




# Klinefelter Bone Microarchitecture Evolution with Testosterone Replacement Therapy

A. Piot<sup>1,2</sup> · I. Plotton<sup>3,5,6</sup> · S. Boutroy<sup>2</sup> · J. Bacchetta<sup>2,4</sup> · S. Ailloud<sup>1</sup> · H. Lejeune<sup>5,6</sup> · R. D. Chapurlat<sup>1,2</sup> · P. Szulc<sup>2</sup> · C. B. Confavreux<sup>1,2,7</sup> 

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## Abstract

Klinefelter Syndrome (KS) patients, defined by a 47 XXY karyotype, have increased risk of fragility fractures. We have assessed bone microarchitecture by high resolution peripheral quantitative CT (HR-pQCT) at the radius and tibia in young KS patients, naïve from testosterone replacement therapy (TRT). Areal bone mineral density (BMD) and body composition were assessed by dual X-ray absorptiometry (DXA). Total testosterone (tT) was measured at baseline. Bone measurements have been repeated after 30 months of TRT. We enrolled 24 KS patients and 72 age-matched controls. KS patients were (mean ± SD) 23.7 ± 7.8 year-old. KS patients had significantly lower relative appendicular lean mass index (RALM) and lower aBMD at spine and hip than controls. Ten patients (42%) had low tT level ( $\leq 10.4$  nmol/L). At baseline, we observed at radius a marked cortical (Ct) impairment reflected by lower Ct.area, Ct.perimeter, and Ct.vBMD than controls. At tibia, in addition to cortical fragility, we also found significant alterations of trabecular (Tb) compartment with lower trabecular bone volume (BV/TV) and Tb.vBMD as compared to controls. After 30 months of TRT, 18 (75%) KS patients were reassessed. Spine aBMD and RALM significantly increased. At radius, both cortical (Ct.Pm, Ct.Ar, Ct.vBMD, Ct.Th) and trabecular (Tb.vBMD) parameters significantly improved. At tibia, the improvement was found only in the cortical compartment. Young TRT naïve KS patients have inadequate bone microarchitecture at both the radius and tibia, which can improve on TRT.

**Keywords** HR-pQCT · Hypogonadism · Osteoporosis · Sex steroids · Cortical

## Introduction

Klinefelter syndrome (KS) is a congenital condition due to an extra X chromosome (47 XXY). Despite being the most frequent sex chromosome disorder (1/660 male births), it is frequently under-diagnosed [1]. Patients have substantial morphotypic symptoms that are inconsistently present such as tall stature, broad hips, narrow shoulders, sparse body hair, gynecomastia, and small testes with azoospermia leading to infertility [2]. KS patients also have an increased risk of fracture with higher morbidity and mortality [3, 4]. KS patient bone phenotype has been essentially explored by areal bone mineral density (aBMD) [5]. The increased fracture risk may be related to lower aBMD, as previously reported in cross sectional studies [6–16]. It is estimated that 42.4% of the KS patients present with osteopenia and osteoporosis [17], but this finding is not constant [18–20]. This raises the possibility of impairment in bone quality in addition to the decrease of aBMD. Bone histology reports of KS patients are scarce. In humans, Delmas and Meunier

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A. Piot and I. Plotton have contributed equally to this work.

✉ C. B. Confavreux  
cyrille.confavreux@chu-lyon.fr

- <sup>1</sup> Département de Rhumatologie, Hospices Civils de Lyon, Lyon, France
- <sup>2</sup> INSERM UMR 1033, Université de Lyon, Lyon, France
- <sup>3</sup> Hormonologie et Endocrinologie Moléculaire et Maladies Rares, Hospices Civils de Lyon, Lyon, France
- <sup>4</sup> Service de Néphrologie Rhumatologie et Dermatologie Pédiatriques, Hospices Civils de Lyon, Lyon, France
- <sup>5</sup> Département de Médecine de la Reproduction, Hospices Civils de Lyon, Lyon, France
- <sup>6</sup> INSERM UMR 1208, Université de Lyon, Lyon, France
- <sup>7</sup> Service de Rhumatologie Sud, Centre Hospitalier Lyon Sud, 165, Chemin du Grand Revoyet, 69310 Pierre Bénite, France

reported histomorphometric analysis of iliac crest biopsies in 5 KS patients. They found rarefied spongy bone and a strong reduction of osteoblastic activity with decreased osteoid thickness and low appositional rate [21]. A reduced bone volume has also been found in the XXY mice model but not the osteoblastic depression [22]. In 3D  $\mu$ CT, these mice displayed reduced trabecular thickness [22].

High-resolution peripheral quantitative computed tomography (HR-pQCT) allows for non invasive bone microarchitecture analysis and distinguishes the trabecular and cortical compartments. Up to now, only one cross-sectional study using HR-pQCT has been performed in KS patients. This study found impaired cortical and trabecular compartments at the tibia in KS patients [23]. Nevertheless, this study included a large proportion of patients previously treated with testosterone replacement therapy (TRT) that may have affected their bone phenotype. Thus, the description of bone microarchitecture in TRT-naïve KS patients remains timely, along with the determination of the effect of TRT on bone microarchitecture.

We therefore conducted a study to assess bone microarchitecture by HR-pQCT in TRT-naïve non-mosaic KS patients, and prospectively examined its evolution over 30 months of TRT.

## Subjects and Methods

### Participants

FERTIPRESERVE is a clinical research program lead (IP) in Lyon, France (NCT01918280). This program, started in 2012, aims to preserve fertility in KS patients with low sperm count. It has been approved by the local ethics committee “Comité de Protection des Personnes Lyon Sud-Est II—CHU de Lyon, France” (study number 2010-017-2 on June 17th 2010 with amendments on September 9th 2015 and October 10th 2017). The study has been performed in agreement with the Helsinki Declarations of 1975 and 1983. All patients, and parents in case of minors, provided written consent for the study. To be included in FERTIPRESERVE, patients had to be aged  $\geq 16$  years, have a Tanner score  $> 5$  and a homogenous karyotype assessed on 200 cells. Exclusion criteria were other impaired spermatogenesis causes such as testicular medical history (torsion, radiotherapy, trauma, or orchitis), and medical history of any treatment with gonadal toxicity, including chemotherapy, psychotropic and neuroleptic drugs. Patients with a personal history of bone disease (e.g. Paget bone disease, osteomalacia, primary hyperparathyroidism...) were also excluded. Briefly, in FERTIPRESERVE, before any hormonal TRT, young KS patients were proposed to undergo a surgical testicular biopsy to extract mature spermatozooids by the Testicular

Sperm Extraction (TESE) method. The spermatozooids obtained were frozen in liquid nitrogen for future in vitro fertilization via Intra Cytoplasmic Sperm Injection (ICSI) [24]. Once testicular biopsy had been performed, patients underwent the bone evaluation and then received TRT. A second bone evaluation was proposed after 30 months on TRT. The current analysis has been approved by the national data protection commission (CNIL). For this bone analysis, each KS patient was aged-matched ( $\pm 1$  year) to 3 healthy controls from French male cohorts (STRAMBO for adults [25], and VITADOS, NTC01832623, for teenagers [26]).

### Clinical Evaluation and Testosterone Replacement Therapy

At baseline, body weight and height were measured using calibrated scale and height gauge. Personal and familial history of fracture, and lifestyle habits were recorded. Leisure physical activity, current smoking, alcohol consumption (occasional, moderate 1–3 units/day,  $> 3$  units/day) were self-reported. Once TRT had been introduced, patients were regularly followed-up in outpatient clinics (IP) as recommended by the Endocrine Society clinical practice guideline [27]. TRT used injectable testosterone enantate (Bayer<sup>®</sup>) 250 mg/1 mL by intramuscular injection that was delivered according to the following routine protocol. Initiation of treatment was performed at a dose of 0.2 mL weekly during the first month, subsequently increased to a dose of 0.3 mL. After 6 months of this regimen, injections were performed every 15 days with an adjusted dose ranging between 0.3 and 0.5 mL to maintain plasma testosterone level within the normal range of the laboratory for young men (total testosterone  $> 12$  nmol/L and bioavailable testosterone between 2.25 and 10.7 nmol/L). Biological controls of total testosterone (tT) and bioavailable testosterone (bio-T) were carried out, before and 48 h after the injection, at 3 and 6 months and then every 6 months.

### Dual-Energy X-ray Absorptiometry (DXA)

Areal BMD was measured by DXA (Discovery<sup>®</sup>, Hologic, Inc., Waltham, MA, US) at the total hip, femoral neck and lumbar spine. The CV of daily measurements of the Hologic phantom was 0.35%. Whole body composition was also assessed by DXA; relative appendicular lean mass index (RALM) corresponded to the sum of lean mass of both arms and legs divided by (body height)<sup>2</sup>. DXA was performed at baseline and repeated after 30 months of TRT.

### Bone Microarchitecture

Volumetric Bone Mineral Density (vBMD) and microarchitecture were assessed at the non-dominant distal radius and

right distal tibia by HR-pQCT (XtremeCT-1<sup>®</sup>, Scanco Medical, Brüttisellen, Switzerland) at baseline [28].

Obtained cortical parameters were: cortical vBMD (Ct.vBMD), cortical perimeter (Ct.Pm), cortical area (Ct.Ar) and cortical thickness (Ct.Th). Ct.Th was calculated as the cortical volume divided by the outer bone surface.

Regarding trabecular compartment, we obtained trabecular vBMD (Tb.Vbmd) and trabecular bone volume (BV/TV) assuming fully mineralized bone to have mineral density of 1200 mg HA/cm<sup>3</sup>. Trabecular elements were identified by the mid-axis transformation, and the distance between them was assessed by the 3D distance transformation (Tb.Sp). Trabecular number (Tb.N, 1/mm) was defined as the inverse of Tb.Sp. Trabecular thickness (Tb.Th,  $\mu$ m) was derived from BV/TV and Tb.N. Intra-individual distribution of Tb.Sp (Tb.Sp.SD,  $\mu$ m) reflects heterogeneity of trabecular network and was quantified using the standard deviation of the distance between the mid-axes.

After 30 months of TRT, bone microarchitecture was reassessed. To ensure a reliable analysis during longitudinal analysis, strict positioning rules are used to perform acquisition. Member (leg or forearm) is placed in a brace with a fixed position. The new scout view is systematically compared to the baseline printed one to correctly replace the region of analysis. Only HR-pQCT slices with at least 80% of common volume of interest between baseline and follow-up acquisitions were used to perform the longitudinal analysis as previously described [29].

Controls and patients were assessed in a single bone research center (Inserm UMR1033, Hôpital Edouard Herriot, Lyon, France) on the same HR-pQCT and DXA machines with regular calibration overtime to ensure reliable and fully comparable data among patients and during follow-up. Controls from VITADOS cohort were assessed contemporaneously to the KS patients (2013–2015) and STRAMBO cohort were measured between 2006 and 2008.

### Serum Measurements

At baseline, before any testicular surgery, a fasting-state blood sample was drawn from KS patients at 8 a.m. Serum was stored at  $-80^{\circ}\text{C}$ . Standard bone laboratory tests were performed using the routine procedure. Total testosterone was measured by tritiated radioimmunoassay (RIA) after diethylether extraction with a lower limit of detection of 0.06 nmol/L and inter-assay coefficient of variation (CV)  $\leq 10\%$ . Bioavailable testosterone was assayed by RIA after ammonium sulfate precipitation. Total 17 $\beta$ -estradiol was measured by RIA with a detection limit of 17 pmol/L and an inter-assay CV  $\leq 8\%$ . Hypogonadism cut-off for each parameter was defined according to the normal values in our laboratory: 10.4 nmol/L for tT, 2.25 nmol/L bio-T, and 66.0 pmol/L for 17 $\beta$ E2.

### Statistical Analysis

All calculations were performed using R version 3.4.4 software (Copyright 2017 The R Foundation for Statistical Computing). The distribution of variables was assessed using the Shapiro–Wilk's test. Tb.Sp and Tb.Sp.SD had skewed distribution and were log-transformed. At baseline, comparisons between patients with KS and controls were performed using the Student *t*-test for continuous variables. Patients with KS were stratified according to: age, steroids (tT and bio-T), RALM (median = 7.61 kg/m<sup>2</sup>), fat mass (median = 22.5%) and growth plate status. Each stratum was compared to the controls using the Dunnett–Hsu test. A paired Student *t*-test was used to compare aBMD and bone microarchitecture between baseline and follow-up time points. Data are presented as mean  $\pm$  SD, or median (interquartile range, IQR). Statistical significance was set at  $p < 0.05$ .

## Results

### Baseline Characteristics

Between February 2014 and November 2015, a total of 33 KS patients were screened. All patients entered the care pathway through infertility. Most of the time, infertility was associated with low body hair and muscle mass. Among these, 9 were excluded (8 had previously received in the past significant amount of TRT, 1 had a mosaic mutation) so 24 patients were included in the bone substudy. KS patients were age-matched with 72 male controls (66 from the STRAMBO cohort and 6 from the VITADOS cohort).

The mean  $\pm$  SD age of KS patients was  $23.7 \pm 7.8$  years (see age distribution in Supplemental 4A) and the mean BMI was normal ( $20.9 \pm 3.4$  kg/m<sup>2</sup>). Body weight, height and BMI did not differ significantly between cases and controls. One patient had a personal history of fragility fracture (non-traumatic wrist fracture during adolescence). Among KS patients, 11 (46%) practiced regular physical activity, and 11 (46%) were current smokers. None of the patients received calcium-vitamin D supplementation. The mean  $\pm$  SD level of tT was  $11.5 \pm 5.3$  nmol/L, that of bio-T was  $2.0 \pm 1.1$  nmol/L, and that of 17 $\beta$ E2 was  $61.5 \pm 33$  pmol/L (Table 1).

### Bone Mass and Bone Microarchitecture

KS patients had significantly lower aBMD at lumbar spine, femoral neck, and total hip ( $p < 0.001$  for each) than controls. KS patients also had a significantly lower RALM ( $p < 0.001$ ) and greater proportion of fat mass ( $p < 0.001$ ;

**Table 1** Baseline characteristics of the patients with Klinefelter syndrome and controls

		KS (n=24)	KS follow-up (n=18)	Controls (n=72)
Clinical data	Age (years)	23.7±7.8	22.7±7.6	25.6±6.7
	Age range (years)	[16–37]	[16–37]	[15–37]
	Weight (kg)	71.8±16.1	72.1±17.4	76.6±12.3
	Height (cm)	178.9±7.8	178.9±8.3	177.0±6.9
	BMI (kg/m <sup>2</sup> )	22.5±5.3	22.6±5.5	24.5±4.0
Fracture history	Personal history	1 (4.2)	1(5.5)	–
	Familial history	3 (12.5)	3 (16.7)	–
Physical activity	Low	9 (37.5)	7 (38.9)	–
	Moderate	4 (16.7)	4 (22.2)	–
	High	11 (45.8)	7 (38.9)	–
Smoking	Current	11 (45.8)	7 (38.9)	–
Alcohol	Occasional	16 (66.7)	11 (61.1)	–
	Moderate	3 (12.5)	2 (0.11)	–
	> 3 U/day	0	0	–
Sex steroids	tT (nmol/L)	11.5±5.3	11.21±4.86	–
	bio-T (nmol/L)	2.0±1.1	2.01±1.11	–
	17βE2 (pmol/L)	61.5±33.0	51.63±35.82	–
Bone metabolism	Calcium (mmol/L)	2.5±0.1	2.46±0.07	–
	Phosphorus (mmol/L)	1.1±0.1	1.08±0.14	–
	25OHD (nmol/L)	62.0±28.2		–
	Bone ALP (μg/L)	20.1±12.0	21.5±12.9	–
	CTX (pg/mL)	669.8±321.9	716.1±329.2	–
	PINP (ng/mL)	153.6±189.6	163.8±210.8	–
	PTH (ng/L)	41.1±13.4	40.9±14.3	–
	TSH (mUI/L)	1.1±0.5	1.2±0.6	–

KS Klinefelter Syndrome, BMI Body Mass Index, > 3 U/day > 3 units of alcohol per day, tT total testosterone, bio-T bioavailable testosterone, 17βE2 17β estradiol, Bone ALP bone alkaline phosphatase, CTX Cross-linked C-telopeptide, PINP Procollagen I Intact N-Terminal, PTH parathyroid hormone; TSH thyroid-stimulating hormone

**Table 2** Bone mineral density and body composition in patients with Klinefelter Syndrome and controls at baseline

		KS (n=24)	Controls (n=72)	p
aBMD	Lumbar spine (g/cm <sup>2</sup> )	0.949±0.110	1.079±0.148	<0.001
	Femoral neck (g/cm <sup>2</sup> )	0.875±0.134	0.990±0.138	<0.001
	Total hip (g/cm <sup>2</sup> )	0.987±0.124	1.120±0.142	<0.001
Whole body DXA	RALM (kg/m <sup>2</sup> )	7.53±1.28	8.65±1.05	<0.001
	Fat mass (%)	22.3±6.8	14.1±6.2	<0.001

Data is expressed as mean ± SD. Student *t*-test was used for analysis

DXA dual-energy X-ray absorptiometry, KS Klinefelter Syndrome, aBMD areal bone mineral density, RALM relative appendicular lean muscle mass

Table 2). This is all the more true that the KS patients are hypogonadal (Table 3).

At the distal radius, we observed a marked cortical deterioration with lower Ct.Ar, Ct.Pm, and Ct.vBMD, as compared to controls. At the tibia, in addition to cortical fragility, we also found a significant impairment of trabecular parameters with lower BV/TV and Tb.vBMD (Table 4).

## Hypogonadism and Bone Microarchitecture

Ten (42%) and 15 (63%) patients were hypogonadal according to tT and bio-T. At the radius, testosterone hypogonadal patients had significantly lower cortical parameters (CtPm, Ct.Ar) than control patients whereas eugonadal patients had not (Table 5). There were no significant trabecular

**Table 3** Bone mineral density and body composition in patients with Klinefelter Syndrome, according to total testosterone level

		KS tT ≤ 10.4 nmol/L (n = 10)	KS tT > 10.4 nmol/L (n = 14)	<i>p</i>
aBMD	Lumbar spine (g/cm <sup>2</sup> )	0.953 ± 0.099	0.946 ± 0.126	0.89
	Femoral neck (g/cm <sup>2</sup> )	0.846 ± 0.120	0.996 ± 0.144	0.38
	Total hip (g/cm <sup>2</sup> )	0.987 ± 0.099	0.988 ± 0.144	0.99
Whole body DXA	RALM (kg/m <sup>2</sup> )	7.75 ± 1.67	7.36 ± 0.95	0.47
	Fat mass (%)	26.5 ± 7.0	19.3 ± 4.9	<b>&lt; 0.05</b>

Bold corresponds to significant results

Datas are expressed as mean ± SD. Student *t*-test was used for analysis

DXA dual-energy X-ray absorptiometry, KS Klinefelter Syndrome, aBMD areal bone mineral density, RALM relative appendicular lean muscle mass

**Table 4** Bone microarchitecture in Klinefelter Syndrome patients and controls at baseline

	Radius			Tibia		
	KS	Controls	<i>p</i>	KS	Controls	<i>p</i>
Ct.Pm (mm)	<b>77 ± 7</b>	<b>81 ± 6</b>	<b>&lt; 0.05</b>	111 ± 10	112 ± 7	0.37
Ct.Ar (mm <sup>2</sup> )	<b>56 ± 18</b>	<b>68 ± 18</b>	<b>&lt; 0.01</b>	<b>122 ± 28</b>	<b>162 ± 29</b>	<b>&lt; 0.001</b>
Tb.Ar (mm <sup>2</sup> )	271 ± 54	283 ± 52	0.31	672 ± 144	658 ± 110	0.56
Tt.vBMD (mg/cm <sup>3</sup> )	327 ± 66	346 ± 52	0.16	<b>312 ± 49</b>	<b>358 ± 46</b>	<b>&lt; 0.001</b>
Ct.vBMD (mg/cm <sup>3</sup> )	<b>793 ± 94</b>	<b>827 ± 76</b>	<b>0.05</b>	<b>844 ± 59</b>	<b>879 ± 48</b>	<b>&lt; 0.01</b>
Ct.Th (mm)	0.73 ± 0.3	0.84 ± 0.22	0.06	1.12 ± 0.29	1.46 ± 0.28	<b>&lt; 0.001</b>
BV/TV (%)	17.5 ± 2.9	17.7 ± 2.6	0.73	<b>17.2 ± 2.4</b>	<b>18.8 ± 2.5</b>	<b>&lt; 0.01</b>
Tb.vBMD (mg/cm <sup>3</sup> )	210 ± 36	212 ± 31	0.73	<b>207 ± 29</b>	<b>226 ± 30</b>	<b>&lt; 0.05</b>
Tb.N (1/mm)	1.98 ± 0.23	2.03 ± 0.22	0.33	1.90 ± 0.25	2.03 ± 0.28	0.07
Tb.Th (μm)	88.7 ± 14.6	87.7 ± 10.7	0.69	91.4 ± 13.4	93.3 ± 10.6	0.48
Tb.Sp.Sd (μm)	168 ± 31	165 ± 29	0.71	196 ± 42	182 ± 40	0.15

Bold highlights significant results

Data are expressed as mean ± SD. The Student *t*-test was used for analysis

HR-pQCT high resolution peripheral quantitative computed tomography, KS Klinefelter syndrome, BV/TV bone volume/tissue volume, Ct.Pm cortical perimeter, Ct.Ar cortical area, Tb.Ar trabecular area, vBMD volumetric bone mineral density, Tt.vBMD total vBMD, Ct.vBMD cortical vBMD, Tb.vBMD trabecular vBMD, Tb.N trabecular number, Ct.Th cortical thickness, Tb.Th trabecular thickness, Tb.Sp.SD trabecular spacing standard deviation (trabecular distribution)

differences. In contrast, at the tibia, tT hypogonadal patients not only displayed lower cortical parameters but also lower BV/TV and lower Tb.vBMD parameters than controls. KS patients with normal tT had only significant lower cortical parameters in comparison to controls (Table 5). These results were independent of muscle mass since all differences remained statistically significant after adjustment for RALM.

### Muscle Mass, Fat Mass, and Bone Microarchitecture

At both skeletal sites, patients with low RALM (Table 6) had impaired cortical parameters in comparison to controls. In addition, at the tibia, patients with low RALM had also

lower Tb.vBMD compared to the controls. All comparisons remained significant after adjustment for bio-T.

At the radius, there was no difference according to fat mass group (Supplemental Table S1). At the tibia, all KS patients had lower Tt.vBMD and Ct.Th. The phenotype was even more severe in the high fat mass group with a significant reduction of BV/TV, Ct.vBMD and Tb.vBMD.

### Change in Bone Microarchitecture After Testosterone Replacement Therapy

A total of 18 KS patients (75%) were reassessed after median of 30.4 (29.7–31.0) months of TRT. Others declined. Over this period, none of them underwent any fragility fracture. aBMD increased significantly at the lumbar spine (0.949 g/



**Table 5** Bone microarchitecture in Klinefelter Syndrome patients and controls at baseline according to hypogonadism status

	Controls <i>n</i> = 72	KS according to tT		KS according to bio-T	
		tT ≤ 10.4 nmol/L <i>n</i> = 10	tT > 10.4 nmol/L <i>n</i> = 14	bio-T ≤ 2.25 nmol/L <i>n</i> = 15	bio-T > 2.25 nmol/L <i>n</i> = 9
<b>Radius</b>					
Ct.Pm (mm)	<b>81 ± 6</b>	<b>76 ± 8*</b>	79 ± 6	<b>76 ± 8*</b>	79 ± 4
Ct.Ar (mm <sup>2</sup> )	<b>68 ± 18</b>	<b>52 ± 20*</b>	59 ± 18	<b>54 ± 17*</b>	61 ± 21
Tb.Ar (mm <sup>2</sup> )	283 ± 52	259 ± 50	281 ± 58	264 ± 58	286 ± 48
Tt.vBMD (mg/cm <sup>3</sup> )	346 ± 52	316 ± 63	336 ± 69	322 ± 67	336 ± 67
Ct.vBMD (mg/cm <sup>3</sup> )	<b>827 ± 76</b>	783 ± 111	801 ± 83	793 ± 94	793 ± 100
Ct.Th (mm)	0.84 ± 0.22	0.70 ± 0.26	0.76 ± 0.25	0.71 ± 0.24	0.77 ± 0.28
BV/TV (%)	17.7 ± 2.6	16.6 ± 3.0	18.1 ± 2.9	17.1 ± 3.3	18.3 ± 2.3
Tb.vBMD (mg/cm <sup>3</sup> )	212 ± 31	199 ± 36	218 ± 35	205 ± 39	220 ± 28
Tb.N (1/mm)	2.03 ± 0.22	2.04 ± 0.22	1.93 ± 0.23	1.92 ± 0.23	2.09 ± 0.2
Tb.Th (μm)	87.7 ± 10.7	81.4 ± 11.3	94.4 ± 14.6	89.2 ± 16.1	87.9 ± 12.0
Tb.Sp.Sd (μm)	165 ± 29	167 ± 35	169 ± 29	176 ± 34	147 ± 19
<b>Tibia</b>					
Ct.Pm (mm)	112 ± 7	110 ± 7	111 ± 11	109 ± 9	114 ± 11
Ct.Ar (mm <sup>2</sup> )	<b>162 ± 29</b>	<b>121 ± 25***</b>	<b>123 ± 32***</b>	<b>120 ± 20***</b>	125 ± 40
Tb.Ar (mm <sup>2</sup> )	658 ± 110	652 ± 96	686 ± 172	647 ± 120	715 ± 176
Tt.vBMD (mg/cm <sup>3</sup> )	<b>358 ± 46</b>	<b>301 ± 41***</b>	<b>320 ± 55*</b>	<b>305 ± 43***</b>	324 ± 60
Ct.vBMD (mg/cm <sup>3</sup> )	<b>879 ± 48</b>	<b>844 ± 49*</b>	<b>844 ± 68*</b>	<b>844 ± 49*</b>	844 ± 77
Ct.Th (mm)	<b>1.46 ± 0.28</b>	<b>1.11 ± 0.22**</b>	<b>1.12 ± 0.34***</b>	<b>1.11 ± 0.19***</b>	<b>1.13 ± 0.42**</b>
BV/TV (%)	<b>18.8 ± 2.5</b>	<b>18.5 ± 2.0**</b>	19.4 ± 2.7	<b>18.2 ± 2.3**</b>	19.3 ± 2.2
Tb.vBMD (mg/cm <sup>3</sup> )	<b>226 ± 30</b>	<b>195 ± 31**</b>	216 ± 25	<b>200 ± 31**</b>	219 ± 22
Tb.N (1/mm)	2.03 ± 0.28	1.85 ± 0.22	1.94 ± 0.27	<b>1.80 ± 0.22**</b>	2.07 ± 0.18
Tb.Th (μm)	93.3 ± 10.6	88.1 ± 12.7	93.8 ± 13.8	93.1 ± 14.4	88.6 ± 11.6
Tb.Sp.Sd (μm)	182 ± 40	205 ± 44	189 ± 40	207 ± 46	164 ± 28

Bold highlights significant results

Datas are expressed as mean ± SD. Dunnett–Hsu were performed to compare subgroups of patients with Klinefelter syndrome according to levels of sex steroids to controls

*HR-pQCT* high resolution peripheral quantitative computed tomography, *KS* Klinefelter syndrome, *BV/TV* bone volume/tissue volume, *Ct.Pm* cortical perimeter, *Ct.Ar* cortical area, *Tb.Ar* trabecular area, *vBMD* volumetric bone mineral density, *Tt vBMD* total vBMD, *Ct vBMD* cortical vBMD, *Tb.vBMD* trabecular vBMD, *Tb.N* trabecular number, *t.Th* cortical thickness, *Tb.Th* trabecular thickness, *Tb.Sp.SD* trabecular spacing standard deviation (trabecular distribution), *tT* total testosterone, *bio-T* bioavailable testosterone

*p* value: \* < 0.05, \*\* < 0.01, \*\*\* < 0.001

cm<sup>2</sup> ± 0.11 to 0.993 g/cm<sup>2</sup> ± 0.11; *p* < 0.01) but no significant change was observed at the femoral neck (*p* = 0.38). During this period, there was also a significant increase in muscle mass (RALM from 7.53 ± 1.28 to 8.02 ± 1.42 kg/m<sup>2</sup> (*p* < 0.01)).

At the radius, both the trabecular and cortical compartments significantly improved on TRT. At the tibia, the improvement was found only in the cortical compartment (Ct.Ar, Ct.vBMD, Ct.Th) (Table 7).

Regarding hypogonadism (hypogonadal patients with tT ≤ 10.4 nmol/L vs eugonadal patients tT > 10.4 nmol/L), a significant gain was observed at both sites (radius and

tibia) in both groups on TRT. Nevertheless, the gain looks larger in the hypogonadal group (Table 7).

To take into account the young age of our KS patients, growth plates were scored at baseline into three groups (Supplemental 2). The subgroup of patients with fully closed growth plate (*N* = 14 at radius and *N* = 9 at tibia) still presented a significant increase in bone microarchitecture parameters. Supplemental 3A. Furthermore, patients with open growth plate experimented on TRT a significant larger increase of bone microarchitecture parameters than the one with closed growth plate. Supplemental 3B.

Bone microarchitecture parameters were not so much different—except the Ct vBMD— at baseline between the youngest

**Table 6** Bone microarchitecture in Klinefelter Syndrome patients and controls at baseline according to RALM

	Controls <i>n</i> = 72	KS according to RALM	
		RALM ≤ 7.61 kg/m <sup>2</sup> <i>n</i> = 12	RALM > 7.61 kg/m <sup>2</sup> <i>n</i> = 12
<b>Radius</b>			
Ct.Pm (mm)	<b>81 ± 6</b>	<b>77 ± 5*</b>	78 ± 8
Ct.Ar (mm <sup>2</sup> )	<b>68 ± 18</b>	<b>48 ± 19***</b>	67 ± 12
Tb.Ar (mm <sup>2</sup> )	283 ± 52	278 ± 52	263 ± 58
Tt.vBMD (mg/cm <sup>3</sup> )	<b>346 ± 52</b>	<b>302 ± 64*</b>	360 ± 56
Ct.vBMD (mg/cm <sup>3</sup> )	<b>827 ± 76</b>	<b>753 ± 103**</b>	845 ± 48
Ct.Th (mm)	<b>0.84 ± 0.22</b>	<b>0.63 ± 0.29*</b>	0.83 ± 0.17
BV/TV (%)	17.7 ± 2.6	177 ± 23	18.2 ± 2.7
Tb.vBMD (mg/cm <sup>3</sup> )	212 ± 31	200 ± 35	222 ± 34
Tb.N (1/mm)	2.03 ± 0.22	1.95 ± 0.22	2.01 ± 0.25
Tb.Th (μm)	87.7 ± 10.7	85.6 ± 17.3	91.6 ± 11.6
Tb.Sp.Sd (μm)	165 ± 29	170 ± 31	166 ± 32
<b>Tibia</b>			
Ct.Pm (mm)	112 ± 7	110 ± 11	111 ± 8
Ct.Ar (mm <sup>2</sup> )	<b>162 ± 29</b>	<b>106 ± 20***</b>	<b>145 ± 22*</b>
Tb.Ar (mm <sup>2</sup> )	658 ± 110	688 ± 165	651 ± 113
Tt.vBMD (mg/cm <sup>3</sup> )	<b>358 ± 46</b>	<b>296 ± 39***</b>	334 ± 55
Ct.vBMD (mg/cm <sup>3</sup> )	<b>879 ± 48</b>	<b>826 ± 57**</b>	870 ± 55
Ct.Th (mm)	<b>1.46 ± 0.28</b>	<b>0.97 ± 0.24***</b>	1.26 ± 0.26
BV/TV (%)	18.8 ± 2.5	17.0 ± 2.6	17.5 ± 2.3
Tb.vBMD (mg/cm <sup>3</sup> )	<b>226 ± 30</b>	<b>205.0 ± 29.3*</b>	209.8 ± 29.7
Tb.N (1/mm)	2.03 ± 0.28	1.94 ± 0.27	1.85 ± 0.22
Tb.Th (μm)	93.3 ± 10.6	89.6 ± 14.9	93.2 ± 12.0
Tb.Sp.Sd (μm)	182 ± 40	18.9 ± 4.7	20.2 ± 3.7

Bold highlights significant results

Data are expressed as mean ± SD. The Dunnett–Hsu test was used to compare subgroups of patients with Klinefelter syndrome stratified according to the median of RALM and controls

KS Klinefelter Syndrome, RALM relative appendicular lean muscle mass, HR-pQCT high resolution peripheral quantitative computed tomography, BV/TV bone volume/tissue volume, Ct.Pm cortical perimeter, Ct.Ar cortical area, Tb.Ar trabecular area, vBMD volumetric bone mineral density, Tt.vBMD total vBMD, Ct.vBMD cortical vBMD, Tb.vBMD trabecular vBMD, Tb.N trabecular number, Ct.Th cortical thickness, Tb.Th trabecular thickness, Tb.Sp.SD trabecular spacing standard deviation (trabecular distribution)

*p* value: \* < 0.05, \*\* < 0.01, \*\*\* < 0.001

KS patients (< 20) and the oldest one (≥ 20 years old). Nevertheless, the bone parameters absolute variations tended to be higher in youngest group than in the oldest one. Supplemental 4 B and 4C.

## Discussion

We described for the first time the bone microarchitecture in TRT naïve young KS patients and its evolution on TRT treatment. We found that young adult KS patients did not achieve cortical mass and architecture comparable with the age-matched healthy controls. At the tibia, trabecular bone was also impaired. After 30 months of TRT, patients

experienced at both sites an improvement of their aBMD, RALM and cortical bone status. The trabecular bone status also improved at the radius. Altogether, these data suggest a benefit of TRT treatment in young adult KS patients for a better bone health.

One issue may be the risk of supraphysiologic testosterone serum levels on TRT. Testosterone was administered every 2 weeks. The choice of intermittent regimen rhythm limits supraphysiologic testosterone levels. Moreover, patients were clinically evaluated at initiation and during follow-up to look for “supraphysiologic” symptoms such as aggressiveness and sexual compulsions. In addition, total testosterone and bioavailable testosterone were assessed before injection and at the peak (48 h after injection).

**Table 7** HR-pQCT bone microarchitecture evolution in patients with Klinefelter Syndrome (KS) after 30-months of testosterone replacement therapy

	KS patients with follow-up All <i>n</i> = 18		Testosterone levels ≤ 10.4 nmol/L <i>n</i> = 8		Testosterone levels > 10.4 nmol/L <i>n</i> = 10	
	T0	T1	T0	T1	T0	T1
<b>Radius</b>						
Ct.Pm (mm)	77 ± 7	77 ± 7***	75 ± 8	76 ± 8*	<b>77.66 ± 6.97</b>	<b>78.16 ± 7*</b>
Ct.Ar (mm <sup>2</sup> )	57 ± 17	65 ± 1*	49 ± 19	64 ± 6*	62.82 ± 14.24	65.7 ± 14.2
Tb.Ar (mm <sup>2</sup> )	266 ± 55	259 ± 57*	261 ± 52	249 ± 54*	269.69 ± 60.84	267.64 ± 61.92
Tt.vBMD (mg/cm <sup>3</sup> )	325 ± 67	352 ± 61**	304 ± 57	346 ± 50**	<b>342.58 ± 71.96</b>	<b>355.96 ± 71*</b>
Ct.vBMD (mg/cm <sup>3</sup> )	786 ± 77	835 ± 46***	758 ± 92	832 ± 44*	<b>807.76 ± 57.87</b>	<b>836.53 ± 50**</b>
Ct.Th (mm)	<b>742.78 ± 237.63</b>	<b>850.00 ± 183.08*</b>	<b>647.50 ± 240.10</b>	<b>846.25 ± 130.38*</b>	819 ± 217.48	853 ± 223.76
Tb.vBMD (mg/cm <sup>3</sup> )	209 ± 36	214 ± 34**	198 ± 33	205 ± 30*	<b>217.37 ± 38.02</b>	<b>221.78 ± 37*</b>
Tb.N (1/mm)	2.0 ± 0.2	2.0 ± 0.2	80.6 ± 11.7	82.8 ± 9.1	93.9 ± 15.5	93.7 ± 17.51
Tb.Th (µm)	88 ± 15	89 ± 15	75 ± 8	76 ± 8	<b>78 ± 7</b>	<b>78 ± 7*</b>
<b>Tibia</b>						
Ct.Pm (mm)	108 ± 10	109 ± 10*	109 ± 9	109 ± 9	108.14 ± 11.37	108.35 ± 11.32
Ct.Ar (mm <sup>2</sup> )	121 ± 26	131 ± 25***	118 ± 24	133 ± 17*	<b>122.9 ± 28.41</b>	<b>129.93 ± 30.72**</b>
Tb.Ar (mm <sup>2</sup> )	647 ± 139	637 ± 143***	651 ± 109	638 ± 119*	<b>643.78 ± 164.53</b>	<b>636.48 ± 165.86**</b>
Tt.vBMD (mg/cm <sup>3</sup> )	310 ± 51	327 ± 51***	297 ± 41	323 ± 44	<b>320.97 ± 57.64</b>	<b>330.16 ± 58.64**</b>
Ct.vBMD (mg/cm <sup>3</sup> )	842 ± 49	865 ± 46**	832 ± 40	868 ± 30*	<b>850.2 ± 56.52</b>	<b>862.34 ± 57.99**</b>
Ct.Th (mm)	<b>1123.33 ± 263.97</b>	<b>1221.11 ± 272.39**</b>	<b>1087.50 ± 200.70</b>	<b>1226.25 ± 203.11*</b>	<b>1152.00 ± 313.43</b>	<b>1217.00 ± 328.67**</b>
Tb.vBMD (mg/cm <sup>3</sup> )	204 ± 29	208 ± 28	196 ± 35	204 ± 33	210.48 ± 23.54	211.14 ± 24.02
Tb.N (1/mm)	1.9 ± 0.3	1.7 ± 0.6	1.9 ± 0.3	2.0 ± 0.4	1.9 ± 0.3	1.5 ± 0.8
Tb.Th (µm)	91 ± 14	91 ± 13	89 ± 14	86 ± 9	92.1 ± 14.63	95.2 ± 13.77

Bold highlights significant results

Data are expressed as mean ± SD. Student *t* test was used for analysis; T0 corresponds to baseline; T1 corresponds to bone microarchitecture after 30 months of testosterone replacement therapy

HR-pQCT high resolution peripheral quantitative computed tomography, KS Klinefelter Syndrome, BV/TV: bone volume/tissue volume, Ct.Pm cortical perimeter, Ct.Ar cortical area, Tb.Ar trabecular area, vBMD volumetric bone mineral density, Tt.vBMD total vBMD, Ct.vBMD cortical vBMD, Tb.vBMD trabecular vBMD, Tb.N trabecular number, Ct.Th cortical thickness, Tb.Th trabecular thickness

*p* value: \* < 0.05, \*\* < 0.01, \*\*\* < 0.001

Therapeutic objectives were to obtain testosterone levels at physiologic levels (total testosterone between 10 and 24 nmol/L and bioavailable testosterone between 2.25 and 10.70 nmol/L). Estradiol level was also assessed and no excessive serum level (< 100 pmol/L) was observed under TRT. We believe that this monitoring of TRT in KS patients reduce as much as possible the risk of over testosterone replacement in patients.

Our results are consistent with the reduced bone volume observed in XYY mice [19]. They are also consistent with microarchitecture data reported by Shanbogue et al. who conducted a cross sectional study in 31 KS patients aged 35.8 ± 8.2 years [23]. In addition to their findings, we observed an impaired bone geometry in our cohort (significantly lower Ct.Pm and Ct.Ar). The difference may stem

from distinct study populations because more than two-thirds of their population (21/31) had previously received TRT [23], and we presently show that bone microarchitecture of KS patients is particularly sensitive to TRT. Moreover, our study highlights the precocity of bone alterations that are already present in these young otherwise healthy KS patients without fracture. The greater differences observed here in this young group of KS patients may suggest a delayed peak bone mass acquisition in these patients.

Regarding longitudinal analysis, to the best of our knowledge, there is no other study in humans investigating the change in bone microarchitecture after TRT. The only available data were obtained using aBMD [10, 30] or total vBMD [8]. Interestingly, it seems that TRT did not improve aBMD [7, 18, 31] except if initiated before the age of attainment



of peak bone mass [13], supporting early TRT treatment for these patients to optimize bone architecture and peak bone mass. Herein, after a median 30 months on TRT, our patients experienced a significant increase in Ct.Pm, Ct.Th and Ct.vBMD at both skeletal sites and a significant decrease in Tb.Ar. These findings suggest that the observed bone gain during TRT may be related to both endocortical apposition and periosteal apposition.

The FERTIPRESERVE project is in line with the Endocrine Society clinical practice guideline for TRT in men with hypogonadism [27] and the recent guidelines of the European Academy of Andrology (EAA) on KS [32]. These guidelines, by their holistic approach of the KS from birth to adulthood and from pathophysiology to comorbidities, are a major contribution to the field. The guidelines recommend to introduce TRT not before the end of adolescence and after treatment of fertility disorders by semen collection, cryopreservation or even testicular biopsy for TESE [32]. The recommendations stress the importance of vitamin D supplementation and suitable calcium intake. They also highlight that patients with KS are at risk of low bone mineral density and fractures independently of their serum levels of testosterone. Our bone sub-protocol echoes to the lack of bone data underlined by the guidelines [27, 32]. The major contribution of our study is to describe the bone microarchitecture in a very homogenous group of non treated young adult KS patients and to follow their response to TRT. We have observed that independently of their hormonal status, the whole group benefit from the TRT, even if the gain was larger in the hypogonadal group. This finding provide new data to discuss in the next KS guidelines since the current ones restrict TRT introduction to hypogonadal patients after fertility consideration.

Mechanisms underlying bone fragility in KS patients remain unclear. First, we observed that bone alterations were more severe in hypogonadal KS patients suggesting that sex steroid deficit may play an important role. In KS patients, tT serum levels are normal in prepubertal boys, but increase inadequately during puberty, leading to hypogonadism in 65–85% of adults [33]. In our patient group, less than half of the KS patients were tT hypogonadal which was less than expected for a KS population [33]. This may be due to the young age of the included patients, since testicular function declines with age in KS patients after puberty [34]. Indeed, testicular failure is observed during puberty, with hyalinization and fibrosis of seminiferous tubes, leading to small testes and spermatogenesis failure and hypoleidigism with a reduction of androgen secretion despite an elevated secretion of LH. Our finding that the most severe bone alterations were present in hypogonadal patients, is in line with earlier studies that investigated the association between tT and bone parameters measured via aBMD [9–11, 29, 35]. Nevertheless, it was not the case for the studies using aBMD reported

by Bojesen et al. [36], and Ferlin et al. [17], or the study reported by Wong et al. using QCT [37].

Total testosterone, includes biologically inactive testosterone bound to Sex Hormone Binding Globulin (SHBG). Unfortunately, data on free testosterone, bioavailable testosterone (bio-T), or 17 $\beta$ -estradiol (17 $\beta$ E2) and bone in these KS patients are scarce. Except a few studies [10, 12, 18, 36], most of them [7–9, 13, 17, 23, 30, 31, 35, 38] only assess the relationship between aBMD or bone microarchitecture and tT. Therefore, we also measured bio-T. The deterioration of bone microarchitecture of bio-T hypogonadal KS patients was in line with the results of tT but more profoundly impaired suggesting that the assessment of bio-T may be relevant in future studies.

Estradiol is also a key component of bone health in males since estrogen play a major role in bone physiology during puberty and in preventing bone loss in aging healthy men. In KS patients, some studies found normal [12, 36, 39] or elevated 17 $\beta$ E2 levels [17, 18, 40, 41] compared to controls. Herein, nearly three-quarters of patients had decreased 17 $\beta$ E2 concentrations beside their increased fat mass. However, this might be expected since concentrations of 17 $\beta$ E2 and bio-T are strongly correlated; 85% of estradiol comes from peripheral aromatization of testosterone [42]. Thus, TRT may act directly on cortical apposition through testosterone and indirectly on endocortical apposition through estradiol. Interestingly, Shanbogue et al. found a positive association between 17 $\beta$ E2 and Ct.Th at the radius and Tt.vBMD at the tibia, but not with tT [23] which supports the role of both bio-T and 17 $\beta$ E2 in these patients.

Because some KS patients had normal sex steroid levels, other mechanisms may be responsible for bone fragility. This is supported by an *in vivo* study from Liu et al. [22], where the XXY mice did not fully recovered a normal bone phenotype when treated by TRT. In our cohort, we also observed a partial bone improvement since at the tibia, all patients on TRT improved, whereas at the radius, the improvement was mainly observed in hypogonadal KS patients. The discrepancy between the two sites radius and tibia provides a clue for further investigation to explore the role of mechanical loading in KS patients. Furthermore, as reported with aBMD [36], microarchitecture deterioration was also more severe in KS patients with low RALM suggesting that muscle has a significant role in sustaining bone microarchitecture in these patients. In another cohort of both KS and patients with anorchia, lower RALM was associated with poorer Ct.Ar and Ct.Th at the tibia as assessed by QCT [37]. After TRT, our patients had a significant increase of RALM. This is consistent with a double-blind placebo-controlled randomized trial of TRT for 6 months, that found a significantly increased muscle mass and decreased fat mass in 274 older hypogonadal men [43]. An indirect effect of TRT on the

impaired muscle/fat ratio [19, 36] is possible but has never been fully explored.

In addition to its association with testosterone, bone-muscle crosstalk may be involved not only through its mechanical effect (load and strength applied). The potential involvement of dystrophin, present on X chromosome, has never been studied in KS patients. Further investigations on bone-muscle interactions in KS might be helpful.

Other genetic aspects should also be considered. It has been described that some KS patients present a substantial genetic variation of *Androgen Receptor (AR)* gene with repeated CAG polymorphisms of the first exon. This is of interest since CAG length is inversely proportional to the AR function and that the X chromosome with fewer CAG polymorphisms is preferentially inactivated [44]. AR function may therefore be reduced and thus explaining bone fragility despite normal levels of sex steroids. Selective inactivation of the shorter X chromosome may also be responsible of a lower bone response to TRT [45, 46].

Regarding bone turnover, serum biomarkers in young KS patients naïve from TRT are scarce but worth to be shown. The limited number of patients and the absence of controls in our study prevent too much interpretation. Nevertheless, the data we observed were in the normal range of our laboratory and we did not observe any trend between hypogonadal (low tT) and eugonadal patients. In the literature, Bojeson et al. have also assessed bone turnover markers in 70 KS patients and 71 controls. No difference was observed suggesting that KS patients, at least through classical serum biomarkers evaluation, had no bone turnover alteration [36].

The present study has some limitations. Sex steroid measurements were only measured at baseline and were performed by RIA. At that time LC-MS was not routinely available in our Institution. Because of insufficient materials, we were able to re-assess only some patients by LC-MS. In these low value range, results are consistent ( $R^2=0.93$ ) between the two technics. This is in line with the comparative test between RIA and LC-MS performed in 180 patients to set the method (Supplemental 5). Thus, we remain confident in our findings. Hip aBMD was not available in the controls from the VITADOS cohort. Indeed, VITADOS cohort did not plan hip aBMD since it enrolled children down to 12 years old with still bone immaturity. In addition, because of the image acquisition protocol used for HR-pQCT, measurement site may vary with arm and leg length at baseline. Another bias in this longitudinal study of a young population could be related to re-positioning, which was minimized by recruiting only patient with Tanner stage V or more that do not grow anymore. Finally, our study remains an exploratory research work on bone microarchitecture in KS patients. We acknowledged that bone microarchitecture

is an intermediate criterion that does not totally recapitulate general bone health of subjects and that our study was not design to explore fracture events. We observed that even in a homogenous population of young, never treated, non-mosaic KS patients, there were some microarchitecture heterogeneity among patients. Growth plate status, testosterone serum level, and age were important contributors for baseline status and evolution on TRT. The small sample size of the subgroups and the absence of control group call for further studies and preclude from drawing firm conclusions to treat patients. For the moment, physicians should still stick to the current guidelines.

In conclusion, we found that TRT-naïve without mosaics KS patients have an early bone impairment at both radius and tibia, that is more severe when they are hypogonadal and/or with low muscle mass. TRT over a median 30 months, in addition to the previously known benefits, also improves bone microarchitecture at both skeletal sites. Our findings suggest a beneficial effect of an early TRT initiation in KS patients to optimize their peak bone mass acquisition and improve their bone microarchitecture. Nevertheless, this is uncontrolled data involving a difficult analysis of longitudinal HR-pQCT data and with possible confounders such as the young age of some subjects without full skeletal maturity. Further studies of early TRT vs placebo are required to clarify the natural gain expected in these patients and the boost effect provided by TRT.

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**Author Contributions** Study design: IP, SB and CC. Study conduct: IP and CC. Data collection: IP, SA, SB, JB, HL and CC. Data analysis: AP, CC, MP, PS. Data interpretation: AP, CC, PS. Drafting manuscript: AP, CC, PS. Revising manuscript content: IP, SA, SB, JB, HL and RC. Approving final version of manuscript: AP, PS, IP, SA, SB, JB, HL, RC and CC. AP and CC take responsibility for the integrity of the data analysis.

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**Data Availability** The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Declarations

**Conflict of interest** A. Piot, I. Ploton, S. Boutroy, J. Bacchetta, S. Ailoud, H. Lejeune, R. D. Chapurlat, P. Szulc and C. B. Confavreux declare that they have no conflict of interest concerning this study.

**Ethical Approval** It has been approved by the local ethics committee “Comité de Protection des Personnes Lyon Sud-Est II—CHU de Lyon, France” (study number 2010-017-2 on June 17th 2010 with amendments on September 9th 2015 and October 10th 2017). The study has been performed in agreement with the Helsinki Declarations of 1975 and 1983.

**Informed Consent** All patients, and parents in case of minors, provided written consent for the study.

**Human and Animal Rights statement** The authors certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

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