrane receptors that are essential for the B *versus* T cell fate decision and the transition between the steps of early T cell differentiation [12, 31]. Wnt genes encode lipid-modified secreted glycoproteins that regulate cell fate specification and progenitor cell proliferation, and it has been shown that elevated Wnt signaling levels alter the differentiation of epithelioreticular cells and, therefore, disrupt T cell differentiation [32]. Hedgehog (Hb) signaling control the homeostasis and maturation of thymocytes by influencing cell survival and proliferation [13].

The several steps of T cell differentiation occur in distinct intrathymic locations and are characterized by the expression of differential combinations of the cell surface molecules CD34, CD2, CD7, CD3, CD4, CD8 and the TCR, to name only the most common (Figure 8).

Most thymocytes develop into conventional $CD4^+$ or $CD8^+$ T cells, expressing αβ TCRs. Some thymocytes develop into other cell lineages, including lymphoid dendritic cells, natural killer T cells (NKT), regulatory T cells (Treg), or intraepithelial lymphocytes (IELs), but the differentiation of these subsets of T cells is beyond the scope of this thesis.

The $CD34^+$ stem cells originated from the bone marrow enter the thymic medulla via post-capillary venules, and then migrate to the cortex, in the periphery of the thymic pseudolobuli [33]. It has been shown that these cells have the ability to differentiate not only in T cells, but also NKT cells, NK cells, B cells, dendritic cells as well as myeloid lineage cells [34, 35]. Definitive commitment to the T cell lineage is marked by CD1a expression. Initially, cells express CD2 and CD7 on their surface, but not CD4 or CD8 [immature double-negative (DN) cells]. As maturation progresses, the cells begin

assembling their TCR by V(D)J recombination and differentiate to $CD3^+CD4^+CD8^-$ cells [immature single positive (SP) cells], and then to $CD3^+CD4^+CD8^+$ cells [double positive (DP) cells], which are presented with self and foreign antigens by type II and III epithelioreticular cells. If the thymocyte recognizes a self- or foreign-peptide/MHC complex with an intermediate/high-affinity interaction, then it will survive the selection (positive selection); if not, the cell will die by neglect [36]. Cells that pass the positive selection leave the thymic cortex and enter the medulla. Here they undergo another selection process in which cells directed with high affinity to self-antigen displayed by MHC are eliminated (negative selection) [37, 38].

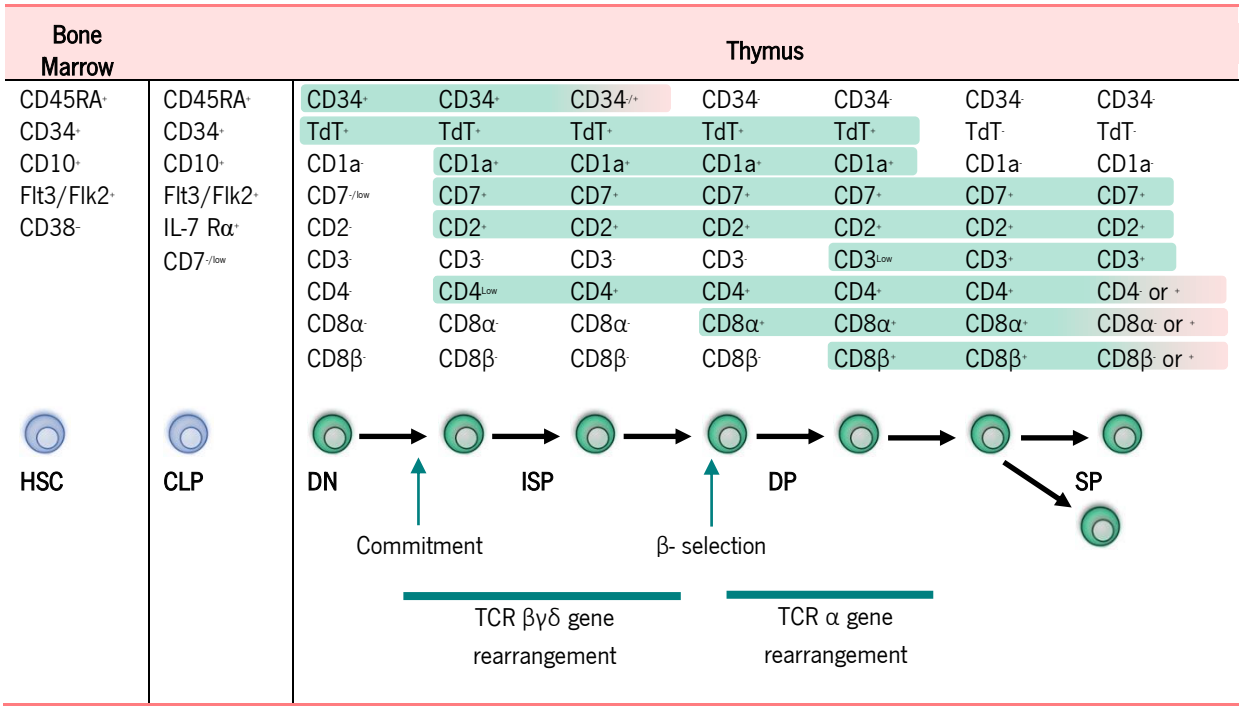

Figure 8 – Major steps of T cell differentiation in the thymus.
* CD7 is expressed on most but not all recent thymic emigrants. CLP, common lymphoid progenitor; DN, double negative; DP, double positive; HSC, hematopoietic stem cell; ISP, immature single positive; SP, single positive; TdT, terminal deoxynucleotidyl transferase. Adapted from [39].

Approximately 98% of the developing thymocytes die within the thymus by apoptosis. Cells that survive become either cytotoxic $CD8^+$ T cells or helper $CD4^+$ T cells (mature single-positive cells), leaving the thymus to circulate in the periphery [40, 41].

Blood T cells repeatedly leave the bloodstream and migrate to secondary lymphoid organs, where they may encounter their specific foreign antigen and become activated. Activation leads to clonal expansion and differentiation from naïve T cells to effector T cells, at the peak of the primary response. Effector T cells are attracted to sites of infection where they can induce the death of infected cells or activate macrophages; other effector T cells are attracted into B cell areas where they help to activate an antibody response. Memory T cells develop over time in response to diverse antigen exposure and comprise a heterogeneous population, including stem central memory T cells, central memory T cells, effector memory T cells and tissue resident memory T cells [42-44] (Figure 9). Each subpopulation of memory T cells is characterized by the expression of distinct homing receptors and also by unique effector properties.

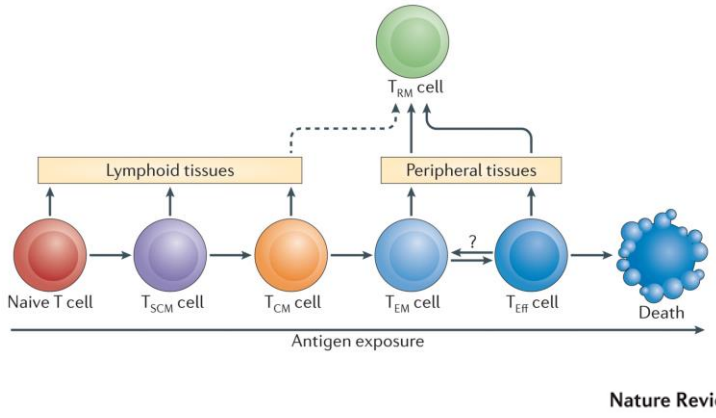

Figure 9 – Heterogeneity within the memory T cell subpopulation.
CM, central memory; Eff, effector; EM, effector memory; RM, resident memory; SCM, stem central memory. Reproduced from [44].

3. Thymic function assessment

The thymus plays an important role in several physiological and pathological conditions, in particular when *de novo* production of T cells is compromised or needed (*e.g.* in primary immunodeficiencies, in chronic HIV infection, during and after chemotherapy, and after hematopoietic stem-cell transplantation). Assessment of thymic function in these scenarios

is essential to aid to both diagnosis and management of the patients, however there is not a single direct method to do it. Instead, several indirect methods are used, each one with its own advantages and limitations [45]. These methods focus on the thymic structure or metabolism, on the cells exported by the thymus, called recent thymic emigrants (RTE), or on the TCR repertoire [46]. The main techniques to assess these parameters in humans are: 1) imaging; 2) molecular biology techniques; and 3) flow cytometry (Table 5).

Other techniques are useful to assess the impact of peripheral proliferation on the thymic function surrogates [*e.g.* Ki67 expression analysis; measurement of telomere length; bromodeoxy-uridine (BrdU), deuterated water ($^{2}H_2O$) or deuterated-glucose (D-glucose) labeling].

Table 5 – Techniques to assess the thymic function in humans and the respective surrogates.

	Advantages	Limitations
1. Imaging		
CT scan, MRI or US		
• Thymic volume • Thymic index	- Widely available - Estimation of the amount of normal thymic tissue, in most cases	- Possible dissociation between volume and function
PET		
• ^{18}F-FDG uptake	- Information in real time	- Expensive - Limited availability - Inaccurate images
2. Molecular biology techniques		
• TRECs	- TRECs measure intra-thymic events and are very stable (good for follow-up)	- TRECs do not reflect the actual but the past activity, and are affected by peripheral division and cell death (poor value as an isolated measurement) - Inexistence of a gold standard method; potential bias when comparing results
• TCR repertoire	- TCR repertoire diversity as a qualitative measure of thymic function	
3. Flow cytometry		
• Recent thymic emigrants	- Easy to measure - Reflects recent thymic function	- Difficulty to separate RTE from other naïve T cells

CT, computed tomography; ^{18}F-FDG, fluorine-18 fluorodeoxyglucose; MRI, magnetic resonance imaging; RTE, recent thymic emigrants; TCR, T cell receptor; TREC, T cell receptor excision circles; US, ultrasonography.
Adapted from [47].

3.1 - Imaging

Imaging evaluation of the thymus can be performed as an estimation of thymic function, assuming that thymic morphology and its evolution are correlated with the organ output, which is still not consensual. Many techniques have been used, including magnetic resonance imaging (MRI) and ultrasonography (US). However, CT scanning has been regarded as the imaging modality of choice for assessment of a mediastinal structures and has proved to be superior to US in determining thymus volume in HIV-infected patients [48, 49]. At least two thymic parameters can be analyzed by CT: volume and index.

Thymic volume is determined by the delineation of the thymic outline throughout a series of contiguous sliced images, all with the same thickness and length. The manual delineation of the thymus shape is automatically converted by software in a quantitative measure (cm^3) [50, 51]. The most striking limitation of thymic volume as a parameter of thymic activity is the fact that it does not provide information about the evolution of thymic structure and the quantity of adipose tissue. In the absence of a thymic biopsy, we cannot be sure that an increase in thymic volume results mainly from an increase of thymic cortex and medullary tissue and not of adipose or connective tissue. Despite its limitations, thymic volume and its evolution have been related to thymic activity in multiple studies. In a multivariate analysis including TREC quantification, naïve T cell analysis and thymic volume alterations of a prospective cohort of HIV-infected patients under ART, only thymic volume at baseline was independently associated to $CD4^+$ T cell recovery [52].

Thymic index, unlike the volume, provides information about thymic structure in a categorical manner. According to McCune et al., an index scoring scale may be used to measure the presence of thymic tissue (brighter areas in the CT), as opposed to adipocyte tissue [50]. This index ranks from a score of 0 to 5: 0, no soft tissue, with thymus entirely replaced by fat; 1, minimal, barely recognizable soft tissue; 2, minimal soft tissue, more obvious; 3, moderate soft tissue; 4, moderate soft tissue of greater extent, almost mass like; 5, mass-like appearance, that raises concern for a thymoma (Figure 10). Thymic

index complements the information provided by thymic volume in the sense that it better reflects the variation of thymic structure.

^{18}F-fluorodeoxyglucose-positron emission tomography (FDG-PET) is a noninvasive technique that identifies tissues with increased glucose metabolism. The main advantage of using FDG-PET to assess the thymic glucose uptake as an estimate of thymic function, is the acquisition of real time information. Limitations rely on the costs, restricted availability and image inaccuracy due to *physics*-related factors, namely positron range and photon noncollinearity, which limit the spatial resolution of PET.

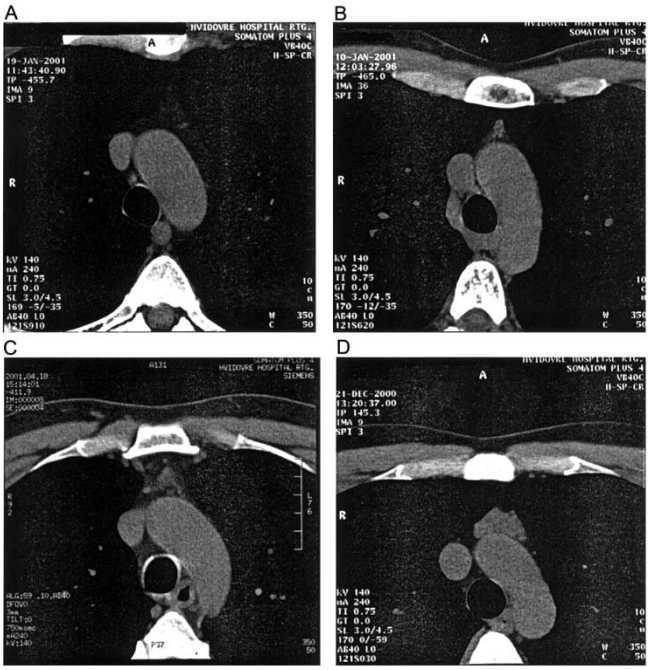

Figure 10 – CT scan images of patients with different thymic indices.
Representative image of a thymic index of 1 **(A)**, 2 **(B)**, 3 **(C)** and 4 **(D)**.
Reproduced from [53].

3.2 – Molecular biology techniques

3.2.1 – Quantification of T-cell receptor excision circles (TRECs)

TREC are extrachromosomal DNA byproducts, produced during the TCR-gene rearrangement that occurs in intrathymic differentiation of T cells. To explain the production of TREC, TCR-gene rearrangement will be briefly described.

TCR is a membrane bound heterodimer composed of two polypeptide chains. The vast majority of peripheral T cells express TCRα and TCRβ chains (αβ T cells), whereas only 1-10% of peripheral T cells express the alternative TCRγ and TCRδ chains (γδ T cells). Each of these chains includes one constant (C), one variable (V) and one transmembrane region (Figure 11A).

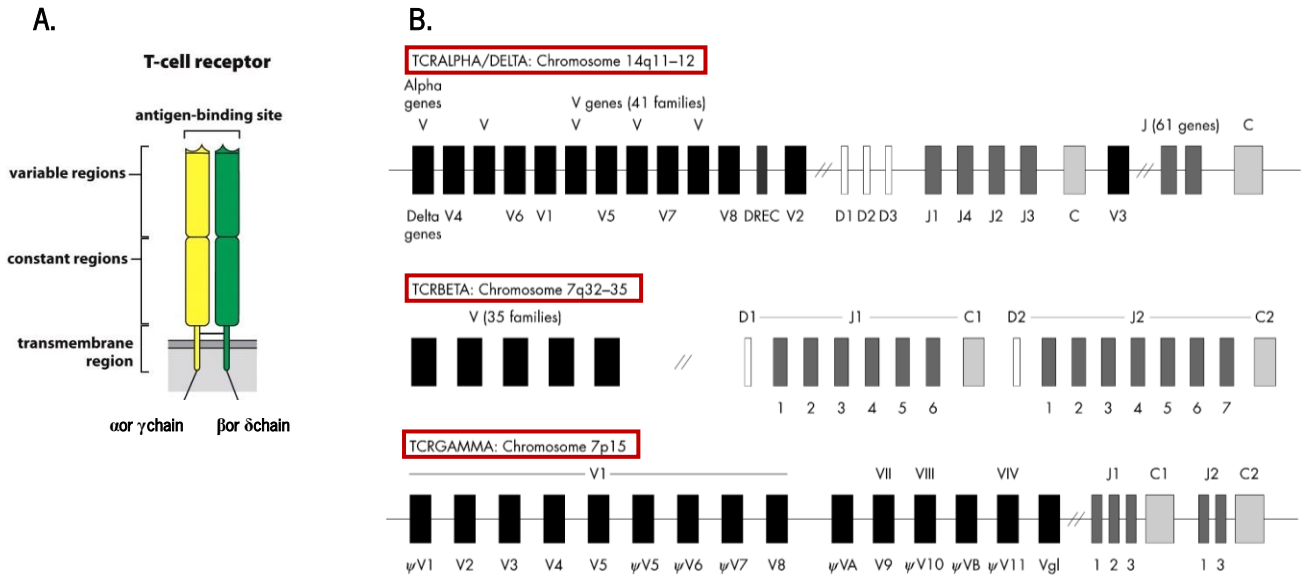

Figure 11 – Structure of the T cell receptor (TCR) and the germline organization of its genes.
A. Heterodimeric structure of TCR. **B.** The genes encoding the TCRα chain are located within the TCRA loci on chromosome 14q11–12, whereas the TCRβ and TCRγ genes (TCRB and TCRG) are located at chromosomal positions 7q32–35 and 7p15, respectively. The TCRD locus is located within the TCRA locus. Adapted from [54].

TCR genes are located on chromosomes 7 and 14 (TCRA, TCRB, TCRD and TCRG loci). The V region of the α and γ chains is encoded by a V and a joining (J) gene segment, whereas the V region of the β and δ chains is additionally encoded by a diversity (D) gene segment (Figure 11B). V(D)J gene recombination is the process responsible for the diversity of the αβ and γδ TCR repertoire. This complex end-to-end fusion of gene segments is mediated by recombination-activating genes (RAG) 1 and 2 that recognize "heptamer–spacer–nonamer" recombination signal sequences (RSSs) flanking each V, D and J gene segment. Generation of a coding TCR chain results in the excision of stable extrachromosomal DNA circles composed of the intervening genomic sequences present between both rearranged segments, the so-called TRECs. TRECs are not replicated during cell division and are passed on to one of the two daughter cells.

During differentiation, αβ T cells rearrange first the TCR β-chain, at the double-negative level, resulting in the excision of a DβJβ-TREC (β-TREC). Several divisions later, at the double-positive stage, cells rearrange their TCR α-chain and the signal-joint (sj) TREC is formed. Then, three to four divisions later, the coding-joint (cj) TREC is formed (Figure 12).

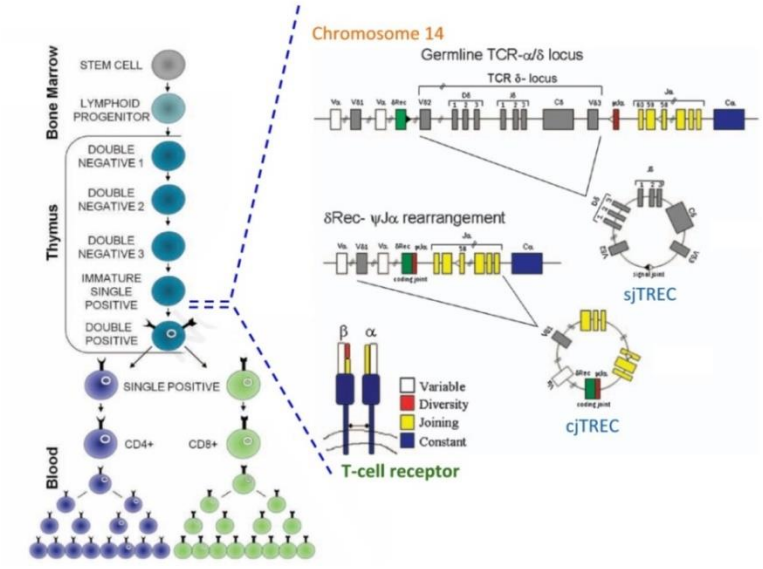

Figure 12 – T cell receptor excision circles (TREC) formation.
cjTREC: coding-joint TREC; sjTREC: single-joint TREC; TCR: T cell receptor.
Adapted from [55, 56].

Because of the enormous diversity of TCRA-VJ and TCRB-V(D)J recombination events, and thus of the TREC produced, no single but the total number of TRECs can be used as a marker to assess overall thymic function. However, a common requirement for productive rearrangement of the TCRA locus is deletion of the TCRD locus which it encompasses. The two rearrangement events that occur during this process are identical in 70% of αβ T cells. Thus the TREC generated are common to most αβ T cells and can be used to detect and quantify thymic output of αβ T cells in clinical samples [57].

TREC measurements are typically expressed as the number of TRECs per cell and are called the average TREC content. Measuring the total number of TRECs in a population of cells is a much better measure of thymic output, but requires estimates of total cell numbers, which is not possible in humans.

Arellano et al. have compared the levels of TRECs in the thymus and in peripheral blood mononuclear cells (PBMCs) from thirty-three patients who underwent cardiac surgery. They found a positive correlation between these two parameters and a negative correlation between each TREC level (in the thymus and in PBMCs) and patients' age [58]. Recently, a population study in which 1000 age- and sex-stratified healthy adults were enrolled, showed that TREC levels decrease with age, and are higher in women and in individuals with the single-nucleotide polymorphism rs2204985a, a common variant within the TCRA-TCRD locus [59].

TREC data should be interpreted with caution since confounding factors, such as cell division, cell death, the longevity of naïve T cells and intracellular degradation, are known to affect TREC levels, together with the fact that the precise half-life of TREC has not been established (Figure 13) [60, 61]. Different mathematical models were proposed to obtain quantitative insights into thymic production from TREC measurements [56, 60, 62-68].

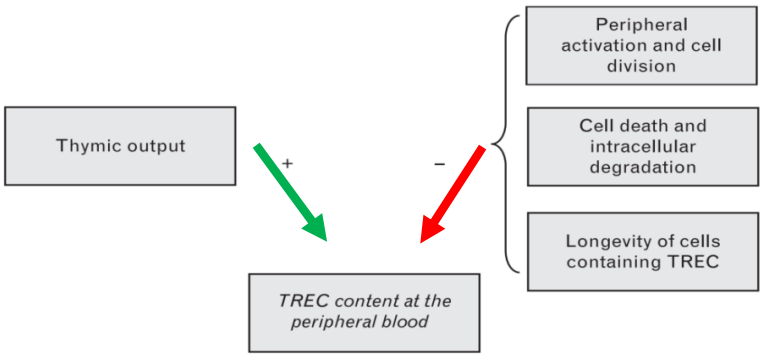

Figure 13 – Factors that influence the quantity of TREC in the peripheral blood.
TREC, T cell receptor excision circles. Adapted from [61].

In 2004, Dion et al. described a robust, quantitative index of intrathymic cell proliferation: the sj/β TREC ratio [69]. Given that αβ T cells rearrange first the TCR β-chain, and then, rearrange their TCR α-chain, the quantification of the TREC generated at these 2 checkpoints allows estimating the intrathymic proliferative history of the lymphocytes (Figure 14). Assuming that the confounding factors above-mentioned affect equally β-TREC and sj-TREC levels, their ratio stays constant. Using this ratio, Dion et al. showed that sj/β TREC ratio is negatively correlated with age. In the same study, the authors also showed that there is a significant reduction of intrathymic proliferation in recently HIV-infected individuals [69].

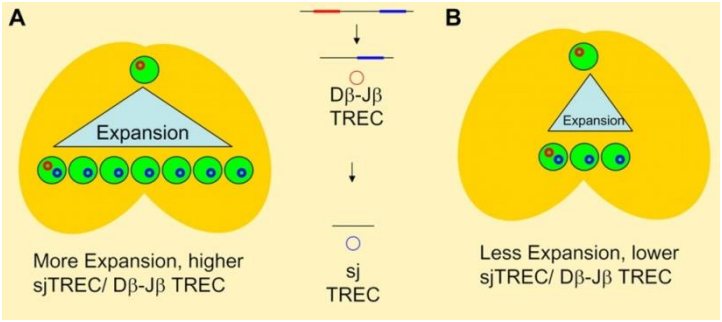

Figure 14 – Effect of intrathymic proliferation in sj/β TREC ratio.
Thymocytes rearrange the TCR β-chain at the double-negative level, producing a Dβ-JβTREC (β-TREC). Then, cells proliferate to the double-positive stage (expansion). Finally, cells rearrange their TCR α-chain, producing a sj-TREC. High **(A)** and low **(B)** intrathymic proliferation levels result in different sj/β TREC ratios.
Reproduced from [70].

Real time quantitative PCR (RTqPCR) is the preferred assay to quantify TREC because it is sensitive and accurate, based on specific detection of the amplified target sequences during each PCR cycle. TREC content can be measured on PBMCs, purified $CD4^+$ and $CD8^+$ T cells or isolated naïve T cells [61].

3.2.2 – TCR repertoire analysis

The TCR repertoire corresponds to the sum of all TCR recognition specificities. It is generated during the first two decades of life and maintained through life via homeostatic proliferation of T cells. The TCR repertoire diversity is exclusively created during T cell differentiation, in the thymus. Therefore, analysis of TCR repertoire may be used to estimate, or at least to compare, the thymic function of different individuals.

Recognition specificity depends on the variable regions of the α- and β-chains, which contain three loops, called complementarity-determining regions (CDRs) 1, 2, and 3, that directly contact with the ligand. The hypervariable CDR3 loops are generated during the V(D)J gene recombination, while the CDR1 and CDR2 loops are encoded within the V gene segment. Hence, the peptide specificity of αβ T cells is primarily determined by the CDR3 loops of the α- and β-chain variable domains [71].

The diversity is greatest in the pre-selection repertoire, within the thymus. Positive and negative selection in the thymus leads to a peripheral naïve repertoire that is substantially less diverse by purging the pre-selection repertoire of most specificities. In the periphery, antigen exposure further narrows the repertoire over time leading to clonal expansion of antigen-specific populations (Figure 15) [72-74].

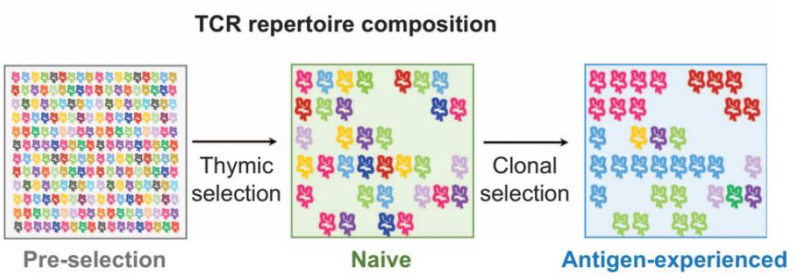

Figure 15 – Representation of TCR repertoire composition.
TCR diversity is greatest in the pre-selection repertoire (gray); peripheral naïve repertoire is substantially less diverse (green); antigen exposure further narrows the repertoire over time leading to clonal expansion of antigen-specific populations (blue). TCR, T cell receptor.
Reproduced from [72].

TCR repertoire in humans is estimated to contain approximately 10^8 distinct specificities, despite of the theoretical individual diversity being much higher (10^{18}) [75-78]. To determine all this diversity is a huge technical challenge [79]. In the past decades, different techniques were used: from the use of monoclonal antibodies to analysis of specific V gene subgroups by fluorescence microscopy or flow cytometry, to quantitative PCR strategies, in parallel with spectratyping [80]. The initial approaches consisted in extrapolating the entire repertoire diversity from the results of exhaustive capillary-based sequencing of rearranged TCRα and TCRβ genes expressed in limited subsets of the repertoire [77]. Nowadays, it is possible to use next generation sequencing (NGS) platforms, such as the Illumina® systems, which present several advantages, including ultra-high throughput, scalability, and speed. Biosoftwares and sophisticated machine learning algorithms for comprehensive analysis of the high-dimensional data generated by these platforms are being developed [81-84].

Importantly, some studies suggested that TCR β diversity is relatively stable from early human adulthood until the age of 70 years [85, 86], while others suggested a roughly linear decrease with age [87]. So, age and other factors, such as peripheral selection and B cell compartment, may influence the TCR repertoire and need to be taken in consideration while interpreting the TCR repertoire diversity as a surrogate of thymic function [88, 89].

3.3 – Flow cytometry analysis

Thymic function may be determined by quantifying the number of T cells exported by the thymus, which are called recent thymic emigrants (RTE). RTE consist of a phenotypically and functionally subset of naïve T cells, present in the peripheral blood. They are enriched in TREC content, and may be identified by the expression of platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31), detected by flow cytometry (Figure 16) [90].

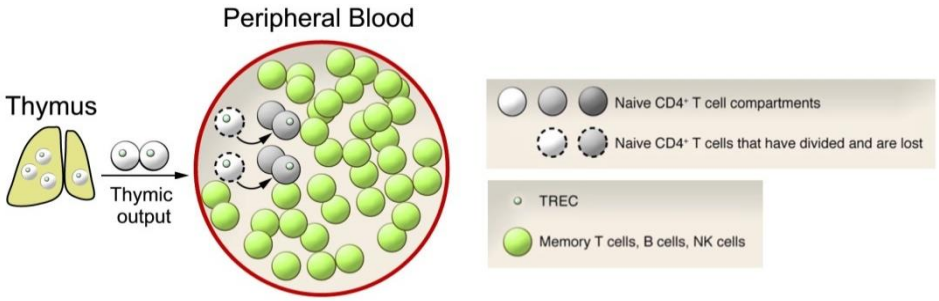

Figure 16 – Thymic output, recent thymic emigrants and other T cell subsets.
Recent thymic emigrants (RTE), a subset of naïve T cells, are represented in light grey, containing T cell receptor excision circles (TREC). Adapted from [91].

Although RTE contain a higher number of TRECs than the other naïve T cells, it is important to notice that: 1) not all TREC$^+$ cells are RTEs; and 2) not all RTE are TREC$^+$, due to the nonreplicative nature of TREC.

Identifying CD31$^+$ cells among naïve CD4$^+$ T cells (CD45RA$^+$CD4$^+$CD3$^+$ lymphocytes) is a canonical strategy for analyzing RTE, as this marker is highly expressed by cells recently exported from thymus and it is progressively lost during post-thymic maturation of naïve T cells, in humans [92]. Some limitations of this strategy include: 1) TCR-induced activation down-regulates CD31 expression [93]; 2) there is not a clear differentiation between RTE and naïve T cells generated by homeostatic proliferation [86, 94]; 3) thymectomised individuals maintain a population of CD31$^+$ T cells in circulation [95]; and 4) at least in the context of HIV infection, only CD4$^+$ RTE, and not CD8$^+$ RTE, are identified [96].

In an attempt to overcome some of these limitations, one may analyze exclusively $CD4^+$ T cells with high expression of CD31, which are associated with a higher TREC content [90]. Unfortunately, the definition of high expression is not consensual, and the traditional methods of manual gating are subjective and inherently inaccurate. It is very important to describe precisely the gating strategies used in data analysis to ensure reproducibility.

Ten years ago, a study has identified human RTE by the expression protein tyrosine kinase 7 (PTK7) in T cells [97]. More recently, Bains et al. combined a mathematical survival analysis approach with data from healthy and thymectomised humans, to understand the apparent persistence of populations of "veteran" $PTK7^+$ T cells in thymectomised individuals. The conclusions were that, despite the immature phenotype, $PTK7^+$ cells do not necessarily represent a population of RTE, and probably the average post-thymic age of $PTK7^+$ T cells will increase linearly with the age of the host [98].

4. Strategies to modulate thymic function

Thymic function is essential in the maintenance of a stable and balanced immune system, *i.e.* an immune system able to discriminate self- from non-self-antigens, efficient in defending the host against potential harmful agents, while limiting the extent of dysfunction caused by inflammation and avoiding immune responses against harmless commensal agents, particularly abundant in the gut and in the skin, or environmental factors. A healthy immune system needs to be unresponsive to certain stimuli that could elicit an immune response in specific conditions. This unresponsiveness or tolerance is of paramount importance, not only in the pathophysiology of autoimmune disorders, but also in the context of infection [99].

Manipulation and boost of thymic function could be useful in the treatment of all conditions characterized by immune dysfunction, from immunosenescence, autoimmune diseases and transplantation, to HIV infection and other immunodeficiencies. This section summarizes the available strategies to modulate and improve thymic function, and reviews

the main findings, both in the pre-clinical and clinical settings, for the development of new strategies (Table 6).

Table 6 – Strategies to modulate thymic function.

1. **Sex steroid ablation**
2. **Hormones and growth factors**
   - Growth hormone and insulin-like growth factor 1
   - Prolactin
   - Thyroid hormones
   - Leptin
   - Ghrelin
   - Corticotrophin-releasing hormone
   - Keratinocyte growth factor
   - Fibroblast growth factor 21
   - FMS-like tyrosine kinase 3 ligand
3. **Cytokines**
   - Interleukin (IL) -7, -2, -4, -22, -12
4. **Thymic transplantation**
5. **Intrathymic injection of thymocyte progenitors**
6. **Forkhead box N1**
7. **Micro RNAs**
8. **Nutritional changes**
9. **Natural compounds**

4.1 – Sex steroid ablation

Thymic involution starts after the first year of life, but it accelerates abruptly in puberty. This rapid involution was associated with the dramatic increase in circulating sex hormones that characterizes this developmental period, given that administration of sex steroids (androgens or estrogens) to young pre-pubertal mice results in thymic atrophy [100-102]. Mice models showed that surgical and chemical castration promotes a rapid and synchronous expansion of the different thymocyte subsets in the thymus, and that thymus regeneration is inhibited by re-administration of sex steroids [103-108]. Castration in mice results not only in alterations within the thymus, but also in an increase of the number of T cells in the periphery [108, 109].

Androgen and estrogen receptors are expressed in both thymocytes and thymic stromal cells, in rodents and humans [101, 110, 111]. Using transgenic mice, two studies suggested that functional androgen receptors in thymic epithelial cells (TEC or erc), and not in thymocytes, are required for androgen-mediated thymic atrophy [112, 113]. The mRNA levels of some factors known to promote thymic regeneration [IL-7, TGF-β, or fibroblast growth factor (Fgf)-7] were not significantly altered in the thymic stroma of mice after castration [109]. The precise mechanism responsible for the thymic regeneration promoted by sex steroid ablation remains unknown.

Sex steroid ablation may be achieved permanently through surgical removal of both testicles (bilateral orchiectomy) or in a transient and reversible manner, using luteinizing hormone releasing hormone (LHRH) agonists, androgen receptor antagonists or androgen receptor degradation enhancers (Figure 17).

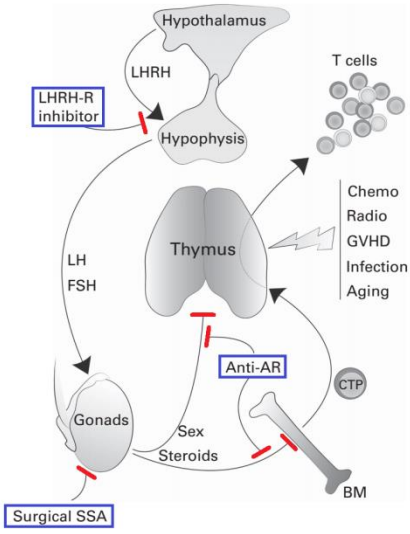

Figure 17 – Overview of the hypothalamic–pituitary–gonadal axis and its relation with thymic function.

Anti-AR, anti-androgen receptors; BM, bone marrow; Chemo, chemotherapy; CTP, circulating T cell progenitors; GVHD, graft-versus-host disease; LHRH-R, luteinizing hormone-releasing hormone receptor; Radio, radiotherapy; SSA, sex steroid ablation. Adapted from [114].

In humans, bilateral orchiectomy presents severe systematic and psychologic effects, being performed almost exclusively in cancer patients with no other treatment option.

Although chemotherapy, often in conjunction with radiotherapy, is the common "standard of care" to treat prostate and breast cancer patients, LHRH-agonists and anti-androgen receptors are also used (*e.g.* Lupron®, ASC-J9®, Goserelin). It has been shown that patients with prostate cancer on LHRH-agonists treatment present a significant increase in T cell numbers and in naïve $CD4^+TREC^+$ cells [109]. Also, administration of goserelin before allo- or auto-HSCT resulted in a significant enhancement of naïve $TREC^+$ T cells [115]. These studies demonstrate the efficacy of sex steroid ablation in promoting thymic regeneration, although a careful risk-benefit analysis needs to be performed to apply this strategy to patients with compromised thymic function.

4.2 – Hormones and growth factors

Besides the androgens and estrogens, thymic function is also influenced by other hormones. For example, thymocyte proliferation and migration are enhanced by growth hormone (also called somatotropin) and prolactin, whereas apoptosis of these developing cells is heightened by glucocorticoids [116]. Furthermore, thymic epithelial cells also secrete "self-hormones" [thymosins, thymopoietin (also known as thymopentin), thymulin and thymic humoral factor (THF)] and "atopic" hormones, such as oxytocin, melatonin or insulin [117-120], underlining the interplay between the thymus and the endocrine system. Hormone-based therapies are being developed as a strategy to enhance thymic function during aging and in immunodeficiency disorders.

- **Growth hormone (GH) and insulin-like growth factor 1 (IGF-1)**

GH, also known as somatotropin, is a hormone produced by the pituitary gland (hypophysis) with anabolic effects. It promotes cell growth, division, and regeneration, and it is involved in glucose and lipids metabolism. A large proportion of GH effects in the body are mediated by the production and release of IGF-1.

Thymocytes are able to produce and secrete GH [121]. Thymic microenvironment is modulated by GH through an increase in thymulin, chemokines, and cytokines levels

[121]. Mice models showed that thymocytes migrate towards sources of stromal cell-derived factor 1 (also called CXC chemokine ligand 12) and laminins, which deposition is enhanced by GH. So, GH also modulates thymocyte development *in vivo* through a combined action of these proteins [122].

IGF-1 is expressed in the thymic stroma, in particular by thymic epithelial cells and fibroblasts, while the IGF-1 receptor is expressed not only by the stromal component but also by the thymocytes [123, 124]. The IGF-1 receptor is expressed in some types of thymic tumors [125]. One study showed that the expression of this receptor in the tumor was associated with worse progression-free survival [126].

Transgenic mice overexpressing GH, and mice and humans treated with recombinant GH have enlarged thymi and increased TCR diversity [127, 128]. Antibodies against GH, IGF-1 or IGF-1 receptor abrogated the positive effects of GH in thymus [121, 123]. Mice treated with IGF-1 showed increased thymopoiesis reflected by higher frequency of RTE [124]. Additionally, administration of IGF-1 to cats infected feline immunodeficiency virus resulted in thymic cortical regeneration and reduction of inflammation [129].

Recombinant human GH (rhGH) is commonly used in the treatment of pediatric growth disorders and of adult GH deficiency. The interruption of rhGH treatment in adults with GH deficiency resulted in a decrease in thymic output and in intrathymic cell proliferation [127]. Clinical trials tested the efficacy of rhGH to enhance thymic recovery and immune reconstitution and to revert visceral adipose tissue (VAT) accumulation in HIV-infected patients with promising results [130-132]. More recently, the safety and efficacy of GH in improving immune reconstitution post unrelated cord blood transplant was assessed in a phase 1 clinical trial, but the results are not available yet (Clinical Trial-NCT00737113).

Recombinant human IGF-1 (rhIGF-1) is used to treat patients with severe primary IGF-1 deficiency (Laron syndrome) and patients unresponsive to endogenous or administered GH (*e.g.* patients with GH receptor mutations or GH-inactivating antibodies) [133]. The effects of rhIGF-1 on the thymus of these patients have not been reported. Due to the risk of

severe adverse side effects (*e.g.* hypoglycemia), rhIGF-1 administration should be limited to cases with no other therapeutic options [133].

- **Prolactin**

Prolactin is a pituitary hormone, best known for being responsible for milk production during breast-feeding. Numerous extrapituitary tissues and cells express prolactin (*e.g.* mammary gland gonads, skin, adipocytes, endothelial cells, and immune cells) [134]. This broad expression explains the involvement of prolactin in many different processes such as reproduction, metabolism, platelet aggregation, angiogenesis, hematopoiesis and behavior. Prolactin may also be classified as a growth factor, given that it shares several features with hematopoietic growth factors, including multiple sites of synthesis, receptors structure and distribution, and signal transduction pathways that promote cell survival and growth [134].

In vitro studies showed decreased proliferation and the production of IL-2 and IL-4 by human T cells knockdown for the prolactin receptor [135]. Prolactin receptors are expressed by thymic epithelial cells in rats, and administration of prolactin to hypophysectomised rats resulted with increased thymic weight [136, 137]. However, both prolactin knockout and prolactin receptor knockout mice showed no significant thymic alterations [138]. These apparently contradictory results may be explained by the existence of compensatory mechanisms. More studies are needed to determine the impact of prolactin on thymic function in physiological conditions, and to explore its potential as a therapeutic target.

- **Thyroid hormones**

Thyroid hormones include thyroxine (T4), the most abundant in peripheral blood, and triiodothyronine (T3), the most potent. T3 nuclear receptors are expressed by thymocytes and thymic epithelial cells [139]. It is unknown if thymic cells are able to produce thyroid hormones, but expression of thyrotropin (thyroid stimulating hormone, TSH) was detected

by immunohistochemistry in thymic epithelial cells, in the subcapsular region and cortex of human thymi [140]. Differential expression of TSH receptor was also detected in human thymi by gene expression profiling, which suggests a potential role for the TSH receptor during T cell differentiation [141]. Additionally, thyrotropin-releasing hormone (thyroliberin, TRH), which stimulates the synthesis and release of TSH, is transcribed in the thymus of rats [142].

Some patients with Grave's disease, a form of hyperthyroidism, present thymic hyperplasia, and the normalization of thyroid hormones levels led to thymic hyperplasia resolution [143, 144]. Consistent with this, injection of T3 in mice resulted in an increase in the thymic volume and cellularity, and in higher proliferation of thymocytes [145]. Moreover, mice lacking functional TSH receptor have lower numbers of DP and SP thymocytes than wild-type mice, which suggests that TSH functions as a growth factor for thymocytes [141]. More studies are needed to completely unravel the importance of the TRH – TSH – T3/T4 cascade in regulation of thymic function.

- **Leptin**

Leptin, a cytokine-like hormone, mainly produced by adipocytes, is structurally similar to IL-6 and it is involved in the control of appetite, metabolism, and T cell function. Leptin promotes the production of pro-inflammatory cytokines, enhances $CD4^+$ T cell proliferation, and prevents $CD4^+$ T cell apoptosis [146-148].

Leptin administration augmented thymopoiesis in leptin-deficient obese mice (ob/ob mice), but not in wild-type mice [149]. In a mice model of stress, which induces thymic atrophy, leptin was able to prevent thymic weight loss [149].

Ex vivo studies showed that isolated human Treg cells express high amounts of both leptin and the leptin receptor (ObR), and that administration of leptin can act as a negative signal for Treg proliferation [150]. Leptin-deficient mice have increased numbers of Treg, which can be reduced by administration of leptin, suggesting that the leptin pathway acts as a negative signal Treg proliferation [150]. Given the importance of Treg to inhibit

autoimmunity and inflammation, studies in nonhuman primates are needed to infer the safety of leptin administration.

- **Ghrelin**

Ghrelin, also known as lenomorelin, is a peptide hormone produced by cells in the gastrointestinal tract, particularly in the stomach. Together with leptin, ghrelin is responsible for the control of appetite. Ghrelin also stimulates the production of GH and it is involved in the regulation of the immune system, by inhibiting inflammatory responses [151].

Ghrelin and ghrelin receptor are expressed in the thymus, and their expression diminishes with progressive aging [152]. Ghrelin-deficient mice display reduced thymopoiesis, contraction of stem cells and major perturbations in the TCR repertoire of peripheral T cells [153]. Administration of ghrelin to old mice reverted thymic involution, restoring thymic architecture, increasing the number of thymocytes and RTEs, and improving TCR diversity of peripheral T cell subsets [153]. These studies suggest that ghrelin and its receptor play a role in thymic biology and suggest a possible therapeutic benefit of harnessing this pathway in immunocompromised patients.

- **Corticotrophin-releasing hormone (CRH)**

CRH is a hormone produced in the hypothalamus, and it stimulates the secretion of the adrenocorticotropic hormone (ACTH) by the pituitary gland, being an important component of the hypothalamic-pituitary-adrenal axis in stress conditions.

Stress is known to induce thymic involution [154]. In mice exposed to stress, CRH gene expression in the hypothalamus has been reported to be associated with acute thymic involution [155]. Furthermore, thymic involution induced by lipopolysaccharide was reduced by intracerebroventricular administration of recombinant CRH (rCRH) in mice. However, no changes in thymopoiesis were seen with intraventricular administration of

rCRH in control animals. Future research may help to unravel the thymorestorative or thymoprotective nature of CRH.

- **Keratinocyte growth factor (KGF)**

KGF, the seventh member of the fibroblast growth factor family (fibroblast growth factor-7, FGF-7), is a secreted signaling molecule and it is a potent epithelial cell mitogen [156-158]. KGF receptor is expressed on thymic epithelial cells and induces the proliferation of thymic epithelial cell progenitors and mature epithelial cells in fetal thymic organ cultures [159]. Subcutaneous administration of KGF enhanced thymopoeisis in old mice [160]. Administration of KGF and pifithrin, a small molecule that transiently inhibits p53, improved thymic epithelial cell recovery and enhanced T cell reconstitution after murine HSCT [161]. In rhesus macaques submitted to autologous HSCT, KGF treatment led to increased frequencies of naïve T cells in their lymph nodes and of RTE in circulation compared with controls [162]. Transient p53 inhibition combined with KGF administration promote rapid and durable thymic and peripheral T cell recovery after HSCT.

Palifermin is a recombinant form of KGF used in the treatment of cancer patients receiving chemo or radiotherapy, to help to repair their mucosal barrier. A randomised double blind placebo controlled clinical trial was performed to verify if palifermin improves the immune recovery of HIV-infected patients on ART. No significant differences were found between palifermin-treated patients and controls in either the numbers of RTEs, naïve T cell or thymic size [163]. Further studies, testing different doses and schedules, may lead to the development of strategies to use recombinant KGF to improve thymic function in HIV infection or other diseases.

- **Fibroblast growth factor 21 (FGF-21)**

FGF-21 is a growth factor secreted in the liver, with mitogenic activities. It plays a critical role in the regulation of systemic glucose and lipid metabolism [164].

In mice, FGF-21 is expressed in the liver, and also in thymus, at lower levels [165]. Recently, it was reported that overexpression of FGF-21 improves the number of cortical thymic epithelial cells and thymocyte progenitors, and reduces aging-related thymic involution in mice [166]. Young FGF-21-deficient mice show a significant reduction in the percentage of SP thymocytes without obvious alteration in thymic epithelial cells [167]. Old FGF-21-deficient mice present accentuated thymic involution compared to wild-type mice. FGF-21 deficiency in mice increases lethality post-irradiation and delays the reconstitution of thymus [166].

FGF-21 has been attracting attention as a drug candidate for the treatment of obesity and metabolic syndrome, but its therapeutic potential may be expanded to pathologic conditions characterized by compromised thymic function.

- **FMS-like tyrosine kinase 3 (FLT3) ligand**

FLT3 ligand acts like a growth factor by binding to the functional group of FLT3. FLT3 is a cell surface receptor expressed by early progenitors with lymphoid and myeloid potential (EPLM) in the bone marrow. FLT3 is involved in the pathogenesis of acute myeloid leukemia, aplastic anemia, brain cancer and malaria.

In mice thymi, a fraction of hematopoietic early progenitors expresses FLT3, while FLT3 ligand is expressed by thymic stromal cells [168]. FLT3-deficient mice express higher levels of FLT3 ligand around the thymic blood vessels than wild-type mice. Interaction between FLT3-expressing thymic cells and the FLT3 ligand is important for the physiological maintenance of early T cell development [169, 170].

In mice subjected to bone marrow transplantation, treatment with FLT3 ligand enhanced thymic function, as reflected by increased thymic cellularity and number of TRECs. On the other hand, administration of FLT3 ligand to resulted in enhanced peripheral expansion in athymic mice subjected to T cell depletion, showing that FLT3 ligand acts in T cell homeostasis via thymic-dependent and thymic-independent pathways [171].

Daily injection of human FLT3 ligand in mice resulted in a dramatic increase in the number of EPLM in the bone marrow, and also in the number the dendritic cells in several tissues, including the bone marrow and the thymus [172, 173]. Given that dendritic cells participate in the intrathymic negative selection of auto-reactive T cells contributing to central immune tolerance, one may say that targeting FLT3 and/or FLT3 ligand is a potential strategy to manipulate not only the "quantity" but also the "quality" of thymopoiesis.

4.3 – Cytokines

Cytokines are small proteins involved in cell signaling. They play a major role in the control of immune responses, being secreted mainly by immune cells, but also by epithelial cells and fibroblasts. Although the function of some cytokines in the thymus is not completely understood, it is consensual that cytokines secreted by thymic epithelial cells are essential to the development of T cells (reviewed in [174]). Administration of cytokines is one possible strategy to modulate thymic function and T cell differentiation, and several cytokines have been tested in pre-clinical and clinical studies.

- **Interleukin-7 (IL-7)**

IL-7 is a type 1 short chain cytokine produced by the stromal cells of primary and secondary lymphoid organs, playing a role at multiple stages of T and B cells development. The interaction between IL-7 and its receptor, composed by the two subunits IL-7 receptor-α (CD127) and common-γ chain receptor (CD132), promotes survival and proliferation of single-positive thymocytes before their exportation from the thymus [175, 176]. IL-7 also contributes to the diversity of TCRs generated in the thymus [177], and it is essential for the homeostasis of peripheral T cells. Although several cytokines are able to enhance the immune recovery in certain conditions, no other compares to IL-7 in terms of potency and breadth of effects [178].

The addition of IL-7 to neonatal human thymic cultures increased the number of immature and mature T cells [179]. Treatment of young mice with anti-IL-7 led to severe thymic atrophy [180], while treatment of old mice with IL-7 resulted in restoration of thymic output and improvement in peripheral T cell function [175, 181]. In healthy cynomolgus monkeys and rhesus macaques infected by simian immunodeficiency virus (SIV), IL-7 administration caused an increase in both $CD4^+$ and $CD8^+$ peripheral T cells, and a decrease in the blood TREC levels, probably due to dilution by cell proliferation [182]. Treatment with recombinant simian IL-7 was then tested in old rhesus macaques prior to vaccination with influenza, and it led to higher thymic output and increased haemagglutinin titers compared to controls [183].

Based on the *in vitro* and *in vivo* findings, clinical trials phase I and phase II were conducted to test the benefit of recombinant IL-7 treatment in lymphopenic and non-lymphopenic patients with cancer [184-186]. Recombinant IL-7 was also administered to patients infected by HIV, hepatitis C or hepatitis B virus, and after allogeneic HSCT (reviewed in [178]). These trials showed an increase in $CD4^+$ and $CD8^+$ peripheral T cells associated with recombinant IL-7 administration, with few severe side-effects [185]. Unfortunately, in HIV-infected patients, IL-7 therapy amplified the HIV reservoir by increasing the number of T cells harboring integrated HIV DNA, limiting the use of this cytokine in the context of HIV infection [187, 188].

Despite the importance of IL-7 during thymopoiesis, the results of the clinical trials underlined the effects of IL-7 in the periphery and not within the thymus. In fact, administration of IL-7 was associated with increased TCR repertoire diversification mainly as a result of the preferential post-thymic proliferation of naive T cells (Figure 18).

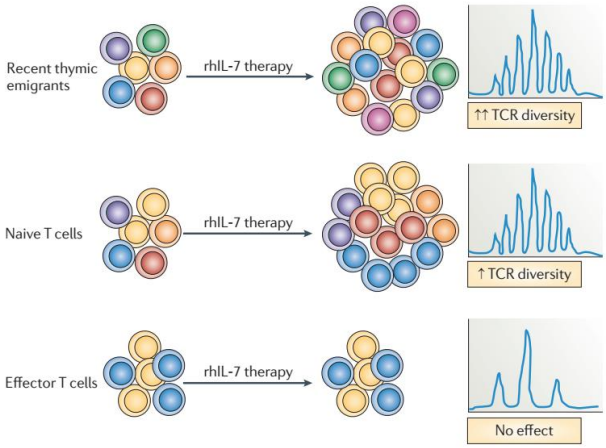

Figure 18 – Diversification of TCR repertoire after therapy with recombinant IL-7 depends mostly on expansion of recent thymic emigrants and other naïve T cells.
rhIL-7, recombinant human interleukin-7; TCR, T cell receptor. Reproduced from [178].

- **Interleukin-2 (IL-2)**

IL-2 is particularly important for the production, function and homeostasis of Treg, but it is also involved in the differentiation of other T cells [189, 190]. Administration of IL-2 led to significant increases of Treg in patients with cancer [191, 192]. In HIV-infected patients, intermittent administration of IL-2 increased the number of $FOXP3^+$ $CD4^+$ T cells and had minimal impact on $CD8^+$ T cells [193, 194].

- **Interleukin-4 (IL-4)**

IL-4, initially called B cell stimulatory factor 1, contributes to activation of B cells and their differentiation into plasma cells, as well as to T cell proliferation [195, 196]. It is produced in large amounts by invariant natural killer T (iNKT) cells during infection. It is also produced by iNKT cells in the thymic medulla in physiological conditions [197, 198]. A recent study showed that a subset of highly-specialized thymic epithelial cells, similar to the tuft cells found at mucosal barriers, mediates the production of IL-4 by iNKT cells, interfering with T cell differentiation [199].

IL-4 also inhibits the proliferation of human renal cell carcinoma cells lines [200]. For that reason, recombinant human IL-4 was administered to patients with kidney cancer by daily

subcutaneous injection, at a dose of 5 mcg/kg, for 28 days. Toxicity in this dose and schedule was significant, with nausea, vomiting, and diarrhea as the most common side effects [201].

- **Interleukin-22 (IL-22)**

IL-22 was discovered in 2000, and it was initially called named IL-10-related T cell–derived inducible factor (IL-TIF) [202]. It is mainly produced by Th17 cells and innate lymphoid cells (ILCs), but it is also produced by γδ T cells and NKT cells [203]. The primary targets of IL-22 are epithelial and stromal cells, modulating tissue responses during inflammation and promoting cell proliferation (reviewed in [204]).

Intrathymic levels of IL-22 increase in mice after thymic insult, and IL-22-deficient mice show limited thymic regeneration after thymic damage compared to wild-type mice [205]. However, the impact of IL-22 in thymic regeneration and immune recovery requires further research.

- **Interleukin-12 (IL-12)**

IL-12 belongs to the IL-12 family, which is comprised by three other elements: IL-23, IL-27 and IL-35. IL-12 is primarily produced by antigen presenting cells (APCs) and targets T and NK cells. It plays an important role in the defense against intracellular pathogens, but it also is involved in the genesis of some immune disorders, such as multiple sclerosis, Crohn's disease, allergy and asthma [206]. IL-12 also presents anti-angiogenic effects, which seem to be mediated by lymphocyte-endothelial cross talk [207].

Aged IL-12-deficient mice exhibited accelerated thymic involution compared with wild-type mice. This involution was associated with a higher number of double negative thymocytes, degeneration of extracellular matrix and blood vessels and increase apoptosis in aged mice, but not in young mice. IL-12 exposure did not enhance the thymocyte proliferation *in vitro*. However, IL-12 with IL-7 or IL-2 administration *in vivo* induced intense thymocyte proliferation, both in aged IL-12-deficient and aged wild-type mice [208].

Recombinant IL-12 was tested in patients with cancer and infection by *Mycobacterium tuberculosis*, however the toxicity associated with systemic administration of IL-12 limits the doses tolerated by patients. Effective and less toxic alternatives are being explored [209].

4.4 – Thymic transplantation

Transplantation of allogeneic thymic tissue has been successful in treating children born without a thymus (*e.g.* FOXN1 deficiency associated with severe combined immunodeficiency; DiGeorge syndrome). Thymic transplantation results in diverse and functional T cell production with activation of B cells. A possible complication of this procedure is the occurrence of autoimmune diseases, specially involving the thyroid [210-212].

Experiments in swine models showed that transplantation of aged/involuted thymic tissue into juvenile, thymectomized animals led to regeneration of the transplanted tissue, suggesting that extrinsic, rather than intrinsic factors to the thymus play a predominant role in thymic involution [213]. Also in swines, transplantation of thymic tissue and renal allografts from a single donor to thymectomized animals induced tolerance across fully MHC-mismatched barriers [214]. Regarding induction of graft tolerance, in a rat model of diabetes mellitus, intrathymic injection of pancreatic islets induced donor-specific immune tolerance, which allowed permitted the survival of a second islet allograft transplanted to an extrathymic site [215]. These studies may help to optimize thymic transplantation procedures.

The effect of combining thymic transplantation with ART was studied in a clinical trial [216]. Eight patients were randomized into thymic transplantation and control arms. In the four patients that received postnatal cultured allogeneic thymic transplants, the grafts were rejected within 2 months. This clinical trial showed no beneficial effects of thymic transplantation on the immune recovery of HIV-infected patients, but there were several study limitations, including the absence of HLA matching and a small sample size.

Bio-engineering strategies are being developed to improve the production of T cells in artificial thymic organoids [217]. One hurdle in thymic culture systems is the loss of thymic epithelial cells to support the T cell development [218]. One solution that is being studied is to revert adult mouse thymic epithelial cells to a precursor phenotype [219]. Another strategy being explored is the use of decellularized thymus scaffolds [217]. Recently, Seet et al. described a serum-free, artificial *in vitro* system that recapitulates thymopoiesis, efficiently supporting T cell differentiation from hematopoietic stem and progenitor cells [220]. It is possible that transplantation of "artificial thymi" become a reality in the future.

4.5 – Intrathymic injection of thymocyte progenitors

Intrathymic injection of thymocyte progenitors has been investigated as method for thymic regeneration and to accelerate the immune recovery following HSCT. Murine models showed that, in the absence of myeloablative conditioning, thymopoiesis by transplanted thymocyte is sustained after intrathymic but not intravenous administration [221]. After irradiation, association of this procedure with KGF and IL-7 showed synergistic effects, with increased thymic cellularity and increased immature T cell production [210].

4.6 - Forkhead box N1 (FOXN1)

FOXN1, a transcription factor, is master regulator of epithelial cell differentiation. Accordingly, children with a homozygous FOXN1 mutation present alopecia, nail dystrophy and athymia. The absence of thymus leads to severe T cell immunodeficiency in these children, with severe, recurrent, life-threatening infections occurring in the first months of life [222]. These patients present aberrant T cells in circulation, with an activated memory-like phenotype, impaired function, an oligoclonal repertoire and with overrepresentation of $CD4^{-}CD8^{-}$ (DN) T cells and of $FOXP3^{+}$ regulatory-like T cells [223]. The origin of these cells may be a dysfunctional thymic rudiment or extrathymic tissues, such as lymph nodes, tonsils or gut-associated lymphoid tissue [223-225].

Using static and inducible genetic model systems and chromatin studies, it has been shown that Foxn1 regulates the expression of genes involved in antigen processing and thymocyte selection [226]. A better understanding FOXN1 expression in thymic epithelial cells and its functions during T cell differentiation may be useful to develop strategies of thymic regeneration or modulation centered in this transcription factor.

4.7 – Micro RNAs (miRNAs)

Micro RNAs (miRNAs) are approximately 22 nucleotide-long non-coding RNA molecules that control the cellular RNA machinery, being essential in maintaining hematopoiesis and a balanced immune system. miRNAs are transcribed mainly by RNA polymerase II, which produces a primary transcript containing the mature miRNA sequence and a varying amount of flanking region. Then, a Drosha and DGCR8 complex cleaves that primary RNA into a precursor miRNA that is exported from the nucleus, and then processed by the Dicer nuclease in the cytoplasm [227].

In mice, Dicer deletion and miR-29a, a cluster of the Dicer dependent microRNA network, deletion resulted in the degeneration of thymic architecture and function, inducing heightened sensitivity of the thymic epithelium to involution signals during infection [228]. Furthermore, mice with DGCR8-deficient thymic epithelial cells present alterations in thymic architecture and exhibit a dramatic loss of thymic cellularity. These mice have a severe reduction of thymic epithelial cells expressing autoimmune regulator (AIRE) transcription factor, with loss of central immune tolerance [229]. Also in mice, generation of T cells in a miRNA-free thymic epithelial cells environment elicited autoimmunity, possibly due to the reduction of self-antigens expression in the thymic medulla associated with AIRE expression reduction, leading to export of autoreactive T cells from the thymus [230].

New therapeutic interventions may be developed based on the major role of miRNAs in thymic epithelium function and maintenance.

4.8 – Nutritional changes

Nutrition is a key player in human health. It not only impacts the quality of life, but also the life expectancy. Nutrition changes influence the function of endocrine glands, the nervous system, the immune system, and probably all the other organ systems [231, 232]. Caloric restriction is able to increase the lifespan of certain animal species, and it can enhance thymopoiesis and prevent age-related thymic involution in mice, restoring diversity of TCR repertoire diversity [233, 234]. On the contrary, thymic involution, increased thymocyte apoptosis, decreased TREC levels and reduced TCR diversity are seen in obese mice [235]. The effects of caloric restriction in humans are not clear. Caloric restriction, through intermittent fasting or restricting feeding, may be useful in the treatment of obesity. In non-obese healthy individuals, caloric restriction resulted in weight loss and significantly reduced thyroid axis activity and reactive oxygen species, but its effects on thymopoiesis were not reported [236]. The negative effects of caloric restriction, or other nutritional changes, need to be carefully evaluated before applying this strategy to immuno-compromised patients.

4.9 – Natural compounds

Some natural compounds limit thymic involution induced during infection. For example, betulinic acid, found in different plant species, was administered to mice increasing the number of thymocytes and the thymic weight, inducing maturation of DP thymocytes to SP thymocytes and reducing the apoptosis of thymocytes induced by dexamethasone [237-239]. Curcumin, a yellow pigment found in *Curcuma longa* plants, inhibited deltamethrin-induced apoptosis in mouse thymocyte suspensions, and reduced tumor-induced thymocyte apoptosis in mouse models of cancer, showing thymic protective effects in both *in vitro* and *in vivo* studies [240, 241]. Interestingly in the context of this thesis, some studies suggested that curcumin has antiviral properties against HIV, by inhibiting the viral protease or integrase. However, a clinical trial found no evidence that curcumin reduced viral load or increased $CD4^+$ T cell counts in HIV-infected patients [242-245].

5. Thymic function and HIV infection

The major hallmark of HIV infection is the depletion of $CD4^+$ T cells, which are produced in the thymus. Therefore, the involvement of thymus in HIV pathogenesis was rapidly foreseen and, soon after the report of the first clinical cases of HIV infection (between 1983 and 1984), 11 HIV-infected patients received thymic transplants, in an attempt to avoid the rapid fatal course of the disease at that time [246]. Unfortunately, 10 of these patients died soon after transplantation, while 1 patient was lost to follow up [246]. Shortly after, two clinical trials tried to improve the outcome of patients by the administration of calf thymus extracts, thymosins or thymostimulin [247-250]. Again, this strategy was unsuccessful. Simultaneously to these experimental therapies, researchers were intensively investigating the effects of HIV infection in the thymus. Infection of human thymocytes and thymic epithelial cells by HIV was reported in *post mortem* studies, *in vitro* studies using thymic epithelial cultures or human fetal-thymus organ cultures (HF-TOC), and *in vivo* studies using animal models such as the SCID-hu mice [251, 252].

In this section, the thymic alterations caused by HIV and the influence of the thymus on the clinical evolution of HIV infection, before and during antiretroviral therapy, will be reviewed (Figure 19).

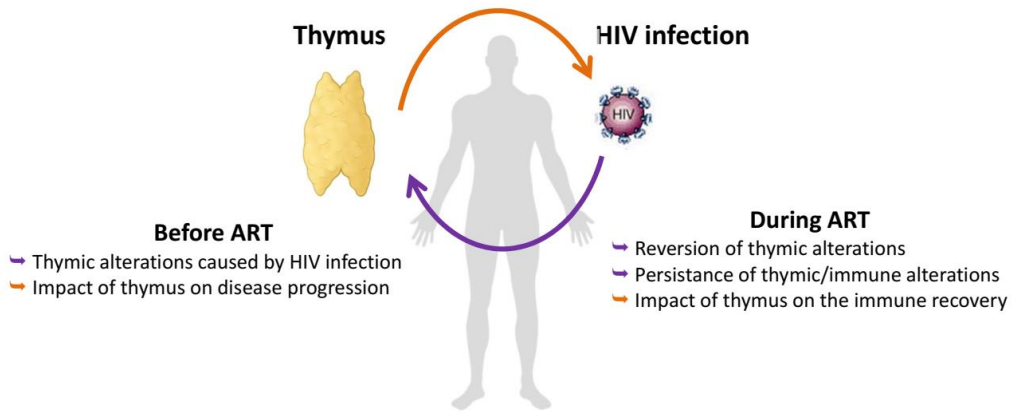

Figure 19 – Interplay between thymus and HIV infection.

5.1 – Thymus and HIV pathogenesis

Alterations in the thymus associated with HIV infection were evaluated by histology, imaging techniques, determination of the TREC levels and the sj/β TREC ratio; analysis of the TCR repertoire diversity; and evaluation of RTE by flow cytometry. As thymus suffers a natural process of involution during aging, it is important to compare thymic alterations caused by HIV both in children and in adults.

Changes in the histology of thymi from HIV-infected adults include: marked involution with disruption of thymic architecture, depletion of thymocytes and epithelial cells, loss of the normal corticomedullary demarcation, patchy fibrosis, reduction/disappearance of the Hassal's corpuscles and alterations in the epithelial cells, which become spindled with pyknotic nuclei [253]. In some instances thymitis may occur, with infiltration of the thymus by plasmacytic cells. These changes are similar to those described in HIV-infected children, except for a predominance of plasmacytic infiltrates in adults [254-257]. Some authors attribute these changes to an accelerated aging process caused by HIV [258, 259]. Depletion of thymic cells is mainly caused by three distinct mechanisms: 1) reduction of progenitor cells; 2) death of HIV-infected cells; and 3) death of bystander cells mediated by viral and host pathogenic factors [260, 261].

Most studies in HIV-infected patients in which thymic volume and index were assessed using imaging techniques compared different time points or explored the relation of these parameters with the immune recovery (Tables 7a and 7b). There is not so much data comparing untreated HIV-infected patients to healthy individuals. Untreated HIV-infected children present smaller thymi and lower thymic indices compared to aged-matched healthy controls [262-264]. Some studies reported no differences in thymic index between HIV-infected adults and healthy controls, although thymic index correlated with $CD4^+$ T cell counts in HIV patients [50, 53].

Table 7a – Studies in HIV-infected individuals with thymic evaluation by CT scan.

Authors, Publication Year	Results focusing on CT scan data	Ref
McCune et al., 1998	Some HIV-infected patients present abundant thymic tissue.	[50]
Smith et al., 2000	Abundant thymic tissue is associated with higher naïve CD4$^+$ T cell counts.	[265]
Tei xeira et al., 2001	Poor immune response to ART may be caused by thymic impairment.	[266]
Napolitano et al., 2002	Growth hormone treatment is associated with an increase in thymic mass.	[267]
Franco et al.,2002	Patients with low TRECs levels at ART initiation present significant increases in thymic volume and in TRECs number, contrary to patients with high TRECs levels.	[51]
Kolte et al., 2002	Larger and abundant thymi are associated with higher CD4$^+$ T cell counts, higher TREC frequencies and broader immunologic repertoires.	[53]
Delgado et al., 2002	Thymic volume correlates negatively with age and positively with TRECs and naïve CD4 and CD8 T cells in adults with virologic treatment failure.	[268]
Mussini et al., 2002	Some patients present high immune recovery independently of thymic changes.	[269]
De la Rosa et al., 2002	Baseline thymic volume is a predictor of early CD4 cell repopulation.	[270]
Rubio et al., 2002	The thymic volume increase is related to the rise of both total and naïve CD4$^+$ cell counts.	[271]
Ruiz-Mateos et al., 2003	In MVA, only thymic volume at baseline was associated with CD4$^+$ T cell recovery.	[52]
Kalayjian et al., 2003	Reduced thymic volumes are associated with age and with HIV infection among younger, but not older, subjects.	[272]
Ruiz-Mateos et al., 2003	There is an inverse correlation between IL-7 levels and thymic volume at baseline; and a direct correlation between IL-7 levels at baseline and thymic volume change.	[273]
Manfredi et al., 2003	Thymic index increase is associated with higher CD4$^+$ T cell count changes.	[274]
Lu et al., 2003	Patients receiving interleukin-2 present increases in CD4$^+$ T cell counts but not in thymic size.	[275]
Ruiz-Mateos et al., 2004	Thymic volume at baseline is associated with the magnitude of short- and long-term CD4$^+$ T cell recovery.	[276]
Smith et al., 2004	Patients with thymic size above the median had a tendency for higher increases in naïve CD4 T cells compared to patients with thymic size below the median.	[277]
Vallejo et al., 2005	Increased thymic volume was associated with higher plasma viral load and greater CD4 count decline early after ART interruption.	[278]
Harris et al., 2005	Thymic size correlates with naive T cell numbers in patients and uninfected individuals, and with naïve T cell TREC frequencies in uninfected individuals.	[45]
Molina-Pinelo et al., 2005	Patients with complete response to ART present higher thymic volume at baseline.	[279]
Kalayjian et al., 2005	Thymic index at baseline correlated with early naïve CD4$^+$ T cell changes.	[280]
Fernandez et al., 2006	Thymic volume is correlated with both naive CD4$^+$ and naïve CD8$^+$ T cell numbers.	[281]
Lee et al., 2006	Long term pediatric survivors have thymic volume and activity approximating uninfected youths.	[282]
Monila-Pinelo et al., 2006	Thymic volume predicts CD4$^+$ T-cell decline after prolonged treatment interruption.	[283]
Fernandez et al., 2006	Immune activation correlates with poor CD4$^+$ T-cell recovery in patients with a small or undetectable thymus.	[284]
Soriano-Sarabia et al., 2007	Thymic volume is similar between HIV-mono- and HIV/HCV co-infected patients.	[285]
Hazra et al., 2007	There was no significant thymic volume change in children on ART.	[286]
Hansen et al., 2009	Growth hormone administration is associated with higher thymic index and area.	[287]
Tanaskovic et al., 2010	There was a marginal association between thymus volume and CD45RA$^-$CD31$^+$ cells proportion among CD4$^+$ T cells.	[96]
Smith et al., 2010	IGF-1 change was positively correlated with thymic index change in patients receiving rhGH and in the control group.	[288]
Plana et al., 2011	The administration of rGH increased thymic volume; changes in thymic volume were not associated with the levels of IL-7 or IGF-1.	[289]
Kalayjian et al., 2013	Correlation between immune activation reduction and naïve, memory and total CD4$^+$ T cell increase is attenuated in patients with small thymi.	[290]
Herasimtschuk et al., 2013	Patients receiving low-dose rhGH show thymic density increase.	[131]
Torres et al., 2014	Thymic volume did not change significantly after 48 weeks of ART.	[291]
Jacobson et al., 2014	Thymus size did not change significantly with plalifermin administration.	[163]
Aguilera-Sandoval et al., 2016	An interim analysis indicated futility to detect differences in thymic volume between patients and uninfected controls with the planned sample size.	[292]
Rosado-Sánchez et al., 2017	Baseline thymic volume was associated with the CD4/CD8 ratio normalization.	[293]

ART, antiretroviral therapy; CT scan, computed tomography scan; IGF-1, insulin-like growth factor 1; IL-7, interleukin-7; MVA, multivariate analysis; rhGH, recombinant human growth hormone; TRECs, T-cell receptor excision circles; US, ultrasonography.

Table 7b – Studies in HIV-infected individuals with thymic evaluation by other imaging techniques.

Authors, Publication Year	Technique	Results focusing on imaging data	Ref
Meyers et al., 1992	Radiography	Decreased thymus size on X-ray is a sign of pediatric HIV infection.	[262]
Meyers et al., 2001	Radiography	Early thymic involution is associated with rapidly progressive disease in HIV-infected children.	[264]
Chevalier et al., 2002	US	HIV-infected children present decreased muscle mass and early thymic involution.	[263]
Kolte et al., 2002	US, CT scan	CT scan is preferable to US when evaluating thymic volume during ART.	[49]
Vigano et al., 1999	MRI	Thymus volume correlates with the progression of vertical HIV infection.	[294]
Clerici et al., 2002	MRI	Thymic volume predicts long-term immune reconstitution in children on ART.	[295]
Miguez-Burbano et al., 2008	MRI	Thymic volume increase during ART relates to cognition and is affected by alcohol consumption.	[296]
Miguez-Burbano et al., 2009	MRI	Thymic volume changes during ART depend on the amount and the type of alcohol comsumption.	[297]
Hardy et al., 2004	^{18}F-FDG PET	Thymic activity assessed by ^{18}F-FDG uptake may increase during ART.	[298]
Lee et al., 2008	^{18}F-FDG PET	Mediastinal PET positivity after chemotherapy in a patient with HIV-associated Hodgkin's lymphoma was due to thymic reconstitution.	[299]
Tanaskovik et al., 2011	^{18}F-FDG PET	Poor immune responders present no evident thymic tissue.	[300]
Lelievre et al., 2012	^{18}F-FDG PET	Baseline thymic ^{18}F-FDG uptake correlated with TREC levels and naive CD4^{+} T cells numbers.	[301]

Note: Studies are ordered by imaging technique and year of publication.
^{18}F-FDG PET, positron emission tomography with ^{18}F-labeled fluoro-2-deoxyglucose; ART, antiretroviral therapy; CT scan, computed tomography scan; MRI, magnetic resonance imaging; US, ultrasonography.

Kalayjian *et al.* reported that untreated HIV-infected patients under 45-year-old had lower thymic indices than healthy controls, but above 45-year-old, patients and controls presented similar thymic indices [272]. It is possible that thymic involution induced by HIV infection is more evident in children and young adults, in which aging-related thymic involution has not yet occurred or is not so pronounced.

Harris *et al.* compared virologically suppressed HIV-infected adults to age-matched uninfected individuals and, contrary to the expected, found that the former and not the latter had higher thymic indices [45]. These results could be explained by a rebound effect just as it has been observed in adults after myeloablation and after correction of Cushing's disease [302-309].

Thymic output, estimated by the number of TRECs, is decreased in HIV-infected patients, with or without treatment, both children and adults. In general, TREC levels in PBMCs, in $CD4^+$ T cells and in $CD8^+$ T cells are lower in these patients compared with age-matched healthy controls [57, 310, 311]. However, one study reported differences in TREC levels in in purified $CD4^+$ and $CD8^+$ T cells between patients in different CDC stages (early-stage or stage 1, ≥ 500 $CD4^+$ T cells/μL; intermediate-stage or stage 2, 200-499 $CD4^+$ T cells/μL; and late-stage or stage 3, < 200 $CD4^+$ T cells/μL). While TREC levels in $CD4^+$ T cells were higher in patients in stage 1, and lower in patients in stage 3, compared to uninfected individuals, TREC levels in $CD8^+$ T cells were severely reduced in patients compared to uninfected individuals, regardless of the CDC stage [312]. Mathematical models suggested that a decreased production of cells by the thymus is the major contributing factor to the decline in TREC concentration within $CD4^+$ T cells, while in $CD8^+$ T cells both increased peripheral T cell division and decreased thymic production contribute to the decline in TRECs levels [313].

In a prospective cohort of hemophilic patients, TRECs were quantified in 131 patients with known HIV-1 seroconversion dates and TREC levels were significantly lower in patients that developed AIDS compared to patients in earlier stages of the infection, suggesting an association between lower baseline thymic output and faster progression of the disease [314].

Several studies showed that the sj/β TREC ratio, an estimate of the intrathymic cell proliferation described in section 3 of this chapter, is reduced in some but not all HIV-infected patients [69]. For example, Yang et al. showed that elite controllers (patients who control HIV replication without ART) with low $CD4^+$ T cell counts and non-elite-controller patients had lower sj/β TREC ratios than age-matched elite controllers with normal $CD4^+$ T cell counts or uninfected individuals [315]. Some authors reported a lower sj/β TREC ratio in HIV patients with rapidly progressive disease compared to slow progressors, while other authors found no differences [316, 317]. Additionally, HIV-infected patients with high sj/β

TREC ratio (> 10) presented higher nadir $CD4^+$ T cell counts and a slower $CD4^+$ T cell decline compared to those with a low ratio (< 10) [318].

The diversity of TCR repertoire may be considered a qualitative measure of thymic function. The TCR repertoire was investigated in HIV-infected individuals in many studies, using DNA hybridization, quantitative PCR or flow cytometry, showing decreased expression of certain V genes [319-322]. Also, spectratyping analysis studies showed skewing of CDR3 length distributions in certain V families [323-325]. Heather et al. used a novel quantitative high-throughput sequencing pipeline to characterize the TCR repertoires, and showed that HIV-infected individuals have highly altered TCR repertoires (Figure 20): some sequences are highly overrepresented and TCR diversity is significantly decreased; there is altered distribution of V gene usage, depletion of public TCR sequences, and disruption of TCR networks [326].

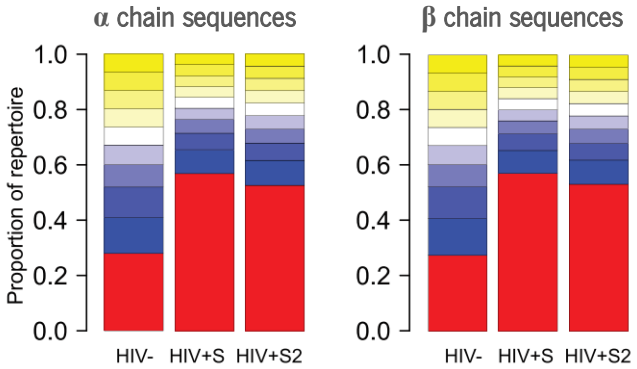

Figure 20 – TCR repertoire alterations in HIV infection before and during ART.
The plots show the proportion of alpha or beta sequences in the HIV-uninfected (HIV-) and HIV-infected patients prior to ART (HIV+S) or after ART initiation (HIV+S2). The percentile ranges are shown in decreasing order from the largest 10% (red) to the smallest 10% (yellow).
ART, antiretroviral therapy; TCR, T cell receptor. Reproduced from [406].

RTE may be identified among $CD4^+$ and $CD8^+$ T cells by the expression of CD31, in healthy individuals [96]. In HIV-infected patients, caution is needed while interpreting the expression of CD31 among naïve T cells as a surrogate of thymic output, since TREC levels and thymic size correlated with the percentage of $CD31^+$ cells among $CD4^+$ T cells,

but did not correlate with percentage of $CD31^+$ cells among $CD8^+$ T cells in these patients [96].

In children with perinatally acquired HIV infection, the percentage of RTE among $CD4^+$ T cells correlated with the $CD4^+$ T cell count, independently of the viral load [327]. This suggests that thymic output significantly impacts the disease severity in these patients, in accordance with a study published twenty years earlier, that compared infants with rapid progression to AIDS with infants with slow or no progression to AIDS [328]. Furthermore, RTE and non-RTE naïve T cells are similarly infected by HIV, and both contribute to the persistence of HIV reservoirs in patients on ART [329, 330].

Different HIV strains present different rates of replication and cytopathogenic effects within the thymus [331]. One explanation is the use of the co-receptor CXCR4 or CCR5 by the different strains, since CXCR4 is expressed by the majority of thymocytes, while CCR5 is only expressed by < 5 % of total thymocytes [332, 333]. Although HIV is able to infect both thymocytes and thymic epithelial cells, there is little evidence that thymus itself contributes to the persistence of HIV reservoirs during suppressive ART. In studies with non-human primates on ART, neither SIV DNA, replication-competent virus or multiply spliced HIV RNA were detected in the thymus, while Gag RNA was detected in the thymus in only one of six animals [334-336].

HIV infection in the thymus may impact the clinical course of the disease not only by contributing to $CD4^+$ T cell depletion, but also through the export of altered proportions of Treg, a subset of $CD4^+$ T cells with suppressive properties that express the X chromosome-encoded transcription factor *forkhead box P3* (FOXP3). It has been shown that HIV-infected patients present higher proportions but lower absolute counts of Treg than uninfected controls [337-341].

Summing up, thymopoiesis is suppressed by HIV infection, precluding regeneration of the peripheral T cell compartment [342]. However, the impact of thymic alterations in the

clinical course of the disease depends on multiple viral and host factors, such as CCDR5 or CXCR4 viral tropism, patient age and comorbidities, and, of course, the initiation of ART.

5.2 – Thymus, ART and the immune recovery of HIV-infected patients

In 1999, Haynes et al. reported three clinical cases of HIV patients previously thymectomized for myasthenia gravis treatment that started ART [343]. The clinical course of these patients was very heterogeneous: the first patient, with $CD4^+$ T cell of 99 cells/μL at ART initiation, progressed rapidly to AIDS; the second patient, with approximately 110 $CD4^+$ T cells/μL at ART initiation, progressed to AIDS seven years after seroconversion; and the third patient remained asymptomatic for more than seven years on ART, with $CD4^+$ T cell counts ranging from 285 to 572 cells/μL. Despite being thymectomized, the last two patients showed an increase in $CD4^+$ T cell counts upon ART initiation, raising the possibilities of production of T cells in vestigial thymic tissue or in extrathymic sites, or restoration of $CD4^+$ T cell numbers based on peripheral proliferation without production of new T cells. The last scenario might explain an increase in $CD4^+$ T cell number but leads to an impaired immune recovery (*e.g.* narrow and skewed TCR repertoire). The contribution of thymic output is essential for an adequate immune reconstitution not only in HIV-infected patients on ART, but also after chemotherapy or HSCT [282, 344-347].

Thymic alterations present in untreated HIV-infected patients may be reverted, at least partially, during ART (Figure 21). Several studies, in pediatric and adult patients, showed that ART is able to induce an increase in thymic volume and index [51, 265, 271, 273, 348]; TREC levels [51, 349-352]; sj/β TREC ratio [69, 317] and TCR repertoire diversity [353-357]. Regarding Treg, their proportion and absolute number tend to normalize during ART, although alterations within Treg subpopulations, namely a lower proportion of $CD31^+$ Treg and higher proportion of $Ki67^+$ Treg, may persist during up to 24 months of ART in patients that started ART with severe lymphopenia (< 200 $CD4^+$ T cells counts) [341].

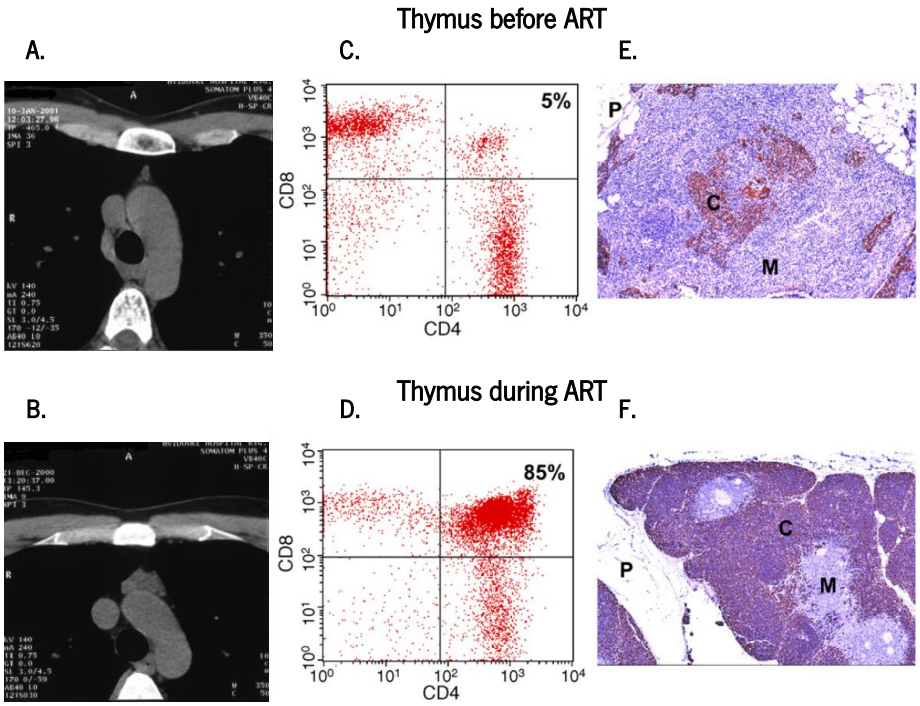

Figure 21 – Reversal of thymic changes associated with HIV infection during ART.
Representative images, from different patients, of chest computed tomography scans, CD4/CD8 FACS plots of isolated thymocytes and H&E stained tissue sections of thymus biopsies. **A)** Thymic index of 2. **B)** Thymic index of 4. **C)** Low percentage of double positive thymocytes. **D)** High percentage of double positive thymocytes. **E)** Altered thymic architecture, with infiltration of the thymic perivascular space with peripheral inflammatory cells. **F)** Thymic biopsy performed 6 months after ART initiation, showing normal thymic architecture. ART, antiretroviral therapy; C, thymic cortex; M, thymic medulla; P, thymic perivascular space. Adapted from [344] and [53].

The thymic "restorative" changes associated with ART do not occur in all patients. For example, some patients on ART have no thymic volume or index increase [274, 286, 291]; show no or only transient changes in TREC levels [273, 287, 358, 359]; present a stable sj/β TREC ratio [69, 360] or maintain the TCR repertoire alterations [360, 361]. The persistence of thymic alterations during ART restrains the immune recovery, particularly in patients in which the restoration of $CD4^+$ T cell homeostasis is more dependent on thymopoiesis.

In fact, several studies showed a correlation between thymic function surrogates and different parameters of immune recovery of HIV-infected patients. For example, thymic

volume of adult patients at ART initiation correlated positively with $CD4^+$ T cell counts and was independently associated with the normalization of the CD4/CD8 ratio during treatment [52, 270, 293]. The increase of thymic volume also correlated positively with the $CD4^+$ T cells counts [348]. Additionally, higher thymic indices were associated with higher counts of naïve $CD4^+$ T cells [265].

Although one cross-sectional study showed similar sj/β TREC ratios between patients with poor immunological response (PIR) (*i.e.* with low $CD4^+$ T cell counts despite virological suppression) and patients with adequate immunological response (AIR, *i.e.* with virological suppression and high $CD4^+$ T cells counts) to ART [362], in another study, patients with PIR presented lower sj/β TREC ratios in comparison to patients with AIR or to healthy individuals [316]. These apparently contradictory results are probably due to different inclusion criteria: while in the first study PIR was defined as a total $CD4^+$ T cell count increase < 200 cells/μL, in the second study, PIR was defined as a $CD4^+$ T cell count increase per year < 100 cells/μL. It has been shown that patients with poor immune recovery presented smaller thymi, were more likely to have no detectable thymus, had lower number of naïve T cell, TREC levels, and percentages of RTE compared to patients with adequate responses to ART [266, 281, 363].

Importantly, reports showed that patients with poor immunological responses to ART tend to be older than patients with adequate responses [364-366], and HIV-infected children have earlier and greater increases in naïve $CD4^+$ T cells than adults, which is probably related with the aging-related involution of thymus [367].

Patients with prolonged virological suppression and adequate responses to ART may be candidates to scheduled ART interruption in the context of clinical trials. Few reports shed some light on the impact of thymus on the change of $CD4^+$ T cell counts during these interruptions. In children undergoing scheduled ART interruption, although the $CD4^+$ T cell counts decreased abruptly, the ratio between $CD45RA^+$ T cells and $CD45RO^+$ T cells, which is influenced by thymic function, remained constant, and these children were able to rapidly restore $CD4^+$ T cells counts after ART reintroduction [368]. In adults, thymic volume

was the only variable in univariate analysis with statistical significance to predict a decrease in $CD4^+$ T cell count to less than 350 cells/μL after ART interruption [283]. Nadir CD4 count, age, sex, HCV co-infection, TREC levels and early viral load rebound were not different between patients with $CD4^+$ T cell counts that declined below this threshold and the other patients [283].

Concluding, innumerous studies have shown that thymic alterations associated with HIV may be reversed during ART, at least partially, in both adults and children. In general, patients with higher estimates of thymic function present higher $CD4^+$ T cell counts and better immune recoveries. A balanced immune system, with normal T cell counts and normal frequencies in their subpopulations, and a fully and diverse immune repertoire, will only be restored during ART if thymus function is entirely reconstituted. Further research is needed to identify those who would benefit the most from each strategy to enhance thymic function and, consequently, their immune recoveries.

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PART II | Results

CHAPTER 3.
Immunological non-responders

Definition of Immunological Non-Response to Antiretroviral Therapy: a Systematic Review

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Definition of Immunological Non-Response to Antiretroviral Therapy: a Systematic Review

1. Abstract

BACKGROUND: A subset of HIV-infected patients on antiretroviral therapy (ART) fail to achieve satisfactory $CD4^+$ T cell counts, despite full virologic suppression. Terminologies and criteria to identify this condition are extremely diverse. This diversity precludes data comparison and aggregation from distinct sources and restricts knowledge expansion. Achieving consensual term and criteria is urgent and for that a compressive revision is a first necessary step.

METHODS: We performed a systematic review using the original research articles from PubMed containing a set of predefined terms related with the concept of immunological non-response, published in English between January 1, 2009, and September 30, 2018. The representative terminology and criteria were extracted and evaluated.

FINDINGS: The search retrieved 1360 studies, of which 103 were eligible for analysis. These used 22 different terms and 73 distinct criteria for the definition. The most frequent term was "immunological non-responders", followed by "discordant immunological response" and "suboptimal $CD4^+$ T-cell recovery". Of the 73 criteria, only 9 were used by more than one team. The most frequent criterion, included in 8 studies from 4 independent research groups, was a $CD4^+$ T cell count < 350 cells/μL after ≥ 24 months of virologic suppression.

INTERPRETATION: This review is expected to foster the discussion necessary to achieve a consensual definition to identify the patients with unsatisfactory $CD4^+$ T cell recovery. This is an essential step to boost knowledge on the mechanisms underlying this unfavorable outcome and to early identify this vulnerable group of patients in need of tighter medical monitoring.

2. Introduction

Antiretroviral therapy (ART) transformed the course of human immunodeficiency virus (HIV) infection, improving patient prognosis, quality of life and life expectancy [1, 2]. After ART initiation, plasma viral load drops to undetectable levels in patients with good adherence and without resistance-associated mutations [3], and $CD4^+$ T cell count increases rapidly initially, followed by a longer period of slower rise [4]. Some authors describe a $CD4^+$ T cell count plateau, while others report increases even after several years of therapy, depending on different factors such as age, pre-ART $CD4^+$ T cell count and viral load [5-8]. However, some individuals are unable to restore satisfactory $CD4^+$ T cell counts despite full suppression of viral replication (virologic suppression; usually < 50 copies/mL [9, 10]), and have higher mortality and morbidity [4, 11]. The proportion of patients with reduced immunological response despite viral suppression has been reported to be between 9 % and 45 % depending on the cohorts and criteria used. However, the heterogeneity of the criteria makes prevalence estimates problematic.

In the absence of a consensual criterion, it is difficult to compare and combine coherent and solid knowledge. In clinical trials aiming to improve $CD4^+$ T cell recovery [12-21], variability in selection criteria precludes definitive conclusions [22]. A consensual definition is difficult to achieve but is absolutely required to be able to combine data from distinct studies/patients cohorts.

In this systematic review, we analyze the diversity in terminologies and criteria applied to HIV-infected patients with full virologic response to ART but impaired immune response and establish the relative frequency of terms and criteria used. We also discuss possibilities for consensual definition and criteria. For convenience, we use along the text the term “immunological non-response” (INR), as it was the most frequently used term on the included studies.

3. Material and Methods

This review was conducted according to PRISMA practice standards. The manuscript follows PRISMA and MOOSE checklists [23, 24], adapted as needed. The review protocol was not registered in PROSPERO because it reviewed definitions rather than outcomes, not fitting the database format.

3.1 – Search strategies

Records were retrieved from PubMed using the interface of the R-based tool Adjutant [25]. The search strategy combined medical subject headings (MeSH terms) and other expressions commonly used to describe HIV-infected individuals with impaired immune recovery despite virologic suppression during ART. The full expression used was:

(HIV[mh] OR HIV Infections[mh]) ***AND***
(antiretroviral therapy, highly active[mh] OR ART OR antiretrovir)* ***AND***
(((discordan OR impair* OR incomplet* OR insufficient* OR low OR low-level OR poor OR suboptimal OR unfavorabl* OR unsatisfactor* OR weak)* ***AND*** *(immune OR immunologic* OR CD4-Positive T-Lymphocytes[mh] OR CD4 Lymphocyte Count[mh] OR (CD4 AND (T-cell OR T-lymphocyt*))) AND (gain* OR reconstitut* OR recover* OR repopulat* OR respon* OR restorat*))* ***OR*** *((immune OR immunologic*) AND (discordan*)))*
AND *"english"[Language]* ***AND***
("2009/01/01"[Date - Publication] : "2018/09/30"[Date - Publication])

Additional publications from Web of Science® were included after expert recommendation or scrutiny of reference lists.

3.2 – Eligibility criteria

This review included all studies and reports meeting the following requirements:

a) Use of the concept of INR, with or without an explicit definition;
b) Publication date: between January 2009 and September 2018;

c) Publication type: original research articles; other types of articles were excluded (meeting abstracts, case reports, reviews and commentaries);

d) Language: written in English;

e) Study population: HIV-1 infected patients on ART over 15 year-old; in studies including patients with and without treatment only the group of patients under ART was considered;

f) Outcome measures: "virologic suppression", implicitly or explicitly defined as plasma HIV RNA copies below a threshold; "poor immune response to ART", explicitly defined as not reaching a $CD4^+$ T cell count, $CD4^+$ T cell count change or slope, CD4/CD8 ratio or other immune recovery surrogate threshold.

All studies conducted on pregnant women or on patients co-infected by HIV-2 were excluded.

3.3 – Screening procedure

The list of publications obtained through Adjutant was exported to Microsoft Excel 2010 (Redmond, Washington, USA). This dataset had the following fields: PubMed Unique Identifier (PMID), year of publication, journal name, authors, title, abstract, article type, language, PMC citation count, PubMed Central identifier (pmcID), digital object identifier (DOI) and MeSH terms. A filter was applied to exclude records published before 2009 or not written in English. Publications retrieved from other sources were manually added to the dataset. Two researchers independently screened all titles and abstracts to assess whether the record should be included in the analysis, according to predefined criteria. All records with at least one favorable opinion transitioned to full-text screening.

Full-text pdf. files were obtained from the journal websites or by email request to the authors, and assessed using the reference manager Mendeley Desktop v1.19.1 (Mendeley, London, UK). Each full text was screened by one of two independent researchers to retrieve the following information: study characteristics (study type, recruitment period, number of HIV-infected and INR patients enrolled), demographic characteristics of participants (age, gender, country), terminology (as explained in Data

Analysis subsection) and criteria for INR definition [viral load threshold used to define virologic suppression, immune recovery surrogate, threshold and time point(s) used to assess immune response to ART]. The two researchers discussed dubious cases and/or consulted a third researcher until reaching consensus.

3.4 – Data analysis

3.4.1 – Country classification

Countries were categorized into low, lower-middle, upper-middle or high-income countries according to the Gross National Income per capita, as defined by the World Bank for 2017 [26].

3.4.2 – Terminology

Many articles presented different terms to designate INR patients. To analyze the diversity between publications, the term associated with the definition criteria stated in the methods section was selected as the most representative in each publication. When a unique term was not clear, the most prevalent term throughout the manuscript or the term used in the figures/tables or title was selected. Records were grouped by similar defining terms, and a table listing the distinct terms was produced.

3.4.3 – INR criteria

In each study analyzed, authors had defined one or more criteria to identify INR patients. These criteria included at least one immune recovery surrogate ($CD4^+$ T cell count, its increase from baseline, its slope, CD4:CD8 ratio or other) and at least one threshold value for that surrogate. Criteria were considered as single when including only one surrogate and one threshold value, and as combined when including more than one surrogate or more than one threshold value, simultaneously or alternatively, independently of the assessment time point. Time points to assess INR status were expressed in time after ART initiation or time of virologic suppression. When the authors defined the time point as time "on suppressive ART", it was considered that patients presented virologic suppression

during that period. Authors expressed this period in weeks, months or years. To simplify comparison between studies, time points were first converted to months, as follows: 24 weeks equals 6 months; 36 weeks equals 9 months; 48 and 52 weeks equal 12 months; and 96 weeks equals 24 months. Records were then grouped by similar criteria, and two tables listing single and combined criteria were produced.

3.4.4 – Independent publications

To complement the analysis of terminology and criteria diversities, the number of publications with no authors in common (ni) was assessed for each term and each criterion found. In publications based on large national or international collaborations, i.e. with more than 20 authors listed, only the named authors were considered for this analysis.

4. Results

4.1 – Study selection

On October 26th, 2018, the PubMed search using Adjutant interface yielded 1360 documents sourced from 345 journals, and published in English from January 2009 to September 2018. Fifteen additional documents were included based on expert recommendations. These publications were not found in the search probably due to their very recent publication date. Two independent reviewers screened titles and abstracts of the 1375 documents and excluded 1251 that were beyond the scope of this review. Twenty-one additional documents were excluded after full-text assessment due to: absence of virologic suppression (n = 6); severe lack of information (n = 2); and definition criteria not explicit (n = 12). Final analyses of INR definition included 103 publications (Figure 1; Table S1 of Supplementary Material).

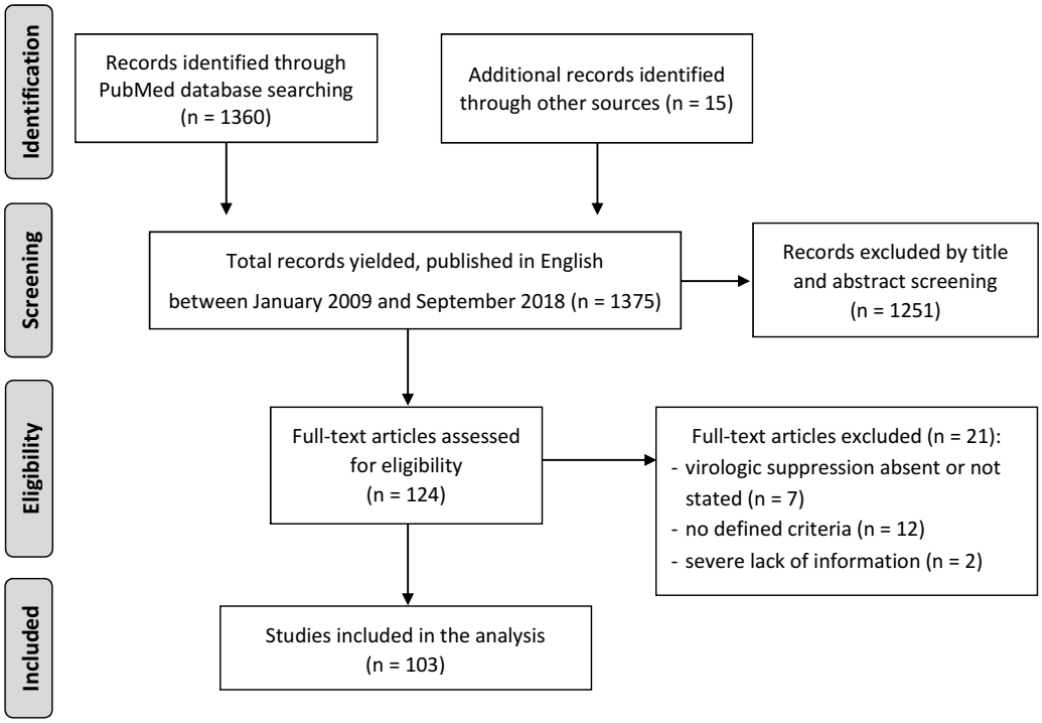

Figure 1 – PRISMA flow diagram of identification and selection of studies for the systematic review. Adapted from Moher *et al.* [23].

4.2 – Characteristics of studies included in the final analyses

The 103 included studies were published in 41 different peer reviewed journals. Four journals contributed with 50 publications (49 % of publications) but most journals (29 of 41) contributed with only one publication (Table S2 of Supplementary Material). Journals were mainly focused on Immunology, Infectious Diseases and/or Virology (Scopus sub-subject areas). Most studies (78 %) were from journals ranked Q1 by CiteScore in at least one of their attributed sub-subjects in 2017.

The number of studies published over time was stable with approximately 10 studies per year (Figure 2). Most studies (n = 48, 47 %) were longitudinal, 32 (31 %) were cross-sectional and 23 (22 %) were experimental. Nine studies focused exclusively on men (n = 8, 8 %) or women (n = 1, 1 %). All other studies (n = 94, 91 %) included participants of

both sexes, with a male percentage between 24 and 97 % (median 79 %, IQR 19 %). All publications included patients aged ≥ 16 years, as defined by the eligibility criteria.

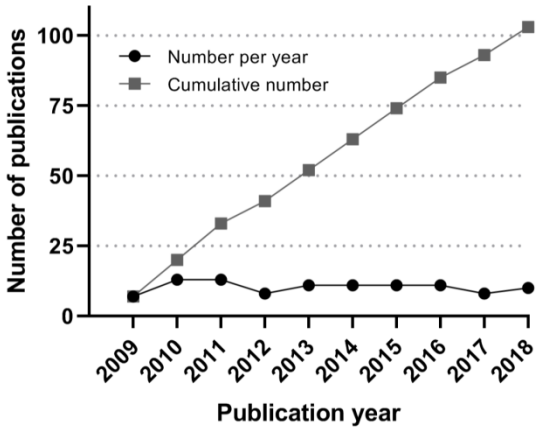

Figure 2 – Number of publications per year and cumulative sum (2009-2018).

Seven of the 103 publications (7 %) corresponded to international projects, 4 of which were large collaborations/consortia (TAHOD and Australia; ART-LINC of IeDEA; EuroSIDA Study Group; and ART-CC and COHERE Cohort Collaborations; Table S3 of Supplementary Material). The other 96 studies (93 %) enrolled patients from 30 different countries. These were mostly (n = 25, 83 %) upper-middle- and high- income countries according to the 2017 World Bank classification (Table S4 of Supplementary Material). The percentage of males included in all studies from upper-middle and high-income countries was higher than 50 %, while in all studies from low and lower-middle income countries it was lower than 50 % (Figure 3).

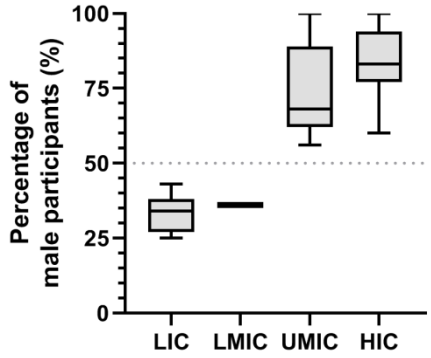

Figure 3 – Percentage of male participants in each study by country classification according to 2017 income.
HIC, high-income countries; LIC, low-income countries; LMIC, lower-middle-income countries; UMIC, upper-middle-income countries.

4.3 – Terminology to define patients with immunological non-response

All terms applied to INR in the 103 publications presented two elements: 1) an adjective (e.g., "discordant", "inadequate", "immunological", "impaired", "poor", "suboptimal") and 2) a noun to describe the alterations in patients' immunologic state during ART (e.g., "reconstitution", "recovery", "response", "restoration"). Terms that included "$CD4^+$" (e.g., "$CD4^+$ T cell-recovery", "$CD4^+$ T cell repopulation") were separated from the terms that used a less specific expression (e.g., "immune reconstitution", "immunological response"). Terms with the common adjectives or nouns were then grouped, resulting in 22 different categories (Table 1). When all orthographic variations and synonyms were considered, more than 50 different expressions were obtained. Considering only the set of 22 categorical terms (T.), the 3 that appeared most often were: "immunological non-responders" (n = 24, T.6 in Table 1); "discordant immunological response" (n = 18, T.3 in Table 1) and "suboptimal $CD4^+$ T-cell recovery" (n = 9, T.20 in Table 1).

To understand if the same authors contributed with multiple instances of the same term, the frequency of each term in publications with no authors in common was also analyzed (ni in Table 1). In this case, the most frequent expressions were still "immunological non-responders" (ni = 15, T.6 in Table 1) and "discordant immunological response" (ni = 9, T.3 in Table 1), followed by "suboptimal $CD4^+$ T-cell recovery" and "poor $CD4^+$ T cell

recovery" (both with ni = 7, T.18 and T.20 in Table 1, respectively). As expected, publications with authors in common tended to share similar terms. In a few cases, publications with authors in common used different terms (e.g., "immunological non-responders", "low CD4 T-cell reconstitution" and "poor $CD4^+$ cell recovery" in [13, 27, 28]).

4.4 – Criteria to define patients with immunological non-response

The 103 studies included 73 different criteria to classify INR patients. Of these, 48 (66 %) were single criteria, defined as not reaching a single threshold of a single surrogate, and 25 (34 %) were combined criteria, defined as multiple conditions considered simultaneously (AND) or alternatively (OR). All criteria used $CD4^+$ T cell counts to evaluate immune recovery during ART, either as absolute number, in 59 of the 73 criteria (81 %; C.1-C.35 in Table 2a, and C.49-C.68 and C.70-C.73 in Tables 2b), or as the change in $CD4^+$ T cell count compared to a baseline value, in the remaining 14 criteria (19 %; C.36-C.48 in Table 2a and C.69 in Table 2b). Of the 59 criteria that used absolute $CD4^+$ T cell count as surrogate of the immune recovery, 38 (64 %) presented a single definition. In the other 21 (36 %), it was combined with $CD4^+$ T cell count change and/or CD4/CD8 ratio.

Fifty-eight criteria were unique, and only 15 were used in more than one study. Of these, only 9 were used by groups with no authors in common (C.9, C.10, C.20-C.23, C.33, C.45, C.61 in Table 2a and 2b). The three most used criteria considered $CD4^+$ T-cell counts < 350 cells/μL after ≥ 24 months of virologic suppression (n = 8, C.22 in Table 2a); < 250 cells/μL after ≥ 24 months of virologic suppression (n = 5, C.12 in Table 2a); and < 350 cells/μL after ≥ 24 months of ART (n = 4, C.21 in Table 2a).

The most frequently used thresholds were "< 350 cells/μL" (n = 35) and "< 200 cells/μL" (n = 22), evaluated "at or after at least 12 months" (n = 36) of ART or virologic suppression and "at or after at least 24 months" (n = 35) of ART or virologic suppression.

Table 1 - Terminology used to designate patients with limited immune recovery despite virologic suppression in the included publications.

Term (*T*)	Publications	n	n_i	Term code
$CD4^+$ T-cell deficiency	[48]	1	1	*T.1*
Discordant response of $CD4^+$ T cells	[49]	1	1	*T.2*
Discordant immunological response [a)]	[39, 42, 43, 45, 46, 50-62]	18	9	*T.3*
Failure to achieve CD4 response/ complete immunological response	[63]	1	1	*T.4*
Immune reconstitution failure [b)]	[64-66]	3	3	*T.5*
Immunological non-response [c)]	[18, 27, 37, 47, 67-86]	24	15	*T.6*
Impaired CD4 cell recovery	[87, 88]	2	2	*T.7*
Inadequate $CD4^+$ T cell recovery	[89]	1	1	*T.8*
Inadequate immunological responder	[38]	1	1	*T.9*
Incomplete $CD4^+$ T cell recovery	[17, 90-92]	4	2	*T.10*
Incomplete immune reconstitution	[93]	1	1	*T.11*
Insufficient $CD4^+$ T cell response	[94]	1	1	*T.12*
Insufficient immunological restoration	[95]	1	1	*T.13*
Low-level CD4 T cell repopulation	[96-99]	4	1	*T.14*
Low CD4 T-cell reconstitution [d)]	[13, 36, 100-102]	5	2	*T.15*
Nonimmune reconstituters	[103]	1	1	*T.16*
Paradoxical immunological response	[104]	1	1	*T.17*
Poor $CD4^+$ T cell recovery [e)]	[28, 105-110]	7	7	*T.18*
Poor immunological responders [f)]	[111-116]	6	6	*T.19*
Suboptimal $CD4^+$ T-cell recovery [g)]	[16, 20, 44, 117-122]	9	7	*T.20*
Suboptimal immune responders [h)]	[14, 123-129]	8	3	*T.21*
Virologic-only response	[33, 130, 131]	3	3	*T.22*
	Total	**103**	**72**	***22***

Note: Terms are alphabetically ordered. ni stands for number of publications with no authors in common.
a) Includes: “discordant immunological and virologic response”, “discordant patients”, “discordant responders”, “immuno-virological discordance”, “immunodiscordant patients”, “immunodiscordant responders”, “immunodiscordant subjects”, “immunologic discordance”, “immunologic discordants”, “immunological cART discordance” and “immunologically discordant”.
b) Includes: “immune failure” and “immunologic failure with virologic success”.
c) Includes: “immune non-responders”, “immune non-responding patients”, “immunologic non-responders”, “immunological non-responders”, “immunologic non-response” and “immunological nonresponder”.
d) Includes: “low CD4 group” and “low CD4-recovery subjects”.
e) Includes: “poor CD4 lymphocyte recovery”, “poor CD4 recovery”, “poor CD4+ T cell response” and “poor recovery of CD4 T cells”.
f) Includes: “immunologically poor responder”, “poor immune recovery”, “poor immunological recovery” and “poor immunological response”.
g) Includes: “sub-optimal CD4 recovery”, “suboptimal CD4 reconstitution”, “suboptimal CD4 recovery”, “suboptimal CD4 response”, “suboptimal CD4 T-cell response” and “suboptimal CD4+ T cell response”.
h) Includes: “suboptimal immune reconstitution”, “suboptimal immune recovery”, “suboptimal immune response” and “suboptimal immunological response”.

Table 2a - Single criteria to select poor immunological responders in the included publications.

	What?	How much?	When?	Publications	n	n_i	Criterion code
CD4+ T-cell count	Absolute number	< 200 cells/μL	≥ 6 mos. of ART	[68]	1	1	*C.1*
			≥ 6 mos. of VS	[28]	1	1	*C.2*
			At 12 mos. of ART	[78]	1	1	*C.3*
			≥ 12 mos. of ART and ≥ 6 mos. of VS	[47]	1	1	*C.4*
			After 24 mos. of ART	[115]	1	1	*C.5*
			≥ 24 mos. of ART and ≥ 6 mos. of VS	[77]	1	1	*C.6*
			≥ 24 mos. of VS	[71]	1	1	*C.7*
			≥ 36 mos. of VS	[70, 87, 90]	3	1	*C.8*
			Other	[55, 59]	2	2	*C.9*
		< 250 cells/μL	≥ 12 mos. of VS	[88, 98]	2	2	*C.10*
			At 24 mos. of ART	[36, 99]	2	1	*C.11*
			≥ 24 mos. of VS	[96, 97, 100-102]	5	1	*C.12*
			At 36 mos. of ART	[81]	1	1	*C.13*
		< 350 cells/μL	≥ 6 mos. of ART [a]	[91]	1	1	*C.14*
			≥ 6 mos. of VS	[79]	1	1	*C.15*
			≥ 9 mos. of VS	[82]	1	1	*C.16*
			At 12 mos. of ART	[118]	1	1	*C.17*
			12-24 mos. of ART [b]	[93]	1	1	*C.18*
			≥ 12 mos. of ART and ≥ 6 mos. of VS	[51]	1	1	*C.19*
			≥ 12 mos. of VS	[92, 104, 121]	3	2	*C.20*
			≥ 24 mos. of ART	[49, 83, 84, 105]	4	3	*C.21*
			≥ 24 mos. of VS	[27, 42, 43, 46, 56, 62, 65, 123]	8	4	*C.22*
			≥ 24 mos. of ART and ≥ 12 mos. of VS	[67, 69, 117]	3	2	*C.23*
			≥ 24 mos. of ART and ≥ 18 mos. of VS	[111] [c]	1	1	*C.24*
			At 48 mos. of ART	[112]	1	1	*C.25*
			Other	[53]	1	1	*C.26*
		< 400 cells/μL	≥ 12 mos. of ART	[13] [d]	1	1	*C.27*
			≥ 24 mos. of ART and ≥ 18 mos. of VS	[85]	1	1	*C.28*
		< 500 cells/μL	≥ 12 mos. of VS	[38]	1	1	*C.29*
			≥ 12 mos. of ART and ≥ 6 mos. of VS	[108]	1	1	*C.30*
			≥ 36 mos. of VS	[72]	1	1	*C.31*
			≥ 48 mos. of VS	[74]	1	1	*C.32*
			≥ 60 mos. of ART	[73, 89]	2	2	*C.33*
		< Median	≥ 12 mos. of ART and ≥ 6 mos. of VS	[48]	1	1	*C.34*
		< pre-ART value	Other	[60]	1	1	*C.35*
	Change	< 50 cells/μL	At 6 mos. of ART	[130]	1	1	*C.36*
			Up to 6 mos. of ART	[125]	1	1	*C.37*
			At 12 mos. of ART	[131]	1	1	*C.38*
			≥ 12 mos. of ART	[113]	1	1	*C.39*
		< 100 cells/μL	At 12 mos. of ART	[54]	1	1	*C.40*
			After 12 mos. of ART	[94]	1	1	*C.41*
			At 6 or 12 mos. of ART	[52]	1	1	*C.42*
		< 150 cells/μL	Until 12 mos. of ART	[122]	1	1	*C.43*
		< 200 cells/μL	At 12 mos. of ART	[109]	1	1	*C.44*
			Other	[114, 116]	2	2	*C.45*
		< 20 %	Within 6 mos. of ART	[80]	1	1	*C.46*
		First quartile	At 48 mos. of ART	[44, 127, 129]	3	1	*C.47*
			At 84 mos. of ART	[126]	1	1	*C.48*
				Subtotal	75	59	*48*

Note: ni stands for number of publications with no authors in common.

a) $CD4^+$ T-cell counts < 350 cells/μL for ≥ 12 mos.
b) First or second ART regimen.
c) $CD4^+$ T-cell count between 101 and 350 cells/μL.
d) $CD4^+$ T-cell count between 101 and 400 cells/μL.

Table 2b - Combined criteria to select poor immunological responders in the included publications.

What and how much?		When?	Publications	n	n_i	Criterion code
CD4· T-cell count < 200 cells/µL	AND CD4· T-cell count change < 30 %	At 12 mos. of VS	[50, 57]	2	1	*C.49*
	AND/OR CD4· T-cell count change < 25 %	≥ 12 mos. of ART	[18]	1	1	*C.50*
	AND CD4· T-cell count change < 200 cells/µL	At 24 mos. of ART	[128]	1	1	*C.51*
	OR CD4· T-cell count change < different cutoff values (< 0, 0-49 or 50-99 cells/µl)	At 6 mos. of ART	[106]	1	1	*C.52*
	OR CD4· T-cell count change < 100 cells/µL	Up to 12 mos. of VS	[64]	1	1	*C.53*
	OR CD4· T-cell count change < 20 %	≥ 12 mos. of ART	[75]	1	1	*C.54*
	OR (CD4/CD8 ratio < 1 AND CD4· T-cell count < 400 cells/µL AND CD4· T-cell count change < 100 cells/µL)	≥ 12 mos. of VS	[58]	1	1	*C.55*
	OR (CD4/CD8 ratio < 1 AND CD4· T-cell count < 400 cells/µL AND CD4· T-cell count change < 100 cells/µL)	≥ 24 mos. of VS	[39]	1	1	*C.56*
CD4· T-cell count < 250 cells/µL	AND slope of annual change [-20;+20] cells/µL	≥ 12 mos. of ART	[16, 20]	2	1	*C.57*
CD4· T-cell count < 300 cells/µL	AND CD4· T-cell count change < 10 %	≥ 12 mos. of VS	[76]	1	1	*C.58*
	OR CD4· T-cell count change < 250 cells/µL	≥ 12 mos. of VS	[103]	1	1	*C.59*
CD4· T-cell count < 350 cells/µL	AND CD4· T-cell count change < 50 cells/µL	≥ 12 mos. of ART and ≥ 9 mos. of VS	[120]	1	1	*C.60*
	AND CD4· T-cell count change < 100 cells/µL	≥ 12 mos. of ART	[17, 119]	2	2	*C.61*
	AND/OR CD4· T-cell count change < 30 %	≥ 12 mos. of ART	[86]	1	1	*C.62*
	AND slope of annual change* < 50 cells/µL	≥ 24 mos. of VS	[95]	1	1	*C.63*
CD4· T-cell count < 500 cells/µL	AND CD4/CD8 ratio < 1	≥ 96 mos. of VS	[37]	1	1	*C.64*
	OR CD4/CD8 ratio < 1	At 120 mos. of ART	[63]	1	1	*C.65*
CD4· T-cell count ≤ pre-ART value OR CD4· T-cell count change < 50 cells/µL		At 12 mos. of ART	[66]	1	1	*C.66*
CD4· T-cell count < different cutoff values (200, 350 or 500 cells/µl)		Up to 60 mos. of ART	[124]	1	1	*C.67*
CD4· T-cell count < different cutoff values ranging from 200 to 600 cells/µl OR CD4· T-cell count change < different values ranging from 50 to 500 cells/µl		≥ 24 mos. of VS	[45]	1	1	*C.68*
CD4· T-cell count change < different cutoff values (< 25 or 25-100 cells/µl)		At 24 mos. of ART	[110]	1	1	*C.69*
CD4· T-cell count < 200 cells/µL after ≥ 12 mos. of ART OR CD4· T-cell count < 350 cells/µL after ≥ 24 mos. of ART			[14]	1	1	*C.70*
(CD4· T-cell count < 350 cells/µL OR change < 50 cells/µL at 6 mos. of ART) OR (CD4· T-cell count < 350 cells/µL OR change < 100 cells/µL at 12 OR 24 mos. of ART)			[33]	1	1	*C.71*
CD4· T-cell count < 200 cells/µL at 12 mos. of ART OR CD4· T-cell count < 500 cells/µL at 30 mos. of ART			[61]	1	1	*C.72*
CD4· T-cell count < 350 cells/µL after ≥ 12 mos. of ART OR (persistently falling CD4· T-cell counts and percentages for ≥ 24 mos.)			[107]	1	1	*C.73*
			Subtotal	28	26	*25*
			Total	103	85	*73*

Note: n_i stands for number of publications with no authors in common.

* Regression slope per year in the original publication.

Table 3 – Summary of geographical setting of included studies.*

Setting:	Publications	
	Number (n)	Percentage (%)
Low-income countries	9	9 %
Lower-middle-income countries	2	2 %
Upper-middle-income countries	12	12 %
High-income countries	73	71 %
Countries in different income groups	7	7 %
Total	103	100 %

* As defined by the World Bank for 2017 [26].

5. Discussion

Terms standardization is as old as human language, and assumes particular relevance in the context of scientific communication [29]. In 1986, to avoid the multiplicity of names used for the "AIDS virus" , the International Committee for the Taxonomy of Viruses recommended the use of the term "human immunodeficiency virus" and of the abbreviation "HIV" to designate this retrovirus [30]. This consensus was essential to expand scientific knowledge on HIV.

People living with HIV on ART who experience poor immune recovery despite full virologic suppression have higher morbidity and mortality [9], but the data on these patients are difficult to combine due to the lack of a standardized case-definition [11, 22]. Although previous reviews mentioned or discussed this problem [22, 31, 32], or addressed clinical outcomes [11], this is the first to systematically analyze INR terminology and defining criteria.

Our results show striking diversity in terms and definitions used in the literature. In the 103 analyzed studies, the authors used more than 50 different expressions that were grouped into 22 distinct categories of defining terms. Some publications included several different expressions used interchangeably throughout the text, from which we only selected the most representative, so the diversity in INR terminology is even higher. Furthermore, 73 different criteria to define INR cases were found encompassing variable thresholds and time points.

"Immunological non-response" (INR) was the most frequent term. The expression "non-response" is used to refer to a weak response and not exactly an absence of response, as most of these patients generally present a small but existent immunological response after ART initiation.

"Discordant immunological response" (DIR) was the second most frequent. However, this term may refer to two different conditions: virologic-only response or immunologic-only response [33, 34].

"Suboptimal $CD4^+$ T-cell recovery" (SCR) was the third most frequent term. "Suboptimal" literally means "below the highest level", which, in this case, excludes the optimal responses, but includes the poor and intermediate levels of immunologic response to ART.

"Poor $CD4^+$ T cell recovery" and "poor immunological responders" (PIR) were relatively frequent. Although the word "poor" may be perceived with a negative connotation and connected to the stigma and discrimination already associated with HIV infection [35], it literarily means "of a low or inferior standard or quality" (Oxford English Dictionary) or "less than adequate" (Merriam-Webster Dictionary), which is accurate for this condition. The expression "$CD4^+$ T cell recovery" is more precise than "immunological response" in that it specifies the parameter used for analysis. "Poor $CD4^+$ T cell recovery" is also used in the "Guidelines for the use of antiretroviral agents in adults and adolescents living with HIV" from the United States Department of Health and Human Services [9], indicating internationally widespread use of this term beyond the scientific literature. Other terms were observed less frequently.

The abbreviations "DIR", "SCR" and "PIR" are not standardly used with other medical meaning, however, "INR" is frequently used for "international normalized ratio", the standardized measure of oral anticoagulant effects.

Seventy-three different criteria to define INR were found. Most studies (81 %) used the $CD4^+$ T cell count value and only 19 % used the change compared to baseline. In a review article, Cenderello & De Maria [22] have explained the limitations of each situation, which are related to grouping together different recovery patterns. When crossing a $CD4^+$ T cell count threshold is considered to define INR, patients who started with very different values (e.g. < 100 and 200-350 cells/μL) but achieved the same $CD4^+$ T cell count at a certain time point will be pooled together, although they present very different $CD4^+$ T cell count changes. When $CD4^+$ T cell count change is considered to define INR, patients who started with very different $CD4^+$ T cell counts and present the same change value will be pooled together, although these patients present different dynamics of immune recovery.

Regarding the 3 most frequent criteria among the 73 found, 2 used the threshold of 350 cells/μL, and 2 used the time point ≥ 24 months of virologic suppression. Theoretically, lower thresholds and distant time points are more restrictive, and tend to narrow the concept of INR. For example, fewer patients are expected to have "$CD4^+$ T-cell count < 250 cells/μL after ≥ 12 months of ART" than those with "$CD4^+$ T-cell count < 350 cells/μL after ≥ 12 months of ART" or "$CD4^+$ T-cell count < 250 cells/μL after ≥ 6 months of ART". However, all these criteria identify groups of patients with higher risk of morbidity and mortality. Earlier time points are less restrictive and may lead to including patients with slower immune response, who may reach normal $CD4^+$ T cell counts after a longer period. This may lead to the expenditure of resources in patients with better prognosis (compared with patients with more restrictive INR criteria), and also to biased conclusions in clinical trials (e.g., underestimating INR impact in the clinical outcome; no benefit of closer follow-up, treatment intensification or new therapeutic strategies in INR compared to controls). Furthermore, earlier time points certainly allow earlier interventions and enhanced impact of these interventions.

To define a standard criterion, one needs to first discuss how restrictive it must be to represent the true clinical outcome of these patients. A standard definition should also clearly state whether the time point is considered from the beginning of ART or of virologic suppression, and in the latter, how virologic suppression should be assessed. "Persistent virologic suppression" is a subjective expression due to the occurrence of blips. A good example of a more clear virologic suppression definition, from Pacheco *et al.*, 2015 [36], is "all viral load measurements during this period [first 2 years of ART] <500 copies/mL, allowing for exceptions during the first 24 weeks", although the threshold should be < 50 instead of < 500 copies/mL and viral blips should also be contemplated.

Additional points must also be considered. Pre-ART $CD4^+$ T cell counts influence the immune recovery pattern, and the lower the baseline $CD4^+$ T cell counts, the more difficult it is to reach the threshold. Moreover, an isolated $CD4^+$ T-cell count measurement may not reflect the patient immune recovery pattern, since many factors, such as co-existing

infections, influence the absolute $CD4^+$ T count at a given time point. Thus, a standard definition should also include the conditions under which patients meet or fail the criteria.

Other parameters might also be useful to classify ART response based on the immune recovery. Cenderello & De Maria suggested including patient age, nadir $CD4^+$ T cell count, CD4/CD8 ratio and presence of AIDS-defining and non-AIDS-defining morbidity in the classification of ART response based on the immune recovery [22]. Other parameters with prognostic value could be: ART status (naïve vs. experienced), baseline viral load, and other immunologic parameters [37-40]. Patients with INR could benefit from a prognostic index including these and/or other parameters, similar to the International Prognostic Index for patients with aggressive non-Hodgkin lymphoma [41].

To close this discussion section, it must be highlighted that the best INR definition depends on the setting it will be applied. When managing people initiating ART, the change in $CD4^+$ T cell count over the first few months is very important, but when managing patients who have been on ART for many years, the absolute count is more relevant, given that it is expected a much rapid $CD4^+$ T cell count increase in the first months of therapy compared to the increase after 24 months of ART [4]. So, our proposal is to establish, for patients initiating ART with $CD4^+$ T cell counts below a specific cutoff (e.g. < 200 cells/μL): 1) a definition based on $CD4^+$ T cell count change assessed at a relatively early time point to predict those who may be at higher risk (e.g. change < 50 cells/μL at 6 months of ART); and 2) a definition based on absolute count after a certain period on ART to exclude those patients with slow but satisfactory immune recoveries (e.g. $CD4^+$ T cell count < 350 cells/μL after ≥ 24 months of ART). These definitions should be validated in large studies.

One strength of this review is the transparent and comprehensive search strategy, encompassing a large set of terms. As limitations, the publication search was restricted to one search engine, and a set of search terms had to be defined a priori, so the existence of other terminologies cannot be excluded. However, the latter limitation was partially overcome by the broadness of the search strategy. In fact, the search included several

terms that, although not present in Table 1, occurred in the full-text of different publications. As an example, "unfavorable" and "unsatisfactory" were used in several full-texts as synonyms of "poor" [42-47] but were not selected as the most representative of those publications. Records published before 2009 were not included since they might include terms and criteria that have fallen into disuse. Although a structured quality analysis of the included studies is not described, the fact that most studies were published in journals ranked Q1 by CiteScore assures, at least in part, the study quality. Future research should also include analysis of nadir $CD4^+$ T cell counts, ART schemes and clinical outcomes. Additionally, two important gaps identified in this review should also be addressed: most studies report data from upper-middle- and high- income countries, as also observed in Kelly *et al.*, 2010 [11]; and patient samples from upper-middle- and high-income countries include more males than samples from low and lower-middle income countries, thus introducing gender differences when comparing these two settings.

This review raises awareness on the diversity of terminologies and criteria for INR found in the literature, and may serve as a basis for decision-makers, researchers and clinicians to discuss this problem. A standard term and defining criteria that are consensual and supported by the HIV clinical community are urgently needed. Standardization of INR terminology and criteria could lead to new interventions, policies and practices to improve patient prognosis.

Ethics statement

This review used published data and ethical approval and consent was not required.

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Supplementary Material (Chapter 3)

TABLE S1 - Data on the 103 publications included in the systematic review.

No.	First author, *Journal*, Year of publication	Country / International	Study type	Male percentage among total participants	Representative INR term	INR criteria (immune recovery surrogate, threshold and time point or period)	Viral load (threshold and time point or period)	Ref.
1	Lee SC, *Sci Rep*, 2018	Malaysia	Cross-sectional	100 %	Suboptimal immune recovery (sIR)	CD4 count < 350 cells/µL after ≥ 24 mos. of VS	< 50 copies/mL for ≥ 24 mos.	[123]
2	De Benedetto I, *JAIDS*, 2018	Italy	Cross-sectional	79 %	Immunological non responders (INRs)	CD4 count ≤ 350 cells/µL after ≥ 24 mos. of ART	< 50 copies/mL for ≥ 12 mos.	[69]
3	Rosado-Sánchez I, *Front Immunol*, 2018	Ivory Coast	Longitudinal	NA	Low CD4-recovery subjects (LR-subjects)	CD4 count < 250 cells/µL after ≥ 24 mos. of VS	NA	[100]
4	Lu W, *Front Microbiol*, 2018	Italy	Cross-sectional	100 %	Immunological non-responders (INRs)	CD4 count < 350 cells/µL after ≥ 24 mos. of ART	Below the detection limit for ≥ 24 mos.	[27]
5	Tasca KI, *J Immunol Res*, 2018	Spain	Cross-sectional	60 %	Inadequate CD4+ T cell recovery (tIR)	CD4 count < 500 cells/µL after ≥ 60 mos. of ART	≤ 50 copies/mL after 60 mos. of ART	[89]
6	Logerot S, *AIDS*, 2018	Germany	Experimental	72 %	Poor immunological responder (PIR)	CD4 count < 350 (101-350) after ≥ 24 mos. of ART[1]	< 50 copies/mL for ≥ 18 mos.	[111]
7	Bandera A, *Front Immunol*, 2018	Italy	Cross-sectional	85 %	Immunological non-responders (INR)	CD4 count ≤ 350 cells/µL ≥ 24 mos. of ART	< 50 copies/mL for ≥ 12 mos.	[67]
8	Wójcik-Cichy K, *Arch Immunol Ther Exp*, 2018	Poland	Cross-sectional	74 %	Suboptimal CD4 recovery	CD4 count < 350 cells/µL after ≥ 24 mos. of ART	< 50 copies/mL for ≥ 12 mo	[117]
9	Mupfumi L, *PLoS One*, 2018	Botswana	Longitudinal	32 %	Immunologic non-response	CD4 change < 20 % within 6 mos. of ART	< 400 copies/mL after 6 mos. of ART	[80]
10	Rodríguez-Gallego E, *AIDS*, 2018	Spain	Longitudinal	78 %	Immunological non-responders (INR)	CD4 count < 250 cells/µL at 36 mos. of ART	NA	[81]
11	Darraj M, *J Infect Public Health*, 2017	Italy	Longitudinal	74 %	Immune reconstitution failure	CD4 count < 200 cells/µL OR CD4 change < 100 cells/µL up to 12 mos. of VS	< 50 copies/mL (time point or period NA)	[64]
12	Gomez-Mora E, *PLoS One*, 2017	Spain	Cross-sectional	77 %	Immunodiscordant	CD4 count < 350 cells/µL on long term ART (median 127 mos.)	< 50 copies/mL (time point or period NA)	[53]
13	Giuliani E, *Immunol Lett*, 2017	Italy	Cross-sectional	75 %	Poor CD4+ T-cell recovery	CD4 count < 350 cells/µL at 24 to 49 (IQR) mos. of ART	Below the detection limit. ART for ≥ 24 mos.	[105]

[1] Information available at: https://clinicaltrials.gov/ct2/show/NCT01241643 accessed at 2019 February 1

TABLE S1 (Continued)

No.	First author, *Journal*, Year of publication	Country / International	Study type	Male percentage among total participants	Representative INR term	INR criteria (immune recovery surrogate, threshold and time point or period)	Viral load (threshold and time point or period)	Ref.
14	Gunda DW*, BMC Res Notes*, 2017	Tanzania	Longitudinal	33 %	Poor immune recovery	CD4 count < 350 cells/µL at 48 mos. of follow-up (ART)	NA	[112]
15	Rosado-Sanchez I, *Antimicrob Agents Chemother*, 2017	Spain	Longitudinal	89 %	Low CD4-recovery subjects (LR subjects)	CD4 count < 250 cells/µL after ≥ 24 mos. of VS; CD4 count at baseline < 200 cells/µL	NA	[101]
16	Rosado-Sanchez I, *Antiviral Res*, 2017	Spain	Longitudinal	100 %	Low CD4-recovery subjects (LR subjects)	CD4 count < 250 cells/µL after ≥ 24 mos. of VS; CD4 count at baseline < 200 cells/µL	NA	[102]
17	Minami R, *J Infect Chemother*, 2017	Japan	Experimental	100 %	Immunological non-responder	CD4 count < 350 cells/µL at 27.5 ± 5.3 (mean ± SEM) mos. of ART	Below the detection limit for ≥ 6 mos.	[79]
18	Raffi F, *J Antimicrob Chemother*, 2017	France	Longitudinal	80 %	Failure to achieve CD4 response/ Failure to achieve complete immunological response	More than one CD4 count < 500 cells/µL in the last 18 months, after 120 mos. of ART / (More than one CD4 count < 500 cells/µL OR more than one CD4:CD8 ratio < 1 in the last 18 months, after 120 mos. of ART)	< 50 copies/mL for ≥ 12 mos. with no more than 1 viral blip (50-500 copies/mL)	[63]
19	Stiksrud B, *JAIDS*, 2016	Norway	Cross-sectional	79 %	Immunological non-responder (INR)	CD4 count < 400 cells/µL after ≥ 24 mos. of ART	≤ 20 copies/mL for ≥ 18 mos.	[85]
20	Kayigamba FR, *PLoS One*, 2016	Rwanda	Longitudinal	38 %	Immunological cART discordance	CD4 change < 100 cells/µL at 12 mos. compared to baseline	< 40 copies/mL at 12 mos.	[54]
21	Perez-Santiago J, *AIDS*, 2016	Spain	Cross-sectional	76 %	Immunodiscordant subgroups	$CD4^+$ T-cell count < different cutoff values ranging from 200 to 600 cells/µl OR $CD4^+$ T-cell count change < different values ranging from 50 to 500 cells/µl after ≥ 24 mos. of VS	< 50 copies/mL for ≥ 24 mos.	[45]
22	Girard A, *JAIDS*, 2016	France	Cross-sectional	86 %	Immunological non-responder (INRs)	CD4 count < 500 cells/µL after ≥ 36 mos. of VS	< 40 copies/mL for ≥ 36 mos.	[72]
23	Menkova-Garnier I, *PLoS* Pathog, 2016	France	Cross-sectional	34 %	Immunological non-responders (INRs)	CD4 count < 500 cells/µL AND CD4:CD8 ratio < 1 after ≥ 96 mos. of VS	Below the detection limit for ≥ 96 mos.	[37]
24	El-Beeli M, *Hum Immunol*, 2016	Oman	Longitudinal	45 %	Poor immune responder	CD4 change < 50 cells/µL per year	< 50 copies/mL at ≥ 12 mos.	[113]

TABLE S1 (Continued)

No.	First author, *Journal*, Year of publication	Country / International	Study type	Male percentage among total participants	Representative INR term	INR criteria (immune recovery surrogate, threshold and time point or period)	Viral load (threshold and time point or period)	Ref.
25	Tincati C, *AIDS*, 2016	Italy	Cross-sectional	90 %	Immunological nonresponders (INR)	CD4 count < 350 cells/µL AND/OR CD4 change < 30 % after ≥ 12 mos. of ART	< 40 copies/mL at ≥ 12 mos.	[86]
26	Nakanjako D, *AIDS*, 2016	International	Longitudinal	NA	Sub-optimal immune responders (SO-IR)	$CD4^+$ T-cell count < different cutoff values (200, 350 or 500 cells/µl) up to 60 mos. of ART	NA	[124]
27	Thiebaut R*, Clin Infect Dis*, 2016	International	Experimental	70 %	Low CD4 T-cell reconstitution	CD4 count < 400 (101-400) cells/µL after ≥ 12 mos. of ART	< 50 copies/mL at ≥ 12 mos.	[13]
28	Valiathan R, *Immunobiology*, 2016	USA	Cross-sectional	70 %	(Immunologically) discordant patients	CD4 count < 200 cells/µL OR (CD4:CD8 ratio < 1 AND CD4 count < 400 cells/µL AND CD4 change < 100 cells/µL) after ≥ 24 mos. of VS	< 50 copies/mL for ≥ 24 mos.	[39]
29	Shive CL, *JAIDS*, 2016	USA	Cross-sectional	NA	Immune failure patients	CD4 count < 350 cells/µL after ≥ 24 mos. of ART	< 50 copies/mL at ≥ 24 mos.	[65]
30	Shmagel NG, *Dokl Biochem Biophys*, 2015	Russia	Cross-sectional	62 %	Discordant response of $CD4^+$ T cells	CD4 count < 350 cells/µL after ≥ 24 mos. of ART	< 50 copies/mL at ≥ 24 mos.	[49]
31	Zhang F, *Biosci Trends*, 2015	China	Longitudinal	NA	Suboptimal CD4+ T-cell recovery	CD4 count < 350 cells/µL at 12 mos. of ART	NA	[118]
32	Cillo AR, *AIDS*, 2015	USA	Experimental	94 %	Suboptimal CD4+ T-cell recovery	CD4 count < 250 cells/µL after 22 to 54 (IQR) mos. of ART AND slope of annual change [-20; +20] cells/µL in the last 12 mos.	< 50 copies/mL	[16]
33	Kye-Hyung K, *Korean J Intern Med*, 2015	South Korea	Longitudinal	85 %	Immunologic non-responder	CD4 count < 500 cells/µL after ≥ 48 mos. of VS	< 50 copies/mL for ≥ 48 mos.	[74]
34	van Lelyveld SFL, *PLoS One*, 2015	Netherlands	Experimental	94 %	Suboptimal immunological response	$CD4^+$ T-cell count < 200 cells/µL after ≥ 12 mos. of ART OR $CD4^+$ T-cell count < 350 cells/µL after ≥ 24 mos. of ART	< 50 copies/mL for ≥ 6 mos.	[14]
35	Massanella M, *J Transl Med*, 2015	Spain	Cross-sectional	88 %	(Immuno)discordant patients	CD4 count < 350 cells/µL after ≥ 24 mos. of ART	< 50 copies/mL for ≥ 24 mos.	[46]
36	Batista G, *Med Mal Infect*, 2015	Senegal	Longitudinal	35 %	Suboptimal immune reconstitution (SIR)	CD4 change < 50 cells/µL up to 6 mos. of ART	< 50 copies/mL up to 6 mos.	[125]

TABLE S1 (Continued)

No.	First author, *Journal*, Year of publication	Country / International	Study type	Male percentage among total participants	Representative INR term	INR criteria (immune recovery surrogate, threshold and time point or period)	Viral load (threshold and time point or period)	Ref.
37	Li T, *HIV Clin Trials*, 2015	China	Experimental	89 %	Immune non-responders	CD4 count < 200 cells/µL OR CD4 count < 20 % increase compared to baseline after ≥ 12 mos.	< 40 copies/mL at ≥ 24 mos.	[75]
38	Pacheco YM, *Antiviral Res*, 2015	Spain	Longitudinal	78 %	Low CD4-group	CD4 count < 250 cells/µL at 24 mos. of ART	< 500 copies/mL for ≥ 24 mos.	[36]
39	Mingbunjerdsuk P, *Jpn J Infect Dis*, 2015	Thailand	Longitudinal	68 %	Immunological discordance	CD4 count < 200 cells/µL at 12 mos. of VS OR CD4 change < 30 % at 12 mos. compared to baseline	< 50 copies/mL up to 12 mos.	[57]
40	Nakanjako D, *Trop Med Int Health*, 2015	Uganda	Experimental	NA	Suboptimal immune responders (SO-IR)	CD4 change < 25th percentile (295 cells/µL) at 84 mos. of ART compared to baseline	< 400 copies/mL from 6 mos. of ART	[126]
41	Somsouk M, *PLoS One*, 2014	USA	Experimental	100 %	Incomplete CD4+ T cell recovery	CD4 count < 350 cells/µL after ≥ 12 mos. of VS AND CD4 change < 100 cells/µL in the last year	< 40 copies/mL for ≥ 12 mos.	[17]
42	Saidakova EV, *Dokl Biol Sci*, 2014	Russia	Cross-sectional	NA	Immunological nonresponders (IN)	CD4 count < 350 cells/µL after 24 mos. of ART	< 50 copies/mL at 24 mos. of ART	[83]
43	Asmelash A, *BMC Infect Dis*, 2014	International	Longitudinal	0 %	Suboptimal CD4 response (SCR)	CD4 count < 350 cells/µL AND CD4 change < 100 cells/µL at 12 mos. of ART compared to baseline	< 400 copies/mL for 12 mos.	[119]
44	Routy JP, *HIV Med*, 2014	Canada	Experimental	95 %	Immune nonresponding HIV-infected patients	CD4 count < 350 cells/µL after ≥ 9 mos. of VS	< 50 copies/mL for ≥ 9 mos.	[82]
45	Jacobson JM, *JAIDS*, 2014	USA	Experimental	91 %	Poor CD4 lymphocyte recovery	CD4 count < 200 cells/µL after ≥ 6 mos. of VS	≤ 200 copies/mL for ≥ 6 mos.	[28]
46	Gaardbo JC, *JAIDS*, 2014	Denmark	Longitudinal	86 %	Immunological nonresponders (INR)	CD4 count < 200 cells/µL after ≥ 24 mos. of VS	≤ 200 copies/mL for ≥ 24 mos.	[71]
47	Takuva S, *J Int AIDS Soc*, 2014	South Africa	Longitudinal	36 %	Poor CD4 recovery	CD4 count < 200 cells/µL OR CD4 change < different cutoffs (< 0, 0-49, or 50-99 cells/µL) at 6 mos. of ART compared to baseline	< 400 copies/mL at 6 mos. of ART	[106]
48	Zoufaly A, *PLoS One*, 2014	International	Longitudinal	72 %	Immuno-virological discordance (ID)	CD4 count < pre-ART value at 0-6, 7-12, 13-18, 19-24 and >24 mos. of VS	≤ 50 copies/mL	[60]

TABLE S1 (Continued)

No.	First author, *Journal*, Year of publication	Country / International	Study type	Male percentage among total participants	Representative INR term	INR criteria (immune recovery surrogate, threshold and time point or period)	Viral load (threshold and time point or period)	Ref.
49	Bayigga L, *BMC Immunol*, 2014	Uganda	Longitudinal	NA	Suboptimal immune responders	CD4 change < 25th percentile (199 cells/μL) after 48 mos. of ART compared to baseline	< 400 copies/mL for ≥ 6 mos.	[127]
50	Saison J, *Clin Exp Immunol*, 2014	France	Cross-sectional	71 %	Inadequate immunological responder group (iIR group)	CD4 count < 500 cells/μL after ≥ 12 mos. of VS	< 50 copies/mL at ≥ 12 mos.	[38]
51	Engsig FN, *Clin Infect Dis*, 2014	International	Longitudinal	NA	Incomplete CD4 recovery	CD4 count ≤ 200 cells/μL after ≥ 36 mos. of VS	≤ 500 copies/mL for ≥ 36 mos.	[90]
52	Asdamongkol N, *Jpn J Infect Dis,* 2013	Thailand	Experimental	68 %	Immunological discordance	CD4 count < 200 cells/μL AND CD4 count increase < 30 % at 12 mos. of VS compared to baseline	< 40 copies/mL for ≥ 12 mos.	[50]
53	Rusconi S, *PLoS One*, 2013	Italy	Experimental	82 %	Immunological non-responders (INRs)	CD4 count ≤ 200 cells/μL AND/OR CD4 count increase < 25 % after ≥ 12 mos. of ART compared to baseline	< 50 copies/mL for ≥ 12 mo	[18]
54	Sennepin A, *AIDS*, 2013	France	Longitudinal	75 %	Immunological nonresponders (InRs)	CD4 count < 350 cells/μL for ≥ 24 mos. of ART	< 40 copies/mL for ≥ 24 mos.	[84]
55	Su QJ, *Int J STD AIDS*, 2013	China	Experimental	56 %	Insufficient CD4+ T-cell response	CD4 count increase < 100 cells/μL after 12 mos. of ART	< 50 copies/mL for ≥ 12 mos.	[94]
56	Rallon N, *J Antimicrob Chemother*, 2013	Spain	Cross-sectional	82 %	Poor immunological response	CD4 change < 200 cells/μL after long-term ART	< 50 copies/mL	[114]
57	Nakanjako D, *BMC Immunol*, 2013	Uganda	Longitudinal	43 %	Suboptimal immune responders	CD4 count increase < 25th percentile (298 cells/μL) after 48 mos. of ART compared to baseline	NA	[129]
58	Negredo E, *J Antimicrob Chemother*, 2013	Spain	Experimental	86 %	Immunodiscordant response	CD4 count < 350 cells/μL after ≥ 24 mos. of VS	NA	[62]
59	Hunt PW, *Blood*, 2013	USA	Experimental	96 %	Incomplete CD4+ T cell recovery	CD4 count < 350 cells/μL for ≥ 12 mos.	NA	[92]

TABLE S1 (Continued)

No.	First author, *Journal*, Year of publication	Country / International	Study type	Male percentage among total participants	Representative INR term	INR criteria (immune recovery surrogate, threshold and time point or period)	Viral load (threshold and time point or period)	Ref.
60	Anude CJ, *BMC Infect Dis*, 2013	Nigeria	Longitudinal	36 %	Immunologic failure with virologic success (VL+/CD4-)	CD4 count ≤ pre-ART value OR CD4 change < 50 cells/µL at 12 mos. of ART compared to baseline	< 400 copies/mL at 12 mos. of ART	[66]
61	Horta A, *PLoS One*, 2013	Portugal	Cross-sectional	79 %	Immunological non responders	CD4 count < 500 cells/µL after ≥ 60 mos. of ART	≤ 50 copies/mL for ≥ 12 mos.	[73]
62	Helleberg M, *AIDS*, 2013	Denmark	Cross-sectional	NA	Poor CD4 response	CD4 change < 25 or [25; 100] cells/µL after 24 mos. of ART	< 400 copies/mL ≥ 12 mos. of ART	[110]
63	Massanella M, *AIDS*, 2012	Spain	Experimental	86 %	Immunodiscordant patients	CD4 count < 350 cells/µL after > 24 mos. of VS	< 50 copies/mL for > 24 mos.	[56]
64	Cuzin L, *JAIDS*, 2012	France	Experimental	92 %	Insufficient immunological restoration	All CD4 counts < 350 cells/µL AND regression slope < 50 cells/µL/year during the last 24 mos.	< 50 copies/mL for 24 mos.	[95]
65	Lichtenstein KA, *Antivir Ther,* 2012	USA	Experimental	97 %	Poor CD4+ T-cell responses	CD4 count < 350 cells/µLfor ≥ 12 mos. of ART OR CD4 change persistently < 0 for ≥ 24 mos.	Median < 48 copies/mL at ≥ 12 mos. of ART	[107]
66	Wilkin TJ, *J Infect Dis*, 2012	USA	Experimental	94 %	Suboptimal CD4+ T-cell recovery	CD4 count < 250 cells/µL AND slope between [-20; +20] cells/µL/year during the last 12 mos.	Below the detection limit for ≥ 12 mos.	[20]
67	Mendez-Lagares G, *J Infect Dis*, 2012	Spain	Cross-sectional	74 %	Low-level CD4 T cell repopulation group (LLR patients)	CD4 count < 250 cells/µL after ≥ 24 mos. of VS	< 50 copies/mL for ≥ 24 mos.	[96]
68	Julg B, *PLoS One*, 2012	South Africa	Longitudinal	39 %	Discordant virological and immunological responses	CD4 count < 200 cells/µL at 12 mos. of ART OR CD4 count < 500 cells/µL at 30 mos. of ART	< 50 copies/mL within the first 12 mos.	[61]
69	Mendez-Lagares G, *J Antimicrob Chemother*, 2012	Spain	Cross-sectional	80 %	Low-level CD4 T cell repopulation (LLR patients)	CD4 count < 250 cells/µL after ≥ 24 mos. of VS	< 50 copies/mL for ≥ 24 mos.	[97]
70	van Lelyveld SFL, *AIDS*, 2012	Netherlands	Longitudinal	83 %	Poor immunological recovery	CD4 count < 200 cells/µL after 24 mos. of ART	< 500 copies/mL within 9 mos. and after 18-24 mos. of ART	[115]

TABLE S1 (Continued)

No.	First author, *Journal*, Year of publication	Country / International	Study type	Male percentage among total participants	Representative INR term	INR criteria (immune recovery surrogate, threshold and time point or period)	Viral load (threshold and time point or period)	Ref.
71	Casotti JAS, *Rev Inst Med Trop Sao Paulo*, 2011	Brazil	Cross-sectional	NA	Discordant immunologic and virologic responses	CD4 counts persistently < 350 cells/µL over the last 6 mos (≥ 12 mos. of ART and ≥ 6 mos. of VS)	< 50 copies/mL for ≥ 6 mos.	[51]
72	Soria A, *PLoS One*, 2011	Italy	Cross-sectional	73 %	Immunological non responders (INR)	CD4 count < 200 cells/µL after > 12 mos. of ART	< 50 copies/mL over the last 6 mos.	[47]
73	Casotti JAS, *BMC Infect Dis*, 2011	Brazil	Cross-sectional	78 %	Paradoxical immunological response (PIR)	CD4 count < 350 cells/µL after ≥ 12 mos. of VS	< 50 copies/mL for ≥ 12 mos.	[104]
74	Fernandez S, *J Infect Dis*, 2011	Australia	Cross-sectional	95 %	$CD4^+$ T-cell deficiency	CD4 count < median (490 cells/µL) after > 12 mos. of ART and ≥ 6 mos. of VS	< 50 copies/mL for ≥ 6 mos.	[48]
75	Byakwaga H, *J Infect Dis*, 2011	Australia	Experimental	92 %	Suboptimal CD4+ T-cell response	CD4 count < 350 cells/µL in the last 6 mos. AND CD4 change < 50 cells/µL in the last 12 mos.	< 50 copies/mL in the last 9 mos.	[120]
76	Choi JY, *JAIDS*, 2011	International	Longitudinal	84 %	Virologic-only response (VR+IR-)	(CD4 count <350 cells/µL OR change < 50 cells/µL at 6 mos. of ART) OR (CD4 count < 350 cells/µL OR CD4 change < 100 cells/µL at 12 or 24 mos. of ART)	< 500 copies/mL at 6, 12 and 24 mos. of ART	[33]
77	Tanaskovic S, *AIDS*, 2011	Australia	Longitudinal	100 %	Poor recovery of CD4+ T cells	CD4 count < 500 cells/µL at > 12 mos. of ART	< 50 copies/mL for ≥ 6 mos.	[108]
78	Hunt PW, *J Infect Dis*, 2011	USA	Experimental	93 %	Incomplete CD4+ T cell recovery	CD4 count < 350 cells/µL for ≥ 12 mos. (after ≥ 6 mos. of ART)	Most patients with < 75 copies/mL	[91]
79	Merlini E, *PLoS One*, 2011	Italy	Longitudinal	77 %	Immunological non responders (INRs)	CD4 count < 200 cells/µL at 12 mos. of ART	< 60 copies/mL for ≥ 12 mos.	[78]
80	Hatano H, *J Infect Dis*, 2011	USA	Experimental	97 %	Suboptimal CD4+ T cell response	CD4 count < 350 cells/µL for ≥ 12 mos. of VS	< 75 copies/mL for ≥ 12 mos.	[121]
81	Nakanjako D, *BMC Infect Dis*, 2011	Uganda	Longitudinal	25 %	Suboptimal CD4 reconstitution	CD4 change < 25th percentile (199 cells/µL) at 48 mos. of ART compared to baseline	< 400 copies/mL for 48 mos.	[44]
82	Zoufaly A, *J Infect Dis*, 2011	Germany	Longitudinal	77 %	Immuno-virological discordance	CD4 count < 200 cells/µL at 0-6, 7-12, 13-24 or > 24 mos. of VS	< 50 copies/mL for 0-6, 7-12, 13-24 or > 24 mos.	[59]

TABLE S1 (Continued)

No.	First author, *Journal*, Year of publication	Country / International	Study type	Male percentage among total participants	Representative INR term	INR criteria (immune recovery surrogate, threshold and time point or period)	Viral load (threshold and time point or period)	Ref.
83	Grabmeier-Pfistershammer K, *JAIDS*, 2011	Austria	Cross-sectional	NA	Nonimmune reconstituters (non-IR)	CD4 count < 300 cells/μL OR CD4 change < 250 cells/μL for ≥ 12 mos. of VS	Below the detection limit (NA)	[103]
84	Bellistri GM, PLoS One, 2010	Italy	Cross-sectional	87 %	Immunological nonresponders (INRs)	CD4 count ≤ 200 cells/μL after ≥ 6 mos. of ART (mean ART length of 29 months)	≤ 50 copies/mL (time point or period NA)	[68]
85	Loutfy MR, *JAIDS*, 2010	Canada	Longitudinal	83 %	immunologic discordance	CD4 count < 200 cells/μL at 12 and 24 mos. of ART	< 50 copies/mL at 12 [9; 15] mos. of ART	[55]
86	Engsig FN, *BMC Infect Dis*, 2010	Denmark	Longitudinal	78 %	Immunological non-responders (INRs)	CD4 count ≤ 200 cells/μL after 36 mos. of VS	< 50 (199 or 399) copies/mL for ≥ 36 mos.	[70]
87	Marchetti G, *AIDS*, 2010	Italy	Cross-sectional	81 %	Immunological nonresponders (INRs)	CD4 count ≤ 200 cells/μL after ≥ 24 mos. of ART; CD4 nadir < 100 cells/μL	< 50 copies/mL over the last 6 months	[77]
88	Hermans SM, *PLoS One*, 2010	Uganda	Longitudinal	34 %	Suboptimal immune response	CD4 count < 200 cells/μL OR CD4 change < 200 cells/μL at 24 mos. of ART	< 400 copies/mL during the first 6-12 mos. of ART	[128]
89	Negredo E, *Clin Infect Dis*, 2010	Spain	Cross-sectional	77 %	Discordant patients	CD4 count always < 350 cells/μL for > 24 mos.	< 50 copies/mL for > 24 mos.	[43]
90	Massanella M, *AIDS*, 2010	Spain	Cross-sectional	77 %	Discordant patients	CD4 count < 350 cells/μL after > 24 mos. of VS	< 50 copies/mL for ≥ 24 mos.	[42]
91	Sachdeva N, *Viral Immunol*, 2010	USA	Longitudinal	78 %	Discordant patients	CD4 count < 200 cells/μL OR (CD4 count < 400 cells/μL, CD4 change ≤ 100 cells/μL and CD4:CD8 ratio < 1) after ≥ 12 mos. of VS	< 50 copies/mL for ≥ 12 mos.	[58]
92	Erikstrup C, *JAIDS*, 2010	Denmark	Longitudinal	94 %	Impaired CD4 cell recovery	CD4 count < 200 cells/μL after 36 mos. of VS	< 50 copies/mL for ≥ 36 mos.	[87]
93	Tuboi SH, *JAIDS*, 2010	International	Longitudinal	39 %	Virologic only response (VR+IR-)	CD4 change < 50 cells/μL at 6 [3; 9] mos. of ART compared to baseline	< 500 copies/mL at 6 mos. of ART	[130]
94	Woelk CH, *AIDS*, 2010	USA	Longitudinal	100 %	Poor CD4+ T-cell recovery group	CD4 change < 200 cells/μL at 12 mos. of ART	< 50 copies/mL during 12 mos.	[109]

TABLE S1 (Continued)

No.	First author, *Journal*, Year of publication	Country / International	Study type	Male percentage among total participants	Representative INR term	INR criteria (immune recovery surrogate, threshold and time point or period)	Viral load (threshold and time point or period)	Ref.
95	Gilson RJC, *HIV Med*, 2010	UK	Longitudinal	75 %	Discordant responders	CD4 change < 100 cells/µL at 6 [6; 10] or 12 [10;14] mos. of ART compared to baseline	< 50 copies/mL within 6 mos. of ART	[52]
96	Magen E, *Int J Infect Dis*, 2010	Israel	Experimental	61 %	Immunological non-responders	CD4 count < 300 cells/µL AND CD4 change < 10% after ≥ 12 mos. of ART compared to baseline	< 400 copies/mL for > 12 mos.	[76]
97	Pacheco YM, *Curr HIV Res*, 2009	Spain	Longitudinal	75 %	low-level CD4 counts repopulation group (Low-group)	CD4 count < 250 cells/µL at 24 mos. of ART	< 1000 copies/mL during the first 24 mos. of ART	[99]
98	Mavigner M, *PLoS One*, 2009	France	Cross-sectional	87 %	Poor immunological responders	CD4 change < 200 cells/µL after 60 to 94 (IQR) mos. of ART	< 50 copies/mL for 84 mos. (median)	[116]
99	Molina-Pinelo S, *J Antimicrob Chemother*, 2009	Spain	Cross-sectional	86 %	Low-level CD4 T cell repopulation patients	CD4 count ≤ 250 cells/µL after ≥ 12 mos. of ART	< 50 copies/mL for ≥ 12 mos.	[98]
100	Stepanyuk O, *AIDS*, 2009	USA	Longitudinal	100 %	Impaired CD4 recovery	CD4 count < 250 cells/µL ≥ 12 mos. of VS	< 50 copies/mL for ≥ 12 mos.	[88]
101	Önen NF, *HIV Med*, 2009	USA	Longitudinal	75 %	Sub-optimal CD4 recovery	CD4 change < 150 cells/µL until 12 mos. of ART compared to baseline	< 400 (before 1999) or < 50 (thereafter) copies/mL at 12 mos.	[122]
102	van Griensven J, *JAIDS*, 2009	Rwanda	Longitudinal	27 %	Virologic-only response (VR+IR-)	CD4 change < 50 cells/µL at 12 mos. of ART compared to baseline	< 40 copies/mL at 12 mos. of ART	[131]
103	Falster K, *JAIDS*, 2009	Australia	Longitudinal	94 %	Incomplete immune response	CD4 counts < 350 cells/µL in the 12–24 months after starting the first or second cART regimen	< 400 copies/mL at ≥ 9 mos.	[93]

Note: Publications are ordered by year of publication.

ART, antiretroviral therapy; cART, combination antiretroviral therapy; CD4 count, $CD4^+$ T cell count; CD4 change, $CD4^+$ T cell count change; INR, immunological non-response; IQR, interquartile range; NA, not available; Ref., reference; SEM, standard error of mean; UK, United Kingdom; USA, United States of America; VS, virologic suppression.

TABLE S2 - List of scientific journals in which the included records were published, and number of publications per journal.

	Journal title (*NLM Abbreviation*)	No. of publications
1	Antimicrobial agents and chemotherapy (*Antimicrob Agents Chemother*)	1
2	Antiviral therapy (*Antivir Ther*)	1
3	Archivum immunologiae et therapiae experimentalis (*Arch Immunol Ther Exp*)	1
4	Bioscience trends (*Biosci Trends*)	1
5	Blood (*Blood*)	1
6	BMC research notes (*BMC Res Notes*)	1
7	Clinical and experimental immunology (*Clin Exp Immunol*)	1
8	Current HIV research (*Curr HIV Res*)	1
9	Doklady biological sciences (*Dokl Biol Sci*)	1
10	Doklady. Biochemistry and biophysics (*Dokl Biochem Biophys*)	1
11	Frontiers in microbiology (*Front Microbiol*)	1
12	HIV clinical trials (HIV Clin Trials)	1
13	Human immunology (*Hum Immunol*)	1
14	Immunobiology (*Immunobiology*)	1
15	Immunology letters (*Immunol Lett*)	1
16	International journal of infectious diseases (*Int J Infect Dis*)	1
17	International journal of STD & AIDS (*Int J STD AIDS*)	1
18	Journal of immunology research (*J Immunol Res*)	1
19	Journal of infection and chemotherapy (*J Infect Chemother*)	1
20	Journal of infection and public health (*J Infect Public Health*)	1
21	Journal of the International AIDS Society (*J Int AIDS Soc*)	1
22	Journal of translational medicine (*J Transl Med*)	1
23	Medecine et maladies infectieuses (*Med Mal Infect*)	1
24	PLoS pathogens (*PLoS Pathog*)	1
25	Revista do Instituto de Medicina Tropical de Sao Paulo (*Rev Inst Med Trop Sao Paulo*)	1
26	Scientific reports (*Sci Rep*)	1
27	The Korean journal of internal medicine (*Korean J Intern Med*)	1
28	Tropical medicine & international health (*Trop Med Int Health*)	1
29	Viral immunology (*Viral Immunol*)	1
30	Antiviral research (*Antiviral Res*)	2
31	BMC immunology (*BMC Immunol*)	2
32	Frontiers in immunology (*Front Immunol*)	2
33	Japanese journal of infectious diseases (*Jpn J Infect Dis*)	2
34	Clinical infectious diseases (*Clin Infect Dis*)	3
35	HIV medicine (*HIV Med*)	3
36	BMC infectious diseases (*BMC Infect Dis*)	5
37	The Journal of antimicrobial chemotherapy (*J Antimicrob Chemother*)	5
38	The Journal of infectious diseases (*J Infect Dis*)	7
39	Journal of acquired immune deficiency syndromes (*J Acquir Immune Defic Syndr, JAIDS*)	14
40	PloS one (PLoS One)	14
41	AIDS - London, England (*AIDS*)	15

TABLE S3 - Countries involved in the large international collaborations/consortia.

International project	Countries/Region
Antiretroviral Therapy Cohort Collaboration (ART-CC)	Austria, Canada, Denmark, France, Germany, Italy, Netherlands, Spain, Switzerland, United Kingdom, United States of America
Antiretroviral Therapy in Lower Income Countries Collaboration (ART-LINC)	Africa (Botswana, Burundi, Cameroon, Democratic Republic of Congo, Côte d'Ivoire, Kenya, Malawi, Morocco, Nigeria, Rwanda, Senegal, South Africa, and Uganda), Brazil, India, Thailand
Collaboration of Observational HIV Epidemiological Research Europe (COHERE)	Argentina, Austria, Belarus, Belgium, Bosnia-Herzegovina, Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Georgia, Greece, Hungary, Iceland, Ireland, Israel, Italy, Latvia, Lithuania, Luxembourg, Netherlands, Norway, Poland, Portugal, Romania, Russia, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Ukraine, United Kingdom
EuroSIDA Study Group	Argentina, Austria, Belarus, Belgium, Bosnia-Herzegovina, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Georgia, Greece, Hungary, Iceland, Ireland, Israel, Italy, Latvia, Lithuania, Luxembourg, Netherlands, Norway, Poland, Portugal, Romania, Russia, Serbia, Slovenia, Spain, Sweden, Switzerland, Ukraine, United Kingdom
TREAT Asia HIV Observational Database (TAHOD)	Australia, China, India, Malaysia, Philippines, Singapore, Taiwan, Thailand, United States of America

TABLE S4 - Countries of patients' enrolment and the respective classification by the income in 2017.

Countries (n = 30)	Country classification by income (Gross National Income per capita in 2017)
Rwanda Senegal Tanzania Uganda	Low-income country ($995 or less)
Nigeria	Lower-middle-income ($996 to $3895)
Botswana Brazil China Malaysia Russia South Africa Thailand	Upper-middle-income ($3896 to $12055)
Australia Austria Canada Denmark France Germany Israel Italy Japan Netherlands Norway Oman Poland Portugal South Korea Spain United Kingdom United States of America	High-income ($12056 or more)

Reference: The World Bank. How we classify countries. Available at: https://datahelpdesk.worldbank.org/knowledgebase/articles/906519-world-bank-country-and-lending-groups. Accessed on November 23rd, 2018.

CHAPTER 4.
HIV Infection and Thymic Function

4.1 – Thymic Function as a Predictor of Immune Recovery in Chronically HIV-Infected Patients Initiating Antiretroviral Therapy

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Thymic Function as a Predictor of Immune Recovery in Chronically HIV-Infected Patients Initiating Antiretroviral Therapy

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Poor immunological responders (PIR) are HIV-infected patients with virologic suppression upon antiretroviral therapy (ART) but persistently low $CD4^+$ T cell counts. Early identification of PIR is important given their higher morbimortality compared to adequate immune responders (AIR). In this study, 33 patients severely lymphopenic at ART onset, were followed for at least 36 months, and classified as PIR or AIR using cluster analysis grounded on their $CD4^+$ T cell count trajectories. Based on a variety of immunological parameters, we built predictive models of PIR/AIR outcome using logistic regression. All PIR had $CD4^+$ T cell counts consistently below 500 cells/μL, while all AIR reached this threshold. AIR showed a higher percentage of recent thymic emigrants among $CD4^+$ T cells; higher numbers of sj-TRECs and greater sj/β TREC ratios; and significant increases in thymic volume from baseline to 12 months of ART. We identified mathematical models that correctly predicted PIR/AIR outcome after 36 months of therapy in 77–87% of the cases, based on observations made until 2–6 months after ART onset. This study highlights the importance of thymic activity in the immune recovery of severely lymphopenic patients, and may help to select the patients that will benefit from closer follow-up or novel therapeutic approaches.

Keywords: poor immunological responders, predictive modeling, immune recovery, $CD4^+$ T cells, thymic function, immune activation, antiretroviral therapy, HIV infection

INTRODUCTION

The majority of patients infected by the human immunodeficiency virus (HIV) show immune recovery upon antiretroviral therapy (ART). This recovery usually occurs with a rapid increase in $CD4^+$ T cell numbers during the first few months, followed by a slower increase toward a plateau (1–3). An adequate immune recovery is defined as the attainment of $CD4^+$ T cell counts within the range observed in healthy adult individuals (i.e., 500–1,500 cells/μL). However, $CD4^+$ T cell counts remain persistently low in 9–45% of patients, depending on the criteria used, despite complete viral

suppression (i.e., undetectable plasma HIV RNA) (4–6). There is no consensual case-definition for these patients, and dozens of different terms are used in the literature to refer to them, such as "poor immunological responders", "immunological non-responders", and "discordant immune responders" (5). In this study, we use the terms poor and adequate immunological responders (PIR and AIR), because these seem to be the terms that best reflect patients' immunologic response to therapy.

Distinct immune recovery trajectories throughout ART result from differences in $CD4^+$ T cell production by the thymus, peripheral proliferation, virus- or immune-mediated cell death and/or ability to obtain survival stimuli. Differences in recovery may also derive from variations in the extent of $CD4^+$ T cell migration from lymphoid tissues to peripheral blood or to the gastrointestinal tract soon after ART initiation (1, 7, 8).

Production of new T cells by the thymus appears to be a major driver of immune recovery during ART, being particularly relevant for patients who start therapy with <200 $CD4^+$ T cells/μL, in whom poor immune responses are more frequent (9–16). Many other factors have been associated with poor immune recovery, such as older age, lower nadir $CD4^+$ T cell counts, residual viral replication, increased T cell death, immune hyperactivation, altered ratio of regulatory T cells (Treg) to Th17 cells, tissue fibrosis [reviewed in (17, 18)] and specific metabolic profiles (19).

PIR have higher morbidity and mortality rates than AIR (20–22). Therefore, there is an urgent need for tools to identify PIR early and improve their prognoses. This study discriminated PIR and AIR among patients with acquired immunodeficiency syndrome (AIDS) and <200 $CD4^+$ T cells/μL at ART onset, not using any of the many clinical criteria reported in the literature, but instead using clustering analysis of longitudinal data. For this analysis, we simply specified that patients should be divided into 2 clusters on the basis of their $CD4^+$ T cell count trajectories. *A posteriori* analysis of the trajectories revealed that one cluster included patients whose trajectories reached higher $CD4^+$ T cell counts, with all patients in that cluster presenting > 500 $CD4^+$ T cells/μL at least at one time point over the first 36 months of therapy (AIR), contrary to patients of the other cluster (PIR). In addition, immunological parameters were compared between PIR and AIR and early alterations were identified as predictors of PIR status after 36 months of therapy.

MATERIALS AND METHODS

Study Participants

Patients infected by HIV, with <200 $CD4^+$ T cells/μL at ART initiation and with ≥36 months of follow-up were selected (n=33) from a prospective cohort of patients (n=100 individuals; **Figure S1**) on medical care at the Centro Hospitalar do Porto, Portugal. The enrolment period ran between April 2010 and October 2012. All patients were provided an explanation of the study and signed an informed consent (local Ethical Committee approval—reference 168/CES); were older than 18 years, chronically infected with HIV-1, ART-naïve at enrolment and with clinical criteria to initiate ART. ART schemes chosen for each individual took into consideration national and international guidelines. All patients were therapy compliant throughout the follow-up; after a median time of 6 months of ART, all patients presented sustained plasma viral loads below 50 copies/mL, except for 4 individuals who had viral blips (**Figure S2**). Clinical information and peripheral blood samples were retrieved at baseline (just before ART initiation) and at 2, 6, 12, 16, 20, 24, 28, 32, 36, 42, 48, 54, and 60 months of ART (median time deviations to each time point was ≤8 days). Individuals were followed for at least 36 months, with median follow-up time of 60 months. $CD4^+$ T cell counts and plasma viral load quantification were assessed at all available time points by a certified laboratory.

Imaging

Sixteen of the 33 patients underwent chest computed tomography (CT) scans at baseline and at 12 months of ART (**Figure S1**). CT scans were performed without contrast in a Siemens Somatom emotion apparatus (16 sections). Thymic volume was considered as the mean of measurements, blindly performed by two independent operators, in cm^3. Thymic index, assessed by one of the operators, was determined by scoring the presence of thymic tissue as opposed to adipose tissue: (0) thymus entirely replaced by fat; (1) minimal, barely recognizable, soft tissue; (2) minimal, but more obvious, soft tissue; (3) moderate soft tissue; (4) moderate soft tissue of greater extent, almost mass like; (5) mass-like appearance that raises concern for a thymoma (23). Both operators were blinded to any demographic or clinical data besides the HIV serostatus.

Blood Processing and Flow Cytometry (FACS) Analysis

For each participant and at each time point, venous blood was collected to K_2EDTA collecting tubes and processed on the same day. A blood aliquot for FACS analysis was taken and, from the remaining blood, PBMCs were isolated by gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, United Kingdom). After PBMCs' enumeration, 2×10^6 cells were used for FACS staining and 1×10^6 cells aliquots were stored at −80 °C for TRECs quantification.

For FACS, three antibody panels were design for evaluation of T cell activation (Panel 1, performed in 100 μL of whole blood), recent thymic emigrants (RTE; Panel 2, performed in 200 μL of whole blood) and Treg (Panel 3, performed in 2×10^6 PBMCs), as previously described (24). A mixture of anti-CD45RA-FITC (HI100), anti-CD69-PE (FN50), anti-CD45RO-PerCP/Cy5.5 (UCHL1), anti-HLA-DR-PeCy7 (L243), anti-CD8-APC (RPA-T8), anti-CD4-APC/Cy7 (RPA-T4), and anti-CD3-Pacific Blue (OKT3) was used for Panel 1; a mixture of anti-CD45RA-FITC (HI100), anti-CD3-PE (OKT3), anti-CD45RO-PerCP/Cy5.5 (UCHL1); anti-CD31-PeCy7 (WM59), anti-CCR7-Alexa Fluor 647 (G043H7), anti-CD4-APC/Cy7 (RPA-T4), and

Abbreviations: ACC, accuracy; AIC, Akaike information criterion; AIR, adequate immunological responders; ART, antiretroviral therapy; AUC, area under the curve; CT, computed tomography; IQR, interquartile range; LRT, likelihood ratio test; PBMCs, peripheral blood mononuclear cells; PIR, poor immunological responders; RTE, recent thymic emigrants; TRECs, T cell receptor excision circles; Treg, regulatory T cells.

anti-CD8-Brilliant Violet 421 (RPA-T8) was used for Panel 2; and a mixture of anti-Ki67-FITC (MOPC-21), anti-FoxP3-PE (PCH101), anti-CD127-PerCP/Cy5.5 (AO19D5), anti-CD31-PeCy7 (WM59), anti-CD25-APC (BC96), anti-CD4-APC/Cy7 (RPA-T4), anti-CD45RA-Pacific Blue (HI100), and anti-CD3-V500 (UCHT1) was used for Panel 3. All antibodies were from BioLegend, except for anti-CD3-V500 (BD Horizon), anti-FoxP3-PE (eBiosciences) and anti-Ki67 (BD Pharmigen). Samples were acquired using a BD LSRII flow cytometer using FACS DIVA software (Becton and Dickinson, NJ, USA); data were analyzed using FlowJo Software (Tree Star, OR, USA). Gating strategies are depicted in **Figure S3**.

Quantification of T Cell Receptor Excision Circles (TRECs)

TRECs were quantified at baseline, 6, 12, 24, and 36 months of ART in 14 of the 33 patients (**Figure S1**). Quantification of sj-TRECs and DJβ1-TRECs was performed by nested polymerase chain reaction (PCR), using primers and standard curve's plasmids as previously described (25, 26). Briefly, 1×10^6 PBMCs were lysed in Tween-20 (0.05%), nonidet P-40 (NP-40; 0.05 %), and Proteinase K (100 μg/mL) for 30 min at 56 °C followed by 15 min at 98 °C. Multiplex PCR amplification was performed using the specific "out" primers for sj-TREC or for each of the 6 DJβ1-TREC, together with the CD3 chain (10 min at 95 °C followed by 22 cycles of 30 s at 95 °C, 30 s at 60 °C and 3 min at 72 °C) using the 5′/3′ out primer pairs. Following the first round of amplification, each PCR product was 100-fold diluted; from the same diluted PCR product, TREC and CD3 were quantified in independent qPCR runs using a LightCycler® system (Roche Diagnostics, Basel, Switzerland). Using the specific "in" primers, qPCR amplification was as follows: 15 min at 95 °C, followed by 45 cycles of 1 s at 95 °C, 15 s at 55 °C and 10 s at 72 °C; fluorescence was measured at the end of each cycle. Results were expressed both as number of TRECs/10^5 cells and number of TRECs/mL.

Statistical Analysis

Statistical analysis was performed in the computing environment R (v3.4.2) using RStudio (v1.1.383). Overall, a p-value <0.05 (α) was considered statistically significant; for multiple comparisons, Bonferroni corrections were applied and differences or effects that remained significant ($<$ α/number of comparisons) are indicated in the Figures with a *.

The R package *traj* (v1.2) was used to identify two clusters of patients based on their $CD4^+$ T cell counts trajectories from baseline to 36 months of ART. The package implements the 3-step procedure proposed by Leffondré et al. (27) to identify clusters of longitudinal trajectories: first, 24 summary measures (**Table S1**) that describe features of the trajectories were calculated (28); second, a principal component analysis was performed on these 24 measures to select measures that best describe the main features of the trajectories, based on eigenvalues (>1); finally, trajectories were classified into clusters based on the previously selected factors.

We used the *nlme* package (v3.1.137) in R to fit nonlinear mixed effects models to the $CD4^+$ T cell count and the CD4/CD8 ratio data, based on the exponential model described by Li et al. (9):

$$X(t) = A + B\,[1 - exp(-t\,/\,\tau)]$$

where $X(t)$ represents $CD4^+$ T cell count or CD4/CD8 ratio at one given time point t; A represents the theoretical $CD4^+$ T cell count or CD4/CD8 ratio at baseline; B represents the theoretical maximum increase that could be reached; t refers to time (in months) since ART initiation; and τ is a constant representing the timescale over which $X(t)$ reaches its maximum value. The arguments used in the $CD4^+$ T cell count models were: fixed = list ($A \sim 1$, $B \sim$ group, $\tau \sim$ group) and random = $A + B \sim 1 \mid$ Patient_ID, where Patient_ID correspond to the identification of each patient. The arguments used in the CD4/CD8 ratio models were: fixed = list ($A \sim 1$, $B \sim$ group, $\tau \sim 1$) and random = $A + B + \tau \sim 1 \mid$ Patient_ID.

Normality of data distributions was assessed by the Shapiro-Wilk test. Correlations were assessed using the Pearson or Spearman rank tests, depending on the underlying distribution. For quantitative data, depending on the underlying distributions, comparisons between two independent groups were performed using an independent t-test or Wilcoxon/Mann-Whitney U-test, unless otherwise stated. Cohen's d or r, respectively, was calculated as a measure of effect size. Within-group comparisons between two time points were performed with the paired-samples t-test (for approximately normally-distributed data); in this case, Cohen's d was calculated based on the standard deviation of the differences. For qualitative data, comparison was performed using Fisher exact test; effects sizes were described with the odds ratio (dichotomous variables) or Cramér's V (nominal variables with >2 categories) (29).

Effect sizes were assessed as follows:

- Cohen's d: small if <0.3; medium if [0.3,0.8]; or large if ≥0.8;
- r: small if <0.3; medium if [0.3, 0.5]; or large if ≥0.5;
- Cramér's V: small if <0.3; medium if [0.3,0.7]; or large if > 0.7;
- Correlation coefficient (r or ρ): negligible if <0.3; low if [0.3; 0.5]; moderate if [0.5; 0.7]; high if [0.7; 0.9]; or very high if [0.9; 1.0].

All statistical tests performed were two-tailed. Extreme outliers were defined as lower than P25 – 3 × Interquartile range (IQR) or higher than P75 + 3 × IQR. All tests were performed with and without extreme outliers to verify their effect on the p-value and all results pointed to the same direction (with significant or non-significant p-values) after exclusion of the extreme outliers. All the results reported included the extreme outliers.

Logistic regression analysis was conducted using the *glm()* function in R (v3.4.3) to identify associations between independent variables, assessed between baseline and 6 months on ART (inclusively), and the dependent variable "Being AIR or PIR" (Y = 1 for "AIR"; Y = 0 for "PIR"). Selection of variables for the multivariate analysis included a univariable logistic regression analysis for each candidate to detect potential predictors (p-value < 0.20). A backward stepwise selection procedure was performed, analyzing the significance of the coefficient for each variable at each step, until no further

improvement was possible. The adequacy of the final model was assessed by means of a likelihood ratio test (LRT, reduced model vs. full model). The Akaike information criterion (AIC) was used to compare models with the same observations. The Hosmer-Lemeshow test for goodness-of-fit was applied to the final model and diagnostic plots were carried out as well, including ROC curve. The area under the curve (AUC) statistic was also calculated as a measure of goodness-of-fit, considering: [0.5; 0.7], poor; [0.7; 0.8], fair; [0.8; 0.9], good; and >0.9, excellent. For each model, all patients were classified as "probably PIR" (probability of being PIR $\geq$0.5) or "probably AIR" (probability of being PIR <0.5). Sensitivity and specificity were calculated as the percentage of "probably PIR" among PIR and the percentage of "probably AIR" among AIR, respectively. Accuracy (ACC) was calculated as the percentage of patients PIR or AIR that were respectively classified as "probably PIR" or "probably AIR" among all patients. Relevant R code is available in **Supplementary Material**.

RESULTS

Analysis of CD4$^+$ T Cell Counts Trajectories Identified Two Clusters of Patients With Distinct Kinetics of Immune Recovery

Demographic and clinical characteristics of the patients are presented in **Table 1**.

The CD4$^+$ T cell count trajectories of the 33 patients were automatically analyzed using R *traj* package, and two clusters were formed (**Figure 1A**; **Table S2**). From the summary measures considered for each trajectory (**Table S1**), the main descriptors of the CD4$^+$ T cell count trajectories were range and change relative to the mean over time.

All patients in cluster 1 ($n = 14$) had at least one CD4$^+$ T cell count above 500 cells/μL during the first 36 months of ART, and were classified as AIR. Patients in cluster 2 ($n = 19$) had all CD4$^+$ T cell counts bellow 500 cells/μL, during the same time period, and were classified as PIR (**Figures 1A**, **S4A,B**).

At baseline, PIR tended to be older, with lower CD4/CD8 ratios, and had significantly lower plasma viral loads; there were no significant differences in baseline CD4$^+$ cell counts between PIR and AIR (**Table 1**). After 12 months of ART, AIR exhibited significantly higher CD4$^+$ T cell counts than PIR. This difference became progressively more evident throughout follow-up (**Figure 1A**). AIR consistently presented higher median rates of increase of CD4$^+$ T cell counts during the first 12 months of ART (**Table 2**). The CD4/CD8 ratio tended to be lower in PIR than AIR at all time points (**Figures 1B**, **S4C,D**).

The adjusted kinetics for CD4$^+$ T cell counts and CD4/CD8 ratio in PIR, obtained using nonlinear mixed effects models, were:

$$\text{CD4 count } (t) = 130.1 + 284.5\,[1 - exp(-t/31.9)\,]$$
$$\text{CD4/CD8 } (t) = 0.163 + 0.475\,[1 - exp(-t/27.620)]$$

and in AIR:

$$\text{CD4 count } (t) = 130.1 + 499.0\,[1 - exp(-t/17.6)\,]$$
$$\text{CD4/CD8 } (t) = 0.163 + 0.644\,[1 - exp(-t/27.620)]$$

These kinetics indicate that on long-term ART, mean CD4$^+$ T cell counts among PIR asymptoted to 414.6 cells/μL and the CD4/CD8 ratio to 0.638; these quantities stabilized at 629.1 and 0.807, respectively, in AIR (**Figures 1C,D**).

CD4$^+$ T Cell Proliferation and Activation Status Did Not Differ Between PIR and AIR

We did not detect differences in CD4$^+$ T cell division rates, as assessed by Ki67 expression (**Figures 2A,B**, **S5A,B**), nor in their activation status (CD69$^+$ cells; **Figure 2C**). Absolute numbers of CD69$^+$CD4$^+$ T cells were higher in AIR at 12 months of ART, reflecting their greater CD4$^+$ T cell counts (**Figures 2D**, **S5C,D**).

Diverse immune recoveries have been associated with differences in CD4$^+$ T cell proliferation and activation (30–32). To assess whether the apparent discrepancy between these associations and our results could be related to CD4$^+$ T cell counts at baseline, we analyzed a group of HIV-infected patients who started ART with CD4$^+$ T cell counts in the range 200–350 cells/μL, who achieved full virologic suppression and adequate immune recovery (referred to as HIV-infected$_{\text{BL}[200-350]}$). These individuals presented lower percentages of Ki67$^+$ cells and of CD69$^+$ cells among CD4$^+$ T cells than PIR or AIR at baseline (**Figure S7**).

No substantial differences were observed between AIR and PIR in either percentage or absolute numbers of Treg (CD25$^{\text{high}}$CD127$^-$Foxp3$^+$ CD4$^+$ T cells; **Figures 2E,F**, **S5E,F**).

The naïve/memory CD4$^+$ T cell ratio (i.e., CD45RA$^+$CD45RO$^-$/CD45RA$^-$CD45RO$^+$) was tendentially higher in AIR than in PIR at baseline and 6 months, and significantly higher at 12, 24, and 36 months of ART (**Figures 2G**, **S4E,F**), which is consistent with naïve cells contributing more to CD4$^+$ T cell recovery in AIR than in PIR.

AIR Presented Higher Thymic Function Since Baseline, Independently of Age Differences

Thymic function was characterized using three distinct metrics: (1) assessment of thymic index (defined in the imaging subsection of the Methods) and volume, at baseline and 12 months of ART; (2) quantification of peripheral blood TRECs at baseline, 6, 12, 24, and 36 months of ART); and (3) quantification of recent thymic emigrants (RTE; CD4$^+$CD3$^+$CD45RA$^+$CD31$^+$) in blood, at baseline, 6, 12, 24, and 36 months of ART. AIR and PIR presented similar thymic volumes at baseline and at 12 months of ART. However, AIR exhibited significant increases in thymic volume during that period, while PIR did not (variations of 6.47 $\pm$ 4.59 cm^3 vs. 1.43 $\pm$ 4.89 cm^3, respectively; **Figure 3A**). PIR tended to present lower thymic index, both at baseline and 12 months of ART (**Figure 3B**). A thymic score was calculated as the product of thymic volume and index means, over the first year of ART. AIR

TABLE 1 | Demographic and clinical characteristics of HIV-infected patients included in the study.

	Total ***n* = 33**	**AIR** ***n* = 14**	**PIR** ***n* = 19**	**AIR vs. PIR**
Gender, male:female, *n* (%)	23:10 (70%: 30%)	9:5 (64%: 36%)	14:5 (74%: 26%)	$p = 0.707$[a], OR = 0.64
Age at baseline, in years				
Mean ± SD	42.2 ± 10.4	38.1 ± 8.6	45.3 ± 10.8	$t_{(31)} = -2.032$, $p = 0.051$[b],
Range [Min; Max]	[23; 67]	[23,51]	[28; 67]	Cohen's $d = 0.72$
HIV viral load at baseline, in log_{10} copies per mL, Median [Min; Max]	5.57 [4.78; 6.98]	5.96 [5.25; 6.98]	5.41 [4.78; 6.84]	**$U = 203$, $p = 0.011$[c], $r = -0.44$**
CD4$^+$ T cell count at baseline, in cells/μL, Median [Min; Max]	67 [8; 193]	93 [12; 193]	61 [8; 182]	$U = 167$, $p = 0.229$[c], $r = -0.21$
CD4/CD8 ratio at baseline,				
Mean ± *SD*	0.122 ± 0.095	0.160 ± 0.113	0.095 ± 0.070	$t_{(31)} = 2.032$, $p = 0.051$[b],
Range [Min; Max]	[0.010; 0.373]	[0.017; 0.373]	[0.010; 0.272]	Cohen's $d = 0.72$
Clinical categories*, *n* (%)				
A (Asymptomatic or acute HIV infection)	3 (9%)	1 (7%)	2 (11%)	
B (not A or C)	14 (42%)	4 (29%)	10 (53%)	$p = 0.262$[a], Cramér's V = 0.27
C (AIDS-defining conditions)	16 (48%)	9 (64%)	7 (37%)	
Co-infections at baseline, *n* (%)				
HCV	9 (27%)	2 (14%)	7 (37%)	$p = 0.241$[a], OR = 3.50
HBV	1 (3%)	1 (7%)	0 (0%)	$p = 0.424$[a], OR = 0.00
HIV subtype, *n* (%)				
B	13 (39%)	5 (36%)	8 (42%)	
G	11 (33%)	4 (29%)	7 (37%)	
C	4 (12%)	2 (14%)	2 (11%)	$p = 0.633$[a], Cramér's V = 0.40
Other	4 (12%)	3 (21%)	1 (5%)	
Unknown	1 (3%)	0 (0%)	1 (5%)	
HIV transmission mode, *n* (%)				
Intravenous drug user	6 (18%)	1 (7%)	5 (26%)	
Men who have sex with men	9 (27%)	5 (36%)	4 (21%)	
Heterosexual	15 (45%)	6 (43%)	9 (47%)	$p = 0.567$[a], Cramér's V = 0.27
Other	2 (6%)	1 (7%)	1 (5%)	
Unknown	1 (3%)	1 (7%)	0 (0%)	
Time from diagnosis to baseline, in months, Median [Min; Max]	5 [0; 128]	23 [1; 85]	3 [0; 128]	$U = 179$, $p = 0.096$[c], $r = -0.29$
ART regimen components, *n* (%)				
2 NRTIs: TDF$^+$FTC	29 (88%)	14 (100%)	15 (79%)	
ABC$^+$3TC	3 (9%)	0 (0%)	3 (16%)	$p = 0.244$[a], Cramér's V = 0.32
AZT$^+$3TC	1 (3%)	0 (0%)	1 (5%)	
3rd Drug: EFV	25 (76%)	10 (71%)	15 (79%)	
DRV/r	6 (18%)	4 (29%)	2 (11%)	$p = 0.473$[a], Cramér's V = 0.30
LPV/r	1 (3%)	0 (0%)	1 (5%)	
NVP	1 (3%)	0 (0%)	1 (5%)	
HIV drug resistance, *n* (%)				
Yes	11 (33%)	3 (21%)	8 (42%)	
No	21 (64%)	11 (79%)	10 (53%)	$p = 0.266$[a], OR = 0.34
Unknown	1 (3%)	0 (0%)	1 (5%)	

**Based on Centers for Disease Control and Prevention (CDC) Classification System.*

[a]Fisher's exact test. [b]Independent t-test. [c]Wilcoxon/Mann-Whitney U test.

Values in bold indicate statistically significant difference ($p < 0.05$) between AIR and PIR. ABC, abacavir; DRV/r, ritonavir boosted darunavir; EFV, efavirenz; FTC, emtricitabine; HBV, Hepatitis B virus; HCV, Hepatitis C virus; LPV/r, ritonavir boosted lopinavir; NRTI, nucleoside or nucleotide analogue reverse transcriptase inhibitors; NVP, nevirapine; OR: odds ratio; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine.

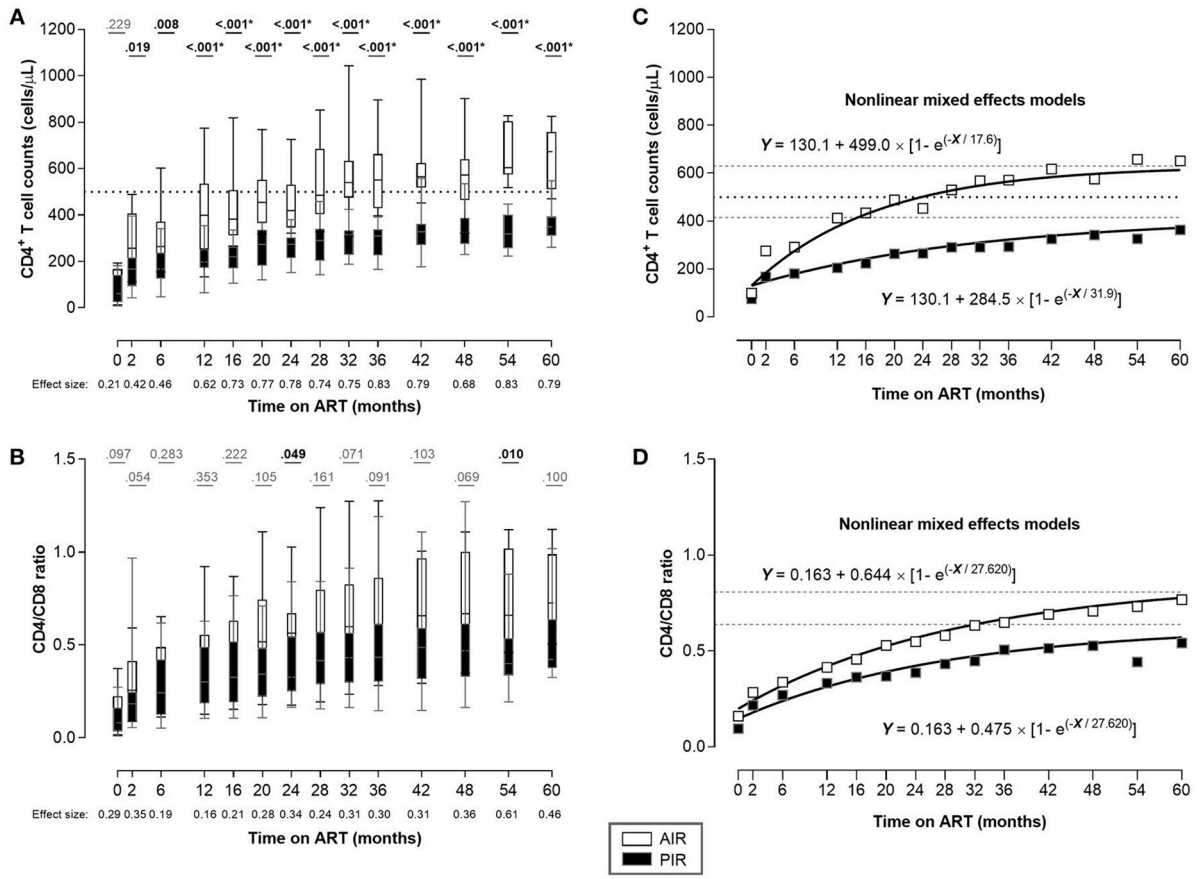

FIGURE 1 | Despite having similar CD4$^+$ T cell counts at ART onset, AIR reached higher CD4$^+$ T cell counts as soon as 2 months after ART initiation. **(A)** CD4$^+$ T cell counts and **(B)** CD4/CD8 ratio were compared throughout ART between AIR and PIR. A Wilcoxon-Mann-Whitney *U*-test was performed to compare the two groups of patients at each time point, and the correspondent *r* is presented as a measure of effect size. *Significant after Bonferroni correction ($\alpha/14 = 0.004$). **(C**,**D)** Nonlinear mixed effects models for CD4$^+$ T cell count and CD4/CD8 ratio evolutions, respectively. White squares represent AIR mean of CD4$^+$ T cell count **(C)**, or CD4/CD8 ratio **(D)** at each time point; black squares represent PIR mean of CD4$^+$ T cell count **(C)**, or CD4/CD8 ratio **(D)** at each time point. Black dotted lines in **(A)** and **(C)** correspond to Y = 500. Gray dashed lines correspond to the asymptotes: Y = 629.1 and Y = 414.6 **(C)**; Y = 0.807 and Y = 0.638 **(D)**, respectively.

TABLE 2 | CD4$^+$ T cell count slopes during ART.

	CD4$^+$ T cell count slope, in cells/μL/month[a], Median [P25; P75]			
Time interval during ART, in months	**Total** ***n* = 33**	**AIR** ***n* = 14**	**PIR** ***n* = 19**	**AIR vs. PIR**
[0; 2]	57 [29; 102]	87 [50; 107]	48 [21; 69]	***U* = 191, *p* = 0.036[b], *r* = 0.36**
[2,12]	7 [2,13]	14 [10,17]	3 [1,7]	***U* = 228, *p* < 0.001[b], *r* = 0.60**
[12,24]	5 [2,10]	6 [-1; 13]	5 [3,10]	*U* = 135, *p* = 0.956[b], *r* = 0.01
[24,36]	3 [1,9]	12 [2,17]	2 [1,4]	***U* = 198, *p* = 0.019[b], *r* = 0.41**
[36,48]	3 [0; 8]	2 [−4; 10]	3 [1,7]	*U* = 68, *p* = 0.544[b], *r* = 0.12
[48,60]	5 [1,9]	7 [1,11]	5 [1,7]	*U* = 23, *p* = 0.830[b], *r* = 0.06

[a] *CD4 count slopes from time point t_i to t_f were calculated for each individual by the least squares estimation method, including all the available CD4 counts between t_i and t_f.*
[b] *Wilcoxon/Mann–Whitney U-test.*
Bold values indicate statistically significant difference ($p < 0.05$) between AIR and PIR.

had a tendency to present higher thymic scores (**Figure 3C**). We also compared thymic volume, index and score between HIV-infected$_{BL[200-350]}$ and AIR and PIR, respectively. Interestingly, AIR, but not PIR, presented higher thymic volume at 12 months of ART and higher thymic score than HIV-infected$_{BL[200-350]}$ (**Figure S8**).

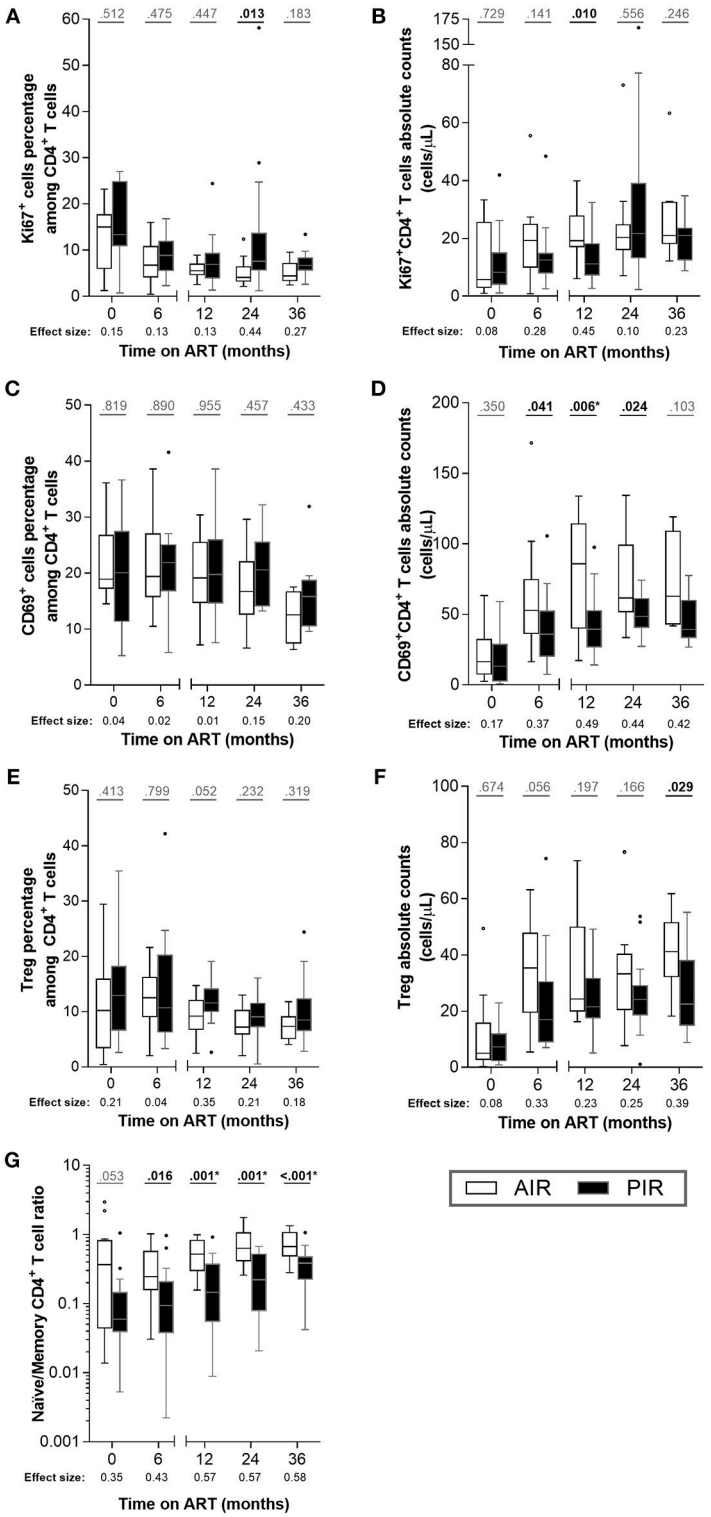

FIGURE 2 | Throughout ART, PIR and AIR presented no major differences in $CD4^+$ T cell activation status, proliferation, Treg numbers, nor naïve/memory $CD4^+$ T cell ratio. **(A)** The percentage among $CD4^+$ T cells and **(B)** absolute number of $Ki67^+$ cells, **(C,D)** $CD69^+$ cells, **(E,F)** Treg ($CD25^{high}CD127^-FoxP3^+CD4^+$ T cells) and **(G)** naïve/memory $CD4^+$ T cell ratio were compared throughout therapy in AIR and PIR. A Wilcoxon-Mann-Whitney *U*-test was performed to compare the two groups of patients at each time point, and the corresponding *r* is presented as a measure of effect size. *Significant after Bonferroni correction ($\alpha/5 = 0.010$).

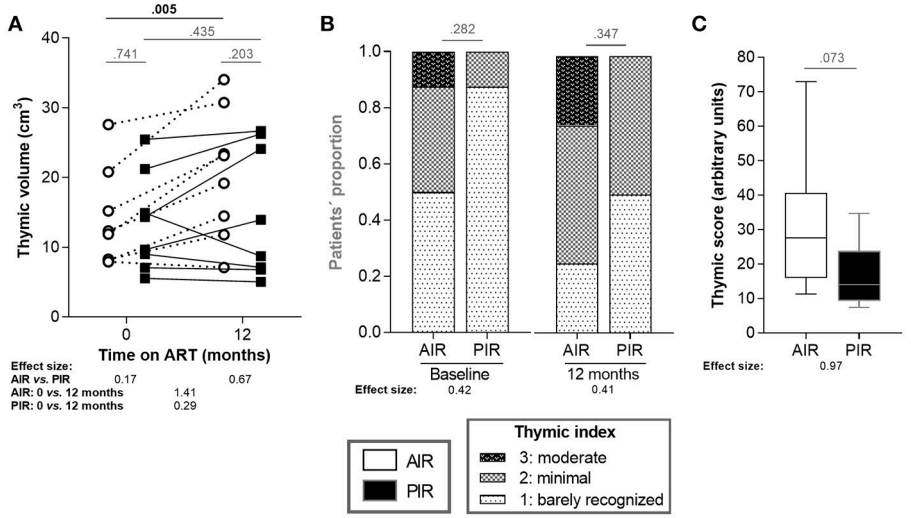

FIGURE 3 | AIR presented thymic volume increase during the first 12 months of ART. **(A)** Thymic volumes and **(B)** thymic indices were compared between AIR and PIR at baseline and at 12 months of ART. Thymic indices ranged from 1 to 3. **(C)** Thymic scores were calculated as the product of thymic volume and index means, over the first year of ART. Thymic volumes and scores **(A,C)** were compared using independent *t*-tests and the correspondent effect size estimates were calculated using for Cohen's *d*. Thymic indices **(B)** were compared using Fisher's exact test and effect size estimates were calculated using Cramér's *V*.

Furthermore, AIR tended to present higher frequencies of sj-TRECs (numbers per 10^5 PBMCs) at 24 months of ART (**Figure 4A**), and significantly higher numbers of sj-TRECs/mL of blood and sj/β TREC ratios at the same time point (**Figures 4B,C**, **S6A,B**; **Table S3**). Absolute numbers and percentage of RTE among $CD4^+$ T cells were higher in AIR than in PIR throughout the follow-up period, except at baseline and, for the percentage of RTE, at 6 months of ART (**Figures 5A,B**, **S6C–F**; **Table S4**).

We found no correlation between patients' age and any of the surrogates of thymic function (**Table S5**). We also found no correlations between thymic volume (at baseline or at 12 months of ART) and RTE absolute number or percentage among $CD4^+$ T cells at any other time point.

Age, HIV Viral Load, and RTE Percentages at Early Stages of ART Had Predictive Value for Immune Recovery, in Addition to $CD4^+$ T Cell Counts

We performed univariate logistic regressions with all variables to identify predictors of PIR or AIR status. The variables that performed the best (considering the lowest LRT *p*-values) were: $CD4^+$ T cell count at 6 months of ART (LRT p-value = 0.003, AUC = 0.774, ACC = 69.7%), $CD4^+$ T cell count at 2 months of ART (LRT p-value = 0.007, AUC = 0.750, ACC = 74.2%) or $CD4^+$ T cell count slope from baseline to 6 months of ART (LRT p-value = 0.010, AUC = 0.756, ACC = 60.6%). Performing combinations of eight elements from the set of variables identified in the univariate analysis with $p < 0.200$, and applying a stepwise selection, four logistic models, with three independent variables and an area under the curve (AUC) >0.80, were obtained (Models 1–4). Each of the models were represented by the following equation:

$$\text{Probability of being PIR} = \frac{1}{1 + e^{(a \ + \ b \times \text{Predictor 1} + c \times \ \text{Predictor 2} + d \times \text{Predictor 3})}}$$

replacing *a*, *b*, *c*, *d* and the predictors by the coefficients and respective variables listed in **Table 3**. Predictors included the following variables in different combinations: age at baseline; viral load at baseline; RTE percentage among $CD4^+$ T cells at 6 months of ART; $CD4^+$ T cell count slope from baseline to 6 months of ART; ratio of RTE percentages among $CD4^+$ T cells at baseline and 6 months of ART; $CD4^+$ T cell counts at 2 and 6 months of ART. The best of these four models regarding the goodness-of-fit (AUC = 0.91) and accuracy (87.1%), combined age, plasma viral load at baseline and $CD4^+$ T cell counts at 2 months of ART (**Table 3**, Model 4). To compare the relative quality of the models using AIC, we used a fixed data set with no missing values. Data of five patients had to be excluded, and four models (Model 1′ to 4′), including the same variables of Models 1–4, were built using data from the remaining 28 patients. Considering the AIC, the best of these models combined age, plasma viral load at baseline and $CD4^+$ T cell counts at 6 months of ART (**Table S6**, Model 3′).

For each of the Models 1–4, patients that failed to be correctly identified (i.e., PIR identified as "Probably AIR" or AIR identified as "Probably PIR") presented values in the included variables that, in combination, led to their inclusion in the opposite patient group (**Table S7**). We found no single variable responsible for the failure of the model in predicting patient's immune recovery outcome.

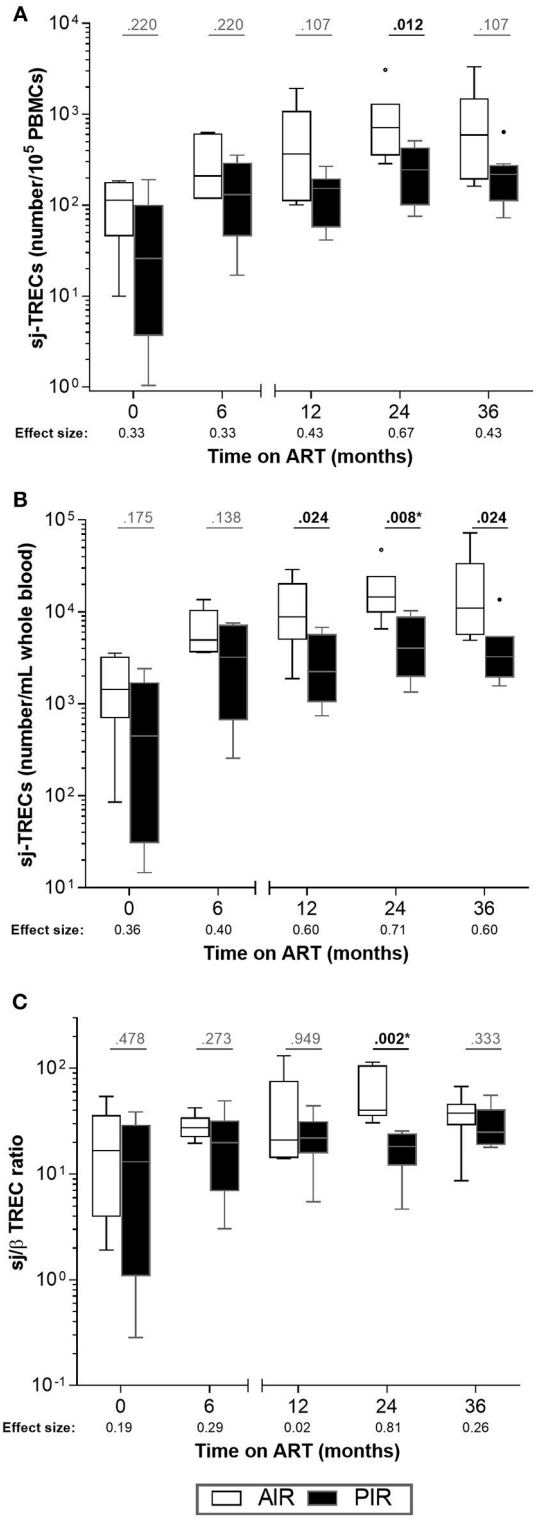

FIGURE 4 | No major differences were observed in intrathymic proliferation between AIR and PIR. **(A)** sj-TRECs/10^5 PBMCs, **(B)** sj-TRECs/mL of whole blood and **(C)** sj/β TREC ratios were compared throughout ART between AIR and PIR. A Wilcoxon-Mann-Whitney *U*-test was performed to compare the two groups of patients at each time point, and the corresponding *r* is presented as a measure of effect size. *Significant after Bonferroni correction ($\alpha/5 = 0.010$).

DISCUSSION

Despite the extensive literature regarding PIR, there is currently no consensus on their definition, hampering their identification and clinical management and contributing to their poorer prognoses compared to other HIV-infected patients. Here, we aimed to distinguish and define PIR and AIR by clustering analysis of the trajectories of $CD4^+$ T cell counts during therapy, and to build logistic models to predict PIR status using parameters assessed during the first months of ART. Although mathematical tools have been previously used to separate PIR and AIR [e.g., Pérez-Santiago et al. used random forest classification and unsupervised clustering to explore different definitions of a discordant immune response (33)], to our knowledge, the use of longitudinal cluster analysis has not been reported in this context. Our results revealed that PIR presented lower HIV viral load, CD4/CD8 ratio, naïve/memory $CD4^+$ T cell ratio and, most importantly, lower thymic function, strengthening the importance of thymus in immune recovery during ART. Furthermore, our logistic models were able to correctly predict PIR/AIR outcome after 36 months of therapy in up to 87% of cases, based on observations made until 2–6 months after ART onset.

This study included only severely lymphopenic patients (<200 $CD4^+$ T cells/μL), in order to limit the effect of variation in counts at baseline, which itself can explain distinct patterns of immune recovery (34). Limiting our sample to severely lymphopenic patients is also of particular interest given that these patients present a higher risk of new AIDS-defining events and death (22, 35).

The importance of the thymus in HIV pathogenesis and in immune recovery during ART is well-recognized. Several studies have shown that both adults and children infected by HIV present thymic alterations that may be reversed during ART; and that higher estimates of thymic function in patients on therapy are associated with higher $CD4^+$ T cell counts and better immune recoveries (36–38). Here we saw that AIR exhibited higher levels of thymic function than PIR by the following metrics: higher percentage of RTE among $CD4^+$ T cells at 12, 24, and 36 months of ART; higher number of sj-TRECs and a greater sj/β TREC ratio at 24 months of ART; and significant increase in thymic volume between baseline and 12 months of ART. The observation regarding RTE percentages suggests that thymic function is superior in AIR before and during therapy, and thymic volume gain in AIR, but not in PIR, points to a higher functional regeneration potential/capacity. Interestingly, our data also suggest that thymic function regeneration is more evident in AIR in comparison to a group of HIV-infected individuals initiating ART with $CD4^+$ T cell counts ranging between 200 and 350 cells/μL. The observed differences in naïve/memory $CD4^+$ T cell ratio and RTE percentage among $CD4^+$ T cells are particularly interesting as they may relate to the mechanism behind the divergent trajectories of immune recovery between PIR and AIR. They point to a scenario in which the two groups present different dynamics within the naïve and memory $CD4^+$ T cell subpopulations, probably related to distinct thymic functions. These results are in accordance with previous studies

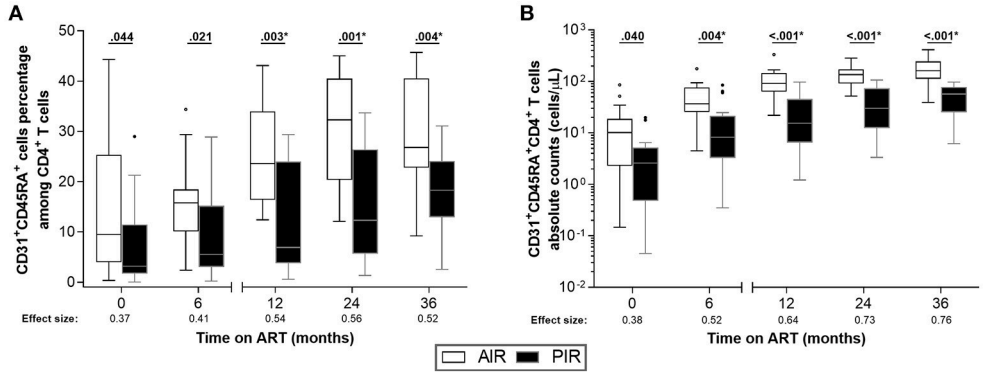

FIGURE 5 | AIR had higher percentages of RTE ($CD31^+CD45RA^+$) among $CD4^+$ T cells since baseline. **(A)** RTE percentage among $CD4^+$ T cells and **(B)** their absolute numbers were compared throughout ART between AIR and PIR. A Wilcoxon-Mann-Whitney *U*-test was performed to compare the two groups of patients at each time point, and the correspondent r is presented as a measure of effect size. *Significant after Bonferroni correction ($\alpha/5 = 0.010$).

TABLE 3 | Statistical comparison of the multivariate logistic regression models.

Logistic regression							
Models	**Coefficients**	**Predictor variables**	**LRT[a]**	***p*-value[b]**	**ROC analysis**		
					AUC	**Accuracy[c]**	**Sensitivity/ Specificity**
Model 1 ($n = 31$)	$a = -8.670$ $b = -0.102$ $c = 1.985$ $d = 0.105$	Age at baseline Log HIV plasma VL at baseline % RTE among $CD4^+$ T cells at 6 mo	$p = 0.537$	0.084 0.019 0.061	0.861	77.4%	76.5/78.6%
Model 2 ($n = 30$)	$a = -15.182$ $b = 2.519$ $c = 0.133$ $d = -2.179$	Log HIV plasma VL at baseline $CD4^+$ T cell count slope [0, 6] mo % RTE among $CD4^+$ T cells ratio $_{(0/6mo)}$	$p = 0.112$	0.051 0.029 0.038	0.871	76.7%	81.3/71.4%
Model 3 ($n = 33$)	$a = -9.703$ $b = -0.103$ $c = 1.897$ $d = 0.013$	Age at baseline Log HIV plasma VL at baseline $CD4^+$ T cell count at 6 mo	$p = 0.885$*	0.071 0.029 0.037	0.902	84.8%	89.5/78.6%
Model 4 ($n = 31$)	$a = -10.971$ $b = -0.160$ $c = 2.610$ $d = 0.012$	Age at baseline Log HIV plasma VL at baseline $CD4^+$ T cell count at 2 mo	$p = 0.333^{\dagger}$	0.038 0.018 0.017	0.908	87.1%	94.1/78.6%

Models are ordered by increasing sensitivity.

[a] *Likelihood ratio test (LRT) was used to compare the goodness of fit of reduced model (three final variables) vs. full model (initial variables); a non-significant p-value means that the reduced model is as good as the full model.*

[b] *p-Value for each of the three predictors of the reduced model.*

[c] *Accuracy corresponds to the percentage of patients PIR or AIR that were respectively classified as "probably PIR" or "probably AIR" among all patients.*

*LRT was performed using only 31 of the 33 patients due to missing values in variables that were excluded during the stepwise selection.

† *LRT was performed using only 29 of the 31 patients due to missing values in variables that were excluded during stepwise selection.*

AUC, area under the ROC curve; LRT, likelihood ratio test; mo, months; p, p-value; ROC, receiver operating characteristic curve; VL, viral load.

focusing on poor immunologic responses to ART, either with case-control (39), cross-sectional (40) or longitudinal designs (21, 41).

RTE were identified as $CD31^+CD45RA^+CD4^+$ T cells, which represent a subset of naïve $CD4^+$ T cells with high TREC content (42). Some limitations in the usage of CD31 have been identified, such as: (1) TCR-induced activation down-regulates CD31 expression (43, 44); (2) some $CD4^+CD45RA^+CD31^+$ cells undergo *in vivo* peripheral proliferation without immediate loss of CD31, resulting in an accumulation of $CD45RA^+CD31^+$ proliferative offspring (45, 46); (3) thymectomised individuals maintain a population of $CD31^+$ T cells in circulation (47). Notwithstanding these limitations, it is still one of the most well established markers of RTE.

A decrease in thymic function is associated with aging (48–50). Even though we found no significant age differences between

PIR and AIR, PIR tended to be older, a tendency that might explain the reduced thymic function presented by this group. This possibility is particularly important given that age at baseline was included in the predictive models discussed below. If age alone could account for the differences in surrogates of thymic function, we would expect an inverse correlation between age and these parameters. Such correlations have been described by others in the context of HIV infection or hematopoietic stem-cell transplantation (16, 51, 52). However, we saw no correlations between age and any surrogates of thymic activity in our cohort, which might be related to the narrow age range of this cohort: all but two AIR and one PIR were over age 30. It might also be that improvement in thymic function during ART is, to a certain extent, independent of patient age, at least for these patients' age range.

PIR presented significantly lower HIV plasma loads at baseline, as reported in other studies (53–56). This association may seem counterintuitive, given that higher plasma viral loads have been associated with disease progression and poorer prognosis (57–59). One possible explanation is that the high HIV plasma loads in AIR are the main factor contributing to the lymphopenia, so the suppression of HIV replication by ART results in an adequate immunological response; while in PIR, typically with low HIV plasma loads, different mechanisms contribute to the lymphopenia and the effectiveness of ART to counterbalance each of these mechanisms is limited.

To build predictive models to identify PIR as early as possible, we focused on parameters at baseline, 2 or 6 months of ART. While the univariate logistic regressions showed that $CD4^+$ T cell counts at 2 or 6 months of ART, and the slope between baseline and 6 months of ART were the single variables with highest predictive value, the multivariate analyses allowed us to build models with greater prediction accuracies. Of the four multivariate models described here, the one including age and plasma viral load at baseline, and RTE percentage among $CD4^+$ T cells at 6 months of ART (Model 1) performed better than any of the univariate models, despite excluding $CD4^+$ T cell counts. Furthermore, the model which included age and plasma viral load at baseline, and $CD4^+$ T cell count at 2 months of ART (Model 4) was able to correctly predict AIR/PIR status in 87.1% of the cases, with high sensitivity and specificity (94.1 and 78.6%, respectively). Although these results are encouraging, it should be highlighted that they were obtained using a relatively small number of patients of a single hospital. Therefore, replication of our analyses in other cohorts or encompassing larger numbers of patients will be of great value.

In contrast with our results, others have shown greater numbers of Treg and higher levels of cell proliferation and activation in PIR (30–32). These differences might be due to the exclusive analysis of patients with $CD4^+$ T cell counts <200 cells/μL at ART onset in our study, or to distinct criteria to define PIR and AIR. It has been shown that severely lymphopenic patients present higher percentage of Treg, lower percentage of Treg with a naïve phenotype and impaired production of Treg by the thymus, a phenotype not observed for patients starting ART with higher $CD4^+$ T cell counts (>350 cells/μL) (60).

The major strengths of this study are the longitudinal prospective design, with several evaluations over at least 36 months of therapy, and the multi-parametric evaluation of thymic activity, since each method alone is indirect and presents its own advantages and limitations [reviewed in (61)]. Our results show that persistent differences in thymic function in severely lymphopenic HIV-infected patients are associated with distinct immune recoveries that diverge soon after ART initiation. The outcome of PIR can be improved by early identification of these patients. This will prompt closer follow-up and possibly, in the future, the administration of strategies to boost thymic function. As pre-clinical and clinical trials aiming to enhance thymic function are currently on-going, studies like the one presented here might help to select the patients that could benefit the most from novel therapeutic approaches.

ETHICS STATEMENT

Peripheral blood samples were provided by patients with chronic HIV-1 infection, who participated in the prospective cohort study authorized by the local ethics committee (reference 168/CES). From all participating patients, an informed written consent was obtained.

AUTHOR CONTRIBUTIONS

RR-S, AH, and MC-N conceptualized the study. CN, AH, and MC-N designed experiments. RR-S, CN, IF, JC-G, and AH performed experiments. RR-S, CA, and EA performed statistical analysis. RR-S and CN prepared the figures. RC, AY, and MC-N supervised research. All authors discussed the results and contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00025/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material (Subchapter 4.1)

RELEVANT R CODE

PART 1: Longitudinal cluster analysis

```
# Import data
library(xlsx, quietly = TRUE)
dfxt <-read.table("CD4_counts_0-36mo.txt",header=T) # The txt file contains CD4+ T cell counts of the 33 patients from baseline to 36 months of ART

Time <-
data.frame(Patient_ID=dfxt$Patient_ID,time.1=rep(0,33),time.2=rep(2,33),time.3=rep(6,33),time.4=rep(9,33),time.5=rep(12,33),time.6=rep(16,33),time.7=rep(20,33),time.8=rep(24,33),time.9=rep(28,33),time.10=rep(32,33),time.11=rep(36,33))

Data<-dfxt
dim(Data)
dim(Time)
LstData<-list(CD4=Data,TimePoints=Time)
head(LstData$CD4)
head(LstData$TimePoints)

Data=LstData$CD4
Time=LstData$TimePoints

# Run step1 (measures), step2 (factors) and step3 (clusters)
s1 = step1measures(Data,Time, ID=TRUE)
s2 = step2factors(s1)
s3 = step3clusters(s2, nclusters = 2)

# Print and plot "traj object"
s3
plot(s3)
summary(s3)

# Plot mean combination trajectories
plotCombTraj(s3)

# Plot mean trajectories
plotMeanTraj(s3)

# Plot median trajectories
plotMedTraj(s3)

# Display measures
head(s1$measurments)

# Plot mean trajectory of all individuals
plot(s1$measurments$ID, s1$measurments$m5)
```

```
# Display factors
head(s2$factors)

# Display "traj object”
plot(s3)
s3$cluster
f <- as.factor(s3$cluster[,2])
table(f)

s3$cluster[which(s3$cluster[,2]==1),1]
s3$cluster[which(s3$cluster[,2]==2),1]
```

PART 2: Logistic regression models

```
# Import data
library(xlsx, quietly = TRUE)
df <- read.xlsx2("File1.xlsx", sheetIndex = 1, stringsAsFactors = FALSE) # File 1 contains all clinical and laboratorial data
df[df == "NA"] <- NA

### Univariate logistic regression analysis
Model.1u<-glm(groups ~ V1, data = df, family = binomial()) # V1, Variable 1
# P(“Being PIR”) = 1 / [1+exp(1)^ (-(b0+b1*V1)) ]
summary(Model.1u)

# Chi-square:
modelChi <- Model.1u$null.deviance - Model.1u$deviance # deviance for the model
chidf <- Model.1u$df.null - Model.1u$df.residual  # degrees of freedom
chisq.prob <- 1 - pchisq(modelChi, chidf)

### Multivariate logistic regression analysis
# Example: Model 1
Model.1m <- glm(groups ~. , data = dfx1, family=binomial)
full.model <- Model.1m # 8 variables: V1 to V8

stepAIC(Model.1m) # V4, V5 and V8 out
redAIC <- glm(subg~V1 + V2 + V3 + V6 + V7,
   data = dfx1, family=binomial)
summary(redAIC) # V7 out

redAIC.2 <- glm(subg~V1 + V2 + V3 + V6,
   data = dfx1, family=binomial)
summary(redAIC.2) # V6 out

redAIC.3 <- glm(subg~V1 + V2 + V3,
   data = dfx1, family=binomial)
summary(redAIC.3)
```

```
reduced.model <- redAIC.3 # 3 variables


# Likelihood ratio test
anova(redAIC.3, Model.1m, test="Chisq") # p-value > 0.05

# Akaike information criterion
summary(reduced.model)

# Hosmer–Lemeshow test
library(MKmisc)
HLgof.test(fit=fitted(reduced.model),obs=dfx2)

# ROC analysis
library(epiDisplay)
lroc(reduced.model)
lroc(reduced.model)$auc

# Sensitivity (pa.PIR), specificity (pa.AIR) and accuracy (pa.total)
pa.PIR <- sum(df.md1[which(df.md1$groups==”PIR”),"Probability_Model.1"]>0.5)/n1
pa.AIR <- sum(df.md1[which(df.md1$groups==”AIR”),"Probability_Model.1"]<0.5)/n2
pa.total <- (sum(df.md1[which(df.md1$groups==”PIR”),"Probability_Model.1"]>0.5) +
sum(df.md1[which(df.md1$groups==”AIR”),"Probability_Model.1]<0.5))/(n1+n2)
n1 # number of PIR in the data set of the model
n2 # number of AIR in the data set of the model
```

TABLE S1 | Measures computed in the first step of the clustering analysis.

***traj* package: Measures computed for of each trajectory**	
Measures	Formula
Measures of change in y_i $(i = 1, ..., k)^a$	
1. Range	$\max y_i$ - $\min y_i$
2. Mean-over-time	$\bar{y} = \frac{1}{k}\sum_{i=1}^{k} y_i$
3. Standard deviation (SD)	$s_y = \sqrt{\frac{1}{k-1}(y_i - \bar{y})^2}$
4. Coefficient of variation (CV)	$100 \times \frac{s_y}{\bar{y}}$
5. Change	$y_k - y_1$
6. Mean change per time unit	$\frac{y_k - y_1}{t_k - t_1 + 1}$
7. Change relative to the first score	$\frac{y_k - y_1}{y_1}$
8. Change relative to the mean over time	$\frac{y_k - y_1}{\bar{y}}$
9. Slope of the linear model $y_i = a + bt_i + \epsilon_i$	$b = \frac{\sum_{i=1}^{k}(y_i - \bar{y})(t_i - \bar{t})}{\sum_{i=1}^{k}(t_i - \bar{t})^2}$
10. R^2:Proportion of variance explained by the linear model $y_i = a + bt_i + \epsilon_i$	$R^2 = b^2 \times \frac{\sum_{i=1}^{k}(t_i - \bar{t})^2}{\sum_{i=1}^{k}(y_i - \bar{y})^2}$
Measures of change in $\Delta_{1,i} = y_{i+1} - y_i$ $(i = 1, ..., k)^*$	
11. Maximum of the first differences	$\max \Delta_{1,i}$
12. SD of the first differences	$s_{\Delta_1} = \frac{1}{k-2}\sum_{i=1}^{k-1}(\Delta_{1,i} - \bar{\Delta}_1)^2$ where $\bar{\Delta}_1 = \frac{1}{k-1}\sum_{i=1}^{k-1}\Delta_{1,i}$
13. SD of the first differences per time unit	$s_{\Delta'_1} = \frac{1}{k-2}\sum_{i=1}^{k-1}(\Delta'_{1,i} - \bar{\Delta}'_1)^2$ where $\Delta'_1 = \frac{\Delta_{1,i}}{t_{i+1} - t_i}$
14. Mean of the absolute first differences	$\lvert\Delta_1\rvert = \frac{1}{k-1}\sum_{i=1}^{k-1}\lvert\Delta_{1,i}\rvert$
15. Maximum of the absolute first differences	$\max \lvert\Delta_{1,i}\rvert$
16. Ratio of the maximum absolute first difference to the mean-over-time	$\frac{(\max \lvert\Delta_{1,i}\rvert)}{\bar{y}}$
17. Ratio of the maximum absolute first difference to the slope	$\frac{(\max \lvert\Delta_{1,i}\rvert)}{b}$
18. Ratio of the SD of the first differences to the slope	$\frac{s_{\Delta_1}}{b}$
Measures of change in $\Delta_{2,i} = \Delta_{1,i+1}\Delta_{1,i}$ $(i = 1, ..., k)^*$	
19. Mean of the second differences	$\bar{\Delta}_2 = \frac{1}{k-2}\sum_{i=1}^{k-2}\Delta_{2,i}$
20. Mean of the absolute second differences	$\lvert\Delta_2\rvert = \frac{1}{k-2}\sum_{i=1}^{k-2}\lvert\Delta_{2,i}\rvert$
21. Maximum of the absolute second differences	$\max \Delta_{1,i}$
22. Ratio of the maximum absolute second difference to the mean-over-time	$\frac{(\max \lvert\Delta_{2,i}\rvert)}{\bar{y}}$
23. Ratio of the maximum absolute second difference to mean absolute first difference	$\frac{(\max \lvert\Delta_{2,i}\rvert)}{\lvert\bar{\Delta}_1\rvert}$
24. Ratio of the mean absolute second difference to the mean absolute first differece	$\frac{(\lvert\bar{\Delta}_{2,i}\rvert)}{\lvert\bar{\Delta}_1\rvert}$

Note: If a measure is equal to zero, it will be set to the smallest, non-zero value of the same measure across the sample during further calculations. If Y_1, the first observation of the trajectory of an individual, is equal to zero, it will also be replaced.

Adapted from the "*User guides, package vignettes and other documentation*" section of R *traj package*.

TABLE S2 | R *traj* package clusters.

Cluster	Patient ID	$CD4^+$ T cell count (cells / µL) at each time point (months on ART)														
		BL	2	6	9	12	16	20	24	28	32	36	42	48	54	60
Cluster 1 (*n* = 14)	AH093	193	404	602	602	774	707	766	726	853	1044	896	986	902	1048	708
	AH083	181	325	353	362	508	467	768	589	503	612	551	562	624	613	619
	AH082	176	218	272	271	370	363	347	409	437	359	646	NA	NA	NA	NA
	AH092	164	461	310	429	628	401	514	428	456	378	424	519	586	589	599
	AH007	147	414	425	NA	534	819	629	679	732	694	681	846	621	828	727
	AH052	107	165	213	199	300	290	360	348	670	308	424	452	558	594	470
	AH095	94	308	222	358	253	266	319	341	397	582	397	488	323	512	505
	AH037	92	163	169	230	133	322	368	322	543	530	NA	534	NA	NA	NA
	AH033	68	270	282	NA	428	512	515	454	736	785	666	NA	NA	NA	NA
	AH044	56	489	463	621	537	505	434	439	488	560	660	599	442	518	736
	AH087	42	142	188	245	335	347	414	511	328	507	600	NA	NA	NA	NA
	AH053	32	244	256	111	518	475	526	337	482	520	501	609	692	796	825
	AH090	20	143	165	244	249	320	404	380	379	542	532	624	527	641	644
	AH066	12	112	155	186	209	274	475	375	405	537	436	565	473	595	526
Cluster 2 (*n* = 19)	AH011	182	176	208	NA	198	220	301	304	287	260	309	319	275	255	261
	AH088	174	227	238	252	180	241	277	274	273	316	310	311	324	378	272
	AH099	161	NA	159	222	185	237	259	267	266	334	326	315	369	451	NA
	AH063	146	262	293	369	248	335	358	300	338	323	338	NA	NA	NA	NA
	AH089	143	396	254	256	328	240	304	263	290	261	325	343	338	223	347
	AH091	97	157	167	191	192	213	247	218	329	236	230	337	309	364	NA
	AH086	92	202	159	174	159	191	165	214	203	226	220	269	230	416	330
	AH100	67	102	109	173	172	170	348	287	458	325	378	392	494	NA	NA
	AH048	63	205	126	169	221	207	236	190	202	188	226	177	261	261	356
	AH074	61	NA	341	220	354	331	337	380	393	412	381	NA	NA	NA	NA
	AH079	47	142	166	145	253	176	226	307	341	344	290	351	326	296	393
	AH006	43	177	274	NA	252	304	367	363	403	328	355	247	299	230	310
	AH073	37	85	118	106	104	106	140	222	188	246	210	558	261	306	323
	AH013	30	60	190	NA	204	329	273	303	375	424	323	NA	NA	NA	NA
	AH035	25	229	178	NA	285	250	360	299	338	351	335	365	440	NA	NA
	AH003	22	135	128	NA	186	159	182	191	190	229	243	276	325	410	349
	AH080	14	167	197	152	197	263	327	302	327	320	392	381	395	447	548
	AH039	9	77	72	89	100	155	121	152	143	198	166	214	276	352	NA
	AH085	8	43	47	91	65	119	174	186	170	189	197	344	535	331	412

Note: Elements of each cluster are ordered by decreasing baseline $CD4^+$ T cell count.

[a] Cells are shaded according to the CD4 count value: red, <200 $CD4^+$ T cells/µL; orange, 200-350 $CD4^+$ T cells/µL; yellow, 350-500 $CD4^+$ T cells/µL; green, >500 $CD4^+$ T cells/µL.

Abbreviations: BL, baseline (just before ART initiation); ID, identification code; NA, not available.

TABLE S3 | Signal joint TRECs and sj/β TREC ratio.

Cluster	Patient ID	**sj-TREC** (number/10^5 cells) at each time point (months on ART)					**sj-TRECs** (number/mL whole blood) at each time point (months on ART)					**sj/β TREC ratio**				
		BL	**6**	**12**	**24**	**36**	**BL**	**6**	**12**	**24**	**36**	**BL**	**6**	**12**	**24**	**36**
AIR (*n* = 6)	AH083	158,5	117,0	203,0	370,3	201,3	3186,1	3603,6	8098,6	10961,6	5837,9	15,9	23,7	13,9	42,8	39,6
	AH082	176,9	611,1	815,2	721,8	882,3	3556,5	13566,7	17772,4	15446,1	21440,9	30,1	42,2	57,9	114,4	67,3
	AH052	69,4	222,2	526,5	727,3	716,2	901,7	4222,0	9529,7	13455,3	11961,2	17,5	31,0	24,9	30,5	38,5
	AH033	57,3	628,6	1924,8	3084,0	3343,0	1214,2	9491,5	28872,7	47494,3	72209,3	1,9	31,5	131,5	37,4	35,8
	AH044	184,2	117,2	113,6	702,1	160,7	1657,2	5693,7	5999,9	17271,3	4900,7	54,1	23,1	17,2	103,9	8,6
	AH066	9,9	195,4	100,3	285,9	469,9	85,2	3634,0	1876,3	6518,1	10009,9	4,6	19,6	14,3	36,8	37,0
PIR (*n* = 8)	AH063	105,4	96,6	133,8	257,5	253,1	1739,3	1390,8	1498,2	3218,3	3214,9	21,6	19,0	30,9	17,3	18,3
	AH048	7,8	249,3	41,3	87,2	102,8	69,4	7554,6	1004,6	1787,1	1696,2	1,8	32,7	5,5	11,5	26,3
	AH074	32,1	110,6	171,2	258,4	227,6	568,3	3118,4	6180,8	7183,7	5463,1	14,7	30,0	44,3	25,5	24,2
	AH079	19,7	150,6	266,0	487,0	283,3	325,8	3313,2	6810,6	10325,1	5439,7	11,5	20,5	31,8	24,2	45,7
	AH073	85,7	306,8	191,1	232,1	204,4	1620,6	7487,0	2961,9	4851,0	3310,9	31,9	15,1	18,1	19,1	25,6
	AH035	190,1	354,1	197,3	512,4	641,2	2414,6	6514,9	4418,5	9479,4	13656,8	38,8	49,4	25,6	24,1	55,7
	AH080	2,3	28,2	51,4	135,4	132,4	17,5	423,7	745,1	2477,7	2622,0	0,8	3,0	15,9	13,8	17,9
	AH039	1,0	16,9	73,5	75,3	72,3	14,6	257,2	1175,4	1340,1	1562,6	0,3	4,2	15,7	4,7	20,7

Notes: AIR and PIR were separated based on clustering analysis of the longitudinal trajectories of $CD4^+$ T cell counts, from baseline to 36 months of ART. Measurement of TRECs was only performed in a subgroup of patients. Elements of each group are ordered by decreasing baseline $CD4^+$ T cell count.

Abbreviations: BL, baseline (just before ART initiation); ID, identification code; NA, not available; TRECs, T-cell receptor excision circles.

TABLE S4 | Absolute number and percentage of $CD4^+CD45RA^+CD31^+$ T cells in peripheral blood.

Cluster	Patient ID	CD4 count (cells / µL) at baseline	$CD4^+CD45RA^+CD31^+$ T cells count (cells / µL) at each time point (months on ART)					Percentage of $CD31^+CD45RA^+$ cells among $CD4^+$ T cells at each time point (months on ART)				
			BL	6	12	24	36	BL	6	12	24	36
Cluster 1 (*n* = 14)	AH093	193,0	85,5	177,0	333,6	283,1	409,5	44,3	29,4	43,1	39,0	45,7
	AH083	181,0	34,4	79,4	119,9	123,1	120,7	19,0	22,5	23,6	20,9	21,9
	AH082	176,0	51,7	93,6	128,0	172,2	285,5	29,4	34,4	34,6	42,1	44,2
	AH092	164,0	6,3	32,6	80,4	51,8	39,1	3,8	10,5	12,8	12,1	9,2
	AH007	147,0	11,9	37,1	NA	134,4	178,4	8,1	8,7	NA	19,8	26,2
	AH052	107,0	8,2	36,4	91,5	NA	126,4	7,7	17,1	30,5	NA	29,8
	AH095	94,0	10,3	24,0	NA	74,0	101,2	11,0	10,8	NA	21,7	25,5
	AH037	92,0	9,9	26,0	21,8	135,2	NA	10,8	15,4	16,4	42,0	NA
	AH033	68,0	2,8	39,2	145,5	177,1	274,4	4,1	13,9	34,0	39,0	41,2
	AH044	56,0	13,4	75,0	66,6	77,7	123,4	24,0	16,2	12,4	17,7	18,7
	AH087	42,0	0,1	4,5	63,0	165,1	160,8	0,35	2,4	18,8	32,3	26,8
	AH053	32,0	10,6	43,0	166,8	115,9	167,8	33,0	16,8	32,2	34,4	33,5
	AH090	20,0	0,8	14,0	NA	171,0	212,3	4,2	8,5	NA	45,0	39,9
	AH066	12,0	0,2	25,9	39,3	103,9	103,3	1,6	16,7	18,8	27,7	23,7
Cluster 2 (*n* = 19)	AH011	182	5,3	6,5	7,5	4,5	NA	2,9	3,1	3,8	1,5	NA
	AH088	174	3,6	1,9	6,8	12,4	11,7	2,1	0,8	3,8	4,5	3,8
	AH099	161	0,0	0,3	11,2	29,9	45,3	0,0	0,2	6,1	11,2	13,9
	AH063	146	19,9	84,7	45,4	46,8	61,9	13,6	28,9	18,3	15,6	18,3
	AH089	143	4,7	60,5	96,4	69,4	90,7	3,3	23,8	29,4	26,4	27,9
	AH091	97	4,9	24,7	51,5	73,5	71,5	5,1	14,8	26,8	33,7	31,1
	AH086	92	2,4	5,2	6,7	20,1	26,4	2,7	3,3	4,2	9,4	12,0
	AH100	67	2,7	17,0	41,3	82,1	96,4	4,1	15,6	24,0	28,6	25,5
	AH048	63	1,0	3,7	15,2	26,0	35,0	1,6	3,0	6,9	13,7	15,5
	AH074	61	17,7	64,4	92,7	105,6	81,9	29,0	18,9	26,2	27,8	21,5
	AH079	47	1,4	17,9	60,7	83,2	70,2	3,0	10,8	24,0	27,1	24,2
	AH006	43	NA	NA	1,5	4,8	NA	NA	NA	0,6	1,3	NA
	AH073	37	0,1	6,5	5,4	12,6	10,0	0,2	5,6	5,2	5,7	4,8
	AH013	30	6,4	11,9	24,1	76,1	73,6	21,3	6,3	11,8	25,1	22,8
	AH035	25	0,3	8,3	28,2	36,8	56,6	1,2	4,6	9,9	12,3	16,9
	AH003	22	NA	NA	1,3	3,3	6,2	NA	NA	0,7	1,7	2,6
	AH080	14	2,4	14,7	29,4	62,2	92,5	17,3	7,4	14,9	20,6	23,6
	AH039	9	0,3	2,7	6,5	10,1	24,4	3,3	3,8	6,5	6,7	14,7
	AH085	8	NA	1,0	1,2	17,0	47,3	NA	2,2	1,9	9,1	24,0

Notes: Clusters were formed based on the longitudinal trajectories of $CD4^+$ T cell counts from baseline to 36 months of ART. Elements of each cluster are ordered by decreasing baseline $CD4^+$ T cell count.

Abbreviations: BL, baseline (just before ART initiation); ID, identification code; NA, not available.

Table S5 | Correlations between age and thymic function surrogates.

Correlation (Patient age, Parameter X_i) [a]							
Parameter X_i	**Time point**	**Total**		**AIR**		**PIR**	
		Cor coeff [b]	*p*	Cor coeff [b]	*p*	Cor coeff [b]	*p*
Thymic volume mean (cm^3) n = 16 (8 AIR, 8 PIR)	0 M	r = 0.508	**.045**	r = 0.690	.058	r = 0.235	.575
	12 M	r = 0.578	**.019**	r = 0.698	.054	r = 0.534	.172
Thymic score (arbitrary units) n = 16 (8 AIR, 8 PIR)	[0;12] M	r_s = 0.334	.206	r = 0.489	.219	r = 0.314	.449
sj-TRECs (per 10^5 cells) n = 14 (6 AIR, 8 PIR)	0 M	r_s = - 0.186	.525	r = 0.085	.873	r = - 0.422	.297
	6 M	r_s = 0.223	.443	r_s = 0.232	.658	r = 0.130	.758
	12 M	r_s = - 0.349	.221	r = - 0.153	.772	r = - 0.387	.344
	24 M	r_s = - 0.144	.624	r_s = - 0.145	.784	r = - 0.368	.370
	36 M	r_s = - 0.175	.551	r_s = 0.029	.957	r_s = - 0.683	.062
sj/β TREC ratio (arbitrary units) n = 14 (6 AIR, 8 PIR)	0 M	r = 0.110	.709	r = 0.433	.391	r = - 0.422	.273
	6 M	r = - 0.026	.930	r = 0.264	.613	r = - 0.239	.569
	12 M	r_s = - 0.261	.368	r_s = 0.232	.658	r = - 0.886	**.003**
	24 M	r_s = - 0.027	.928	r_s = 0.406	.425	r = - 0.600	.116
	36 M	r = 0.087	.768	r = 0.128	.809	r_s = 0.098	.818
Percentage of $CD31^+CD45RA^+$ cells among $CD4^+$ T cells (%) n = 33 (14 AIR, 19 PIR)	0 M	r_s = - 0.215	.254	r_s = 0.121	.680	r_s = - 0.271	.309
	6 M	r_s = - 0.193	.299	r = 0.128	.663	r_s = - 0.256	.322
	12 M	r = - 0.289	.121	r = - 0.025	.941	r_s = - 0.148	.545
	24 M	r = - 0.280	.121	r = - 0.112	.716	r = - 0.116	.635
	36 M	r = - 0.285	.126	r = - 0.032	.917	r = - 0.252	.329
Absolute number of $CD31^+CD45RA^+$ $CD4^+$ T cells (per / µL) n = 33 (14 AIR, 19 PIR)	0 M	r_s = - 0.255	.175	r_s = 0.053	.858	r_s = - 0.198	.463
	6 M	r_s = - 0.232	.209	r_s = 0.095	.747	r_s = - 0.273	.290
	12 M	r_s = - 0.205	.276	r_s = 0.128	.708	r_s = - 0.104	.672
	24 M	r_s = - 0.324	.070	r = - 0.313	.298	r_s = - 0.120	.623
	36 M	r_s = - 0.259	.167	r = - 0.119	.699	r = - 0.205	.429

[a] Correlation between patient age at baseline (in years) and each of the parameters on the left column. [b] Pearson or Spearman correlation coefficient (r or r_s, respectively), according to Shapiro-Wilk test for normality assessment.

Abbreviations: Cor coeff, correlation coefficient; M, months on antiretroviral therapy; *p*, *p*-value.

Table S6 | Statistical comparison of multivariate logistic regression models.

Logistic regression								
Models	**Coefficients**	**Predictor variables**	**LRT** [a)]	**AIC** [b)]	***p-value*** [c)]	**ROC analysis**		
						AUC	**ACC** [d)]	**Sensitivity / Specificity**
Model 1' (*n*=28)	a = - 6.828 b = - 0.163 c = 2.117 d = 0.124	Age at baseline Log HIV plasma VL at baseline % RTE among $CD4^+$ T cells at 6 mo	*p*=.193	31.946	.047 .026 .060	0.878	78.6 %	78.6 / 78.6%
Model 2' (*n*=28)	a = -14.957 b = 2.391 c = 0.178 d = - 2.193	Log HIV plasma VL at baseline $CD4^+$ T cell count slope [0, 6] mo % RTE among $CD4^+$ T cells ratio $_{(0/6\ mo)}$	*p*=.043*	30.279	.081 .052 .044	0.872	78.6 %	71.4 / 85.7%
Model 3' (*n*=28)	a = -10.578 b = - 0.154 c = 2.211 d = 0.020	Age at baseline Log HIV plasma VL at baseline $CD4^+$ T cell count at 6 mo	*p*=.654	27.857	.046 .044 .047	0.918	82.1 %	78.6 / 85.7%
Model 4' (*n*=28)	a = - 9.720 b = - 0.160 c = 2.439 d = 0.011	Age at baseline Log HIV plasma VL at baseline $CD4^+$ T cell count at 2 mo	*p*=.390	29.725	.038 .018 .017	0.898	85.7 %	78.6 / 92.9%

Note: Models are ordered by increasing accuracy and specificity.

[a)] Likelihood ratio test (LRT) was used to compare the goodness of fit of reduced model (3 final variables) *vs.* full model (initial variables); a non-significative *p*-value means that the reduced model is as good as the full model.

[b)] AIC is an estimator of the relative quality of statistical models. Given two models for the same data set, the preferred model is the one with the minimum AIC value.

[c)] *p*-value for each of the 3 predictors of the reduced model.

[d)] Accuracy corresponds to the percentage of patients PIR or AIR that were respectively classified as "probably PIR" or "probably AIR" among all patients.

*) The full model has better goodness of fit than the reduced model considering $\alpha = .050$.

Abbreviations: ACC, accuracy; AUC, area under the ROC curve; LRT, likelihood ratio test; mo, months; p, p-value; ROC, receiver operating characteristic curve; VL, viral load.

Table S7 | "Probability of being PIR" among AIR and PIR, according to each multivariate logistic regression model.

Cluster	Patient ID	Age at BL	Log HIV plasma VL at BL	% RTE among $CD4^+$ T cells at 6 mo	$CD4^+$ T cell count slope [0, 6] mo	% RTE among $CD4^+$ T cells ratio (0/6 mo)	$CD4^+$ T cell count at 2 mo	$CD4^+$ T cell count at 6 mo	"Probability of being PIR"			
									Model 1 (*n*=31)	Model 2 (*n*=30)	Model 3 (*n*=33)	Model 4 (*n*=31)
AIR (*n* = 14)	AH007	43	6,15	8,73	40,11	0,93	414,00	425,00	0,48	0,03	0,04	0,04
	AH033	33	6,52	13,90	31,00	0,29	270,00	282,00	0,09	0,01	0,05	0,02
	AH037	28	5,71	15,40	11,21	0,70	163,00	169,00	0,19	0,70	0,39	0,20
	AH044	46	5,25	16,20	57,21	1,48	489,00	463,00	0,78	0,08	0,18	0,22
	AH052	35	6,03	17,10	16,86	0,45	165,00	213,00	0,18	0,22	0,29	0,24
	AH053	51	6,98	16,80	32,43	1,96	244,00	256,00	0,15	0,08	0,17	0,12
	AH066	46	6,15	16,70	21,96	0,09	112,00	155,00	0,35	0,05	0,68	0,72
	AH082	51	5,34	34,40	16,82	0,85	218,00	272,00	0,42	0,80	0,78	0,93
	AH083	23	5,59	22,50	22,29	0,84	325,00	353,00	0,08	0,49	0,04	0,02
	AH087	32	6,42	2,38	17,63	0,15	142,00	188,00	0,26	0,05	0,16	0,09
	AH090	37	5,48	8,47	22,25	0,50	143,00	165,00	0,66	0,38	0,73	0,71
	AH092	42	5,33	10,50	10,88	0,36	461,00	310,00	0,78	0,75	0,47	0,15
	AH093	31	6,60	29,40	54,06	1,51	404,00	602,00	0,01	0,00	0,00	0,00
	AH095	36	5,90	10,80	14,25	1,02	308,00	222,00	0,38	0,66	0,34	0,09
PIR (*n* = 19)	AH003	44	5,41	NA	14,89	NA	135,00	128,00	NA	NA	0,91	0,91
	AH006	61	5,08	NA	37,30	NA	177,00	274,00	NA	NA	0,94	1,00
	AH011	36	4,90	3,11	4,86	0,93	176,00	208,00	0,91	0,99	0,80	0,86
	AH013	57	6,84	6,25	27,50	3,41	60,00	190,00	0,56	0,85	0,53	0,82
	AH035	37	5,29	4,64	20,04	0,26	229,00	178,00	0,81	0,44	0,76	0,58
	AH039	43	5,58	3,80	8,82	0,87	77,00	72,00	0,83	0,86	0,93	0,91
	AH048	47	5,56	2,97	6,18	0,55	205,00	126,00	0,89	0,82	0,91	0,82
	AH063	32	4,78	28,90	23,01	0,47	262,00	293,00	0,36	0,75	0,53	0,62
	AH073	41	4,95	5,55	11,68	0,03	85,00	118,00	0,92	0,77	0,95	0,97

	AH074	30	5,64	18,90	36,62	1,53	NA	341,00	0,19	0,37	0,09	NA
	AH079	41	5,03	10,80	17,44	0,28	142,00	166,00	0,85	0,69	0,90	0,94
	AH080	41	6,38	7,44	24,07	2,33	167,00	197,00	0,36	0,73	0,32	0,25
	AH085	28	4,94	2,17	5,28	NA	43,00	47,00	0,82	NA	0,93	0,89
	AH086	49	5,33	3,28	7,08	0,81	202,00	159,00	0,94	0,93	0,93	0,92
	AH088	67	5,60	0,80	4,80	2,60	227,00	238,00	0,99	1,00	0,95	0,99
	AH089	53	5,57	23,80	10,57	0,14	396,00	254,00	0,63	0,51	0,78	0,54
	AH091	48	6,33	14,80	5,36	0,34	157,00	167,00	0,37	0,33	0,61	0,56
	AH099	61	5,43	0,22	4,76	0,13	NA	159,00	0,98	0,76	0,97	NA
	AH100	44	4,87	15,60	5,46	0,26	102,00	109,00	0,86	0,94	0,97	0,98

Note: Cells are shaded in green if "Probability of being PIR" < 0.50 in AIR (true negatives) or ≥ 0.50 in PIR (true positives). Cells are shaded in red if "Probability of being PIR" ≥ 0.50 in AIR (false positives) or < 0.50 in PIR (false positives). **Abbreviations:** BL, baseline (just before ART initiation); ID, identification code; mo, months of ART; NA, not available.

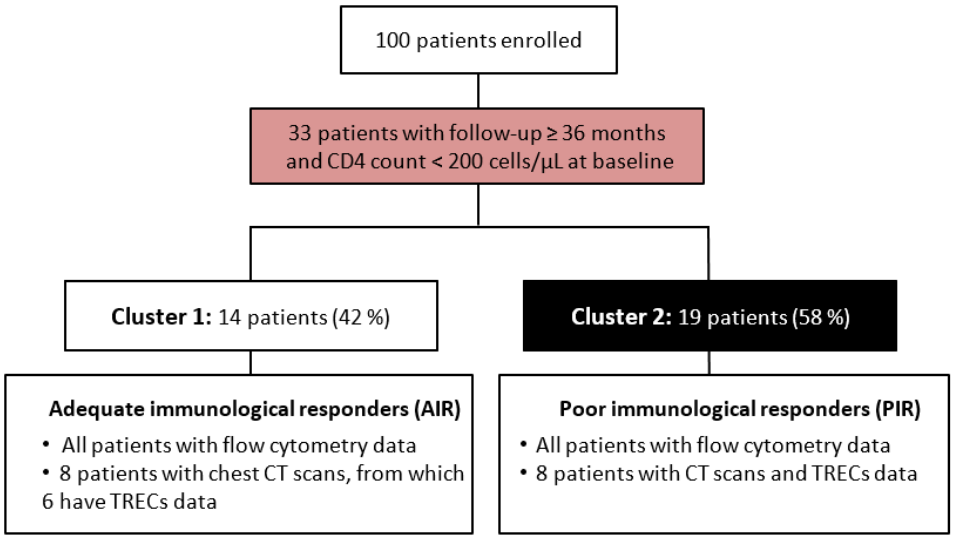

Figure S1 | Patient selection and clustering process. Thirty-three HIV-infected individuals, with $CD4^+$ T cell counts over 200 cells/µL and with at least 36 months of follow-up since ART initiation, were selected from a prospective cohort of 100 individuals. Selected individuals were clustered according to their $CD4^+$ count T cell trajectories during the 36 months of ART using *traj package* and classified as AIR (at least one $CD4^+$ T cell count above 500 cells/µL during the first 36 months of ART) or PIR (all $CD4^+$ T cell counts bellow 500 cells/µL).

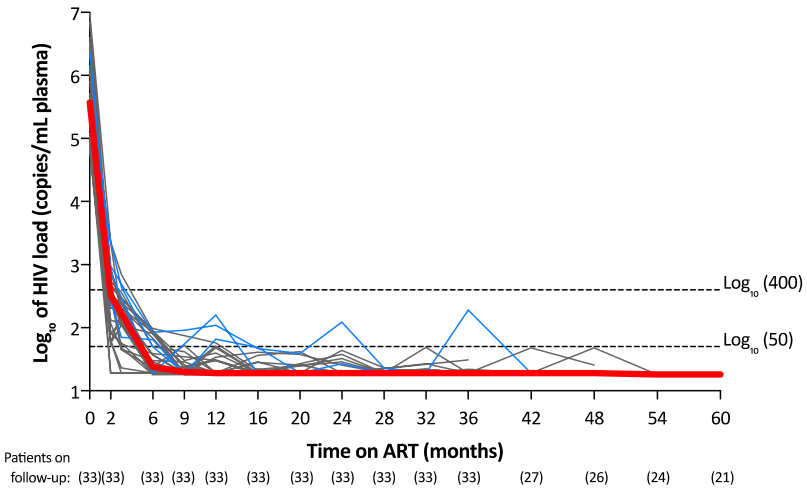

Figure S2 | Viral load levels throughout the follow-up period. Each grey or blue line represents a single patient; blue lines stand for four patients who presented, each one, a virological blip, defined as single HIV RNA values between 50 and 400 copies/mL after 6 months of follow-up; the red line represents the median of all patients' HIV plasma loads over time. The detection limit of the technique was 19 copies/mL of plasma [$Log_{10}(19)=1.28$].

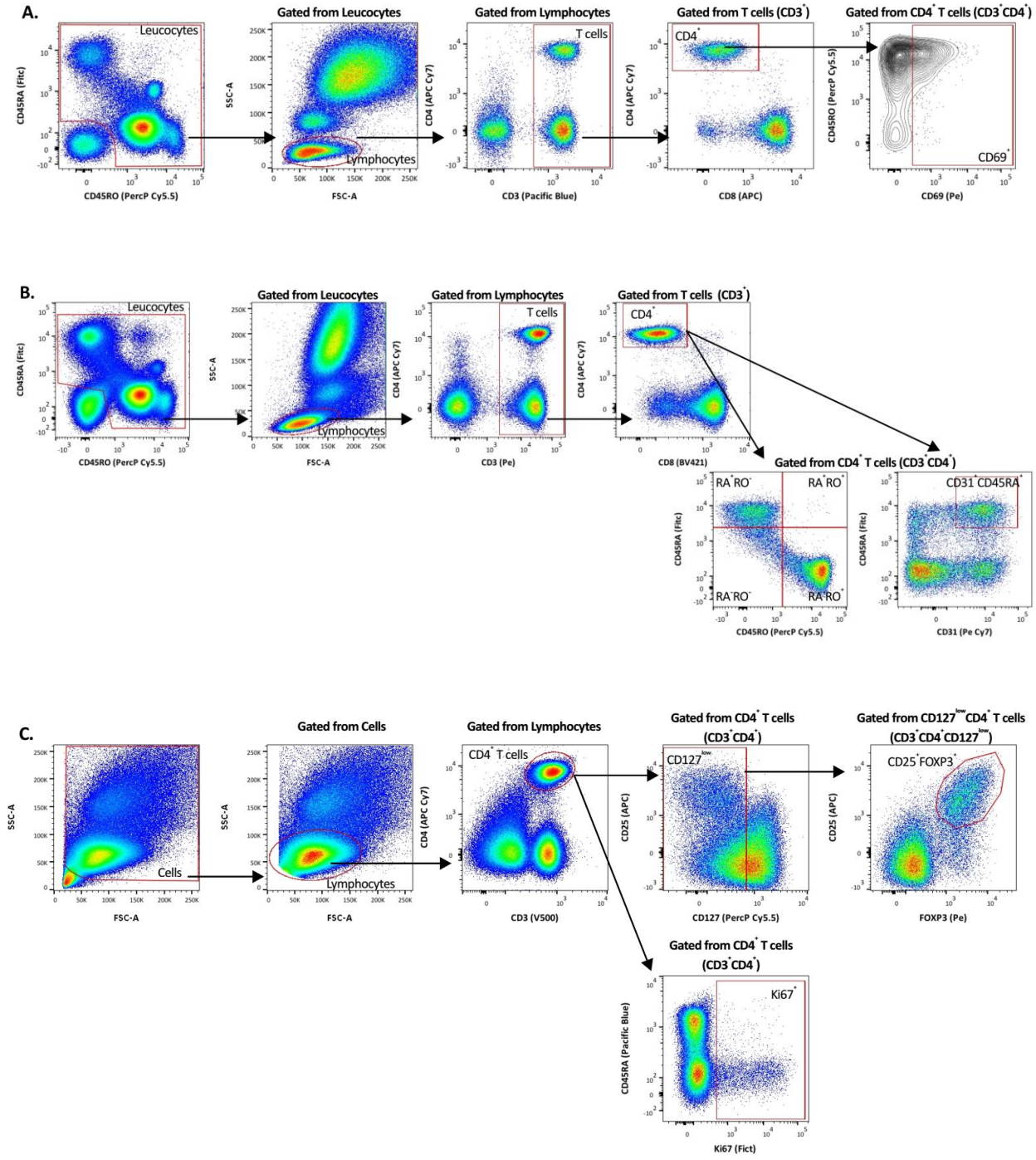

Figure S3 | Flow cytometry gating strategy. (A) Identification of $CD69^+$ and $HLA\text{-}DR^+$ (activation markers) cells among $CD4^+$ T cells (panel 1); **(B)** Identification of $CD45RA^-CD45RO^+$ (memory), $CD45RA^+CD45RO^-$ (naïve) and $CD45RA^+CD31^+$ (recent thymic emigrants; RTE) cells among $CD4^+$ T cells (panel 2). **(C)** Identification of $CD3^+CD4^+CD127^{low}CD25^{high}FOXP3^+$ cells (regulatory T cells; Treg) and of $Ki67^+$ (proliferation marker) cells among $CD4^+$ T cells.

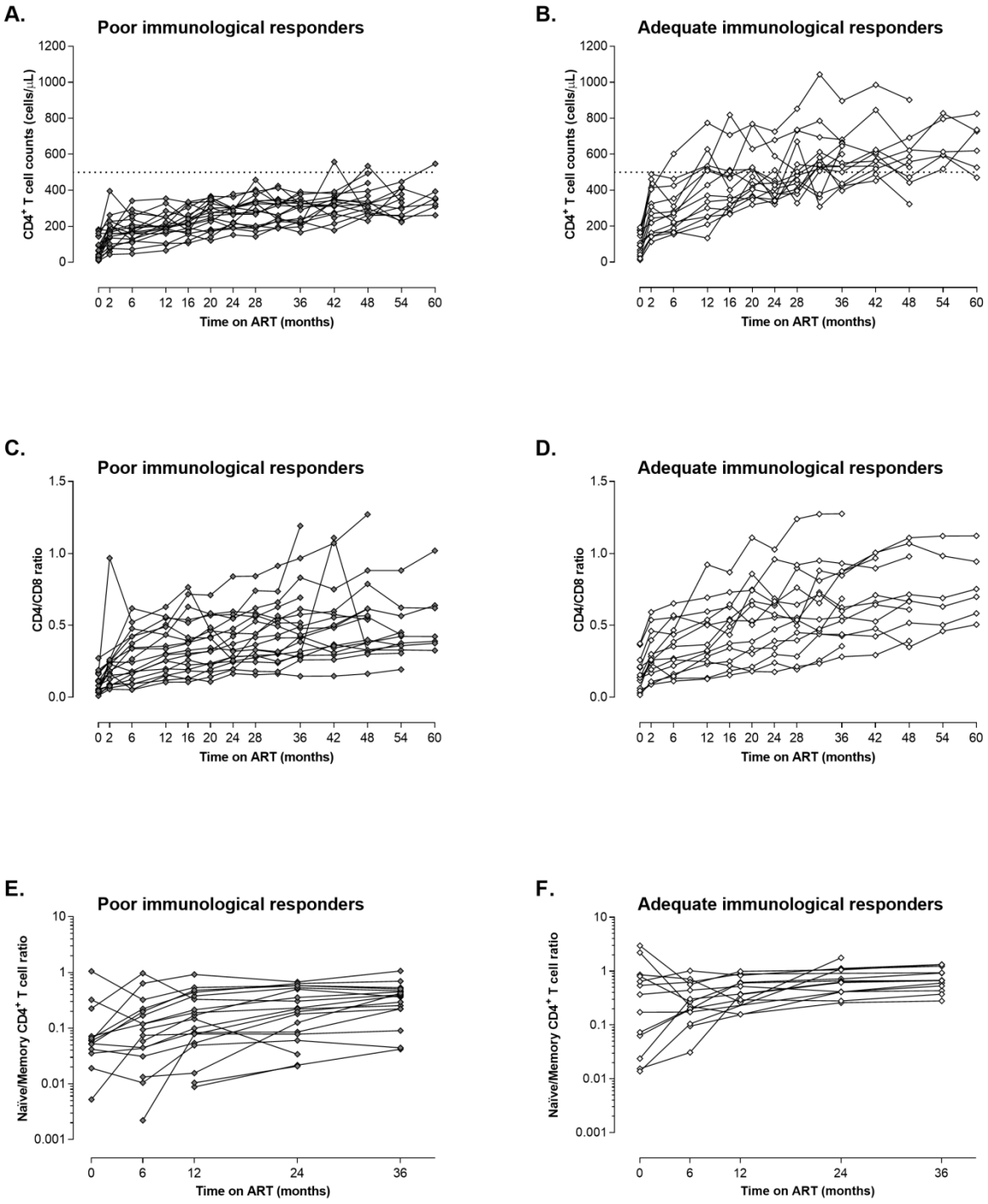

Figure S4 | Spaghetti plots. (A, B) $CD4^+$ T cell count evolution of each patient in PIR and AIR group, respectively. **(C, D)** CD4/CD8 ratio evolution of each patient in PIR and AIR group, respectively. **(E, F)** Naïve/Memory $CD4^+$ T cell ratio evolution of each patient in PIR and AIR group, respectively.

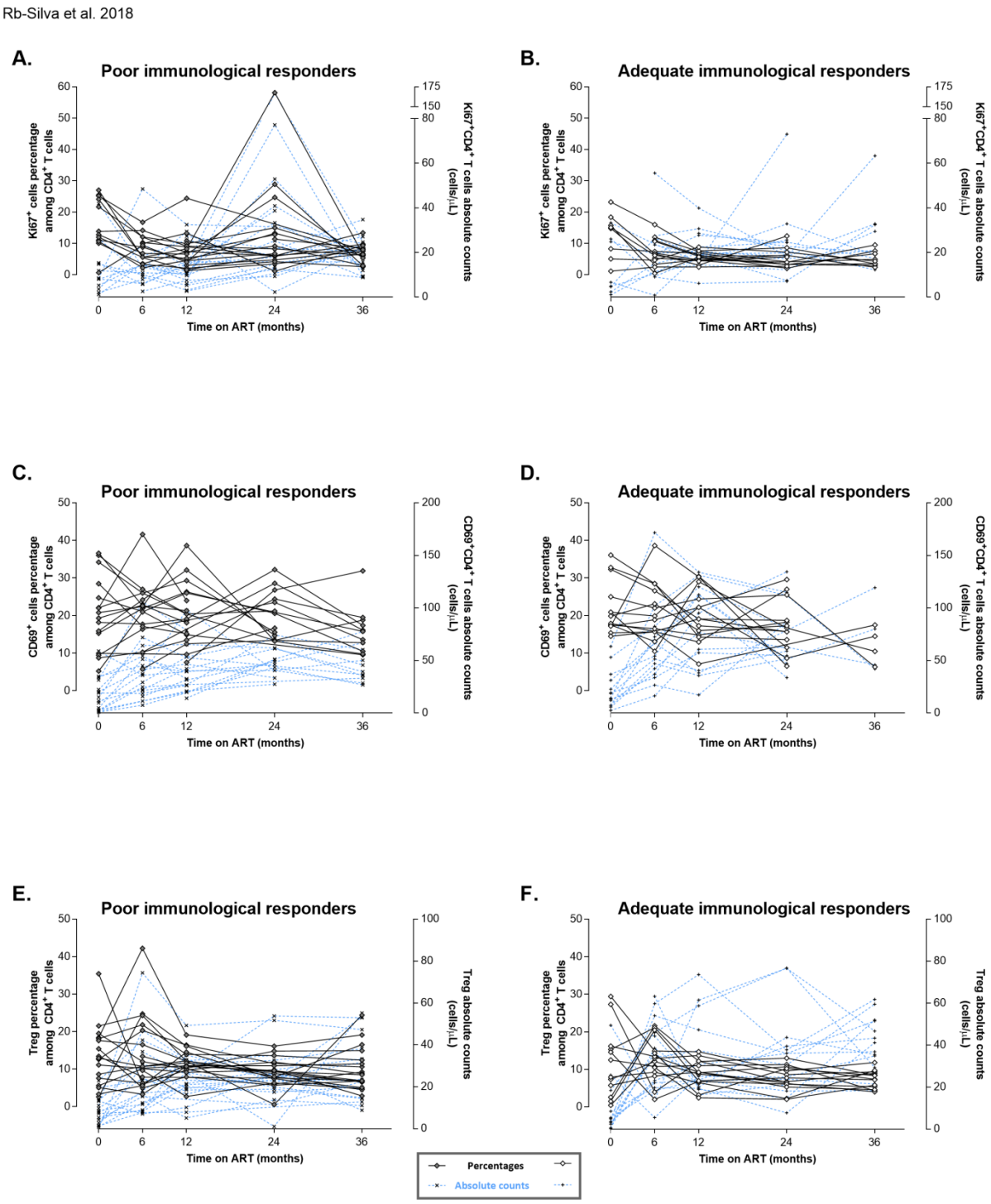

Figure S5 | Spaghetti plots. (A, B) Percentage among $CD4^+$ T cells and absolute number of $Ki67^+$ cells in PIR and AIR group, respectively. **(C, D)** Percentage among $CD4^+$ T cells and absolute number of $CD69^+$ cells in PIR and AIR group, respectively. **(E, F)** Percentage among $CD4^+$ T cells and absolute number of regulatory T cells (Treg) in PIR and AIR group, respectively.

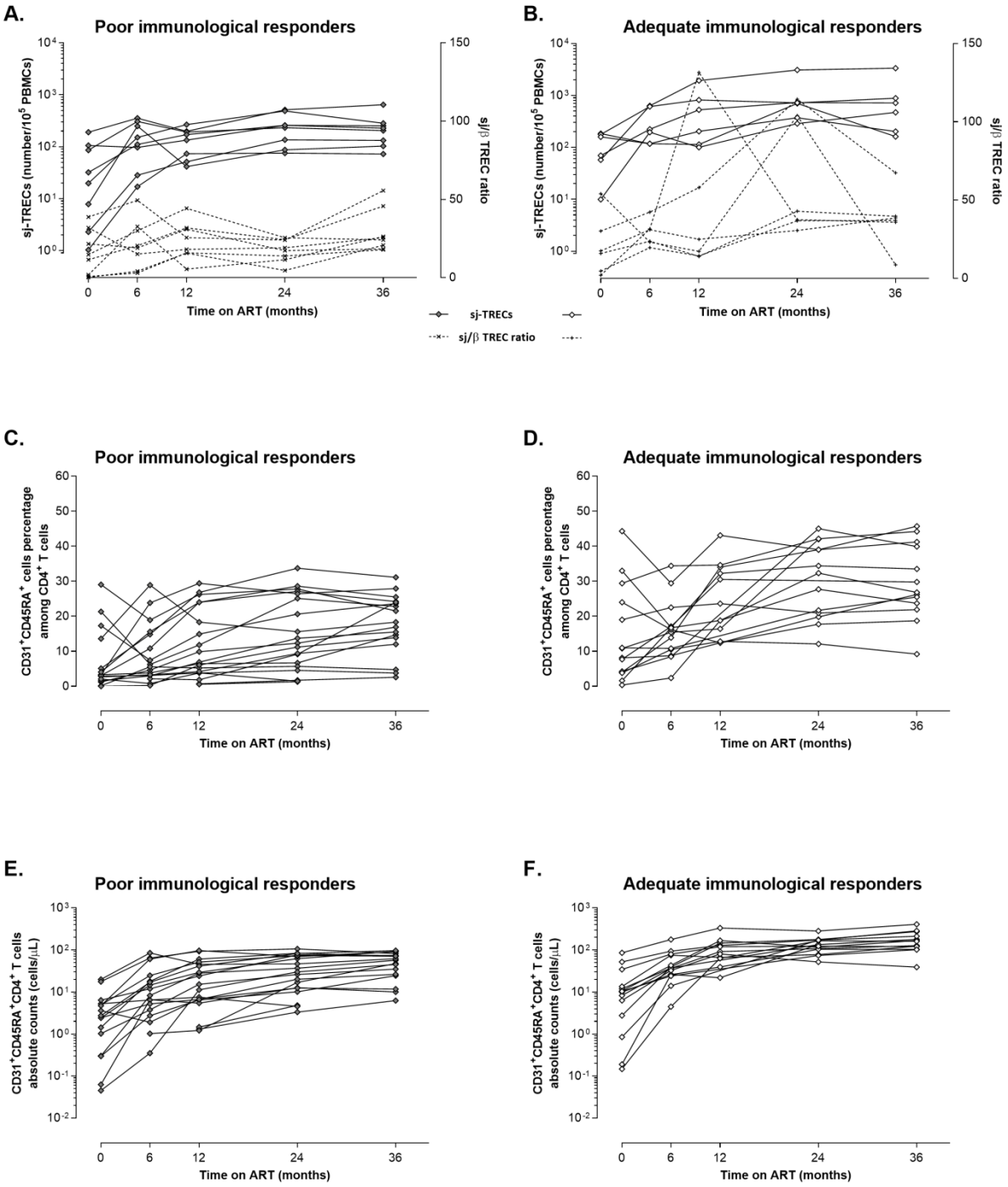

Figure S6 | Spaghetti plots. (A, B) Number of sj-TRECs in copies/10^5 PBMCs and sj/βTREC ratio evolution for each patient in PIR and AIR group, respectively. **(C, D)** Percentage of $CD31^+CD45RA^+$ cells among $CD4^+$ T cells evolution for each patient in PIR and AIR group, respectively. **(E, F)** Absolute number of $CD31^+CD45RA^+CD4^+$ T cells evolution for each patient in PIR and AIR group, respectively.

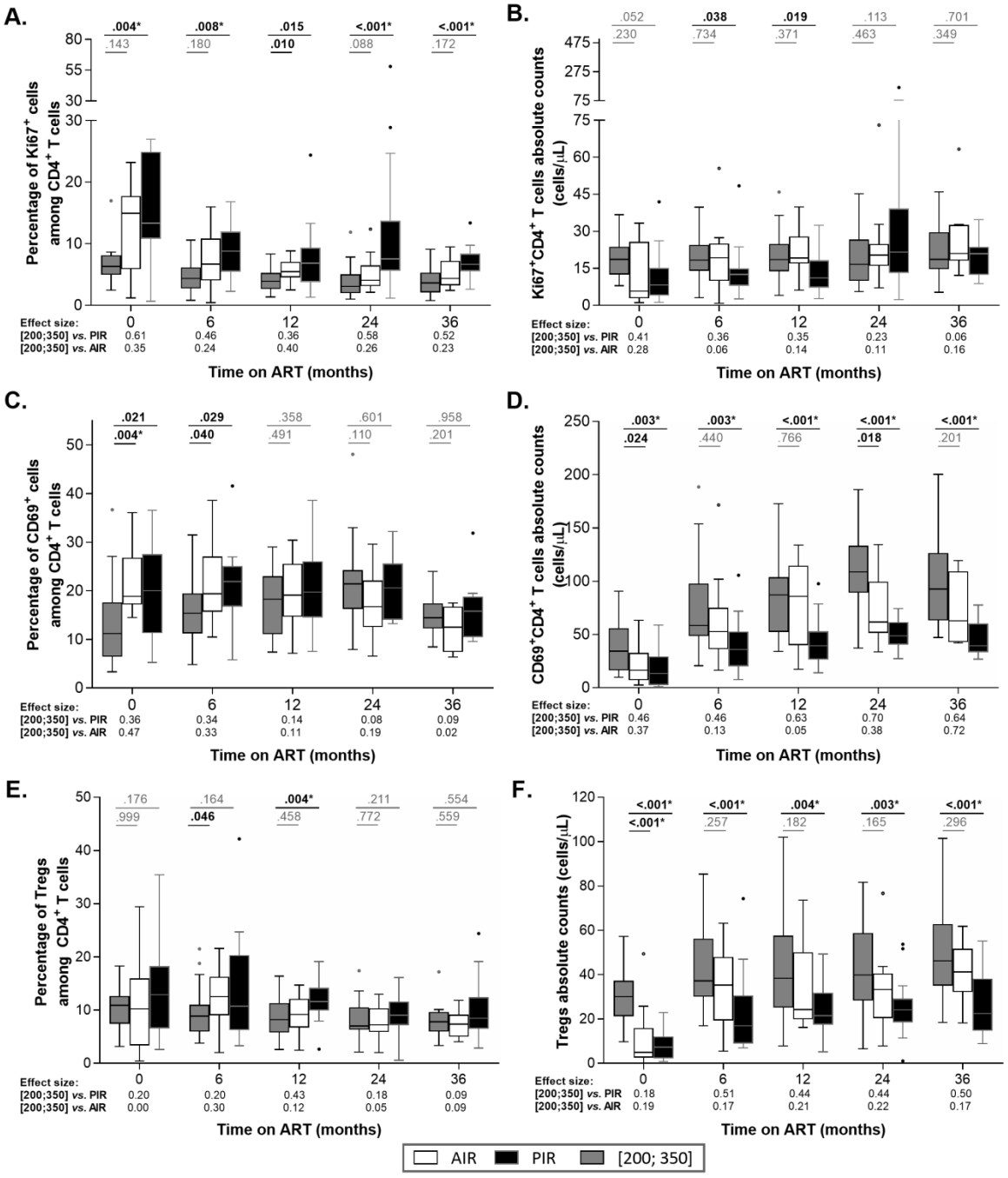

Figure S7 | Comparison of FACS data between patients with CD4$^+$ T cell count of [200; 350] cells/µL and patients with CD4 count <200 cells/µL (AIR and PIR) at baseline, throughout antiretroviral therapy. (A) Percentage of Ki67$^+$ cells among CD4$^+$ T cells; **(B)** Absolute numbers of Ki67$^+$CD4$^+$ T cells; **(C)** Percentage of CD69$^+$ cells among CD4$^+$ T cells; **(D)** Absolute numbers of CD69$^+$CD4$^+$ T cells; **(E)** Percentage of HLA-DR$^+$ cells among CD4$^+$ T cells; **(F)** Absolute numbers of HLA-DR$^+$CD4$^+$ T cells. Grey boxplots represent patients with [200; 350] cells/µL; white boxplots represent AIR; black boxplots represent PIR. Comparisons were performed using Wilcoxon/Mann-Whitney U-tests, and the correspondent r is presented as a measure of effect size. *, significant after Bonferroni correction (α / 5 = .010).

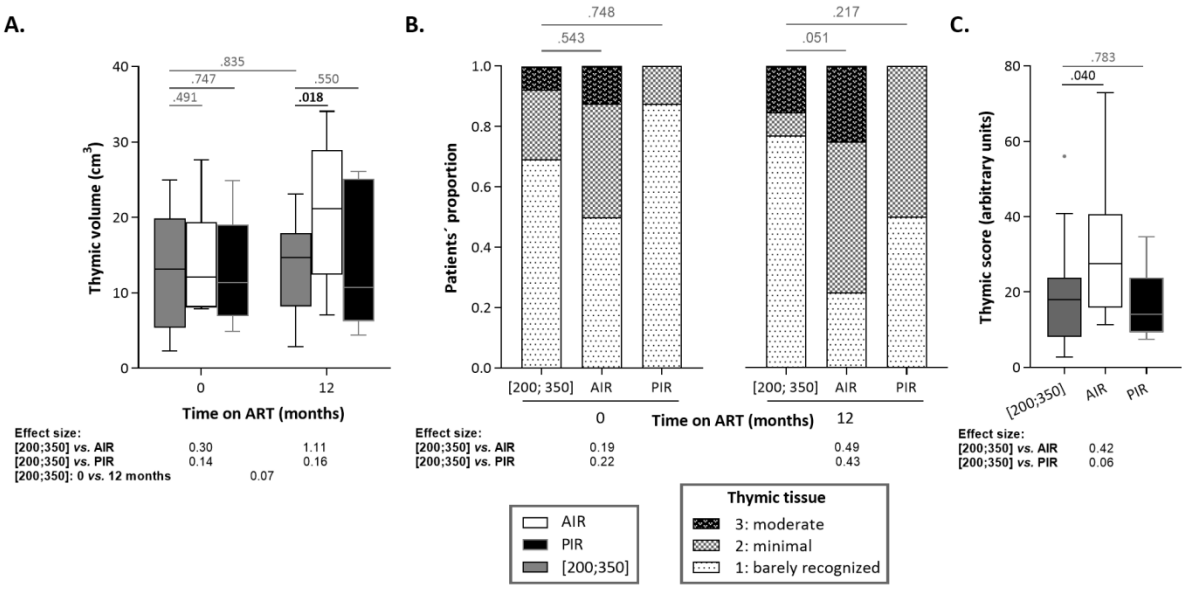

Figure S8 | Comparison of imaging data between patients with CD4$^+$ T cell count of [200; 350] cells/µL and patients with <200 cells/µL (AIR and PIR) at baseline, throughout antiretroviral therapy. (A) Thymic volume and **(B)** thymic index were compared between patients with CD4$^+$ T count of [200; 350] cells/µL and AIR and PIR, respectively, at baseline and at 12 months of ART. **(C)** Thymic scores were calculated as the product of thymic volume and index means, over the first year of ART, and were also compared between those patients. Thymic volumes **(A)** were compared using independent t-tests; Cohen's d were calculated as a measure of effect size. Thymic indices **(B)** were compared using Fisher exact test and effect size estimates were calculated using Cramér's V. Thymic scores **(C)** were compared using independent Wilcoxon-Mann-Whitney U-tests, and the correspondent r is presented as a measure of effect size.

4.2 – Exploring data from HIV-infected patients using mathematics and programming

"... mathematics is not just useful but essential for understanding biological systems and (...) the immune system more than most needs a mathematical approach if our understanding is to move forward beyond the purely descriptive."

Robin E. Callard & Andrew J. Yates
From "Immunology and mathematics: crossing the divide" [1]

"Computing has revolutionized the biological sciences over the past several decades, such that virtually all contemporary research in molecular biology, biochemistry, and other biosciences utilizes [programming]."

Berk Ekmekci, Charles E. McAnany, Cameron Mura
From "An Introduction to Programming for Bioscientists" [2]

Mathematical modeling is an important tool for biomedical research, particularly in the field of Immunology. As early as 1993, Perelson *et al.* presented a mathematical model for the interaction of HIV with $CD4^+$ T cells [3]. The following year, Essunger & Perelson presented another model regarding $CD4^+$ T cell subpopulations (naïve, activated and memory) [4]. In 1998, Kirschner *et al.* presented a mathematical model of HIV infection of thymus, which allowed comparing disease progression between adults and children [5]. Since then, thymic function has been extensively evaluated in HIV-infected patients, using

one or several of the techniques mentioned in the previous section. However, in most studies, data from each technique were analyzed independently. Mathematical models are interesting tools by allowing the combination of data from distinct methods.

Besides the work presented in Chapter 3 and in the subchapter 4.1, two other topics were addressed in the scope of this thesis: 1) thymic score; and 2) model for the thymic output/peripheral proliferation ratio. These topics have in common the application of mathematical tools and programming to explore the data obtained from the cohort of HIV-infected patients. This subchapter presents the results that were achieved in each of these topics.

4.2.1. Thymic score

1. Introduction

Thymic imaging provides a useful contribution to thymic function estimation. Although not consensual, thymic visual features, and their evolution, were suggested to correlate with the production of new T cells [6, 7]. The majority of studies on this topic in the context of HIV infection present data from chest computed tomography (CT) (Tables 7a and b in Chapter 3). At least two thymic parameters can be analyzed by CT: volume and index. Thymic volume is determined based on manual delineation of the thymus in series of images of contiguous slices with the same thickness and length [6, 8]. Thymic index corresponds to a categorical measure of thymic tissue (brighter areas in the CT images) as opposed to adipocyte tissue (less bright in the CT images), as described in the section "3. Thymic function assessment" in Chapter 2. To recap, thymic index is determined by visual inspection of the CT scan images, and it ranges from 0 to 5: 0 - thymus entirely replaced by fat; 1 - minimal, barely recognizable thymic tissue; 2 - minimal thymic tissue, more obvious; 3 - moderate thymic tissue; 4 - moderate thymic tissue of greater extent, almost mass like; 5 - mass-like appearance, that raises concern for thymoma [8]. Thymic index is most frequently used to evaluate the thymus in the context of HIV infection. It has also been used in other contexts, such as HSCT [9, 10], but its application is not widely spread.

Thymic volume and index give independent information on different morphologic features, and it would be useful to combine the information from both in a single parameter. Given that it is expected that a bigger volume and a higher index correspond to higher thymic activity, in theory, a patient with a smaller thymus but a higher index may have a thymic output close to a patient with a bigger thymus but a lower index. Therefore, we hypothesize that the product of thymic volume and thymic index, here named thymic score, evens the comparison between small but very active thymi and big but less active thymi. We explored

this hypothesis by analyzing data on thymic function and immune recovery from a prospective cohort of HIV-infected patients on ART using R software.

2. Material and Methods

Study Participants

Forty-six individuals from a prospective cohort study of HIV-infected patients were selected for this study.[a)] Characteristics of the cohort study (inclusion criteria, ethical approval) are available in the Materials and Methods section of subchapter 4.1. All individuals (n = 46) were followed for at least 12 months, with median follow-up time of 60 months. All patients were therapy compliant throughout the follow-up. Clinical information and peripheral blood samples were retrieved at baseline (just before ART initiation) and at 6, 12, 24, 36, 48, and 60 months on ART (median time deviations to each time point was ≤ 8 days). $CD4^+$ T cell counts and plasma viral load quantification were assessed at all available time points by a certified laboratory.

Immune recovery assessment

The immune recovery was evaluated by $CD4^+$ T cell counts at baseline, 6, 12, 24, 36, 48, and 60 months on ART, their fold-change [defined as $(X_{Time\ point}-X_{Baseline})/X_{Baseline}$] at 6, 12, 24, 36, 48 and 60 months on ART, and their slope (calculated by the least squares estimation method [11]) over the first 12, 24, 36, 48 and 60 months on ART, and by CD4/CD8 ratio at 6, 12, 24, 36, 48 and 60 months on ART.

[a)] The group of patients analyzed in this study ("4.2.1. Thymic score") is different from the group selected for the study presented in subchapter 4.1 (paper Front Immunol. 2019; 10: 25). The former group is composed by the 46 HIV-infected patients submitted to CT scan (whose $CD4^+$ T cell counts at ART initiation varied from 9 to ≥ 500 cells/μL), while the latter group is composed by 33 patients with $CD4^+$ T cell counts < 200 cells/μL at ART initiation and ≥ 36 months of follow-up. All patients were enrolled in the prospective longitudinal study between April 2010 and October 2012 (ethical approval reference 168/CES October 2009).

Thymic function assessment

Thymic function was estimated by three different techniques: imaging (CT scan), molecular biology (TREC quantification) and flow cytometry (RTE analysis). Detailed description of these procedures is available in the Materials and Methods section in the subchapter 4.1.

All patients (n = 46) were submitted to a chest CT scan at baseline and at 12 months on ART. Blood samples from all patients were used for flow cytometry (FACS) analysis (at baseline, 6, 12, 24, 36, 48, and 60 months on ART). In a subset of 37 patients, TRECs were quantified in isolated PBMCs (at baseline, 6, 12, 24 and 36 months on ART).

Thymic score in the first year of ART was calculated by multiplying the mean thymic volume (average between thymic volume at baseline and at 12 months on ART) by the mean thymic index (average between thymic index at baseline and at 12 months on ART).

Statistical analysis

The statistical analysis was performed in the computing environment R v3.5.2, using RStudio v1.1.463. Graphs were built using GraphPad Prism v8.1.0.325.

Continuous numerical variables were presented as mean and SD, if normally distributed, or as median and range percentile 25th to percentile 75th [P25, P75], if not normally distributed. Categorical and ordinal variables were presented as absolute numbers and percentages. Normality of data distribution was assessed by Shapiro-Wilk test. Correlations between two continuous numerical variables were assessed using Pearson (r, for normal distributions) or Spearman rank test (ρ, for non-normal distributions). Correlation coefficient (r or ρ) was considered negligible/low if ≤ 0.39; moderate if [0.4;0.7[; high if [0.7; 0.9[; or very high if [0.9; 1.0]. Comparison of quantitative data between two independent groups were made by using independent t-test (for data with normal distribution) or Mann–Whitney U test (for data with non-normal distributions). For more than two groups, these comparisons were performed using one-way independent analysis of variance (ANOVA), when ANOVA assumptions were met, or Kruskal-Wallis test. For qualitative data, comparison between two groups was performed using Fisher exact test

(one- or two-tailed, as appropriate). For more than two groups, comparisons were performed using Qui-squared (X^2) test. Comparison of quantitative data of the same group between two time points were made by paired-samples t-test (for normal distributions) or Wilcoxon signed-rank test (for non-normal distributions). Other longitudinal comparisons were performed using repeated-measures ANOVA or Friedman test, based on assumptions met.

Missing data were handled by complete case analysis (removing patients with missing information). Severe outliers were defined as lower than P25-3×IQR or higher than P75+3×IQR. A p-value < 0.05 was considered statistically significant. Effect size determined by Cohen's *d* was considered small if < 0.3; medium if [0.3,0.8]; or large if ≥ 0.8; effect size determined by *r* was considered small if < 0.3; medium if [0.3, 0.5]; or large if ≥ 0.5.

Thymic volume, index and score analysis in R

An Excel file with all data regarding the demographic and clinical characteristics of patients, as well as the thymic function surrogates and the immune recovery parameters (Table S1 in Supplementary Material), was imported to R environment as a data frame called "df.46". Data structure and content were carefully verified, including: names and types of the variables; range of the values in each variable or the set of possible values; and existence of missing /unknown values.

To analyze the correlations between CT scan data (thymic volume, index or score) and the immune recovery parameters, two data frames were created selecting two subsets of variables from "df.46": "Group1.df", with all the CT scan variables; and "Group2.df", with the immune recovery variables (R code I in Supplementary Material). A data frame called "Correlation.df" was created to list: i) the results of Shapiro-Wilk tests for each CT scan variable and each parameter of immune recovery; ii) the correlation test used for each combination between a CT scan variable and a parameter of immune recovery, and iii) the respective p-value and correlation coefficient (R code II in Supplementary Material). Severe

outliers, defined as explained in the Statistical analysis section, were determined (R code III in Supplementary Material). All correlations were initially calculated with all data and recalculated after exclusion of severe outliers, and if the exclusion led to different results, that information was cleared stated.

The same strategy (*i.e.* the procedure described in the previous paragraph) was applied to analyze the correlations between CT scan data (thymic volume, index or score) and the other thymic function surrogates (TRECs levels and RTE percentages and counts).

3. Results

Patients' demographic and clinical characteristics.

Summary demographic and clinical characteristics of the 46 patients are available in Table 4.

Most patients (n = 36, 78 %) started ART with emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF), associated with efavirenz (EFV). After a median time of 6 months on ART, all patients presented sustained plasma viral loads below 50 copies/mL, except five individuals who had viral blips, defined as single HIV RNA value between 50 and 400 copies/mL preceded and proceeded by values < 50 copies/mL, if patient still on follow-up. Evolution of viral load and $CD4^+$ T cell count during the follow-up are presented in Figures 6 and 7.

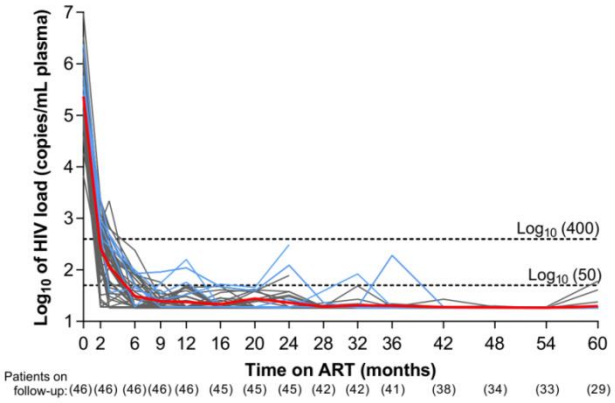

Figure 6 – Viral load evolution for each patient, throughout ART.

Each grey or blue line represents a single patient; blue lines stand for patients who presented virological blips; the red line represents the median of all patients' HIV plasma loads over time. The detection limit of the technique was 19 copies/mL of plasma [$Log_{10}(19)=1.28$].

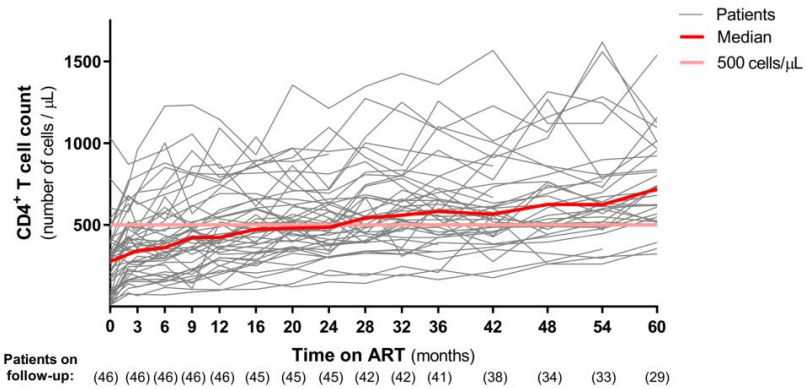

Figure 7 – $CD4^+$ T cell evolution for each patient, throughout ART.

Each grey line represents a single patient; the red line represents the trajectory of the medians of $CD4^+$ T cell counts over time.

Table 4 - Demographic and clinical characteristics of the patients (n = 46).

HIV-infected patients	Total
Gender, male : female, n (%)	34 : 12 (74% : 26%)
Age at baseline, in years	
Mean ± SD	40.0 ± 10.0
Range	[22; 63]
Clinical categories*, n (%)	
A (Asymptomatic or acute HIV infection)	17 (37 %)
B (not A or C)	22 (48 %)
C (AIDS-indicator conditions)	7 (16 %)
CD4+ T-cell categories, n (%)	
1 (> 500 CD4+ T cells/µL)	5 (11 %)
2 (200-499 CD4+ T cells/µL)	23 (50 %)
3 (< 200 CD4+ T cells/µL)	18 (39 %)
HIV subtype, %	
B	23 (50%)
G	12 (26%)
C	7 (15%)
Other/Unknown	4 (9%)
HIV viral load at baseline, in log10 copies per mL, Mean + SD	5.36 ± 0.61
CD4+ T cell count at baseline, in cells/µL Median [Min, Max]	278 [9; 1033]
HIV transmission mode, %	
Intravenous drug user	6 (13 %)
Men who have sex with men	14 (30 %)
Heterosexual	21 (46 %)
Other/Unknown	5 (11 %)
ART regimen components, n (%)	
2 NRTIs: TDF+FTC	36 (78 %)
ABC+3TC	10 (22 %)
3rd Drug: EFV	37 (80 %)
DRV/r	9 (20 %)

*According to the Centers for Disease Control and Prevention (CDC) Classification System.
ABC, abacavir; CDC, Centers for Disease Control and Prevention; DRVr, ritonavir boosted darunavir; EFV, efavirenz; FTC, emtricitabine; IQR, interquartile range; NRTI, nucleoside or nucleotide analogue reverse transcriptase inhibitors; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine.

Thymic volume increased during the first year on ART and the volume change was correlated with body weight change.

At baseline, thymic volume was not significantly correlated with age, HIV plasma load, $CD4^+$ T cell count or CD4/CD8 ratio (Figure 8). Female and male patients presented similar ages (41 ± 9 years *versus* 39 ± 10 years; t(23.7) = 0.637, *p*-value = 0.530), but female patients presented lower thymic volumes at baseline than male patients (7.2 [4.7; 10.7] cm^3 versus 13.9 [9.6; 21.0] cm^3, W = 90; *p*-value = 0.004; *r* = - 0.43) (Figure 9).

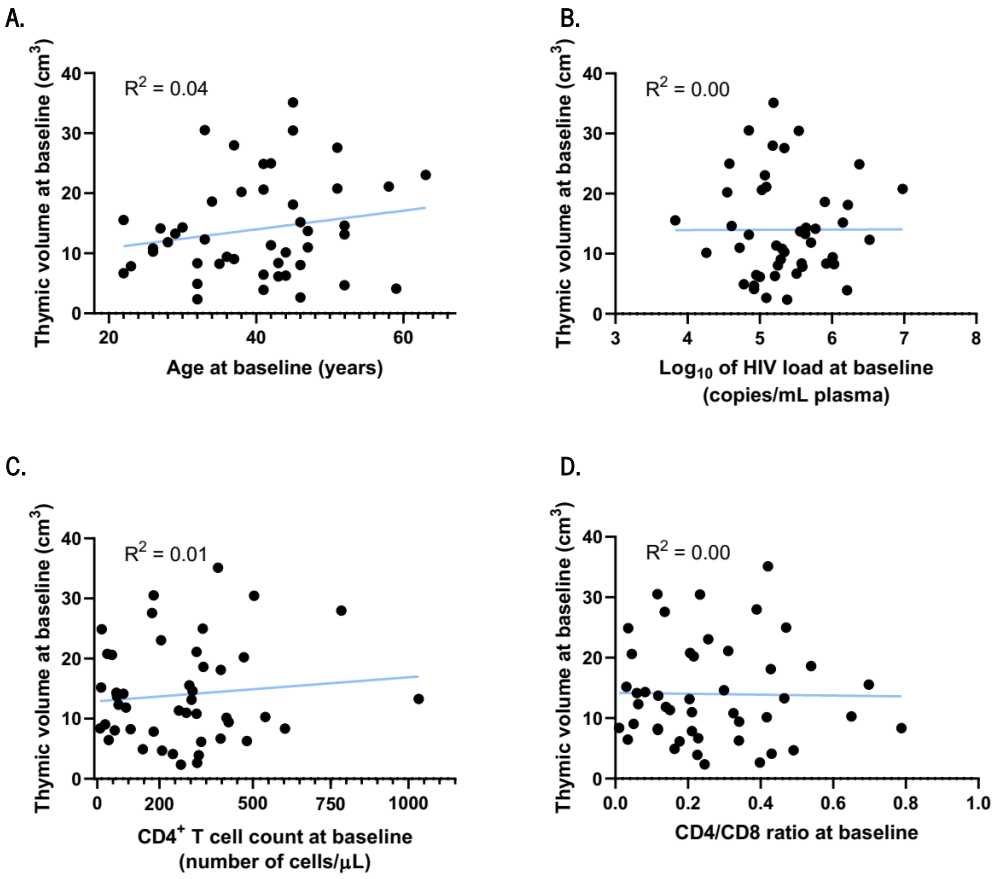

Figure 8 – No correlation between thymic volume and age (A), HIV plasma load (B), $CD4^+$ T cell count (C) or CD4/CD8 ratio (D) at baseline. Each dot represents a single patient. The blue line corresponds to the linear regression line.

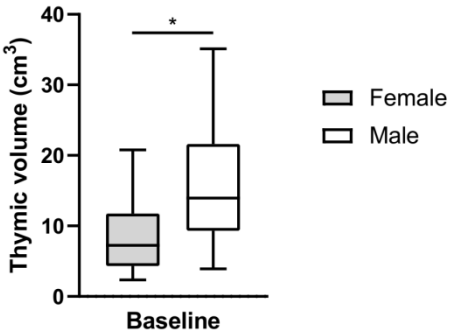

Figure 9 – Comparison of thymic volumes at baseline between female and male patients.
*, *p*-value < 0.05.

Thymic volume was positively correlated with body weight (S = 6481, p < 0.001, ρ = 0.60) and with body mass index (S = 5478, p < 0.001, ρ = 0.52) at baseline (Figure 10). Importantly, female patients presented lower body weight compared to male patients (57.5 ± 9.9 Kg versus 71.9 ± 12.7 Kg; t(24.7) = - 4.028, p < 0.001, Cohen's d = - 1.20) but no difference was found regarding body mass index (22.4 ± 3.8 Kg/m^2 versus 24.5 ± 3.9 Kg/m^2; t(15.7) = - 1.546, *p*-value = 0.142) at baseline.

Altogether, thymic volume was lower at baseline (12.1 [8.1; 19.8] cm^3) compared to 12 months on ART (14.6 [8.7; 21.3] cm^3) (W = 304; *p*-value = 0.009; *r* = - 0.39) (Figure 11a). During the first year on ART, thymic volume increased in 26 patients (57 %); it was maintained (variation < 5 %) in 9 patients (20 %), and it decreased in 11 patients (24 %) (Table 5). There were no differences in thymic volume change regarding patient sex (W = 228, *p*-value = 0.562) (Figure 11b). There was no correlation between thymic volume change and age (S = 16949, *p*-value = 0.765, ρ = - 0.05) (Figure 12a). However, there was a positive correlation between thymic volume change and weight change (defined as the difference between weight at 12 months on ART and baseline) (S = 6816, *p*-value < 0.001, ρ = 0.58) (Figure 12b).

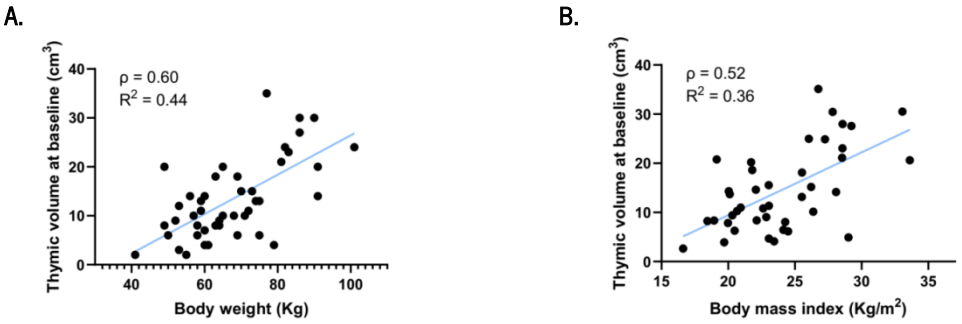

Figure 10 – Positive correlation between thymic volume and both body weight (A) and body mass index (B) at baseline. Each dot represents a single patient. The blue line corresponds to the linear regression line.

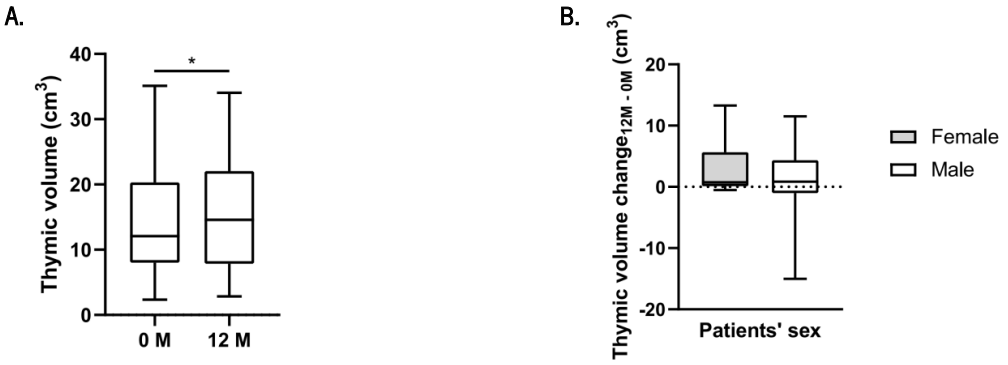

Figure 11 – Comparison between thymic volumes at baseline and 12 months on ART (A) and between thymic volume change in female and male patients (B). *, *p*-value < 0.05.

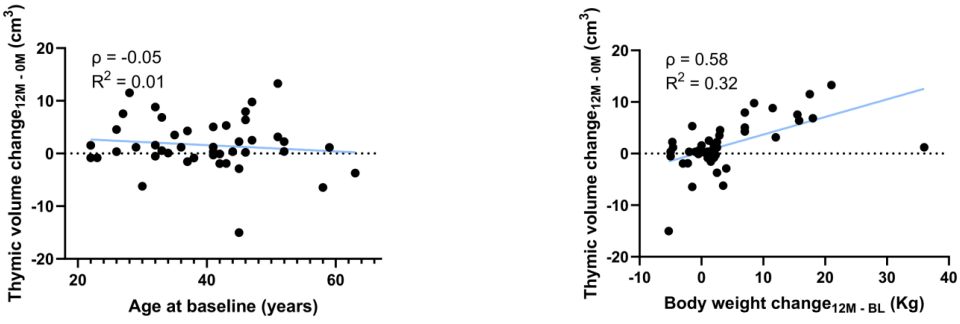

Figure 12 – Correlation between thymic volume change and age at baseline (A), and between thymic volume change and body weight change from baseline to 12 months on ART (B).
Each dot represents a single patient. The blue lines correspond to the linear regression lines.

Table 5 – Thymic volumes and indices at baseline and 12 months of ART.

Sex	Patient ID	Thy. volume 0M	Thy. volume 12M	Thy. index 0M	Thy. index 12M	Thy volume change	Thy index change
Female	AH033	12,30	19,16	2	3	6,86	+ 1
	AH044	8,05	14,43	1	1	6,39	0
	AH045	6,29	6,66	1	3	0,36	+ 2
	AH047	2,65	2,86	1	1	0,20	0
	AH049	10,16	10,50	1	1	0,35	0
	AH052	8,24	11,75	1	2	3,50	+ 1
	AH053	20,78	34,06	1	2	13,27	+1
	AH060	18,63	18,72	3	3	0,09	0
	AH063	4,92	4,41	2	2	-0,51	0
	AH070	2,34	3,93	1	1	1,59	0
	AH073	6,45	6,17	1	2	-0,28	+ 1
	AH084	4,12	5,27	1	1	1,14	0
Male	AH034	14,63	16,89	2	1	2,26	- 1
	AH035	9,05	13,34	1	2	4,29	+ 1
	AH037	11,85	23,38	2	1	11,53	- 1
	AH039	8,38	6,50	1	1	-1,88	0
	AH040	9,42	10,59	3	3	1,17	0
	AH041	18,10	15,23	2	1	-2,87	- 1
	AH042	11,34	11,27	1	1	-0,07	0
	AH046	35,11	20,11	2	1	-15,00	- 1
	AH048	13,73	23,52	1	1	9,78	0
	AH050	13,16	13,56	1	1	0,40	0
	AH051	27,99	26,46	2	2	-1,54	0
	AH054	10,99	13,53	1	1	2,53	0
	AH055	14,16	21,70	1	1	7,54	0
	AH058	3,91	4,21	2	2	0,30	0
	AH059	13,28	14,47	2	2	1,18	0
	AH061	24,98	23,08	1	1	-1,90	0
	AH062	23,06	19,35	1	1	-3,70	0
	AH064	8,36	17,18	1	3	8,82	+ 2
	AH065	10,82	15,36	1	2	4,54	+ 1
	AH066	15,17	23,12	1	2	7,95	+ 1
	AH067	30,46	32,71	1	2	2,25	+ 1
	AH068	10,27	10,63	2	2	0,36	0
	AH069	6,15	11,47	1	1	5,32	0
	AH071	6,68	5,87	2	1	-0,81	- 1
	AH074	14,33	8,12	1	1	-6,21	0
	AH075	21,12	14,68	1	1	-6,44	0
	AH076	15,54	17,11	2	3	1,56	+ 1
	AH077	20,21	19,35	3	3	-0,86	0
	AH078	4,67	5,06	2	1	0,39	- 1
	AH079	20,62	25,67	1	2	5,04	+ 1
	AH080	24,88	26,10	1	1	1,22	0
	AH081	30,53	31,09	2	2	0,56	0
	AH082	27,59	30,75	2	3	3,15	+ 1
	AH083	7,86	7,03	3	2	-0,83	- 1

Note: Thymic volumes are presented in cm^3. Thymic volume change corresponds to the difference between thymic volume at baseline and thymic volume at 12 months on ART. Patient ID, patient identification code.

Thymic index at 12 months on ART was equal to the baseline in most patients.

Thymic index did not change between baseline and 12 months on ART in 26 patients (57 %). In 13 patients (28 %) there was an increase, and in 7 patients (15 %) there was a decrease in thymic index at 12 months on ART compared to baseline (Table 5). There were no significant differences between female and male patients regarding the thymic index at baseline, at 12 months on ART or the thymic index change. There was no significant difference in the thymic volume between patients with distinct thymic indices at baseline or at 12 months on ART (Figure 13).Thymic index at baseline correlated positively with $CD4^+$ T cell count (S = 10540, *p*-value = 0.017, ρ = 0.35) and CD4/CD8 ratio at baseline (S = 11022, *p*-value = 0.030, ρ = 0.32) (Table 6).

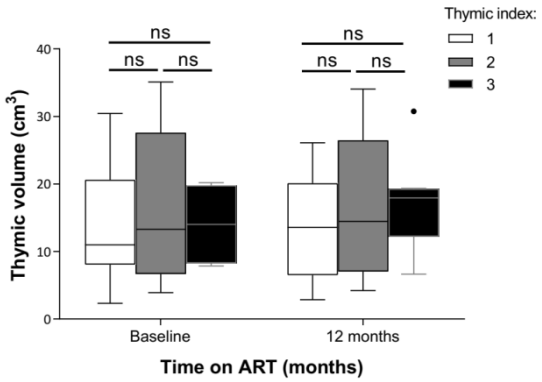

Figure 13 – Comparion of thymic volume between patients with different thymic indices.
ns, not significant.

Thymic score correlated with less parameters of immune recovery compared to thymic index.

Correlations between thymic volume, thymic index or thymic score and each of the parameters of the immune recovery of HIV-infected patients on ART were analyzed (Table 6). The thymic volumes at baseline and at 12 months were positively correlated with $CD4^+$ T cell count slopes on the first 24 and 36 months on ART on ART. Thymic volume did not correlate with any other immune recovery parameter. Thymic score correlated significantly with $CD4^+$ T cell counts at 36 and 48 months on ART, and also with the $CD4^+$ T cell count

slopes in the first 24 and 36 months on ART. Thymic index mean correlated with $CD4^+$ T cell counts at baseline, 12 and 48 months on ART (Table 6).

Table 6 – Correlation matrix: correlations between thymic volume, index or score and several parameters of immunological recovery during ART.

		Thymic volume				Thymic index			Thymic score [a]
		0 mo.	12 mo.	Change	Mean	0 mo.	12 mo.	Mean	[0, 12] mos.
$CD4^+$ T cell count	0	ρ = 0.03	ρ = -0.10	ρ = -0.32	ρ = -0.02	ρ = 0.35	ρ = 0.18	ρ = 0.31	ρ = 0.16
	6	ρ = 0.16	ρ < 0.01	ρ = -0.32	ρ = 0.10	ρ = 0.20	ρ = 0.09	ρ = 0.18	ρ = 0.19
	12	ρ = 0.10	ρ = 0.05	ρ = -0.12	ρ = 0.11	ρ = 0.30	ρ = 0.29	ρ = 0.35	ρ = 0.28
	24	ρ = 0.19	ρ = 0.07	ρ = -0.28	ρ = 0.16	ρ = 0.28	ρ = 0.18	ρ = 0.27	ρ = 0.29
	36	ρ = 0.23	ρ = 0.24	ρ = -0.06	ρ = 0.26	ρ = 0.30	ρ = 0.23	ρ = 0.28	ρ = 0.40
	48	ρ = 0.20	r = 0.17	ρ = -0.18	r = 0.24	ρ = 0.28	ρ = 0.24	ρ = 0.35	ρ = 0.34
	60	ρ = 0.15	r = 0.13	ρ = -0.13	r = 0.21	ρ = 0.19	ρ = 0.21	ρ = 0.24	ρ = 0.27
$CD4^+$ T cell count fold change	[0, 6]	ρ = 0.15	ρ = 0.22	ρ = 0.22	ρ = 0.19	ρ = -0.3	ρ < 0.01	ρ = -0.15	ρ = 0.08
	[0, 12]	ρ = 0.11	ρ = 0.22	ρ = 0.30	ρ = 0.17	ρ = -0.23	ρ = 0.06	ρ = -0.09	ρ = 0.08
	[0, 24]	ρ = 0.10	ρ = 0.25	ρ = 0.32	ρ = 0.17	ρ = -0.26	ρ = -0.08	ρ = -0.18	ρ = 0.03
	[0, 36]	ρ = 0.13	ρ = 0.26	ρ = 0.31	ρ = 0.2	ρ = -0.24	ρ = 0.02	ρ = -0.12	ρ = 0.10
	[0, 48]	ρ = 0.05	ρ = 0.23	ρ = 0.34	ρ = 0.15	ρ = -0.39	ρ = -0.04	ρ = -0.19	ρ = 0.02
	[0, 60]	ρ = 0.02	ρ = 0.20	ρ = 0.34	ρ = 0.12	ρ = -0.32	ρ = -0.10	ρ = -0.24	ρ = -0.02
$CD4^+$ T cell count slope	[0, 12]	ρ = 0.10	ρ = 0.19	ρ = 0.24	ρ = 0.18	ρ = 0.06	ρ = 0.30	ρ = 0.24	ρ = 0.26
	[0, 24]	ρ = 0.34	r = 0.34	ρ = 0.13	r = 0.33	ρ = 0.15	ρ = 0.23	ρ = 0.23	ρ = 0.45
	[0, 36]	ρ = 0.37	r = 0.42	ρ = 0.09	r = 0.44	ρ = 0.13	ρ = 0.21	ρ = 0.19	ρ = 0.48
	[0, 48]	ρ = 0.16	r = 0.33	ρ = 0.05	r = 0.38	ρ = -0.02	ρ = 0.33	ρ = 0.26	ρ = 0.32
	[0, 60]	ρ = 0.22	r = 0.31	ρ = -0.04	r = 0.37	ρ = -0.11	ρ = 0.25	ρ = 0.14	ρ = 0.29
CD4/CD8 ratio	0	ρ = -0.03	ρ = -0.16	ρ = -0.29	ρ = -0.08	ρ = 0.32	ρ = 0.08	ρ = 0.23	ρ = 0.07
	6	ρ = 0.09	ρ = -0.01	ρ = -0.17	ρ = 0.06	ρ = 0.12	ρ = 0.05	ρ = 0.11	ρ = 0.10
	12	ρ = 0.01	ρ = -0.09	ρ = -0.22	ρ = -0.03	ρ = 0.22	ρ = 0.15	ρ = 0.23	ρ = 0.11
	24	ρ < 0.01	ρ = -0.13	ρ = -0.27	ρ = -0.06	ρ = 0.25	ρ = 0.09	ρ = 0.20	ρ = 0.08
	36	ρ = 0.03	ρ = -0.07	ρ = -0.22	ρ = -0.01	ρ = 0.21	ρ = 0.11	ρ = 0.19	ρ = 0.10
	48	ρ = 0.05	ρ = -0.07	ρ = -0.27	ρ < 0.01	ρ = 0.20	ρ = 0.05	ρ = 0.17	ρ = 0.09
	60	ρ = -0.01	r = -0.22	ρ = -0.37	r = -0.06	ρ = 0.10	ρ = -0.04	ρ = 0.07	ρ = -0.05

Note: The correlation coefficient between each pair of variables (respective column and row) was calculated using the Pearson or Spearman rank tests (r or ρ), depending on the data distributions. Cells are shaded according to the *p*-value: dark pink, *p*-value ≤ 001; light pink, *p*-value > 0.01 and ≤ 0.05.

[a] Thymic score = Volume Mean $_{[0,12]}$ × Index Mean $_{[0,12]}$

Table 7 – Correlation matrix: correlations between thymic volume, index or score and other thymic function surrogates.

			Thymic volume				Thymic index			Thymic score [a]
			0 mos.	12 mos.	Change	Mean	0 mos.	12 mos.	Mean	[0, 12] mos.
Recent thymic emigrants (CD31+CD45RA+CD4+ T cells)	cells/µL	0	ρ = 0.07	ρ = 0.16	ρ = 0.03	ρ = 0.03	ρ = 0.38	ρ = 0.29	ρ = 0.39	ρ = 0.23
		6	ρ = 0.13	ρ = 0.10	ρ = 0.16	ρ = 0.16	ρ = 0.36	ρ = 0.32	ρ = 0.40	ρ = 0.29
		12	ρ = 0.18	ρ = 0.22	ρ = 0.10	ρ = 0.10	ρ = 0.37	ρ = 0.45	ρ = 0.49	ρ = 0.41
		24	ρ = 0.23	ρ = 0.20	ρ = 0.19	ρ = 0.19	ρ = 0.43	ρ = 0.46	ρ = 0.54	ρ = 0.48
		36	ρ = 0.27	ρ = 0.29	ρ = 0.23	ρ = 0.29	ρ = 0.37	ρ = 0.49	ρ = 0.50	ρ = 0.53
	Percentage amnong CD4+ T cells	0	ρ = 0.17	r = -0.07	ρ = -0.17	r = 0.22	ρ = 0.32	ρ = 0.33	ρ = 0.38	ρ = 0.32
		6	ρ = 0.09	r = 0.01	ρ = -0.07	r = 0.14	ρ = 0.41	ρ = 0.42	ρ = 0.50	ρ = 0.32
		12	ρ = 0.23	r = 0.12	ρ = 0.02	r = 0.25	ρ = 0.34	ρ = 0.56	ρ = 0.54	ρ = 0.48
		24	ρ = 0.22	r = 0.21	ρ = 0.09	r = 0.20	ρ = 0.43	ρ = 0.50	ρ = 0.56	ρ = 0.49
		36	ρ = 0.24	r = 0.28	ρ = 0.12	r = 0.29	ρ = 0.36	ρ = 0.58	ρ = 0.56	ρ = 0.54
sj-TREC	number/10^6 cells	0	ρ = 0.02	ρ = 0.06	ρ < 0.01	ρ = 0.04	ρ = 0.38	ρ = 0.51	ρ = 0.55	ρ = 0.26
		6	ρ = 0.17	ρ = 0.28	ρ = 0.27	ρ = 0.22	ρ = 0.24	ρ = 0.54	ρ = 0.48	ρ = 0.41
		12	ρ = -0.01	ρ = 0.05	ρ = 0.15	ρ< 0.01	ρ = 0.31	ρ = 0.76	ρ = 0.69	ρ = 0.32
		24	ρ = -0.06	ρ = 0.04	ρ = 0.24	ρ = -0.02	ρ = 0.17	ρ = 0.71	ρ = 0.58	ρ = 0.27
		36	ρ = 0.12	ρ = 0.20	ρ = 0.28	ρ = 0.15	ρ = 0.08	ρ = 0.77	ρ = 0.59	ρ = 0.40
	number / mL	0	ρ = -0.08	ρ = -0.04	ρ = 0.05	ρ = -0.06	ρ = 0.31	ρ = 0.50	ρ = 0.52	ρ = 0.19
		6	ρ = 0.16	ρ = 0.29	ρ = 0.32	ρ = 0.23	ρ = 0.22	ρ = 0.53	ρ = 0.46	ρ = 0.41
		12	ρ = 0.03	ρ = 0.10	ρ = 0.20	ρ = 0.06	ρ = 0.33	ρ = 0.72	ρ = 0.68	ρ = 0.36
		24	ρ = 0.03	ρ = 0.14	ρ = 0.26	ρ = 0.08	ρ = 0.20	ρ = 0.73	ρ = 0.61	ρ = 0.36
		36	ρ = 0.11	ρ = 0.18	ρ = 0.26	ρ = 0.14	ρ = 0.19	ρ = 0.76	ρ = 0.62	ρ = 0.41
sj/β TREC ratio		0	ρ = 0.04	ρ = 0.04	ρ = -0.07	ρ = 0.05	ρ = 0.22	ρ = 0.39	ρ = 0.38	ρ = 0.20
		6	ρ < 0.01	r = 0.15	ρ = 0.31	r = 0.09	ρ = 0.23	ρ = 0.53	ρ = 0.43	ρ = 0.31
		12	ρ = -0.02	ρ = 0.03	ρ = 0.16	ρ = -0.02	ρ = 0.35	ρ = 0.61	ρ = 0.57	ρ = 0.32
		24	ρ = 0.03	ρ = 0.11	ρ = 0.18	ρ = 0.07	ρ = 0.34	ρ = 0.64	ρ = 0.59	ρ = 0.40
		36	ρ = 0.18	ρ = 0.28	ρ = 0.25	ρ = 0.22	ρ = 0.03	ρ = 0.68	ρ = 0.51	ρ = 0.44

Note: Each cell in the table contains the correlation coefficient between two variables (respective column and row), calculated by the Pearson or Spearman rank tests (r or ρ), depending on the underlying distributions. Cells are shaded according to the *p*-value: dark pink, *p*-value ≤ 001; light pink, *p*-value > 0.01 and ≤ 0.05.

[a] Thymic score = Volume Mean $_{[0,12]}$ × Index Mean $_{[0,12]}$

Mean thymic index correlated better with the other thymic function surrogates than the thymic volume or score.

Thymic volume (at baseline, at 12 months on ART, its change or mean) did not correlate with any other thymic function surrogates, except for a correlation between thymic volume change and the absolute number of RTE ($CD31^+$ $CD45RA^+$ $CD4^+$ T cells) at baseline (Table 7). Thymic score correlated positively with the absolute number and the percentage of RTE among $CD4^+$ T cells at 12, 24 and 36 months of ART. It also correlated positively with the levels of sj-TRECs (in number/ mL), but not with the sj/β TREC ratio (Table 7). Contrary to thymic volume or score, thymic index (at 12 months on ART or thymic index mean) correlated significantly with all the other thymic function surrogates (Table 7).

Discussion of the results presented in "4.2.1 Thymic score" is integrated in the Part III of this dissertation. To summarize the results: thymic score over the first year of ART correlated with the immune recovery after 24-36 months of therapy, but not with the immune recovery during the first 12 months of ART. Regarding the other thymic function surrogates, thymic score correlated with RTE absolute number and frequency among $CD4^+$ T cells and with sj-TREC levels but not with sj/β TREC ratio. Contrary to thymic score, thymic index showed significant correlations with all the other thymic function surrogates and with more parameters of immune recovery.

These findings suggest that thymic index has more potential than thymic score as an estimate of thymic function in the context of immunological recovery during ART. Further studies are needed to evaluate the predictive value of thymic index in the outcome of HIV-infected patients on ART. The association between thymic volume changes during ART and the changes in body weight and/or body mass index should be further investigated.

4.2.2. Thymic output/peripheral proliferation ratio model

1. Introduction

Several mechanisms contribute to the increase of $CD4^+$ T cell counts after ART initiation, as reviewed in the section "6. Immunological recovery during antiretroviral therapy" in Chapter 1. Migration of memory $CD4^+$ T cells from the lymphoid tissues to the bloodstream is the main contributor to the initial increase in $CD4^+$ T cell number [12], while, after few months of therapy, other mechanisms become more relevant, including thymic output of new $CD4^+$ T cells, peripheral proliferation, extension of cell half-life and decrease of cell death [13, 14]. Each of these mechanisms may be more or less predominant, depending on multiple factors, such as age, presence of co-infections or levels of immune activation and inflammation. We showed that thymic function is particularly relevant for the immune recovery of patients that start ART with < 200 $CD4^+$ T cells/L (subchapter 4.1). In the era of personalized medicine, it is useful to determine the relative contribution of each mechanism for the immune recovery, in order to select or adapt the most adequate therapeutic strategies for each patient. Based on the previous model of thymic output described by Bains *et al.* [15], we developed a model for the ratio between thymic output and peripheral cell proliferation in the context of HIV infection.

2. Material and Methods

Study Participants

Thirty-seven individuals from a prospective cohort study of HIV-infected patients were selected for this study.[b)] Characteristics of the cohort study (inclusion criteria, ethical approval) are available in the Materials and Methods section of subchapter 4.1. All individuals (n = 37) were followed for at least 36 months. All patients were therapy compliant throughout the follow-up. Clinical information and peripheral blood samples were retrieved at baseline (just before ART initiation) and at 6, 12, 24, and 36 months on ART (median time deviations to each time point was ≤ 8 days). $CD4^+$ T cell counts and plasma viral load quantification were assessed at all available time points by a certified laboratory.

Blood samples processing and analysis

Blood samples were collected into Na_2-EDTA collecting tubes and processed on the same day, at baseline, 6, 12, 24 and 36 months on ART. These samples were used for flow cytometry analysis and TREC quantification. Detailed description of these procedures is available in the Materials and Methods section in subchapter 4.1..

Mathematical modeling

The ordinary differential equation models presented in this work were analyzed in the computing environment R (v3.4.2) using R Studio (v1.1.383).

[b)] Data used in "Thymic output/peripheral proliferation ratio model" is from a different group of patients than the group selected for the study described in subchapter 4.1 (paper Front Immunol. 2019; 10: 25). The former group is composed by the 37 HIV-infected patients in which TREC levels were quantified in PBMCs. The $CD4^+$ T cell counts at ART initiation in this group varied from 9 to ≥ 500 cells/μL. In the study described in subchapter 4.1, 33 patients were selected based on $CD4^+$ T cell counts < 200 cells/μL at ART initiation and follow-up ≥ 36 months. Sixteen patients are common between the two groups. All patients were enrolled in the prospective longitudinal study between April 2010 and October 2012 (ethical approval reference 168/CES October 2009).

The dynamics of naïve T cells in the whole body depend on the production of new cells by the thymus, on the peripheral cell proliferation and on the cell death. This dynamics can be represented by the equation from Bains *et al.* [15]:

$$\frac{dN(t)}{dt} = \theta(t) + \rho(t)N(t) - \delta(t)N(t)$$

Equation 1

Where:

$\frac{dN(t)}{dt}$, change of total naïve T cell population during the period of time t, in the whole body;

θ (t), thymic output, number of cells leaving the thymus during the period of time t;

ρ (t), per cell addition rate to the total naïve T cell population through peripheral cell division;

δ (t), average rate of total naïve T cell population loss (cell death);

N, total naïve T cells in the whole body.

Similarly, the dynamics of the TREC content in the total naïve T cells depend on the production by the thymus of new naïve T cells contending TRECs and on the loss of TRECs by cell death, considering that intracellular degradation of TRECs should be negligible. The changes in total TREC content of naïve T cells are described by the equation from Bains *et al.* [15]:

$$\frac{dT(t)}{dt} = c(t)\theta(t) - \delta(t)T(t)$$

Equation 2

Where:

T, TREC content in total naïve T cells (N);

$\frac{dT(t)}{dt}$, change in the total naïve T cell TREC content during the time period t;

c(t), average TREC content of total cells emerging from the thymus;

θ (t), thymic output, number of T cells leaving the thymus during the period of time t;

δ (t), average loss rate of TREC containing naïve T cells (cell death rate).

The average number of TRECs per naïve T cell (τ) varies over time. The changes in that variable can be defined by combining the equations 1 and 2 [15]:

$$\frac{d\tau(t)}{t} = \frac{d}{dt}\left(\frac{T}{N}\right) = \frac{\left(\frac{dT}{dt}.N(t) - \frac{dN}{dt}.T(t)\right)}{N(t)^2}$$

$$\frac{d\tau(t)}{dt} = \frac{\theta(t)}{N(t)}.\left(c(t) - \tau(t)\right) - \rho(t)\tau(t)$$

Equation 3

Where:

$\frac{d\tau(t)}{d(t)}$, change in the average number of TREC per naive T cell during the time period t;

θ (t), thymic output, number of T cells leaving the thymus during the period of time t;

N(t), total naïve T cells in the whole body;

c(t), average TREC content of total cells emerging from the thymus;

τ or T/N , average number of TREC per naïve T cell;

ρ (t), per cell addition rate to the total naïve T cell population through peripheral cell division.

Thymic output (θ) can be defined, according to Bains *et al.* [15], as follows:

$$\frac{d\tau(t)}{dt} = \frac{\theta(t)}{N(t)}.\left(c(t) - \tau(t)\right) - \rho(t)\tau(t)$$

$$\theta(t) = \left(\frac{d\tau(t)}{dt} + \rho(t)\tau(t)\right).\frac{N(t)}{c(t) - \tau(t)}$$

$$\theta(t) = \left(\frac{d\tau(t)}{dt}N(t) + \rho(t)\tau(t)N(t)\right).\frac{1}{c(t) - \tau(t)}$$

Equation 4

Where:

θ (t), thymic output, number of T cells leaving the thymus during the period of time t;

$\frac{d\tau(t)}{d(t)}$, change in the average number of TREC per naïve T cell during the time period t;

N(t), total naïve T cells in the whole body;

ρ (t), per cell addition rate to the total naïve T cell population through peripheral cell division;

τ or $\frac{T}{N}$, average number of TREC per naïve T cell;

c(t), average TREC content of total cells emerging from the thymus.

The determination of the number of dividing cells can be performed based on the expression of Ki67, as follows [16]:

$$\rho(t)N(t) \approx \frac{KN(t)}{\Delta}$$

Equation 5

Where:

ρ (t)N(t), contribution of peripheral cell division (cells/day);

K, fraction of naïve T cells expressing Ki67:

$$\frac{\text{blood naïve T cells expressing Ki67 + tissue naïve T cells expressing Ki67}}{Total\ naïve\ T\ cells};$$

Δ, Ki67 expression duration.

By combining equations 4 and 5, we get:

$$\theta(t) = \left(\frac{d\tau(t)}{dt}N(t) + \tau(t)\frac{KN(t)}{\Delta}\right) \cdot \frac{1}{c(t) - \tau(t)}$$

Equation 6

Considering that:

$$\frac{d\tau(t)}{dt}N(t) \ll \tau(t)\frac{KN(t)}{\Delta}$$

and solving the equation 6, we get:

$$\theta(t) = \left(\tau(t)\frac{KN(t)}{\Delta}\right) \cdot \frac{1}{c(t) - \tau(t)}$$

Equation 7

For each HIV-infected patient, the ratio thymic output/ peripheral cell proliferation (R_θ or R*Theta*) may be determined as follows:

$$\mathrm{R}Theta = \frac{\theta(t)}{\rho(t)N(t)} = \frac{\theta(t)}{\frac{KN}{2\Delta(1-\mathrm{K})}}$$

$$\mathrm{R}Theta = \frac{\tau}{c-\tau}\left(1 + \frac{2\Delta(1-K)}{K} \times \frac{d(\ln\tau)}{\mathrm{dt}}\right)$$

Equation 8

Where:

R*Theta*, ratio thymic output to peripheral cell proliferation;

τ or $\frac{T}{N}$, average number of TREC per naïve T cell;

c(t), average TREC content of total cells emerging from the thymus (0.25, according to [17]);

Δ, Ki67 expression duration;

K, fraction of naïve T cells expressing Ki67;

$\frac{d(\ln\tau)}{\mathrm{dt}}$, slope of the natural logarithm of τ.

3. Results

Patients' characteristics.

From the 37 patients, 26 (70 %) were male. At baseline, mean age was 40 ± 9 years, mean viral load, in log10 copies per mL, was 5.30 ± 0.59, and median $CD4^+$ T cell count was 269 cells/μL (min = 9, max = 784).

At baseline, the median of number of sj-TRECs in all patients was 105 copies/10^5 cells (min = 2, max = 1036); the median of the percentage of naïve T cells among total T cells was 52 % (min=17 %, max = 89 %); the median of the percentage of $Ki67^+$ cells among naïve T cells was 3 % (min = 0 %, max = 24 %).

As the equation 8 considers the number of sj-TRECs, the number of naïve T cells and the percentage of $Ki67^+$ cells among naïve T cells, we analyzed the evolution of these parameters in each patient. Results for all patients are depicted in Figure 14.

Ratio thymic output to peripheral cell proliferation (R_θ) determination

R_θ was calculated for each patient at baseline, 6, 12, 24 and 36 months on ART (Table 8).

R_θ were expected to vary between 0 (no contribution of thymic output to the increase of naïve T cells) and $+\infty$ (negligible contribution of peripheral cell proliferation to the increase of naïve T cells). However, some patients presented negative R_θ values at different time points (Table 8).

R_θ negative values at baseline and 36 months on ART may be explained by the inaccuracy in the determination of $\frac{d(\ln \tau)}{dt}$ at these time points, given that no data before ART initiation and after 36 months was available. Exploring the data, we found that patients with negative R_θ presented relatively low percentages of $Ki67^+$ cells among naïve T cells despite the very negative $\frac{d(\ln \tau)}{dt}$.

There was no correlation between the values of R_θ at the each different time point and the respective $CD4^+$ T cell counts (Figure 15).

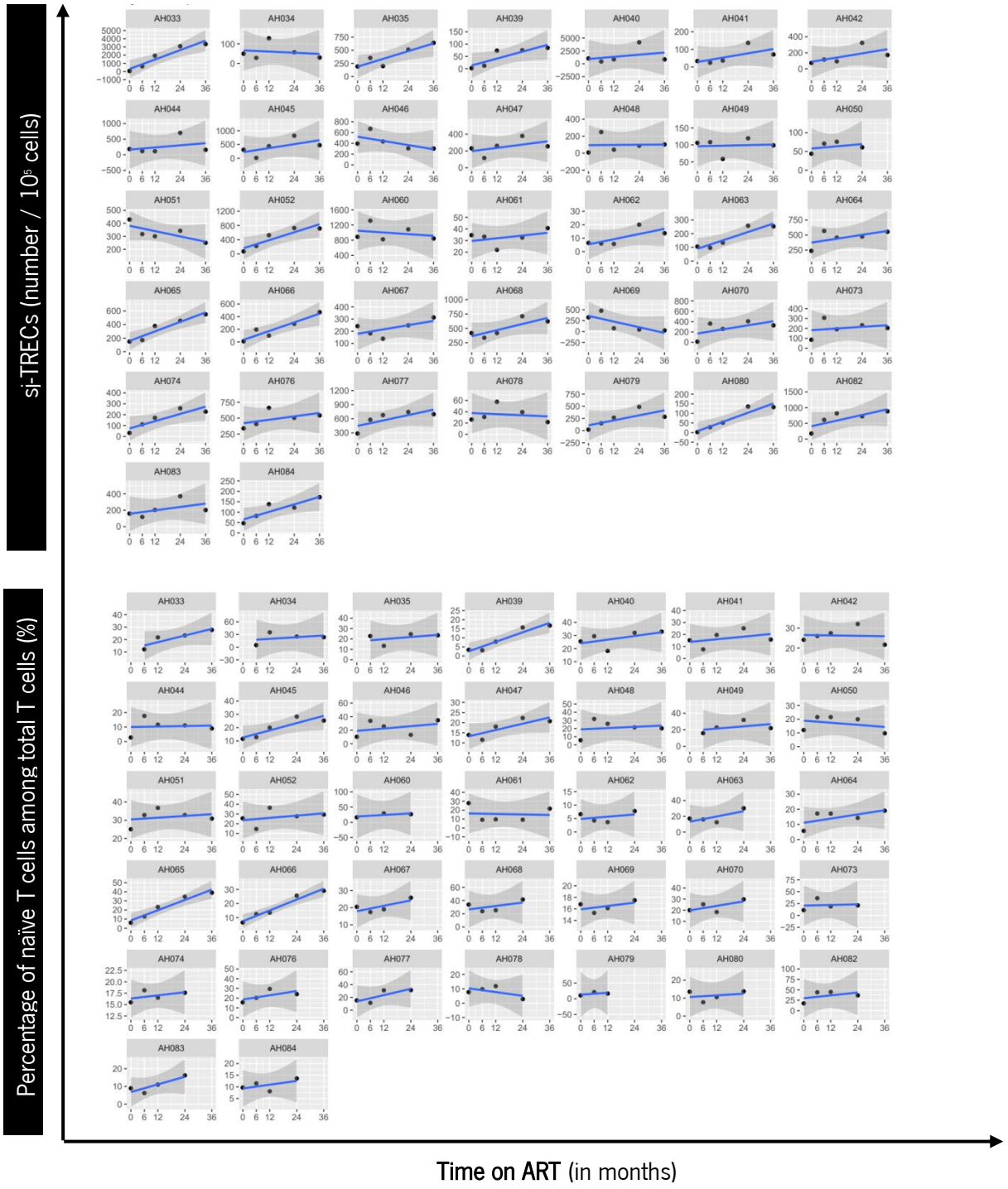

Figure 14 – Evolution of sj-TREC number (A), percentage of naïve T cells (B) and percentage of Ki67⁺ cells among naïve T cells (C) throughout ART, for each patient.

Naïve T cells were identified as CD45RA+CD45RO- cells. Each graph represents a single patient, identified by the patient ID (code AHXXX). Each blue line represents a linear regression line.

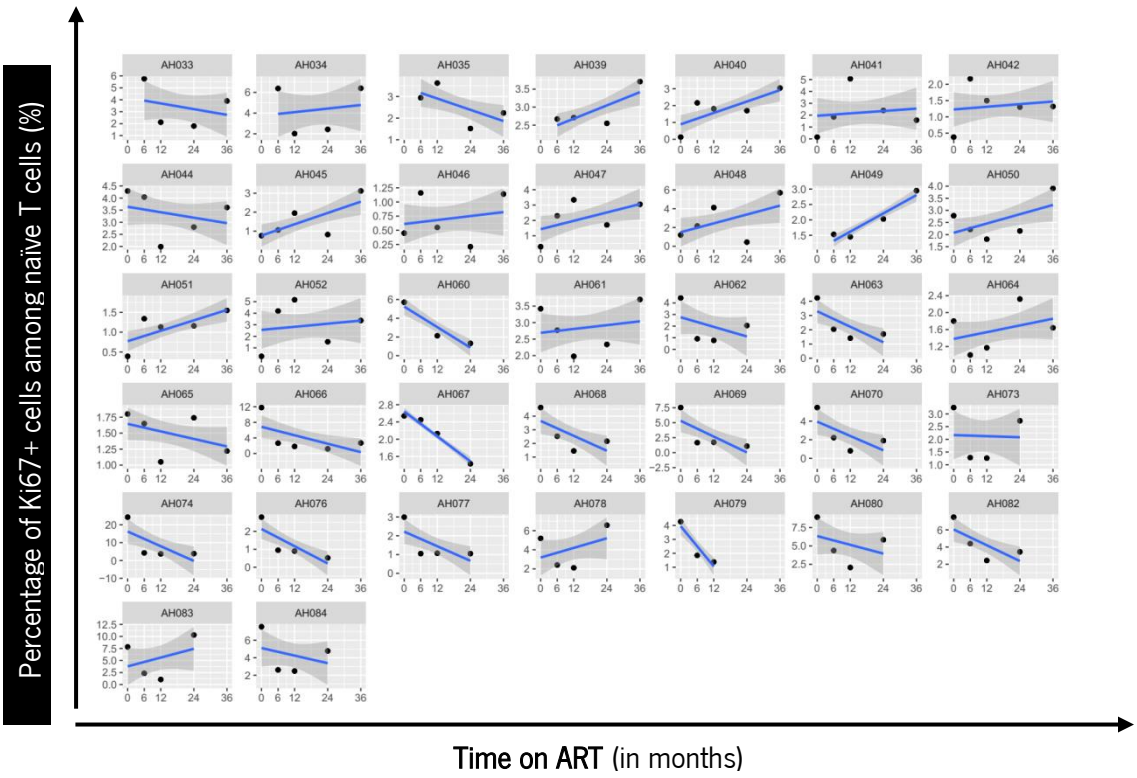

Figure 14 – Continuation.

Table 8 – Ratio thymic output to peripheral cell proliferation (R_θ).

Patient ID	0 mos.	6 mos.	12 mos.	24 mos.	36 mos.
AH033	0,592	1,187	1,360	1,029	1,029
AH034	- 0,023	0,000	0,001	0,000	0,000
AH035	0,087	0,085	0,133	0,149	0,149
AH039	NA	0,103	0,040	0,099	0,099
AH040	0,200	0,528	0,368	- 1,553	- 1,553
AH041	NA	0,012	0,034	0,015	0,015
AH042	0,015	0,026	0,092	0,028	0,028
AH044	0,010	0,136	0,612	- 0,139	- 0,139
AH045	- 0,006	0,286	0,133	0,069	0,069
AH046	- 0,047	0,101	NA	- 0,175	- 0,175
AH047	0,053	0,085	0,073	0,035	0,035
AH048	0,052	0,006	NA	0,034	0,034
AH049	- 0,044	0,006	0,020	0,019	0,019
AH050	0,014	0,018	0,008	NA	NA
AH051	- 0,008	0,040	0,043	0,017	0,017
AH052	0,089	0,064	0,149	0,105	0,105
AH060	- 0,027	0,169	0,195	0,218	0,218
AH061	0,031	0,012	0,013	0,002	0,002
AH062	0,020	0,023	0,018	0,023	0,023

Patient ID	0 mos.	6 mos.	12 mos.	24 mos.	36 mos.
AH063	0,045	0,057	0,044	0,074	0,074
AH064	0,003	0,138	0,159	0,079	0,079
AH065	0,029	0,069	0,050	0,063	0,063
AH066	0,229	0,018	0,087	0,110	0,110
AH067	0,023	0,117	2,643	- 1,632	- 1,632
AH068	0,180	0,046	0,075	0,070	0,070
AH069	- 0,474	- 0,015	0,003	0,011	0,011
AH070	0,264	0,058	0,051	0,047	0,047
AH073	0,050	0,062	0,049	0,046	0,046
AH074	0,066	0,113	0,107	0,043	0,043
AH076	0,103	0,084	0,099	0,149	0,149
AH077	0,728	0,011	0,123	- 0,051	- 0,051
AH078	0,012	NA	0,055	0,015	0,015
AH079	0,132	0,314	0,071	0,049	0,049
AH080	0,040	0,043	0,054	0,023	0,023
AH082	0,076	0,093	0,107	NA	NA
AH083	0,119	0,111	0,107	0,071	0,071
AH084	0,068	0,106	0,048	0,048	0,048

Note: Grey shaded cells indicate negative values. NA, not available.

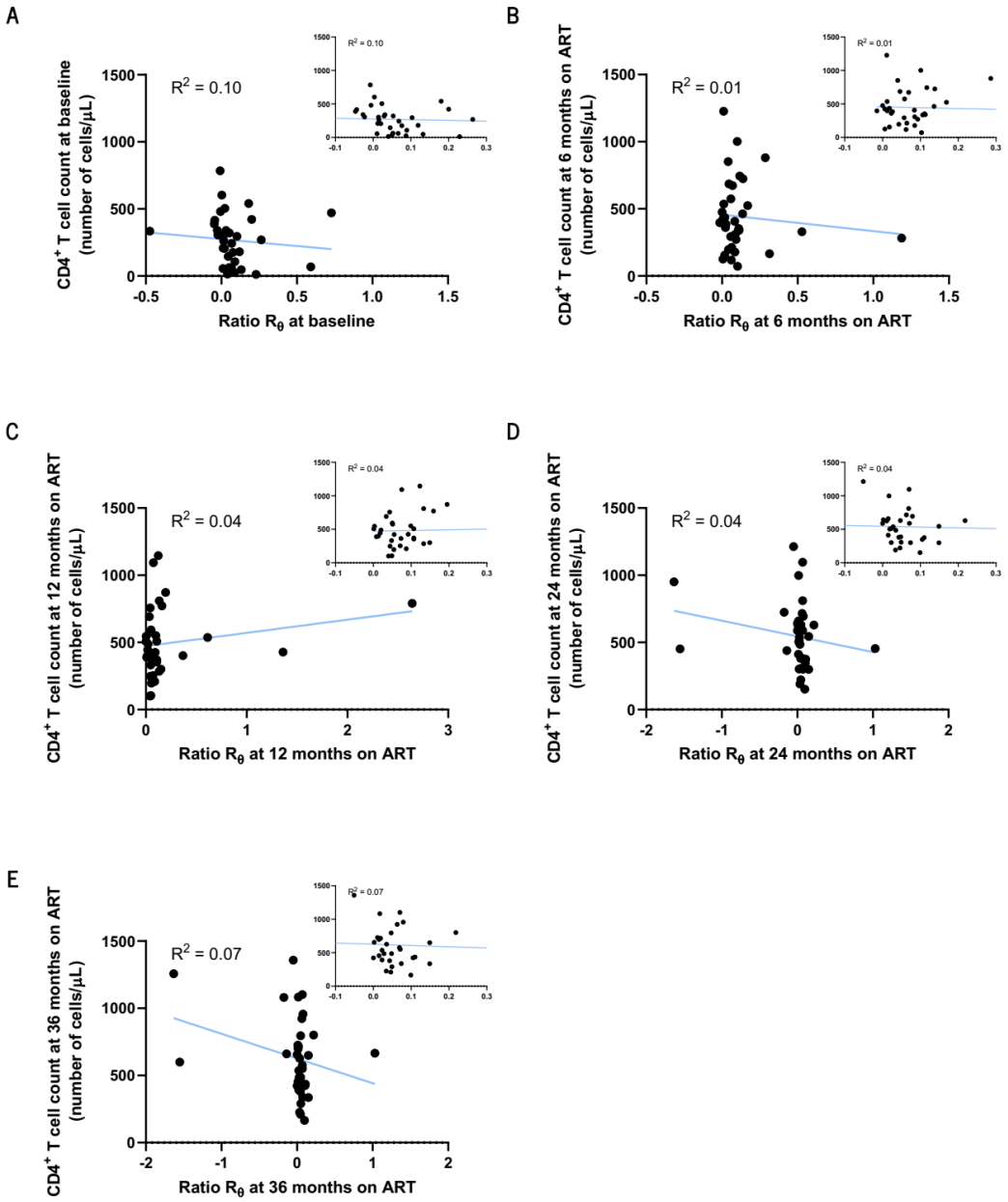

Figure 15 – There was no correlation between the ratios thymic output to peripheral cell proliferation (R_θ) and CD4+ T cell counts, at any time point.

Each graph represents a time point: baseline, just before ART initiation **(A)**, 6 months **(B)**, 12 months **(C)**, 24 months **(D)** and 36 months on ART **(E)**. Each small graph next to the graphs A-E corresponds to an *inset* of the areas with the greatest density of dots. Each dot in the different graphs represents a single patient. Each blue line represents a linear regression line.

The model of the ratio thymic output/peripheral proliferation presented herein did not perform as well as expected. Several reasons might explain the obtention of negative values, as discussed in detail in Part III. Further analyses are needed to verify the causes of the poor performance of the model.

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Supplementary Material (Subchapter 4.2)

Table S1 – List of variables in the Excel file "Thyfunction_Immrecov.xlsx".

1. Thymic function surrogates:
   - thymic volume at baseline ("Volume_0M");
   - thymic volume at 12 months of ART ("Volume_12M");
   - thymic volume mean ("Volume_BL.12_mean");
   - thymic volume change ("Volume_diff'' = "Volume_12M" – "Volume_0M");
   - thymic volume percentage of change (''Volume_perc_change'' = ("Volume_diff" × 100)/ "Volume_0M_mean"));
   - thymic index at baseline ("Index_0M");
   - thymic index at 12 months of ART ("Index_12M");
   - thymic index mean ("Index_BL.12_mean");
   - thymic score ("Thymic_score");

   1.1. For each of the time points 0, 6, 12, 24 and 36 months of ART:
   - number of sj-TRECs /10^5 cells (e.g. "sjTREC_Median_0M")
   - number of sj-TRECs /mL (e.g. "sjTREC.per.mL_0M")
   - number of β-TRECs /10^5 cells (e.g. "bTREC_Median_0M")
   - sj/ β TREC ratio (e.g. "TREC_ratio_0M")

   1.2. For each of the time points 0, 2, 3, 6, 9, 12, 16, 20, 24, 28, 32, 36, 42, 48, 54 and 60 months of ART:
   - percentage of RTE among CD4+ T cells (e.g. "RTE_PERC.of.CD4_0M")
   - number of RTE per μL (e.g. "RTE_per.uL_0M")

2. Immune recovery parameters:
   - CD4+ T cell count at 0, 6, 12, 24, 36, 48, and 60 months of ART (e.g. "CD4_Count_0M");
   - CD4+ T cell count fold-change at 6, 12, 24, 36, 48 and 60 months of ART (e.g. "CD4_FolCh_6M.BL");
   - CD4+ T cell count slope the first 12, 24, 36, 48 and 60 months of ART (e.g. "Slope_0.6M");
   - CD4/CD8 ratio at 0, 6, 12, 24, 36, 48 and 60 months of ART (e.g. "CD4_CD8_Ratio_0M").

Relevant R code

R code I

```
df.46 <- read_excel("Thyfunction_Immrecov.xlsx", na = "NA")
Group1.df <- df.46[,1:9)]

names(Group1.df)
# "Volume_0M"   "Volume_12M"   "Volume_BL.12_mean"   "Volume_diff"
# "Volume_perc_change", "Index_0M" , "Index_12M" ,  "Index_BL.12_mean" , "Thymic_score"

Group2.df <- df.46[,10:33)]
names(Group2.df)
# "CD4_Count_0M"  "CD4_Count_6M"  "CD4_Count_12M"  "CD4_Count_24M"
# "CD4_Count_36M" ."CD4_Count_48M"  "CD4_Count_60M"
# "CD4_FolCh_6M.BL"  "CD4_FolCh_12M.BL"  "CD4_FolCh_24M.BL"  "CD4_FolCh_36M.BL"
# "CD4_FolCh_48M.BL"  "CD4_FolCh_60M.BL"
# "Slope_0.12M"  "Slope_0.24M"  "Slope_0.36M"  "Slope_0.48M"
# "Slope_0.60M"
# "CD4_CD8_Ratio_0M"  "CD4_CD8_Ratio_6M"  "CD4_CD8_Ratio_12M"
# "CD4_CD8_Ratio_24M" # "CD4_CD8_Ratio_36M"  "CD4_CD8_Ratio_48M"
# "CD4_CD8_Ratio_60M"
```

R code II

```
Correlation.df <- data.frame(matrix(ncol = 7, nrow = (dim(Group1.df)[2]*dim(Group2.df)[2])))
Column_names <- c("Group1_element", "Group2_element", "Shapiro.test_G1_element",
"Shapiro.test_G2_element","Corr_test", "p_value", "corr_coeff" )
colnames(Correlation.df) <- Column_names

#1. Fill column "Group1_element": repeat each element of group 1 xx times, being xx the number
of elements in group 2
```

```
a <- vector()
for(i in c(1:dim(Group1.df)[2])){
  a <- c(a, rep(names(Group1.df)[i],dim(Group2.df)[2]))
}

Correlation.df$Group1_element <- a

#2. Fill column "Group2_element": repeat all elements of group 2 xx times, being xx the number of elements in group 1
Correlation.df[,c("Group2_element")] <- rep(names(Group2.df),dim(Group1.df)[2])

#3. Fill column "Shapiro.test_G1_element":
for(i in c(1:dim(Correlation.df)[1])){
  Correlation.df[i,"Shapiro.test_G1_element"] <- round(shapiro.test(Group1.df[,Correlation.df[i,"Group1_element"]])$p.value, digits = 3)}

#4. Fill column "Shapiro.test_G2_element":
for(i in c(1:dim(Correlation.df)[1])){
  Correlation.df[i,"Shapiro.test_G2_element"] <- round(shapiro.test(Group2.df[,Correlation.df[i,"Group2_element"]])$p.value, digits = 3)}

#5. Fill column "Corr_test":
for(i in c(1:dim(Correlation.df)[1])){
  if(Correlation.df[i,"Shapiro.test_G1_element"]>0.05 & Correlation.df[i,"Shapiro.test_G2_element"]>0.05)(Correlation.df[i,"Corr_test"] <- "pearson")
  else(Correlation.df[i,"Corr_test"] <- "spearman")
}

#6. Fill columns "p_value" and "corr_coeff":
for(i in c(1:dim(Correlation.df)[1])){
  a <- cor.test(Group1.df[,Correlation.df[i,"Group1_element"]],Group2.df[,Correlation.df[i,"Group2_element"]], method = Correlation.df[i,"Corr_test"])
    Correlation.df[i,"p_value"] <- a$p.value
    Correlation.df[i,"corr_coeff"] <- a$estimate
}
```

R code III

```
# Determining severe outliers
Severe_Outliers.df <- data.frame(matrix(ncol = 2, nrow = dim(Group1.df)[2]+dim(Group2.df)[2]))
Column_names <- c("Variable", "Number_of_Sev_Outliers")
colnames(Severe_Outliers.df) <- Column_names

Variables <- c(names(Group1.df),names(Group2.df))
Severe_Outliers.df$Variable <- Variables

for(i in c(1:dim(Group1.df)[2])){
 if(length(boxplot(Group1.df[,Severe_Outliers.df$Variable[i]],range=3)$out)==0)
  {Severe_Outliers.df$Number_of_Sev_Outliers[i]<- "0"}
 else
 {Severe_Outliers.df$Number_of_Sev_Outliers[i]<-
boxplot(Group1.df[,Severe_Outliers.df$Variable[i]],range=3)$out}
}

for(i in c(1+dim(Group1.df)[2]:(dim(Group1.df)[2]+dim(Group2.df)[2])){
 if(length(boxplot(Group2.df[,Severe_Outliers.df$Variable[i]],range=3)$out)==0)
 {Severe_Outliers.df$Number_of_Sev_Outliers[i]<- "0"}
 else
 {Severe_Outliers.df$Number_of_Sev_Outliers[i]<-
boxplot(Group2.df[,Severe_Outliers.df$Variable[i]],range=3)$out}
```

PART III | Discussion and Conclusion

CHAPTER 5.
General Discussion, Conclusion and Future Perspectives

The work presented in this dissertation focuses on two aspects of the immunological recovery of HIV-infected patients during ART: the immunological non-response (INR) and the role of the thymus in the process of recovery. The main achievements of this work are: 1) the review of the diversity of terms and criteria applied to INR patients and the proposal of an uniform term and criteria; 2) new evidence supporting a major contribution of thymic function for the immunological recovery of severely lymphopenic patients at ART initiation, associated with predictive models of the outcome of these patients; and 3) the development of an enduring interdisciplinary collaboration to explore the thymic score and the mathematical model for the ratio thymic output to peripheral cell proliferation (R_θ). In this chapter, we present a broad and in-depth critical analysis of the results, assess the strengths and limitations of this work, and explore the possible implications of what we have found.

Helping to achieve a consensus regarding the terminology and criteria applied to "Immunological non-responders"

HIV-infected patients with impaired immunological response to ART despite virologic suppression present higher risk of both AIDS and non-AIDS events (non-AIDS cancers and cardiovascular, end-stage renal, and liver diseases) and higher mortality than patients with an adequate immune recovery during ART [1-7].

The diversity of terms and criteria used to identify and classify INR patients hampers their study, by limiting data aggregation and knowledge accumulation, and hinders cooperation between research groups. Therefore, there is an urgent need to find a consensual term

and criteria [8-11]. This would allow to accurately determine the number of patients suffering from this condition, to identify risk factors, to clarify the contribution and interplay between different pathophysiological mechanisms, and to develop the effective strategies to treat these patients, improving their outcome. Although the exact number of patients suffering from this condition is unknown, it will most probably grow in the next years, as INR is associated with older age, and the average age of people living with HIV (PLHIV) is progressively increasing [12]. In fact, the number of PLHIV aged 50 years and over raised from 8% in 2000 to 16% in 2016, and it is still raising [13].

We conducted the first systematic review on the terminology and criteria applied to INR. The 22 categoric terms and 73 criteria used in the 103 included studies demostrate the high heterogeneity of definitions used. The determination of the most frequent terms and criteria may help the community of researchers on HIV to converge into a standardized definition. Our proposal to combine two criteria has the advantage of identifying INR patients few months after ART initiation and also on long-term ART. However, it was not our aim to define a consentual definition as reccomendations should be elaborated by internationally recognised authorities in the field, such as the HIV/AIDS societies, after intense discussion envolving all the interested parts. Our intention is to promote and reinforce a discussion to support the adoption of new recommendations. One possible solution is that all the professionals that show interest in this question are involved and enabled to provide feedback, in a similar process as the one undertook by the Sedentary Behavior Research Network (SBRN) for standardization of the terminology and definitions related to sedentary behaviors [14].

One advantage of our systematic review, described in Chapter 3, is the transparent and wide search strategy. This strategy resulted in a realistic overview of the heterogeneity of terms and criteria in use. One limitiation of the review is the restriction of the search to PubMed. In future systematic reviews, we will consider to conduct the search in other search engines besides PubMed, such as Web of Science and EMBASE.

It must be highlighted, once again, that all clinical trials performed in the context of INR did not show a consistent improvement of $CD4^+$ T cell counts or a evident decrease in morbidity or mortality [15-22]. A major downside of these clinical trials was their heterogeneous selection criteria. We expect our review to foster the discussion on the definition of a consensual INR definition, so that this review helps, albeit indirectly, to improve the planning of future clinical trials.

Clarifying the role of thymic function in the immunological recovery of HIV-infected patients during ART

There is plenty of evidence that the thymus is important for the immunological recovery during ART, but its precise role in this process is still not clear. A major obstacle to the study of the role of thymus in any context is the absence of a direct method to assess the activity of this organ. Each of the available indirect methods only gives a partial perspective of what is happening in the thymus. The estimation of thymic function using several of these indirect methods may result in apparently contradictory results, as some correlations between the different methods are weak. In this case, it is necessary to undertake a carefull analysis of the results to understand the factors that lead to their divergence.

The 33 severely lymphopenic HIV patients studied were divided in two groups with different immunological recoveries (PIR = 19 and AIR = 14) by clustering analysis of the longitudinal $CD4^+$ T cell count trajectories. In this way, we did not privilege one INR criterion over the others described in the literature, and the similarity of the trajectories of $CD4^+$ T cell counts within each group was guaranteed. In the following paragraphs, we discuss the results that we obtained for each thymic function surrogate, linking these data with prior works and focusing on aspects that were not discussed in the Part II of this dissertation.

Starting with the thymic volume and its evolution, previous studies had already showed that ART is able to induce a thymic volume increase in some but not all patients. For

example, Vigano *et al.* reported that thymic volume increases during ART [23], while Hazra *et al.* found no volume change in another group of patients on ART with similar age [24]. However, it must be highlighted that the median thymic volume increased from 8.9 cm^3 at baseline to 16.9 cm^3 at 12 months of ART in the first study, while in the second study the median thymic volume at baseline was 16.3 cm^3. In our study, AIR and PIR had similar thymic volumes at baseline, and AIR, but not PIR, showed an increase of thymic volume from baseline to 12 months of ART (increase of 6.47 ± 4.59 cm^3 in AIR vs. 1.43 ± 4.89 cm^3 in PIR). AIR and PIR showed similar body weigth changes from baseline to 12 months of ART (10.0 ± 6.6 Kg in AIR *versus* 8.5 ± 10.6 Kg in PIR). However, there was no statistically significant difference in the thymic volume at 12 months of ART between AIR and PIR. When compared to a group of HIV-infected patients with $CD4^+$ T cell counts at baseline between 200 and 350 cells/μL (HIV-infected$_{BL[200,350]}$), AIR but not PIR presented significantly higher thymic volume than HIV-infected$_{BL[200,350]}$ patients at 12 months of ART, despite the thymic volumes at baseline was similar between the three groups. The reason for a significant difference on thymic volume at 12 months on ART between AIR and HIV-infected$_{BL[200,350]}$ but not between AIR and PIR may be the small sample sizes and/or the significant dispersion of values in PIR. Post-mortem analysis of healthy individuals aged from 20 to 64 years, revealed thymic volume means ranging from 22 to 28 cm^3, depending on their age group [25]. Altough these values were not determined by CT scan, we extrapolate that thymic volume tended to get close to the thymic volume of age-matched healthy individuals during ART in AIR, while in PIR and HIV-infected$_{BL[200,350]}$ patients the thymic volume remained lower.

Regarding thymic index, McCune *et al.* showed that both healthy and HIV-infected adult individuals present thymic indices varying from 1 to 4 [26]. In that study, only 12% of HIV-infected patients (total n = 99) presented a score of 4; additionally, higher thymic indices were associated with higher $CD4^+$ T cell counts. Given these observations, it is not surprising that in our cohort, patients' indices ranged from 1 to 3 since these were evaluated in a total of 16 individuals (8 AIR and 8 PIR) whose basal $CD4^+$ T cell counts

were very low (<200 cells/uL). Despite the small sample size and the absence of statistically significant differences between AIR and PIR, AIR tend to have higher thymic indices than PIR. As aging is associated with a decrease in thymic index, in HIV-infected and uninfected individuals [26], it is possible that the trend for lower thymic indices in PIR compared to AIR is related to the older age in PIR. On the other hand, Kalayjian *et al.* reported that untreated HIV-infected patients aged ≤ 30 years presented lower thymic indices than age-matched healthy controls, but similar indices were found between patients over 45 years old and age-matched healthy controls [27]. So, it is possible that HIV induces a more intense decrease of thymic index in young adults than in older adults; and that ART is able to revert that decrease in the former but not in the latter. To test the first part of this hypothesis, the ideal would be to compare the thymic index before and after HIV seroconversion in a cohort of patients stratified by age. Two obstacles to that study are the irradiation exposure associated with CT scans, and the costs of following a large number of individuals from which only a very small percentage will eventually get infected by HIV. To test the hypothesis of different effects of ART on the thymic index depending on patients' age, we would like to increase the number of patients in our cohort, to stratify them by age, and control for the $CD4^+$ T cell counts at ART initiation.

AIR presented significantly higher numbers of sj-TRECs and greater sj/β TREC ratios only at 24 months of ART. There were no significant differences between AIR and PIR regarding these two thymic function surrogates at the other time points; however, when we look at the distribution of values in each time point, the minimum, median and maximum values of sj-TRECs and sj/β TREC ratios are higher in AIR than in PIR in all time points. The lack of significant differences between the groups may be associated with the small sample size and wide ranges of TREC levels in each group at each time point, which are similar to those reported in the literature. Delobel *et al.* reported similar sj/β TREC ratios between two groups of HIV-infected patients with different immunological recoveries, however, contrary to our study, in their study the two groups had different $CD4^+$ T cell counts at baseline (median $CD4^+$ T cell count of 73 *versus* 155 cells/μL) and the duration of ART in

each group was not reported [28]. One explanation may be that they compared patients with similar thymic functions in different stages of HIV infection, and the different immunological recoveries were due to the different immunological starting point. In contrast, a cross-sectional study compared HIV-infected patients on ART with different immunological recoveries to healthy individuals found that patients with an adequate immunological recovery presented greater sj/β TREC ratio than the ones with impaired immunological recoveries, and also than the healthy individuals [29]. It would be useful to include more patients in our study, in order to compare TREC levels between AIR and PIR over time with a larger sample. It would be important to also include a group of gender and age-matched uninfected individuals, to verify if the alterations in TREC levels and/or sj/β TREC ratio observed in patients normalize with ART. Obstacles to accomplish that are the time and resources needed, as sj- and β-TREC measurements are laborious, expensive and time-consuming [30].

In respect to RTE, assessed by flow cytometry, AIR showed higher percentage of RTE among $CD4^+$ T cells than PIR at all time points. This is in accordance with previous results from a longitudinal study that compared two groups of HIV-infected patients with different immunological recoveries [31]. One difference between that study and ours is that the two groups had different $CD4^+$ T cell counts at baseline (29 ± 33 *versus* 164 ± 78 cells/μL). Despite that, in both studies the $CD4^+$ T cell count mean at baseline in the two groups of patients was < 200 $CD4^+$ T cells/μL. Interestingly, the percentages of RTE that we found in AIR patients are close to the percentages Li *et al.* described in a group of healthy volunteers [31].

Taken together, our results support that among severely lymphopenic patients starting ART, the ones with a higher thymic function and/or a higher capacity of thymic regeneration have a better immunological response to therapy. We hypothezise that, in AIR but not in PIR, severe lymphopenia or an associated factor drive the increase of thymic volume and of the other thymic function surrogates, which ultimately results in a better

immunological recovery. The clinical implications of this are: first, it is important that HIV-infected patients start ART before the thymus is severely affected by the infection, given that some alterations may be irreversible; second, thymic function may be a good therapeutic target to improve the outcome of patients showing impaired immunological recovery; and, finally, patients with a compromised thymic function and/or a low thymic regenerative capacity should be timely identified, so that their prognosis can be improved.

Our predictive models were able to correctly distinguish, in up to 87% of the cases, the immunological recovery pattern at 36 months of ART based on assessments performed until 6 months of therapy. These models should be tested in larger cohorts, and if validated, their sensitivity and specificity could be improved by including additional predictive factors and adjusting the threshold to consider a patient as "probably PIR" or "probably AIR". It is expected that, in larger studies, RTE frequency assessed by flow cytometry at early stages of ART significanlty increases the predictive value of the models. RTE assessment by flow cytometry has two advantages in relation to other thymic function surrogates: unlike CT sacan, it does not require irradiation exposure, and it is simpler, less expensive and less time consuming than using molecular biology techniques.

If clinicians could timely identify the HIV patients with high risk of maintaining a low thymic activity during ART hampering their immunological recovery, they could adapt their approaches in order to optimize the outcome of those patients: patients with higher probability of being PIR would be submitted to a closer follow-up, with scheduled consultations every n weeks; additional tests could be performed to exclude risk factors (*e.g.* presence of co-infections); and, finally, new therapeutic strategies to improve the prognosis of these patients could be developed and implemented.

Interdisciplinarity: new perspectives and novel approaches

After the enrolment of 100 patients in our cohort study, while the follow-up period was still on going, we verified that patients' immunological recoveries and also thymic function

estimates were quite heterogeneous, which could mask thymus role in the course of the disease, which we were trying to unravel. We decided to examine the thymic function in the subset of patients with the worst recoveries, and therefore with the worst prognoses. However, there were multiple criteria in the literature used to define the "patients with unsatisfactory $CD4^+$ T cell restoration despite full virological suppression", and no comparison between those criteria. As we were trying to select the most adequate criteria among the ones described in the literature, another solution has emerged from informal conversations with scientists from other areas of research, mathematicians and statisticians, who suggested and helped us to apply their tools in our work. From the clustering analysis of longitudinal trajectories to the development of different mathematical models, we worked as a interdisciplinary team to explore the data that have been collected. Although we consider that applying forefront mathematical and computing methodologies to our data is extraordinarily useful, it takes extra time and effort, as researchers from different fields need to develop advanced communication skills to be able to discuss scientific results using a non-restricted and non-specialized language while maintaining the degree of scientific rigor. New developments are awaited, but for the moment, we limited this work to relatively simple and well-estabilished methodologies to explore the thymic score and the R_θ.

Given that each surrogate of thymic function only gives a partial perspective of the activity of the thymus, it would be of great interest to combine all thymic function surrogates in a single equation that would give a global estimate of thymic function. This simple idea, shared by some researchers in the field, requires an extraordinary effort to be put into practice. The main reason for that is the variability of each surrogate and the weak correlations between the surrogates in a diverse population. To better illustrate the problem, it is worth taking a look to an example from other context, such as the relation between human body weight and height. Although there are tall overweighted people and tall underweighted people, in general, taller people weight more than short people. Therefore, there is a positive but not perfect correlation between human weight and height,

and both could be combined in a single parameter, the body mass index. This useful index allows to estimate if a person has the adequated weight for her or his height. Similarly, it would be interesting if we could determine, using the thymic function surrogates, if the thymic function of a patient is the adequate or expected for his/her age and clinical condition.

In our cohort, patients with different thymic indices (1, 2 or 3) showed similar thymic volume distributions, both at baseline and 12 months of ART. This shows the need to combine the information of each parameter. We multiplied the thymic index mean by the thymic volume mean, to get a single parameter of the morphological features of thymus, the thymic score. We expected that thymic score would estimate the thymic function of each patient over the first year of ART.

Contrary to our initial expectations, the results regarding thymic score were not promising. First, thymic index mean, but not thymic score, was positively correlated with all the thymic function estimates by other techniques (RTE numbers, sj-TREC levels and sj/β TREC ratio). Secondly, thymic score did not correlate with most of the immunological recovery parameters. Third, contrary to thymic index, thymic score did not correlate with RTE frequencies, sj-TREC levels or sj/β TREC ratio.

One possibility is that thymic index is a better estimate of thymic function than thymic volume and there is no added benefit in combining the two. We found that thymic volume change was associated with the body weight change, and it is known that malnutrition induces thymic atrophy [32, 33]. So, the thymic volume increase may be caused not only by reversion of HIV-induced alterations by suppression of the viral replication, but also by an improved nutritional status, and it may not reflect accurately the changes in the thymic output during ART. In fact, although several papers reported thymic volume and RTE data during HIV infection, we only found one cross-sectional study showing a marginal correlation between thymic volume and RTE frequency ($r = 0.46$, p-value = 0.06) [34]. However, several studies showed a correlation between thymic volume and immunological

recovery during ART [35-38]. It would be useful to compare thymic volume between groups of HIV patients stratified according to the $CD4^+$ T cell count at ART initiation and their weight and healthy individuals matched for age, gender and weight.

We have to consider that many factors interfere with the thymic output during HIV infection: altered migration of cells from the bone marrow, altered thymic microenvironment, etc. It would be useful to explore thymic score in healthy individuals, but, once again, we need to weigh the pros and cons of exposing healthy individuals to CT scan irradiation.

Although the thymic score did not show to be advantageous when estimating thymic function in the context of HIV infection, its scrutiny helped us to increase our computing skills. In fact, exploring the relation of thymic score with each of other thymic function surrogates and the immunological recovery parameters, at each time point, would had been exhaustive and very time-consuming if we had not used programming. Now that we have a ready-to-use R code, with just few alterations, we can explore the relation between other surrogates and try new combinations, as, for example, the combination of RTE frequencies and thymic index.

R_θ, the ratio thymic output to peripheral cell proliferation, was determined for each of 37 patients at baseline, 6, 12, 24 and 36 months of ART (total n = 185). Theoretically all R_θ values should be positive, as conceptually thymic output and peripheral cell proliferation range from zero to $+\infty$. However, 18 of the obtained values were negative. Potential explanations for these results are:

1) In our model, R_θ is calculated by multiplying two factors (Equation 8 in page 209) each of which have a significant error attached to them. Especially the estimations of the gradient of τ at each time point include a high error since they are based on measurements performed over months;

2) Our model assumes that $Ki67^{high}$ and $Ki67^{low}$ cells die at the same rate, which is probably not be the case. It has been shown that malignant tumor cells with high or low Ki67

expression are associated with different prognosis, so $Ki67^{high}$ and $Ki67^{low}$ cells are biologically different and probably present different death rates [39, 40];

3) Another issue is that the model assumes naïve T cells as a homogeneous population, which is not the case. Several subpopulations can be identified among naïve T cells based on their phenotype, function, dynamics and differentiation status [41]. It is likely that our model is oversimplified, and so the calculated R_θ values are not trustworthy, *i.e.* do not retain correct information about the real value of the thymic output to peripheral cell proliferation ratio in each patient at each time point;

4) The model assumes that $TREC^+$ T cells are only lost by cell death. However, it is possible, although improbable, that patients with negative R_θ present a not negligible intracellular degradation of TRECs. In fact, the half-life of TRECs is assumed to be very long because TRECs can be detected in T cell subpopulations of individuals who were thymectomized up to 39 years earlier, but it has not been determined [42, 43]. A higher intracellular degradation of TRECs in these patients would explain a decrease of the average number of TRECs per naïve T cell independent of the peripheral cell proliferation, which was not considered in the elaboration of the model.

To test the several scenarios, we could combine data from different methods of estimating cell proliferation and thymic output; shorten the intervals between the measurements and/or increase the number of time points; try re-deriving the equation for R_θ including distinct death rates between $Ki67^{high}$ and $Ki67^{low}$ cells; create more complex models considering the different subpopulations among naïve T cells, particularly the RTE; and/or include an additional term to the R_θ equation to take into consideration the possible intracellular degradation of TRECs.

Main conclusions and future perspectives

In the work presented in this dissertation, we demonstrate the high heterogeneity of INR definitions, regarding both the terms and the criteria applied in the literature to identify this condition. By analyzing the different terms and criteria, we expect to foster the discussion for the usage of a consensual definition. This will greatly improve the ability to compare and combine results from distinct studies, contributing to the identification of interventions to reduce the mortality and morbidity in this vulnerable group of patients.

This work also provides new evidence supporting the major role of thymic function for the immunological recovery of severely lymphopenic patients starting ART. We showed not only that patients with an adequate immunological response have, in general, higher thymic function levels than patients with a poor immunological response to ART, but that one of the thymic function surrogates (the frequency of RTE) has predictive value to the patients' outcome.

In the medium-term future, we would like to increase the number of patients in our cohort study, by colaborating with different hospitals; to validate the predictive models of the immunological recovery of severely lymphopenic individuals at ART initiation, using independent cohorts; and to further explore the combination of data from the several thymic function surrogates and its relation with the immunological recovery during ART. In a long-term perspective, we would like to create a mathematical model of thymic function reflecting all the information from the different surrogates. Such a model would be useful to identify and select patients that could benefit from strategies to modulate the thymic function.

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PART IV | Annexes

Annex 1

Ethical Committee approval for the prospective cohort study

Hospital de Joaquim Urbano – Centro Hospitalar do Porto

Reference 168/CES 2009

Ministério da Saúde
HOSPITAL DE JOAQUIM URBANO
Administração Regional de Saúde do Norte

Exmª Senhora
Drª Ana Aboim Horta
agaboim@hotmail.com

Hospital de Joaquim Urbano

Sua referência	Data	Nossa referência	Data	Processo
		168/CES	21.10.09	

ASSUNTO: **Pedido de autorização de realização do projecto de investigação "Reconstituição e Homeostasia do Sistema Imune na Infecção por VIH", da Drª Ana Maria Lacerda Morgado Fernandes de Carvalho Aboim**

Na sequência da reunião da Comissão de Ética do Hospital de Joaquim Urbano de 23 de Setembro de 2009, relativamente ao pedido de parecer sobre o projecto de investigação "Reconstituição e Homeostasia do Sistema Imune na Infecção por VIH", da Drª Ana Maria Lacerda Morgado Fernandes de Carvalho, depois de analisadas todas as alterações solicitadas foi dado parecer favorável à sua realização.

Com os melhores cumprimentos,

Porto e Hospital Joaquim Urbano, 2 de Outubro de 2009

A Comissão de Ética

Dr.ª Elizabete Maria das Neves Borges

Mod 104-A

Rua Câmara Pestana, 348 – 4369-004 PORTO – Tel. 225 899 550 – Fax 225 106 160 – hju@hjurbano.min-saude.pt

Annex 2

Addendum to the Ethical Committee approval

Hospital de Joaquim Urbano Unit– Centro Hospitalar do Porto

Reference 127/12(NA-DEFI/089-CES)

centro hospitalar
do Porto

Hospital Santo António | Hospital Maria Pia | Maternidade Júlio Dinis | Hospital Joaquim Urbano

Largo Prof. Abel Salazar
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Exm.ª Sr.ª
Dr.ª Ana Maria Lacerda Morgado Aboim Horta
Serviço de Doenças Infecciosas
Unidade Hospital Joaquim Urbano

ASSUNTO: Trabalho Académico – Doutoramento - "Reconstituição e homeostasia do sistema imune na infecção por VIH"- N/ REF.ª 127/12(NA-DEFI/089-CES)
- Adenda

O Conselho de Administração do CHP **autoriza** a adenda ao estudo de investigação acima mencionado nesta Instituição, no Serviço de Doenças Infecciosas da Unidade Hospital Joaquim Urbano, sendo Investigador Principal, a Dr.ª Ana Maria Lacerda Morgado Fernandes de Carvalho de Aboim Horta.
A adenda ao estudo de investigação foi previamente analisada pela Comissão de Ética para a Saúde, bem como pela Direcção Clínica, tendo obtido Parecer Favorável.

Cumprimentos,

CONSELHO DE ADMINISTRAÇÃO
Dr. SOLLARI ALEGRO — Presidente
Dr.ª ÉLIA GOMES — Vogal Executiva
Dr. PAULO BARBOSA — Director Clínico
Dr. PORTO GOMES — Vogal Executivo
Enf.º EDUARDO ALVES — Enfermeiro Director

* Em todas as eventuais comunicações posteriores sobre este estudo é indispensável indicar a nossa ref.ª.