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Osteoporose em pacientes adultos infetados pelo Vírus da Imunodeficiência Humana, sob terapêutica anti-retrovírica / Osteoporosis in Human Immunodeficiency Virus infected adult patients under antiretroviral therapy

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DESIGNAÇÃO DA ÁREA DO PROJECTO

Endocrinologia

TÍTULO DISSERTAÇÃO/MONOGRAFIA (riscar o que não interessa)

Osteoporosis in Human Immunodeficiency Virus infected adult patients under antiretroviral therapy

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Osteoporosis in Human Immunodeficiency Virus infected adult patients under antiretroviral therapy

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Abstract

Background: HIV-infected patients are at higher risk of developing osteoporosis. Our objective was to determine the prevalence of osteoporosis in a population of HIV-infected patients under cART and to evaluate its relationship with several risk factors, including body composition [lipodystrophy defined by Fat Mass Ratio (FMR) and different groups of body fat distribution].

Methods: Cross-sectional study of 126 HIV-infected patients on cART. The BMD was measured at the lumbar spine, hips and total body using DXA. Lipodystrophy defined by FMR was defined as the ratio of the percentage of trunk fat mass to the percentage of lower limb fat mass by DXA. We compared demographic, HIV-related, anthropometric, metabolic, hormonal and renal factors for their associations with osteoporosis using the chi-square or Fisher's exact test and for their associations with total, lumbar spine and femur BMD using the Spearman correlation coefficients.

Results: Osteoporosis was present in 15.9% of HIV-infected patients. Osteoporotic patients had lower hip circumference, higher total testosterone and higher urea levels. Lipodystrophy defined by FMR and the different groups of body composition did not present significantly differences concerning osteoporosis/osteopenia and total, lumbar spine and femur BMD. Total BMD was positively correlated with diastolic blood pressure, weight, height, neck circumference, hip circumference, FMR and negatively correlated with SHBG levels. Spine BMD was positively correlated with systolic blood pressure, weight, height, BMI, neck circumference, upper-arm circumference, waist circumference, hip circumference, FMR, total testosterone, free testosterone and negatively correlated with total cholesterol, HDL cholesterol, SHBG and 25-

OH vitamin D3. Femur BMD was positively correlated with weight, neck circumference, abdominal circumference, hip circumference, FMR and negatively correlated with age, duration of HIV infection, length of cART, SHBG and urea levels. SHBG was independently associated with total and lumbar spine BMD, while age and urea were independently associated with femur BMD.

Conclusions: Osteoporotic HIV-infected patients have lower hip circumference than non-osteoporotic patients. Lipodystrophy and different groups of body fat distribution do not seem to play an independent role in the development of osteoporosis. SHBG is independently related to total and spine BMD, while older age and higher urea levels are independent risk factors for decreased femur BMD.

Keywords: Osteoporosis, body composition, lipodystrophy

Background

Over the past two decades, there have been substantial advances in the treatment of Human Immunodeficiency Virus (HIV) infection. Increased accessibility and widespread combination antiretroviral therapy (cART) has significantly prolonged life expectancy in HIV-patients but, with that, also long-term exposure to cART toxicities and the virus itself. As a consequence, a large number of metabolic comorbidities have emerged, including lipodystrophy, insulin resistance, diabetes, dyslipidemia, cardiovascular disease, and

osteoarticular manifestations such as osteoporosis, osteopenia and fractures [1, 2].

There is clear evidence from cross-sectional studies that those with HIV are at higher risk of developing reduced bone mineral density (BMD) compared with non-HIV-infected individuals [3]. Multiple risk factors are involved but underlying mechanisms are still incompletely elucidated. In addition to traditional risk factors that have been characterized in the general population - older age, low body mass Index (BMI), inadequate physical activity, previous fracture, smoking, alcohol abuse, glucocorticoid-therapy, hypogonadism, menopause, hypovitaminosis D - there are risk factors specifically associated with HIV infection: cART, low CD4 count, chronic inflammation, co-infection with hepatitis C or hepatitis B, renal disease or diabetes [1, 2, 4-7]. There are also some evidences describing that low BMD could be related to lipodystrophy [8-11], but it is still controversial [4].

This study aims to determine the prevalence of osteoporosis in a population of HIV-infected adult patients under cART, in a single-site hospital and to evaluate its relationship with several risk factors, including lipodystrophy defined by fat mass ratio (FMR) and different groups of body fat distribution.

Methods

Subjects

As part of a cross-sectional cohort study, 126 HIV-infected Caucasian adults, 64 men and 62 women, non-institutionalized, were evaluated at the Endocrinology

Outpatient Department of São João Hospital. Patients were consecutively referred from the Infectious Diseases Department between January/2008 to March/2016 and were included in the first visit. The patients were on cART. The study protocol was approved by the Hospital Ethics Committee for Health.

Clinical assessment

For all patients, the following information was collected, using a standardized protocol: sex, age, time since HIV diagnosis, risk factor for HIV transmission, transmission mode, type and duration of cART exposure, $CD4^+$ plasmatic count, smoking history (current, past, or never) and alcohol consumption (yes or no). The "Centers for Disease Control and Prevention" criteria were used for classifying the degree of infection [12].

Height was measured in the standing position, using a wall stadiometer (Holtain Limited Crymych, Dyfed®) and body weight was measured using the TANITA (Tanita®, model TBF 300) scale. Circumferences of neck, waist, hip, thigh and arm, and blood pressure were measured using the current recommendations [13, 14].

BMI was calculated as weight divided by height squared (kg/m^2). Clinical lipodystrophy was assessed by both patient and practitioner, and was defined as peripheral lipodystrophy, with or without central fat accumulation [14]. Abdominal prominence (central obesity) was defined by a waist circumference >102 cm for men and > 98 cm for women, as defined by NCEP-ATP III modified criteria for metabolic syndrome [15]. All clinical assessments were performed by the same observer.

Evaluation of body composition

Patients were classified into four different groups: (1) No lipodystrophy (patients without clinical lipoatrophy and without abdominal prominence); (2) Isolated central fat accumulation (patients without clinical lipoatrophy and with abdominal prominence); (3) Isolated lipoatrophy (patients with clinical lipoatrophy and without abdominal prominence) and (4) Mixed forms of lipodystrophy (patients with clinical lipoatrophy and with abdominal prominence) [13].

BMD was assessed with whole-body dual–energy X-ray absorptiometry (DXA – Lunar Expert XL). DXA measurement was performed while the patient was in a supine position, with standard position of the arms and feet. BMD was measured on specific anatomic sites: the lumbar spine (L1-L4) and the hips (femoral neck), and it was reported as T and Z scores. The World Health Organization (WHO) classification for bone demineralization was used for diagnosis purposes of osteoporosis [T score <-2.5 standards deviation (SDs)] and for the diagnosis of osteopenia (T score between -1 and -2.5 SDs) [16, 17]. Regional fat mass values were grouped and analyzed for the following anatomical regions: arms, legs, trunk and total body. The FMR is the ratio of the percentage of the trunk fat mass to the percentage of the lower limb fat mass (FMR = % of the trunk fat mass/ % of the lower limb fat mass) [18]. We define lipodystrophy by FMR using the cut-off value of 1.961 threshold for men and 1.329 for women [19]. The quantification of total, visceral and peripheral fat was performed with a 64-slice computed tomography scanner (Siemens Sensation 64 Cardiac) with the same technique as previously described [20]. All values were expressed in cm^2 rounded to the nearest centesimal.

Laboratory analysis

A venous blood sample was drawn after a 12-hour overnight fast. All the samples were analyzed at the central laboratory of our hospital. The $CD4^+$ cells count was determined by flow cytometry. Glycosylated hemoglobin (HbA1c) was measured by Variant II Turbo (*Bio-Rad*). We measured total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, lipoprotein (a), urea, creatinine, calcium, phosphate and magnesium using Olympus AU5400 (*BecKman Coulter*). Total testosterone, free testosterone, sex hormone binding globulin (SHBG), luteinizing hormone (LH), follicle-stimulating hormone (FSH), parathyroid hormone (PTH) and 25-OH vitamin D3 were determined using Cobas e411 (*Roche Diagnostic*). Leptin was measured using bead-based multiplex assay (*Luminex*). Fibrinogen was measured using Cobas t411 *(Roche Diagnostic*). Insulin-growth factor 1 (IGF-1) was measured using Immulite 2000 (*Siemens*). Thyroid-stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4) were measured using Architect i2000 SR (*Abbot*). These were the criteria for the definition of gonadal status: testosterone deficiency was defined based on a serum total testosterone below 2.8 ng/mL and/or a calculated free testosterone levels, according to Vermeulen formula, below 6.5 pg/mL [21].

Measurements of insulin resistance

Insulin resistance was defined by the homeostasis model assessment of insulin resistance (HOMA), using the following formula:

HOMA – IR index = (insulin x glucose)/22.5 [22].

Statistical analysis

Data were described as mean and SD for quantitative variables, and were compared using Student-t and ANOVA or Mann–Whitney and Kruskall-Wallis tests, as appropriate. Categorical variables were described as counts and proportions, and compared using the chi-square or Fisher's exact test. To study the association between BMD and clinical and metabolic characteristics, Spearman correlation coefficients were estimated. Multiple linear regression models were computed in order to estimate the associations between BMD and clinical and metabolic characteristics, independently of selected confounders. Statistical analysis was performed using SPSS version 24.0 software (SPSS Inc., Chicago, Illinois, USA). All probabilities were two tailed and p values of <0.05 were regarded as significant.

Results

The study comprised 126 patients under cART, including 64 males (50.8%) and 62 females (49.2%), with a mean age of 49.3 years. Firstly, we divided our sample according to the presence of osteoporosis and 20 patients (15.9%) were found to have this disease. The results are shown in Table 1.

No significant differences were observed between osteoporotic and non-osteoporotic patients, regarding the sex, age, duration of HIV infection, HIV stage, length of cART, class of antiretroviral drugs used, CD4 count, risk factors for HIV transmission, drugs used, smoking history, alcohol consumption, co-infection hepatitis C, hypertension, systolic and diastolic blood pressures.

Patients with osteoporosis had lower hip circumference (p=0.004). No differences were observed concerning weight, height, BMI, neck circumference, upper-arm circumference, abdominal circumference, waist circumference, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, lipoprotein (a), HbA1c, HOMA, leptin and fibrinogen.

Osteoporotic patients had higher total testosterone levels (p=0.001). However, no differences were found about the presence of hypogonadism, free testosterone, SHBG, LH and FSH.

Urea levels were higher in osteoporotic patients (p=0.028), but no differences were found about creatinine, magnesium, calcium, phosphate, PTH, 25-OH vitamin D3 and IGF-1.

No differences were found between osteoporotic and non-osteoporotic patients regarding TSH, free T3 and free T4.

FMR was estimated for 75 patients, from whom, 23 (30.7%) patients were classified as lipodystrophic and 52 (69.3%) patients were classified as non-lipodystrophic. No differences were observed between HIV-infected patients with and without FMR defined lipodystropy, regarding the presence of osteopenia or osteoporosis and total, femur and spine BMD. Also, no differences were found between the four groups of body fat distribution (no lipodystrophy, isolated central fat accumulation, isolated peripheral lipoatrophy and mixed forms of lipodystrophy) regarding the presence of osteoporosis or osteopenia and total, femur and spine BMD (Table 2).

Lower total BMD was associated with lower diastolic blood pressure, lower weight, lower height, lower neck circumference, lower hip circumference, lower

FMR and higher SHBG (Table 3). There was no association between total BMD and age, duration of HIV infection, length of cART, CD4+ count, systolic blood pressure, BMI, upper-arm circumference, abdominal circumference, waist circumference, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, lipoprotein (a), HbA1c, HOMA, leptin, fibrinogen, total testosterone, free testosterone, LH, FSH, urea, creatinine, magnesium, calcium, phosphate, PTH, 25-OH vitamin D3, IGF-1 and TSH.

Lower spine BMD was associated with lower systolic blood pressure, lower weight, lower height, lower BMI, lower neck circumference, lower upper-arm circumference, lower waist circumference, lower hip circumference, higher total cholesterol, higher HDL cholesterol, lower FMR, lower total testosterone, lower free testosterone, higher SHBG and higher 25-OH vitamin D3. No association was observed between spine BMD and age, duration of HIV infection, length of cART, CD4+ count, diastolic blood pressure, abdominal circumference, LDL cholesterol, triglycerides, lipoprotein (a), HbA1c, HOMA, leptin, fibrinogen, LH, FSH, urea, creatinine, magnesium, calcium, phosphate, PTH, IGF-1, TSH, free T3 and free T4.

Lower femur BMD was related with older age, longer duration of HIV infection, longer cART, lower weight, lower neck circumference, lower abdominal circumference, lower hip circumference, lower FMR, higher SHBG and higher urea. No correlation was found between femur BMD and CD4 count, systolic and diastolic blood pressure, weight, height, BMI, upper-arm circumference, waist circumference, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, lipoprotein (a), HbA1c, HOMA, leptin, fibrinogen, total

testosterone, free testosterone, LH, FSH, creatinine, magnesium, calcium, phosphate, PTH, 25-OH vitamin D3, IGF-1, TSH, free T3 and free T4.

In the multivariate linear regression, SHBG was associated to total BMD after adjusting for diastolic blood pressure, weight and neck circumference (Table 4, model 1). When a model was tested for the adjustment on the association between total BMD, weight and FMR, only weight remained significantly associated to total BMD (Table 4, model 2).

Regarding spine BMD, in the multivariate linear regression, SHBG was associated even after adjustment for BMI, neck circumference and total cholesterol (Table 5, model 1). Another model was tested adjusting for FMR instead of neck circumference and BMI and SHBG remained associated to spine BMD, while FMR did not (Table 5, model 2).

Regarding femur BMD, age and urea were associated after adjusting for weight, neck circumference and SHBG (Table 6, model 1). When adjustment for age, weight and FMR was tested, age and weight remained significantly to femur BMD, while FMR did not (Table 6, model 2).

Discussion

Osteoporosis is one of the major problems facing HIV-infected patients. Prevalence of osteoporosis in HIV-infected patients varies in many studies in relation to the demographic characteristics. In a meta-analysis of these studies, a prevalence of 6-16% of osteoporosis was estimated [3], which is similar with the prevalence of 15.9% found in our study.

Several factors may increase the risk of osteoporosis in HIV-infected patients. There are findings implicating the cART on the mechanism of osteoporosis and specific antiretroviral drugs such as protease inhibitors (PIs) are more strongly associated with bone loss [3, 7, 23, 24], but we did not find any difference regarding the cART classes.

HIV-related risk factors such as duration of HIV infection and current CD4+ cells count play a role in the bone loss [23]. Our research could not find an association between osteoporosis and these factors, such as other recent study [7].

Conventional risk factors like older age and environmental exposures (smoking, alcohol consumption) have been reported as related to osteoporosis in HIV-infected patients [7, 25, 26]. We could not find a relationship between osteoporosis and these variables. Although, our results show a correlation between lower femur BMD and older age, longer duration of HIV infection and longer length of cART, proving that the first bone loss in HIV-infected patients occurs in the femur. Moreover, we also found older age as an independent risk factor for decreased femur BMD.

Body weight is an important determinant of BMD and in many studies BMI appears as an independent risk factor for low BMD in HIV-infected patients [23, 26, 27]. We did not find a difference between osteoporotic/non-osteoporotic patients regarding weight and BMI, but we found that a lower weight is related to lower total, spine and femur BMD, while lower BMI is only related to lower spine BMD.

BMI, weight and lipodystrophy are related to body circumferences (neck, upper-arm, abdominal, waist and hip circumferences) and there are no studies comparing the risk of osteoporosis in HIV-infected adult patients according to these measures. To our knowledge, our study is the first to report that HIV-osteoporotic patients have significantly lower hip circumference than HIV-non osteoporotic patients. In addition, lower hip circumference is also related to lower total, spine and femur BMD. Hip circumference is related positively to subcutaneous and femoral-gluteal fat [28], which means this fat tissue acts like a protective factor for bone loss.

Lipodystrophy syndrome has been used to describe an increasingly common fat-redistribution syndrome in HIV-patients, and it affects up to half or even more HIV-infected patients receiving cART [29]. Some studies have described that low BMD could be related to lipodystrophy [8-11] but it is still controversial [4]. We studied lipodystrophy defined by FMR and four groups of body fat distribution (no lipodystrophy, isolated central fat accumulation, isolated lipoatrophy and mixed forms of lipodystrophy). In our analyze, lipodystrophic HIV-patients (defined by FMR) and non lipodystrophic HIV-patients (defined by FMR) did not present different BMD. Lower FMR was also related to lower total, spine and femur BMD, but when we used the multivariate linear regression, FMR was not an independent risk factor to bone loss, which means it is expected that lipodystrophy (defined by FMR) acts in bone mediated by weight.

Concerning the different groups of body fat distribution, we are the first to study the relationship between clinical lipodystrophy and osteoporosis in HIV-infected patients and according to our results body composition is not related to BMD alterations. This issue is still a matter of controversy, since in healthy patients

there is evidence that centrally located body fat is associated with lower BMD [30] but other results suggest that central and peripheral fat body mass have an independent and protective effect on the presence of osteoporosis or osteopenia [31].

Hypogonadism is common in HIV-infected men, but according to recent data deficiency of testosterone is not related with decreased BMD in HIV-patients [32, 33] . Moreover, estradiol levels seem to be more important than testosterone in preventing decreased BMD in HIV-infected men [32] , but we could not measure this hormone. In contrast, our data show that osteoporotic HIV-patients have higher total testosterone levels than non-osteoporotic patients and SHBG emerged as an independent risk factor for decreased total and spine BMD. This may happen because, in HIV-infected patients, SHBG levels are elevated and consequently total testosterone as well, decreasing the free fraction of this hormone.

There is a high prevalence of chronic kidney disease in HIV population [34]. We found higher urea in osteoporotic HIV-patients comparing with non-osteoporotic HIV-patients. We also found that higher urea levels are an independent risk factor to decreased femur BMD.

25-OH vitamin D has one of the most important roles in calcium homeostasis and bone health but we did not find any difference between osteoporotic and non-osteoporotic HIV-patients regarding it. However we found that an increase in 25-OH vitamin D levels is related to lower spine BMD. This is not according with the literature and it is difficult to explain [2, 35].

Our study has a few limitations. First, because it is a cross-sectional study, we could not establish a causal relation between HIV infection and decreased BMD. Also, its statistical power is not very high because of the small number of our sample and the low heterogeneity of the enrolled patients. We did not measure estradiol levels, we do not have the information about supplementation of 25-OH vitamin D3, neither the time of measurements of it, and there were no enough patients to study free T3 and free T4 according to total BMD. In addition, we do not have data on specific cART regimens over time, namely duration of tenofovir disoproxil fumarate.

Conclusion

Osteoporotic HIV-infected patients have lower hip circumference than non-osteoporotic patients. Clinical lipodystrophy and different groups of body fat distribution do not seem to play an independent role in the development of osteoporosis. SHBG is independently related to total and spine BMD, while older age and higher urea levels are independent risk factors for decreased femur BMD.

List of abbreviations

95%CI – 95.0% confidence interval for β

BMD – bone mineral density

BMI - body mass index

cART - combined antiretroviral therapy

FMR - fat mass ratio

FSH – follicle stimulation hormone

HbA1c - glycosylated hemoglobin

HDL– high density protein

HIV - human immunodeficiency virus

HOMA - homeostasis model assessment of insulin resistance

IGF-1 - insulin-like growth factor 1

II - integrase inhibitor

IQR – interquartile range

LDL cholesterol - low-density lipoprotein

LH - luteinizing hormone

NNRTI - non-nucleoside reverse transcriptase inhibitor

*N*RTI - nucleoside reverse transcriptase inhibitor

PI - protease inhibitor

PTH - parathyroid hormone

SD – standard deviation

SHBG - sex hormone binding globulin

T3 – triiodothyronine

T4 – thyroxine

TSH – thyroid stimulating hormone

WHO - World Health Organization

β – unstandardized coefficient

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Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author [FM]. The data are not publicly available because they contain information that could compromise research participant privacy/consent.

Authors' contributions

FM conceived the study, participated in its design, in the acquisition of data and drafted the manuscript; ARC participated in the acquisition of data; APS participated in the acquisition of data and in the manuscript revision; ACS performed the statistical analysis and revised critically the manuscript; AJM performed the CT scans and reviewed the data; JP performed DXA scans and reviewed the data; AS critically revised the manuscript; DC critically revised the manuscript; PF conceived the study, participated in its design, in the acquisition of data and critically revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

All patients provided written informed consent and the study protocol was approved by the São João Hospital's Ethics Committee for Health.

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Table 1

Clinical, anthropometric, metabolic, hormonal and renal parameters of the total sample according to the presence or absence of osteoporosis.

	Total (n=126)	No osteoporosis (n= 106)	Osteoporosis (n= 20)	p value
Sex [n(%)]				0.254
Female	62 (49.2)	55 (51.9)	7 (35.0)	
Male	64 (50.8)	51 (48.1)	13 (65.0)	
Age [years, mean (SD)]	49.3 (11.3)	48.8 (11.6)	51.7 (9.0)	0.293
Duration of HIV infection [years, median (IQR)]	9 (7)	9 (7)	11.5 (11)	0.271
HIV stage [n (%)]				0.297
A	62 (54.9)	55 (57.3)	7 (41.2)	
B	4 (3.5)	3 (3.1)	1 (5.9)	
C	44 (38.9)	36 (37.5)	8 (47.1)	

cART [years, median (IQR)]	7 (7.0)	7 (7.0)	8.5 (10.5)	0.177
NRTI [n (%)]	110 (90.9)	95 (93.1)	15 (78.9)	0.070
NNRTI [n (%)]	64 (52.9)	53 (52)	11 (57.9)	0.822
PI [n (%)]	50 (41.3)	43 (42.2)	7 (36.8)	0.859
II [n (%)]	11 (9.1)	9 (8.8)	2 (10.5)	0.683
CD4 count [cells/mm^3, median (IQR)]	499.5 (305.0)	488.5 (341)	544 (159)	0.354
HIV risk factor [n(%)]				0.296
Injecting drug user	8 (23.5)	6 (24)	2 (22.2)	
Heterosexual contact	23 (67.6)	18 (72)	5 (55.6)	
Homosexual contact	1 (2.9)	0 (0)	1 (11.1)	
Others	2 (5.9)	1 (4)	1 (11.1)	
Drugs [n (%)]				

Statins [n (%)]	31 (24,6)	25 (23.6)	6 (30)	0.575
Fibrates [n (%)]	21 (16.7)	20 (18.9)	1 (5)	0.230
Antihypertensive Agents [n(%)]	25 (19.8)	21 (19.8)	4 (20)	0.999
Oral Antidiabetic Agents [n(%)]	12 (9.5)	9 (8.5)	3 (15)	0.404
Insulin [n (%)]	4 (3.2)	3 (2.8)	1 (5)	0.504
Smoking history [n (%)]				0.226
Current	57 (46.7)	51 (49.5)	6 (31.6)	
Former	48 (39.3)	37 (35.9)	11 (57.9)	
Never	17 (13.9)	15 (14.6)	2 (10.5)	
Alcohol consumption [n (%)]	39 (32)	6 (4.9)	33 (27.1)	0.999
Co-infection Hepatitis C [n (%)]	24 (19.4)	19 (79.2)	5 (20.8)	0.538
Arterial hypertension [n (%)]	42 (33.3)	37 (29.3)	5 (4)	0.546

Systolic blood pressure [mmHg, median (IQR)]	120 (25)	120 (25)	121 (25)	0.801
Diastolic blood pressure [mmHg, median (IQR)]	71 (14)	70 (15)	72.5 (12)	0.286
Weight [kg, mean (SD)]	68.5 (13.0)	69.3 (13.4)	63.8 (9.5)	0.080
Height [m, mean (SD)]	1.64 (0.1)	1.64 (0.1)	1.63 (0.1)	0.896
BMI [kg/m^2, median (IQR)]	25.4 (5.6)	25.6 (5.4)	24.8 (6.0)	0.631
Underweight [n (%)]	9 (7.1)	7 (6.6)	2 (10.0)	
Normal weight [n (%)]	44 (34.9)	36 (34)	8 (40.0)	
Overweight [n (%)]	56 (44.4)	47 (44.3)	9 (45.0)	
Obesity [n (%)]	17 (13.5)	16 (15.1)	1 (5.0)	
Neck circumference [cm, median (IQR)]	36.8 (6.1)	37.0 (6.0)	36.5 (8.5)	0.914

Upper-arm circumference [cm, median (IQR)]	28.0 (4.0)	28 (3.9)	27.5 (5)	0.167
Abdominal circumference [cm, median (IQR)]	94.0 (10.0)	94.0 (10.0)	90.0 (13.0)	0.101
Waist circumference [cm, SD)]	91.7 (11.2)	92.2 (11.2)	88.4 (11.2)	0.226
Hip circumference [cm, SD]	47.6 (7.0)	48.0 (4.6)	43.0 (4.8)	0.004
Total cholesterol [mg/dL, SD]	222.2 (58.0)	222.5 (58.3)	208.2 (55.9)	0.270
LDL cholesterol [mg/dL, mean (SD)]	141.0 (43.0)	143.0 (41.3)	128.3 (51.5)	0.191
HDL cholesterol [mg/dL, median (IQR)]	47.0 (20)	47.5 (20)	47.0 (21)	0.870
Triglycerides [mg/dL, median (IQR)]	166.0 (115.0)	169.0 (120)	130 (74)	0.097

Lipoprotein a [mg/dL, median (IQR)]	17.5 (44)	18.9 (45)	13.0 (14.5)	0.263
HbA1c [%, median (IQR)]	5.4 (0.8)	5.4 (0.8)	5.5 (0.7)	0.055
HOMA [median, (IQR)]	2.2 (2.8)	2.1 (2.6)	3.2 (9.6)	0.625
Leptin [ng/mL, median (IQR)]	5.8 (9.0)	7.3 (9.2)	2.7 (5.5)	0.190
Fibrinogen [mg/dL, median (IQR)]	347 (120)	348 (117)	270 (181)	0.276
Male hypogonadism [n (%)]	9 (18)	8 (88.9)	1 (11.1)	0.999
Total testosterone [ng/mL, median (IQR)]	3.0 (5.6)	1.6 (5.0)	6.7 (5.3)	0.001
Free testosterone [ng/dL, median (IQR)]	3.5 (12.5)	1.71 (11.79)	12.6 (9.86)	0.084
SHBG [nmol/L, median (IQR)]	62.5 (58.8)	61.3 (59.1)	76.5 (60.7)	0.543

LH [mIU/mL, median (IQR)]	5.9 (5.2)	6.42 (5.53)	5.24 (5.16)	0.824
FSH [mIUmL, median (IQR)]	7.2 (9.3)	7.6 (9.75)	5.71 (4.82)	0.303
Urea [g/L, median (IQR)]	0.34 (0.13)	0.31 (0.12)	0.37 (0.14)	0.028
Creatinine [mg/L, median (IQR)]	8.7 (2.3)	8.65 (2.4)	8.7 (2.6)	0.730
Magnesium [mmol/L, SD]	1.65 (0.15)	1.6 (0.1)	1.7 (0.2)	0.708
Calcium [mmol/L, median (IQR)]	4.60 (0.30)	4.65 (0.30)	4.60 (0.25)	0.481
Phosphate [mg/L, median (IQR)]	31.5 (6.45)	31.8 (6.6)	30.75 (5.35)	0.985
PTH [pg/mL, median (IQR)]	42.2 (22.2)	43.4 (22)	37.3 (23.5)	0.095
25-OH vitamin D3 [ng/mL, median (IQR)]	25 (19)	21.5 (17)	31 (22)	0.181
IGF-1 [nmol/L, (SD)]	142.2 (60.8)	146.2 (60.6)	107.1 (52.6)	0.053
TSH [mIU/L, median (IQR)]	1.70 (1.34)	1.68 (1.28)	2.34 (1.78)	0.285

Free T3	3.27 (0.62)	3.25 (0.61)	3.49 (0.71)	0.209
Free T4	0.96 (0.11)	0.96 (0.11)	0.97 (0.30)	0.246

Abbr: HIV - human immunodeficiency virus; cART - combined antiretroviral therapy; *N*RTI - nucleoside reverse transcriptase inhibitor; NNRTI - non-nucleoside reverse transcriptase inhibitor; PI - protease inhibitor; II - integrase inhibitor; BMI - body mass index; LDL cholesterol - low-density lipoprotein cholesterol; HDL cholesterol – high density protein cholesterol; HbA1c - glycosylated hemoglobin; HOMA - homeostasis model assessment of insulin resistance; SHBG - sex hormone binding globulin; LH - luteinizing hormone; FSH – follicle stimulation hormone; PTH - parathyroid hormone; IGF-1 - insulin-like growth factor 1; TSH – thyroid stimulating hormone; T3 – triiodothyronine; T4 – thyroxine; SD – standard deviation; IQR – interquartile range.

Table 2

BMD according to the presence of lipodystrophy defined by FMR and according to the presence of different groups of body composition.

	Lipodystrophy defined by FMR			Groups of body fat distribution				
	No Lipodystro-phy (FMR defined) (n= 52)	Lipodystro-phy (FMR defined) (n= 23)	p value	No lipodystro-phy (n= 8)	Isolated central fat accumula-tion (n=37)	Isolated peripheral lipoatrophy (n=33)	Mixed forms lipodystro-phy (n=34)	p value
Normal BMD [n(%)]	32 (61.5)	13 (56.5)	0.927	4 (40.0)	20 (54.1)	13 (39,4)	16 (47.1)	0.305
Osteopenia [n(%)]	16 (30.8)	8 (34.8)		4 (40.0)	16 (43.2)	13 (39.4)	13 (38.2)	
Osteoporosis [n(%)]	4 (7.7)	2 (8.7)		2 (20.0)	1 (2.7)	7 (21.2)	5 (14.7)	
Total BMD	1.17 (0.11)[a]	1.18 (0.1)[a]	0.487	1.20 (0.13)[b]	1.18 (0.09)[b]	1.13 (0.15)[b]	1.18 (0.16)[b]	0.181
Spine BMD	0.9 (0.13)[a]	0.99 (0.17)[a]	0.252	1.01 (0.20)[b]	0.96 (0.18)[b]	0.93 (0.16)[b]	1.02 (0.28)[b]	0.081
Femur BMD	1.21 (0.15)[a]	1.25 (0.15)[a]	0.277	1.19 (0.46)[b]	1.22 (0.21)[b]	1.11 (0.23)[b]	1.05 (0.33)[b]	0.290

Abbv: BMD – bone mineral density; FMR - fat mass ratio.

[a] [g/cm^2, mean (SD)], SD – standard deviation. [b] [g/cm^2, median (IQR)], IQR – interquartile range

Table 3

Correlation of baseline and clinical parameters with total, spine and femur bone mineral density.

	Total BMD		Spine BMD		Femur BMD	
	r	p	R	p	r	p
Age	-0.2	0.082	-0.059	0.527	-0.299	0.001**
Duration of HIV infection	-0.039	0.737	0.067	0.473	-0.316	0.000**
Duration of cART	0.012	0.916	0.054	0.560	-0.27	0.002**
CD4 count	0.134	0.250	0.101	0.290	-0.084	0.376
Systolic blood pressure	0.119	0.301	0.267	0.004**	-0.114	0.216
Diastolic blood pressure	0.305	0.007**	0.179	0.052	0.063	0.498
Weight	0.318	0.005**	0.445	0.000**	0.227	0.013*
Height	0.270	0.018*	0.348	0.000**	0.07	0.447
BMI	0.165	0.151	0.245	0.007**	0.169	0.065

Neck circumference	0.289	0.017*	0.420	0.000**	0.238	0.031*
Upper-arm circumference	0.209	0.085	0.297	0.007**	0.204	0.065
Abdominal circumference	0.147	0.211	0.167	0.105	0.240	0.019*
Waist circumference	0.188	0.104	0.242	0.012*	0.182	0.061
Hip circumference	0.290;	0.012*	0.323	0.002**	0.320	0.002**
Total cholesterol	-0.119	0.301	-0.211	0.023*	0.141	0.127
LDL cholesterol	-0.065	0.576	-0.169	0.071	0.125	0.179
HDL cholesterol	-0.137	0.235	-0.215	0.021*	-0.028	0.765
Triglycerides	0.017	0.882	-0.075	0.428	0.152	0.101
Lipoprotein (a)	-0.186	0.126	-0.201	0.082	-0.113	0.329
HbA1c	-0.023	0.845	0.024	0.806	-0.099	0.311
HOMA	0.115	0.347	0.209	0.083	0.148	0.217
Leptin	-0.008	0.957	-0.110	0.461	-0.054	0.721
Fibrinogen	0.125	0.312	0.077	0.540	0.044	0.725

FMR	0.254	0.028*	0.295	0.011*	0.281	0.014*
Total testosterone	0.224	0.064	0.322	0.002**	0.034	0.751
Free testosterone	0.209	0.092	0.395	0.000**	0.086	0.432
SHBG	-0.395	0.002**	-0.481	0.000**	-0.226	0.039*
LH	-0.036	0.777	0.059	0.586	-0.130	0.221
FSH	-0.088	0.480	0.007	0.951	-0.151	0.151
Urea	-0.113	0.333	-0.056	0.556	-0.268	0.004**
Creatinine	0.101	0.383	0.030	0.754	0.066	0.486
Magnesium	-0.030	0.796	-0.043	0.648	-0.085	0.357
Calcium	0.067	0.560	0.112	0.240	0.084	0.377
Phosphate	-0.017	0.882	0.056	0.560	-0.122	0.201
PTH	0.017	0.897	0.019	0.861	0.003	0.980
25-OH vitamin D3	-0.5	0.667	-0.363	0.027*	-0.198	0.234
IGF-1	0.126	0.297	0.033	0.756	0.014	0.891

TSH	0.014	0.908	-0.032	0.752	-0.074	0.458
Free T3	-----	-----	-0.197	0.368	-0.138	0.519
Free T4	-----	------	-0.220	0.227	-0.244	0.170

Abbr: BMD – bone mineral density; HIV - human immunodeficiency virus; cART - combined antiretroviral therapy; BMI - body mass index; LDL cholesterol - low-density lipoprotein cholesterol; HDL cholesterol – high density protein cholesterol; HbA1c - glycosylated hemoglobin; HOMA - homeostasis model assessment of insulin resistance; FMR – fat mass ratio; SHBG - sex hormone binding globulin; LH - luteinizing hormone; FSH – follicle stimulation hormone; PTH - parathyroid hormone; IGF-1 - insulin-like growth factor 1; TSH – thyroid stimulating hormone; T3 – triiodothyronine; T4 – thyroxine; r – correlation coefficient; p – sig. (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

Table 4

Multivariate linear regression analysis of factors associated with total bone mineral density

Total BMD	β	95%CI
Model 1		
Diastolic blood pressure	0.002	-0.001/0.005
Weight	0.003	-0.013/0.006
Neck circumference	-0.004	-0.014/0.005
SHBG	-0.001	-0.001/0.000
Model 2		
FMR	0.019	-0.015/0.054
Weight	0.003	0.001/0.005

Abbv: SHBG – sex hormone binding hormone; FMR – fat mass ratio; β – unstandardized coefficient; 95%CI – 95.0% confidence interval for β.

Table 5

Multivariate linear regression analysis of factors associated with spine bone mineral density

Spine BMD	β	95%CI
Model 1		
BMI	0.005	-0.003/0.131
Neck circumference	0.007	-0.001/0.015
Total cholesterol	-0.001	-0.001/0.000
SHBG	-0.001	-0.002/0.000
Model 2		
BMI	0.008	-0.002/0.000
FMR	0.027	-0.020/0.074
Total cholesterol	0.000	-0.001/0.000
SHBG	-0.001	-0.002/0.000

Abbv: BMI – body mass index; SHBG – sex hormone binding hormone: FMR – fat mass ratio; β – unstandardized coefficient; 95%CI – 95.0% confidence interval for β.

Table 6

Multivariate linear regression analysis of factors associated with femur bone mineral density

Femur BMD	B	95%CI
Model 1		
Age	-0.006	-0.009/-0.003
Weight	0.004	-0.001/0.009
Neck circumference	0.004	-0.009/0.018
SHBG	-5.242E-5	-0.001/0.001
Urea	-0.763	-1.143/-0.383
Model 2		
Age	-0.004	-0.007/-0.002
FMR	0.033	-0.009/0.075
Weight	0.006	0.003/0.009

Abbv: SHBG – sex hormone binding hormone: FMR – fat mass ratio; β – unstandardized coefficient; 95%CI – 95.0% confidence interval for β.

ANEXOS

Parecer da Comissão de Ética para a Saúde e Autorização do Concelho de Administração do Centro Hospitalar São João

Normas da revista Biomed Central Infectious Diseases

196-16

Unidade de Investigação

Tomei conhecimento. Nada a opor.

2 de Novembro de 2016

A Coordenadora da Unidade de Investigação

(Prof.ª Doutora Ana Azevedo)

DIRECÇÃO CLÍNICA
22/11/2016

Aprovado. Ao CA.

(Prof.ª Doutora Ana Azevedo)

AUTORIZADO

CONSELHO DE ADMINISTRAÇÃO (R) REUNIÃO DE 30 NOV 2016

Presidente do Conselho de Administração

Director Clínico | Enfermeira Directora | Vogal Executivo | Vogal Executivo

Exmo. Senhor

Presidente do Conselho de Administração do

Centro Hospitalar de S. João – EPE

Assunto: Pedido de autorização para realização de estudo/projecto de investigação

Nome do Investigador Principal: Flávia Andreia Pinto Moreira

Título do projecto de investigação: Osteoporosis in HIV-infected adults patients under antiretroviral therapy

Pretendendo realizar no(s) Serviço(s) de Endocrinologia do Centro Hospitalar de S. João – EPE o estudo/projecto de investigação em epígrafe, solicito a V. Exa., na qualidade de Investigador/Promotor, autorização para a sua efectivação.

Para o efeito, anexa toda a documentação referida no dossier da Comissão de Ética do Centro Hospitalar de S. João respeitante a estudos/projectos de investigação, à qual endereçou pedido de apreciação e parecer.

Com os melhores cumprimentos.

Porto, 06 / Julho / 2016

O INVESTIGADOR/PROMOTOR

Flávia Moreira

Comissão de Ética para a Saúde do HSJ

Parecer

Projecto de investigação: "Osteoporosis in HIV-infected adult patients under antiretroviral therapy".

Promotores:
- Não aplicável.

- Pertinência do estudo

- Trata-se de um estudo de coorte, retrospectivo, a realizar no âmbito da tese de Mestrado Integrado em Medicina na Faculdade de Medicina da Universidade do Porto (FMUP), que tem como objectivo principal, avaliar a prevalência de osteoporose nos doentes com infecção VIH sob terapêutica antiretrovírica.
  - Serão incluídos todos os indivíduos com infecção VIH seguidos na consulta de Endocrinologia do Centro Hospitalar de S. João, entre janeiro de 2004 e março de 2016.
  - O estudo terminaria em dezembro de 2015 e não terá nem precisará de qualquer apoio financeiro. *Dada a submissão deste protocolo à CES em julho de 2016, o prazo indicado terá que ser alargado.*
  - Todos os dados a colher de forma a anónima (sócio-demográficos, antropométricos, clínicos, virológicos, analíticos e terapêuticos) são pertinentes e adequados aos objectivos do estudo. *No protocolo submetido à CES não figura a metodologia usada para diagnosticar a osteoporose.*
    - O estudo é pertinente, importante e está bem fundamentado.
    - O protocolo de estudo, os critérios de inclusão e de exclusão estão suficientemente detalhados e não levantam quaisquer questões do foro ético.
    - A Investigadora Principal, Flávia Moreira, estudante do 6º ano do curso de Medicina da FMUP, tendo como elo de ligação (e orientadora da Tese) a Médica especialista de Endocrinologia, a Professora Paula Freitas (especialista do Serviço de Endocrinologia do Hospital de S. João EPE), dispõe das competências técnica e científica para a realização do estudo.
    - O estudo será realizado no Serviço de Endocrinologia do Hospital de S. João, EPE e dispõe da autorização para a sua realização pelo seu Director, Professor Davide Carvalho. O serviço proponente dispõe das condições necessárias para a realização do estudo.

– Beneficio/Risco

- Dada a natureza retrospectiva do estudo, não haverá riscos, incómodos ou benefícios para os participantes.

– Respeito pela liberdade e autonomia do sujeito do ensaio

- Dada a natureza retrospectiva do estudo, não há necessidade de proceder à obtenção do consentimento informado.

– Confidencialidade dos dados

- A confidencialidade e a privacidade dos dados são garantidas.

– Indemnização por danos

Não aplicável.

– Continuação do tratamento

Não aplicável.

- Propriedade dos dados

Não aplicável.

Conclusão

Em face da análise do protocolo de "Osteoporosis in HIV-infected adult patients under antiretroviral therapy", proponho a sua aprovação pela CES do HSJ/FMUP, depois de obtidas as respostas às questões em sublinhado e em itálico.

Porto, 22 de julho de 2016

O Relator
Prof. Manuel Vaz Silva

Todas as questões foram esclarecidas

Pedro Bento

20/09/16

7. **SEGURO**

a. *Este estudo/projecto de investigação prevê intervenção clínica que implique a existência de um seguro para os participantes?*

SIM ☐ (Se sim, junte, por favor, cópia da Apólice de Seguro respectiva)
NÃO ☒
NÃO APLICÁVEL ☐

8. **TERMO DE RESPONSABILIDADE**

Eu, Flávia Andreia Pinto Moreira, abaixo-assinado, na qualidade de Investigador Principal, declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (com as emendas de Tóquio 1975, Veneza 1983, Hong-Kong 1989, Somerset West 1996 e Edimburgo 2000) e da Organização Mundial da Saúde, no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo no decurso do actual internamento ou da mesma consulta.

Porto, 07 de Julho / 2016

27/07/16

A Comissão de Ética para a Saúde tendo aprovado o parecer do Relator, aguarda que o Investigador/Promotor esclareça as questões nele enunciadas para que possa emitir parecer definitivo.

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

Flávia Moreira

O Investigador Principal

	PARECER DA COMISSÃO DE ÉTICA PARA A SAÚDE DO CENTRO HOSPITALAR DE S. JOÃO/FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO
emitido na reunião plenária da CES de ____/_____/_____	Centro Hospitalar **São João**. CONSIDERADOS QUE FORAM COMO SATISFATÓRIOS OS ESCLARECIMENTOS PRESTADOS PELO(A) INVESTIGADOR(A), A CES APROVA POR UNANIMIDADE O PARECER DO RELATOR, PELO QUE NADA TEM A OPOR À REALIZAÇÃO DESTE PROJETO DE INVESTIGAÇÃO. 30/07/16 Prof. Doutor Filipe Almeida Presidente da Comissão de Ética

Normas da revista BioMed Central Infectious Diseases

Research article

Criteria

Research articles should report on original primary research, but may report on systematic reviews of published research provided they adhere to the appropriate reporting guidelines which are detailed in our editorial policies. Please note that non-commissioned pooled analyses of selected published research will not be considered.

Preparing your manuscript

The information below details the section headings that you should include in your manuscript and what information should be within each section.

Please note that your manuscript must include a 'Declarations' section including all of the subheadings (please see below for more information).

Title page

The title page should:

- present a title that includes, if appropriate, the study design e.g.:
    - "A versus B in the treatment of C: a randomized controlled trial", "X is a risk factor for Y: a case control study", "What is the impact of factor X on subject Y: A systematic review"
    - or for non-clinical or non-research studies a description of what the article reports

- list the full names, institutional addresses and email addresses for all authors
    - if a collaboration group should be listed as an author, please list the Group name as an author. If you would like the names of the individual members of the Group to be searchable through their individual PubMed records, please include this information in the “Acknowledgements” section in accordance with the instructions below
- indicate the corresponding author

Abstract

The Abstract should not exceed 350 words. Please minimize the use of abbreviations and do not cite references in the abstract. Reports of randomized controlled trials should follow the CONSORT extension for abstracts. The abstract must include the following separate sections:

- Background: the context and purpose of the study
- Methods: how the study was performed and statistical tests used
- Results: the main findings
- Conclusions: brief summary and potential implications
- Trial registration: If your article reports the results of a health care intervention on human participants, it must be registered in an appropriate registry and the registration number and date of registration should be in stated in this section. If it was not registered prospectively (before enrollment of the first participant), you should include the words

'retrospectively registered'. See our editorial policies for more information on trial registration

Keywords

Three to ten keywords representing the main content of the article.

Background

The Background section should explain the background to the study, its aims, a summary of the existing literature and why this study was necessary or its contribution to the field.

Methods

The methods section should include:

- the aim, design and setting of the study
- the characteristics of participants or description of materials
- a clear description of all processes, interventions and comparisons. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses
- the type of statistical analysis used, including a power calculation if appropriate

Results

This should include the findings of the study including, if appropriate, results of statistical analysis which must be included either in the text or as tables and figures.

Discussion

This section should discuss the implications of the findings in context of existing research and highlight limitations of the study.

Conclusions

This should state clearly the main conclusions and provide an explanation of the importance and relevance of the study reported.

List of abbreviations

If abbreviations are used in the text they should be defined in the text at first use, and a list of abbreviations should be provided.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

- Ethics approval and consent to participate
- Consent for publication
- Availability of data and material
- Competing interests
- Funding
- Authors' contributions
- Acknowledgements
- Authors' information (optional)

Please see below for details on the information to be included in these sections.

If any of the sections are not relevant to your manuscript, please include the heading and write 'Not applicable' for that section.

Ethics approval and consent to participate

Manuscripts reporting studies involving human participants, human data or human tissue must:

- include a statement on ethics approval and consent (even where the need for approval was waived)
- include the name of the ethics committee that approved the study and the committee's reference number if appropriate

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If your manuscript does not report on or involve the use of any animal or human data or tissue, please state "Not applicable" in this section.

Consent for publication

If your manuscript contains any individual person's data in any form (including individual details, images or videos), consent for publication must be obtained from that person, or in the case of children, their parent or legal guardian. All presentations of case reports must have consent for publication.

You can use your institutional consent form or our consent form if you prefer. You should not send the form to us on submission, but we may request to see a copy at any stage (including after publication).

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If your manuscript does not contain data from any individual person, please state “Not applicable” in this section.

Availability of data and materials

All manuscripts must include an ‘Availability of data and materials’ statement. Data availability statements should include information on where data supporting the results reported in the article can be found including, where applicable, hyperlinks to publicly archived datasets analysed or generated during the study. By data we mean the minimal dataset that would be necessary to interpret, replicate and build upon the findings reported in the article. We recognise it is not always possible to share research data publicly, for instance when individual privacy could be compromised, and in such instances data availability should still be stated in the manuscript along with any conditions for access.

Data availability statements can take one of the following forms (or a combination of more than one if required for multiple datasets):

- The datasets generated and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS]
- The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.
- All data generated or analysed during this study are included in this published article [and its supplementary information files].

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- The data that support the findings of this study are available from [third party name] but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of [third party name].
- Not applicable. If your manuscript does not contain any data, please state 'Not applicable' in this section.

More examples of template data availability statements, which include examples of openly available and restricted access datasets, are available here.

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Hao Z, AghaKouchak A, Nakhjiri N, Farahmand A. Global integrated drought monitoring and prediction system (GIDMaPS) data sets. figshare. 2014. http://dx.doi.org/10.6084/m9.figshare.853801

With the corresponding text in the Availability of data and materials statement:

The datasets generated during and/or analysed during the current study are available in the [NAME] repository, [PERSISTENT WEB LINK TO DATASETS].[Reference number]

Competing interests

All financial and non-financial competing interests must be declared in this section.

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Please use the authors initials to refer to each author's competing interests in this section.

If you do not have any competing interests, please state "The authors declare that they have no competing interests" in this section.

Funding

All sources of funding for the research reported should be declared. The role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript should be declared.

Authors' contributions

The individual contributions of authors to the manuscript should be specified in this section. Guidance and criteria for authorship can be found in our editorial policies.

Please use initials to refer to each author's contribution in this section, for example: "FC analyzed and interpreted the patient data regarding the hematological disease and the transplant. RH performed the histological examination of the kidney, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript."

Acknowledgements

Please acknowledge anyone who contributed towards the article who does not meet the criteria for authorship including anyone who provided professional writing services or materials.

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system and also include collaborating author names as the last paragraph of the “Acknowledgements” section. Please add authors in the format First Name, Middle initial(s) (optional), Last Name. You can add institution or country information for each author if you wish, but this should be consistent across all authors.

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Endnotes

Endnotes should be designated within the text using a superscript lowercase letter and all notes (along with their corresponding letter) should be included in the Endnotes section. Please format this section in a paragraph rather than a list.

References

All references, including URLs, must be numbered consecutively, in square brackets, in the order in which they are cited in the text, followed by any in tables or legends. The reference numbers must be finalized and the reference list fully formatted before submission.

Examples of the BioMed Central reference style are shown below. Please ensure that the reference style is followed precisely.

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Web links and URLs: All web links and URLs, including links to the authors' own websites, should be given a reference number and included in the reference list rather than within the text of the manuscript. They should be provided in full, including both the title of the site and the URL, as well as the date the site was accessed, in the following format: The Mouse Tumor Biology Database. http://tumor.informatics.jax.org/mtbwi/index.do. Accessed 20 May 2013. If an author or group of authors can clearly be associated with a web link (e.g. for blogs) they should be included in the reference.

Example reference style:

Article within a journal

Smith JJ. The world of science. Am J Sci. 1999;36:234-5.

Article within a journal (no page numbers)

Rohrmann S, Overvad K, Bueno-de-Mesquita HB, Jakobsen MU, Egeberg R, Tjønneland A, et al. Meat consumption and mortality - results from the European Prospective Investigation into Cancer and Nutrition. BMC Med. 2013;11:63.

Article within a journal by DOI

Slifka MK, Whitton JL. Clinical implications of dysregulated cytokine production. Dig J Mol Med. 2000; doi:10.1007/s801090000086.

Article within a journal supplement

Frumin AM, Nussbaum J, Esposito M. Functional asplenia: demonstration of splenic activity by bone marrow scan. Blood 1979;59 Suppl 1:26-32.

Book chapter, or an article within a book

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology. London: Academic; 1980. p. 251-306.
OnlineFirst chapter in a series (without a volume designation but with a DOI)
Saito Y, Hyuga H. Rate equation approaches to amplification of enantiomeric excess and chiral symmetry breaking. Top Curr Chem. 2007. doi:10.1007/128_2006_108.

Complete book, authored

Blenkinsopp A, Paxton P. Symptoms in the pharmacy: a guide to the management of common illness. 3rd ed. Oxford: Blackwell Science; 1998.

Online document

Doe J. Title of subordinate document. In: The dictionary of substances and their

effects. Royal Society of Chemistry. 1999. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999.

Online database

Healthwise Knowledgebase. US Pharmacopeia, Rockville. 1998. http://www.healthwise.org. Accessed 21 Sept 1998.

Supplementary material/private homepage

Doe J. Title of supplementary material. 2000. http://www.privatehomepage.com. Accessed 22 Feb 2000.

University site

Doe, J: Title of preprint. http://www.uni-heidelberg.de/mydata.html (1999). Accessed 25 Dec 1999.

FTP site

Doe, J: Trivial HTTP, RFC2169. ftp://ftp.isi.edu/in-notes/rfc2169.txt (1999). Accessed 12 Nov 1999.

Organization site

ISSN International Centre: The ISSN register. http://www.issn.org (2006). Accessed 20 Feb 2007.

Dataset with persistent identifier

Zheng L-Y, Guo X-S, He B, Sun L-J, Peng Y, Dong S-S, et al. Genome data from sweet and grain sorghum (Sorghum bicolor). GigaScience Database. 2011. http://dx.doi.org/10.5524/100012.

Preparing main manuscript text

Quick points:

- Use double line spacing
- Include line and page numbering
- Use SI units: Please ensure that all special characters used are embedded in the text, otherwise they will be lost during conversion to PDF
- Do not use page breaks in your manuscript

Preparing tables

When preparing tables, please follow the formatting instructions below.

- Tables should be numbered and cited in the text in sequence using Arabic numerals (i.e. Table 1, Table 2 etc.).
- Tables less than one A4 or Letter page in length can be placed in the appropriate location within the manuscript.
- Tables larger than one A4 or Letter page in length can be placed at the end of the document text file. Please cite and indicate where the table should appear at the relevant location in the text file so that the table can be added in the correct place during production.

- Larger datasets, or tables too wide for A4 or Letter landscape page can be uploaded as additional files. Please see [below] for more information.
- Tabular data provided as additional files can be uploaded as an Excel spreadsheet (.xls) or comma separated values (.csv). Please use the standard file extensions.
- Table titles (max 15 words) should be included above the table, and legends (max 300 words) should be included underneath the table.
- Tables should not be embedded as figures or spreadsheet files, but should be formatted using ‘Table object’ function in your word processing program.
- Color and shading may not be used. Parts of the table can be highlighted using superscript, numbering, lettering, symbols or bold text, the meaning of which should be explained in a table legend.
- Commas should not be used to indicate numerical values.