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Evaluation of T-wave alternans activity under stress conditions after 5 d and 21 d of sedentary head-down bed rest

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Abstract

It is well known that prolonged microgravity leads to cardiovascular deconditioning, inducing significant changes in autonomic control of the cardiovascular system. This may adversely influence cardiac repolarization, and provoke cardiac rhythm disturbances. T-wave alternans (TWA), reflecting temporal and spatial repolarization heterogeneity, could be affected. The aim of this work was to test the hypothesis that 5 d and 21 d head-down (−6°) bed rest (HDBR) increases TWA, thus suggesting a higher underlying electrical instability and related arrhythmogenic risk.

Forty-four healthy male volunteers were enrolled in the experiments as part of the European Space Agency's HDBR studies. High-fidelity ECG was recorded during orthostatic tolerance (OT) and aerobic power (AP) tests, before (PRE) and after HDBR (POST). A multilead scheme for TWA amplitude estimation was used, where non-normalized and T-wave amplitude normalized TWA indices were computed. In addition, spectral analysis of heart rate variability during OT was assessed.

Both 5 d and 21 d HDBR induced a reduction in orthostatic tolerance time (OTT), as well as a decrease in maximal oxygen uptake and reserve capacity, thus suggesting cardiovascular deconditioning. However, TWA indices were found not to increase. Interestingly, subjects with lower OTT after 5 d HDBR also showed higher TWA during recovery after OT testing, associated with

unbalanced sympathovagal response, even before the HDBR. In contrast with previous observations, augmented ventricular heterogeneity related to 5 d and 21 d HDBR was not sufficient to increase TWA under stress conditions.

Keywords: electrocardiogram (ECG), T-wave alternans (TWA), arrhythmias, ventricular repolarization, head-down bed rest, microgravity

(Some figures may appear in colour only in the online journal)

1. Introduction

It is well known that gravity plays an essential role in determining the distribution of hydrostatic pressure gradient and plasma volume within the cardiovascular system. In particular, prolonged weightlessness results in a redistribution of body fluids from the lower half of the body toward upper regions, leading to cardiac deconditioning and inducing significant changes in both autonomic and cardiovascular systems (Convertino and Hoffler 1992). This results in orthostatic intolerance episodes once gravity is re-established, decrease in plasma volume and reduced aerobic capacity, together with bone and muscle loss.

Potentially, cardiac electrical activity could also be affected by this process, as suggested by some anecdotal data of cardiac arrhythmias and conduction disorders during spaceflight (Hawkins and Zieglschmid 1975, Charles *et al* 1994). Recent studies have also shown that long-duration spaceflight, but not short-duration flight, led to the prolongation of the QTc interval in crewmembers (D'Aunno *et al* 2003, Mitchell and Meck 2004), thus potentially generating an increased risk for arrhythmia susceptibility and related sudden cardiac death (SCD).

T-wave alternans (TWA) is defined as a consistent beat-to-beat alternation in the amplitude, duration or morphology of the ST-segment and/or the T wave. It reflects temporal and spatial heterogeneity of ventricular repolarization and it is regarded as a noninvasive risk marker for predicting SCD and ventricular vulnerability (Verrier *et al* 2011). Therefore, TWA could be affected in the context of microgravity.

The head-down (−6°) bed rest (HDBR) maneuver represents a well-established model on Earth for inducing and studying the effects of sustained exposure to simulated microgravity on the cardiovascular system, as well as to test potential countermeasures to prevent its deconditioning (Pavy-Le Traon *et al* 2007).

In this context, only one study (Grenon *et al* 2005) reported results relevant to TWA induced by 9 to 16 d of HDBR in 24 healthy male subjects, suggesting that this prolonged immobilization head-down condition could lead to the development of sustained alternans. However, the heterogeneity of the observed subjects' response, and of the HDBR duration, did not allow drawing final conclusions about the potential negative effects of HDBR on cardiac electrical stability.

More recently, changes in temporal and spatial heterogeneity of ventricular repolarization induced by short (5 d) (Caiani *et al* 2013) and long duration (90 d) (Sakowski *et al* 2011) HDBR were investigated using different approaches by the computation of the spatial ventricular gradient (SVG) (Burger 1957) and of the QRS-T angle (Scherptong *et al* 2008), and by measuring the T-wave amplitude. Both studies confirmed that HDBR induced a reversible increase in ECG repolarization heterogeneity by an increase in QRS-T angle accompanied by a decrease in SVG, thus supporting the hypothesis of increased ventricular arrhythmic risk. In addition, the T-wave amplitude was found to be markedly reduced with HDBR.

Based on these observations, we hypothesized that HDBR induces reversible increase in ventricular repolarization heterogeneity that could be manifested through TWA as a result of increased ventricular arrhythmic risk.

Consequently, our aim was to assess the effects of short- (5 d) and mid- (21 d) duration HDBR on TWA, computed from the ECG tracings obtained during orthostatic tolerance (OT) testing induced by 80° head-up tilt (HUT) and during peak aerobic power (AP) exercise testing, performed both before and immediately after termination of HDBR.

In addition, autonomic nervous system (ANS) activity during the OT test was studied to evaluate its possible relationship with alterations in TWA.

2. Materials and methods

2.1. Experimental protocol

In the context of the European Space Agency (ESA) HDBR strategy, an all-male population was recruited, after multiple screening and psychological tests, for two sedentary short-term (SHORT, 5 d) and two mid-term (MID, 21 d) duration HDBR campaigns. The choice of including only males was driven by the ESA standardization plan.

Subjects had no history of cardiovascular disease and were not taking medications of any kind. One SHORT (12 subjects, age range 21–41 years) and one MID (12 subjects, age range 20–44 years) campaign (ESA acronyms: BR-AG1 and MNX, respectively) were performed at the Institut de Médecine et de Physiologie Spatiales (MEDES) facility, at the University Hospital of Rangueil, Toulouse, France. One SHORT (10 subjects, age range 25–44 years) and one MID (10 subjects, age range 23–42 years) campaign (ESA acronyms: SAG and MEP, respectively) were performed at the German Aerospace Center (DLR) at Cologne, Germany. All volunteers provided written informed consent to participate in the study approved by the respective Ethical Committee for Human Research at both hosting institutions.

For all campaigns, the protocol was designed as a cross-over study: every subject repeated the HDBR two (MEP) or three times (BR-AG1, SAG and MNX), one with no intervention (control) and one or two with specific interventions applied during HDBR, with a washout period (1.5 months for SHORT and 4 months for MID duration campaigns) between the end of one repetition and the onset of the next one. The order of inclusion in the intervention group was randomly assigned to each subject. Applied interventions to be tested as countermeasures consisted in: BR-AG1, (i) continuous 30 min or (ii) intermittent 6 min × 5 min short-arm daily centrifugation periods; SAG, (i) 25 min of daily upright quiet standing and (ii) 25 min of locomotion replacement training; MNX, (i) resistive vibration exercise (RVE) and (ii) RVE + high protein intake (1.8 g (kg body weight)$^{-1}$ d^{-1} + 0.6 g (kg body weight)$^{-1}$ d^{-1} of whey protein) and alkaline salt; MEP, (i) high protein intake (1.2 g (kg body weight)$^{-1}$ d^{-1} + 0.6 g (kg body weight)$^{-1}$ d^{-1} of whey protein (0.6 g (kg body weight)$^{-1}$ d^{-1}) and alkaline salt.

In this paper, to allow for comparison despite the different campaigns and countermeasures adopted, only the data obtained when the subjects were included in the sedentary control group will be analyzed, both for SHORT and MID HDBR.

The protocol for each subject included 5 or 8 d of acclimation to the bed rest facility (referred to as PRE period). Those days are denoted as BDC-5 to BDC-1 in SHORT campaigns and BDC-8 to BDC-1 in MID campaigns, where the number after BDC stands for the day before the beginning of the provoking maneuver (i.e. the uninterrupted HDBR period). During this period, all subjects adhered to a monitored, strict −6° head-down tilt bed rest, 24 h a day for 5 or 21 d for SHORT or MID campaigns, respectively, with a strictly controlled diet to prevent body weight changes. Subjects were awakened at 6:30 am and prompted to

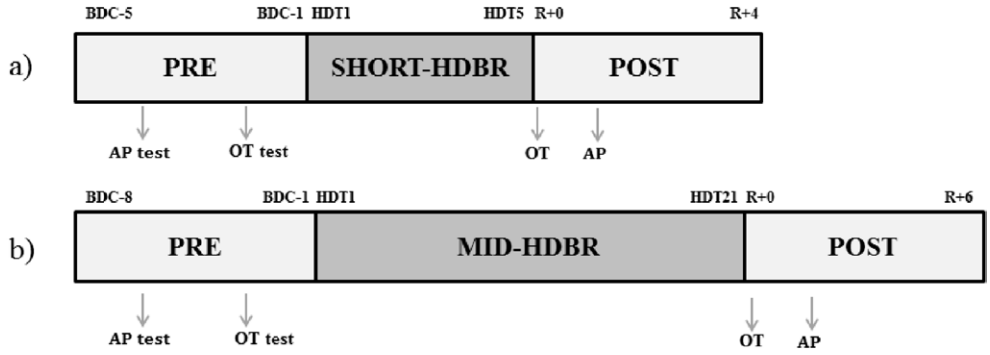

Figure 1. Schematic and timing of the OT and AP tests, for both the SHORT (a) and MID (b) duration HDBR campaigns.

start sleeping at 11:00 pm each day, with no napping allowed during the day. After completing the HDBR portion of the study, subjects remained in the facility for an additional 5 or 7 d (referred to as POST period). Those days are denoted as R + 0 to R + 4 in SHORT and as R + 0 to R + 6 in MID, where R + 0 is the day that started with termination of the immobilization period by the orthostatic tolerance (OT) test. During the PRE and POST periods, lying in bed during the day was not allowed. Figure 1 schematically shows the protocols of SHORT and MID campaigns.

Decisions regarding the specific experimental protocol implemented for each campaign were driven by the ESA standardization plan, also based on the need to accommodate multiple experiments conducted by different researchers.

2.1.1. Orthostatic tolerance (OT) test. At R + 0, once the cardiovascular monitoring equipment was connected, the subjects first spent a period of at least 5 min in a supine position on a tilt board with their lower body enclosed in a lower body negative pressure (LBNP) chamber, which was initially unpressurized. Thereafter, the tilt angle was changed to 80° HUT and maintained at this angle for 30 min in SHORT, and for 15 min in MID campaigns.

After this time, if the orthostatic test was not already ended, the pressure in the LBNP chamber was changed by −10 mmHg decrements at 3 min intervals until the test was terminated. Termination criteria included signs of pre-syncope (tunnel vision, pallor, sweating or malaise, sudden bradycardia, hypotension or undue lack of subject response to questions) and/or at the request of the subject (Linnarsson *et al* 2014). Orthostatic tolerance time (OTT) was defined as the time from HUT until stop criteria were reached. At the end of the test, supine position was restored.

The same protocol was performed during the ambulatory period (BCD-2) before the HDBR to assess OTT at baseline.

2.1.2. Peak aerobic power (AP) test. Peak aerobic power was determined as $\dot{V}O_{2peak}$ during incremental dynamic leg exercise on a cycle ergometer (Ergometrics 800S, Ergoline, Bitz, Germany), before (BDC-5 or BDC-7, respectively for SHORT and MID) and after HDBR (at R + 1, at least 26 h after discontinuation of HDBR, except for the SHORT BR-AG1 campaign, in which it was performed at R + 0, 6 h after the OT test). Breath-by-breath $\dot{V}O_2$ was recorded with an Oxycon Pro metabolic cart (E Jaeger, Hochberg, Germany). $\dot{V}O_{2peak}$ was determined during the subject selection. The sitting subject exercised for 5 min at power outputs estimated

to require 25%, 50% and 75% of $\dot{V}O_{2peak}$. Thereafter, the power output was increased by 25 W every minute until exhaustion, i.e. when the required cycling cadence of >70 min^{-1} was no longer maintained.

For each $\dot{V}O_2$ recording, $\dot{V}O_{2peak}$ was calculated to be the highest value in a 60 s moving average window.

The timing of the OT and AP tests for both the SHORT (a) and MID (b) duration HDBR campaigns is shown in figure 1.

2.2. ECG acquisition

The ECG signal was acquired during both OT and AP tests using a 12-leads 24 h Holter digital recorder (H12+, Mortara Instrument Inc., Milwaukee, WI) with 1000 Hz sampling frequency, at PRE (BDC-2 and BDC-5, respectively) and at the end of the HDBR period (R + 0 and/or R + 1, as specified above).

2.3. TWA analysis

From the acquired ECG, three specific intervals were selected for further processing:

- for OT test: (1) baseline (BAS): the 4 min preceding the HUT; (2) TILT: the first 4 min of HUT; (3) recovery (REC): the first 4 min once supine position was restored at test termination.
- for AP test: (1) baseline (BAS): the 5 min preceding the start of the test (baseline), while the subject was already instrumented and seated on the cycloergometer but not pedaling; (2) EX1: the 5 min of exercise at 25% of workload; (3) EX2: from minute 5 to minute 10 of the test at 50% of workload. The analysis was limited up to a heart rate of 130 beats min^{-1}, to avoid the possible mechanical interference at twice of the pedaling cadence (variable, but $\geqslant$70 rpm) in the ECG signal, which could induce mechanical alternant components at the TWA frequency (Bailón *et al* 2013).

Preprocessing of ECG recordings included QRS detection using a wavelet-based ECG delineator (Martínez *et al* 2004). Baseline wander was removed in each lead using a cubic spline interpolation technique. Finally, the ECG was low-pass filtered (with cut-off frequency of 15 Hz) to remove noise from the TWA frequency range and down-sampled to 125 Hz to reduce the computational cost of TWA analysis.

The resulting filtered ECG signal was processed for automated TWA analysis in segments of 32 consecutive beats with a 50% overlap. To exclude from this analysis possible transients present in the signal, a stability criterion (Monasterio *et al* 2012) based on the heart rate (HR) was defined as follows:

- difference between the maximum and minimum instantaneous HR in the segment less than 20 beats min^{-1};
- at least 75% of the beats labeled as sinus beats, with the difference between the ith and the $(i-1)$th RR intervals less than 150 ms.

Automatic TWA analysis was then performed on the suitable 32-beat segments using a multilead scheme based on periodic component analysis (πCA) (Monasterio *et al* 2010) combined with the Laplacian likelihood ratio method (LLRM) (Martínez and Olmos 2005). A block diagram of the analysis performed in this work is shown in figure 2.

This approach uses πCA to find the optimal linear transformation from eight standard leads (V1–V6, I and II) to a new set of leads (T1–T8) where the two-beat periodicity of the ST–T

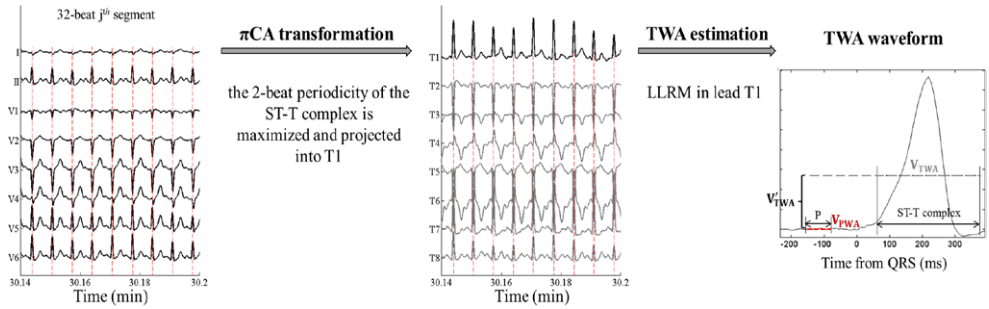

Figure 2. Block diagram of TWA analysis.

complex, which is the periodicity corresponding to the TWA, is maximized. As a result of this transformation, the most two-beat periodic components remain projected into the first transformed lead, T1(Saul and Allen 2001). Figure 2 illustrates this effect.

The Laplacian likelihood ratio (LLR) method (Martínez and Olmos 2005) was then applied to the transformed lead T1 to estimate the TWA waveform, representing a distribution of the alternans amplitude within the ST–T complex.

The TWA amplitude in the 32-beat jth segment, $V_{\mathrm{TWA}}(j)$, was defined as the absolute value of the mean of the TWA estimated waveform. To eliminate from the TWA amplitude the potential amount of noise and other non-alternant ECG components, which could appear and interfere in $V_{\mathrm{TWA}}(j)$ (especially under stress test), we defined a new measurement $V'_{\mathrm{TWA}}(j) = V_{\mathrm{TWA}}(j) - V_{\mathrm{PWA}}(j)$, where $V_{\mathrm{PWA}}(j)$ can be interpreted as the alternant component present in the P-wave of the ECG, and was computed similarly to $V_{\mathrm{TWA}}(j)$. Assuming noise as uniformly distributed within the whole beat, $V_{\mathrm{PWA}}(j)$ represents an estimate of the noise level in the TWA measurement. Therefore, $V'_{\mathrm{TWA}}(j)$ measures TWA amplitude over the noise level.

Based on previous observations that HDBR induces modifications in the T-wave morphology, in particular by reducing the maximum T-wave amplitude and T-wave area (Sakowski *et al* 2011, Caiani *et al* 2013, 2014b), we also introduced a normalized index of TWA to take into account possible effects of these changes on TWA obtained at PRE and at POST bed rest. The normalized TWA amplitude at each segment was defined as $V_{\mathrm{TWAn}}(j) = V'_{\mathrm{TWA}}(j)/V_{\mathrm{T}}(j)$, where $V_{\mathrm{T}}(j)$ represents the mean value of the first principal component in the ST–T complex computed by applying the principal component analysis (PCA) over the same eight leads.

Finally, TWA and TWAn indices were computed by averaging all suitable segments at each defined interval.

2.4. Power spectral analysis of heart rate variability

For the ECG data collected during the OT test, the instantaneous HR variability (HRV) series, $d_{\mathrm{HRV}}(n)$, was derived from the QRS detection marks obtained in the preprocessing stage (Hernando *et al* 2011).

For HRV spectral analysis, three intervals of 3.5 min duration were extracted starting from the previously defined intervals for TWA analysis during OT test, but excluding HR abrupt transient changes: (1) baseline (BAS), from 4 min up to 30 s before the tilt; (2) TILT, from 30 s to 4 min after HUT; (3) recovery (REC), from 30 s after supine position was restored to minute 4 of recovery.

For each interval, the power spectral density (PSD) of $d_{\mathrm{HRV}}(n)$ was computed by using the periodogram estimator. The power in the LF (0.04–0.15 Hz) and HF bands (0.15–0.4 Hz), P_{LF}

and P_{HF} respectively, was obtained by integrating the power spectrum in the corresponding frequency bands. Then, the normalized P_{LFn} and the ratio P_{LF}/P_{HF} were computed (Task Force 1996).

2.5. Statistical analysis

Data are presented as median and 25th and 75th percentiles, unless otherwise specified. To evaluate significance of changes induced by HDBR, in each corresponding interval of the OT and AP protocols (BAS, TILT and REC, and BAS, EX1 and EX2, respectively) a non-parametric Wilcoxon rank test was applied between PRE and POST values.

In addition, to evaluate significance of changes induced by the OT or by the AP test, a non-parametric Wilcoxon rank test was also applied to the relevant BAS condition, for PRE and POST separately.

To evaluate potential differences in the observed phenomena related to orthostatic deconditioning and on the effective application of the LBNP during the OT test, we subdivided the subjects into two subgroups, separately for SHORT and MID duration campaigns, based on the OTT at POST:

- LOW-OTT, including subjects for SHORT with OTT $\leqslant$ 30 min, and for MID with OTT $\leqslant$ 15 min (i.e. no LBNP was needed to induce OT test termination);
- HIGH-OTT, including subjects for SHORT with OTT $>$ 30 min, and for MID $>$ 15 min (i.e. OT test termination only after LBNP activation).

Comparisons between LOW-OTT and HIGH-OTT subgroups were performed using the Mann–Whitney test.

For all tests, the null hypothesis was rejected when $p \leqslant 0.05$.

3. Results

For the SHORT HDBR, all 22 subjects in sedentary HDBR completed the experiments. Due to technical problems, data during AP test from both the PRE and POST were available for 20 out of 22 subjects only.

For the MID HDBR, only 20 out of 22 subjects in sedentary HDBR completed the experiments (one withdrawal in MNX and one in MEP). However, for technical problems during AP test, paired data from PRE and POST were available in 18 subjects only.

3.1. Orthostatic tolerance test

For the SHORT HDBR, where LBNP was applied after the first 30 min of HUT, OTT was significantly reduced between PRE and POST (38.7 (36.18; 43) versus 8.3 (5.37; 32.5) min, $p < 0.001$). Also for the MID HDBR, where LBNP was applied after 15 min of HUT, OTT after sedentary HDBR was reduced (24.1 (21; 28.7) versus 13 (5.9; 20.1) min, $p < 0.001$).

3.1.1. Results of 5 d duration HDBR (SHORT). In SHORT, when comparing PRE versus POST, an increase in HR was found at BAS and at TILT. Despite this increment, a significant decrease in TWA at BAS was present, while TWAn did not change (figure 3, top panels). From spectral analysis of HRV, P_{LF} and P_{LF}/P_{HF} increased at POST during BAS and REC but not during TILT, evidencing a shift in the ANS balance toward sympathetic activation induced by HDBR in these two phases (table 1).

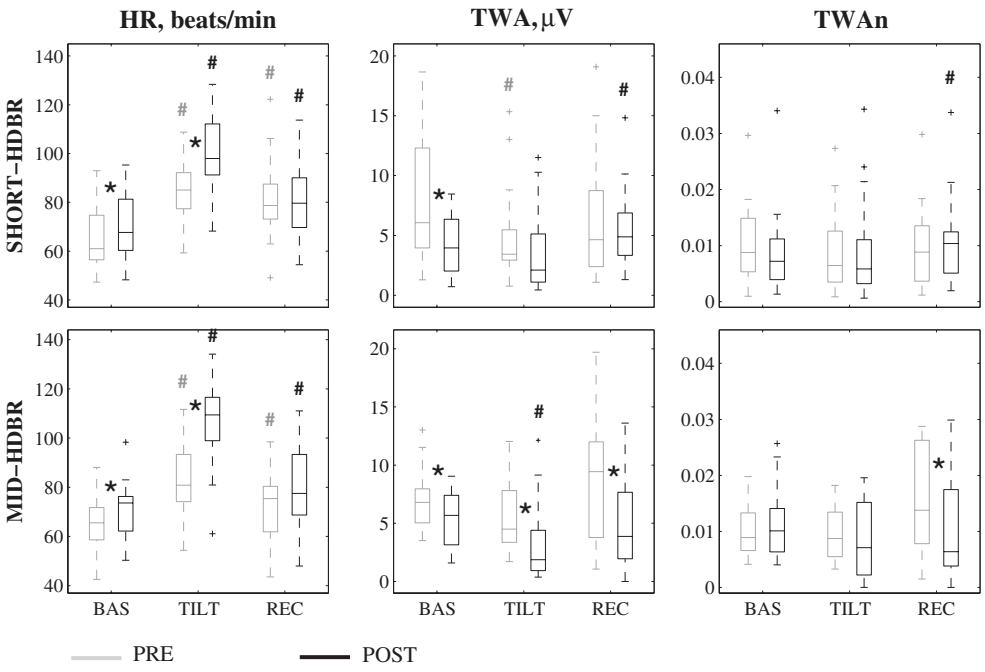

Figure 3. Cumulative results for HR and TWA amplitudes, reported as absolute and normalized (TWAn), obtained in SHORT (top) and in MID (bottom) campaigns during orthostatic tilt test (*: $p < 0.05$, PRE versus POST, #: $p < 0.05$ versus BAS).

When considering LOW-OTT and HIGH-OTT subgroups, we observed that already at PRE three subjects terminated the OT before LBNP was activated (OTT: 7.9 min, 22 min and 24.3 min, respectively), thus showing earlier pre-syncopal symptoms even before HDBR. To avoid confusing effects, these subjects were excluded from the following comparison between subgroups. Accordingly, the LOW-OTT_{SHORT} subgroup was composed of 12 subjects, whereas the HIGH-OTT_{SHORT} subgroup included the remaining seven subjects.

When comparing these two subgroups at POST (figure 4(B), bottom) during TILT, higher TWA and TWAn, though not significant ($p = 0.056$ and $p = 0.1$, respectively), were observed in LOW-OTT_{SHORT}, suggesting that a lower orthostatic tolerance to HUT could be related to an increased electrical instability measured in terms of higher TWA. Interestingly, in both PRE and POST at REC, TWAn was significantly higher in LOW-OTT_{SHORT} compared to HIGH-OTT_{SHORT}.

As regards HRV spectral analysis, before HDBR (figure 4(A), top), in LOW-OTT_{SHORT}, both P_{LF} (0.68 (0.62; 0.79) versus 0.91 (0.85; 0.94) a.u.) and P_{LF}/P_{HF} (2.13 (1.68; 4.11) versus 10.97 (5.58; 15.92) a.u.) significantly ($p = 0.002$) increased at TILT compared to BAS. This phenomenon was not observed in HIGH-OTT_{SHORT}, thus suggesting that sympathetic drive during orthostatic stimulation increased particularly in the LOW-OTT_{SHORT} subgroup. Conversely, after HDBR, the increase in sympathetic response was visible in both subgroups (figure 4(A), bottom).

3.1.2. Results of 21 d duration HDBR (MID). As in SHORT, a significant increase in HR was found at BAS and at TILT when comparing PRE versus POST, accompanied by a reduction in TWA in BAS, TILT and REC (figure 3, bottom). However, when considering TWAn, this reduction maintained its significance only at REC.

Table 1. HRV parameters expressed as median (25th–75th percentiles) computed at PRE and POST after 5 (SHORT-HDBR) and 21 d (MID-HDBR) of HDBR for each tilt phase, BASE, TILT and REC.

		PRE-HDBR			POST-HDBR		
		BAS	TILT	REC	BAS	TILT	REC
SHORT-HDBR	P_{LF} (ms^{-2})	1146 (778; 1321)	1760[b] (1159; 2553)	1229 (769; 2867)	847[a] (568; 1125)	792[a] (566; 1652)	1532[b] (891; 1846)
	P_{HF} (ms^{-2})	456 (258; 728)	269[b] (143;422)	555[b] (269; 1632)	259[a] (180; 342)	126[a,b] (59; 213)	360[b] (199; 560)
	P_{T} (ms^{-2})	1864 (1178; 2542)	2610[b] (1713; 3536)	2443[b] (1295; 5234)	1323 (872; 1674)	1869 (732; 2599)	2472[b] (1355; 3024)
	P_{LFn}	0.70 (0.62; 0.81)	0.85[b] (0.80; 0.93)	0.71 (0.60; 0.80)	0.76[a] (0.68; 0.81)	0.87[b] (0.84; 0.93)	0.78[a] (0.69; 0.84)
	P_{LF}/P_{HF}	2.38 (1.61; 4.27)	5.62[b] (4.08; 14.27)	2.43 (1.48; 4.08)	3.17[a] (2.12; 4.30)	6.84[b] (5.29; 13.30)	3.58[a] (2.23; 5.39)
MID-HDBR	P_{LF} (ms^{-2})	990 (579; 1846)	1625[b] (1003; 3000)	1664 (971; 2247)	817 (460; 1399)	633[a] (412; 1656)	1142 (654; 2628)
	P_{HF} (ms^{-2})	390 (230; 588)	240[b] (146; 315)	511[b] (310; 812)	237 (153; 366)	84[a,b] (31; 216)	213 (110; 489)
	P_{T} (ms^{-2})	1651 (1027; 2592)	2387[b] (1376; 3609)	2782[b] (1884; 3564)	1629 (813; 2482)	1121[a] (629; 2148)	1272 (1011; 3637)
	P_{LFn}	0.73 (0.67; 0.82)	0.87[b] (0.84; 0.92)	0.78 (0.70; 0.82)	0.79 (0.67; 0.85)	0.90[b] (0.84; 0.94)	0.82[a] (0.74; 0.88)
	P_{LF}/P_{HF}	2.68 (2.04; 4.58)	6.86[b] (5.21; 12.25)	3.60 (2.35; 4.54)	3.69 (2.04; 5.96)	9.16[b] (5.43; 16.48)	4.67[a] (2.84; 7.09)

[a] $p < 0.05$, PRE versus POST,
[b] $p < 0.05$ versus BAS.

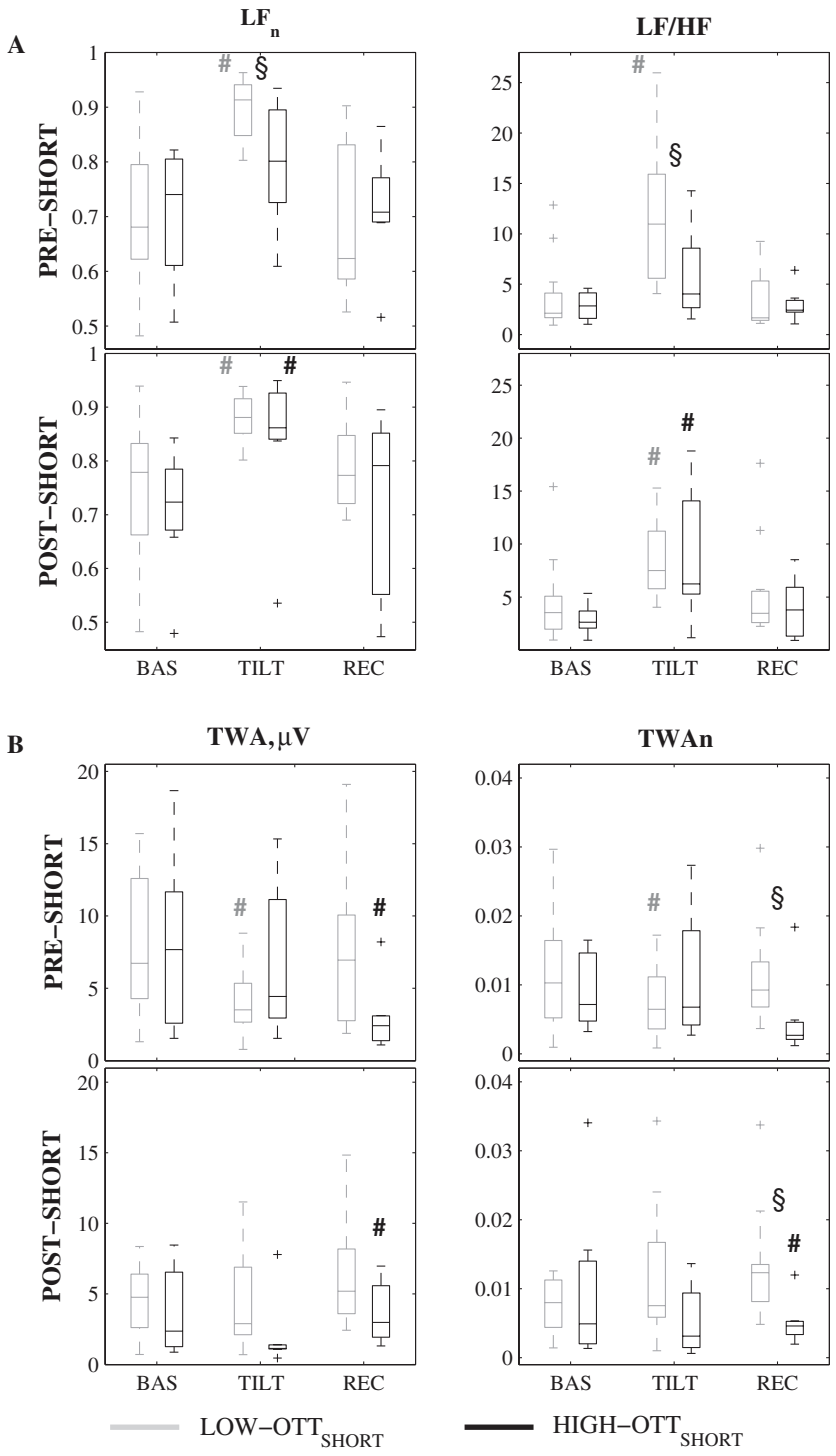

Figure 4. HRV (A) and TWA (B) indices computed for LOW-OTT_{SHORT} (gray) and HIGH-OTT_{SHORT} (black) groups, before and after the SHORT-HDBR (top and bottom panels, respectively). #: $p < 0.05$, versus BAS. §: $p < 0.05$, LOW-OTT_{SHORT} versus HIGH-OTT_{SHORT}.

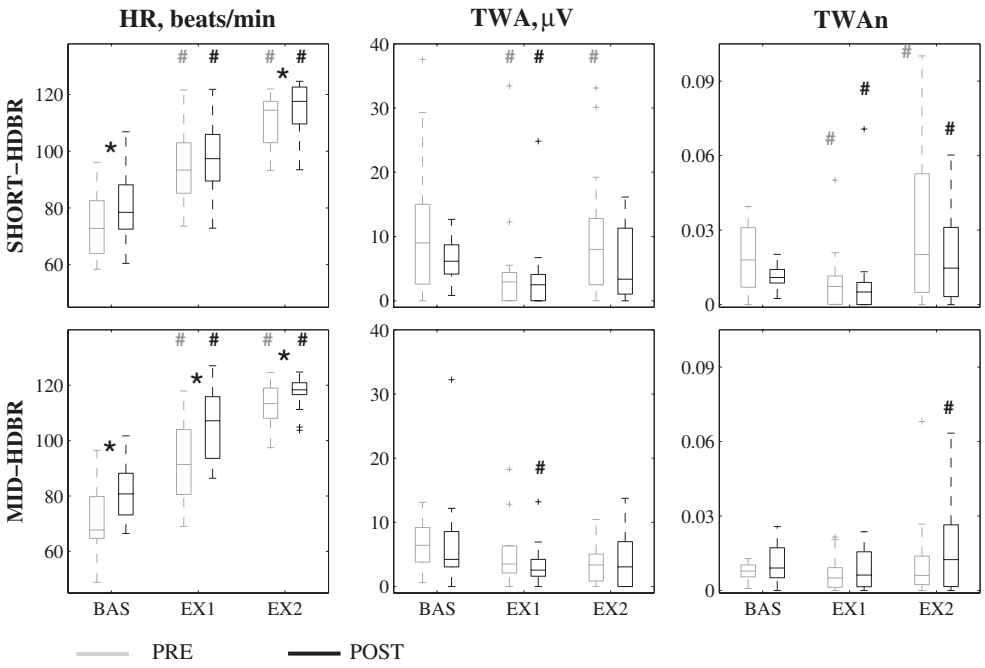

Figure 5. HR and TWA amplitude distribution at each AP test step (BAS, EX1, EX2), computed before (gray) and after (black) 5 d (top panels) and after 21 d (bottom panels) of HDBR. *: $p < 0.05$, PRE versus POST. #: $p < 0.05$, versus previous step.

As regards HRV spectral analysis, P_{LF} and P_{LF}/P_{HF} increased after HDBR at REC, evidencing higher sympathetic drive at POST during this phase (see table 1).

When considering LOW-OTT and HIGH-OTT subgroups, only one subject at PRE terminated the OT test before LBNP was activated (OTT: 12.1 min); consequently, it was excluded for the following subgroups comparison. Accordingly, the LOW-OTT_{MID} subgroup was composed by 11 subjects, whereas the HIGH-OTT_{MID} subgroup included the remaining eight subjects. From this comparison, neither TWA nor TWAn appeared to be different both at PRE and at POST. In both subgroups, P_{LF} and P_{LF}/P_{HF} increased during TILT when compared to BAS, suggesting a higher sympathetic ANS response with tilt both before and after HDBR.

3.2. Peak aerobic power test

HDBR-induced significant changes on aerobic power after both 5 and 21 d. In particular, HR_{peak} was increased from 179 (171; 186) beats min^{-1} to 184 (175; 187) beats min^{-1} in SHORT, and from 183 (179; 189) beats min^{-1} to 190 (180; 195) beats min^{-1} in MID, while $\dot{V}O_{2peak}$ was diminished from 3187 (2739; 3626) ml min^{-1} to 3115 (2766; 3473) ml min^{-1} in SHORT, and from 3355 (2507; 3787) ml min^{-1} to 2564 (2234; 3250) ml min^{-1} in MID.

3.2.1. Short duration HDBR. Results of HR and TWA analysis are presented in figure 5, upper panels. As expected, both at PRE and at POST, HR increased progressively during the AP test (EX1 versus BASE, EX2 versus EX1, $p < 0.001$). When compared to PRE values, HDBR induced an increase in HR, which was significant in BAS and EX2, though not in EX1. As

regards TWA indices, both TWA and TWAn showed a similar trend during the AP test, with a reduction in EX1 compared to BAS and subsequent slight increase in EX2. No differences between PRE and POST were evidenced.

Also when subgrouping subjects as HIGH-OTT$_{SHORT}$ and LOW-OTT$_{SHORT}$ based on the previous OT test, no differences between subgroups were visible in TWA and TWAn.

3.2.2. Mid-duration HDBR. Results of HR and TWA analysis are presented in figure 5, bottom panels. As in SHORT, both at PRE and at POST, HR increased progressively during the AP test (EX1 versus BASE, EX2 versus EX1, $p < 0.001$). Compared to PRE, HR was significantly higher at POST in each stage of the test. The analysis of TWA and TWAn, both as a whole or considering subgroups, did not show any difference induced by the test or by HDBR (figure 5, bottom).

4. Discussion and conclusions

Both 5 d and 21 d sedentary HDBR induced a reduction in orthostatic tolerance, as well as a decrease in maximal oxygen uptake and reserve capacity to perform physical work, thus suggesting cardiovascular deconditioning.

When considering the repolarization phenomenon, despite previous observations (Caiani *et al* 2013), the absence of any significant increase in normalized TWA indices between PRE and POST HDBR during OT and AP tests suggests that neither 5 d nor 21 d exposure to simulated microgravity were able to alter ventricular repolarization heterogeneity under stress conditions enough to the point of increasing TWA amplitude.

Our results are not concordant with the only study assessing TWA PRE and POST HDBR during incremental dynamic leg exercise on a cycle ergometer, where the number of subjects resulting positive at the TWA test, with an onset HR higher than 110 bpm, was found to have increased after 9–16 d of HDBR (Grenon *et al* 2005). In that study, four out of 24 subjects (17%) were classified as TWA$^+$ already at PRE, becoming 10/24 (41.7%) at POST. However, it has to be remarked that two TWA$^+$ subjects at PRE exhibited TWA$^-$ at POST, without a clear explanation.

In our study, we evaluated the potential effects of SHORT and MID HDBR duration on TWA using a different computational method, potentially more robust than spectral based methods (Monasterio *et al* 2009, Orini *et al* 2014). In addition, we introduced a normalization in TWA computation, to evaluate if changes in T-wave amplitude, known to be elicited by HDBR, could impact on TWA measurement. Those changes in T-wave have been related to the loss of fluids and hypovolemia, resulting in diminished plasma volume and shrinking of heart cavities (Caiani *et al* 2014a). Our findings from AP test showed the expected increase in HR during the considered exercise phases (i.e. BAS, EX1, EX2) with higher values at POST compared to PRE in agreement with the observed decrease in maximal oxygen uptake and reserve capacity. No HDBR-related increment in TWA was noticed, neither in absolute nor in normalized values, for both SHORT and MID.

A possible cause of discrepancy with Grenon *et al* could be due to the fact that AP test was performed in our study within 26 h after the end of HDBR, while in (Grenon *et al* 2005) it was performed within 6 h after its conclusion. The fact that subjects had maintained in the upright position for a considerable period before the test could have mitigated some of the HDBR-induced effects.

In addition to evaluating TWA during AP testing, we performed the same analysis also during OT testing. To the best of our knowledge, this is the first study attempting to describe

the TWA phenomenon under this condition. We also performed spectral analysis of HRV during OT testing to elucidate potential autonomic nervous system imbalance in relation to TWA. We observed a trend of decrease in TWA with TILT compared to BAS, significant only for SHORT at PRE and for MID at POST, together with a statistically significant increase in sympathetic tone with respect to baseline values (BAS) during OT, for both SHORT and MID campaigns. In previous studies on Long QT syndrome patients, TWA has been shown to be provoked by emotional stress, suggesting that sympathetic stimulation may play an important role (Schwartz and Malliani 1975). However, this sympathetic activation did not influence TWA amplitude in patients with structural heart disease or ventricular arrhythmias (Kaufman *et al* 2000). Interestingly, when considering normalized amplitudes (TWAn) this decrease at TILT is less evident and even non-significant. This different behavior in TWA indices could be explained by the fact that sympathetic stimulation induces T-wave flattening with increasing tilt angles, as reported in (Baumert *et al* 2011), thus being considered with the normalization step. Nevertheless, the relationship between these phenomena remains still controversial and further research would be needed to better elucidate the potential stabilization of repolarization with increased sympathetic tone in healthy subjects.

As regards TWA, an apparent decrease between PRE and POST was visible for both SHORT and MID; however, when considering TWAn, this effect disappeared, thus probably being related to a decrease of the amplitude of the whole T wave at the surface ECG, supporting the hypothesis of no visible changes in TWA with HDBR also when computed during OT testing.

As regards spectral HRV analysis, the main effect of a 5 d HDBR at BAS (i.e. PRE versus POST) was a decrease in absolute power for both LF and HF bands, together with an increase in P_{LF} and P_{LF}/P_{HF}. Conversely, for 21 d HDBR these changes were visible but not significant.

In previous studies, a decrease in absolute P_{LF} and P_{HF} power, but no changes in P_{LF} and P_{LF}/P_{HF} was observed after 30 d of HDBR during controlled breathing (0.25 Hz) (Sakowski *et al* 2011). Also, after 14 d HDBR, a decrease in P_{HF} power with an increase in P_{LF}/P_{HF} were found (Iwasaki *et al* 2005).

At POST, TILT increased BAS levels of P_{LF} and P_{LF}/P_{HF} and decreased P_{HF} for both SHORT and MID in the sedentary control group after 21 d HDBR, similar to what was previously reported (Stenger *et al* 2012).

Interestingly, when we subdivided subjects according to their OTT at POST, higher TWA values during REC were observed in subjects with OTT < 30 min (LOW-OTT_{SHORT}) after 5 d of HDBR, suggesting higher electrical instability associated to a more important orthostatic intolerance due to cardiac deconditioning. In addition, these subjects presented a significant increase with TILT in both P_{LF} and P_{LF}/P_{HF} already at PRE campaign, while in the HIGH-OTT_{SHORT} group this effect was not visible. Moreover, the increase in sympathetic tone observed during TILT was accompanied by a reduced TWAn, in agreement with previous studies on patients with syncopal and postural orthostatic tachycardia syndrome, in which the reaction of sympathetic tone to orthostasis was more severe compared to control subjects (Duplyakov *et al* 2011). However, we did not find such differences after 21 d of HDBR. This discrepancy could also be due to the different protocol, since LBNP was applied after only 15 min, instead of 30 min as in SHORT.

The observed relationship between higher TWA during REC and lower OTT is a novel finding of this study, hence additional research would be needed to better elucidate the involved mechanisms and confirm these results.

Also, longer HDBR (60 d) could give a better insight on the effective presence or not of changes in TWA related to higher ventricular repolarization's heterogeneity, as a 21 d period did not increase TWA parameters measured by surface ECG. Also, further testing of

continuous 24 h ECG recordings would help in the assessment of arrhythmogenic risk related to simulated microgravity exposure.

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