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RESEARCH ARTICLE

Bone Deleterious Effects of Different NRTIs in Treatment-naïve HIV Patients After 12 and 48 Weeks of Treatment

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Abstract: ***Background*:** Bone alterations have been observed in the course of HIV infection, characterized by a marked decrease in bone mineral density (BMD) and an increase in the frequency of fractures as a result of fragility. We aim to evaluate early changes in bone metabolic profile and the possible association with tenofovir and other nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) in treatment-naïve HIV patients.

***Methods*:** We conducted a prospective study in naïve HIV-infected adults (under 50 years), separated into three groups according to NRTI therapy: tenofovir disoproxil fumarate (TDF); tenofovir alafenamide (TAF) and abacavir (ABC). BMD and epidemiological, immunological and metabolic bone parameters were evaluated. Bone markers were analyzed in plasma at baseline, 12 and 48 weeks after initiating treatment.

***Results*:** Average age of patients was 34.8 years (± 9.6). 92.4% of them with CD4 count > 200 cel/μL. At week 12 after starting treatment, both TDF [increase in PN1P (31.7%, p = 0.004), TRAP (11.1%, p = 0.003), OPN (19.3%, p = 0.045) and OC (38.6%, p = 0.001); decrease in OPG (-23.4%, p = 0.003)] and TAF [increase in 42.6% for CTX (p = 0.011), 27.3% for OC (p = 0.001) and 21% for TRAP (p = 0.008); decrease in OPG (-28.8%, p = 0.049)] presented a deep resorption profile compared to ABC, these differences in bone molecular markers, a tendency to equalize at week 48, where no significant differences were observed. Patients treated with TDF showed the greatest decrease in Z-score in both lumbar spine (LS) and femoral neck (FN) at week 48 without statistically significant differences.

***Conclusion*:** Treatment-naïve HIV patients have a high prevalence of low bone density. Treatment with TDF is associated with greater bone deterioration at 12 and 48 weeks. TAF seems to present similar early bone deterioration at 12 weeks which disappears at 48 weeks.

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1. INTRODUCTION

The emergence and development of combination antiretroviral treatment (cART) have increased not only the life expectancy of people living with Human Immunodeficiency Virus (PLHIV), but also associated comorbidities, the so-called serious non-AIDS events (SNAEs) [1, 2]. HIV infection affects tissues and organs such as the heart, kidney and liver, as well as the central nervous system and the musculoskeletal system, with changes similar to those observed in aging [3]. In recent years, the incidence of bone alterations during the course of HIV infection has increased, characterized by a notable decrease in bone mineral density (BMD) in the lumbar spine (LS) and femoral neck (FN), and an increase in the frequency of fractures [4]. Both the risk factors associated with HIV infection (malnutrition, low body weight, high tobacco and alcohol consumption, and low levels of vitamin D) and the disease itself induce bone

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remodeling through a complex interaction of T cells with osteoclasts and osteoblasts. In adults [3, 5-8], a decrease in BMD has also been associated with prolonged antiretroviral treatment, principally tenofovir disoproxil fumarate (TDF), indicating that the initial loss of BMD after beginning cART may be due to effects of the medication [5, 6]. However, several studies have shown bone damage in therapy-naïve HIV-infected patients [3, 9, 10], cases in which the main guidelines do not specifically recommend evaluating BMD [11-13].

Nucleoside and nucleotide reverse-transcriptase inhibitors (NRTIs) are included in most of the recommended regimens for treating HIV infection. Among these, TDF has been widely used for many years based on its established efficacy and tolerance, as demonstrated in clinical trials and real-life studies [14-17]. It is widely used as first-line treatment of HIV infection, for pre- and post-exposure therapy, and for the treatment of hepatitis B virus infection. Adverse effects of TDF include proximal tubular kidney dysfunction, Fanconi syndrome and renal insufficiency [18, 19], in addition to the bone damage mentioned anteriorly in patients taking TDF for years [20, 21]. Recently, new drugs with an improved metabolic profile that apparently does not affect bone remodeling, such as tenofovir alafenamide (TAF), have been developed as an alternative to TDF [18, 19, 22].

Many clinical trials have demonstrated a tenofovir-associated bone loss in HIV patients [14, 15, 17, 20-24], both in therapy-naïve patients and in patients who have formerly received other antiretrovirals. These studies provide evidence for a direct role of tenofovir in BMD loss, as well as changes in bone remodeling biomarkers: C-terminal telopeptide of collagen type 1 (CTX), osteocalcin and procollagen type 1 N propeptide (P1NP), osteoprotegerin (OPG), sclerostin (SOST gene) and receptor activator for NF-κB ligand (RANKL) [25] but most of them show changes after 48, 96 or more weeks of treatment [26-29].

The aim of this study is to evaluate the early changes in bone remodeling in HIV patients without prior treatment during the first 12 and 48 weeks of treatment, analyzing a complete panel of bone profile markers and the changes in BMD observed by DXA.

2. MATERIALS AND METHODS

2.1. Subject and Design Inclusion and Exclusion criteria and Ethical Aspects

From May 2016 to September 2017, 110 treatment-naïve male patients under 50 years with a new diagnosis of HIV infection were included in the study. Before enrollment, the treatment was chosen by their physicians, in accordance with the standard of care based on current national and international guidelines.

Of the 110 therapy-naïve patients originally enrolled, 18 patients were switched to different regimens due to gastrointestinal intolerance or bone involvement and were excluded from the analysis (Fig. **1**). The 92 remaining patients were treated with TDF based regimen (elvitegravir/cobicistat/TDF/emtricitabine [Stribild®] available in hospital in 2016), TAF-based regimen (elvitegravir/cobicistat/TAF/emtricitabine [Genvoya®] available in hospital in 2017) and ABC based regimen (dolutegravir/ABC/lamivudine [Triumeq®]). Patients were followed for up to 12 months (48 weeks) after starting treatment. Immunological and bone turnover markers were evaluated at 0, 12 and 48 weeks after treatment initiation. DXA was done baseline and after 48 weeks from starting antiretroviral treatment.

Patients with the following criteria were excluded from the study: age over 50 years, previous antiretroviral treatment, bone-targeting treatment (denosumab, vitamin D), diabetes, treatment with corticosteroids, rheumatic diseases, renal failure, thyrotoxicosis, advanced liver disease, malabsorption syndrome, or neoplasia; based on a bone remodeling probably affected by these conditions.

Ethics committee approval was obtained from the institutional review board (PIC 155/16). All research was performed according to the right to privacy, guaranteed as stipulated in organic Regulation (EU) 2016/679 of the European Parliament and of the Council of April 27, 2016 on data protection (GDPR), on the protection of personal data, and the Declaration of Helsinki. All patients provided signed informed consent before being included in the study.

2.2. Measurements and Reference Values

Epidemiological aspects, and lifestyle habits, including exercise, calcium intake, and toxic habits (alcohol, tobacco, and drug consumption), were registered using a physician-administered questionnaire. Tobacco use was defined as any previous or current history of smoking, alcohol abuse as an intake of more than 30 grams of alcohol per day, and drug abuse as consumption of recreational drugs once a week or more. The exercise was defined as physical activity three or more times a week. A calcium-rich diet was defined as at least three rations per day of milk, yogurt, cheese, and/or vegetables [30]. The patients' height and weight were measured at the DXA appointment, and their body mass index (BMI) was calculated, with low weight defined as BMI < 20 kg/m^2 [31, 32].

A fasting blood test was ordered before starting treatment (baseline), and at 12 and 48 weeks after treatment initiation. Hematologic parameters related to phospho-calcium metabolism, such as 25-OH-Vitamin D (30-50 ng/ml), parathyroid hormone (PTH) (10-70 pg/ml) [33, 34], thyroid hormone (TSH) (0.35-5.5μUI/ml), and renal function were measured using the Advia 2400 system (Siemens®, Munich, Germany). Immunovirological parameters, such as CD4 and CD8 were measured by flow cytometry, and the HIV-1 viral load was measured by PCR (Roche, Basel, Switzerland). Other parameters related to bone and calcium metabolism were measured, including osteocalcin (OC) (14-46 ng/dl) (measured by luminescence immunoassay), carboxy-terminal telopeptide of type 1 collagen (CTX) (0.064-0.5 ng/ml), procollagen type 1 N-terminal propeptide (P1NP) (10.4-62 ng/dl) (both were quantified by Electrochemiluminescence

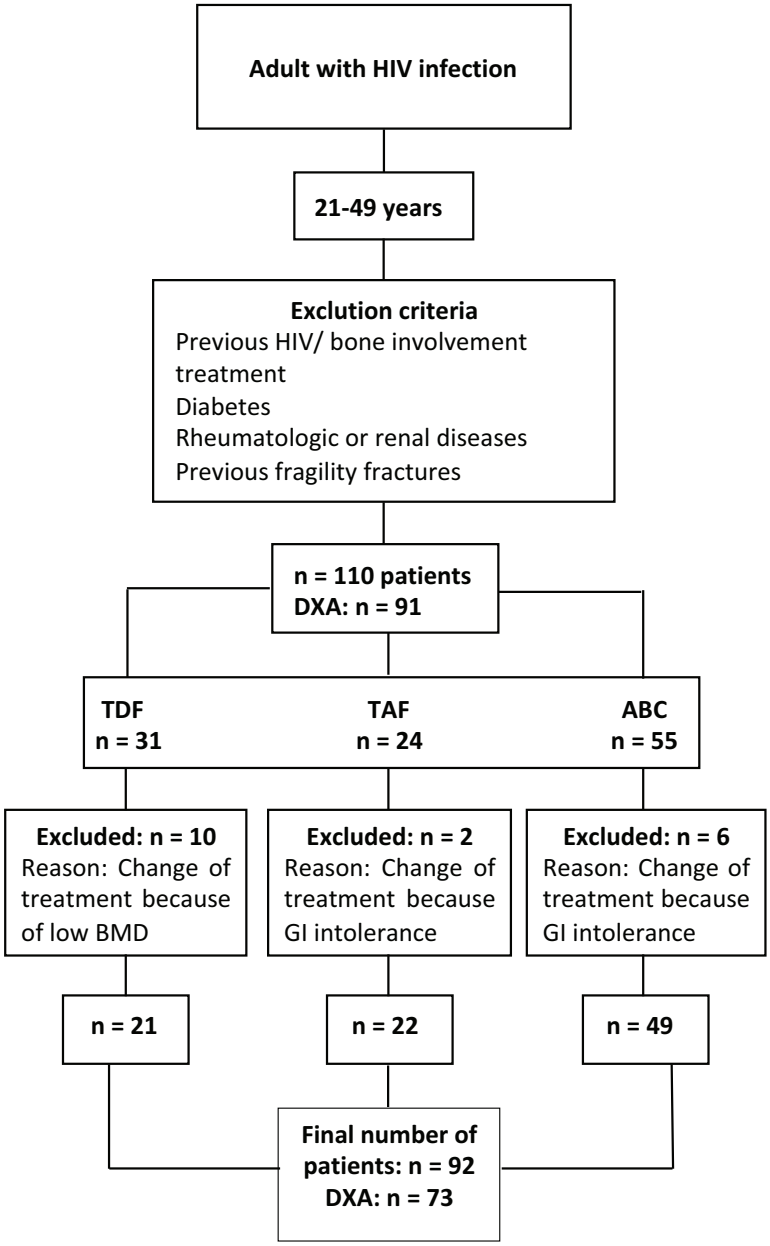

Fig. (1). Diagram of patients included in the study based on the antiretroviral treatments applied. GI (gastrointestinal), HIV (human immunodeficiency virus), TDF (tenofovir disoproxile fumarate), TAF (tenofovir alafenamide), ABC (abacavir).

immunoassay (ECLIA) and tartrate-resistant acid phosphatase (TRAP) (<4.6 U/L) (analyzed by kinetic assay). All of them were performed in the Department of Clinical Analysis and Biochemistry at Hospital Fundación Jiménez Díaz.

Hip and spine bone DXA (Dual X-ray absorptiometry) scanning (HOLOGIC QDR 4500C, Marlborough, MA, USA) was carried out at baseline and after 48 weeks from starting antiretroviral treatment. BMD was measured at the LS (L2-L4), FN and right TH (total hip). As participants were all under 50 years of age, the Z-score was considered, estimating values below -2.0 as a low BMD for their age [35, 36].

2.3. Bone Marker Measurement by Luminex

Luminex multiplex assays are designed to simultaneously detect and quantitate multiple secreted proteins (*e.g.*, cytokines and growth factors), and produce results comparable to conventional ELISA with greater efficiency and throughput [37]. Millipore`s MILLIPLEX MAP Human Bone Magnetic Bead Multiplex kit and MILLIPLEX MAP Human RANKL Magnetic Bead-Single Plex kit (Merk Millipore, Billerica, MA, USA) were used to analyze the different bone markers studied in plasma samples of patients. After centrifugation (15 min at 2500× g), plasma aliquots were kept at -80°C for a short period until used. ELISA multiplex was performed following the manufacturer's protocol, including

bead preparation and standard and quality controls. Each sample was analyzed in duplicate by LUMINEX MAGPIX® technology (Luminex Corp., Austin, TX, USA). The limits of detection were as follows: DKK-1 (Dickkopf-1), 5 pg/mL; OPG (Osteoprotegerin), 7 pg/mL; OPN (Osteopontin), 98 pg/mL; SOST (Sclerostin), 24 pg/mL; and RANKL, 4.88 pg/mL. Intra-assay coefficients of variation for the plasma bone markers studied were less than 10%; inter-assay coefficients of variation were less than 15% for both multiplex kits.

2.4. Statistical Analysis

Qualitative variables were summarized by frequencies and percentages, and quantitative by the mean and standard deviation or by median and quartiles, depending on the skewness of the data distribution. To study the potential relations between variables, Chi-squared test or Fisher's exact test was used for qualitative variables, and Spearman's rank correlation coefficient was used for quantitative variables. Relative changes in metabolic parameters were calculated by the expression 100x (follow-up visit - baseline visit)/baseline visit. For densitometries, due to the presence of zeroes, absolute changes were calculated by the expression (follow-up visit-baseline visit). Statistical significance of changes in each treatment group was evaluated by the Wilcoxon signed-rank test. To compare the changes between treatments, Wilcoxon rank-sum test was used. In order to study if changes in densitometries could be affected by changes in BMI, ANCOVA models were used to compare treatments adjusting by changes in BMI.

Finally, two-way ANOVA models were performed for densitometries and biomarkers, including time, treatment and its interaction. Statistical analysis was carried out using R, software version 3.6.0 (R Core team (2020); R Foundation for Statistical Computing, Vienna, Austria).

3. RESULTS

3.1. Cohort Analysis and Lifestyle

All of 92 patients included in the study 100% were male. The main characteristics of subjects are shown in Table **1**. The average age was 34.8 years (± 9.6). 69% were European and 29% Latin American. All of them had acquired HIV infection through sexual intercourse with other men. At the time of diagnosis, 45.6% were in CDC stage 1 [38] (6.5% with CD4 <200 cell/μL). Regarding the NRTIs-based regimen, 21 subjects received TDF-based regimen, 22 TAF-based regimen and 49 ABC-based regimen.

Table 1. Baseline demographics and disease characteristics.

Variables	N	%
-	**Age**	-
-	34.8 ± 9.6	-
-	**Gender**	-
Male	92	100
-	**Region of Birth**	-
Europe	63	68.5
Latin American	27	29.3
Africa	1	1.1
Asia	1	1.1
-	**Physical Activity**	-
+ 3 times per week	46	59.7
- 3 times per weeks	31	40.3
-	**BMI (kg/m^2)**	-
-	23.6 ± 3.0	-
-	< 20	7.6
-	**Tobacco**	-
Yes	49	57
No	34	39.5
Former smoker	3	3.5
-	**Alcohol**	-
Yes	4	4.7
No	81	95.3
-	**Drugs**	-
Yes	30	34.9
No	55	64.0
Former drug user	1	1.2
-	**HIV Adquisiton**	-

(Table 1) contd....

Variables	N	%
Homosexual	92	100
-	**HIV Infection Stage Based on CD4+ T-lymphocyte Count**	-
Stage 1	42	45.6
Stage 2	43	46.3
Stage 3	7	7.6
-	**CD4 Median**	-
-	505.73 cells/µl	-
-	**CD4/CD8 Ratio**	-
< 0.4	37	40.2
-	**Viral Load >100.000**	-
-	18	19.5
-	**Vitamin D**	-
< 30ng/ml	78	85
-	**HBV**	-
HBV Ag B-	91	98.9
HBV Ag B+	1	1.1
-	**HCV**	-
IgG -	90	97.8
IgG+	2	2.2
-	**Baseline Z-score**	-
Normal	74	81.3
Low BMD for age	17	18.6
-	**48-week Z-score**	-
Normal	59	80.2
Low BMD for age	14	19.1

Table 2. Comparison of baseline data of the biomarkers studied by treatment group. Data are presented as median and quartiles (percentiles of 25% and 75%) and were compared using the Wilcoxon rank test. Statistical significance was considered for $p < 0.05$. *p*: TDF *vs* TAF *vs* ABC. *p1*: TDF *vs* TAF; *p2*: TDF *vs* ABC; *p3*: TAF *vs* ABC).

Variables	TDF	TAF	ABC	*P*	*P1*	*P2*	*P3*
Ca	9.40 (9.02, 9.5)	9.30 (9.00, 9.6)	9.5 (9.20, 9.8)	0.132	0.733	0.076	0.152
P	3.50 (3.10, 4.00)	3.20 (2.70, 3.60)	3.20 (2.85, 3.55)	0.361	0.241	0.190	0.858
25 OH Vit D	22.5 (19.2, 26.8)	15.8 (12.7, 23.6)	20.2 (15.7, 24.0)	0.083	0.049	0.136	0.175
CD4	550 (340, 630)	472 (376, 667)	465 (334, 595)	0.701	0.709	0.419	0.682
CD8	1122 (792, 1225)	893 (808, 1116)	986 (769, 1276)	0.692	0.356	0.553	0.795
CD4/CD8 ratio	0.47 (0.31, 0.72)	0.48 (0.34, 0.66)	0.48 (0.36, 0.67)	0.957	0.903	0.804	0.844
Cholesterol	157 (139, 194)	164 (147, 176)	147 (128, 173)	0.371	0.601	0.338	0.222
TG	106 (90.0, 158)	88.0 (68.5, 111)	96.5 (72.5, 143)	0.204	0.063	0.268	0.393
CTX	0.28 (0.20, 0.38)	0.33 (0.21, 0.46)	0.27 (0.22, 0.37)	0.420	0.473	0.604	0.201
P1NP	46.3 (31.0, 56.6)	44.8 (37.3, 50.4)	41.5 (31.1, 51.9)	0.936	0.767	0.751	0.929
OC	15.8 (13.2, 21.4)	14.2 (11.2, 20.6)	15.9 (13.0, 20.4)	0.774	0.450	0.716	0.682
TRAP	3.00 (2.40, 3.50)	3.05 (2.52, 3.75)	2.95 (2.50, 3.73)	0.851	0.543	0.686	0.894
PTH	31.9 (22.2, 41.3)	36.4 (25.5, 56.3)	39.5 (27.0, 49.6)	0.556	0.315	0.456	0.604
DKK1	436 (261, 670)	324 (249, 565)	454 (276, 650)	0.574	0.545	0.777	0.302
OPG	216 (119, 334)	235 (121, 303)	236 (157, 316)	0.882	0.989	0.681	0.704
OPN	11025 (4435, 18313)	12158 (7897, 17304)	14171 (10203, 19242)	0.229	0.737	0.150	0.202
SOST	1591 (1019, 2225)	1158 (687, 2298)	1468 (885, 2369)	0.495	0.239	0.746	0.361
RANKL	87.7 (35.9, 121)	88.2 (4.88, 188)	52.9 (4.88, 131)	0.654	0.677	0.562	0.424
OPG/RANKL	2.27 (0.89, 5.72	2.15 (1.07, 22.37)	3.25 (1.20, 41.78)	0.512	0.562	0.319	0.461

Table 3. Changes in parameters of plasma samples between TDF, TAF and ABC after 12 weeks of treatment. Data are presented as median and quartiles (percentiles of 25% and 75%) and were compared using the Wilcoxon rank test. Statistical significance was considered for p <0.05. p: 12 weeks *vs* baseline; $p1$: TDF *vs* TAF (12 weeks *vs* baseline); $p2$: TDF *vs* ABC (12 weeks *vs* baseline); $p3$: TAF *vs* ABC (12 weeks *vs* baseline).

Parameters Increments Baseline: 12 Weeks After Initiating Treatment									
Variable	**TDF**		**TAF**		**ABC**		**-**		
	Median (*Q1*, *Q3*)	***p***	**Median (*Q1*, *Q3*)**	***p***	**Median (*Q1*, *Q3*)**	***p***	***p1***	***p2***	***p3***
Ca	0.53 (-0.53, 5.53)	0.286	2.06 (-2.59, 5.53)	0.374	0.00 (-3.06, 5.29)	0.858	0.745	0.643	0.802
P	-2.86 (-21.8, 18.4)	0.629	3.70 (-2.63, 18.4)	0.182	2.94 (-4.18, 25.3)	0.166	0.256	0.165	0.962
25 OH Vit. D	-10.8 (-25.9, 156)	0.980	47.8 (1.88, 156)	**0.003**	2.73 (-28.6, 21.3)	0.336	**0.044**	0.403	**0.031**
CD4	26.5 (-4.91, 46.9)	**0.016**	29.2 (2.00, 46.9)	**0.001**	43.9 (22.6, 41.0)	**<0.001**	0.961	0.521	0.518
CD8	-11.6 (-22.8, 28.6)	0.744	5.24 (-29.1, 28.6)	0.798	-3.47 (-21.2, 3.53)	0.779	0.683	0.521	0.825
CD4/CD8 ratio	49.3 (15.9, 87.7)	**0.001**	30.4 (7.58, 87.7)	**0.001**	43.6 (10.8, 35.3)	**<0.001**	0.635	0.476	0.975
Cholesterol	-1.90 (-12.0, 39.3)	1.000	27.3 (10.2, 39.3)	**<0.001**	13.5 (1.76, 19.0)	**0.002**	**0.001**	**0.021**	**0.046**
Triglycerides	-0.96 (-6.50, 57.7)	0.404	34.9 (10.6, 57.7)	**0.003**	-0.08 (-25.9, 41.2)	0.224	0.124	0.823	0.138
CTX	62.1 (-6.56, 73.7)	0.064	42.6 (-0.36, 73.7)	**0.011**	54.2 (-3.13, 53.0)	**0.002**	0.635	0.723	0.523
P1NP	31.7 (25.7, 38.0)	**0.004**	3.79 (-15.9, 38.0)	0.243	17.2 (4.16, 35.0)	**0.001**	0.075	0.128	0.255
OC	38.6 (22.2, 62.7)	**0.001**	27.3 (10.8, 62.7)	**0.001**	13.9 (0.97, 67.6)	**0.001**	0.433	0.096	0.435
TRAP	11.1 (7.69, 45.5)	**0.003**	21.0 (-3.45, 45.5)	**0.008**	20.0 (2.08, 15.5)	**0.002**	0.564	0.939	0.824
PTH	-1.88 (-37.4, 27.4)	1.000	-5.76 (-18.0, 27.4)	0.934	11.5 (-25.3, 114)	0.674	0.800	0.627	0.581
DKK1	-21.0 (-58.0, 25.3)	0.098	-15.7 (-60.1, 25.3)	0.515	-16.8 (-47.7, 37.6)	**0.007**	0.707	0.736	0.766
OPG	-23.4 (-41.5, 2.54)	**0.003**	-28.8 (-40.4, 2.54)	**0.049**	-11.9 (-37.6, 60.7)	0.294	0.683	0.114	0.292
OPN	19.3 (-3.53, 45.1)	**0.045**	13.6 (-6.93, 45.1)	0.104	0.75 (-14.3, 30.5)	0.670	0.552	0.107	0.269
SOST	-9.8 (-26.3, 15.1)	0.074	-30.5 (-47.8, 15.1)	0.216	-8.77 (-24.7, 52.5)	0.646	0.880	0.268	0.227
RANKL	17.7 (-29.5, 23.2)	0.610	0.00 (-0.19, 23.2)	0.675	0.00 (-12.4, 16.8)	0.162	0.859	0.853	0.534
OPG/RANKL	-23.08 (-62.23, 23.67)	0.413	-33.67 (-79.32, 71.60)	0.922	-23.24 (-90.89, 29.21)	0.165	0.875	0.625	0.437

Table 4. Changes in parameters of plasma samples between TDF, TAF and ABC after 48 weeks of treatment. Data are presented as median and quartiles (percentiles of 25% and 75%) and were compared using the Wilcoxon rank test. Statistical significance was considered for *p* <0.05. *p*: 48 weeks *vs* baseline; *p1*: TDF *vs* TAF (48 weeks *vs* baseline); *p2*: TDF *vs* ABC (48 weeks *vs* baseline); *p3*: TAF *vs* ABC (48 weeks *vs* baseline).

Parameters Increment Baseline: 48 Weeks After Initiating Treatment									
Variable	**TDF**		**TAF**		**ABC**		**-**		
	Median (*Q1*, *Q3*)	***p***	**Median (*Q1*, *Q3*)**	***p***	**Median (*Q1*, *Q3*)**	***P***	***p1***	***p2***	***p3***
Ca	2.17 (1.06, 4.48)	**0.005**	2.11 (-1.59, 4.48)	0.095	1.52 (-2.28, 1.05)	0.182	0.367	**0.030**	0.278
P	-5.30 (-20.2, 18.2)	0.404	-3.03 (-7.69, 18.2)	0.660	0.00 (-6.27, 25.0)	0.465	0.345	0.153	0.655
25 OH Vit. D	29.4 (-9.24, 44.9)	0.083	15.1 (-11.7, 44.9)	**0.041**	26.8 (-14.8, 42.6)	**0.021**	0.823	0.914	0.607
CD4	24.4 (-10.1, 98.1)	0.074	29.4 (8.92, 98.1)	**0.001**	45.6 (26.4, 162)	**<0.001**	0.523	0.120	0.349
CD8	-28.8 (-41.3, 20.6)	0.051	-0.27 (-44.6, 20.6)	0.922	-12.2 (-38.5, 16.0)	0.516	0.367	0.117	0.987
CD4/CD8 ratio	102 (26.7, 130)	**<0.001**	75.0 (35.1, 130)	**<0.001**	65.3 (33.4, 170)	**<0.001**	0.756	0.732	0.912
Cholesterol	10.7 (0.53, 31.6)	**0.025**	19.4 (11.1, 31.6)	**0.001**	19.2 (-0.40, 31.5)	**0.001**	0.333	0.604	0.592
Triglycerides	-1.19 (-24.2, 49.3)	0.860	34.8 (-17.7, 49.3)	**0.029**	6.38 (-19.2, 142)	0.198	0.088	0.134	0.975
CTX	92.3 (17.9, 81.8)	**<0.001**	31.6 (10.3, 81.8)	**0.027**	45.0 (12.8, 122)	**<0.001**	0.139	0.615	0.166
P1NP	81.4 (47.5, 53.4)	**<0.001**	17.8 (6.68, 53.4)	**0.017**	21.4 (2.84, 70.7)	**0.001**	**0.038**	**0.015**	0.878
OC	49.6 (42.7, 50.7)	**<0.001**	1.65 (-10.0, 50.7)	**0.024**	20.1 (-9.34, 126)	**0.011**	**0.033**	0.083	0.275
TRAP	51.1 (23.1, 244)	**0.002**	120 (31.5, 244)	**<0.001**	68.1 (23.3, 56.5)	**<0.001**	0.091	0.797	**0.052**
PTH	35.8 (12.3, 54.7)	0.078	47.6 (-7.11, 54.7)	**0.027**	22.2 (-8.25, 82.8)	0.055	0.817	0.469	0.468
DKK1	67.4 (-18.2, 17.8)	**0.049**	-12.3 (-50.5, 17.8)	0.241	-8.08 (-37.8, -17.7)	0.582	**0.046**	0.096	0.647
OPG	20.1 (-12.2, 54.1)	0.244	0.67 (-38.5, 54.1)	0.623	-11.8 (-32.5, 67.8)	0.218	0.472	0.330	0.904
OPN	63.5 (16.5, 39.5)	**0.013**	23.4 (-19.0, 39.5)	0.123	-1.59 (-27.1, 5.47)	0.645	0.132	**0.015**	0.222
SOST	20.2 (-30.0, 95.5)	0.497	16.9 (-37.3, 95.5)	0.296	-12.3 (-20.8, 78.6)	0.275	0.943	0.814	0.890
RANKL	980 (588, 1425)	**0.004**	302 (-72.2, 1425)	0.164	1067 (325, 1619)	**0.001**	0.278	0.769	0.258
OPG/RANKL	-84.91 (-91.90, -59.94)	0.105	-62.41 (-85.96, 597.6)	0.750	-85.42 (-95.92, 31.37)	0.275	0.500	0.500	0.500

Regarding other life habits and risk factors associated with a lower BMD, 48.9% consumed dairy products on a regular basis, and only 2.17% used steroids for bodybuilding.

We do not see significant changes between the treatment groups in the baseline sample for the measured parameters (Table **2**), having a similar bone deterioration at baseline, regardless of the treatment group.

3.2. Comparative Evolution of Bone Metabolism at 12 Weeks After Initiating Treatment

12 weeks after initiating antiretroviral treatment, a significant increase in PN1P (31.7%, $p = 0.004$), TRAP (11.1%, $p = 0.003$), OPN (19.3%, $p = 0.045$) and OC (38.6%, $p = 0.001$) and a non-significant increase in CTX (62.1%, $p = 0.064$) were observed in those patients who received TDF when compared to baseline. Patients on TAF regimen presented a significant increase in the following proteins when compared to baseline: 42.6% for CTX ($p = 0.011$), 27.3% for OC ($p = 0.001$) and 21% for TRAP ($p = 0.008$). Patients who received ABC therapy presented a 54.2% increase in CTX (p=0.002), 17.2% in P1NP (p=0.001), 13.9% in OC (p =0.001) and 20% in TRAP ($p = 0.002$) when compared to baseline. In contrast, there were no significant changes in OPN after 12 weeks of treatment with ABC (Table **3**). We observed a significant decrease in OPG both in patients taking TDF (-23.4%, $p = 0.003$) and TAF (-28.8%, $p = 0.049$), with non-significant decrease in the ABC group (-11.9%; $p = 0.294$) when compared with baseline measurements. DKK1 levels decreased significantly in patients treated with ABC (-16.8%, $p = 0.007$), but presented a non-significant downward trend in both the TDF and TAF groups (-21% with TDF, -15.7% with TAF, p>0.05) (Table **3**). To verify treatment-induced changes in bone remodeling, we calculated the OPG/RANKL ratio, which tended to decrease [more with TAF (-33.67%, $p = 0.922$)] in all three treatment groups by 12 weeks of treatment, thus suggesting an appreciable increase in bone resorption. These and other bone parameters studied are shown in Table **3**.

3.3. Comparative Evolution of Bone Metabolism at 48 Weeks After Initiating Treatment

We observed a significant increase in CTX (92.3%, p <0.001), P1NP (81.4%, p <0.001), OC (49.6%, p <0.001) and TRAP (51.1%, $p = 0.002$) in the group treated with TDF when compared to baseline. These increases were 31.6% ($p = 0.027$) for CTX, 17.8% ($p = 0.017$) for P1NP, 1.65% ($p = 0.024$) for OC and 120% for TRAP (p <0.001) in those who took TAF. Finally, in the ABC group we observed an increase of 45% (p <0.001) for CTX, 21.4% (p <0.001) for P1NP, 20.1% (p =0.001) for OC and 68.1% for TRAP (p <0.001) (Table **4**).

Both DKK1 and OPN were significantly increased in TDF-treated patients (67.4%, $p = 0.049$ and 63.5% ($p = 0.013$) respectively) when compared to baseline. However, non-significant changes were observed for patients treated with TAF and ABC (Table **4**). Regarding OPG changes at 48 weeks, we found non-significant differences in patients with TDF or TAF (increase) and ABC decrease) (Table **4**). Finally, RANKL was increased in all three treatment groups, with values 980 times greater than baseline ($p = 0.004$) in patients taking TDF, and 1067 times greater (p=0.001) in those taking ABC (Table **4**), while patients taking TAF presented measurements up to 302 times greater than baseline ($p = 0.164$) (Table **4**). Regarding changes in the OPG/RANKL ratio, the downward trend observed at 12 weeks was still present at 48 weeks from the start of treatment, with the greatest decrease in TDF and ABC groups (Table **4**). Other measurements are provided in supplementary (Tables **1**-**3**).

When we analyzed absolute changes in bone parameters between 12 or 48 weeks and baseline, we observed changes with statistical significance in CTX and P1NP with time (ANOVA) at 12 weeks of treatment (supplementary Table **4**), and also in CTX at 48 weeks with time and interaction, where we also find significant changes in P1NP, OC, TRAP and RANKL (supplementary Table **5**).

3.4. Comparing Treatment-Related Changes in Z-scores During the 48-week Study

We observed a general deterioration in bone quality in HIV patients after 48 weeks, regardless of the treatment received. Baseline and 48 weeks after treatment DXAs were completed in 73 out of 92 patients (patients lost to follow-up included 6 from the TDF, 5 from the TAF, and 8 from the ABC group). As all our patients were under 50 years old, we calculated the Z-score. We found that 18.6% of our patients had a Z-score below -2 at the beginning of the study, being similar (19.1%) at the end of the study (Table **1**). We analyzed the differences in Z-score at the LS and FN sites between baseline and 48 weeks for the three treatments (Table **5**). We observed that patients treated with TDF had the greatest decrease in Z-score in both locations (LS and FN), with significant changes compared to baseline. However, there were no statistically significant changes Z- score across different treatment groups (Fig. **2**, Table **5**). No significant differences were found across different groups and no correlation was found between changes in Z-score and changes in BMI in any group (Supplementary Table **6**-**7**). Spearman´s rank correlation coefficient analyses showed an association between CD4 and CD4/CD8 ratio with the Z-score at 48 weeks in the total cohort (Supplementary Table **8**). No statistically significant changes were found for Z-score neither on time, treatment or interaction (Supplementary Table **9**). No changes in weight and height between baseline and 48 weeks post-treatment were observed.

4. DISCUSSION

Our findings corroborate the negative impact of antiretroviral treatment on the bone profile of therapy-naive HIV patients under 50 years, appearing as early as 12 weeks after initiating therapy, and becoming more significant at 48 weeks. To the best of our knowledge, this is one of the first times that a complete panel of bone profile markers has been analyzed in HIV patients under 50 years, in the context of naïve treatment with different most used NRTIs, providing

Table 5. Change in Z-score values after 48 weeks of treatment. Data are presented as mean ± SD and were compared using Student's t-test. Statistical significance was considered as $p < 0.05$. *p*: 48 weeks *vs* baseline (paired Student's t-test); *p1*: TDF *vs* TAF (48 weeks *vs* baseline); *p2*: TDF *vs* ABC (48 weeks *vs* baseline); *p3*: TAF *vs* ABC (48 weeks *vs* baseline) (unpaired Student's t-test).

DXA Changes Baseline: 48 Weeks After Initiating Treatment									
Variable	TDF		TAF		ABC		-		
	Mean±SD	*p*	Mean±SD	*p*	Mean±SD	*p*	*p1*	*p2*	*p3*
Z-score LS	-0.140 ± 0.427	0.225	-0.059 ± 0.654	0.716	-0.117 ± 0.352	**0.039**	0.985	0.841	0.731
Z-score FN	-0.214 ± 0.343	**0.030**	-0.095 ± 0.275	0.175	-0.136 ± 0.302	**0.006**	0.290	0.415	0.626

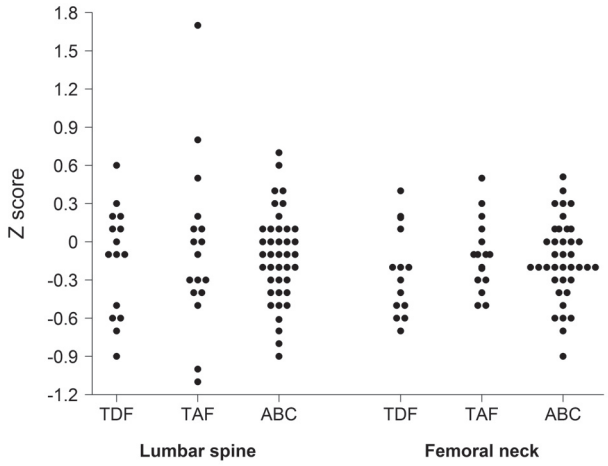

Fig. (2). Changes in Z-score at 48 weeks. Graphs indicate increase or decrease in Z-score values between baseline and 48 weeks DXA scanning in lumbar spine (LS) and femoral neck (FN).

us with an early predictive value that is not considered in the vast majority of studies carried out in HIV patients treated with the same antiretrovirals featured here [14, 18, 19, 22, 39]. A few studies exist on early bone comorbidity in HIV patients treated with TDF or ABC [27, 29, 40, 41], but these are limited to only some bone remodeling markers. Stellbrink *et al.* [42] found similar findings in a 24 weeks analysis (CTX, P1NP and OC), comparing abacavir/lamivudine and tenofovir/emtricitabine (both in fixed-dose combination daily) plus efavirenz; however, our research contributes with a wide variety of bone turnover markers, such as TRAP, DKK1, OPG, OPN, SOST and RANKL, from as early as 12 weeks after the initiation of cART. Vlot *et al*. [26] studied BMD using DXA, in addition to measuring several serum bone remodeling markers (CTX, P1NP, OC), with long-term follow-up of patients (60 and 96 weeks), but also with less bone markers that we include.

Bone damage has been fundamentally associated with TDF [41-43] and, because emtricitabine (FTC) and lamivudine (3TC) are very similar molecules, TDF's standard of comparison for bone damage has classically been abacavir. In recent years, a new tenofovir molecule, tenofovir alafenamide (TAF), has been introduced, widely replacing TDF, with better bone and renal profiles [40, 44]. We have observed that the changes observed in CTX and P1NP between 12 and 48 weeks are greater for TDF than for TAF, and that OC decreases at 48 weeks compared to 12 weeks in patients receiving TAF. In addition, we observed that DKK1 increased for TDF and decreased for TAF after 48 weeks of treatment. These trends in some bone markers might corroborate the reported better profile of TAF regarding bone quality compared to TDF, as DeJesus *et al.* [45] found in a randomized clinical trial. Seto *et al.* [46] also demonstrated that TAF is associated with a superior bone safety profile than that of TDF at more than 96 weeks of treatment, even in patients with risk factors associated with bone loss and higher initial FRAX scores. Our study demonstrates that these bone changes are observed even earlier, at the beginning of treatment (12 weeks), and in adult patients younger than 50 years, a population with a high rate of bone affectation even before treatment initiation [8, 47-49]. Therefore, PLHIV of all ages may potentially benefit from avoiding therapies containing TDF, which has repeatedly been shown to cause BMD loss [16, 26, 48, 50-52] .

Data exist suggesting bone health improvement in patients with low BMD treated with ABC during 48 weeks, after switching from TDF [25, 27, 53]. Our research detects no short-term differences between treatment groups, suggesting that more specific and comprehensive studies are needed (Tables **2**-**3**). Both the 12 and 48-week OPG/RANKL ratios seem to indicate that changes in bone remodeling, and specifically changes in bone resorption, are stimulated more rapidly with TAF (as shown by a steeper downward trend at 12 weeks of treatment), and are more prolonged in the case of TDF and ABC, with patients in these groups reaching very considerable levels of resorption at 48 weeks of treatment.

The precise mechanism of BMD reduction associated with tenofovir is unclear; however, HIV patients treated with TDF have shown increases in bone turnover markers that suggest a higher activity of both osteoblasts and osteoclasts [28]. Recently, Conesa-Buendía *et al.* [54] have demonstrated that tenofovir directly promotes osteoclast differentiation and function (increasing the expression of bone markers such as cathepsin K, RANKL and NFATc1), including activation of MAPK and NFkB pathways.

It has been demonstrated that HIV alters -directly or indirectly- both osteoblasts and osteoclasts, affecting both osteoblastogenesis and osteoclastogenesis, and it has also been described that antiretroviral drugs can have an effect on bone cells, through alterations in RANK/RANKL levels, cytokine production, mitochondrial function, phosphate metabolism and vitamin D metabolism [55]. We observed that patients treated with TAF experienced early changes in

bone remodeling (OPG/RANKL ratio), with a greater decrease compared to baseline at 12 weeks than in the case of the other treatments. However, although this ratio still tends to decrease at 48 weeks, the greatest resorption is seen in patients treated with TDF and ABC. The OPG / RANKL ratio is a widely used parameter in clinical studies to measure equilibrium in the OPG / RANK / RANKL system [56]. Previous research has confirmed the involvement of OPG / RANK / RANKL system dysregulation in the pathogenesis of reduced bone density among HIV-infected patients [57, 58]. The increase in the bone turnover characteristic of ART initiation in HIV patients seems to be associated with changes in systemic concentrations of OPG and RANKL. In our sample, we found that RANKL increased after initiating ART, with this increase being clearly evident after 48 weeks of treatment, while OPG levels decreased in plasma in the short-term (12 weeks), remaining more or less constant upon measurement at 48 weeks. However, we did not observe major changes in the OPG/RANKL ratio's downward trend between treatment groups throughout the study. These findings seem to suggest that changes in bone turnover upon ART initiation (regardless of the treatment regimen) may be independent of the OPG / RANK / RANKL system, and that we need to correlate and assess other markers of bone resorption in order to arrive at a definitive conclusion [59-62].

Some studies correlate antiretroviral therapy with changes in bone and inflammatory markers [63-65] and compare TDF and TAF regimens. In our study, changes observed in bone resorption markers at 12 weeks were greater in patients treated with TDF compared with those receiving ABC. However, at 48 weeks, TDF and ABC showed more similar behavior, with TAF being slightly -though not significantly- less resorptive. In addition, we observed that the highest percentage of the average increase in plasma CTX and P1NP occurred in patients treated with TDF, with clinically relevant decreases in BMD of the hip and spine at week 48 with respect to baseline. It is interesting to note that in patients receiving TDF, temporal changes in CTX, a bone resorption marker, compared to changes in bone formation markers, suggest a stage in which bone destruction seems to overcome bone formation. Together, our results support the concept of reduction in systemic exposures to TDF being responsible for the minimal changes in bone renewal and the smaller decreases in BMD observed in patients receiving TAF compared to those receiving TDF [66-69].

Most of the studies and clinical trials focus on longer courses of treatment (starting at 48 weeks) [22, 70]. Our study provides data from the first year of treatment, and could help to understand how the choice of cART can influence the prevention and improvement of cART-derived bone comorbidities from early on. TDF has shown greater bone damage, but despite the fact that TAF and ABC have a better profile, in both, we can observe an increase in bone resorption markers. They can also be reasons for the bone deterioration in the first weeks of antiretroviral treatment, regardless of drugs, due to immune reconstitution or other factors. As recent data has demonstrated cART efficacy when only two drugs are used [71], it could be an option consider to avoid the use of any drug (TDF, TAF and ABC) when possible, with the intention to prevent and reduce bone deterioration as early as possible, while remembering to make the greatest possible emphasis on modifiable risk factors (principally treatment and lifestyle habits).

There are some limitations to our study. First of all, the sample size, which limits the power of some of the comparisons made between the groups. This might explain why no statistically significant changes between treatment groups were found for the DXA results at 12 months. Another reason might be the fact that the size of the ABC group was s more than double that of the TDF or TAF groups. Although we are aware that the limited (48-week) follow-up time could underestimate long-term changes, our data, which shows the presence of osteopenia/osteoporosis as early on as 48 weeks, plausibly indicate future outcomes. A second limitation concerns the lack of a study group consisting of individuals not infected with HIV; nonetheless, our results can be contrasted with well-established findings from studies conducted in the general population. Though our cohort consists of individuals who had lived with HIV infection for a short time, a certain bias may have been introduced in this regard, as we cannot rule out a slight impact of HIV infection on bone metabolism in the first stages of infection. However, the fact that over 90% of patients had CDC stage 1 and 2 of the disease indicates an appropriate degree of homogeneity. Finally, 20% of the patients could not have both DXA studies, due to having a result outside the window protocol. Women were not included due to a very low prevalence of new HIV infection in women in our setting.

Despite these limitations, we believe that our work offers some very novel data to the current literature by describing systemic markers of bone remodeling and, most importantly, evaluating short-term outcomes (12 and 48 weeks). There are not many studies assessing initial (12 weeks from starting treatment) bone involvement in HIV infected adults (under 50 years) [72-75] out of clinical trials, and, moreover, there are scarcely studies that evaluate the changes in a full panel of molecular bone markers.

CONCLUSION

In conclusion, we observe that treatment-naïve HIV patients have a high prevalence of low bone density. Furthermore, treatment with TDF is associated with greater bone deterioration at 12 and 48 weeks, while TAF appears to present similar early bone deterioration at 12 weeks that disappears at 48 weeks. Further studies are necessary to confirm whether our observations are also related to long-term clinical outcomes, for example, the incidence of bone fractures and other associated aspects.

AUTHORS' CONTRIBUTIONS

AM, ACU and MG designed the experiments, analyzed and interpreted the results, wrote and revised the manuscript. PA was responsible for managing the patient database, clinical visits, treatment distribution, and writing and editing the manuscript. F.M.C.-B. was primarily responsible for carrying out all experimental procedures, analyzed

and interpreted the results, and wrote and revised the manuscript. P.Ll.-G collected samples and performed some experimental procedures and edited and revised the manuscript. P. RP-T, BAA, ICA, LPP, RAP, MDC helped to recruit patients, performed clinical visits and revised the manuscript. RL and GH-B interpreted, revised and edited the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval of this study was obtained from The Medical Ethics Committee of the Fundación Jiménez Díaz University Hospital (Approval no. PIC155-016) at the Fundación Jiménez Díaz Medical University Hospital Ethics Committee, Community of Madrid, Spain.

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All humans research procedures were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

The studied participants were informed about the present research, and a written consent form was taken from all of them before their enrollment.

STANDARDS OF REPORTING

The study conforms to the STARS (www. stard-statement.org) and TRIPOD guidelines (www. tripod-statement.org).

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the results and findings of this study are available within the article.

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CONFLICT OF INTEREST

The authors declare the following conflict of interest, financial or otherwise:

AM has filed a patent on the use of adenosine A2AR agonists to prevent prosthesis loosening (pending) and a separate patent on the use of A2AR agonists and agents that increase adenosine levels to promote bone formation/regeneration. PLl-G has filed a patent on the use of Lipocalin-2 as a treatment for abdominal aortic aneurysms, RL and GH-B have filled a patent on the use of 6-shogaol in osteoarthritis. ACU reports grants and personal fees from ViiV Healthcare, personal fees from Gilead, personal fees from Janssen, personal fees from Merck, outside the submitted work. MG reports grants and personal fees from ViiV Healthcare, personal fees from Gilead, personal fees from Janssen, outside the submitted work. BA reports personal fees from Gilead and ViiV Healthcare, outside the submitted work.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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