Effects of nandrolone decanoate on telomere length and clinical outcome in patients with telomeropathies: a prospective trial

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Abstract

Androgens have been reported to elongate telomeres in retrospective and prospective trials with patients with telomeropathies, mainly with bone marrow failure. In our single-arm prospective clinical trial *(clinicaltrials gov. Identifier: NCT02055456*), 17 patients with short telomeres and/or germline pathogenic variants in telomere biology genes associated with at least one cytopenia and/or radiologic diagnosis of interstitial lung disease were treated with 5 mg/kg of intramuscular nandrolone decanoate every 15 days for 2 years. Ten of 13 evaluable patients (77%) showed telomere elongation at 12 months by flow-fluorescence *in situ* hybridization (average increase, 0.87 kb; 95% confidence interval: 0.20-1.55 kb; *P*=0.01). At 24 months, all ten evaluable patients showed telomere elongation (average increase, 0.49 kb; 95% confidence interval: 0.24-1.23 kb; *P*=0.18). Hematologic response was achieved in eight of 16 patients (50%) with marrow failure at 12 months, and in ten of 16 patients (63%) at 24 months. Seven patients had interstitial lung disease at baseline, and two and three had pulmonary response at 12 and 24 months, respectively. Two patients died due to pulmonary failure during treatment. In the remaining evaluable patients, the pulmonary function remained stable or improved, but showed consistent decline after cessation of treatment. Somatic mutations in myeloid neoplasm-related genes were present in a minority of patients and were mostly stable during drug treatment. The most common adverse events were elevations in liver function test levels in 88%, acne in 59%, and virilization in 59%. No adverse events grade ≥4 was observed. Our findings indicate that nandrolone decanoate elongates telomeres in patients with telomeropathies, which correlated with clinical improvement in some cases and tolerable adverse events.

Introduction

Telomeres are hexameric repetitive DNA sequences and associated proteins that protect the ends of linear chromosomes against DNA damage.[1] Chromosome erosion occurs during mitosis, with loss of approximately 50 to 100 base pairs (bp) of telomeric DNA at each cell division.[2] When telomeres reach critically short length, cells undergo proliferation arrest or apoptosis,[3] and stem cell fate is skewed towards differentiation over self-renewal.[4,5] Highly replicative cells express telomerase, an enzyme that actively maintains telomere length (TL) by adding DNA hexanucleotides to the 3' end of the leading DNA strand, lessening telomere attrition.[6] Pathogenic germline variants in the telomerase complex or other telomere biology genes impair telomere maintenance, causing excessive telomere loss, estimated at approximately 120 bp per year,[7] and eventually impacting cell proliferation and tissue regeneration.[8] Tissues with high proliferative index or exposed to persistent injury, such as the bone marrow, lung, liver, and skin, appear to be particularly susceptible to a telomere repair defect, and they feature prominently in the clinical spectrum of telomere diseases.[9]

Telomeropathies are multi-organ diseases with pleiotropic phenotype, including interstitial lung disease,[10] bone marrow failure,[8] cirrhosis[11,12] and mucocutaneous abnormalities, which may be observed either in isolation or in combination and variable severity, even within pedigrees.[13] Asymptomatic patients may display subclinical organ dysfunction.[14] Curative therapies are few and supportive

measures are the usual standard of care. When patients develop severe organ dysfunction, such as aplastic anemia, allogeneic hematopoietic stem cell transplantation (HSCT) may be considered, but pulmonary and hepatic dysfunction associates with treatment-related morbidity and mortality.[15,16] Conversely, lung and liver transplantation are frequently contra-indicated due to co-existing cytopenias, and outcomes are poor.[17,18]

Growth factors and hormones are involved in controlling telomerase function.[19] In a mouse model of aplastic anemia with very short telomeres, testosterone upregulated telomerase and attenuated cytopenias.[20] In healthy human hematopoietic cells, exposure to androgens increases telomerase activity *in vitro*; in cells from patients carrying telomerase mutations, androgens ameliorated telomerase function, providing a biological basis for the use of sex hormones to treat telomeropathies.[21] In a previous phase I-II prospective trial in telomere diseases, danazol at 800 mg per day for 2 years was associated with telomere elongation in peripheral blood leukocytes.[22] Hematological response was observed in 79% of patients, and lung function as well as pulmonary fibrosis were stable during treatment. The most common adverse event (AE) was elevated liver enzyme levels in 41% of patients.

We conducted a phase I-II single-center prospective trial in patients with telomere diseases, to assess the safety and the clinical and biological effects of the male hormone nandrolone decanoate, a parenteral synthetic steroidal androgen with lower expected hepatic toxicity than typical for orally administered hormones (*clinicaltrials gov. Identifier: NCT02055456*).

Methods

Patients and treatment

Male patients >2 years and female patients >16 years were eligible for enrollment at the Ribeirao Preto Medical School University Hospital (Ribeirao Preto, SP, Brazil). Entry criteria were age-adjusted mean TL <1st percentile and/or identified germline pathogenic variants in telomere biology genes associated with at least one cytopenia and/or radiologic diagnosis of interstitial lung disease (ILD) (see details in the *Online Supplementary Appendix*).[22,23] The local Research Ethics Committee approved the protocol and all patients provided written informed consent (CAAE, 19116913.4.0000.5440). Patients were treated with 5 mg/kg of intramuscular nandrolone decanoate every 15 days for 2 years. Patient monitoring is detailed in the *Online Supplementary Appendix*.

Pulmonary evaluation

Pulmonary involvement was assessed by high-resolution computed tomography (HRCT) of the chest and pulmonary function testing for diffusing capacity for carbon monoxide (DLCO) at baseline and at 24 months. Patients with lung disease had an additional pulmonary evaluation at 12 months. Predicted forced vital capacity (FVC) and predicted DLCO values, corrected for anemia,[25] were used to evaluate lung disease progression.

Telomere length measurement and massively parallel targeting sequencing panel

Peripheral blood leukocyte TL was measured by flow-fluorescence *in situ* hybiridization flow-FISH[26] at landmark time points: enrollment, 12 and 24 months of nandrolone treatment.

Patients were screened for somatic mutations in peripheral blood at landmark time points by an error-correcting DNA sequencing panel covering 60 genes associated with myeloid malignancies and clonal hematopoiesis (ArcherDX customized panel; *Online Supplementary Appendix; Online Supplementary Table S2)*.

End points

Primary objectives of the study were safety and nandrolone decanoate activity in slowing telomere attrition in patients. The primary biologic end point was a reduction of ≥20% in the annual rate of telomere attrition in patients with telomere disease (to ≤96 bp/year)[7,27] during nandrolone administration. The primary safety end point was the occurrence of AE throughout the treatment period.

Secondary end points were hematologic and pulmonary responses (*Online Supplementary Appendix*). Other secondary end points were the incidence of clonal hematopoiesis and hematologic relapse. Changes in chest HRCT scan quantitative measures were exploratory end points.

Statistics

Summary statistics were used to describe the primary biologic and secondary end points. Linear regression models with mixed effects were used to obtain pairwise comparisons of the means of the variables of interest between different periods. These models include a random effect that accounts for dependence between values from the same individual (paired data). Assumption of normality of residuals was visually checked for the models using normal probability plots. The presence of outliers and influential observations were verified by graphs of the studentized residuals and by the Cook's D statistics. The SAS version 9 (proc mixed) was used , considering a level of significance of 0.05.[28]

Results

Patients

From May 2014 to October 2017, 20 consecutive patients

were screened for participation and 17 were enrolled. Seven patients withdrew from the study before the end of 2 years (Figure 1). Median age was 36 years (range, 4–59 years), and five patients (29%) were female. All patients were diagnosed with bone marrow failure, seven patients were additionally diagnosed with ILD, and four patients also had liver involvement: radiologic findings of cirrhosis in all four and gastroesophageal varices in three. Five patients displayed cutaneous features of dyskeratosis congenita. At the time of enrollment, one patient had already been submitted to hematopoietic stem cell transplantation due to aplastic anemia in another center (severity not classified) and was eligible for the study due to lung disease. Clinical characteristics are summarized in Table 1. Germline pathogenic variants in telomere biology genes were identified in all but three patients (Table 2). After study completion, patients received treatment according to the physician's discretion. Some kept supportive care and others received androgens according to local availability (danazol or restarted nandrolone).

Adverse events

The most common AE associated with nandrolone were elevations in liver function test levels in 88%, acne in 59%, and virilization in 59% (Table 3). Severe AE (grade 3) possibly related to drug occurred in seven instances and injection complications were the most common (cutaneous abscess in UPN #14 and sciatic nerve injury in UPN #15). Additionally, three patients had mild (grade 1) injection site reactions: pain in two (UPN# 6 and UPN#7) and local hematomas in one thrombocytopenic patient (UPN #17). No AE ≥ grade 4 related to nandrolone was observed. Dose reduction to 3.5 mg/kg was necessary in five cases due to severe or moderate AE, as a result of acne (in 2 patients, UPN #1 and UPN #15), depression (1 patient, UPN #8), progressive erythrocytosis (1 patient, UPN #4), or progressive signs of early puberty in a 4-year-old child (UPN #9). An additional reduction to 2.5 mg/kg was necessary in three cases due to symptom persistence (UPN #1, UPN #8 and UPN #9). Seven patients withdrew from the study before the end of 2 years: two patients halted therapy due to grade 3 adverse events (acne and depression - UPN #1 and UPN #8, respectively), one patient sought alternative therapies (UPN #16), and four patients died during the period of nandrolone administration (2 from progressive respiratory failure due to ILD – UPN #3 and UPN #13; and 2 from intracranial hemorrhage – UPN #11 and UPN #14). Additionally, three patients died after treatment completion, of progressive ILD in two (UPN #1 and #2) and complication of cytopenias in one (UPN #9), for a total of seven deaths (41%) by September 2020. No deaths were attributed to nandrolone.

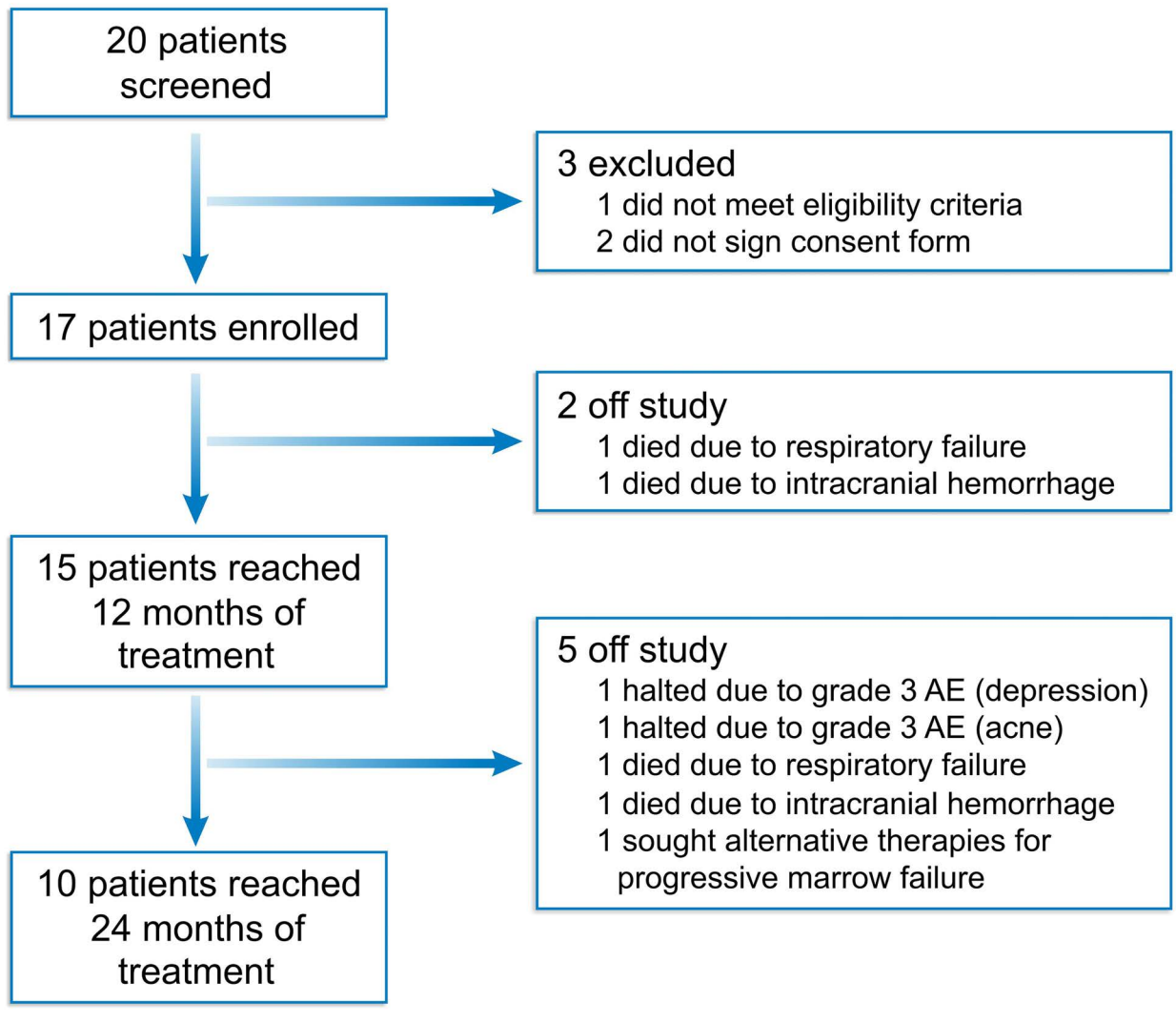

Figure 1. Flowchart of consecutive patients with telomeropathy screened for nandrolone treatment and enrolled in the study.
AE: adverse event.

Table 1. Baseline characteristics of enrolled patients.

Characteristic	All patients (N=17)	Patients with identified genetic telomere-biology variants					No variant identified (N=3)
		TERT (N=7)	*RTEL1* (N=4)	*TERT&TERC* (N=1)	*TERC* (N=1)	*TINF2* (N=1)	
Age in years, median (range)	36 (4-59)	40 (21-59)	34 (10-54)	12	30	4	40 (18-40)
Female sex, N (%)	5 (29)	3	2	0	0	0	0
Bone marrow failure, N (%)							
Moderate AA	14 (82)	7	4	0	1	1	2
Severe AA	1 (6)	0	0	1	0	0	0
Myelodysplastic Syndrome	1 (6)	0	0	0	0	0	1
Transfusion dependency, N (%)							
Red cells	1 (6)	1	0	0	0	0	0
Platelets	3 (18)	1	0	0	0	1	1
Red cells and platelets	4 (24)	0	1	1	1	0	1
Pulmonary fibrosis, N (%)							
Present	7 (41)	3	3	0	1	0	0
Absent	10 (59)	4	1	1	0	1	3
Hepatic involvement, N (%)							
Present	4 (24)	2	2	0	0	0	0
Absent	13 (76)	5	2	1	1	1	3
Mucocutaneous features, N (%)	5 (29)	1	2	0	1	1	0

AA: aplastic anemia.

Telomere attrition

Of the 17 patients enrolled, 15 reached 12 months of treatment and 13 were evaluable for primary end point at 12 months. One patient received a HSCT before study inclusion (UPN #10) and, thus TL was not evaluated; for one patient the telomere sample was missing at 12 months. Ten of 13 patients (77%) met the primary efficacy end point at 12 months and showed consistent telomere elongation (Figure 2A). The average increase in TL at 12 months was 0.87 kb (95% confidence interval [CI]: 0.20-1.55; *P*=0.01). Ten patients reached 24 months of treatment and were evaluable. All of them met the end point criteria and showed telomere elongation at 2 years (Figure 2A). At 24 months the average increase in TL from baseline was 0.49 kb (95% CI: 0.24-1.23; *P*=0.18).

Hematologic response

In intention-to-treat analysis, eight of 16 patients (50%) with marrow failure (UPN #10, who received HSCT previous to study entry was excluded) reached hematologic response at 12 months, and ten of 16 patients (63%) reached hematologic response at 24 months (Figure 2C). Mean hemoglobin increase was 1.65 g/dL (95% CI: 0.65-2.63; *P*<0.01) and 2.09 g/dL (95% CI: 0.99-3.18; *P*<0.01) from baseline to 12 and 24 months, respectively. Absolute reticulocyte count remained stable at 12 months but increased by a mean of 34,487/μL at 24 months (95% CI: 6,051-62,922; *P*=0.02) (*Online Supplementary Figure S1*). Five of eight transfusion-dependent patients became transfusion-independent, and one patient showed a reduction in transfusion requirements >50%. Two patients relapsed during the study period: UPN #9 relapsed 3 months after first dose reduction and eventually died of cytopenia complications 1 year after completing the study; and UPN #11 died due to intracranial hemorrhage 4 months after relapse. Platelet and neutrophil counts did not significantly change during nandrolone treatment (*Online Supplementary Figure S1*). Four patients did not show hematologic response at any time during nandrolone treatment.

Lung disease and pulmonary response

Pulmonary disease (ILD) was identified at baseline in seven patients on HRCT scan, with variable radiologic features. Usual interstitial pneumonia (UIP) pattern was observed in one patients (UPN #3), indeterminate for UIP in three (UPN #2, UPN #4 and UPN #13); non-specific interstitial pneumonia (NSIP) in one (UPN #1); pleuroparenchymatous fibroelastosis (PPFE) in one (UPN #10); and interstitial lung abnormalities without fibrosis in one (UPN # 17). Radiologic patterns did not correlate with any specific genetic lesion (*data not shown*).

In this ILD subgroup, two patients died during nadrolone use due to respiratory failure at two and 16 months, without pulmonary function re-assessment (considered as non-responders in intention-to-treat analysis). In the five

Table 2. Germline and somatic genetic variants in enrolled patients.

Germline variants in telomere biology genes							Somatic gene variants			
							At enrollment		At end of treatment	
Patient N Phenotype	Gene	Transcript (RefSeq)	DNA change	Protein change	Zygosity	ACMG classification	Gene/ location	VAF%	Gene/ location	VAF%
1 AA/ILD	*TERT*	NM_198253	c.193C>A	p.Pro65Thr	homo	P	None		None	
2 AA/ILD/ LD	*RTEL1*	NM_001283010	c.3054C>T	p.Arg743X	het	P	None		None	
3 AA/ILD	*RTEL1*	NM_001283010	c.3054C>T	p.Arg743X	het	P	None		Not sequenced	
4 AA/ILD/ LD	*TERT*	NM_198253	c.2594G>A	p.Arg865His	het	P	*POT1* c.56G>A:p.Gly19Asp	4.0	*POT1* c.56G>A:p.Gly19Asp	6.3
							PPM1D c.1281G>A:p.Trp427Ter	0.54	*PPM1D* c.1281G>A:p.Trp427Ter	0.9
							-		*CBL* c.1348G>A:p.Ala450Thr	0.54
							-		*TERTp* c.-57A>C	0.2‡
5 AA	*TERT*	NM_198253	c.2594G>A	p.Arg865His	het	P	None		None	
6 AA	*TERT*	NM_198253	c.3234C>G	p.Pro1078Leu	het	LP	*TERTp* c.-146A>C	0.6	*TERTp* c.-146A>C	1.5
							PPM1D c.1654C>T:p.Arg552Ter	0.86	*PPM1D* c.1654C>T:p.Arg552Ter	0.5
							PPM1D c.1262C>G:p.Ser421Ter	1.0	*PPM1D* c.1262C>G:p.Ser421Ter	2.0
							POT1 c.1505+3A>G	0.5	-	-
7 AA	*TERT*	NM_198253	c.2594G>A	p.Arg865His	het	P	None		None	
8 AA	None*	-	-	-	-	-	-		*EZH1* c.2098+2T>G	4.3
9 AA/SK	*TINF2*	NM_001099274	c.844C>T	p.Arg282Cys	het	P	None		None§§§	
10 AA/ILD/ LD/SK	*RTEL1*	NM_001283009	c.3257A>G	p.Tyr1086Cys	het	LP	None		None	
	RTEL1	NM_001283009	c.3775_3776del	p.Ala1259fsX2	het	P				

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Germline variants in telomere biology genes							Somatic gene variants			
							At enrollment		At end of treatment	
Patient N Phenotype	Gene	Transcript (RefSeq)	DNA change	Protein change	Zygosity	ACMG classification	Gene/ location	VAF%	Gene/ location	VAF%
11 AA	None	-	-	-	-	-	*ASXL1* c.2858_2859insTGAC: p.Leu953fs	30.0	*ASXL1* c.2858_2859insTGAC: p.Leu953fs§§	6.7
							RUNX1 c.965C>G:p.Ser322X	33.0	*RUNX1* c.965C>G:p.Ser322X§§	7.5
12 AA/SK	*RTEL1*	NM_001283009	c.3257A>G	p.Tyr1086Cys	het	LP	None		None	
	RTEL1	NM_001283009	c.3775_3776del	p.Ala1259fsX2	het	P				
13 AA/ILD/ LD/SK	*TERT*	NM_198253	c.2146G>A	p. Ala716Thr	het	P	*TERTp* c.-124A>C	0.85	*TERTp* c.-124A>C§§	1
14 MDS	None	-	-	-	-	-	*U2AF1* c.101C>T:p.Ser34Phe	26.0	*U2AF1* c.101C>T:p.Ser34Phe§	28.0
15 AA	*TERC*	NR_001566.1	r.94C>T	na	het	P	None		None	
	TERT	NM_198253	c.2329G>A	p.Val777Met	het	P				
16 AA	*TERT*	NM_198253	c.2154C>A	p.Asp718Glu	het	P	*PIGA* c.486delC:p.Val162fs	2.9	*PIGA* c.486delC:p.Val162fs§§	12.6
17 AA/ILD/ SK	*TERC*	NR_001566.1	r.110_113del-GACT	na	het	P	None		None§§	

AA: aplastic anemia; ILD: interstitial lung disease; LD: liver disease; MDS: myelodysplastic syndrome; na: not applicable; SK: skin abnormalities; homo: homozygous; het: heterozygous; VAF: variant allele frequency; ACMG classification, Joint Consensus Recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology for the interpretation of sequence variants (Richards *et al.* Genet Med, 2015); P: pathogenic; LP: likely pathogenic; VUS: variant of uncertain significance. *No telomere biology variant was identified, but the patient was later found to be heterozygous for the SAMD9 gene variant c.740T>C:p.Ile247Thr (NM_001193307), classified as a VUS according to the ACMG guidelines. ‡Detected 2 years after end of treatment at VAF of 0.55%. Retrospectively identified in the sample from the end of treatment at VAF below the assays' limit of detection (VAF of 0.2%). §Sequencing performed 6 months after enrollment; §§sequencing performed 12 months after enrollment; §§§Sequencing performed 18 months after enrollment.

Table 3. Adverse events during nandrolone treatment.

Adverse events	All grades N (%)	Grade 1 N (%)	Grade 2 N (%)	Grade 3 N (%)
Abnormal elevated serum levels				
Transaminases	14 (82)	12 (70)	1 (6)	1 (6)
Canalicular enzymes	10 (59)	7 (41)	3 (18)	0
Bilirubin	3 (18)	3 (18)	0	0
Cholesterol	1 (6)	1 (6)	0	0
Triglycerides	1 (6)	0	1 (6)	0
Prostate-specific antigen	1 (6)	1 (6)	0	0
Creatinine	4 (24)	4 (24)	0	0
Acne	10 (59)	7 (41)	2 (12)	1 (6)
Virilization				
Voice deepening	5 (29)	5 (29)	0	0
Hirsutism	5 (29)	5 (29)	0	0
Erythrocytosis	6 (35)	3 (18)	3 (18)	0
Injection site reactions				
Pain	2 (12)	2 (12)	0	0
Hematoma	1 (6)	1 (6)	0	0
Local infection	1 (6)	0	0	1 (6)
Sciatic nerve injury	1 (6)	0	0	1 (6)
Irritability	5 (29)	5 (29)	0	0
Muco-cutaneous bleeding	4 (24)	4 (24)	0	0
Alopecia	4 (24)	4 (24)	0	0
Hypertrichosis	3 (18)	3 (18)	0	0
Hypertension	2 (12)	0	1 (6)	1 (6)
Depression	2 (12)	1 (6)	0	1 (6)
Systemic allergic reactions	2 (12)	1 (6)	0	1 (6)
Insomnia	2 (12)	2 (12)	0	0
Abdominal pain	2 (12)	2 (12)	0	0
Cramps	2 (12)	2 (12)	0	0
Precocious puberty	1 (6)	0	1 (6)	0
Vulvar synechia	1 (6)	1 (6)	0	0
Clitoromegaly	1 (6)	1 (6)	0	0
Headache	1 (6)	1 (6)	0	0
Prostatism	1 (6)	1 (6)	0	0
Edema	1 (6)	1 (6)	0	0

No adverse event ≥ grade 4 was observed.

remaining evaluable patients, the pulmonary function improved in three or remained stable in two patients during nandrolone therapy (Figure 2B): mean percentage of forced vital capicity (FVC%) was 68.0% (95% CI: 46.8-89.2) at baseline and 69.6 (95% CI: 47.5-91.7) at 2 years (*P*=not significant); and mean percentage of diffusing capacity of carbon monoxide (DLCO%) was 43.7 (95% CI: 27.4-59.8) at baseline and 49.8 (95% CI: 32.8-66.6) at 24 months (*P*=not significant) (Table 4). In aggregate, pulmonary response was observed in two patients (29%) at 12 months and in three patients (43%) at 24 months of treatment (Figure 2C). However, after treatment completion, DLCO was consistently reduced in all five cases with available PFT at 6-12 months post-treatment (Figure 2B) and two more patients died due to respiratory failure 1 and 3 years after stopping nandrolone (Figure 2C).

In three patients with ILD, HRCT visual fibrosis scores remained stable during nandrolone, but quantitative measures obtained at the end of the 2-year treatment showed disease progression in two cases. In the first patient (UPN #4), PV was reduced (5,401 mL at baseline to 5,146 mL at 24 months), and lung density increased (MPD, -733 HU to -726 HU; and P90, -259 HU to -236 HU at baseline and 24 months, respectively), in agreement with a reduction in FVC, as a percentage of predicted values (FVC%) (102% at baseline to 86% at 24 months) observed in PFT. His DLCO, as a percentage of predicted values (DLCO%), remained stable (71% at baseline and 72% at 24 months). The second patient (UPN #10) also showed mild increase in lung density (MPD, -678 HU to -667 HU; and P90, -275 HU to -272 HU, at baseline and 24 months, respectively) but improvement in PV (2,798 mL at baseline

to 3,086 mL at 24 months) and in all functional parameters in PFT (FVC%: 51% to 57% and DLCO%: 28% to 46%, at baseline and 24 months, respectively). This patient ultimately became oxygen-independent during nandrolone use. The third patient (UPN #17) showed improvement in both quantitative HRCT scores (PV, 2,398 mL to 4,246 mL; MPD, -609 HU to -717 HU; and P90, -243 HU to -288 HU, at baseline and 24 months, respectively), with sustained FVC% (83% at baseline and 91% at 24 months), and DLCO% (40.4% at baseline and 40.5% at 24 months).

Three patients (UPN #1, UPN #2 and UPN #13) with ILD showed worsening of both visual scores and quantitative measures during treatment (mean PV, 3,581.7 mL to 2,924.3 mL; mean MPD, -706.3 HU to -624 HU, and mean

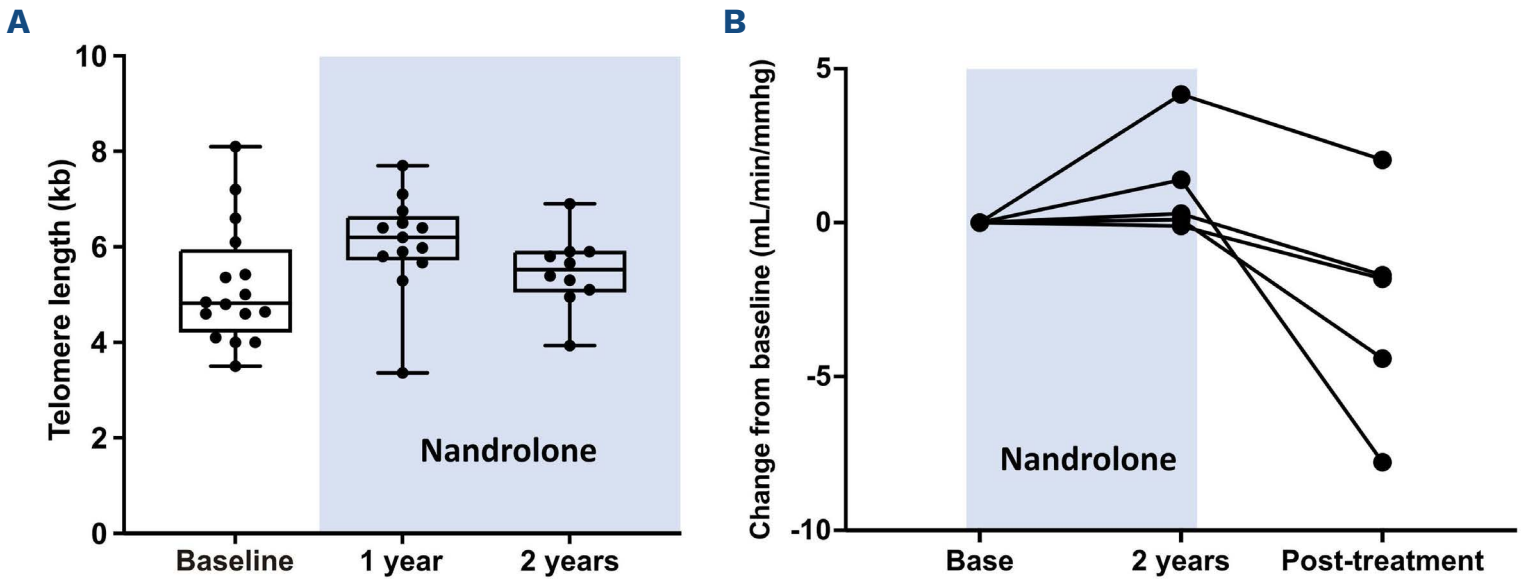

Figure 2. Biologic and clinical response to nandrolone treatment. (A) Telomere length measured by flow-fluorescense *in situ* hybridization at baseline and during 12 and 24 months of treatment. (B) Spider graph with the changes in hemoglobin corrected diffusing capacity of the lungs for carbon monoxide for patients with interstitial lung disease treated with nandrolone. (C) Hematologic and pulmonary response in patients treated with nandrolone decanoate. HSCT: hematopoietic stem cell transplantation.

Table 4. Lung involvement evaluated by pulmonary function testing and quantitative measures in high-resolution computed tomography of the chest in seven patients with interstitial lung disease at baseline and at end-of-treatment.

	Baseline Mean (95% CI)	24 months Mean (95% CI)	*P* value
Pulmonary function test (N=7)			
FVC%	68.0 (46.8-89.2)	69.6 (47.5-91.7)	ns
DLCO%	43.7 (27.4-59.8)	49.8 (32.8-66.6)	ns
High resolution computed tomography (N=7)			
PV (mL)	3,453.7 (2,403.4-4,504.0)	3,483.8 (2,375.2-4,592.3)	ns
MPD (HU)	-678 (-727 to -628.9)	-663.7 (-716.6 to -610.7)	ns
P90 (HU)	-262.6 (-303.2 to -221.8)	-234.1 (-278.0 to -190.1)	ns

FVC%: percentage of predicted forced vital capacity; DLCO%: percentage of predicted diffusing capacity for carbon monoxide; PV: pulmonary volume; MPD: mean pulmonary density; P90: 90th percentile of lung density; HU: Hounsfield unit; ns: not significant.

P90, -280.6 HU to -204.3 HU, at baseline and 24 months, respectively), and in spite of some improvement in PFT in two of them (UPN #1, FVC%, 77% to 79%, DLCO%, 38.9% to 46.4%; and UPN #2, FVC%, 53% to 58%, DLCO%, 56% to 58%), all three required supplementary oxygen and died due to progressive pulmonary disease 1 and 3 years after nandrolone discontinuation and at 16 months of treatment, respectively.

An additional patient with pulmonary disease (UPN #3), who was oxygen-dependent at baseline died in the second month of the study due to acute ILD exacerbation, and PFT and HCRT at follow-up were not performed.

Somatic mutations

Genetic somatic mutations were identified in peripheral blood leukocytes in six patients at baseline (Table 2; Figure 3). Clones bearing mutations in genes associated with myeloid neoplasms remained stable (*PPM1D* mutations in UPN #4 and UPN #6; and *U2AF1* mutation in UPN #14) or decreased (*ASXL1* and *RUNX1* mutations in UPN #11) during nandrolone use. Of note, germline mutations in telomere-biology genes were not identified in two instances (UPN #11 and UPN #14), and despite the transient hematologic response, both prematurely died due to complications of thrombocytopenia.

In patient UPN #4, a *POT1* mutation present at baseline increased in clone size and new low-frequency myeloid mutations (*PPM1D* and *CBL*) emerged in the course of treatment. The patient showed late hematologic response at 24 months.

TERT promoter (*TERT*p) mutations also were observed at baseline (UPN #6 and UPN #13), or emerged during the study (UPN #4). In all of them, the variant allele frequency (VAF) remained stable during nandrolone.

The only somatic clone that significantly increased during study treatment was a PIGA mutation in UPN #16. He did not show improvement in blood counts and was sent for alternative donor HSCT.

The patient UPN #8 carried a *SAMD9L* germline mutation. At the end of treatment, an EZH1 somatic mutation (VAF, 4.3%) was identified; he showed hematologic improvement and remained stable after drug discontinuation (due to depression).

Discussion

In this single-arm prospective clinical trial, treatment with intramuscular nandrolone decanoate for 2 years led to telomere elongation in peripheral blood leukocytes of patients with telomere diseases. Telomere elongation was associated to a certain degree of hematologic response that, in some cases, was delayed and transient and to a tendency to a stable lung function in patients with previous lung involvement. Somatic mutations associated with myeloid malignancies were present in a minority of patients and did not appear to be influenced by the pharmacologic intervention.

Many androgen formulations have been used to treat acquired and inherited bone marrow failure for decades, with variable outcomes and adverse events.[29] In a multisystem, rare, and severe disease lacking effective and definitive therapies, androgen and their derivatives have shown to be safe and to mitigate marrow failure in retrospective[30] and prospective[22] trials. Since the first observation by Sanchez-Medal[31] on the use of anabolic androgens to treat patients with aplastic anemia, the improvement in neutrophils and platelets was less marked and slower. Androgens also are known to stimulate the expansion of the red blood mass via erythropoietin.[32,33] These findings may explain the more robust response in the red cell lineage in the current trial. Our present study expands the knowledge of androgen use for telomere diseases in at least four aspects. First, androgen toxicities

are frequent irrespective of specific formulation, especially in liver function tests and clinical virilization. In our study, nandrolone decanoate led to an elevation in liver enzyme levels (88%) more frequently than reported for danazol (41%)[22] but adverse events were mild in most cases. That nandrolone decanoate is given intramuscularly, bypassing the hepatic first passage of oral formulations, would suggest less propensity for hepatotoxicity, but our prospective trial does not support this prediction. However, neither severe liver AE was observed nor was drug administration interrupted due to liver toxicity; in the four patients with liver disease at enrollment, it was stable during the pharmacologic intervention. The AE profile appears to be similar to that reported for patients with Fanconi anemia or dyskeratosis congenita treated with androgens.[29] The occurrence of two intracranial hemorrhages is of potential concern, since it is not a commonly reported in telomeropathies. In both cases, trauma, hyper-

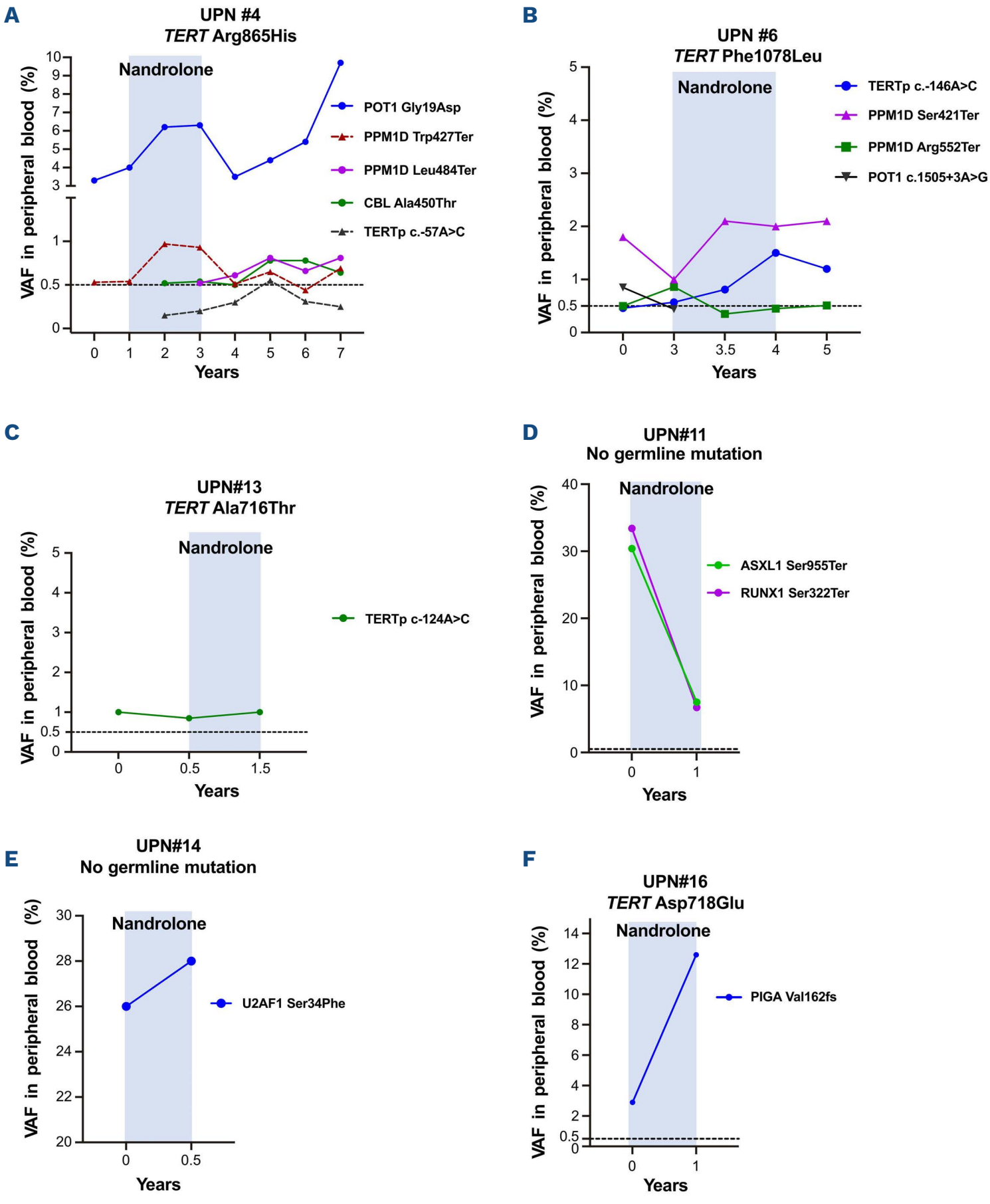

Figure 3. Somatic mutations' dynamics during nandrolone use. (A) patient UPN #4; (B) patient UPN #6; (C) patient UPN #13; (D) patient UPN #11; (E) patient UPN #14 and (F) patient UPN #16. VAF: variant allele frequency.

tension, or increased hematocrit were not observed. The intracranial bleeding events were correlated with the very low platelet counts (<10,000/μL). However, we cannot exclude an unlikely association with the study drug.

Second, all evaluable patients met endpoint criteria at 24 months and showed a reduction in telomere erosion. Indeed, most patients exhibited telomere elongation during the pharmacologic intervention, more significant at 12 months (Figure 2), confirming previous observations in the trial using danazol.[22] One major strength of our study is that TL was determined using the flow-FISH technique, which is more accurate and reproducible than quatitative polymerase chain reaction.[26,34] Although flow-FISH also has some variability, samples from the same patients at different time points were run together at the end of the study to avoid inter-experiment variability, and the elongation pattern was consistent. Flow-FISH has a limitation to detect small differences in TL between paired samples and differences in the annual rate attrition may be difficult to detect for a given individual. However, as most patients showed the same telomere elongation pattern, flow-FISH was able to detect elongation. Our study, using a different male hormone and a different TL measurement technique, confirms that androgens elongate telomeres of peripheral blood leukocytes of patients with telomeropathies. Additional non-controlled studies also have suggested that androgens elongate telomeres.[30] In aggregate, there is retrospective and prospective evidence that androgens elongate telomeres of telomere disease patients *in vivo*.

How androgens elongate telomeres is not entirely clear. Androgen exposure increases telomerase expression and activity *in vitro* in hematopoietic cells.[21] Murine models of telomerase deficiency also have shown telomere elongation under androgen treatment that is telomerase-dependent.[20] Additionally, our results suggest that androgen therapy influences stem cell mobilization, based on the clonal hematopoiesis findings (Table 2; Figure 3). There is evidence that danazol treatment may modulate *TERT*p clones, which operate using promoter regions different from sex hormones.[21,35,36] Recent findings indicate that short telomeres imbalance stem cell fate towards differentiation at the self-renewal expense.[4,5] It is possible that androgens engage quiescent hematopoietic stem cells with longer telomeres and restore hematopoietic stem cell expansion and differentiation.

Third, to the best of our knowledge, this is the first comprehensive analysis of an androgen effects on clonal hematopoiesis. Clones containing mutations associated with myeloid neoplasms remained stable or decreased in size during nandrolone intervention, whereas somatic mutations in telomere biology genes, which may function as somatic genetic rescue,[37] appeared in small-size clones (<5%) and remained stable, except for one patient in which a *POT1*-mutant clone doubled in size during nandrolone and contracted after the intervention period (Table 2; Figure 3). Clonal hematopoiesis was present in a minority of patients and did not appear to be significantly influenced by nandrolone. Of note, we identified one particular patient with a germline pathogenic variant in *TERT* (Asp718Glu) in whom a somatic mutation in the *PIGA* gene was found (Val162fs). The presence of a glycosylphosphatidylinositol (GPI)-negative clone was confirmed in two cell types by flow cytometry at different time points.[38] In our cohort of 87 cases with a GPI-negative clone, this is the only patient with a telomerase mutation in whom a PNH clone was detected by flow cytometry and sequencing, although others have demonstrated frequent minor PNH clones (<0,1%) in inherited marrow failure syndromes.[39] On the other hand, the patient had a germline *TERT* variant predicted to be deleterious *in silico* and located at the same codon as a previously reported variant predicted to be pathogenic and to reduce telomerase activity to 44%.[40] The patient also had a family history of idiopathy pulmonary fibrosis. PNH clones are usually reported in immune aplastic anemia and are thought to be selected by immune escape.[41] Although an immune-mediated mechanism cannot be excluded in this case, it is possible that the GPI-negative clone may be the result of genetic drift and not immune selection.[41]

Forth, the present study extends the observations of the androgen effects on pulmonary function. Seven patients in our study were diagnosed with ILD at baseline; four eventually died from respiratory failure, two of them during nandrolone treatment, emphasizing the severity of the disease when lungs are affected.[9,24] There was significant discordance among HRCT scan visual scores and quantitative measurements, PFT parameters, and clinical outcomes during nandrolone intervention, indicating the difficulty in identifying reliable markers for disease progression. Among patients who completed the 2-year intervention, DLCO remained stable or increased in evaluable patients and consistently decreased when they were off study (Figure 4). Although the number of patients with ILD in the present study is small, the PFT results are comparable to those of the danazol study,[22] suggesting that androgens may decrease lung disease progression. In aggregate, these findings support prospective studies using androgens specifically in patients with ILD associated with telomeropathies.

This study has limitations. The number of patients is relatively small, but compatible with a very rare disorder in a single-center study. Recruitment of patients from other centers and states was disfavored by patient commute, especially in a large country such as Brazil. Additionally, there is no consensus parameter for lung function in patients with pulmonary fibrosis and telomere disease for appropriate follow-up, especially in smaller trials.

In conclusion, nandrolone decanoate elongates telomeres

in patients with telomeropathies, and elongation associates with clinical improvement. These results expand the knowledge on the effects of androgens on telomere maintenance, showing in the clinic that different male hormone formulations are capable of telomere elongation and clinical benefit with tolerable adverse events. As transplant modalities are restricted, nandrolone decanoate may be used to improve hematopoiesis and perhaps stabilize lung function in telomeropathy patients.

Disclosures

No conflicts of interest to disclose.

Contributions

DVC and RTC designed the study. DVC, LFBC, LGDJ, ETV and RTC recruited, treated and followed the patients. FGR, FSD, ALP, BST and BAS performed telomere length measurements and massively parallel targeting sequencing panels. NSY provided and analyzed the massively parallel targeting sequencing data. MKS reviewed high-resolution computed tomography. JBM reviewed pulmonary function testing for diffusing capacity for carbon monoxide. DVC, EZM, NSY and RTC analyzed the data. DVC, NSY and RTC wrote the manuscript; and RTC supervised study conduction. All authors revised the manuscript.

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Data-sharing statement

Original data and protocol are available upon email request to the corresponding author.

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