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Primary prevention of cardiovascular and metabolic disease in HIV positive patients:

prospective study of risk factors and early damage markers (PRECAD STUDY)

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1.1 BACKGROUND: CARDIOVASCULAR RISK IN HIV POSITIVE PATIENTS

Morbidity and mortality from non-AIDS-related events have surpassed those from AIDS-related events with more effective and widespread treatment of HIV in resource-rich settings (1–3). In particular, cardiovascular disease has emerged as an important cause of death in patients with HIV relative to the decreasing incidence of opportunistic disease. Several lines of evidence, from modeling of calculated cardiovascular risk to clinical studies evaluating such hard endpoints as stroke, myocardial infarctions (MIs), and sudden cardiac death have cumulatively supported this finding (4–6).

Several studies have analyzed large clinical databases and cohorts, mainly in the United States, Canada, and Europe, but also in more resource-limited settings, to compare the incidence of cardiovascular disease in patients with and without HIV (7–12).

Although some of these studies are limited by low number of events, short follow-up, and incomplete assessments of other cardiac risk factors, they consistently report a 1.5- to 2-fold increase in the rate of cardiovascular events in individuals with HIV compared with control populations. As an example, in a systematic review of such studies that comprised nearly 800,000 people with HIV with 3.4 million person-years of follow-up, the incidence of cardiovascular disease was 62 events per 10,000 person-years, and the risk ratio compared with people without HIV was 2.16 (1.79 for myocardial infarction and 2.56 for stroke) . From 1990 to 2015, the fraction of cardiovascular disease attributable to HIV infection increased from 0.36 to 0.92 percent; the highest attributable fractions were in sub-Saharan Africa, where HIV was estimated to account for more than 15 percent of the cardiovascular disease burden.

In the United States, one of the largest of these cohort studies evaluated California state-sponsored health insurance claims data, which included 28,513 patients with HIV and 3,054,696 patients without HIV (13). The incidence of coronary heart disease (CHD) (including acute MI, other ischemic heart disease, and coronary atherosclerosis) in patients between the ages of 18 and 24

years was low overall but increased in those infected with HIV compared with the uninfected (relative risk [RR] 6.76, 95% CI 3.36–13.58 for men and 2.47, 95% CI 1.23–4.95, for women). The relative risk of CHD was the most increased in patients with HIV over the age of 45 years compared with uninfected populations.

Other analyses have found that the risk of MI alone is elevated in patients with HIV across a wide range of ages. In a cohort of 27,350 age-matched predominantly male veterans with HIV and 55,109 without HIV followed for a median of six years, HIV infection was associated with a greater risk of acute MI overall, even after adjusting for Framingham risk factors and other comorbidities (adjusted hazard ratio [HR] 1.48, 95% CI 1.27-1.72)(10). When the cohort was analyzed by 10-year age groups, HIV infection was associated with a greater MI risk among patients aged 40 to 69 years but not among younger or older patients. Similarly, in a cohort from Kaiser Permanente Northern California, the age-adjusted rate of hospitalization for MI was 4.3 versus 2.9 events per 1000 person-years in men with and without HIV, respectively (14). However, a subsequent update from the Kaiser study demonstrated a decline in rates of MI among the HIV group leading to a rate similar to that in the group without HIV (12). Even when adjusted for age, gender, race, hypertension, diabetes mellitus, and cholesterol, HIV infection remained associated with a significantly higher incidence of MI (RR 1.75, 95% CI 1.51–2.02). Among women, the relative risk for MI compared with controls was further increased. Similar findings were reported from cohorts in France and Denmark (8,9).

The Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) Study Group prospectively studied the incidence of MI in 23,468 patients with HIV residing in Australia, Europe, and the United States (15,16). The overall incidence was low at 3.5 events per 1000 person-years, but the incidence increased with cumulative exposure to ART (age-adjusted RR 1.26, 95% CI 1.12–1.41, for each additional year of exposure). The association between ART and MI persisted in models that controlled for total cholesterol and triglyceride levels, suggesting that ART may negatively affect cardiovascular risk independent of its effects on lipid profile. Nevertheless, several studies

suggest that higher CD4 cell counts and lower HIV RNA levels are associated with decreased MI risk (17), and in one trial, interruption of ART led to increased cardiovascular events (18), suggesting that treating HIV with ART clearly has beneficial effects on MI risk.

The full extent of cardiovascular risk in treated patients with HIV will become more discernible as many of the initial cohorts continue to follow patients over time. Since the population with HIV is relatively young and actual cardiovascular events are infrequent, many investigators have used surrogate measures of coronary artery disease, such as intima media thickening of the carotid and coronary arteries, as indirect endpoints.

1.2 PATHOGENESIS OF VASCULAR DISEASE

The development of atherosclerosis that underlies cardiovascular disease (CVD and events is a complicated and multifactorial process that involves inflammation and immune dysregulation, endothelial dysfunction, and plaque rupture, as well as the traditional risk factors of hypertension, diabetes, dyslipidemia, and smoking (Figure 1).
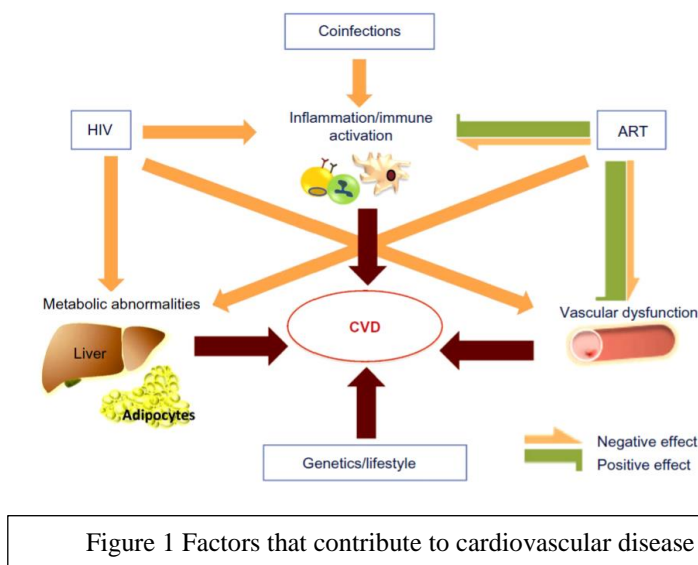

Figure 1 Factors that contribute to cardiovascular disease (CVD)

Inflammation plays a pivotal role in the development of atherosclerosis in general and is an area of active research with regards to HIV infection. Active inflammation may adversely affect endothelial cells and promote a prothrombotic environment that leads to atherosclerosis and plaque rupture. HIV infection has been linked to several markers of inflammation, including C-reactive protein (CRP) and interleukin (IL)-6, which in turn have been associated with cardiovascular events.

Through positron emission tomography (PET) imaging, arterial inflammation can be detected by increased uptake of a radiolabeled glucose analog, fluorodeoxyglucose (FDG), in blood vessels. In one study of 27 HIV-infected patients (mean age, 52 years) on antiretroviral therapy (ART) and without known cardiac disease, PET imaging was performed and compared with the findings in uninfected patients who underwent PET imaging for other reasons (19). Arterial inflammation in the aorta among HIV-infected patients was higher compared with uninfected patients who also had

no known cardiac disease and were matched by age, sex, and Framingham risk score but was similar to that seen in sex-matched uninfected patients (mean age, 69 years) who had known atherosclerotic disease. Although aortic inflammation was not associated with levels of CRP and D-dimer, it did correlate with sCD163, a soluble marker of monocytes and macrophage that has been associated with atherosclerosis in uninfected populations.

1.2.1 SOLUBLE MARKERS

C-reactive protein (CRP) is the most extensively studied of numerous inflammatory biomarkers potentially linked to underlying atherosclerosis in the general population, among whom elevations in CRP are associated with an increased risk of cardiovascular events independent of traditional risk factors. HIV infection has been associated with increased CRP levels when compared with uninfected controls (20). Furthermore, in a study of 922 HIV-infected patients followed for five years, a high CRP was independently associated with increased overall mortality (21). With regards to the specific morbidity of cardiovascular disease, the association between CRP levels and myocardial infarction risk in HIV-infected patients was evaluated in a study of a large hospital database that included 487 HIV-infected and 69,870 uninfected patients (22). High CRP (any value exceeding the upper limit of normal of the standard assay or any value in the highest quantile of the high sensitivity CRP assay) was found more frequently among HIV-infected patients (59 versus 39 percent). In an adjusted model controlling for age, sex, race, hypertension, diabetes and dyslipidemia, the risk for acute myocardial infarction was increased more than fourfold among patients with HIV infection and increased CRP when compared with patients with neither risk factor. There was no association between CRP level and HIV viral load or CD4 cell count, but protease inhibitor use was associated with high CRP levels. The effect of ART in general on CRP is unclear, with some studies showing an increase (23) and others showing a decrease (24).

Interleukin-6 (IL-6) Increased levels of the proinflammatory cytokine IL-6 are thought to have a direct causal role in the development of coronary heart disease among the general population and have been observed in patients with HIV infection, particularly with more advanced disease. However, there was a greater association with increased IL-6 levels compared with the uninfected in the setting of an HIV viral load ≥500 copies/mL (odds ratio [OR] 1.54, 95% CI 1.14-2.09) or a CD4 cell count <200 cells/microL (OR 2.25, 95% CI 1.60-3.16). Furthermore, in post hoc analyses of participants in the SMART trial, which evaluated ART continuation versus treatment interruption based on CD4 cell count, increased levels of IL-6 were associated with HIV RNA viremia, cardiovascular events, and all-cause mortality (25). A subsequent small study suggested that elevations in IL-6 were associated with endothelial dysfunction as reflected by changes in small artery elasticity and endothelial adhesion molecules (26). Finally, in a post hoc analysis of samples from two completed HIV trials, IL-6 was a stronger predictor of cardiovascular events than D-dimer (27).

Activation of T cells is a hallmark of HIV infection and levels of activated CD4 and CD8 T cells have been linked to rates of disease progression. During effective ART, levels of activation fall, but not back to pre-HIV levels in most patients, at least during the first few years of treatment. Studies examining the relationship between T-cell activation and atherosclerosis in HIV infection have yielded inconsistent results. Cross-sectional studies have demonstrated associations between the proportion of activated and senescent CD8 T cells and carotid intima media thickness and carotid lesions (28,29).

Monocyte activation — Soluble CD163, the extracellular component of the hemoglobin-haptoglobin receptor CD163, is released from activated monocytes and macrophages during inflammation. Both aortic inflammation and soft plaque volume in HIV-infected patients have correlated with sCD163(19,30). In addition, sCD14 levels were associated with progression of carotid intima media thickness (IMT) in a small study (31) .

In a clinical trial of treatment-naïve subjects randomly assigned to initiate various ART regimens, there was a rapid and significant improvement in flow-mediated dilation of the brachial artery, a measure of endothelial function, during the first 24 weeks of therapy (32). This improvement in flow-mediated dilation was seen regardless of regimen, suggesting that suppression of HIV, and not the specific drugs themselves, led to improved endothelial function (33). After adjusting for risk factors, including the Framingham 10-year cardiovascular disease risk score, those with HIV infection demonstrated significantly less elasticity in both large and small arteries when compared with the uninfected group. These data suggest that chronic HIV infection, independent of its pharmacologic treatment, induces alterations of endothelial function (34).

However, there is evidence for endothelial dysfunction in HIV-infected patients on therapy as well. Increased pulse wave velocity, which may be an early marker for atherosclerosis, has been noted in two small studies of HIV-infected patients taking ART compared with uninfected controls (35) . Cumulative duration of ART exposure was associated with an incremental increase in velocity, supporting a role of HIV therapy in the pathogenesis of endothelial dysfunction.

Plasma markers of endothelial function — In a longitudinal study of patients newly infected with HIV, measures of endothelial activation (ICAM-1 and VCAM-1) were increased early after HIV acquisition (36). Additionally, in the absence of ART during chronic infection, plasma VCAM-1 levels were independently associated with time to HIV progression or death.

Several studies have described increased plasma levels of endothelial cell products, including von Willebrand factor and soluble thrombomodulin, and the fibrin degradation product D-dimer in those living with HIV, and these levels are noted to inversely correlate with CD4 counts (37). Moreover, increased D-dimer levels in the setting of treatment interruption and increased fibrinogen levels have been associated with increased mortality in HIV-infected patients (21,38)

Tissue factor (TF) and tissue factor pathway inhibitor (TFPI) may play a role in the pathogenesis of atherosclerosis in HIV infection (39). Chronic immune stimulation increases TF expression on the surface of monocytes. In a study of 121 HIV-infected adults, higher concentrations of TF were associated with subclinical atherosclerosis (assessed by carotid intima media thickness) and correlated with longer duration of ART and lower nadir CD4 counts (40).

Brain natriuretic peptide (BNP) is a natriuretic hormone released from myocardial cells in response to volume expansion and possibly increased wall stress. The N-terminal fragment, N-pro-BNP, is also released into the circulation. Serum BNP and N-pro-BNP are increased in patients with heart failure and are predictors of death and cardiovascular events in asymptomatic patients without heart failure (41).

Trimethylamine-N-oxide (TMAO), produced through intestinal microbial metabolism of dietary lecithin, appears to contribute to the development of atherosclerotic plaques through its interactions with macrophages and foam cells (42). Ingested phosphatidylcholine (which is present in very high amounts in foods such as red meat, cheese and eggs) is metabolized in the gut to choline-containing nutrients and, in the large bowel by the intestinal microbiota, to trimethylamine (TMA); the latter is then oxidized to trimethylamine-N-oxide (TMAO) by hepatic flavin-containing monooxygenases. Plasma TMAO levels have been shown to decline following the suppression of intestinal microorganisms with oral broad-spectrum antibiotics and then return to near previous levels following the withdrawal of antibiotics (43) Figure 2.
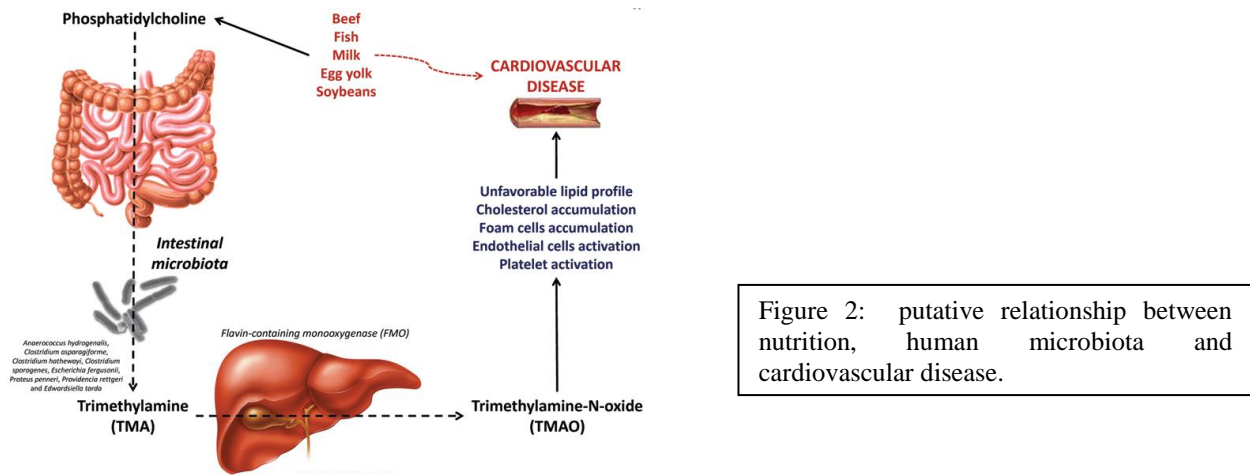
Figure 2: putative relationship between nutrition, human microbiota and cardiovascular disease.

In general population increased levels of plasma TMAO were associated with significantly greater risk of death, MI, or stroke (adjusted HR for highest versus lowest quartile 1.88, 95% CI 1.44-2.44) (44) and have also been correlated with increased atherosclerotic plaque burden (45).

Given the association between TMAO levels and atherosclerotic events as well as the potential for modification of TMAO levels based on intestinal microbiota, TMAO levels may be a future target for therapies aimed at lowering the risk of atherosclerotic events. Prior to this, however, these findings will need to be replicated in other populations.

In PLWH, serum TMAO levels were similar to those in healthy controls, although higher serum concentrations were observed in those receiving combination antiretroviral treatment (cART) (46). Higher TMAO levels have been associated with carotid plaques, endothelial dysfunction and silent cardiac ischemia in HIV-positive subjects (46–50). A recent longitudinal study showed that participants with higher serum TMAO levels had an increased risk of carotid plaque and this was partially mediated by biomarkers of monocyte activation and inflammation (sCD14, sCD163) (50). In addition, PLWH with type 2 diabetes showed the highest endothelial dysfunction (and the effect was associated with TMAO concentrations) (51).

Asymptomatic infection with cytomegalovirus (CMV) has been associated with cardiovascular morbidity in both the HIV-infected and general population (52) tudies evaluating the pathogenesis

of this association have suggested a potential role in atherogenesis; in particular decreased HIV viral replication may be mediating the relationship between an enhanced immune response against CMV and vascular disease (53)

Other viruses that have been implicated in the development of subclinical cardiovascular disease in HIV-infected patients include herpes simplex virus 2 (HSV-2) (54). Coinfection with Hepatitis C virus (HCV) has been linked to endothelial dysfunction (55) and modulates known risk factors for cardiovascular disease in HIV-infected patients, but it is not clear if HCV increases their risk of myocardial infarction.

Vitamin D deficiency in HIV-infected patients has been linked to indirect markers of subclinical atherosclerotic cardiovascular disease, including carotid IMT and brachial artery flow-mediated dilation (56).

1.3 HUMAN MICROBIOME IN HIV

The human gastrointestinal tract hosts a complex microbial community that plays essential homeostatic roles such as energy harvest, pathogen exclusion, and immune regulation. Changes in the composition of the microbiome have been shown to affect these functions (57).

Even in condition of viremic suppression under ART, people living with HIV infection (PLWH) show a chronic immune activation/hyper-inflammation, whose pathogenesis can be due to many factors, such as disruption of the gut associated lymphoid tissue (GALT) and gut mucosa damage; the latter, in particular, allows the translocation of antigenic material from the gut into the hematic circulation and, thus its diffusion at a systemic level (58).

The GALT, in particular CD4+ T lymphocytes residing in the GALT, is one of the main sites in HIV infection which constitute a long-term reservoir site. Once infection has occurred, HIV rapidly depletes CD4+ T cells from the GALT as a larger percentage of these cells expresses elevated level of CCR5 HIV coreceptor, compared to peripheral blood. CD4+ T cell destruction, associated with immune activation in the gut, leads to high levels of CD8+ T cell infiltration and epithelial cellular damage (59).

There is increasing interest in the gut microbiome in PLWH in terms of its modifications and the consequences of microbiome changes after pre/probiotic supplementation (60,61). Several studies compared gut microbiota composition in HIV-infected patients to that of HIV-uninfected control subjects and found an enrichment of Erysipelotrichaceae, Enterobacteriaceae, Desulfovibrionaceae and Fusobacteria and a depletion of Lachnospiraceae, Ruminococceae, Bacteroides, and Rikenellaceae. However, the interaction between HIV and gut microbiome seems to be modulated by lifestyle and behavioural factors (including sexual habits) as well as intestinal wall structural and functional modifications [13]. Some of the aforementioned studies also found correlations between these gut perturbations and markers of inflammation and HIV disease progression, including

kynurenine-to-tryptophan ratios, IL-6, sCD14, IL-1β and peripheral T-cell activation. Recently, gut microbiome traits, after adjusting for confounders, have been associated with metabolic syndrome and coronary heart disease in PLWH (62,63).

The metabolic profile of HIV associated microbiota is characterized by overexpression of the pathways related to resistance to oxidative stress and under-expression of well-known antiinflammatory short chain fatty acids (SCFA) biosynthesis pathways (64,65). Butyrate is a SCFA that represents a crucial energy source for colonocytes, an inducer of inter-enterocyte tight junction proteins that promote gut barrier function (66), and augments gut regulatory T cells which constitute a lymphocyte population critical for mitigating pathologic inflammation (67,68). (Fig. 3)
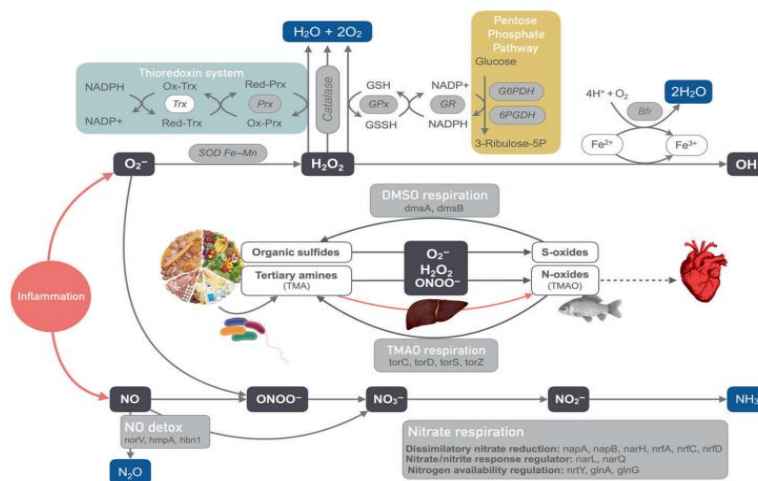

Fig. 3 Summary of enzymes and metabolic processes related with ROS/RNS clearance and enriched in subjects with LGCs (gray boxes). Bacteria enriched in LGC reduce ROS and RNS and generate less toxic metabolites that are eventually excreted from the body. Moreover, different Proteobacteria utilize S-oxides and N-oxides as electron acceptors for anaerobic DMSO and TMAO respiration. S-oxides derive from dietary organic sulfides, which are oxidized by ROS. The N-oxide trimethylamine N-oxide (TMAO) is generated by oxidation of diet-derived tertiary amines like trimethylamine (TMA), either by hepatic flavin-containing monooxigenases or by the direct effect of ROS. TMAO concentrations, however, are also high in fish, from which it can be directly absorbed. Plasma TMAO levels have been epidemiologically linked (dashed line) to severe cardiovascular disease and metabolic disorders, which are also prevalent in HIV-1-infected subjects. G6PDH glucose-6- phosphate dehydrogenase; 6PGDH 6-phosphogluconate dehydrogenase; GR glutathione reductase; GPx glutathione peroxidase; Trx thioredoxin reductase; Prx peroxiredoxin; SOD superoxide dismutase, Fe–Mn family; Bfr bacterioferritin; NO nitric oxide; TMA trimethylamine; TMAO trimethylamine oxide; DMSO dimethyl sulfoxide.

A concomitant decline in butyrate-producing gut microbiota members belonging to the Lachnospiraceae and Ruminococcaceae families (69) was observed in PLWH compared to HIV-seronegative controls.

HIV-associated microbiota has a significantly higher abundance of pathways and metabolites related to resistance to oxidative stress. For taxonomic composition, the ratio of Firmicutes/Bacteroidetes increased significantly in HIV-1-infected patients. Important factor in the maintenance of systemic inflammation is the depletion of anti-inflammatory bacteria, such as Bacteroides. Nevertheless, *Prevotella, Acidaminococcus, Streptococcus* and *Bacteroides* were identified as distinctive members of HIV-associated microbiota in both metagenomics and metatranscriptomic data sets (64,70,71). Prevotella and Acidominococcus species in particular present a gene content that seems involved in all of the pathways that characterize the dysbiotic metabolism of HIV-associated microbiota like those related to oxidative stress resistance. Therefore, this bacterial community is well adapted to gut inflammation caused by HIV infection, which is in turn maintained by a high abundance of Gram-negative bacteria composed of HIVassociated microbiota (64,65). It is possible that the extent of immune damage (proportional to the extent of CD4 T cell loss, i.e. CD4 nadir) dictates the degree of dysregulation of microbiota composition but is itself the cause of later age-associated comorbidities, with the effects on the microbiota contributing little to pathology. However, extent of dysbiosis seems to correlate with comorbidities independently of CD4 nadir, suggesting that the link between dysbiosis and disease progression is not merely (58) an innocuous consequence of prior events but may impact later comorbidity occurrence. Indeed, immune activation may constitute a link between the comorbidities measured herein, each of which have been linked to immune activation levels in PLWH (25,72), and the gut microbiome.

1.4 MICROBIOME CONFOUNDERS IN HIV STUDIES: SEXUAL BEHAVIOR.

Earlier cross-sectional studies examining the gut microbiota in PWH that were not matched for microbiota-associated con- founding variables such as MSM status (primarily having an over-representation of MSM in the PWH group) reported shifts from *Bacteroides* to *Prevotella* predominance following HIV-1 infection (71,73–77). Recent seminal studies have reported an increased abundance of *Prevotella* and depletion of *Bacteroides* in the gut microbiota among men who have sex with men (MSM) as compared to men who have sex with women, independently of HIV infection status (78–80). Because PLWH in many resource-rich settings is predominantly comprised of MSM, selection of seronegative controls from the general population without matching for MSM could therefore have confounded prior studies examining the impact of HIV infection on the gut microbiota. Stratifying participants by sex/sexual practice, Vujkovic-Cvijin and colleagues found that enrichment of *Prevotella* and depletion of *Bacteroides* are not features of HIV infection per se but instead characteristics of the MSM-associated microbiota. However, they also found evidence that treated HIV infection does 13 exert a distinct effect on the microbiota that is largely distinct from that of MSM status and is characterized by a reduction in overall diversity, enrichment of *Gammaproteobacteria*, and depletion of *Clostridiales* members. Interestingly, in their study bacterial taxa associated with MSM either did not overlap with taxa of the HIV-associated microbiota signature or when overlap did occur these taxa exhibited abundance trends opposite that of HIV infection. Finally, their results reveal that antiretroviral-treated HIV infection is associated with distinct gut microbiota features independently of sex/sexual practice, in the form of a reduction in alpha diversity, overall shifts in community structure as denoted by beta diversity analyses, and consistent shifts in abundance of specific bacterial taxa common across participant groups (81). The bacterial shifts observed in PWH included an enrichment of pro-inflammatory taxa belonging to *Enterobacteriaceae* and *Desulfovibrionaceae* consistent with other cohorts (60,77,79).

1.5 TRADITIONAL RISK FACTORS

Overall, the classic cardiovascular risk factors of dyslipidemia, hypertension, diabetes, and smoking are common among populations with HIV, although the frequency of these comorbidities is not sufficient to explain the overall increased incidence of cardiovascular disease observed in the setting of HIV infection (82).

<u>Dyslipidemia</u> Compared with controls without HIV, individuals with HIV more frequently had low high-density lipoprotein (HDL) cholesterol and elevated triglycerides. Among men with HIV, 2 percent had moderate and 17 percent had high predicted CHD risk, in contrast with men without HIV (5 and 11 percent, respectively). Among women with HIV, 2 percent had moderate and 12 percent had high predicted CHD risk, similar to women without HIV. The risk of CHD was significantly lower in patients who were treatment-naïve compared with those patients taking protease inhibitors (PIs; odds ratio [OR] 0.57). Predictors of elevated risk also included low-income status and elevated body mass index (BMI).

Protease inhibitor (PI) regimens that require a lower dose of ritonavir for boosting (eg, darunavir) tend to have less unfavorable effects on the lipid profile (83). Treatment with nonnucleoside reverse transcriptase inhibitors (NNRTIs) is associated with an increase in the HDL cholesterol (84,85). Rilpivirine is associated with a more favorable lipid profile than efavirenz (86). Overall, integrase inhibitors are also associated with a favorable lipid profile (87,88).

<u>Smoking</u> A high prevalence of modifiable risk factors was present in the study participants. For example, 40 percent were current smokers. In addition, >40 percent of men with HIV and >60 percent of women with HIV met criteria for being overweight or obese. However, rates of obesity were less than in individuals without HIV(89)

Insulin resistance and diabetes mellitus — Exposure to older ART agents was associated with insulin resistance and an increased incidence of diabetes mellitus (90). Antiretroviral agents from several classes have been implicated in insulin resistance in particular PIs.One possible explanation for the association is the observation that PIs can direct down-regulation of the glucose transporter

isoform GLUT4, the major transporter of glucose into fat cells and cardiac and skeletal muscle (91)

Several studies have also noted an association between cumulative exposure to NRTIs and greater

insulin resistance and diabetes risk compared with no NRTI exposure (92).

Patients with HIV may have higher rates of hypertension compared with those without HIV (93).

1.6 RISK MODIFIERS

The lipodystrophy syndrome refers to abnormal fat redistribution with lipoatrophy and/or

lipohypertrophy that can occur in patients with HIV on antiretroviral therapy (ART), which is

sometimes associated with metabolic syndrome. The exact prevalence of this phenomenon is

unclear, and there may be different risks for fat loss versus accumulation, suggesting discrete

processes. Nevertheless, although patients with HIV and abnormal fat redistribution may not have

overt obesity, it is often associated with significant metabolic abnormalities, including dyslipidemia

and insulin resistance, and thus increased cardiovascular risk. As an example, 10-year coronary

heart disease (CHD) risk estimates were compared in 91 patients with HIV and patients without

HIV from the Framingham Offspring Study who were matched for age, gender, and body mass

index (BMI)

Since treatment of HIV infection is associated with central fat accumulation, dyslipidemia, and

insulin resistance, studies that evaluate populations with HIV for the presence of metabolic

syndrome have found high rates (94). In the INITIO trial, a study of 881 patients with HIV who

initiated ART, the prevalence of metabolic syndrome at baseline was approximately 8 percent (95).

During three years of follow-up, the incidence of metabolic syndrome was 8 to 12 cases per 100

person-years, depending on the criteria used. Both baseline and incident metabolic syndrome were

associated with the development of diabetes.

The effect of coinfection with hepatitis C virus (HCV) on cardiovascular disease risk in patients

with HIV is uncertain, with studies yielding conflicting results. Several studies in men have

suggested a greater risk of cardiovascular disease in the setting of HCV infection (96–98).

Additionally, in an analysis of data prospectively collected from 32,395 individuals with HIV in the Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) study, the risk for development of MI was similar for patients with HIV-HCV coinfection compared to patients with HIV monoinfection after adjusting for potential confounders (99).

There are a few studies that suggest that increased cholesterol levels may be blunted in patients coinfected with HCV (100).

Substance use — Cardiovascular disease has been associated with cocaine and marijuana use in patients with HIV.

Cocaine has been linked to coronary calcification (101). In a study of 165 African American patients with HIV, significant coronary stenosis (≥50 percent) was detected in 15 percent of participants (102). Those patients with long-term cocaine use (>15 years) had the highest prevalence of stenosis (42 percent).

Marijuana use has been linked to cardiovascular disease in patients with HIV. In a prospective cohort study of 558 men with HIV between the ages of 40 and 60 years, long-term heavy marijuana use (defined as daily or weekly use reported at ≥50 percent of biannual visits) was independently associated with cardiovascular events (odds ratio [OR] 2.5, 95% CI 1.3-5.1) (103).

1.7 SUBCLINICAL ATHEROSCLEROSIS

The available studies have generally demonstrated an increased prevalence of subclinical atherosclerosis in HIV-infected compared with uninfected individuals. Such measurements include intima media thickness (IMT) and intraluminal arterial plaque as assessed by ultrasound and evaluation of coronary artery calcification and plaque by computed tomography (CT).

Evidence of increased subclinical atherosclerosis among HIV-infected elite controllers compared with uninfected controls has also been observed, suggesting that an increased risk of atherosclerosis can occur in the absence of antiretroviral therapy (ART), detectable viremia, or overt immunodeficiency (104,105).

Most, but not all studies of IMT of the carotid artery and coronary arteries in asymptomatic adults have found an increase in measurement with HIV infection (104,106,107). Among the 13 studies that compared carotid IMT in HIV-infected versus uninfected patients, all except for two reported a trend towards higher carotid IMT with HIV. However, the weighted mean difference between HIV-infected and uninfected patients was very small, 0.04 mm (95% CI 0.02-0.06). Moreover, there was significant statistical and clinical heterogeneity between the studies, including differences in measurement technique and underlying traditional cardiovascular risk factors. In a study of over 1700 HIV-infected patients, carotid IMT was higher compared with age-matched uninfected controls among individuals aged 6 to 29 years but was similar to controls among adults 30 years and older, among whom IMT correlated more strongly with traditional risk factors (108).

The effect of ART on IMT appears to vary by agent. As an example, in a randomized trial, atazanavir was associated with a slower rate of progression of carotid IMT compared with darunavir; this difference was hypothesized to be related to the fact that atazanavir increases bilirubin, which may exert an antioxidant effect that slows the progression of atherosclerosis (109). The effect of raltegravir was intermediate.

Studies suggest that HIV-infected individuals may not have a greater burden of coronary artery calcification than uninfected patients after controlling for traditional cardiovascular risk factors but are more likely to have non-calcified atherosclerotic plaque, which may be more likely to rupture.

Additional studies have evaluated coronary artery calcification, which is detected by CT, in HIV-infected patients. In the meta-analysis discussed above, there was no difference in coronary artery calcification between HIV-infected and uninfected patients in the pooled results from five studies (106). However, some but not all (110) subsequent studies using CT angiography to assess overall plaque burden have reported a higher prevalence among HIV-infected patients compared with uninfected patients with similar Framingham risk scores (111). Specifically, HIV-infected individuals had a higher adjusted prevalence and extent of non-calcified plaque, which was associated with advanced age, lower nadir CD4 cell count, and longer duration of ART. The majority of the HIV-infected men in this study were on ART.

1.8 RISK SCORES AND MANAGEMENT

The international guidelines on antiretroviral management of patients with HIV infection from the US Department of Health and Human Services suggest checking lipids every 6 to 12 months and glucose levels every three to six months during ART use (112,113).

Results of the risk factor assessments can be used to estimate an individual's risk for the development of coronary heart disease using established multivariate risk models (114,115). Various models exist, including the Framingham risk score, the American Heart Association/American College of Cardiology (AHA/ACC) Pooled Cohort Equations CV Risk Calculator (PCE), and the Data Collection on the Adverse Effects of Anti-HIV Drugs (DAD) cohort risk calculator that includes measure of HIV disease (116,117).

Interpretation of the results of the risk calculation should be informed by other risk factors not accounted for. Additional factors that may increase the cardiovascular risk above the calculated risk include hepatitis C virus coinfection; metabolic syndrome, lipodystrophy, or fatty liver disease; HIV treatment failure or nonadherence; low CD4 cell count (less than 350 cells/microL); or a history of prolonged HIV viremia or delayed initiation of ART (118).

As an example, in a large cohort study of almost 30,000 veterans with HIV, current smoking, low high-density lipoprotein (HDL) levels, and elevated triglyceride levels were more common compared with an age- and race-matched cohort of over 50,000 veterans without HIV, but the median Framingham risk score was the same and intermediate in both groups (10). Nevertheless, the incidence of acute myocardial infarction was higher among the patients with HIV infection, even after adjusting for Framingham risk factors and other potential contributors (adjusted hazard ratio 1.48, 95% CI 1.27-1.72).

The cardiac risk model based upon data from the large international Data Collection on the Adverse Effects of Anti-HIV Drugs (DAD) cohort, which consisted of patients with HIV infection who were

followed longitudinally for cardiac events, performed better than the Framingham risk score among patients in that cohort (116).

The strategies for minimizing the risk of CVD include statin therapy, blood pressure control, and management of diabetes (119).

Despite the growing importance of cardiovascular morbidity in patients with HIV infection, strategies to minimize cardiovascular disease are often not optimally implemented. As an example, in a study of nearly 14,000 male veterans with HIV, of whom 51 percent had an indication for statin therapy, nearly one-quarter of those with indications were not receiving a statin (120). This finding highlights the importance of improving awareness of cardiovascular disease risk reduction interventions in the primary care of patients with HIV infection.

Exercise and weight reduction should be given emphasis in any preventive cardiovascular program and as part of a healthy lifestyle for all patients with HIV infection (121). These interventions can improve other risk factors that contribute to cardiovascular disease.

One study compared the dietary intake of 362 individuals with and 164 individuals without HIV (122). Compared with the uninfected participants, a significantly greater number of patients with HIV infection had a dietary history above the United States recommended allowances for total fat, saturated fat, and cholesterol intake. The investigators postulated that the increased intake of saturated fat and cholesterol contributed to the elevated triglycerides that were also noted in this patient population. These data suggest the potential benefit of dietary counseling in individuals with HIV.

The World Health Organization has released revised guidance in the 2016 Antiretroviral Therapy Guidelines that includes a conditional recommendation that the assessment and management of cardiovascular risk should be provided for all individuals living with HIV according to standard protocols for the general population (123). In this guidance, reference is made to the Package of Essential Noncommunicable Disease Interventions for the general population that targets those who are >40 years old, smokers, people with known hypertension or diabetes mellitus, those with waist

circumference >90 cm in women and >110 cm in men, and those with family history of diabetes mellitus or premature cardiovascular disease.

Data on the effect of statins on measures of inflammation are emerging. Observational studies had suggested that statins were associated with decreases in inflammatory markers, such as high-sensitivity C-reactive protein (hsCRP), tumor necrosis factor (TNF)-alpha, and interleukin (IL)-6 (124,125).

AIMS OF THE STUDY

Considering the above presented data, the aim of this project was to screen patients living with HIV on cART and without previous cardiovascular major events in order to define a high risk group (integrating risk factors and bioumoral markers).

The secondary aims of the pilot intervention study were:

1) To verify the impact of diet on microbiota composition and plasma TMAO concentrations;

2) To investigate possible biomarkers, including TMAO concentrations, associated with fecal microbial shift following a nutritional dietary intervention;

3) To investigate the shift in gut microbiota diversity with CD4 lymphocytes current and nadir values.

MATERIALS AND METHODS

- **Study design**
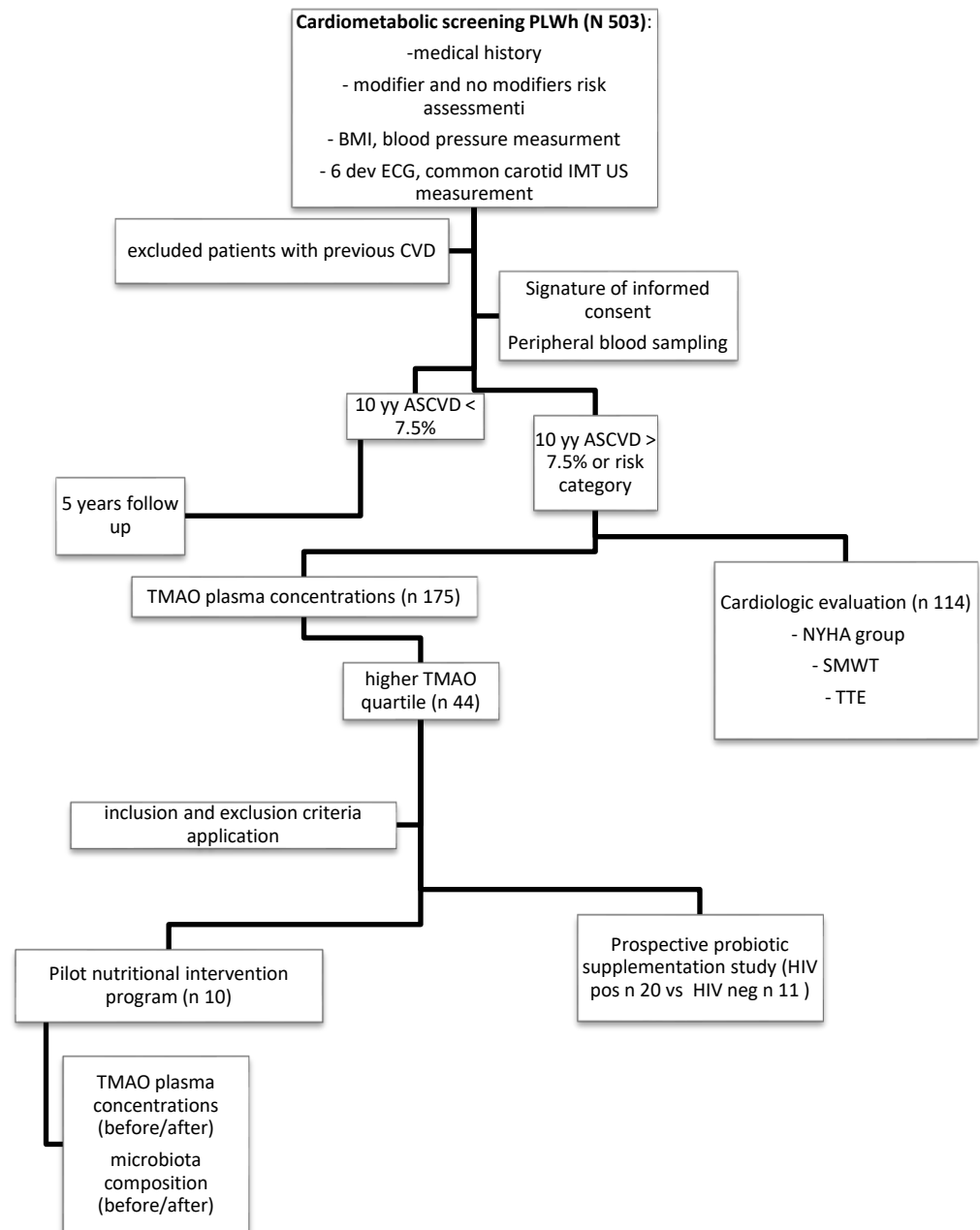
The flowchart of the project is represented in Fig. 1.

Fig. 1 The PRECAD flow diagram of the clinical study

- **Recruiment**
- The first part of the study included consecutive cART treated HIV-positive patients with no previous CVD in a primary prevention program. Since december 2012 to may 2018 visit at outclinic Ospedale Amedeo di Savoia Torino were performed. Anthropometric, clinical and biochemical data were recorded; 10-year cardiovascular risk score was calculated according to the Framingham, ASCVD/AHA/ACC and CUORE algorithms. ASCVD algorithm categorize patient in low [L] <7.5% intermediate [I] 7.5-20 high [H] >20%. EACS targets were <140 mmHg for systolic blood pressure (SBP), 6.5% HbA1C for glucose tolerance, <190 mg/dL and <115 mg/dL for total (TC) and LDL cholesterol (LDL-C). The measurement of common carotid IMT was performed by the same operator at 1 cm from carotid bifurcation as the average of three measurements (abnormal >0.9 mm). Multimorbidity and polypharmacy were defined as the presence of ≥3 comorbidities and ≥5 comedications. Available N-terminal pro-brain natriuretic peptide (nt-proBNP; abnormal >100 pg/mL) measured within 3 months of study visit were recorded.
- Classyfing the all population according to ASCVD score, those with low risk were postponed to a 5 years follow up visit. The group with intermediate, high risk or including in risk category were referred to cardiologist evaluation. In this context, symptoms of heart failure were classified according to New York Heart Association (NYHA) Functional Classification. Electrocardiogram (ECG), transthoracic echocardiography (TTE) and six minute walking test (SMWT) were performed. Left ventricle (LV) function was expressed by ejection fraction (EF). Right ventricle dysfunction (RVD) was defined as tricuspidal annular plane excursion (TAPSE) <13 mm and fractional area change (FAC) <30; pulmonary artery pressure (PAPs) was recorded (normal <30 mmHg).
- Furthermore, the second group was included in a cross-sectional analysis of the determinants of serum TMAO concentrations. Data were obtained from a cross-sectional study in Torino and from the baseline of an interventional pilot trial in Rome: both protocols included PLWH receiving cART.
- TMAO concentrations were also measured in patients enrolled in a prospective probiotic supplementation study conducted in Rome (the CONSORT trial (126)).

- **Nutritional pilot intervention substudy**

Inclusion criteria

- Age > 18 years;
- HIV positive test confirmed by Western-blot test;
- Serum TMAO concentration > 4,0 µmol/l.
- Good self reporting ART adherence
- Viral load <50 cp/ml in the last two measurements

Exclusion criteria

- Concomitant AIDS defining pathology or cancer in chemotherapy
- concomitant intake of potentially cardiotoxic drugs (example taxole, antracycline);
- Active drug addiction;
- concomitant intake of probiotics;
- concomitant intake of antibiotics in the last three months;
- concomitant chronic intestinal disease (ulcerative colitis, Chron disease, irritable colon disease, bacterial overgrowth syndrome);
- concomitant pregnancy or breastfeeding

Considering the inclusion and exclusion criteria, patients were enrolled and proposed a diet intervention pilot study in which baseline and 6-month TMAO serum concentrations and microbiota composition were compared.

This last group were referred to dietologist evaluation were received nutritional information.

According to BMI and blood test values, they received a diet scheme of 1400-1660 kcal/die with recommended daily amount of fiber of 20-30 g according to the Levels of Reference Intake of Nutrients and Energy for the Italian Population (LARN) the Italian Society of Human Nutrition (SINU) (127).

It was recommended a proportion of daily amount respectively 20% of kcal/daily in protein, 25% of lipids and 55% of carbohydrates. Moreover, the group receveid a weekly diet scheme that included 3-4 times meet consumption and 1-2 times sliced meats, 3 times fish, once a week eggs, 3-4 times legumes, 2 times cheese. It was also recommended consumption of brown cereals, fruit ad 2 times per day, 3 portions daily of vegetable, prefering olive oil instead animal fats.

All patients were advised to drink at least 1.5-2.0 litres of water / day, to abolish or reduce alcohol consumption, to continue or increase physical activity (at least 30 '/ day of walking).

The diet adherence was checked by dietician during follow up visit without food frequency questionnaires.

Patients signed a written informed consent and ethics approval was obtained from each institution's ethics committee.

- **Blood specimen collection**

Serum was obtained after blood centrifugation within 3 months of the clinical visit and stored at -80°C until analysis. To quantify TMAO plasma concentrations, 100 µL of plasma were added with 50 µL of internal standard working solution and 90 µL of acetonitrile. After protein precipitation, 5 µL of the supernatants were directly analysed through a validated Hydrophilic Interaction Liquid Chromatography (HILIC) and Tandem Mass Spectrometric (MS/MS) method, with a UHPLC-MS/MS instrument, settled in positive electrospray ionization.

- **DNA extraction and sequencing**

Stool samples were self-collected at home by patients and transferred to sterile sampling containers. The samples were immediately refrigerated at 4°C and within the next 2 h stored in a refrigerator at the temperature of 80°C. Nucleic acid was extracted from the feces collected. Total DNA from the samples was extracted using the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy) following the manufacturer's instructions. One microliter of RNase (Illumina Inc. San Diego. CA) was added to digest RNA in the DNA samples, with an incubation of 1h at 37 °C. DNA was quantifed using the QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) and standardized at 5ng/µL. 16S rRNA amplicon target sequencing. DNA directly extracted from fecal samples was used to assess the microbiota by the amplifcation of the V3-V4 region of the 16S rRNA gene using the primers and protocols described by Klindworth et al. (128). PCR amplicons were cleaned using Agencourt AMPure kit (Beckman Coulter, Milan, Italy) and the resulting products were tagged by using the Nextera XT Index Kit (Illumina Inc. San Diego. CA) according to the manufacturer's instructions and sequenced in paired end mode (2X250bp) on a MiSeq platform

Bioinformatic data analysis after sequencing, raw reads were analyzed by using the Quantitative Insights into Microbial Ecology QIIME2 (129). Primers and adapters were first trimmed by using Cutadapter and then quality filtered using the DADA2 algorithm, removing low-quality bases, chimeric sequences, and sequences shorter than 300 bp by using the dada2 denoise-paired plug in of QIIME2. Amplicon Sequence Variants (ASVs) generated by DADA2 were used for taxonomic

assignment using the qiime feature-classifier plugin against the Greengenes 16S rRNA gene database. QIIME2 diversity script was used to perform alpha diversity analysis.

- **Statistical analysis**

Data are described using number (percentage) or median (interquartile ranges) and analysed through non-parametric tests; a multivariate linear regression analysis was performed including variables with bivariate *P*-values <0.05.

All analyses were performed through SPSS version 24 for Mac (IBM Inc). Wilcoxon's test was used to compare 6-month TMAO serum concentrations and microbiota composition to baseline values (in the intervention study).

The α diversity of the intestinal microbiota was evaluated by the Chao1 index, which estimated the number of different taxa, and the Shannon diversity index, a quantitative measure of community richness calculated using the diversity of the vegan package (130) in R environment. The Observed counts of features (OTU) represent observed abundances of taxa in the sample. The ASVs table was used to build a principal component analysis (PCA) according to the sampling time using the made package of R.

Weighted unifrac distance matrix was generated by QIIME2 and used both to build the Principal Coordinate Analysis (PCoA) and to perform the permutational multivariate analysis of variance (ANOSIM) by the "vegan" package in R environment.

The comparison between groups was performed using Student's t test or the Mann-Whitney U test in the case of non-normally distributed variables. The comparison within the same group was evaluated with the t test for paired data or the Wilcoxon matched pairs test in the case of non-normally distributed variables. A simple correlation analysis between anthropometric and laboratory variables and the individual ASVs (Spearman correlations) was performed. The significant associations were then further evaluated by multiple regression after adjustment for age, BMI, and probiotic use.

No multiple comparison were used for the microbiome analysis for limitated amount of partecipants.

RESULTS

503 patients were enrolled: age, gender (% male) and ethnicity (% Caucasian) were 49.7 years (42.4-57.4), 78.1% and 93.8%, respectively. 460 patients (91.4%) were on HAART [PI-based (40.7%), NNRTI-based (39.5%), INSTI-based (28.5%), ABV containing regimen (13.9%)]. Current and nadir CD4+ cell count were 554/uL (374-718) and 198/uL (87-316.7); treatment duration was 9.8 years (3-16.3); in 388 patients (80.3%) HIV RNA was <50 copies/mL, as showed in table 1. Anthropometric parameters are showed in table 2, highlighting those non attaining suggested targets.

Table 1 – Population characteristics (n 503)		
age years (med, IQR)	48.9	41-57
gender (male: n, %)	384	76.5
race (caucasian: n, %)	449	94.8
Smokers (n,%)	198	42
HCV positive (n,%)	125	26.9
Experienced-patients (n,%)	430	90.9
Naïve-patients (n,%)	43	9.1
Current HIV RNA<20 copies/ml (n,%)	316	70.5
>20 cp/ml (n,%)	132	29.5
PIs-based regimen	189	40
NNRTIs-based regimen	176	37.2
INSTI-based regimen	112	23.7
CCR5R antagonist- based regimen	25	5.3
ABV containing regimen	76	17.8
NRTIs sparing regimen (n, %)	57	13.4
Current CD4 count (n/ml: med, IQR)	532	332-723
Nadir CD4 count (n/ml: med, IQR)	206	86-328
Treatment duration (years: med, IQR)	7.7	2.7-14.3

	Cut off	Med (IQR)	Not to target population (n %)
BMI	18.5-24.99 kg/m2	24,2 (21.8-27.2)	181 (43.2)
Abdominal circumference Male Female	<94 cm <89 cm	90 (84-100) 91 (84-97.5)	116 (38.5) 60 (61.2)
Systolic blood pressure	<140 mmHg	120 (110-130)	81 (18.4)
Total cholesterol	<190 mg/dL	195 (163-222)	250 (55.4)
HDL cholesterol	>39 mg/dL	44 (35-55)	165 (36.3)
LDL cholesterol	<130 mg/dL	123 (99-143)	175 (39.9)
Trigycerides	<180 mg/dL	121 (86-171)	105 (23.2)
eGFR MDRD	>60 ml/min	81 (70-93)	37 (8.6)

Table 2 - Median, interquartile ranges and number (prevalence) of patients not attaining reccomeneded values in BMI, abdominal circumeference, systolic blood pressure and lipids.

10yy ASCVD risk strata were: L 58.5%, I 27.3% and H 14.3%. 244 (49.6%) patients were active smokers; among these, 29 (15%) were classified as having a H CVR (Fig. 1).
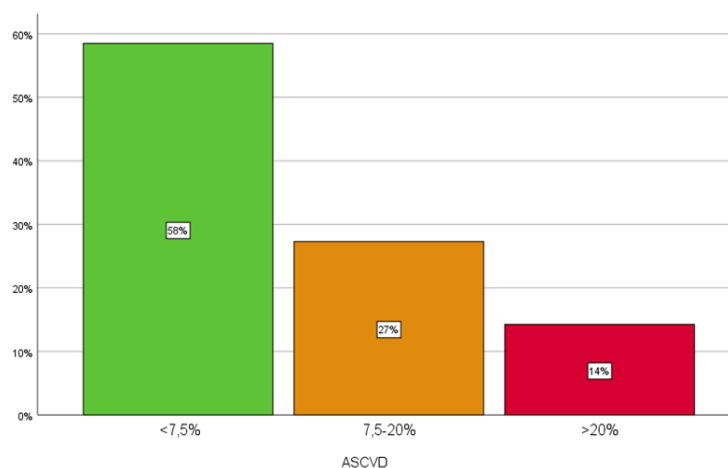

Fig. 1 - 10 year cardiovascular risk score strata according to the ASCVD/AHA/ACC algorithm.

Despite 156 patients (31.5%) were receiving lipid lowering treatment TC and LDL-C targets were not attained in 128 (82%) and 118 (77.6%)[p<0.0001]. This percentage was higher in patients in the high CVR strata (respectively 80% and 67%) (Fig. 2 and 3)
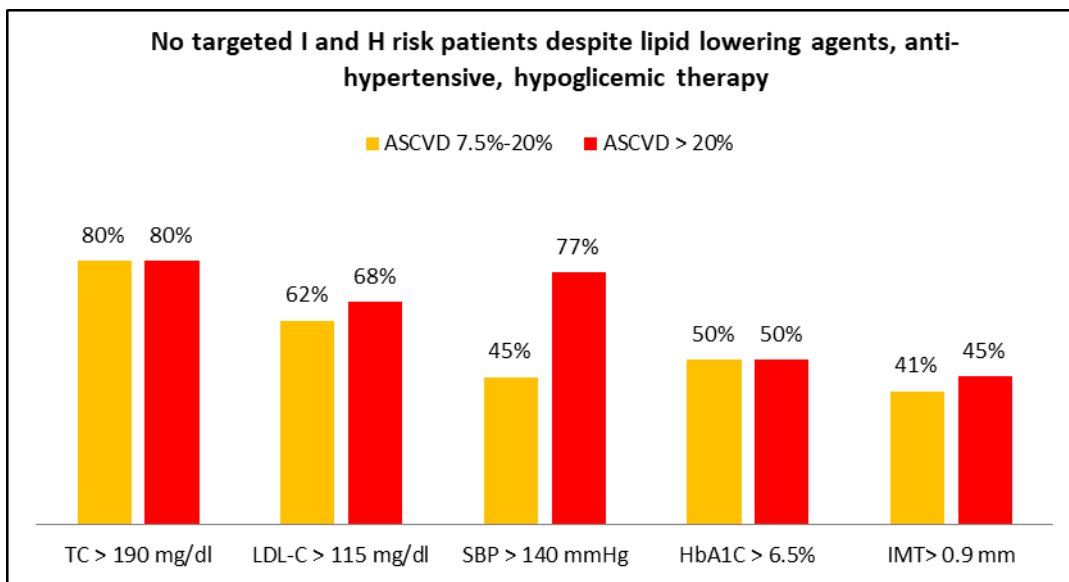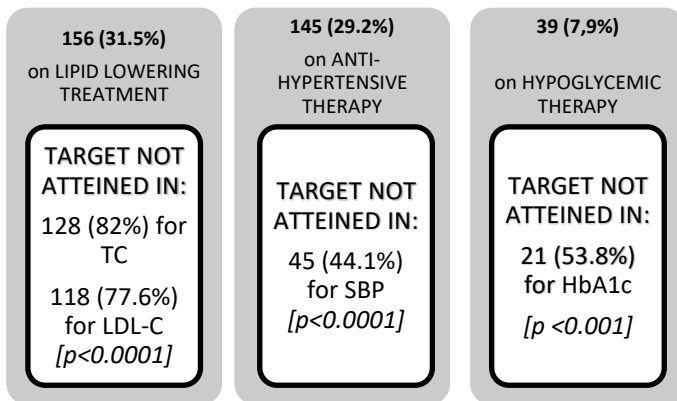
Fig. 2 – 3: Not targeted population despite anti-hypertensive, hypoglicemic or lipid lowering treatment (high) according to ASCVD score (low).

Additionally, despite 145 patients (29.2%) were on anti-hypertensive therapy, 45 (44.1%) were not on target for SPB (44.4%, p<0.0001) in particular in H risk strata (73.3%). Out of 39 patients (7.9%) treated with hypoglycemic therapy, 21 (53.8%) were not atteined HbA1C target, 50% in H risk group (p<0.001).

Out of 325 IMT performed, abnormal values were recorded in 91 (28%) participants: thicker IMT values were observed more frequently in patients in H risk strata (44.9% vs.40.5% I vs. 14.5% L, p<0.001) (Fig. 3).

Median nt-proBNP was 30 pg/ml (15-57) and values >100 pg/mL were observed in 26 (out of 199) patients (13.1%); increased nt-proBNP values were observed in 17.6%, 3.1% and 0% in low, intermediate and high risk strata (p=0.010) (Fig 5).
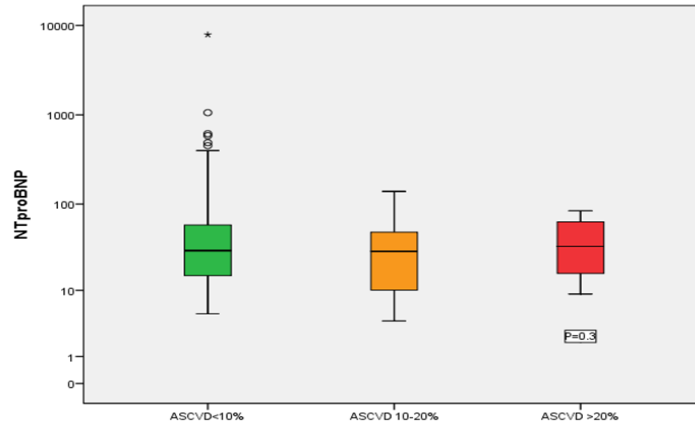

Figure 5: NTproBNP values according to 10 yy ASCVD strata: wide values variabilty in low risk group.

114 (22.6%) completed the whole cardiological follow up, 101 (88.6%) with TTE. According to cardiological examination, 50 patients (45%) were classified in NYHA group 1, 14.4% in group 2, 1.8% in 3. Repolarization abnormalities at ECG were recorded in 20.4%. SMWT was stopped prematurely by 9.8% for hypertension (53%), desaturation (33%) or other symptoms. EF<50% are reported in 4.5% cases without association with NT-proBNP. Measured PAPs in 56 patients (55.4%), increased values were observed in 16 participants (28.6%) Right ventricular dysfunction was observed in 30 patients (32%) and it was associated with a CD4 cell count <200/mcl (p 0.002).

PLASMA T-MAO CONCENTRATIONS

The cross-sectional study enrolled 175 participants: demographic and clinical characteristics of study participants are shown in Table 3.

	number or median	% or IQR
Age: years	50	44-55
Male gender: n (%)	139	79.4%

European ancestry: n (%)	139	79.4%
CD4+ T lymphocyte: Cells/uL	564	389-760
CD4/CD8 ratio:	0.9	0.5-1.1
CD4+ T lymphocyte nadir: Cells/uL	190	85-331
Plasma HIV RNA <50 copies/mL	156	90.7%
Active smokers*	64	42.7%
Hypertension*	20	13.2%
Type 2 Diabetes*	9	5.9%
Dyslipidemia*	13	8.6%
Number of comorbidities:*	1	1-2
Multimorbidity:* n(%)	19	12.5%
10 year Framingham CVR: %	8.6	4.6-17.5
5 year D:A:D CVR: %	3.2	1.2-6.8
10 year ASCVD CVR: %	5.1	2.4-12.2
10 year CUORE CVR: %	4.0	1.6-10
ARV classes: number of participants using		
NRTIs	142	81.1%
NNRTI	50	28.6%
PI	89	50.9%
INSTI	77	44%
R5-inhibitors	7	4%
Two drug regimens	26	14.9%
INSTI + PI	18	10.2%
Lamivudine + PI	4	2.3%
Lamivudine + INSTI	1	0.6%
NNRTI + INSTI	2	1.1%
NNRTI + PI	1	0.6%
Number of comedications	2	1-4
Polypharmacy: n (%)	17	11.2%

*detailed information were available in 151 participants

Table 3. Participants' characteristics. Data are expressed as numbers (percentage) or median values (interquartile ranges). "CVR", cardiovascular risk; "D:A:D", Data collection on Adverse Effects of Anti-HIV Drugs Study; "ASCVD", Atherosclerotic Cardiovascular Disease from the American Heart Association/American College of Cardiology; "ARV", antiretroviral; "NRTIs", nucleos(t)ide reverse transcriptase inhibitors; "NNRTI", non-nucleoside reverse transcriptase inhibitors; "PI", protease inhibitors; "INSTI", integrase strand transfer inhibitors.

Participants were mostly male (79.4%) and of European ancestry (79.4%) with a median age of 50 (44-55) years. The majority of patients had undetectable plasma HIV RNA and CD4 cell count above 500/mm3. Although there was a high prevalence of active smokers (42.7%), other CVR factors were less common (with 13.2% showing hypertension), thus generating median 10-year

CVR scores around 5% (4-8.6% according to the different algorithms). Comorbidities were observed in several participants and multimorbidity was present in 12.5%.

Median serum TMAO concentrations were 165 (103-273) ng/mL, and were higher in older individuals (rho=0.164, P=0.031), in participants with higher serum creatinine (rho=0.181, P=0.030), in non-smokers (205 vs. 153 ng/mL, P=0.007), in those receiving <3 antiretrovirals (244 vs. 157 ng/mL, P=0.009) and >5 comedications (250 vs. 144 ng/mL, P=0.004) and in those with multimorbidity (rho=0.343, P=0.002). No significant association was observed with antiretroviral or concomitant medications, except for higher TMAO levels in patients receiving acetylsalicylic acid (n=6; 299 vs. 146 ng/mL, P=0.004). A bivariate correlation was observed between CVR scores and serum TMAO levels (except for the ASCVD algorithm and with the highest correlation with the D:A:D score): patients presenting higher D:A:D risk score strata had higher TMAO concentrations (143 vs. 144 vs. 265 ng/mL, P=0.018). Fewer drug regimens were more common in patients with high CVR (28.5 vs. 10.1% in the low CVR strata), thus potentially explaining the effect observed at univariate analysis. At linear logistic regression (adjusting for age), multimorbidity (presenting ≥3 comorbidities) was independently associated with higher TMAO concentrations (P=0.015) (Fig. 6-7 left).
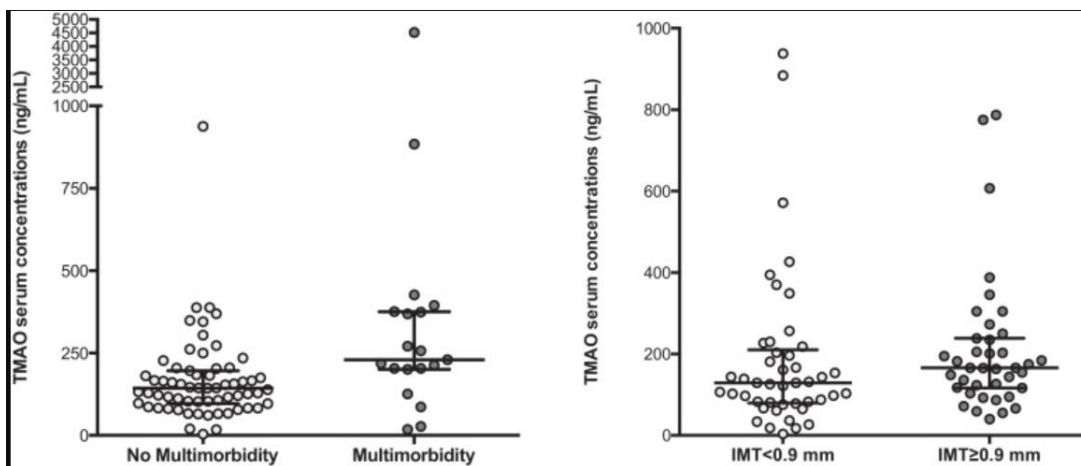

Fig. 6 - 7. Serum trimethylamine-N-oxide concentrations according to the presence/absence of multimorbidity (left) or abnormal carotid intima media thickness (right).

IMT was measured in 84 participants and the mean was 0.85 (0.71-1.21) mm; 39 participants (46.4%) had IMT measurements above the limit of 0.9 mm or carotid plaques. A trend towards

higher serum TMAO concentrations in patients with abnormally high IMT values was observed (166 vs. 129 ng/mL, *P*=0.087) (Fig. 7 right).

A total of 25 participants were enrolled in the prospective probiotic supplementation study in Rome. Participants were mostly male (18, 72%), with a median age of 47 (42-52) years; 21 (84%) participants had plasma HIV RNA <50 copies/mL and median CD4 cell count was 580 (521-716). Clinical and therapeutic characteristics did not differ from the whole cohort (data not shown). Serum TMAO levels at baseline were 95 (57-180) ng/mL and 24 weeks later were 166 (90-259) ng/mL, which was not significantly different (Wilcoxon paired *P*=0.220) (Fig. 8).
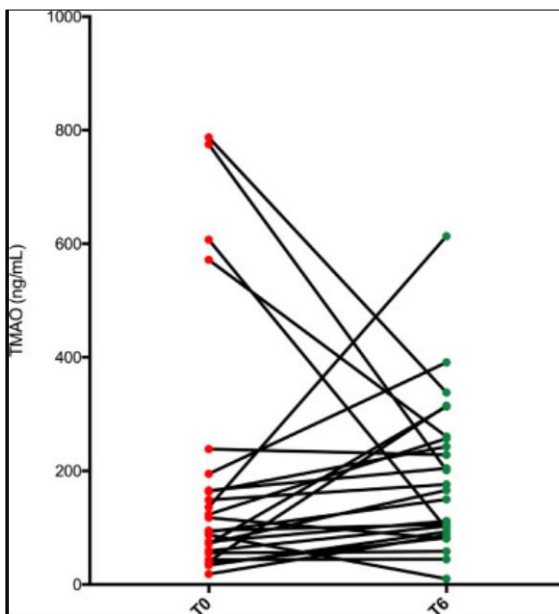

Fig. 8. Serum trimethylamine-N-oxide concentrations at baseline and 6 months after probiotic supplementation in 25 participants.

CHANGES IN THE GUT MICROBIOTA COMPOSITION AFTER DIET: NUTRITIONAL PILOT INTERVENTION SUBSTUDY

Characteristics of the participants - Ten participants were enrolled and were evaluated before and six months after dietologist evaluation for a person-tailored nutritional intervention. HIV infection

was under control in all partecipants and high CD4+ T-cell counts 568/mm^3 (398-625). Table 1 shows baseline and T6 values and paired test *p* values.

From enrolment to the study end, weight and Body Mass Index (BMI) decreased; the majority of metabolic and inflammatory parameters remained stable (systolic and diastolic BP, fasting glucose, CRP), some improved (fasting insulin and HOMA index), some worsened (total and HDL cholesterol, triglycerides). TMAO concentrations increased significantly (p 0.05). Table 4 and Fig 9
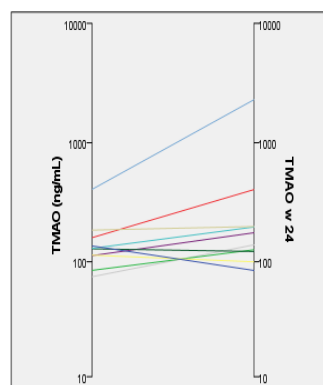

Fig 9- BL to W24 TMAO trend in 10 patients PLWH

Adherence to the dietary recommendations. After the dietary counselling, about 75% of participants declared to be adherent to the given dietary recommendations. Generally patients showed reduced totally intakes (*p* 0.022) in particular of carbohydrates (*p* 0.074), satured fat (*p* 0.007), cholesterol (*p* 0.011), protein (*p* 0.074) and alcohol (*p* 0.068). Table 4.

	At enrolment n 10 (med/IQR)	Study end n 10 (med/IQR)	p
Age years	60.5 (54-69)		
Weight Kg	79.1 (66.5-89.3)	77.3 (68.9-91.7)	.441
BMI kg/m^2	27.5 (22.2-29.15)	26.8 (21.9-29.6)	.678
Systolic BP (mmHg)	132.5 (120-140)	132.5 (120-142.5)	.587
Diastolic BP (mmHg)	80 (77.5-82.5)	80 (77.5-81.2)	.660
Fasting glucose (mg/dL)	97 (88-115)	97.5 (87.7-123.7)	.953
Fasting insulin (microU/ml)	12.9 (7.7-15.5)	11.9 (6.1-14.4)	.386
HOMA – IR (mmol/L*mclU/ml)	3.26 (1.67-4.08)	2.55 (1.78-14.4)	.508

Total cholesterol (mg/dL)	180.5 (154.7-207.25)	187 (167-202)	.61
HDL-cholesterol (mg/dL)	53 (42.2-57)	44.5 (41.7-50.5)	.12
Triglycerides (mg/dL)	118 (96-201)	137 (100.7-247)	.575
CRP (mg/dL)	0.4 (0.4-0.45)	0.4 (0.4-0.4)	.655
TMAO (ng/mL)	**128.9 (105.48-165.81)**	**157.1 (116.4-249.6)**	**.05**
eGFR (ml/min)	88.5 (74.7-94.2)	87.5 (66.5-104.7)	0.91
ASCVD score (%)	**11.7 (7.2-21.2)**	**12.6 (9.8-21-65)**	**0.01**
Dietary intakes			
Energy (kcal/die)	1572 (1421-1694)	1571 (1434-1714)	.093
Carbohydrates (% total kcal)	229.2 (188-274.7)	200 (141.2-220)	.074
Lipid (%)	65.2 (54.5-80.75)	62.5 (53.7-65)	.114
SFA (%)	**27 (17.7-42.85)**	**14 (13-15.5)**	**.007**
MUFA (%)	34 (27-43.6)	41.5 (34.7-45.6)	.153
Polyunsatured fatty acids (%)	8.35 (5-10)	7 (5.75-7)	.173
Cholesterol (mg/die)	**203.5**	**173 (85-180)**	**.011**
Sodium (mg/die)	1535 (1295-2270)	1225 (1165-1348)	.093
Protein (g/die)	75 (60.5-84.75)	57.5 (53.7-70)	.074
Fiber (g/day)	18 (14.25-21)	17.5 (15.7-21.2)	.953
Alcohol (kcal/day)	56.5 (0-142.5)	44 (0-102.75)	.068
Tot intake (Kcal/die)	**1937 (1667-2316)**	**1602 (1475-1718)**	**.022**
Muscolar mass (Kg)	30.9 (26.8-34.8)	30.9 (27.2-35-3)	.046
Fat free mass (%)	55.6 (48.7-61.3)	55.6 (49.2-62.2)	.093
Total body water (%)	40.8 (35.7-45)	40.8 (36.1-45.7)	.046
Body fat %	28.8 (24.5-33.65)	28.6 (20.7-33.6)	.721
PLWH parameters			
Current CD4 count (cell/mcl)	568 (398-625)	558.5 (431-735)	.327
CD4/CD8 ratio	0.85 (0.3-1.5)	0.8 (0.35-1.3)	.68
INIs (n)	5/10	5/10	
PIs (n)	5/10	5/10	
NNRTIs (n)	3/10	3/10	
NRTIs (n)	8/10	8/10	
CCR5 inibitors (n)	2/10	1/10	

Table 4 - Characteristics of the participants at enrolment and at the study end. BMI=body mass index, BP=blood pressure, HOMA-IR=Homeostasis Model Assessment-Insulin Resistance, HDL=high density lipoprotein, LDL=lowdensity lipoprotein, CRP=C-reactive protein, SFA=saturated fatty acids, MUFA=polyunsatured fatty acids, PUFA=polyunsaturated fatty acids, Values are expressed as median (interquartile range) and p values expressed by Wilcoxon matched pairs test.

Microbiota composition at enrolment and at the study end

The gut microbiota composition before the diet presented high prevalence of *Bacteroides*, *Clostridiales*, *Lachnospiraceae* and *Ruminococcaceae* relative abundance (Fig. 10).
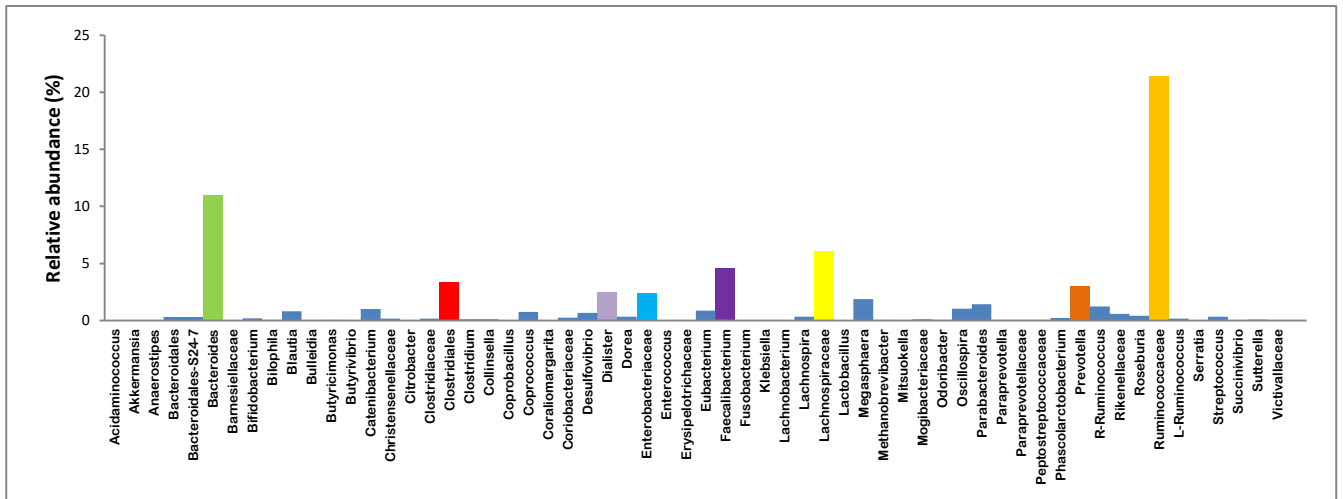
Fig. 10 Gut microbiota composition at enrolment (median value of relative abundance percentage).

The microbiota α-diversity values were not significantly different among subjects at enrolment when compared to subjects at the end of the study (p 0.33). Fig 11

The difference among T0 and W24 beta diversity is represented in Fig. 12 and is not significant according to ANOSIM test (p value 0.54).
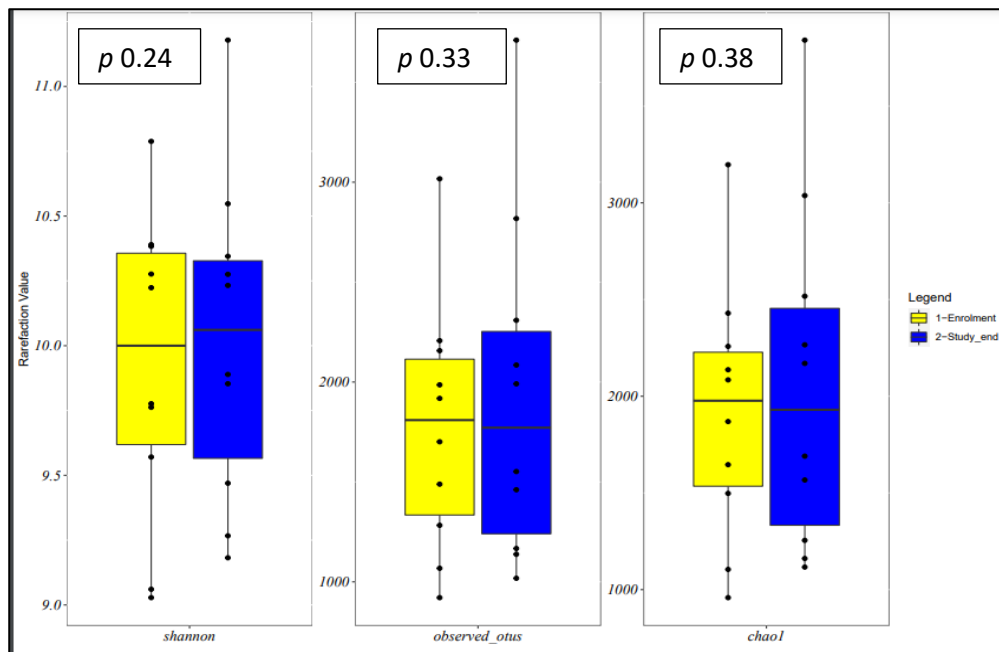

Fig 11 - Boxplots to describe α-diversity measures of fecal microbiota of PLWH patients at enrolment (yellow bars) and study end (blue bars) with Wilcoxon matched pairs test. Individual points and brackets represent the richness estimate and the theoretical standard error range, respectively.
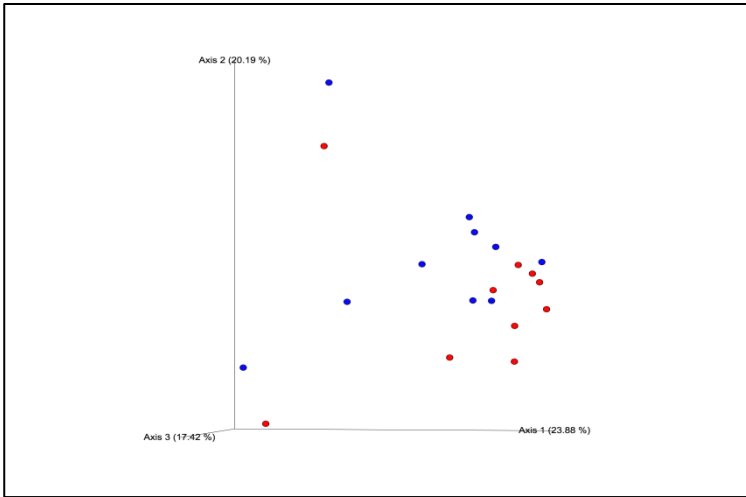
Fig. 12 - Principal component analysis (PCoA) showing beta diversity measured through Weighted UniFrac distance (T0 red; blue T1)

The analysis of microbial taxa abundance at phylum level showed a significant reduction of *Firmicutes* at the study end (p 0.047) and an increase in *Proteobacteria* (p 0.028) and *Bacteroidetes* (p 0.2) (Fig. 13).
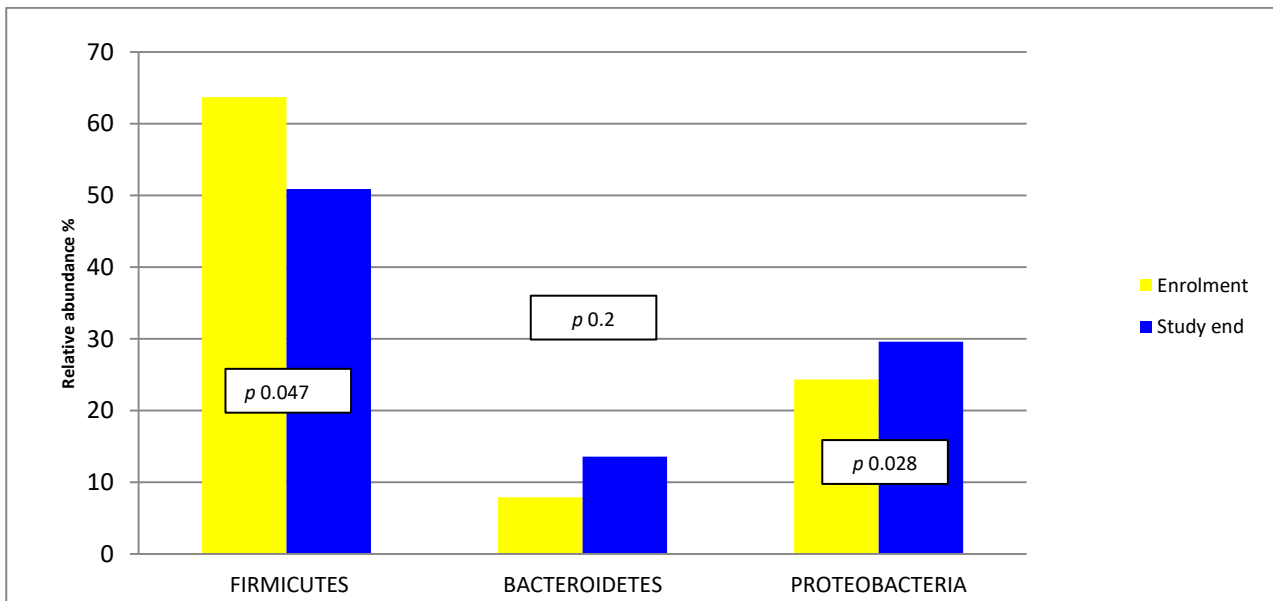
Fig. 13 . Boxplots showing the relative abundance of *Firmicutes*, *Bacteroidetes* and *Proteobacteria* phyla in fecal samples of PLWH patients at enrolment (yellow bars) and study end (blue bars).

Boxplot at genus level showed a signifcant reduction in the abundance of *Ruminococcaceae* (*p* 0.007) and a significant increase of *Prevotella* (*p* 0.036), *Succinvibrio* (*p* 0.028) and Lachnospiraceae (*p* 0.13) (Fig 14 a-b-c) at the study end compared to baseline (BL). Considering

family level there is a significant reduction of *Ruminococcaceae*, including *Faecalibacterium*, *Oscillospora*, *R- Ruminococcus* and *Ruminococcaceae* family (p 0.028) and order of *Clostridiales* (p 0.005) (Fig 14 d-e-f) at W24 compared to BL.

TMAO and Chao-1 average variation increase progressively as Fig. 15 shows.

Fig. 14 a-b-c-d-e-f Relative abundance of *Ruminococcaceae* (*p* 0.007), *Prevotella* (*p* 0.036), *Succinvibrio* (*p* 0.028), *Lachnospiraceae* (*p* 0.13) and *Clostridiales* (*p* 0.005) at baseline to W24.
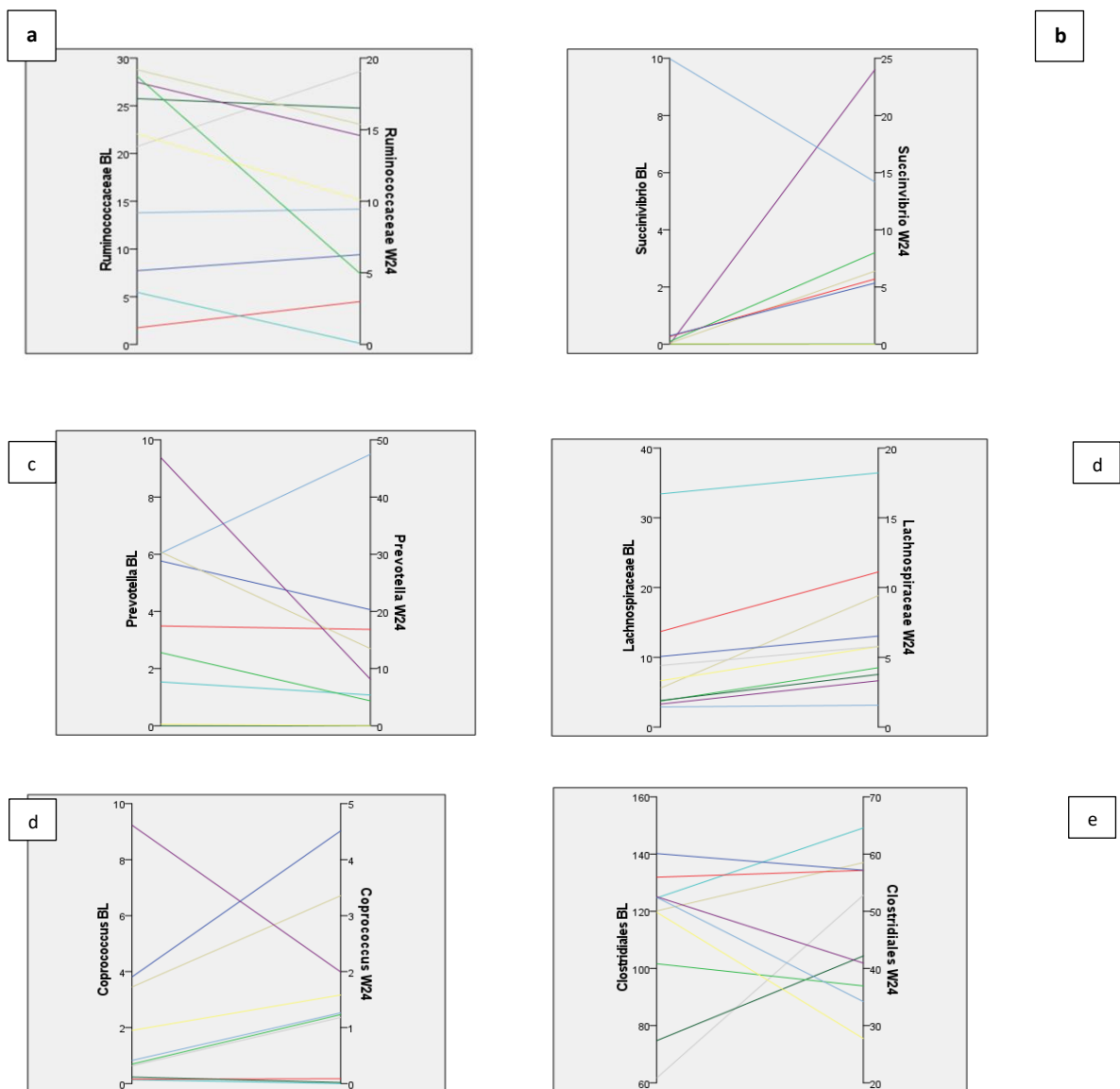
Fig. 15 Bland-Altman plot shows the correlation between CHAO1 and TMAO average variation.
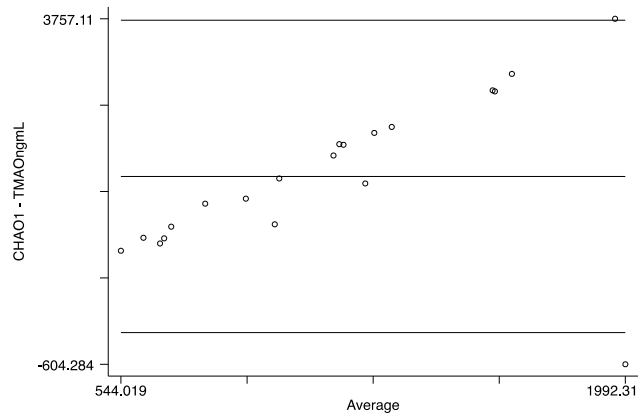
At study end α diversity positive correlated with liver function (AST p 0.021 rho 0,71 ALT p 0.015 rho 0,73) and cART duration (p 0.038 rho -0,6) (Fig. 16 a - b)
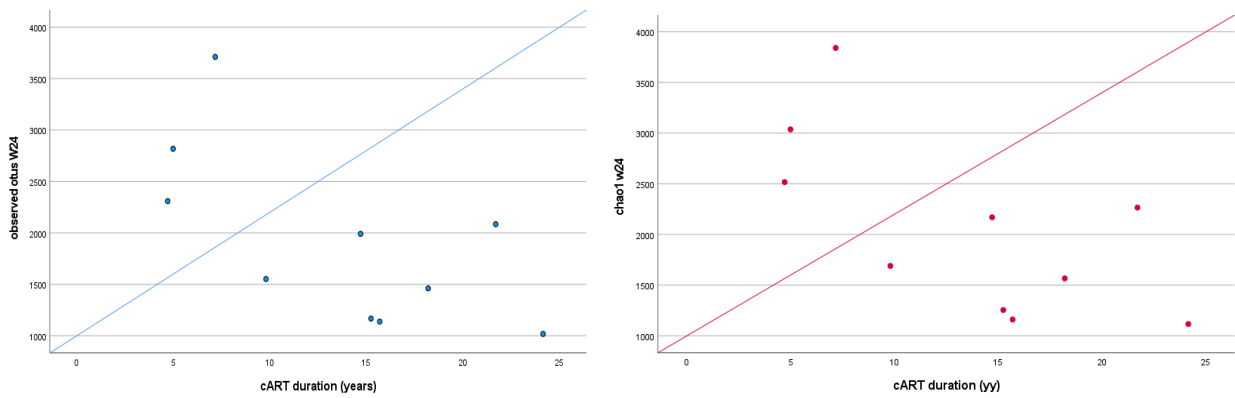
Fig. 16 a Study end observed otus correlates with cART treatment duration (in years) on the left Fig. 16 b Chao 1 inversely correlated with cART duration.

Fig 17 - Heatmap showing Spearman' rho values between changes in microbiota and anthropometric, metabolic, and lifestyle variables from baseline to end of study. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GGT, g-glutamyltransferase; HOMA-IR homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein (Red rho -1; blu rho + 1).

In summary, there were positive (direct) associations between *Akkermansia* and changes in carbohydrates T0 – T24 intake (delta - Δ); *Coprococcus* and *Paraprevotella* with Δ weight and BMI; *Bifidobacterium* and *Rikenellaceae* respectively with CD4 ratio and cell count; *Anaerostipes*, *Barnesiellaceae*, *Collinsella*, *Dialister*, *Dorea*, *L-Rumicoccus* and *Sutturella* with T and HDL CHOL, *Aneorostipes* and *Mogibacteriaceae* with tryglicerides, *Butyricimonas*, *Eubacterium*, *Odoribacter*, *Paraprevotella* and *Streptococcus* with Δ glucose levels, *Lachospiraceae* and *Megasphaera* with fasting insulin and consequently HOMA index.

Butyrivibrio, *Clostridiaceae*, *Coriobacteriaceae*, *Eysipelotrichaceae*, *Methanobreviacter*, *Mitsukella*, *Ruminococcaceae*, *Victivallaceae* were directly associated with CRP variation; *Bacterioidales*, *Catenibacterium* with AST difference, *Coproccoccus* and *Desulfivibrio* even with ALT; *Faecalibacterium* and *Serratia* with 25 OH vitamin D levels; *Clostridium*, *Phascolarctobacterium* and *Serratia* with *e*GRF function.

Considering the nutritional variation, *Bacteriodes* and *Peptostreptococcus* were associated with energy intake, muscolar mass, total body water and fat free mass; *Anaerostipes*, *Collinsella*, *Dialister*, *Erysipelotrichaceae* and *L- Ruminococcus* with MUFA, *Butyrivibrio*, *Coriobacteriaceae*, *Ruminococcaceae* with lipids and SFA, *Clostridiaceae* and with SFA and *Methanobrevibacter* and *Victivallaceae* generically with lipids intake; *Akkermansia* e *Roseburia* with carbohydrates, *Coprococcus* and *Desulfovibrio* with cholesterol intake, *Bacterioidales* and *Mogibacter* with sodium; *Anaerostipes*, *Bilophila* and *Streptococcus* with protein; *Faecalibacterium, Oscillospira, L- Ruminococcus* and *Sutterella* with fiber; *Klebsiella* and *Roseburia* with alcohol intake.

TMAO concentrations and ASCVD score were positively associated with *Barnesiellaceae*, *Clostridium* (fig 17), *Enterococcus, Lachnospira, Lactobacillus* and negatively with *Eubacterium, Odoribacter, Paraprevotella* and *Streptococcus.*

Conversely, negative (inverse) associations were found between *Citrobacter* and *Succinvibrio* with weight and BMI variation from T0 to T24; *Serratia* with CD4 ratio and cell count; *Bacterioidales, Coprabacillus* and *Peptostreptococcus* with T- HDL and tryglicerides Δ; *Clostridium, Enterobacteriaceae, Enterococcus, Lachnospira, Lactobacillus* with glucose level; *Bifidobacterium* and *Klebsiella* with fasting insulin and HOMA index. *Mogibacteriaceae, Prevotella and Roseburia* with CRP Δ. *Citrobacter, Succinvibrio, Lachnospiraceae*

and *Mogibacteriaceae* were inversely correlate with liver function markers, *Mogibacteriaceae* and *Rekinellaceae* with 25 OH vitamin D levels. About diet, *Collinsella* was relate with energy intake, muscolar mass,total body water, fat free mass; *Prevotella* with lipids in particular SFA intake; bacteroidea with carbohydrates and fiber variation; *Citrobacter, Megapherae and Peptostreptococcus* with sodium intake; *Lactobacillus* with protein; *Bacteroides, Coprobacillus* and *Parabacteroides* with fiber intake variation.
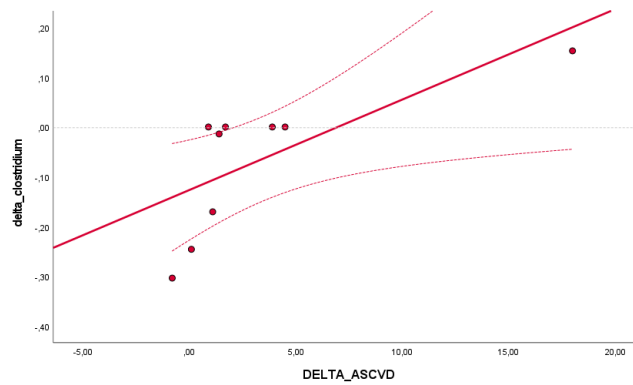

Fig. 18 ASCVD score variation associated with *Clostridium.*

DISCUSSION

Mounting data suggest that HIV infection leads to an excess incidence of cardiovascular disease. Risk factors for cardiovascular disease should be evaluated at the initial visit and continually assessed over time according to international guidelines (131). The optimal approach to cardiovascular risk reduction in PLWH is not precisely defined, but it is widely accepted that, in addition to suppressing HIV viremia, the same risk reduction strategies that are used in uninfected individuals should apply (132).

The aim of this project was to screen PLWH on cART and without previous cardiovascular major events in order to characterize a high-risk group.

We enrolled 503 HIV-positive patients: 91.4% on cART and 80% with undectectable plasma HIV RNA, high current CD4 count (554/ul) (despite a nadir CD4 count of 200 cells/mm3) after about 10 years of exposure to cART. According to 10yy ASCVD algorythm, we classified our participants' risk in low (58.5%), intermediate (27.3%) and high (14.3%).

Those who are classified as intermediate and high risk (41.6%) presented some peculiar feactures in comparison to low risk group. First af all, they did not reach adequate control of TC, LDL-C, Hb1Ac and SBP despite the use of lipid-lowerting (82%), anti-hypertensive (44.1%) and/or hypoglycemic (50%) co-medication (*p*<0.0001). Conversely, the use of several co-medications has been associated with unfavourable outcomes in elderly HIV-negative individuals and increasing data have been reported in ageing HIV-positive individuals (133). More then two thirds were active smokers with prevalence of 49% in comparison with 20-21% in general population (134). Furthermore, thicker IMT values were observed more frequently in patients in H risk strata (44.9% vs.40.5% I vs. 14.5% L, p<0.001). Increased nt-proBNP (> 100 pg/ml) values were observed of high risk strata (p=0.010): despite potentially being associated with uncontrolled hypertension this is particularly worrisome since it has been associated with all-cause death in prospective studies.

Future goals would be apply the strategies for minimizing the risk of CVD including blood pressure control, management of diabetes (119), exercise and weight reduction (121) and smoking cessation.

The decision on whether to recommend aspirin for the prevention of cardiovascular disease in the patient with HIV is the same as that for the general population.

The clinical impact of aspirin has not been extensively studied in patients with HIV. A randomized trial of two doses of aspirin (100 and 300 mg) over 12 weeks in adults with HIV on ART failed to demonstrate an impact on endothelial function or immune activation (135).

High-dose statin may be appropriate for a small number of patients who have tolerated it well for many years or who are intolerant of other high-potency statin options (136).

Participants in the I/H risk group were visited by cardiologists (n 114). A significant prevalence of increased pulmonary artery pressure and right-sided cardiac dysfunction was observed in 30 patients (32%) and it was associated with a CD4 cell count <200/mcl (p 0.002). Besides incomplete immunological recovery no biomarker or screening test was predictive of this cardiac impairment.

Serum TMAO levels observed in this study are similar to those observed in other studies; the only exception is the study by Srinivasa et al., where serum TMAO concentrations were low (0.7 μM vs. 1.2-5.7 μM in the others) and not associated with study outcomes (47).

The concentrations were similar to those reported in HIV-negative and in HIV-positive individuals (137). Higher serum values were observed in patients with higher risk of cardiovascular complications who presented with multimorbidity, polypharmacy and subclinical vascular damage measured by abnormal IMT/plaque presence even after adjusting for age, which indicates it may represent a marker of individual frailty or that nutrition may be a relevant factor for both features.

Moreover, higher TMAO concentrations were not associated with CV risk factors but with reduced renal function and, unexpectedly, with the absence of smoking habit.

In this setting, the relative contribution of high serum TMAO levels needs to be demonstrated; however, as serum TMAO levels are a key factor in atherosclerosis and, less certainly, in renal

damage, these should be taken into account when planning preventive intervention in high-risk patients. The trend towards higher carotid IMT in patients with higher serum TMAO levels is in line with previous work in PLWH: the recent report of longitudinal increase in carotid atherosclerosis in patients with high serum TMAO levels indicates this could be a surrogate biomarker to plan tailored interventions. The lack of association with single comorbidities, antiretroviral drugs or comedications supports the complex mechanisms in TMAO formation that include diet, gut microbiome and hepatic oxidation.

Based on these characteristics, we sent part of I/H risk group to cardiologist (n 114). A significant prevalence of increased pulmonary artery pressure and right-sided cardiac dysfunction was observed in 30 patients (32%) and it was associated with a CD4 cell count <200/mcl (p 0.002). Besides incomplete immunological recovery no biomarker or screening test was predictive of this cardiac impairment.

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Two unexpected associations were described. The first was the observation of higher serum TMAO levels in patients receiving less than three antiretroviral drugs. This was largely driven by a higher CVR in individuals who were treated with two-drug regimens to avoid nucleoside reverse transcriptase inhibitors (NRTIs) associated with significant side effects (such as tenofovir disoproxil fumarate and abacavir). The second association, lower serum TMAO levels in HIV-positive smokers, cannot be clearly explained; however, smoking has been associated with changes in intestinal microbiome and local immunity that may affect TMAO formation (138). Finally, no significant change in serum TMAO levels was observed following 6 months of high-dose probiotic supplementation. Dietary interventions may be more effective in contemporarily affecting TMAO and other pathways supporting atherosclerosis (139). In HIV-negative participants, a greater adherence to a Mediterranean diet (and a higher consumption of plant foodstuffs) was associated with beneficial microbiome-related metabolomic profiles with lower serum and urinary TMAO levels (140). Beneficial immunological, inflammatory and neurocognitive changes were observed with high-dose probiotic supplementation in this cohort, thus indicating an effect on different gut microbes or metabolic pathways (126,141).

NUTRITIONAL PILOT INTERVENTION SUBSTUDY

Several studies have suggested that the gut microbiota contributes to HIV-associated inflammation; yet the complete understanding of its role in this process is hampered by an incomplete knowledge of the precise identities of microbiota members that are altered in PWH.

A pilot study was performed in PLWH with higher TMAO level and/or H ASCVD risk despite persistent viral control.

The gut microbiota composition presented a major prevalence of *Firmicutes* phylum, known to be higher in PLWH in comparison to HIV negative population (64) in particular considering the ratio of *Firmicutes/Bacteroidetes*.

Before the diet, the composition showed a high relative abundance of *Bacteroides*, *Clostridiales*, *Lachnospiraceae* and *Ruminococcaceae*. In particular, *Lachnospiraceae* and *Ruminococcaceae* species in the gut have been shown to induce the expansion of regulatory T cells that can down-regulate harmful inflammatory responses (142). Infact, they produced short-chain fatty acid (SCFA) metabolites, derived from commensal bacteria in these clades and others, promoting intestinal barrier integrity via their role in epithelial cell energy metabolism and induction of regulatory T cells (143).

Personal tailored diet intervention was started evaluating carbohydrates, lipids (divided in SFA, MUFA, PUFA), cholesterol, sodium, protein, fiber, alcohol and total daily intake. Diet is known to affect gut microbiota composition. Conversely, HIV gut microbiota survey studies that have examined diet have found evidence that dietary intake in PWH does not correlate with abundances of gut microbes of the HIV-associated microbiota signature as it does in the seronegative population (21–23).

For example, relative abundance of Bacteroides members correlates with meat intake in seronegative subjects (75,145), but this association was not found in PWH (75), suggesting HIV infection may have a greater impact on the abundance of certain microbes than do certain major

components of diet. Furthermore, dietary patterns between PWH and seronegative participants did not differ significantly(75,144), suggesting that diet alone is unlikely to be the driver of the HIV-associated microbiota signature.

Partially adherence was declared by the patient.

Generally patients showed reduced totally intakes (*p* 0.022) in particular of carbohydrates (*p* 0.074), satured fat (*p* 0.007), cholesterol (*p* 0.011), protein (*p* 0.074) and alcohol (*p* 0.068).

We obtained not significant reductions in weight and BMI stability of systolic and diastolic BP. Moreover fasting glucose, CRP, mild fasting insulin and HOMA index increasing and total and HDL cholesterol, triglycerides worsening. TMAO concentrations increased significantly (p 0.05).

It's known that dysbiosis has been implicated in the development of atherosclerosis through metabolism-independent and metabolite-dependent pathways (146). When dysbiosis is induced by a high fat diet, there will be a reduction in the number of Bifidobacterium, which function as an intestinal barrier preventing bacterial translocation (147). Dysbiosis can also reduce the expression of the intestinal tight junctions proteins, further increasing intestinal permeability and allowing LPS to enter circulation, which will promote inflammation (148). Beyond the metabolism-independent pathway, dysbiosis can exert pro-atherosclerotic effects by altering a variety of metabolites, such as trimethylamine-N-Oxide (TMAO), bile acids, and SCFAs (149).

We report here an increase in TMAO concentrations after the diet intervention: able to modify the metabolism of cholesterol and sterol, suppressing the reverse transport of cholesterol (150), correlated with α diversity parameter (Chao 1) and pathogen bacteria relative abundance increase suggests to persue improving the diet adherence in PLWH.

The microbiota α-diversity and β-diversity values were not significantly different among subjects at enrolment when compared to subjects at the study end (respectively *p* 0.33 and 0.54).

The analysis of microbial taxa abundance at phylum level showed a significant reduction of *Firmicutes* at the study end (p 0.047) and an increase of *Proteobacteria* (p 0.028), known to be enriched for producers of SCFA (143), a class of compounds that provide energy for gut epithelial

cells, prevent expansion of proinflammatory and induce differentiation of immunologic tolerance-promoting T regulatory cells (143) and *Bacteroidetes* (p 0.2), which are important factor in the maintenance of systemic inflammation is the depletion of anti-inflammatory bacteria (73–75).

A significant reduction in the abundance of *Ruminococcaceae* (*p* 0.007), *Clostridiales* (p 0.005) and a significant increase of *Prevotella* (*p* 0.036), *Succinvibrio* (*p* 0.028) and *Lachnospiraceae* (*p* 0.13) at the study end compared to enrolment were observed.

Recently it has been reported a decline in the frequencies of *Lachnospiraceae*, *Ruminococcaceae*, *Rikenellaceae* and *Bacteroides*—bacterial taxa linked with anti-inflammatory properties and maintenance of gut homeostasis— in PWH with varying degrees of consistency (22,31–36).

Studies have also described an increased abundance of *Prevotella* in PWH as compared to HIV-uninfected controls (73,75–77), though these taxonomic shifts have been observed with less consistency. Recent seminal studies have reported an increased abundance of *Prevotella* and depletion of *Bacteroides* in the gut microbiota among men who have sex with men (MSM) as compared to men who have sex with women (MSW), independently of HIV-1 infection status (78–80).

Regarding the food an agrarian diet lead to an increase in *Prevotella*, whereas diets rich in proteins and fats lead to an increase in *Bacteroides* and *Clostridiales* (154–156). *L-Ruminococcus* has been positively associated with omnivorous diets and particularly with animal-based food (140).

Although limited by the small sample size we observed tha OTUs and chao1 were higher in those who had been on cART for less time. It is possible that the extent of immune damage (proportional to the extent of CD4 T cell loss, i.e. CD4 nadir) dictates the degree of dysregulation of microbiota composition but is itself the cause of later age-associated comorbidities, with the effects on the microbiota contributing little to pathology (81). There were no correlation with CD4 count and nadir in our group that may confirm this so called "legacy effect".

Furthermore, recent evidences suggest that ART, as well as many other drugs of common use among HIV infected patients for comorbidities control, can exert antibiotic properties on the gut

microbiome, also affecting species with key roles related to healthy status such as major butyrate producers (*E. rectale*, *R. intestinalis, Coprococcus comes*), propionate producers (*B. vulgatus*, *Prevotella copri, Blautia obeum*) and enterotype drivers (*P. copri*) (157–159).

Moreover TMAO and Chao-1 avarage variation increase progressively. It's known how an oxidative environment thus favors the growth of commensals with antioxidant capabilities and higher metabolic versatility (143,160,161). *Bacteroides* spp. or *Proteobacteria*, for example, are ROS/RNS-resistant bacteria able to use a wide range of final electron acceptors during anaerobic respiration, including nitrate, TMAO, dimetil sulphoxide (DMSO) and tetrathionate as Fig 1 intro part represents (162). In a recent study, Guillen et al (163) suggested that genes encoding for enzymes implicated in the utilization of the latter compounds were enriched in low gene counts individuals, and were mainly encoded by *Bacteroides* and *Proteobacteria.*

Finally, we found different direct and indirect correlation among metabolic, clinic, diet delta variation and single bacteria delta variation. Among these some related with diet intervention are known (164,165) for example *Bacteriodes* and *Peptostreptococcus* associated with energy intake, muscolar mass, total body water and fat free mass; *Anaerostipes*, *Collinsella*, *Dialister*, *Erysipelotrichaceae* and *L- Ruminococcus* with MUFA, *Butyrivibrio, Coriobacteriaceae, Ruminococcaceae* with lipids and SFA, *Clostridiaceae* and with SFA and *Methanobrevibacter* and *Victivallaceae* generically with lipids intake; *Akkermansia* e *Roseburia* with carbohydrates, *Coprococcus* and *Desulfovibrio* with cholesterol intake, *Faecalibacterium, Oscillospira, L-Ruminococcus* and *Sutterella* with fiber; *Klebsiella* and *Roseburia* with alcohol intake (166).

At the same tame, TMAO concentrations and ASCVD score changes were directly associated with SCFA producer bacteria as *Lachnospira* and *Eubacterium and some* pathogen bacteria for example *Clostridium*, *Enterococcus* and inversely with *Paraprevotella* and *Streptoccus* well documented sources of bacteremia or comorbidities.

Modulation of microbial metabolism through dietary intervention or direct supplementation might provide an effective strategy for preventing CVD.

LIMITATIONS

The small sample size and the short follow-up represent limitations of the present study, not allowing for a more detailed interpretation of the results. However, these are preliminary data of an exploratory pilot trial to design a larger trial with a longer follow up. Further limitations are the lack of gut microbiota analysis after one year to assess later microbial shifts because microbial communities are resilient and resistant to change (167); the lack of evaluation of psychological and cognitive aspects of participants, because of the known interaction between those characteristics and the gut microbiota (168) ; and the lack of sexual orientation/behavior/dysfunction assessment, which could be modified by the microbiota modulation (169). The sexual preference data was not collected at the beginning. This is a limitation since MSM cohort from non MSM is associated with strong dysbiosis.

CONCLUSION

In this cohort of well-treated PLWH with no prior major cardiovascular event we assessed cardiovascular risk and associated factors.

According to our evaluation, high risk score is related with poor adherence (by patients or physician) to concomitant drugs and strategies and for a lack of effect in the strategies aimed at modifying known risk factors. NT pro BNP and serum TMAO levels were higher in patients with higher CVD risk suggesting common pathways or an independent reciprocal effect. Carotid intima-media thickness may be an early subclinical damage signal.

The small sample size and the short follow-up represent limitations of the present interventional pilot study, not allowing for a more detailed interpretation of the results. Patients will need to be followed up more constantly to check diet adherence.

However, these are preliminary data to design a larger trial with a longer follow up.

Patients will need to be followed up more constantly to check diet adherence.

We reported an high relative abundance of *Bacteroides*, *Lachnospiraceae* and *Ruminococcaceae,* bacterial taxa linked with anti-inflammatory properties and maintenance of gut homeostasis, before the diet. A significant reduction in the abundance of *Ruminococcaceae*, *Clostridiales* and a significant increase of *Prevotella* and *Succinvibrio* after diet as well as TMAO concentrations and ASCVD score increasing suggest that patients will need to be followed up more constantly to check diet adherence.

Tailored diet interventions aimed at reducing the dietary nutrients intake of choline and carnitine (which are found in red meats, cows and sheep milks, dairy products, fatty fish and eggs) should be extended to most of PLWH with increased cardiovascular risk and permanently followed in the HIV outpatient clinic.

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