Review

Biomarkers of monitoring and functional reserve of physiological systems over time in HIV: expert opinions for effective secondary prevention

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SUMMARY
HIV-positive individuals are more vulnerable to poor health than HIV-negative individuals. This vulnerability is characterized by a higher risk of several common, age-related health problems, even after adjustment for established risk factors.

This expert opinion report aims at identifying the optimal biomarkers for monitoring the structural integrity and function of physiological systems at risk across aging in HIV-seropositive subjects. These biomarkers, readily available locally and relatively cost-effective for clinicians in primary and secondary care, should allow early detection of the first preclinical structural and functional changes in renal, brain, cardiovascular, and skeleton systems or apparatus in HIV subjects across aging (Table 8).

A particular interest of this report is the definition of the concept of biomarker of the “organ functional reserve”. This definition emphasizes the fact that some biomarkers for monitoring the molecular, structural and functional integrity of a given organ reflect a level of impairment that is basically irremediable despite effective pharmacological or nonpharmacological intervention.

Key words: Biomarkers of monitoring; functional reserve; HIV; secondary prevention

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Introduction

The increasing life spans of people with human immunodeficiency virus (HIV) reflect enormous treatment successes and present new challenges related to aging. Although some people with HIV live to older ages with relatively few health problems, others accumulate multiple problems earlier in life. Despite complete suppression of viral load and immune recovery, HIV-positive individuals are more vulnerable to poor health than HIV-negative individuals (High et al., 2012). This vulnerability is characterized by a higher risk of several common, age-related health problems, even after adjustment for established risk factors. These conditions, termed HIV-associated non-AIDS (HANA) events include cardiovascular disease (CVD), osteoporosis, metabolic disorders, hepatic and renal diseases, neurocognitive impairment, and some cancers, as well as age-associated immunologic changes and chronic inflammation (Guaraldi et al., 2011, Deeks et al., 2011, Fitch et al., 2013). While factors such as smoking and antiretroviral drug toxicity (Lanf et al., 2010) explain some of the increased risk for these events, elevated immune activation and inflammation (Armah et al., 2012, Boulware et al., 2011, Hunt et al., 2003, Hunt et al., 2011, Kaplan et al., 2011, Kuller et al., 2008, Neuhaus et al., 2010, Sandler et al., 2011, Tien et al., 2010, Duprez et al., 2012) and poor immune restoration (Lang et al., 2012) have been shown to predict the worsening of the global health status. Characterizing the association of immune activation and inflammation with non-AIDS-defining events, after accounting for the effects of the highly active antiretroviral therapy (ART) and traditional risk factors, is critical to identifying interventions that may reduce excess morbidity and mortality in adult ART recipients.

Among people without HIV, aging and the accumulation of age-related health problems are also highly heterogeneous processes. Although people generally accumulate health problems with age, individuals of the same age can experience very different levels of health. Geriatricians introduced the term “frailty” to describe this variability (Clegg et al., 2013, Mitnitski et al., 2013). However, frailty is not yet well understood in the context of the ART era, where most HIV-positive individuals now experience significant immune recovery, undetectable HIV viral load, and primarily HANA events (High et al., 2012). Neither CD4 count nor viral load appear to be useful surrogate markers of vulnerability in this immune-reconstituted population, whereas frailty is strongly associated with HANA conditions and disability (Onen et al., 2009, Althoff et al., 2014) and might be a more sensitive indicator of health changes (Justice et al., 2012, Brothers et al., 2014, Erlandson et al., 2013). For this reason, it is essential to have algorithms that allow us not only to assess the extension of damage, but also to evaluate the prognostic functional residual capacity at the level of different organs or systems.

This expert opinion report (based on arguments) aims at identifying the optimal biomarkers for
monitoring the structural integrity and function of physiological systems at risk across aging in HIV. These biomarkers should allow an early detection of the first preclinical structural and functional changes in renal, brain, cardiovascular, and skeleton systems or apparatus in HIV subjects across aging. A focus of this report is on biomarkers that are informative, readily available locally, and relatively cost-effective for clinicians in primary and secondary care for secondary prevention (i.e. early diagnosis of organ/apparatus pathology across aging). A particular interest of this report is the definition of the concept of biomarker of the “organ functional reserve”. This definition emphasizes the fact that some biomarkers for monitoring the molecular, structural, and functional integrity of a given organ reflect a level of impairment that is basically irremediable despite an effective pharmacological or nonpharmacological intervention (e.g. biomarkers reflecting the loss of nephrons or neurons due to HIV-related processes and/or ART regimen). For this reason, biomarkers of the “organ functional reserve” are especially useful to identify HIV subjects to be monitored.

From a methodological point of view, the present expert opinion report reflects a cross-disciplinary consensus among co-authors from different disciplines (e.g. infectious disease, neurophysiology, cognitive psychology, cardiology, nephrology, and geriatrics). All authors contributed to the development of all sections and sub-sections from their expertise. The review of the literature was limited to the core topics selected by the cross-disciplinary workgroup, with a particular emphasis on longitudinal studies in cohorts of HIV subjects and cross-sectional studies in these individuals with different disease duration and ages.

**HIV and aging**

*HIV replication, inflammation, immune senescence and aging*

Effective therapies have transformed HIV infection into a chronic illness. As people with HIV live longer, aging-related challenges are arising. Despite complete suppression of viral load and immune recovery, HIV individuals are at higher risk of several common age-related health problems. These conditions, termed HANA events, include several apparatus and organ diseases and metabolic disorders as well as age-associated immunologic changes and chronic inflammation (Guaraldi et al., 2011, Deehs et al., 2011, Fitch et al., 2013). Each involves different physiological systems and etiologies yet are all strongly age-associated in the general population. Although HANA events are more common among HIV individuals who are older, have more severe HIV disease, and have longer duration of antiretroviral treatment and toxicity, these factors do not completely explain differences in risk and survival (Deeks et al., 2011, Justice et al., 2012). Therefore, it is important to define what are the markers of inflammation, immune-activation and immune-senescence, as well
as those of viral replication/activity that are useful to identify vulnerable individuals, for organizing care and for comprehensively measuring the impact of illness and treatment on overall health status.

Age-related and HANA events have been associated with immune activation and immune senescence markers, inflammatory circulating cytokines (Brothers et al., 2014, Erlandson et al., 2013, Desai et al., 2010, Deesk et al., 2013), as well as with both CD4 count and viral load (Desquilbet et al., 2009). Although the clinical spectrum of HIV disease differs whether individuals experience immune deficiency or immune activation, frailty might emerge in the context of both profiles. A hypothetical representation of the association between frailty, HANA, and immune system dysregulation is depicted in Figure 1. The presence of frailty is related to AIDS-associated conditions in patients with advanced immune deficiency and with comorbidities, infective or not, in patients with immune activation. Several surrogate markers of progression of HIV disease are suggested in the figure.

Among individuals on ART, multiple factors have been associated with frailty in cross-sectional studies, using different clinical scales (Table 1). Some are traditional HIV measures, including lower current CD4 cell count (measured continuously (Onen et al., 2009, Althoff et al., 2014) and categorically, as <500 cells/mm$^3$, <350 cells/mm$^3$, <200 cells/mm$^3$, and <100 cells/mm$^3$ (Pathai et al., 2012, Piggot et al., 2013, Ianas et al., 2012, Terzian et al., 2009), lower nadir CD4 count (Onen et al., 2009), CD4/CD8 ratio ≤0.29 (Terzian et al., 2009), detectable viral load (Althoff et al., 2014, Piggot et al., 2013), history of AIDS (Althoff et al., 2014) and longer time since diagnosis (Onen et al., 2009), other factors are not necessarily related to HIV such as hepatitis C coinfection (Ianas et al., 2012), low body mass index (BMI) (Onen et al., 2009, Pathai et al., 2012), high BMI (Shah et al., 2012), lipodystrophy (Shah et al., 2012), depressive symptoms (Onen et al., 2009, Piggot et al., 2013), one-year history of multiple falls (Erlandson et al., 2012), and lower cognitive performance (Onen et al., 2009).

However, a diverse array of biomarkers (Neuhaus et al., 2010), including indices of T-cell activation (e.g., HLA-DR and CD38 expression) (Giorgi et al., 1999, Giorgi et al., 1999, Liu et al., 1998), soluble tumor necrosis factor receptor I (sTNFR-I), sTNFR-II, and interleukin 6 (IL-6) (Fahey et al., 1990, Fahey et al., 1998), are elevated in untreated HIV infection and often remain elevated among ART recipients with undetectable viremia (Hunt et al., 2003, Neuhaus et al., 2100, landay et al. 2007, French et al., 2009) (Table 2). Many mechanisms have been proposed to explain this inflammatory state, including the direct effects of HIV replication, HIV-mediated destruction of mucosal barriers with chronic exposure to gut microbial elements, excess burden of coinfections (e.g., cytomegalovirus, HCV), and loss of normal immune regulatory responses (Almeida et al., 2005). Recent studies have shown that morbidity and/or mortality are associated with inflammation.
biomarkers (IL-6, fibrinogen, cystatin C, and C-reactive protein), soluble CD14 (sCD14), and D-dimer independently of viremia and CD4⁺ T-cell count (Armah et al., 2012, Boulware et al., 2011, Kaplan et al., 2011, Kuller et al., 2008, Sandler et al., 2001, Tien et al., 2010, Hunt et al., 2012). Of note, these studies included individuals with variable levels and duration of virological control, and the independent role of these biomarkers after controlling for potential confounders was not consistently defined. Cellular activation is associated with AIDS-defining outcomes in untreated and treated HIV infection (Giorgi et al., 1993, Liu et al., 1998). The impact of cellular immune activation on HANA events is less well studied because of limited stored peripheral blood mononuclear cell (PBMC) availability. In a Ugandan cohort, T-cell activation predicted short-term mortality (Hunt et al., 2011). In a cohort of HIV-infected women, decreased carotid artery distensibility, a marker for future CVD, was associated with T-cell activation (as measured by the percentage of HLA-DR⁺CD38⁺ cells among CD4⁺ and CD8⁺ T cells) and senescence (as measured by the percentage of CD57⁺CD28⁻ cells among CD4⁺ and CD8⁺ T cells) (Kaplan et al., 2011). In a study (Brothers et al., 2014) of HIV adults with a durable virological response to ART, higher levels of IL-6, sTNFR-I, sTNFR-II, and D-dimer at baseline, year one, and pre-event time points, and a higher level of sCD14 at baseline and pre-event time points were associated with the occurrence of HANA events or death. These effects were independent of traditional risk factors, other comorbid conditions, age, treatment regimen, and treatment-mediated changes in CD4⁺ T-cell counts. Collectively, these data suggest that HIV-associated inflammation improves with therapy, but that abnormalities persist and that pathways that involve IL-6, TNF, and D-dimer predict HANA events even when virus replication is controlled. Moreover, this effect appears to be mediated independently of the classic markers of immunodeficiency (e.g., CD4⁺ T-cell count) and other recognized perturbations in the T-cell phenotype that have been linked to HIV immunopathogenesis (specifically, expression of CD38, HLA-DR, PD-1, CD28, and CD57 on circulating cells). This implies that interventions that target inflammation and/or factors associated with inflammation may be needed to decrease the excess morbidity seen despite virological suppression. The biomarker most strongly associated with outcomes is IL-6 (Kuller et al., 2008, Brothers et al., 2014). This suggests a central role for pathways that lead to elevated systemic IL-6 levels in HANA events among HIV persons with viral suppression during ART. IL-6 has a strong association with both mortality and incident CVD across many disease states (Wetmore et al., 2008, Lindmark et al., 2001, Giovannini et al., 2011). The universal nature of this association suggests that the IL-6 pathway may be a final common pathway for many disease processes mediated by inflammation.
The role of HIV replication in driving elevated IL-6 levels is not clear because earlier work could not show consistent relationships between plasma IL-6 and HIV levels (Shive et al., 2012, Boasso et al., 2008). Intervention trials with IL-6 inhibitors are warranted to explore this relationship further. Furthermore, prospective studies incorporating selected biomarkers may help to clarify the relative contributions of the innate and adaptive immune system to the pathogenesis of HANA diseases in HIV-infected persons.

**Low-level viremia and activation of the immune system**

Recently, the indefinite persistence of inflammation during the course of HIV infection (while on ART, which suppresses viremia) has been confirmed (d'Ettorre et al., 2014, Sarmati et al., 2015). The chronic activation of the immune system induced by continuous HIV residual replication and the presence of stable virus reservoirs may contribute to the morbidity and mortality in HIV patients (Freiberg et al., 2013). Two randomized clinical trials in which raltegravir was added to standard therapy in patients with sustained suppressed viremia found a correlation between the persistence of low levels of HIV replication and inflammation (Hatano et al., 2013, Buzòn et al., 2010). This finding suggests the trigger role of the HIV replication on the inflammatory response (Hatano et al., 2013, Buzòn et al., 2010).

The correlation between low level viremia (LLV) and immune activation has been explored by a number of studies (Poizot-Martin et al., 2013, Schuler et al., 2013, Karlsson et al., 2004, Deeks et al., 2004, Ostrowski et al., 2005, Ostrowski et al., 2008, mavinger et al., 2009, Zheng et al., 2014, Dahl et al., 2014). With few exceptions (Poizot-Martin et al., 2013, Schuler et al., 2013), there is a general consensus that HIV replication at low copies is correlated with the persistence of immune activation and, in addition, recent data show a correlation between CD4 + T cell activation and disease progression (Groves et al., 2012). Higher levels of activated CD8+CD38+ and CD8+HLADR+ lymphocytes were frequently detected in patients with a viremia >20 copies/ml compared with those with <20 copies/ml (Deeks et al., 2004). The presence of detectable HIV viremia below 20 copies/ml was correlated with soluble markers of immune activation (Ostrowski et al., 2008). Furthermore, persistent levels of CD4+ and CD8+ T-cell activation were demonstrated in patients with viremia <50 copies/ml who had poor immunological reconstitution (Mavinger et al., 2009). Recently, Zheng and colleagues (Zheng et al., 2014) analyzed a group of 833 HIV patients on fully active ART for more than 96 months. The study demonstrated that residual LLV between 51 and 200 copies/ml is associated with greater CD8 T-cell activation; in patients with viremia <50 copies/ml, a greater CD8 T-cell activation was associated with older age, hepatitis C virus antibody positivity, higher pre-ART CD8 T-cell activation, lower concurrent CD4/CD8 ratios, and lower CD4 T-cell counts. The persistence of HIV replication in the cerebrospinal fluid in
patients who responded to ART has been correlated with elevated levels of neopterin (Groves et al., 2012). This suggests that the HIV replication at low copies, even in biological sanctuaries, correlates with inflammatory persistence.

Very little is known regarding the persistence of LLV and the clinical evolution of HIV infection with respect to the emergence of AIDS-related diseases or survival. One study analyzed a cohort of more than 17,000 European and American virologically suppressed HIV patients on ART for 3-9 months. Patients with LLV (approximately 3.5% of the total, with 50-200 copies/ml of HIV-RNA) had a 4.5-fold increased risk of virological failure but only a 1.2-fold increased risk of developing AIDS or death (Antinori et al., 2015).

From this point of view, the demonstration that INIs may extinguish the kinetics of viral replication faster and to a greater extent justifies the assumption that they are more effective than the other antiretrovirals of acting on the pathogenetic mechanisms underlying the processes of immune reconstitution. This is particularly interesting in regard to the mechanism of T lymphocyte homeostasis, which allows a more complete recovery of immune function. Integrase inhibitors may be able to interact significantly on two essential mechanisms: immune-activation and immune-proliferation, as they are closely related to the mechanisms responsible for immune exhaustion and the depletion of CD4 T lymphocytes related to HIV-1 replication. These effects are mirrored by the CD4 T lymphocyte gain and the improvement in the quality of the immune system as seen in both naïve and drug-experienced patients.

Prospective studies incorporating selected biomarkers may help to clarify the relative contributions of the innate and adaptive immune system to the pathogenesis of non–AIDS-related diseases in HIV-infected persons. The evaluation of the disease in the HIV patient has now become more complex and must be enriched with new markers able to assess the functionality of different organs and systems.

**Renal reserve in HIV**

**Measurement of renal reserve**

Kidney function includes:

a) elimination of catabolic waste products, mainly from protein metabolism;

b) homeostatic regulation of the body fluid composition and acid-base balance;

c) production of hormones and autacoids (active vitamin D, erythropoietin, prostaglandins).

The ability to eliminate waste products is quantitatively represented by the glomerular filtration rate (GFR). In normal conditions, GFR is not a static function since the resting filtration rate can be acutely increased under specific stimuli such as a dietary protein load (mostly from animal origin) or an i.v. infusion of amino acids (Thomas et al., 1994) that seems to induce the secretion of
prostaglandins and nitric oxide that, in turn, produce vasodilation and increased renal blood flow. This specific property is defined as renal functional reserve and is thought to play a role as an aid to kidney function under stress conditions. Renal functional reserve is lower in the elderly (Ronco, Chawla et al., 2016); moreover, the kidney capacity to increase filtration is maintained in chronic kidney disease (CKD), although to a lower extent, when compared to healthy controls (Barai et al., 2010). In this specific setting, we define renal reserve as the equivalent of the preservation of renal function and not the real physiologic entity of renal reserve.

GFR, is generally accepted as the best index of kidney function that can be measured (renal or plasma clearance of endogenous or exogenous substances) or estimated by applying validated formulas such as CKD-EPI consortium that includes serum creatinine, serum cystatin c or both as index markers of renal function (Levey et al., 2009, Inker et al., 2012). Normal values for estimated GFR in young adults range between 90 and 120 ml/min/1.73 m$^2$. One has to consider, however, that renal function declines with senescence accounting for a loss of $\approx$1 ml/min/1.73 m$^2$ per year after 40 years of age (Cohen, 2014). Aside from a reduced eGFR, an increased urinary protein excretion (proteinuria and/or albuminuria) represents one the main markers of kidney damage, which may precede an overt reduction of renal function (Levey et al., 2015). In normal conditions, urinary excretion of proteins does not exceed 150 mg/day and albuminuria represents only a part of the urinary proteins (<30 mg/day). Increased excretion of albuminuria is a strong indicator of chronic renal diseases and is a robust predictor of all-cause or cardiovascular mortality (Matsushida et al., 2015). Proteinuria higher than 150 mg/day can be caused by an alteration of the glomerular barrier or, alternatively, by a tubular dysfunction. In the first case, urinary proteins contain mainly albumin, which generally represent more than 40% of the total amount of proteins excreted in urine. In the second case, urinary proteins include mainly low molecular weight proteins, which normally escape the glomerular barrier and are taken up by the proximal tubular cells. In this condition albuminuria represents less than 40% of the excreted proteins (Samarawickrama et al., 2012). Alteration of renal function can finally determine a modification in the compositions of body fluids, especially for certain components that are highly regulated by a preserved tubular function (uric acid, phosphate, potassium, hydrogen ions). Therefore, eGFR, proteinuria and altered tubular handling of certain anions or cations are considered index parameters of renal damage and altered renal function that accompany chronic nephropathies cumulatively defined as CKD.

**CKD and biomarkers of progressive loss of renal reserve**

CKD involves one of the following signs: urinary abnormalities (proteinuria and/or hematuria) or alterations in renal handling of ions or ultrasonographic alterations to the renal cortex or a sustained reduction of the GFR for a period of at least 3 months. CKD represents a major public health
problem if we consider that global mortality due to this condition doubled from 1990 to 2010, and CKD ranks in 18th position in the Global Burden of Disease Study (Lozano et al., 2012). The lifetime risk of developing CKD is estimated to be 50% (Levey et al., 2015). Usually, from an epidemiologic point of view, the two major indexes utilized to define a loss of renal functional reserve and CKD are proteinuria (either total urinary proteins higher than 150 mg daily or albuminuria higher than 30 mg daily or albumin over creatinine ratio higher than 30 mg/g) and/or an eGFR (estimated GFR) below 60 ml/min/1.73 m² (KDIGO 2012). Concerning the markers of proximal tubular dysfunctions, EACS guidelines have indicated two of the following signs as sufficient for the diagnosis: hypouricemia (fractional excretion of uric acid >0.1), hypophosphatemia (fractional excretion of phosphate >0.2 or >0.1 if serum phosphate <0.8 mmol/L), tubular proteinuria (daily proteinuria >150 mg, with an increased urinary excretion of specific markers such as retinol-binding protein, β2-microglobulin, α1-microglobulin, or cystatin c or, alternatively, an albumin/total protein ratio in urine <0.4) (Samarawickrama et al., 2012), normoglycemic glycosuria, tubular acidosis (serum bicarbonate <21 mmol/L and a urinary pH >5.5), hypokalemia (EACS guidelines). The presence of tubular dysfunction markers is specifically relevant because they may precede an overt renal dysfunction as assessed by a decreased eGFR. Therefore, they may be considered early markers of kidney damage, especially in those patients that are treated with drugs such as tenofovir or atazanavir that are potentially nephrotoxic (Maggi et al., 2012), Table 2.

Prevalence of CKD and risk factors in HIV-infected patients

The prevalence of CKD among HIV-infected people varies in different regions ranging from less than 5% in European countries to more than 20% in India and even higher than 35% in some sub-Saharan countries (Naicker et al., 2010, Szczech et al., 2002, Emem et al., 2008). Different criteria for defining CKD may partially explain the large variability in CKD prevalence. However, using a compound criterion of eGFR<60 ml/min/1.73 m² or proteinuria, Bonjoch et al. reported a prevalence of 33% of CKD in a cohort of Spanish patients (Bonjoch et al., 2014). CKD prevalence may also depend on GFR formula for estimation, whereby the eGFR based on cystatin c tends to overestimate the percentage of patients with CKD (Jones et al., 2008).

The risk of CKD is distributed heterogeneously among ethnicities such that African-Americans have a fourfold risk compared to Americans of European ancestry. This ratio is maintained among HIV-infected people. Besides the genetic predisposition, factors such as diabetes, hypertension and the HIV infection per se, represent predisposing factors to the development of CKD (Kasembeli et al., 2015). Subjects of African ancestry have a specific predisposition to the occurrence of HIV-associated nephropathy (HIVAN), a major cause of evolving CKD. HIVAN is a specific kidney
disease related to the HIV infection due to a direct action of viral products on function of glomerular or tubular cells that manifests as progressive renal failure with severe proteinuria in the nephrotic range, and absence of the typical nephrotic signs and/or hypertension. There is a peculiar ultrasonographic appearance of markedly hyperechogenic kidneys and complete subversion of the normal cortico-medullary pattern. The histopathologic substrate of this disease is represented by a focal segmental glomerulosclerosis of the collapsing variant (Rosenberg et al., 2015).

In recent years, genetic factors that predispose to HIVAN have been identified: the presence of 2 trypanolytic variants of the APOL1 gene, instead of carrying 0 or 1 variant, is highly associated with HIVAN and a spectrum of other CKDs (Kopp 2011, Genovesev 2010, Tzur, 2010).

The relative risk of kidney disease, defined as CKD stage 3 or higher according to eGFR (<60 ml/min/1.73 m²) in people with HIV infection has been reported to be 3.87 (C.I. 2.18-6.85), compared to uninfected individuals with comparable age and after correction for common co-morbidities of renal disease. Patients with a late stage HIV infection have a higher risk of CKD, compared to those with early disease. Of note, ART-exposed patients have a lower relative risk of renal disease or a slower declining eGFR when compared to ART-naïve HIV-infected patients (Islam et al., 2012, Kalayjian et al., 2012).

Host-related factors more commonly associated with a declining kidney function are older age, female gender, diabetes, hypertension, dyslipidemia, HCV co-infection (Lucas et al., 2013), black race or a history of AIDS (Morlat, 2013, Kalayjian, 2012); to some extent, the methodology used to evaluate GFR (measured versus estimated) may affect this association (Margolick et al., 2014). In the MACS cohort, which included mostly virally suppressed patients, risk factors for CKD, as assessed by the eGFRcreat formula, were represented by older age, history of AIDS, higher proteinuria, and lower serum albumin levels, while black race, HCV co-infection, increased AST or alkaline phosphatase levels, showed a strong association with clinical development of CKD, as assessed by eGFRcys formula (Estrella et al., 2011). However, caution should be exerted in interpreting CKD stratification by eGFRcys because cystatin secretion may be increased in inflammatory states (Longenecker et al., 2015). Moreover, serum or urinary biomarkers represented by lower serum albumin (Lang et al., 2014), or increased urinary excretion of α1-microglobulin, a typical marker of renal proximal tubular damage (Jotwani et al., 2016), even in patients not exposed to TDF (Jotwani et al., 2015), are predictors of incident CKD. More sophisticated studies addressing some specific urinary biomarkers in the prevision of declining eGFR have demonstrated that besides the albumin/creatinine ratio, other factors such as urinary IL-18 may predict faster eGFR decline; a weaker association was also evident with urinary levels of KIM-1 (Shlipak et al., 2012).
**CKD and ARV therapy**

ARV therapies may represent a promoting factor of kidney damage and evolution towards CKD. Especially ARV regimens including TDF and/or Atazanavir have been considered potentially nephrotoxic (Tourret et al., 2013, Hara et al., 2015). Studies that have tried to compare TDF in the therapeutic regimen versus non-TDF based regimens have generated variable data, in some cases showing a higher risk of CKD for those under TDF therapy (especially when associated to PI) (Mocroft et al., 2016, Morlat et al., 2013, Fafin 2012, Kalayjian et al., 2012, Sherzer et al., 2012, Santiago et al., 2014) and other not (Islam et al., 2012, reviewed in Tourret, 2013). TDF induces proximal tubular dysfunction (Casado et al., 2016, Chadwick et al., 2015) and produces a decline in eGFR in a significant number of patients in the early phase of treatment (Laprise et al., 2013), therefore reduced measured GFR correlated with previous exposure to the drug (Margolick et al., 2014). In addition, the yearly loss of eGFR tends to reach a steady state after 4-5 years when there is no statistical difference from patients under TDF-free regimens (Estrella et al., 2013). Given the high rate of TDF discontinuation, in the long run the effect of the drug in evolving cases of advanced CKD (stage 4 and 5) is irrelevant (Ryom et al., 2014).

Among factors related to HIV infection, the immunologic state of the host represents a specific risk factor for renal disease. On the one hand, ART-naïve HIV positive patients with a good immunologic status (CD4 >500 mm$^3$) show a low prevalence of CKD (Achhra et al., 2015), whereas patients with a persistent low CD4 count (<200 mm$^3$) show a significantly increased risk of CKD (Islam et al., 2012, Santiago et al., 2014). A low CD4 count overall is associated with proteinuria or renal dysfunction (Bonjoch et al., 2014).

Advanced CKD (stage 4-5) and evolution toward ESRD is associated with common risk factors (hypertension, diabetes, smoking, proteinuria, hypoalbuminemia, reduced eGFR, black race) and specific risk factors (lower CD4 count, less than 200 mm$^3$, higher viral load, >30,000 copies/ml, hepatitis C co-infection) (Jotwani et al., 2012, Ryom et al., 2014). At multivariate analysis and correction for all other factors, the magnitude of proteinuria in different categories of patients with reduced renal function was the stronger predictor of evolution to ESRD (Jotwani, 2012). Patients with advanced CKD are more often off-TDF for discontinuation, even though, none of the ARV drugs is associated with advanced CKD (Ryom et al., 2014). The risk of developing ESRD in HIV patients is still high (Rasch et al., 2014), however there is evidence of a reduced incidence of HIV patients requiring dialysis or transplantation, especially with a more efficient viral suppression (Abraham et al., 2015).

A clinical score for estimating the risk of CKD has been proposed (Mocroft et al., 2015). Although this score can help predict the incoming CKD in a fairly accurate manner, it is incomplete and
reflects some bias in the study population, like the absence of critical pathophysiologic factors (i.e. proteinuria).

In summary, deterioration of renal functional reserve and occurrence of CKD are highly prevalent in many cohorts of HIV patients, there are still some problems with a heterogeneous modality of disease classification. Risk factors for developing CKD are represented by common co-morbidities (hypertension, diabetes, reduced eGFR) and disease-specific factors (mainly reduced CD4 count). ARV may affect eGFR in a significant number of patients, especially when containing TDF or ATZ, however the eGFR reduction and yearly decline tend to stabilize after the first years. Therefore, advanced CKD and ESRD incidence does not correlate with any of the specific ARV drug, while reduced CD4 count is significantly associated with renal insufficiency and the need for dialysis treatment. This finding strongly underline the concept that in order to reduce the occurrence of advanced CKD in people living with HIV, an adequate treatment of HIV infection aimed at obtaining a good immunological status is of critical importance. The renal toxic effects of ARV drugs are amply counterbalanced by the epidemiological benefits of slowing CKD progression in a setting of a well-controlled HIV infection and host immune response.

Most promising biomarkers to probe renal reserve in HIV subjects are reported in Table 3.

Skeleton reserve
Progressive loss of bone reserve in health and HIV-patients
With the ageing population, disorders of bone and mineral metabolism are becoming increasingly relevant to everyday clinical practice. A model from the national Dutch Athena cohort suggests that the median age of HIV patients on ART will increase from 43.9 years in 2010 to 56.6 in 2030, with the proportion of HIV patients aged 50 years or older increasing from 28% in 2010 to 73% in 2030 (Smith et al., 2015). A recent study suggested that HIV infection accelerates biological ageing (Cohen et al., 2016). Consequently, the interest and the need for effective measures to be used in the screening, diagnosis, and follow up of such pathologies have markedly grown. Remodeling of bone continues throughout life and skeletal tissue is replaced every 10 to 12 years on average. In healthy individuals, bone remodeling is determined by the two major cell types: osteoblasts bone forming cells and osteoclasts bone resorbing cells. Osteoblasts are originated from mesenchymal stem cells and determine the constitution of extracellular bone matrix. Osteoclasts differentiate from monocyte or macrophage under the influence of macrophage colony stimulating factor (M-CSF) and receptor activator for nuclear factor kB-ligand (RANKL), including pro-inflammatory cytokines such as TNF-alfa, osteoprotegerin (OPG) vitamin D and calcium metabolism and hormone levels (Borderi et al., 2009; Amorosa 2006). Osteoblasts may also evolve to osteocytes
when embedded in bone matrix, playing an important role in the control of architectural bone structure (Taylor et al., 2007). Osteocytes, the most abundant cells in bone, have long been postulated to detect and respond to mechanical and hormonal stimuli as well as to coordinate the function of osteoblasts and osteoclasts. The balance of the activity between these two type cells is the crucial determinant of bone mineral density (BMD). Bone remodeling and BMD homeostasis is a continuous process balancing bone resorption by osteoclasts alongside bone formation by osteoblasts in a process known as coupling (Pietschmann et al., 2016). A recent study suggested a role of senescent osteocytes and their senescence-associated secretory-phenotype (SASP) as promising therapeutic targets to prevent age-related bone loss (Farr et al., 2016). In addition to its obvious structural role, the skeleton is an important reservoir of calcium serving both to maintain plasma calcium concentrations and to make optimal use of ingested calcium. It serves both functions mainly by adjusting the balance between bone formation and resorption. Thus, a reduction in skeletal calcium reserves is equivalent to reduction in bone mass. These same processes of formation and resorption are what constitute bone structural remodeling or turnover. Bone strength is a function of bone mass which, in turn, is equivalent to the size of the calcium nutrient reserve. At the same time parathyroid hormone (PTH) is responsible for regulating the prevailing level of bone remodeling and is evoked by a fall in calcium intake. The role of PTH consists of resorption of bone by osteoclasts and replacement of the osteoclasts by osteoblasts, which lay down new bone to fill the hole created by osteoclastic resorption and return to the resting state. PTH regulates serum calcium and phosphorous concentrations through its receptor-mediated combined actions of bone, intestinal tract and kidney. Metabolic bone disease is in fact a common complication of CKD and can be manifested by a combination of abnormalities of calcium, phosphorous, PTH and vitamin D metabolism and abnormalities of bone turnover, mineralization, volume and linear growth. In addition, vitamin D is an important mediator of calcium/phosphate regulation and promote the mineralization of osteoid. Vitamin D deficiency in fact is associated with significant loss in the mechanical integrity and toughness of human cortical bone, which can also be explained by tissue aging (Zheng et al., 2016).

Bone alterations have also been observed in the course of HIV disease, representing a pivotal clinical problem in the management of HIV patients, especially for a possible development of bone fractures (Arnsten et al., 2007).

The major bone lesions detectable in HIV patients are related to bone demineralization and impairment of the skeletal microarchitecture (osteopenia, osteoporosis and osteomalacia). A meta-analysis of pooled prevalence data from eleven cross-sectional studies performed between 2000 and 2005 demonstrated an overall prevalence of 67% reduced BMD and 15% osteoporosis in HIV.
individuals when compared to HIV negative age- and sex-matched controls (Brown et al., 2006). Aging leads to osteopenia-related bone fragility and eventually osteoporosis with high risk of fracture. Age-related changes occur in bone, resulting in a decrease in bone density and a relative increase in adiposity (Jiang et al., 2008; Moerman et al., 2004). It is well established that both adipocytes and osteoblasts arise from marrow mesenchymal progenitor cells (MPCs), although *in vitro* studies suggest an age-related lineage switch between osteogenic and adipogenic fates (Singh et al., 2016). There is the possibility that dysregulation of peroxisome proliferator-activated receptor gamma (PPARγ) and the subsequent effect on both osteoblastogenesis and adipogenesis is a contributory factor in the bone abnormalities observed in HIV-1 infection (Cotter et al., 2009).

The pathogenesis of reduced BMD associated with HIV is likely multifactorial. Osteopenia and osteoporosis are correlated with different patient-related risk factors such as sex, age, Caucasian race, low body weight, malabsorption, family history of femur fracture, inadequate physical activity, smoking, alcohol or opiate use, steroid exposure, endocrine disorders, vitamin D deficiency, lipodystrophy, hepatitis C coinfection and CD4 <50/mm³ after HAART initiation (Brown et al., 2006; Ofotokun et al., 2012; McComsey et al., et al, 2010; Grant et al., 2013; Compston et al., 2016). Adequate counselling to modify these risk factors should not be forgotten in any treatment strategy targeting bone disease (Leite et al., 2010; Sambrook et al., 2006; Saccomanno et al., 2011; Chitu-Tisu et al., 2015). In addition, immune dysfunction and persistent inflammation (Brown et al., 2013) as well as ART drugs have been reported to negatively impact on bone health.

**HIV-related direct mechanisms of BMD loss**

The interaction between HIV and/or its protein with osteoblast/osteoclast function and differentiation has been investigated over the past decade. HIV infection itself has complex host interactions and HIV viral proteins can also directly affect osteoclasts (Mc Ginty et al., 2016). The results of some meta-analyses have shown that a direct infection of osteoblasts (reservoir) and osteoclasts is as a possible mechanism for the effects of HIV on bone formation (Stone et al., 2010; Otokofun et al., 2010, Yin et al., 2006). It is now clearly established that HIV infection is an independent risk factor for osteopenia and osteoporosis (Otokofun et al., 2012). Furthermore, as well as increasing age, HIV determines an impairment of osteoblast/osteoclast crosstalk that affects the differentiation of their progenitor cells through an increase in some proteins such as gp120, Tat, pg55gag and P75-soluble TNF-α receptor (Gibellini et al., 2012). In recent years, research has shed more light on the relationship between a chronic inflammatory state and changes in bone metabolism in HIV (Otokofun et al., 2012). Results have shown that cytokines and other soluble immune factors play a major role in the physiology of osteoblast maturation and osteoblastic bone
resorption (Brown et al., 2013). Moreover, despite full virological control with ART, HIV patients exhibit a hyperactivated immune profile. This profile is characterized by increased circulating pro-inflammatory cytokines, which increase the activity of osteoclasts and reduce naive/central memory T-cells subsets in favor of activated/senescent phenotypes (Marchetti et al., 2006). It has also been shown that some pro-inflammatory cytokines could possess osteoclastogenic and/or anti-osteoclastogenic properties and can target osteoclasts directly or via receptor activator of nuclear factor kB (RANK)/RANK ligand (RANKL)/osteoproegerin (OPG) system (Zupan et al., 2013). In HIV patients, high HIV RNA viral load and T-cell activation have been correlated with elevated levels of RANKL, which lead to osteoclast formation and increased bone resorption. Furthermore, tumor necrosis factor (TNF-α) and IL-6 stimulate osteoclast genesis and bone resorption (Gibellini et al., 2007, Castronuovo et al., 2015). These immunological alterations are similar to those observed in the HIV-uninfected elderly in whom several factors regulate bone metabolism (parathyroid hormone (PTH) and calcitonin). The PTH pathway is particularly important as it modulates the production of the proinflammatory cytokine IL-6, TNF-α, IL-1b and RANKL (Desay et al., 2010). These cytokines have long been implicated in other inflammatory conditions such as postmenopausal osteoporosis and rheumatoid arthritis where bone loss and destruction is a feature, and suggest the effect on inflammation on bone occurs in the general population (Gedmintas et al., 2012; Toraldo et al., 2003, Tyagi et al., 2012).

Could drugs such as statins also prove beneficial to their antinflammatory effect? (Negredo et al., 2016). The Saturn-HIV trial investigated the effects of rosuvastatin on BMD and bone biomarkers and inflammatory cytokines in HIV-infected persons compared with placebo. Those randomized to rosuvastatin had a small but significant gain in hip BMD which was strongly associated with baseline and week 48 sTNFR2 levels supporting a potential BMD-mediated beneficial effect through modulation of inflammation (Erlandson et al., 2015; Funderburg et al., 2015). The connections of the pro-inflammatory cytokines with osteoporosis phenotype in a recently published study (Zupan et al., 2013) suggest an osteoimmunological approach.

Finally, the role of HIV as an independent predictor for low BMD and pre-ART disease severity, measured by absolute CD4 T-cells counts and high HIV-RNA, has been associated with a greater subsequent loss of BMD (Brown et al., 2009; Grant et al., 2013).

How should ART be managed in ART-naive and experienced HIV patients at risk of bone disease?

**Bone disease and ART**

The guidelines stress that all antiretroviral drugs, albeit to varying degrees, may have an impact on bone metabolism. Initiation of ART is associated with a 2%-6% decrease in BMD, especially in the
first 12 months of therapy (Mallon et al., 2003) due to increased levels of bone turnover markers (BMT) associated with increased TNFα levels. However, some ART regimens may be associated with more pronounced bone loss. A high incidence of osteopenia/osteoporosis has been associated with both protease inhibitors (PI) and nucleoside reverse transcriptase inhibitors (NRTI) (Carr et al., 2001; Vescini et al., 2003, Brown et al. 2006). Tenofovir (TDF) showed some bone toxicity compared to other antiretroviral compounds (Stellbrink et al., 2010; Haskelberg et al., 2012, Negredo et al., 2015). The long-term use of PI is associated with BMD loss (Duvivier et al., 2009; Gibellini et al., 2012, Moran et al., 2016, Kinai et al., 2014). Finally, the regimens at the highest risk of damage are those comprising the combination of TDF plus a protease inhibitor pharmacokinetically reinforced by ritonavir (PI/r). Indeed, RTV has been show to increase plasma TDF concentration by 32-50% via inhibition of active TDF secretion by the proximal convoluted tubule (Baxi et al., 2014, Cihlar et al., 2007, Gutierrez et al., 2014). Similarly, cobicistat acts like RTV to boost protease inhibitors and has been shown to increase plasma TDF concentration by 24-30% (German et al., 2010, Rockstroh et al., 2013). A switch strategy to avoid the bone toxicity of TDF plus PI would to be simplify the regimen in virologically suppressed patients on a dual therapy (D’Avino et al., 2016). The HIV Upbeat cohort study reporte that exposure to PI/r but not TDF was significantly associated with lower trabecular bone score (TBS), a novel noninvasive measure of bone microarchitecture that can detect differences in bone quality in patients with similar BMD (Mc Ginty et al., 2016). But the real impact of PI on BMD remains unclear. The alterations caused by PI in body fat could subtend an error in dexa spine measurement. Several studies have shown that there is an increase in weight and BMI in patients who use PI/r compared to NNRTIs and have assessed the correlation between changes in fat mass and reading the DEXA highlighting the possibility that the reduction of BMD is an artefact of fat-associated reading (Yu et al., 2012, Madeddu et al., 2015). The use of the new class of integrase inhibitors (INSTIs) produces a lower loss of BMD both compared to regimens containing a PI/r or TDF and in NRTI-sparing regimens as a simplification strategy in selected subjects. A raltegravir-based regimen was associated with significantly less loss of BMD than a standard regimen containing TDF (Bernardino et al., 2015) Similarly, Pozniak et al., reported a significant increase in spine and hip BMD at 48 weeks in HIV-infected after switching from a TDF-containing regimen to E/C/F/TAF (et al., 2015). In addition, dolutegravir in association with abacavir plus lamivudine is associated with less bone turnover than efavirenz/tenofovir/emtricitabine (Tebas et al., 2015). In the SWORD studies the two-drug regimen showed comparable efficacy to three-or-four-drug regimens in virologically suppressed patients: switching from standard antiretroviral therapy to the dual regimen of dolutegravir plus rilpivirine had a beneficial on bone biomarkers (Llibre et al., CROI 2017) Novel drugs such as tenofovir
alafenamide (TAF) have shown promising results with respect to renal and bone parameters. Several studies support switch off tenofovir DF which is know to cause bone loss (Mills et al., 2016, Gallant et al., 2016, Oka et al., 2016).

Measurement of bone reserve in HIV
The guidelines give useful guidance on how to assess bone disease in HIV patients, indicating that screening for the identification of the bone disease risk must always be performed in these patients. In HIV patients, lifestyle habits useful for the prevention of osteopenia/osteoporosis include physical activity with muscle-strengthening exercise, prevention of slimming, cessation of smoking and alcohol abuse, the daily intake of 1 g of calcium for men with 50-70-years of age or 1-2 g for women over 50 and men over 70-years in the diet (when a sufficient dietary intake cannot be achieved). All HIV patients should have plasma 25-OH vitamin D levels dosed preferably in winter and spring. Supplementary vitamin D should be given to HIV-infected patients with vitamin D insufficiency (<20 ng/mL) or deficiency (<10 ng/mL), particularly if the deficiency is associated with compensatory hyperparathyroidism (Brown et al., 2015).

Low BMD and fragility fractures are common in HIV patients on ART. The Veteran Aging Cohort Study Virtual Cohort (VACS-VC) found fracture rates were 24-32% higher compared with HIV negative controls, while the women’s Interagency HIV Study (WIHS) cohort reported that HIV patients had a greater incidence of fragility fractures than HIV-uninfected patients (Womack et al., 2011; Sharma et al., 2015). In a recent guide the management of bone disease in HIV-patients, Brown and 34 HIV specialists from 16 countries suggested a detailed algorithm as well as EACS and US guidelines (Brown et al. 2015). For those patients with a high risk of fragility fracture, men of 40-49 years of age and premenopausal women aged more than 40 years, the use of the Fracture Risk Assessment Tool (FRAX) algorithm is suggested. The FRAX algorithm includes classical risk factors and is used to calculate the 10-year probability of major fracture risk at the hip, spine and humerus; it can be used with or without BMD assessment but may underestimate fracture risk in HIV patients. The algorithm should be used to identify HIV patients who should be assessed with dual energy X-ray absorptiometry (DXA) for low BMD (Kanis et al., 2009, Kanis et al., 2012). DXA is the most commonly used gold standard screening test for measuring BMD. The DXA measurement of “areal” density does not measure true volumetric density and cannot differentiate cortical bone from trabecular bone. Bone strength is dependent on both bone mass and bone microstructure (TBS). A recent study (Sharma et al., 2016) suggested that future research should explore adding TBS to BMD in the FRAX algorithm as a fracture predictor utilizing a software program installed on existing DXA scanners. Routine DXA screening of all HIV-infected patients on ART is not recommended. It should be considered in postmenopausal women and men aged
over 50 years and in patients who have an intermediate-high risk stratification by FRAX. Major alterations in body composition such as with obesity and weight loss have a complex effect on the measurement of BMD by DXA. Fat layering introduces error and decreases the reproducibility of DXA spine and hip BMD measurement (Yu et al., 2012) Quantitative computed tomography (QCT) is another method of measuring BMD, yielding the true volumetric bone density irrespective of bone size.

BMD does not fully capture the fracture risk as the majority of fractures occur in patients with osteopenia. Recently, the microindentation in vivo technique (BMT) has been available to assess altered bone material properties. BMT induces separation of mineralized collagen fibrils and initiation of cracks, independently of bone mineral density, thus directly measuring the mechanical competence of bone tissue to resist fracture (Malgo et al. 2015, Diez-Perez et al. 2010). Besides DXA, TBS and in vivo BMT directly measure cortical bone, trabecular microarchitecture and mechanical properties of bone at a tissue level, respectively. These three techniques provide additional and necessary information on bone health in treated HIV patients and could be routinely apply to assess bone in clinical practice (Guerri-Fernandez et al. 2017). Finally, calcaneal quantitative ultrasound (QUS) can be used as an additional tool for analyzing BMD in HIV-positive patients. It is a noninvasive and inexpensive technique able to reflect bone quality and should be used as an alternative or integrative examination when DXA are less accessible. QUS could discriminate subjects with and without fracture history and predict risk for future fracture but is still not advisable for monitoring therapeutic effectiveness during follow-up (National Osteoporosis Foundation Guidelines 2014). The prevalence of subclinical vertebral fractures is most common in HIV-infected patients. Therefore, height should be measured every 1-2-years in adults >50-years of age and lateral imaging of the thoracic and lumbar spine with conventional X-rays or DXA should be performed to assess a vertebral fracture in patients with height loss of >4 cm, kyphosis, or recent long-term glucocorticoid treatment (Young et al., 2011, Brown et al., 2015, EACS guidelines 2015) also irrespective of BMD (Weiser-Smeke et al., 2016). With the laboratory diagnosis secondary osteoporosis can be identified along with recognized risk factors that allow a correct therapeutic intervention (Es. hypovitaminosis D or secondary hyperparatiroidism). First level biochemical examinations can exclude disorders of forms of secondary osteoporosis in most cases. These include: complete blood count, serum protein electrophoresis, serum calcium levels, serum phosphate levels, total alkaline phosphatase, serum creatinine and 24-h urinary calcium. In clinical suspicion must be the second-level measurement of biochemical tests: serum-25-OH-vitamin D, parathyroid hormone (PTH), ionized calcium, thyroid stimulating hormone (TSH), cortisol levels,
total testosterone in male, serum or urine immunofixation, celiac disease test, other specific investigations for associated diseases (Rossini et al., 2016)

Biomarkers of bone reserve in clinical practice

Can bone turnover markers assist the clinician in identifying patients with bone loss reserve? Bone turnover markers (BMT) are biochemical markers of either bone formation or bone resorption. Commercially marketed tests are available to assess some of these markers in urine and/or serum by high performance liquid chromatography (HPLC) or immunoassay. Several nonrandomized controlled trials also discussed the potential value of BTM (Iki et al., 2006; Garnero et al., 2000, Worsford et al., 2004). BMT are subject to day-to-day and within day variability which may influence clinical interpretation (Vasikaran et al., 2011). More factors limiting the use of BTM in clinical practice are biological sources of variability that are harder to control (Haskelberg et al., 2011): age and sex (BTM levels stabilize during the 3rd decade of life then decrease in men); menopause is associated with bone resorption (79-97%); sex hormones (estrogen and testosterone levels inversely correlated with bone resorption); ethnicity (lower BTM levels in black men) (Leder et al., 2007); diurnal variation (BMT higher in winter compared to summer (Woitge et al., et al., 1998); physical activity; immobility (associated with increased BTM) (Chen et al., 2006); smoking (did not affect BTM in men, increased BTM in women) (Tamaki et al., 2011); alcohol (reduced levels of BTM). These biochemical indices are non-invasive, with improved sensitivity and comparatively inexpensive and when applied and interpreted correctly helpful tools in the diagnostic and therapeutic assessment of metabolic bone disease. Biochemical monitoring of bone metabolism depends upon measurement of enzymes and proteins released during bone formation and of degradation products produced during bone resorption. Although these markers are not recommended for use in diagnosis of osteoporosis yet, they appear to be useful for the individual bone functional reserve and for monitoring of osteoporotic patients treated with antiresorptive agents. Monitoring biphosphonate therapy with bone markers measurement at baseline and at 3 and 6 months can improve the compliance with therapy by 20% at 1 year. It is essential to establish reference ranges in different populations including HIV patients. The recent recommendation by the Bone Marker Standards Working Group aims to standardize research and include a marker of bone resorption cross linked telopeptide of type 1 collagen (CTX) that is secreted by osteoclasts and bone formation amino terminal propeptides of type 1 collagen (P1NP) that appears to be more sensitive during conversion of procollagen to collagen in all future studies. For the prediction of disease, progression bone turnover as P1NP and CTX can be used. Serum CTX has also gained attention as a possible risk indicator for biphosphonate-related osteonecrosis of the jaw. (Pasoff et al., 2013; Fleisher et al., 2010) Telopeptide-N-terminal (NTX) of collagen constitutes a particularly sensitive
marker capable the early detection of changes in bone resorption that occur in physiological conditions. Recently developed methods for measuring serum levels of tartrate-resistant acid phosphatase (TRAP) and ostase bone specific alkaline phosphatase (BAP) represent superior laboratory tools for assessing the hyperactivity of osteoclasts, osteoblasts and bone loss in HIV patients receiving HAART (Aziz et al., 2014). BAP is also useful in assessing the risk of fracture: it is a complementary test to DEXA. Measurements of TRAP and BAP as bone turnover biomarkers are economical and important for monitoring bone metabolism during HAART and the need for osteoporosis treatment. Recent studies found that depleted sclerostin levels (an osteocyte product and a potent anti-anabolic molecule) were associated with lower BMD and could represent a biomarker of advanced bone disease (Morse et al., 2013). HIV infection is associated with reduction in serum sclerostin level but there is no significant correlation between sclerostin and BMT (Almansouri et al., 2016) Pharmacological inhibition of sclerostin, a well-known, natural occurring inhibitor of the Wnt system essential for bone formation, is another potential new approach for the treatment of osteoporosis. A recent study showed the role of microRNA (miRNA), a minimal-invasive biomarker that plays an essential role in the regulation of bone homeostasis and could reflect skeletal as well as non-skeletal risk factors and accurately predict fracture risk. Specific changes in miRNA transcription levels or miRNA secretory levels have been linked to the development and progression of bone disease (Hackl et al., 2016). In summary, the bone formation biomarkers (bone specific alkaline phosphatase (BALP), intact N-terminal propeptide of type 1 procollagen (P1NP) and osteocalcin (OC), bone turnover regulators (25-hydroxy-vitamin D, intact parathyroid hormone, receptor activator of Nkxkb (RANKL) and osteoprotegerin (OPG), and bone resorption biomarkers (cathepsin K, C-terminal telopeptide (CTx) and osteopontin) are also interesting because they allow small changes in bone turnover to be monitored well before they manifest changes in BMD or fracture risk. Nonetheless, patients with low BMD or high markers of the bone turnover values would be at risk of osteoporosis and warrant preventive measures with antiresorptive agents. Whereas the increase in pill burden in an aging population may not be a forward-looking strategy, Zoledronic acid is a particularly attractive strategy in that a single infusion of 5 mg prior to initiating ART with TDF/FTC/ATV/r has long term effects on suppressing bone turnover (Ofotokun et al., 2016). In a general guidance statement, the National Osteoporosis Foundation (NOF) states that biochemical BMT may:

- Predict risk of fracture independently of BMD in untreated patients.
- Predict extent of fracture risk reduction when repeated after 3-6 months of treatment.
- Predict magnitude of BMD increases with FDA-approved therapies.
- Predict rapidity of bone loss in untreated patients.
• Help determine adequacy of patient’s compliance with osteoporosis therapy.
• Help determine duration of “drugs holiday” and when and if medication should be restarted (NOF 2014).

Novel different biomarkers are desirable which can alone or in combination with existing biomarkers provide a better understanding of bone strength and consequently fracture risk in patients. Finally, a recent study created an index (Bone Balance Index) that combined measurement of bone resorption and bone formation that was most useful for predicting bone loss in the bones of the spine (Shieh et al., 2016).

Biomarkers of bone turnover in clinical practice in the assessment of HIV subjects are listed in Table 4.

The most promising biomarkers to probe bone reserve in clinical practice in HIV-patients are listed in Table 5.

**Brain and cognitive reserve**

*Progressive loss of brain reserve in HIV*

The brain is considered a reservoir of HIV due to the blood-brain barrier limiting the influence of cART. At an early stage of the HIV infection, there are minimal replication and genetic variants of the HIV in the brain and a low probability of local virus compartmentalization (Sturdevant et al., 2015). At a later stage, there are:

1) moderate replication and multiple genetic variants of the HIV in the brain;
2) a certain probability of the local virus compartmentalization;
3) higher levels of HIV and lower CD4 count in the blood when compared to the early stage of virus replication (Sturdevant et al., 2015).

As a result of the mentioned HIV neuroinvasion, a progressive loss of brain reserve can be speculated in HIV. Indeed, the majority of HIV subjects can show neurological, cognitive, and behavioral symptoms over time (Reger et al., 2002; Anthony and Bell 2008; Antinori et al., 2007).

In particular, subclinical neuropathy was found in 10-40% of asymptomatic HIV subjects, this rate being increased (53-100%) in AIDS patients (Chavanet et al., 1988; Gastaut et al., 1989). Furthermore, 50-70% of HIV subjects did suffer from neurologic symptoms and cognitive deficits (the so-called HIV-associated neurocognitive disorders, HAND) across time (Selnes, 2005). All these correlates were mitigated in HIV subjects receiving cART (Clifford, 2008), which reduce viral load and recover CD4 cell counts (Graham et al., 1992; Hammer et al., 1997; Hunt et al., 2003; Williams et al., 2012).

Despite the introduction of cART, the prevalence of HIV subjects with neurological and HAND symptoms is not reduced (Cysique et al., 2009; Sevigny et al., 2004). Indeed, cART dramatically
reduces the concentration of HIV in both plasma and cerebrospinal fluid (CSF), but the virus is frequently detectable in the CSF even after many years of beneficial cART. This fact motivates the quest for biomarkers that index the impact of HIV on the brain reserve towards clinical applications as well as drug discovery and monitoring.

A tentative explanation for the effects of HIV on brain reserve is that such action is mediated by neuroinflammation and impaired cerebrovascular flow due to microvascular lesions in the brain gray and white matter. Indeed, HIV neuroinvasion exerts a deleterious effect on brain mononuclear phagocytes such as monocyte-derived macrophages and microglia with paracrine upregulation of immune-secreted bioactive agents such as cytokines, chemokines, quinolinic acid, and arachidonic metabolites (Kaul, 2009). These agents induce a chronic neuroinflammation over time as revealed by the increase in neopterin in the CSF (Dahl et al., 2014), and this effect can be conceptualized as a loss of brain reserve across aging.

**Biomarkers of brain reserve in HIV patients before and after cART regimen**

In the progression of the HIV infection, brain reserve is expected to be affected as cerebral molecular, structural, and functional alterations before the manifestation of HAND. Furthermore, it is expected to show beneficial effects of cART on brain status, at least for most of the regimens. Nowadays, cerebral molecular, structural, and functional alterations can be measured by several neuroimaging modalities providing neuroimaging and neurophysiological biomarkers. In principle, they have the potential to define the brain reserve in individual HIV over time.

Concerning the structural abnormalities of the brain reserve, the neuroimaging modalities typically used in HIV patients are computed tomography (CT) and magnetic resonance imaging (MRI), the latter having a higher spatial resolution. CT and T1-weighted MRI allow the visual detection of the following volumetric alterations of brain reserve in HIV subjects (Thurnher and Donovan, 2008):

1) regional gray matter (i.e. basal ganglia and posterior cortex with generalized volume reduction of white matter);
2) global cerebral atrophy;
3) ventricular expansion;
4) hydrocephalus;
5) thinning of the corpus callosum.

In HIV subjects with HAND, T2-weighted MRI unveils visible white matter vascular lesions (<30%). When dementia is present (HIV-associated dementia, HAD), two main patterns of MRI hyperintensity emerge from visual inspection (Thurnher and Donovan, 2008):

1) diffuse, bilateral, and symmetric high signal intensity in the white matter (butterfly-like);
2) bilateral, scattering, high signal intensity lesions both in the white and gray matter.
This MRI hyperintensity is possibly due to increased water content and delivery of serum proteins as an effect of abnormal vascular permeability induced by neuroinflammatory cytokines. It is noteworthy that brain white matter abnormalities in CT and MRI biomarkers reflected the progression of the neuroinvasion and beneficial effects of cART (Thurnher and Donovan, 2008). These effects typically improve the signs of brain lesion and neurological symptoms in HIV subjects but they could not recover the premorbid status as a potential biomarker of a reduction of the subject’s structural brain reserve (Thurnher and Donovan, 2008; Peluso et al., 2012). Furthermore, no differences over time (26 months of median interval) in deep gray matter volumes and cortical thickness, as revealed by structural MRI, were found in HIV subjects on stable cART, with high CD4+ count and undetectable viral load (Corrêa et al., 2016), as evidence that the control of HIV relatively preserves the structural brain reserve along the time axis. These features make these MRI biomarkers potential predictors of the status of neurocognitive functions across aging in HIV subjects.

Diffusion tensor imaging (DTI) is a promising new MRI procedure for application to the HIV field (Filippi and Ulug, 2001, Tucker et al., 2004, Thurnher et al., 2005, Thurnher and Donovan, 2008, Gottumukkala et al., 2014). DTI measures tissue anisotropy, thus providing details on tissue microstructure, revealing abnormalities in the fiber structure of brain white matter as a biomarker of structural cerebral connectivity, namely a relevant parameter underpinning cognition. Filippi and colleagues (2001) unveiled DTI biomarker abnormalities associated with normal structural MRI in HIV subjects. Indeed, the HIV subjects with advanced HIV disease (high viral load) had the highest abnormalities of DTI biomarkers, thus suggesting a relationship with the effects of brain neuroinflammation on cerebral connectivity. Another study reported abnormal DTI biomarkers in the genu of the corpus callosum in HIV subjects (Thurnher and Donovan, 2008). However, the DTI biomarkers did not correlate with viral load and immunity, thereby revealing a limited sensitivity to major HIV parameters in single individual subjects. Interestingly, deficiencies in the DTI brain networks of HIV subjects exceeded those observed in healthy aging, especially when associated with a well-known genetic risk factor for dementia such as ApoE4 (Jahanshad et al., 2012). This interaction of age, virus, and ApoE4 unveiled DTI alterations in HIV elderly subjects even when they received cART (Jahanshad et al., 2012). This specific feature of the DTI is interesting as no substantial differences were found in HIV subjects between ApoE4 and non-carriers in general adult age for several other MRI biomarkers such as subcortical and cortical gray matter, global white matter, ventricles, as well as MRS concentrations of N-acetyl-aspartate, creatine, myoinositol, and choline in the basal ganglia, frontal gray matter, and frontal white matter (Cooley et al., 2016). Furthermore, no differences in the integrity of white matter, as revealed by
DTI, were observed in HIV subjects on stable successful cART over 26 months as expected by a biomarker sensitive to the effect of HIV in that favorable clinical condition (Corrêa et al., 2016). However, literature evidence is relatively sparse, and more research is needed on DTI biomarkers of the reserve of structural brain connectivity in HIV subjects across aging.

Perfusion MRI (pMRI) detects abnormalities of regional cerebral blood flow (rCBF) that correlated with disease severity in HAND (see Ances et al., 2009 for a review). It has been shown that HIV patients who have severe HAND had a statistically significant decrease of rCBF bilaterally in the inferior lateral frontal cortices and an increase in the posterior inferior parietal white matter. Furthermore, a cross-sectional study with arterial spin labeling MRI (ASL-MRI) measured resting state rCBF in HIV and control groups. A reduction of rCBF was observed soon after seroconversion, possibly reflecting neuronal or vascular injury among HIV individuals not yet expressing HAND. Finally, a recent pMRI study demonstrated lower rCBV values in both HIV naïve and cART-treated patients compared with control subjects, suggesting that this technique enables the assessment of HIV-related early brain dysfunction in asymptomatic subjects but it would be not very sensitive to the effect of cART (Bladowska et al., 2014). Because pMRI is faster and safer than nuclear medicine techniques, it can be speculated that pMRI biomarkers may be used to monitor rCBF changes in patients who have HIV initially (Thurnher and Donovan, 2008, Ances et al., 2009). However, literature evidence is relatively sparse, and more research is needed on these biomarkers of the reserve of the brain mechanism ensuring rCBF as a hemodynamic underpinning of neurocognitive functions in HIV subjects across aging.

Functional MRI in the resting state condition (rs-fMRI) allows the investigation of the effects of HIV on functional dimensions of the brain reserve. The rs-fMRI measures the blood oxygen level dependent (BOLD) signal. In this experiment condition, BOLD changes indirectly reveal an increase in rCBF and oxygen blood supply in a statistically correlated manner in a couple of voxels or regions of interest as a biomarker of brain functional connectivity. There is some interesting new rs-fMRI evidence in HIV subjects (Chang et al., 2001; Thurnher and Donovan, 2008). In a recent meta-analysis (Thomas et al., 2013), HIV subjects showed greater task-related BOLD in the left inferior frontal gyrus and caudate nucleus as a function of HAND. On the other hand, rs-fMRI allowed the evaluation of the functional correlations among brain networks. These networks showed fewer correlations in HIV patients independently of aging. HIV serostatus and age independently affected rs-fMRI measures with no interaction (Ances et al., 2010). Functional brain demands in HIV subjects were equivalent to those of control subjects who were 15-20 years older. Frailty parallels between HIV infection and aging could result from continued immunological challenges depleting resources and triggering increased metabolic demands.
Concerning the use of fMRI during cognitive tasks, HIV subjects showed greater BOLD in the parietal cortex during simple attention tasks, while greater parietal and a frontal increase of BOLD was reported during more engaging attention tasks (Thurnher and Donovan, 2008). More research is needed to evaluate the sensitivity of rs-fMRI to the effect of cART.

Even if CT and MRI are the most popular neuroimaging techniques to enrich the diagnosis of neurological symptoms and HAND in HIV subjects, molecular neuroimaging is supposed to have an integrative potential (Thurnher and Donovan, 2008). Indeed, HAND can be observed before visible structural brain atrophy, ventricular dilatation, and/or focal CNS lesions. To explore that dimension, there are some effective neuroimaging techniques such as MRI spectroscopy (MRS), single-photon emission computed tomography (rs-SPECT), and positron emission tomography (PET).

MRS measures the signal generated by protons of specific molecules in the brain tissue. These molecules comprise the following compounds:
1) n-acetyl aspartate (NAA), a biomarker of neuronal integrity;
2) choline (Cho), a biomarker of inflammatory response and cellular proliferation;
3) myoinositol (MI), a biomarker of gliosis;
4) creatine (Cr), a reference biomarker of brain energy metabolism.

Due to its intrinsic properties, MRS is supposed to be more sensitive to early stages of HIV neuroinvasion, when structural and vascular lesions of the brain are practically negligible at the visual inspection of the structural MRI biomarkers (Chang et al., 2000, Letendre et al., 2011, Ances and Hammoud, 2014). HIV subjects with chronic infection showed a reduction of NAA and a concomitant increase in Cho and MI. MRS biomarkers are related to HAND and progression of the HIV infection. Immediately after seroconversion, MRS metabolites and structural MRI changes reflect inflammation and neuronal injury in both subcortical (basal ganglia) and cortical (parietal grey matter as well as frontal grey and white matter). Interestingly, MRS biomarkers are sensitive to the worsening of brain molecular structure and neurocognitive function over 3-years even in HIV subjects following successful cART as standard serological parameters (Winston et al., 2015). Furthermore, standard cART and ritonavir-boosted protease inhibitor monotherapy did not show a different effect on neurocognitive function and neuroimaging biomarkers such as bicaudate index, hyperintensities of the white matter, medial temporal lobe and global cortical atrophy, or single voxel (basal ganglia) NAA/Creatine, myoinositol/creatinine, and N-acetyl aspartate (NAA)/choline ratios (Arenas-Pinto et al., 2016). These results show the potential of the MRS biomarkers in monitoring the effects of cART on brain integrity and neurocognitive function in HIV subjects over time. However, the volume of the research with this biomarker is relatively sparse to date.
rs-SPECT is the neuroimaging technique used to scan in vivo brain rCBF and metabolism in HIV subjects in the resting state condition. A seminal rs-SPECT study (Samuelsson et al., 2006) in treatment-naïve HIV subjects with an early infection showed abnormal rCBF at group level but not at single patient level. Another application of rs-SPECT in HIV is based on the ligands labeled 123I-FP-CIT and 123I-IBZM, which are used to measure the availability of dopamine transporters (DAT) and dopamine 2 (DA2) receptors, respectively (Kumar et al., 2009, Obermann et al., 2009). The rationale is previous evidence suggesting a depletion of dopamine in the CSF and brain in HIV subjects with dementia. Interestingly, there would be an upregulation of dopamine in the brain in the early stages of the HIV infection in HIV subjects without HAND. It can be speculated that this upregulation in asymptomatic HIV infection may precede a dysfunctional DA system in later stages of HIV infection. However, more research is needed to evaluate the sensitivity of DAT scan to the effect of cART. Furthermore, a general limitation in the use of SPECT is its invasiveness due to the administration of radioactive ligands and the relatively high cost of serial recordings.

PET is the neuroimaging nuclear medicine technique that typically provides true 3D information on amounts of metabolism, neurotransmitters, neuroinflammation, or amyloidosis in scanned regions of the brain in the resting state condition (rs-PET). PET mapping of brain metabolism is typically performed by the injection of fluorodeoxyglucose (FDG) into the patient’s bloodstream (Pascal et al., 1991). The FDG uptakes the active brain neural tissues, while PET detects pairs of gamma rays emitted indirectly by the positron-emitting radionuclide (tracer). Some rs-FDG-PET evidence in HIV subjects demonstrated a widespread hypometabolism in both subcortical and cortical areas, associated with age, cerebral atrophy, and neurocognitive status, in some cases in spite of normal MRI (Pascal et al., 1991). A hypermetabolic state was also found in the striatum in the early stages of the HIV infection as a reflection of abnormal functional connectivity in the subcortical areas (Hinkin et al., 1995). A subsequent switch from the hypermetabolic to hypometabolic states in subcortical areas (e.g. basal ganglia) and parietal lobe was observed in relation to the progression to dementia (von Giesen et al., 2000). Furthermore, FDG-PET evidence showed a relationship of the resting state mesial-frontal metabolic rate with major variables such as disease progression, therapy duration, and plasma levels of neuroinflammatory biomarkers (Andersen et al., 2010). Moreover, even optimally treated HIV subjects present abnormal cerebral glucose metabolism as a reflection of neuronal damage (Rottenberg et al., 1996). Less clear was the sensitivity of FDG-PET to the effect of aging in HIV subjects (Towgood et al., 2013).

Concerning PET mapping of neuroinflammation (Hammoud et al., 2005), the procedure includes the injection of 11C-PK11195 into the patient’s bloodstream to target the translocator protein (TSPO), a mitochondrial receptor upregulated when microglia is activated during...
neuroinflammation (Of note, a genetic polymorphism of the TSPO receptor is confounding variable to taken into account)). 11C-PK11195-PET evidence showed higher uptake in the thalamus, putamen, cerebellum, frontal cortex, and occipital cortex in treatment-naïve HIV subjects with HAD than control non-infected subjects, but no clear differences between HAD and HIV subjects with no HAND (Garvey et al., 2012). Furthermore, there was less uptake of 11C-PK11195 (less neuroinflammation) in the HIV subjects under ART regimen when compared with those without treatment (Garvey et al., 2012). Finally, there was an increased 11C-PK11195 binding in cingulate, temporal, and frontal areas in HIV subjects receiving cART compared with control non-infected individuals (Garvey et al., 2014).

Overall, several PET and SPECT agents were proposed with the aim of distinguishing subgroups of HIV subjects with HAND of different severity. Although some PET results are encouraging, none of the PET and SPECT tracers have invasiveness/cost/benefit features to be warmly recommended for the assessment of molecular brain reserve as underpinning HAND over time with a specific predictive or monitoring value. Tracers more specific for HIV compartmentation may enhance the role of nuclear medicine in the clinical management of HIV subjects.

A non-invasive, repeatable without learning effects, and cost-effective approach for the evaluation of the neurophysiological functional dimension of the brain reserve in HIV subjects is the measurement of electroencephalographic (EEG) rhythms from scalp electrodes in quiet wakefulness such as eyes-closed resting state condition. These rhythms reflect neurophysiological mechanisms that regulate brain arousal in a condition of low vigilance (Pfurtscheller and Lopes da Silva, 1999).

In a condition of eyes-closed and silent resting state, the dominant rhythm of the cerebral cortex shows a frequency around 10 Hz (alpha rhythm) and maximum amplitude on the posterior cortical regions (Pfurtscheller and Lopes da Silva et al., 1999). This dominant alpha rhythm reflects the synchronization of cortical pyramidal neurons mainly due to thalamocortical and cortical signals as a neurophysiological mechanism of cortical idling and relaxation in conditions of low vigilance. Interestingly, this kind of idling synchronized condition is an ideal starting point for desynchronization neurophysiological mechanisms underlying an active sensory, cognitive, and motor information processing in brain circuits to respond to environment stimuli or act internal plans (Pfurtscheller and Lopes da Silva et al., 1999).

The bulk of EEG investigations has shown a general decrease of dominant resting state alpha (8-12 Hz) rhythms in HIV patients when compared to healthy (HIV-negative) subjects with some exceptions (Gruzelier et al., 1996, Baldeweg et al., 1997, Polich et al., 2000). In the early reports, about 20-30% of HIV subjects presented a paradoxical increase in alpha rhythms at the scalp electrodes, possibly associated with their psychiatric status or serological characteristics of the
In parallel to alpha rhythms, spatially distributed abnormalities of resting state EEG rhythms in HIV subjects were also found at frequencies lower than 8-12 Hz, namely at the delta (<4 Hz) and theta (4-7 Hz) rhythms. These low-frequency EEG rhythms (delta, theta) are expected to show a low amplitude in healthy adults set in quiet wakefulness. In contrast, HIV subjects with subclinical symptoms (even without AIDS) presented abnormally high values of delta (<4 Hz) and theta (4-7 Hz) rhythms at several scalp electrodes, similarly to those observed in elderly subjects with cognitive deficits due to other pathological conditions (Itil et al., 1990). This “slowing EEG” effect was even more pronounced in HIV subjects with AIDS (Itil et al., 1990). Furthermore, high values of frontal and frontotemporal delta and theta rhythms (<7 Hz) were observed in 25% of HIV subjects with no AIDS and 30% of those with lymphadenopathy syndrome (Parisi et al., 1989). Some of them exhibited abnormal neuropsychological scores (30%) and mild cerebral atrophy (20%). Finally, those low-frequency EEG rhythms increased in amplitude at a follow-up of 28 months in naïve HIV subjects (no ART), while they remained unchanged in amplitude in the HIV subjects receiving ART (Baldeweg et al., 1995). Overall, these findings confirmed and extended earlier observations performed based on the simple visualization of the EEG waveforms recorded in the resting state condition in HIV subjects with and without AIDS (Enzenberger et al., 1985; Enzenberger et al., 1986; Gabuzda et al., 1988; Prado et al., 1993; Sinha and Satishchandra, 2003).

What about the literature of HIV subjects with a paradoxical alpha source activity before cART? Previous studies have reported that cognitive abilities and resting state posterior alpha activity were lower, on average, in HIV patients compared with control subjects (Polich et al., 2000), with a partial normalization in the alpha activity by cART (Gruzelier et al., 1996; Harrison et al., 1998). Nonetheless, it has been reported that resting state alpha activity was substantially higher in about 20-30% of HIV subjects compared to the mean of healthy (non-infected) subjects (Koralnik et al., 1990; Baldeweg et al., 1995). This paradoxical alpha activity was proposed as one of the features of an abnormal EEG in HIV subjects (Koralnik et al., 1990), although the specificity of this EEG feature was criticized (Nuwer et al., 1991). Furthermore, such paradoxical alpha activity was more frequent in HIV subjects showing more advanced stages of infection such as immune system depression and psychiatric symptoms (Baldeweg and Gruzelier 1997). In a recent EEG study, 20-30% of HIV subjects showed a paradoxical resting state alpha activity. However, we do not know with certainty why some HIV subjects present this paradoxical EEG feature. Future research should test the hypothesis that this is due to a remarkable level of HIV replication in the brain.
Recently, our research group contributed to the field estimating cortical sources of eyes-closed resting state EEG rhythms in HIV subjects by the popular freeware called low-resolution brain electromagnetic tomography (LORETA; Pascual-Marqui and Michel et al., 1994, Pascual-Marqui et al., 1999, 2002). We used the same general methodological approach that successfully unveiled abnormal EEG sources in seniors with cognitive deficits due to several stages of Alzheimer’s disease and other neurological diseases (for a review see Babiloni et al., 2015a; see Figure 2 for an overview of the methodological approach). Specifically, we reported that compared with a control group of healthy subjects, a group of treatment-naïve HIV subjects was characterized by a higher activity of central and parietal sources of delta rhythms (<4 Hz), as well as a lower activity in topographically diffuse sources of alpha rhythms (Babiloni et al., 2012). The abnormality of those EEG sources was less pronounced in a group of HIV patients experiencing a prolonged chronic treatment with ART (Babiloni et al., 2014). Furthermore, the beneficial effects of ART on those EEG sources were confirmed in naïve HIV subjects who received a successful ART for 5 months (Babiloni et al., 2015b), even when they were stratified for different ART regimens where a group was treated with KIVEXA (ViiV Healthcare; Abacavir and Lamivudine as active principles) and a third drug, whereas another group was treated with TRUVADA (Gilead; Tenofovir and Emtricitabine as active principles) and a third drug (Babiloni et al., 2016a). These findings extended previous studies reporting that cognitive abilities and resting state posterior alpha activity were lower, on average, in HIV patients compared with control subjects (Polich et al., 2000), with a partial normalization in the alpha activity by ART (Gruzelier et al., 1996, Harrison et al., 1998).

More recently, we tested a simple statistical procedure based on the computation of z-score (p<0.05, one-tailed) to identify treatment-naïve HIV male individuals having a deviant activity of cortical (LORETA) sources of resting state eyes-closed delta and alpha rhythms (Babiloni et al., 2016b). The ratio of the activity between parietal delta and high-frequency alpha sources served as an EEG biomarker of interest. The z-score of this EEG biomarker disclosed a relatively high percentage (about 40-50%) of treatment-naïve HIV individuals with a statistical difference of EEG activity (p<0.05, one-tailed). These results suggested that this EEG biomarker might enrich the instrumental assessment of HIV effect on brain function in treatment-naïve HIV male individuals. Finally, another study clarified that the mentioned z-score procedure was sensitive to the impact of ART on naïve HIV individuals at follow-up (Babiloni et al., 2016c).

**Biomarkers of brain reserve in clinical practice (first and second choice for monitoring)**

The above concise review of the research data suggests the use of the following biomarkers of brain reserve as a first choice in clinical practice, taking into account practical costs/benefit considerations and the need to reduce the number of examinations for a global view of the organ...
status. For the instrumental assessment of HIV subjects showing severe or moderate cognitive and behavioral deficits (dementia and mild neurocognitive disorders according to the Frascati criteria; Antinori et al., 2007), structural MRI biomarkers may allow the visual detection of the cortical and subcortical atrophy as well as white and gray matter (micro)vascular lesions (Thurnher and Donovan et al., 2008).

For the instrumental assessment of HIV subjects with mild cognitive deficits, (asymptomatic neurocognitive impairment according to the Frascati criteria; Antinori et al., 2007) or very mild cognitive deficits at the border of the lower limit of normal cognition, z-score (p<0.05, one-tailed) of cortical sources of resting state (eyes closed) delta and alpha EEG rhythms may identify HIV individuals with deviant brain neurophysiological mechanisms of quiet wakefulness (Babiloni et al., 2016a, b).

The above concise review of the research data also suggests the use of MRS biomarkers of brain reserve as a second choice in clinical practice, when more resources for the instrumental assessment of HIV subjects are available for clinical decision-making or research. As mentioned above, MRS of neuronal integrity markers (NAA), neuroinflammation (Cho), and gliosis (MI) in both subcortical (basal ganglia) and cortical regions may be sensitive to the neural correlates of cognitive deficits even at mild stages (Chang et al., 2000, Letendre et al., 2011, Ances and Hammoud et al., 2014).

The most promising MRI and EEG biomarkers of cerebral reserve in HIV are reported in Table 6.

**Cardiovascular reserve in HIV**

*Progressive loss of cardiovascular reserve (structural/functional) in HIV*

One of the principal manifestations of aging is atherosclerosis and its complications. Hence, the vascular age of an individual does not necessarily match the chronological age. Whether HIV infection accelerates or accentuates atherosclerosis development is a matter of debate, but it is a well-known fact that HIV patients suffer a disproportionately higher rate of cardiovascular events compared to their counterparts of similar age and sex. In this sense, therefore, they age faster than other people of similar social status and with a similar risk profile.

Through the evolution of the HIV epidemic, CVD has emerged as a major cause of morbidity and mortality among HIV-infected patients. In contemporary observational studies of patients with HIV, the proportion of total deaths caused by CVD ranges from 6.5% to 15%, with HIV infection alone conferring a 61% increased risk compared with uninfected subjects (Stein and Hsue et al., 2012).

As HIV-infected patients continue to live longer, the burden of CVD will simultaneously rise. As such, CVD has become an important health issue for primary providers, and the ability to identify HIV-infected patients who are at risk is now an essential component in their ongoing management.
Esser et al. demonstrated significantly increased rates of CVD in HIV patients, including an elevated frequency of coronary artery disease, myocardial infarction, and peripheral arterial diseases. Furthermore, aging patients exhibited a higher rate of chronic heart failure, predominantly of ischemic etiology. In multivariate analyses, age, smoking, and advanced symptomatic HIV infection were significantly associated with the prevalence of CVD (Esser et al., 2013).

The pathophysiology of CVD in HIV, however, is complex and multifactorial, likely involving the interplay between an increased burden of traditional risk factors, adverse effects of ART, immune activation, and chronic viral-mediated inflammation. As a result, traditional cardiovascular risk algorithms developed for the non-HIV-infected population do not accurately predict cardiovascular risk in HIV-infected patients because they fail to account for factors unique to HIV infections (Friis-Møller et al., 2010).

Mechanisms by which HIV increases cardiovascular risk include: a chronic inflammatory state, particularly in the vasculature, caused by HIV, and ART metabolic dysfunction caused by ART. For example, Zidovudine (azidothymidine) use has been associated with a specific dose-dependent skeletal myopathy attributed to mitochondrial toxicity and with cardiac dysfunction that resolves with discontinuation of the drug (Curri et al., 2003).

Individuals with HIV are also more likely to be smokers or have smoked cigarettes, and have higher rates of other traditional risk factors such as dyslipidemia and insulin resistance. While mortality rates related to CVD have decreased over time among adults with HIV, excess risk of CVD in the HIV-infected population may persist despite highly active antiretroviral therapy (HAART) treatment and aggressive CVD risk factor control. Beyond atherosclerotic CVD, recent studies suggest that HIV infection may be associated with left ventricular systolic and diastolic function, interstitial myocardial fibrosis, and increased cardiac fat infiltration. Thus, with the increasing average age of the HIV-infected population, heart failure and arrhythmic disorders may soon rival coronary artery disease as the most prevalent forms of CVD.

Prior to the advent of combination ART, dilated cardiomyopathy, associated with severe systolic heart failure and poor prognosis, was a striking complication of late-stage HIV infection, having a prevalence as high as 30% in some studies (Sani, 2008; Thienemann et al., 2013). The disorder appeared to be related to possible myocardial or dendritic cell invasion by HIV itself or other viral pathogens, as well as inflammation and autoimmunity (Sani, 2008). Dilated cardiomyopathy is no longer a prominent manifestation of HIV infection in the HAART era. Accumulating data suggest, however, that such severe HIV-associated cardiomyopathy has been replaced by a more insidious form, where mild left ventricular systolic dysfunction is common and left ventricular diastolic dysfunction is pronounced (Cerrato et al., 2013). A recent metanalysis arrived at pooled prevalence
of LV systolic and diastolic dysfunction in HIV-infected persons of 8.3 and 43.4%, respectively (Cerrato et al., 2013). These frequencies of subclinical LV dysfunction are striking given that the mean age of participants studied was 41 years old. Compared with population-based estimates, the prevalence of reduced LV ejection fraction is about threefold higher, and abnormal LV diastolic function two- to threefold greater in HIV-infected individuals than that reported in older American non-HIV cohorts. Such figures are a cause for concern, because they suggest that as the HIV-infected population continues to age there may be an upsurge in HF on the horizon (Redfield et al., 2003).

Butt et al. (2011) demonstrated a relationship between HIV infection and an increased risk of HF after adjusting for traditional coronary heart disease (CHD) risk factors. This risk persisted even considering patients who did not have a diagnosis of CHD, HF, or angina at baseline. HIV itself plays an important and independent role. Even after excluding patients with a baseline history of CHD, HF, and angina, as well as a CHD event in the follow-up period prior to the diagnosis of HF and a history of alcohol abuse or dependence diagnosis, the risk of incident HF was still substantial among the HIV-infected cohort. Ongoing HIV replication appears to play an important role. Compared with HIV-uninfected participants, only participants with an HIV-1RNA level greater than 500 copies/mL had a significantly increased risk of HF (Butt et al., 2011). Antiretroviral therapy was associated with a slightly attenuated risk, although the difference between CART-naive and CART-experienced groups did not reach statistical significance. Hypertension, obesity, alcohol abuse or dependence, and diabetes mellitus are known to be associated with an increased risk of HF, and offer the possibility of interventions to reduce the risk of HF (Vasan et al., 1997; Ingelsson et al., 2005).

Finally, the question of whether HIV infection should be considered in clinical risk stratification has never been resolved, and this question has assumed new importance with recent changes to lipid treatment guidelines for prevention of CVD (Figure above).

**Measurement of cardiovascular reserve in HIV (with reference to general population)**

In clinical practice, a great effort has been made to find biomarkers useful in identifying HIV people at risk of developing heart disease. An important step in establishing a relationship between HIV-associated immunologic perturbations and CVD is demonstrating that specific markers of these pathways predict subsequent events. However, most studied biomarkers, including high sensitivity C-reactive protein (hsCRP), D-dimer, interleukin (IL)-6, and cystatin C, are predominantly released outside of the myocardium and may not represent a direct relationship between HIV infection and CVD. Gupta et al. (2013) tried to define the relation between hsCRP, IL-6, and D-dimer levels and ECG abnormalities in HIV-infected participants, with the aim to
determine whether these biomarkers, which are associated with clinical CVD, also led to an early risk of subclinical CVD as measured by ischemic abnormalities on the electrocardiogram. The prevalence of baseline ECG abnormalities indicative of cardiac ischemia increased with greater biomarker levels, but after adjustment for age and other risk factors none of the associations remained significant. Similarly, those who developed incident ECG abnormalities had greater baseline biomarker levels than those who did not, but associations were not significant on either unadjusted or adjusted analysis (Gupta et al., 2013).

Among subjects without HIV, novel biomarkers primarily expressed or secreted by cardiovascular tissue in response to pathological stress have been predictive of cardiovascular events and mortality. These include soluble ST2, growth differentiation factor (GDF)-15, N-terminal pro-Btype natriuretic peptide (NT-proBNP), and high sensitivity troponin I (hsTnI). Nevertheless, among HIV-infected patients, only ST2 and GDF-15 were associated with both cardiovascular dysfunction and all-cause mortality, and these variables may be useful in identifying those at risk for developing cardiovascular events and death (Secemsk et al., 2015).

The 12-lead electrocardiogram is the first examination for patients to exclude or confirm a cardiac disease, but is also frequently included in routine tests in clinical practice, independently from diseases and clinical conditions. HIV and highly active antiretroviral therapy may affect cardiac conduction, and a higher incidence of sudden death has been recognized in HIV-positive patients. Gili et al. found that a low CD4+ cell count was associated with long cQT independently from HAART in HIV-positive patients; this finding may be useful to correctly stratify arrhythmic risk in these patients (Gili et al., 2016). Moreno et al. found a prolonged QTc syndrome was not uncommon in a cohort of asymptomatic HIV-infected patients with good immunovirological control, and was associated with hyperlipidemia and diastolic dysfunction. The use of atazanavir, compared with other protease inhibitors, was associated with a lower likelihood of having a prolonged QTc (Moreno et al., 2013).

The evaluation of carotid intima-media thickness (by means of Echo-Color Doppler) is an easy and quick method to quantify atherosclerosis and cardiovascular risk. In this study, among both middle-aged (30-49-years) and older adults (50-75-years), HIV-infected participants had intima-media thickness values that were similar to or lower than those in HIV-uninfected participants. Among HIV-infected participants, associations of common cardiovascular risk factors, such as higher systolic blood pressure and lower high-density lipoprotein cholesterol with carotid artery intima-media thickness strengthened with age. The effects of HIV on carotid artery structure may differ across the lifespan, with traditional determinants of CVD burden playing a larger role and HIV playing a lesser role in older adults than in young adults and children (Hanna et al., 2016).
The use of cardiac MRI can be useful to detect a marker of cardiovascular aging: pericardial fat content (volume at the level of the origin of the left main coronary artery (LM) and at the right ventricular free wall). Pericardial fat content is increased in HIV subjects on chronic HAART (>5-years), who demonstrate HAART-related lipo-accumulation and prolonged HIV duration of infection, although further investigation is warranted to determine whether increased pericardial fat is associated with higher cardiovascular risk leading to premature cardiovascular events in this population (Diaz-Zamudio et al., 2015). Cardiovascular MRI has the advantage of allowing a comprehensive and precise assessment of myocardial structure, function, and tissue characterization. Using cardiovascular MRI, Ntusi et al. demonstrated subclinical inflammation and myocardial disease in asymptomatic HIV-infected individuals without known CVD. Compared with controls, subjects with HIV infection had 6% lower left ventricular ejection fraction, 7% higher myocardial mass, 29% lower peak diastolic strain rate, 4% higher short-tau inversion recovery values, and higher native T1 values. Pericardial effusions and myocardial fibrosis were 3 and 4 times more common, respectively, in subjects with HIV infection. Chronic systemic inflammation in HIV, which involves the myocardium and pericardium, may explain the high rate of myocardial fibrosis and increased cardiac dysfunction in people living with HIV (Ntusi et al., 2016). Nevertheless, MRI is time and money wasting, so it should be difficult to warrant for widespread use do define cardiac preclinical aging.

Accurate and timely risk assessment for CHD is an important part of primary care. Identification of modifiable lifestyle risk factors and consideration of cost-effective interventions is critical to preventing progression of disease. One of the most common tools used today in assessing 10-year cardiovascular risk is the Framingham Risk Score (FRS). Although useful, the FRS has been reported to overestimate risk in the general population and underestimate risk in the HIV-infected population. Coronary artery calcification (CAC) scoring by fast computed tomography is proving to be one of the more common and validated methods of risk stratification. Presence of CAC is considered a marker of subclinical atherosclerosis, and it has been shown to predict CHD risk independently of FRS. Absolute CAC has been shown to be superior to age–sex–race/ethnicity percentiles in predicting cardiac events. In this study, people with HIV had a greater risk of having positive CAC than non-HIV patients when adjusting for age, smoking status, diabetes, antihypertensive therapy, BMI, systolic blood pressure, total cholesterol, and HDL cholesterol. Among participants with positive CAC, HIV infection was not associated with larger amounts of CAC. Current HIV viral load, CD4, length of HIV, interleukin 6, fibrinogen, C-reactive protein (CRP), and D-dimer were not associated with the presence or amount of CAC. HIV was independently associated with a positive CAC in men with increased likelihood occurring between
45 and 50-years of age. Current HIV viral load, CD4 count, length of HIV, and inflammatory markers were unrelated to either presence or amount of CAC (Chow et al., 2015).

Coronary microvascular dysfunction is thought to reflect the initiation and early changes in the progression toward coronary artery disease. The use of dynamic (PET)/CT imaging enables the quantification of the absolute myocardial perfusion in mL/g/min by intravenous injection of a perfusion positron-emitting tracer. This leads to the detection of very subtle signs of disease before structural changes occur, thereby guiding a possible preventive therapy. Knudsen et al. assessed the myocardial flow reserve by Rubidium-82 (82Rb) PET, which is the maximal myocardial blood flow during adenosine stress divided by myocardial blood flow at rest. This ratio depicts the vasodilator function of the coronary circulation, and in the general population it has proved to be highly predictive of future cardiovascular events. In this study, myocardial flow reserve was similar between HIV-infected patients with full viral suppression and HIV-uninfected controls, indicating that well-controlled HIV infection does not compromise the function of the myocardial microcirculation significantly (Knudsen et al., 2015).

Autonomic dysfunction in persons with AIDS has been reported. Measures of autonomic function by means of standard autonomic tests did not differ among groups. However, at rest, both HIV seropositive groups exhibited diminished stroke volume and elevated diastolic blood pressure, albeit within normotensive levels. In addition, the ability to sustain a blood pressure response during prolonged challenge and the relationship between stroke volume and baroreceptor/vagal responsiveness were disrupted in the HIV-symptomatic group. Therefore, in the pre-AIDS stages of infection, autonomic functioning appeared intact. Yet alterations in baroreceptor/vagal function associated with depressed myocardial function may be an early warning signal reflecting cardiovascular pathological processes potentially exacerbated by HIV spectrum disease (Brownley et al., 2001). These results are confirmed and better defined in a recent metanalysis that described a cohort of HIV+ adults on ART, finding a general reduction in autonomic function with a shift toward sympathetic dominance. This shift can have a clinical impact, since it may predispose HIV patients to early and elevated risk of arrhythmias, cardiac events, and accelerated HIV disease progression (McIntosh et al., 2016).

Even in healthy subjects, the heart ages along with other organs of the body. A prominent change is progressive left ventricular (LV) diastolic dysfunction, even though LV mass increases slightly during aging. Accordingly, assessment of LV dysfunction can be employed as a surrogate marker of cardiac age. The clinical factors that may accelerate the cardiac aging process include visceral obesity, diabetes mellitus, dyslipidemia, and hypertension. At the molecular level in cardiac myocytes, reactive oxygen species, transforming growth factor-β, mitochondrial function, and
lysosomal function are also related to cardiac age. Furthermore, age-related LV dysfunction has been shown to be one of the main risk factors for future heart failure. Consequently, assessment of LV diastolic function by means of echocardiography, which provides a noninvasive assessment of cardiac structure and function, is mandatory for both preventing cardiac events and assessing cardiac age (Xu and Daimon, 2016).

Biomarkers of cardiovascular reserve in clinical practice (first and second choice for monitoring) are shown in table 7.

Conclusions
HIV-positive individuals are more vulnerable to poor health than HIV-negative individuals. This vulnerability is characterized by a higher risk of several common, age-related health problems, even after adjustment for established risk factors. These conditions, termed HIV-associated non-AIDS (HANA) events include CVD, osteoporosis, metabolic disorders, hepatic and renal diseases, neurocognitive impairment, and some cancers, as well as age-associated immunologic changes and chronic inflammation.

Current antiretroviral medications have improved side effect profiles and afford new opportunities for ART optimization and toxicity management. The availability of the new HIV drug class of INI represents a valuable option for the treatment of HIV infection, due to its unique tolerability and low drug-to-drug interactions. Patients with HIV-1 infection have several comorbidities requiring multiple pharmacotherapies that can increase their risk of polypharmacy and related adverse events. However, little is known about the impact of aging on medication use in HIV-1-infected older individuals, the potential for interactions with cART and administered medications, and the impact of this on therapy tolerability and virological response with aging. Reducing the pill burden, careful titration of medications, and increasing awareness of common drug-to-drug interactions can prevent co-administration of potentially harmful combinations and reduce unnecessary polypharmacy-related adverse events in this population. Moreover, the demonstration that INIs may extinguish the kinetics of viral replication faster and to a greater extent justifies the assumption that they are more effective than the other antiretrovirals of acting on the pathogenetic mechanisms underpinning the processes of immune reconstitution.

This expert opinion report aims at identifying the optimal biomarkers for the monitoring of structural integrity and function of physiological systems at risk across aging in HIV-seropositive subject. These biomarkers, readily available locally and relatively cost-effective for clinicians in primary and secondary care, should allow an early detection of first preclinical structural and
functional changes in renal, brain, cardiovascular, and skeleton systems or apparatus in HIV subjects across aging.

A particular interest of this report is the definition of the concept of biomarker of the “organ functional reserve”. This definition emphasizes the fact that some biomarkers for monitoring the molecular, structural and functional integrity of a given organ reflect a level of impairment that is basically irremediable despite an effective pharmacological or nonpharmacological intervention. For this reason, biomarkers of the “organ functional reserve” could be especially useful in monitoring HIV-positive patients on antiretroviral therapy.
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Table 1 - Main variables associated with frailty in HIV subjects on combination antiretroviral therapy (ART).

<table>
<thead>
<tr>
<th>Demographic and Socio-economic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Education</td>
</tr>
<tr>
<td>Employment</td>
</tr>
<tr>
<td>Economic status</td>
</tr>
<tr>
<td>Ethnicity</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HIV-related</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection duration</td>
</tr>
<tr>
<td>CD4-CD4/8</td>
</tr>
<tr>
<td>CD4 nadir</td>
</tr>
<tr>
<td>Viral load</td>
</tr>
<tr>
<td>HAART regimen</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comorbidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipodystrophy</td>
</tr>
<tr>
<td>Diabetes</td>
</tr>
<tr>
<td>Kidney disease</td>
</tr>
<tr>
<td>Inflammation</td>
</tr>
<tr>
<td>Hepatitis C coinfection</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depression</td>
</tr>
<tr>
<td>Cognition</td>
</tr>
<tr>
<td>Autonomy</td>
</tr>
<tr>
<td>Falls</td>
</tr>
<tr>
<td>Weak upper and lower extremities</td>
</tr>
</tbody>
</table>


**Table 2 - Biomarkers associated with age-related and HANA conditions in the assessment of HIV subjects.** The biomarkers are classified based on the proposal that they are mandatory (very useful as first level analysis, M) vs enriching (provide complementary information, E). The Table specifies if the biomarker of interest derives from immune activation (IA), immune senescence (IS) or inflammatory cytokine (IC) action. See the main text for details and explanations.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Technique</th>
<th>Mandatory/Enriching</th>
<th>Inflammation/Immune activation/Immune senescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble CD14 (sCD14)</td>
<td>Enzyme Immuneassay</td>
<td>M</td>
<td>IA, IC</td>
</tr>
<tr>
<td>CD163</td>
<td>Enzyme Immuneassay</td>
<td>E</td>
<td>IA</td>
</tr>
<tr>
<td>CD16&lt;sup&gt;+&lt;/sup&gt; monocytes,</td>
<td>Enzyme Immuneassay</td>
<td>E</td>
<td>IA</td>
</tr>
<tr>
<td>% HLA-DR&lt;sup&gt;+&lt;/sup&gt;CD38&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;T cells</td>
<td>Flow Cytometry</td>
<td>M</td>
<td>IA</td>
</tr>
<tr>
<td>% HLA-DR&lt;sup&gt;+&lt;/sup&gt;CD38&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;T cells</td>
<td>Flow Cytometry</td>
<td>M</td>
<td>IA</td>
</tr>
<tr>
<td>terminally differentiated CD45RA&lt;sup&gt;+&lt;/sup&gt;CCR7&lt;sup&gt;-&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;T cells</td>
<td>Flow Cytometry</td>
<td>E</td>
<td>IS</td>
</tr>
<tr>
<td>% CD57&lt;sup&gt;+&lt;/sup&gt;CD28&lt;sup&gt;-&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt;T cells</td>
<td>Flow Cytometry</td>
<td>E</td>
<td>IS</td>
</tr>
<tr>
<td>% CD57&lt;sup&gt;+&lt;/sup&gt;CD28&lt;sup&gt;-&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt;T cells</td>
<td>Flow Cytometry</td>
<td>E</td>
<td>IS</td>
</tr>
<tr>
<td>Interleukin 6</td>
<td>Enzyme Immuneassay</td>
<td>M</td>
<td>IC</td>
</tr>
<tr>
<td>Tumor necrosis factor α (TNFα)</td>
<td>Enzyme Immuneassay</td>
<td>E</td>
<td>IC</td>
</tr>
<tr>
<td>Soluble tumor necrosis factor I (sTNFR-I)</td>
<td>Enzyme Immuneassay</td>
<td>E</td>
<td>IC</td>
</tr>
<tr>
<td>Soluble tumor necrosis factor II (sTNFR-II)</td>
<td>Enzyme Immuneassay</td>
<td>E</td>
<td>IC</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Enzyme Immuneassay</td>
<td>E</td>
<td>IC</td>
</tr>
<tr>
<td>Cystatin C</td>
<td>Enzyme Immuneassay</td>
<td>E</td>
<td>IC</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>Enzyme Immuneassay</td>
<td>M</td>
<td>IC</td>
</tr>
<tr>
<td>D-dimer</td>
<td>Enzyme Immuneassay</td>
<td>M</td>
<td>IC</td>
</tr>
</tbody>
</table>
Table 3 - Biomarkers of renal reserve in the assessment of HIV subjects. The biomarkers are classified based on the proposal that they are mandatory (very useful as first level analysis, M) vs. enriching (provide complementary information, E). The Table specifies if the biomarker of interest reflects the status of the kidneys at molecular (M), structural (S) and functional (F) level. See the main text for details and explanations.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Technique</th>
<th>Mandatory/Enriching</th>
<th>Molecular/Structure/Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria</td>
<td>24 h - proteinuria</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>UPCR (Urinary protein/creatinine ratio)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albuminuria</td>
<td>24 h Albuminuria</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>UACR (Urinary albumin/creatinine ratio)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GFR</td>
<td>eGFR calculation from serum creatinine and/or cystatin c</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Renal cortical Echogenicity/thickness</td>
<td>Renal ultrasound</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td>Tubular dysfunction markers</td>
<td>Two or more of the following parameters:</td>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>a) fractional excretion uric acid &gt; 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>b) fractional excretion phosphate &gt; 0.2 (&gt;0.1 if serum Phos &lt; 2.5 mg/dl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c) tubular proteinuria (UACR/UPCR &lt; 0.4 or increased urinary excretion of specific tubular protein markers*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>d) Normoglycemic glycosuria</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>e) tubular acidosis (Serum HCO₃ &lt; 21 mmol/L with urinary pH &gt; 5.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Retinol-binding protein, β2-microglobulin, α1-microglobulin, cystatin.
**Table 4** - The biomarkers are classified based on the proposal that they are mandatory (very useful as first level analysis, M) vs. enriching (provide complementary information, E). The Table specifies if the biomarker of interest derives from collagen (C) and probes the osteoclastic enzymes (OE), osteocyte activity (OA), or bone formation (BF). See the main text for details and explanations. Legend: The N-terminal telopeptide (NTx); C-terminal telopeptide (CTx); Deoxypyridinoline (DPD); Pyridoline (PYD); Tartrate resistant acid phosphatase 5b (TRAP 5b) serum; Cathepsin K; RANKL; Osteoprotegerin (OPG); Bone specific alkaline phosphatase (BALP); Osteocalcin (OC); Procollagen type I N-terminal propeptide (P1NP); Procollagen type I carboxyl terminal propeptide (P1CP).

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Technique</th>
<th>Mandatory/Enriching</th>
<th>Collagen derived / Osteoclastic Enzyme/Osteocyte Activity/Bone Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTx</td>
<td>serum/urine</td>
<td>M</td>
<td>C</td>
</tr>
<tr>
<td>CTx</td>
<td>serum/urine</td>
<td>M</td>
<td>C</td>
</tr>
<tr>
<td>DPD</td>
<td>serum/urine</td>
<td>E</td>
<td>C</td>
</tr>
<tr>
<td>PYD</td>
<td>serum/urine</td>
<td>E</td>
<td>C</td>
</tr>
<tr>
<td>TRAP 5b</td>
<td>serum</td>
<td>M</td>
<td>OE</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>serum</td>
<td>E</td>
<td>OE</td>
</tr>
<tr>
<td>RANKL</td>
<td>serum</td>
<td>E</td>
<td>OE</td>
</tr>
<tr>
<td>OPG</td>
<td>serum</td>
<td>E</td>
<td>OA</td>
</tr>
<tr>
<td>BALP</td>
<td>serum</td>
<td>M</td>
<td>BF</td>
</tr>
<tr>
<td>OC</td>
<td>serum</td>
<td>M</td>
<td>BF</td>
</tr>
<tr>
<td>P1NP</td>
<td>serum</td>
<td>M</td>
<td>BF</td>
</tr>
<tr>
<td>P1CP</td>
<td>serum</td>
<td>E</td>
<td>BF</td>
</tr>
</tbody>
</table>
Table 5 - Promising biomarkers of bone reserve in the assessment of HIV subjects. The biomarkers are classified based on the proposal that they are mandatory (very useful as first level analysis, M) vs. enriching (provide complementary information, E). The Table specifies if the biomarker of interest reflects the status of the bones at molecular (M), structural (S) and functional (F) level.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Technique</th>
<th>Mandatory/Enriching</th>
<th>Molecular/Functional/Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone mineralization</td>
<td>serum calcium levels, serum phosphate levels, total alkaline phosphatase, serum creatinine, PTH and 24-h urinary calcium.</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Bone turnover</td>
<td>Serum CTX, P1NP</td>
<td>M</td>
<td>M/F</td>
</tr>
<tr>
<td>Bone mineral density (BMD)</td>
<td>DEXA-based vertebral fracture assessment (VFA)</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td>Bone microstructure (TBS)</td>
<td>QUS, FRAX adjusted for TBS</td>
<td>E</td>
<td>S</td>
</tr>
<tr>
<td>Fracture risk</td>
<td>FRAX score, QUS, serum miRNAs (PCR)</td>
<td>M</td>
<td>S</td>
</tr>
</tbody>
</table>

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Table 6 - Magnetic resonance imaging (MRI) and resting state eyes-closed electroencephalographic (EEG) biomarkers of cerebral reserve in the assessment of HIV subjects. The biomarkers are classified based on the proposal that they are mandatory (very useful as first level analysis, M) vs. enriching (provide complementary information, E). The Table specifies if the biomarker of interest probes the integrity of the brain at molecular (M) or structural (S) level or reflects the cerebral function (F). See the main text for details and explanations.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Technique</th>
<th>Mandatory/Enriching</th>
<th>Molecular/Structure/Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1-weighted MRI</td>
<td>MRI</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td>T2-weighted MRI</td>
<td>MRI</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td>z-score of delta/alpha</td>
<td>EEG</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>MRS</td>
<td>MRI</td>
<td>E</td>
<td>M</td>
</tr>
</tbody>
</table>
Table 7 - Biomarkers of cerebrovascular reserve in the assessment of HIV subjects. The biomarkers are classified based on the proposal that they are mandatory (very useful as first level analysis, M) vs. enriching (provide complementary information, E). The Table specifies if the biomarker of interest derives from Hormonal (H), Molecular (M), Functional (F), and Autonomic (A) level. See the main text for details and explanations.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Technique</th>
<th>Mandatory/Enriching</th>
<th>Hormonal/Molecular/Functional/Autonomic</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST2 and GDF-15</td>
<td>Blood sample</td>
<td>E</td>
<td>H</td>
</tr>
<tr>
<td>QTc</td>
<td>ECG</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Pericardial fat content</td>
<td>Cardiac MRI</td>
<td>E</td>
<td>M</td>
</tr>
<tr>
<td>Coronary artery calcification (CAC) scoring</td>
<td>Fast CT</td>
<td>E</td>
<td>M</td>
</tr>
<tr>
<td>Myocardial flow reserve</td>
<td>Rubidium-82 (82Rb) PET</td>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td>Left ventricular (LV) diastolic dysfunction</td>
<td>Echocardiography</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Baroreceptor/vagal function</td>
<td>Standard autonomic tests</td>
<td>E</td>
<td>A</td>
</tr>
</tbody>
</table>
Table 8 - Markers of Inflammation/Immune activation and to analyze renal, bone, cerebrovascular and cardiovascular reserve in the assessment of HIV subjects.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Technique</th>
<th>Pathological mechanism analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biomarkers of Inflammation or Immune activation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soluble CD14 (sCD14)</td>
<td>Enzyme Immuneassay</td>
<td>Inflammation and Immune activation</td>
</tr>
<tr>
<td>% HLA-DR&lt;sup&gt;+&lt;/sup&gt;CD38&lt;sup&gt;+&lt;/sup&gt;CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>Flow Cytometry</td>
<td>Immune activation</td>
</tr>
<tr>
<td>% HLA-DR&lt;sup&gt;+&lt;/sup&gt;CD38&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>Flow Cytometry</td>
<td>Immune activation</td>
</tr>
<tr>
<td>Interleukin 6</td>
<td>Enzyme Immuneassay</td>
<td>Inflammation</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>Enzyme Immuneassay</td>
<td>Inflammation</td>
</tr>
<tr>
<td>D-dimer</td>
<td>Enzyme Immuneassay</td>
<td>Inflammation</td>
</tr>
<tr>
<td><strong>Biomarkers of renal reserve</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteinuria</td>
<td>24-h Proteinuria</td>
<td>Structural</td>
</tr>
<tr>
<td></td>
<td>UPCR (Urinary protein/creatinine ratio)</td>
<td></td>
</tr>
<tr>
<td>Albuminuria</td>
<td>24-h Albuminuria</td>
<td>Structural</td>
</tr>
<tr>
<td></td>
<td>UACR (Urinary albumin/creatinine ratio)</td>
<td></td>
</tr>
<tr>
<td>GFR</td>
<td>eGFR calculation from serum creatinine and/or cystatin c</td>
<td>Functional</td>
</tr>
<tr>
<td>Renal cortical Echogenicity/thickness</td>
<td>Renal ultrasound</td>
<td>Structural</td>
</tr>
<tr>
<td><strong>Biomarkers of bone reserve</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone mineralization</td>
<td>Serum calcium levels, serum phosphate levels, total alkaline phosphatase, serum creatinine, PTH and 24-h urinary calcium.</td>
<td>Molecular</td>
</tr>
<tr>
<td>Bone turnover</td>
<td>Serum CTX, P1NP</td>
<td>Molecular and Functional</td>
</tr>
</tbody>
</table>
| Bone Mineral Density (BMD)             | DEXA based vertebral fracture assessment (VFA) | Structural }
<table>
<thead>
<tr>
<th>Bone microstructure (TBS)</th>
<th>QUS</th>
<th>FRAX adjusted for TBS</th>
<th>Structural</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fracture Risk</td>
<td>FRAX score, QUS, serum miRNAs (PCR)</td>
<td>Structural</td>
<td>Molecular</td>
</tr>
</tbody>
</table>

**Biomarkers of cerebral reserve**

<table>
<thead>
<tr>
<th>T2-weighted MRI</th>
<th>MRI</th>
<th>Structural</th>
</tr>
</thead>
<tbody>
<tr>
<td>z-score of delta/alpha</td>
<td>Resting state eyes-closed electroencephalographic</td>
<td>Functional</td>
</tr>
</tbody>
</table>

**Biomarkers of cardiovascular reserve**

<table>
<thead>
<tr>
<th>QTc</th>
<th>G</th>
<th>Functional</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left ventricular (LV) diastolic dysfunction</td>
<td>Echocardiography</td>
<td>Structural and Functional</td>
</tr>
</tbody>
</table>
Figure 1 - Hypothetical association between frailty prevalence, HANA conditions, and immune system dysregulation
Figure 2 - Overview of the methodological approach of Babiloni and colleagues in the analysis of cortical sources of resting state eyes-closed electroencephalographic (EEG) rhythms in HIV subjects in a recent series of experiments (Babiloni et al., 2012, 2014, 2015, 2016a, b, c). These rhythms reflect spontaneous fluctuations of vigilance in quiet wakefulness to probe functional synchronization of cortical neural activity in HIV subjects before and after cART. Four principal stages characterize the general methodology: (1) EEG recording is performed with 19 scalp electrodes placed according with clinical standards, namely 10-20 montage system. (2) Artifact-free EEG segments are selected by a semi-automatic procedure validated by expert electroencephalographists. (3) EEG power density spectrum at scalp electrodes is computed to control the general quality of the artifact-free EEG segments selected for the analysis of the EEG cortical sources. (4) Cortical sources of resting state eyes-closed EEG rhythms (free from artifacts) are estimated and compared between groups of healthy control and HIV subjects at the group and individual levels. For this purpose, a technique available in the public domain for free and validated over time was used, namely the low-resolution brain electromagnetic tomography (LORETA) (http://www.uzh.ch/keyinst/loreta.htm).
Figure 3 - Hypothesis of premature and accelerated cardiovascular system aging in cARV-treated HIV-infected patients (from d’Ettorre et al., 2016).