European Journal of Applied Physiology

Analysis of changes in cardiac circadian rhythms of RR and QT induced by a 60-day Head-Down Bed Rest with and without nutritional countermeasure

--Manuscript Draft--

Manuscript Number:	EJAP-D-19-00984R2
Full Title:	Analysis of changes in cardiac circadian rhythms of RR and QT induced by a 60-day Head-Down Bed Rest with and without nutritional countermeasure
Article Type:	Original Article
Keywords:	Head-Down Bed Rest; heart rate; ventricular repolarization; circadian rhythm; nutrition; cardiac deconditioning
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Funding Information:	Agenzia Spaziale Italiana (2018-7-U.0) — PhD Enrico Gianluca Caiani
Abstract:	Purpose Prolonged weightlessness exposure generates cardiovascular deconditioning, with potential implications on ECG circadian rhythms. Head-down (-6°) tilt (HDT) bed rest is a ground-based analogue model for simulating the effects of reduced motor activity and fluids redistribution occurring during spaceflight. Our aim was to evaluate the impact of 60-day HDT on the circadianity of RR and ventricular repolarization (QTend) intervals extracted from 24h Holter ECG recordings, scheduled 9 days before HDT (BDC-9), the 5th(HDT5), 21st(HDT21) and 58th(HDT58) day of HDT, the first (R+0) and 8th(R+7) day after HDT. Also, the effectiveness of a nutritional countermeasure (CM) in mitigating the HDT-related changes was tested. Methods RR and QTend circadian rhythms were evaluated by Cosinor analysis, resulting in maximum and minimum values, MESOR (a rhythm-adjusted mean), oscillation amplitude (OA, half variation within a night-day cycle), and acrophase (, the time at which the fitting sinusoid's amplitude is maximal) values. Results RR and QTend MESOR increased at HDT5, and the OA was reduced along the HDT period, mainly due to the increase of the minima. At R+0, QTend OA increased, particularly in the control group. The slightly anticipated during HDT, and was delayed at R+0. Conclusion 60-day HDT affects the characteristics of cardiac circadian rhythm by altering the physiological daily cycle of RR and QTend intervals. Scheduled day-night cycle and feeding time were maintained during the experiment, thus inferring the role

	of changes in the gravitational stimulus to determine these variations. The applied nutritional countermeasure did not show effectiveness in preventing such changes.
Response to Reviewers:	

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Editors-in-Chief European Journal of Applied Physiology

Milan, 19th May 2020

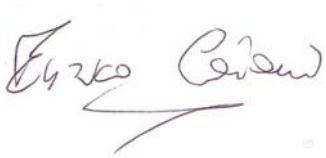
Dear Proff. Westerblad and Westerterp,

Thank you again for the opportunity to further revise and improve our manuscript "*Analysis of changes in cardiac circadian rhythms of RR and QT induced by a 60-day Head-Down Bed Rest with and without nutritional countermeasure*", submitted as Original Paper to European Journal of Applied Physiology.

In the revised version, all comments raised by Reviewer 1 were taken into account, including a revision of the English style from a native speaker. Resulting changes have been highlighted in red in respect to the previous version of the manuscript.

We sincerely hope that this revisied version could be considered suitable for possible publication on your prestigious journal.

Yours sincerely,



Prof. Enrico G Caiani
on behalf of all co-authors.

We would like to thank the Reviewers for their constructive feedback on our manuscript.
We have corrected it accordingly in the revised manuscript, with all changes marked in red.

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Reviewer #1
The manuscript has been significantly improved, and the novel has been highlighted. However, there are still some places need to be revised

We thank the Reviewer for his/her appreciation of our work.

1. Line 5: adapt ...to instead of adapt for.
2. Line 16: "and recent reports described that circadian rhythms irregularities", it may be better to be changed to be "and recent reports demonstrate that circadian rhythms irregularities"
3. Line 22: Unusual expression: keeps us entrained
4. Line 30: " considered as important risk factor " will be better if it is changed to be " considered as an important risk factor "
5. Line 32-33: "The function of various systems, including the autonomic nervous system that regulates heart rate (HR), displays circadian rhythmicity ", may be better if rewritten this way "The functions of various systems are under circadian control, including the autonomic nervous system that regulates heart rate (HR)"
6. Line 40: "Thus, it becomes clear the importance to examine how microgravity could impact cardiac circadian rhythms", this expression is not straightforward, instead it's kind of twist. I suggest it should be revise, for instance, "Thus, it is import to examine the impacts of microgravity on cardiac circadian rhythms"
7. Anyway, there are many such places with unprecise expressions. I suggest the authors get help from a person using native English or from the language editing service.

Thank you for your suggestions. We modified the sentences relevant to comments 1-7 as suggested. In addition, the manuscript underwent review from a native English/American speaking person.

8. Fig 3. If the width of the two panels is narrowed, the rhythmic fluctuation might be more obvious. In this way, the space will be saved and Fig.3 and Fig.4 may be combined.
9. Fig. 6 and Fig. 7 could be combined into four panels in one figure.
11. In the figures, if there are more than one panel, please label them with alphabets, like A, B, C, etc.

Based on these comments, we combined Figure 3 and Figure 4 into one figure with four panels, labeling the panels with letters A, B, C and D, and so we did for Figure 6 and Figure 7. Relevant text in the Results section was modified accordingly.

10. Try to get rid of starting each paragraph by "In Figure 6, the pooled results for OA are reported" or similar expression. Alternative elucidation of the data are recommened to be used.

According to the Reviewer suggestion, we reviewed the Results and modified accordingly the text when needed, by using alternative forms.

12. Discussion:

1) The first and second paragraph: The first one is a single sentence paragraph, I suggest the authors to these two paragraphs. In the meantime, the second paragraph needs to be revised since currently it reads like a laundry list. The authors need to summarize the results here instead of putting everything equally in array here.
3) Also in discussion: There are many sections with only one paragraph, try to avoid this just like to avoid one sentence paragraph, though it is not necessary to have more than one paragraph in every section.

We thank the Reviewer for these observations that led us to modify the Discussion as suggested.

2) About the section "Effects of the nutritional countermeasure" in discussion: After the last revision, this paragraph provides clear background why the authors proposed the experiment with nutritional countermeasures. The idea is novel despite the negative result. Nonetheless it will be better to shorten this section, just remain the basic information helping readers get the basic background and how the authors proposed the idea.

We are glad that now this was clearer. As requested, we shortened this section as suggested, by keeping the information that we believe could be useful to propose an interpretation to our results.

Reviewer #2

I am satisfied that the authors have adequately addressed my concerns and that the changes made have enhanced the clarity of the manuscript.

We thank the Reviewer for his/her appreciation of our work.

Analysis of changes in cardiac circadian rhythms of RR and QT induced by a 60-day Head-Down Bed Rest with and without nutritional countermeasure

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Abstract

Purpose Prolonged weightlessness exposure generates cardiovascular deconditioning, with potential implications on ECG circadian rhythms. Head-down (-6°) tilt (HDT) bed rest is a ground-based analogue model for simulating the effects of reduced motor activity and fluids redistribution occurring during spaceflight. Our aim was to evaluate the impact of 60-day HDT on the circadianity of RR and ventricular repolarization (QTend) intervals extracted from 24h Holter ECG recordings, scheduled 9 days before HDT (BDC-9), the 5th(HDT5), 21st(HDT21) and 58th(HDT58) day of HDT, the first (R+0) and 8th(R+7) day after HDT. Also, the effectiveness of a nutritional countermeasure (CM) in mitigating the HDT-related changes was tested.

Methods RR and QTend circadian rhythms were evaluated by Cosinor analysis, resulting in maximum and minimum values, MESOR (a rhythm-adjusted mean), oscillation amplitude (OA, half variation within a night-day cycle), and acrophase (φ, the time at which the fitting sinusoid's amplitude is maximal) values.

Results RR and QTend MESOR increased at HDT5, and the OA was reduced along the HDT period, mainly due to the increase of the minima. At R+0, QTend OA increased, particularly in the control group. The φ slightly anticipated during HDT, and was delayed at R+0.

Conclusion 60-day HDT affects the characteristics of cardiac circadian rhythm by altering the physiological daily cycle of RR and QTend intervals. Scheduled day-night cycle and feeding time were maintained during the experiment, thus inferring the role of changes in the gravitational stimulus to determine these variations. The applied nutritional countermeasure did not show effectiveness in preventing such changes.

Keywords Head-Down Bed Rest · heart rate · ventricular repolarization · circadian rhythm · nutrition · cardiac deconditioning

Aknowledgments
This work was supported by the Italian Space Agency (contract 2018-7-U.0, recipient E G Caiani).

Abbreviations

BDC	Baseline data collection
CM	Countermeasure
CTRL	Control
ECG	Electrocardiogram
HDT	Head-Down Tilt
HR	Heart Rate
OA	Oscillation Amplitude
RR	Time between two successive R-waves

QTend Time elapsed between the Q wave and the end of the T wave

Introduction

Life on Earth has been adapted to the rotation of our planet. Living organisms, including humans, have an inner biological clock that helps them anticipate and adapt their physiology to the fluctuations in the day. This regular adaptation is referred to as the circadian rhythm, originating from the Latin words *circa*, meaning "around", and *dies*, meaning "day". The inner circadian clock, a hierarchically organised network of structures responsible for generating ~24 h or daily rhythms, is driven in mammals by a circadian pacemaker (or master clock) located in the suprachiasmatic nuclei of the hypothalamus (Bonmati-Carrion et al. 2017). This inner clock regulates critical functions such as behavior, hormone levels, sleep, body temperature and metabolism, having the light/dark cycle as a main input (Erren et al. 2009). Loss of entrainment (i.e., the process whereby the circadian clock actively synchronises to cyclic environmental signals), such as in shift work or chronic jet lag, has been associated with increased risk for a number of negative health outcomes (Buijs et al. 2016). Moreover, circadian misalignment (i.e., a suboptimal form of entrainment) resulting from daylight saving time has been shown to increase risk of heart attack (Kirchberger et al. 2015; Jiddou et al. 2013), similar to the increased risk for cardiovascular diseases reported for shift work and other circadian misalignment conditions (Ohlander et al. 2015). Recent reports demonstrated that circadian rhythms irregularities are also linked to various chronic health conditions, such as sleep disorders, obesity, diabetes, depression, bipolar disorder, seasonal affective disorder, and ageing (Logan and McClung 2019).

In the context of human physiology associated with prolonged microgravity exposure, such as during spaceflight, the human body undergoes several pathophysiological adaptations to the new environment, and thus the regulatory system should maintain its functionality. On Earth, the daily exposure to the light/dark cycle keep physiological processes synchronized around the 24-hour-long day, while astronauts, orbiting around Earth, experience a sunrise or sunset every 45 minutes. Together with light-dark cycle, gravity appears to act as a cue for the circadian timing system (Fuller et al. 1994; Liang et al 2012): the regular alternation between upright (1 Gz) and recumbent (0 Gz) positions within the 24-h impacts all aspects of human function. Eliminating the gravitational component of this cycle, spaceflight additionally challenges the human circadian rhythm (Liang et al. 2014; Watenpaugh 2016). Previous studies showed that astronauts suffer from misalignment of sleep and circadian rhythms in space (Santy et al. 1988; Gundel et al. 1997; Wu et al. 2018), a condition that could result in impairments of health, alertness, and performance during spaceflight (Flynn-evans et al. 2016), and it is therefore considered as an important risk factor during long-term spaceflight by the National Aeronautics and Space Administration.

The functions of various physiological systems are under circadian control, including the autonomic nervous system that regulates heart rate (HR) (Chan et al. 2012). Prolonged exposure to weightlessness associated with spaceflight is known to generate cardiovascular deconditioning, causing significant changes in both autonomic and cardiovascular systems (Convertino and Hoffler 1992), as well as possibly inducing cardiac rhythm disorders (Caiani et al. 2016) and increasing the ventricular repolarization heterogeneity, thus leading to increased risk of arrhythmia susceptibility when a gravity field is restored (Martín-Yebra et al. 2019). Studies on shift workers, exposed to rhythms disruption, showed a possible correlation between circadian misalignment and increased risk of developing hypertension, inflammation and cardiac disease (Morris et al. 2016; Manfredini et al. 2018).

For these reasons, it is important to examine the impact of microgravity on cardiac circadian rhythms, to evaluate how this could affect astronauts' health. To this aim, in simulated microgravity conditions, the evaluation of how the human circadian timing system changes its performance and phase of entrainment (i.e., the relationship between the internal clock and the external day time) could help in understanding how the systems' dynamics are modified in absence of gravity.

Head-down (-6°) tilt (HDT) bed rest is a ground-based space analogue model of chronic circulatory unloading which simulates the effects of reduced motor activity, the elimination of the regular alternation between 1 Gz and 0 Gz, and the fluids redistribution occurring during sustained exposure to microgravity; it also represents a valid platform for testing the effects of potential countermeasures. We hypothesized that HDT bed rest could be used to examine the changes in the human circadian timing system associated with prolonged exposure to microgravity through the analysis of cardiac electrical activity by ECG recordings.

Based on our recent results, showing an increase in ventricular repolarization instability in terms of T-wave alternans induced by long-term HDT bed rest (Martín-Yebra et al. 2019), we also hypothesized that the modifications in circadian rhythms induced by this condition could interfere with ventricular repolarization interval (QT) duration and its coupling with HR. Importantly, a first mechanistic link between endogenous circadian rhythms and cardiac electrical instability through changes in QT duration, most often associated with sudden cardiac death in humans, has been demonstrated in an animal model (Jeyaraj et al. 2012), thus suggesting the need to further evaluate this aspect also in humans. Accordingly, our main aim was to evaluate the impact of long duration (60-day) HDT bed rest on the circadianity of the rhythms extracted from 24-hour Holter ECG signals, relevant to the HR and, for the first time in this setting, to the QT duration.

A secondary aim was to assess the potential effectiveness of a novel nutritional countermeasure (CM), consisting of anti-oxidant and anti-inflammatory supplements including Omega-3, in preventing or mitigating the effects induced by

long term physical inactivity on RR and QT circadianity. Indeed, nutrition is a critical aspect of spaceflight, and it plays a fundamental role in maintaining crew's physiological and psychological health. Microgravity causes muscles to weaken and decrease in mass and volume (Rambaut et al. 1977; Gopalakrishnan et al. 2010), and induces bone and calcium loss (Smith and Heer 2002), requiring adequate nutritional and physical exercise protocols to counteract these undesired effects. Nutrition also plays a role in maintaining cardiovascular performance: Omega-3 fatty acid intake has been shown effective in beneficially impacting the cardiovascular health on Earth (Mozaffarian et al. 2006), although these effects during microgravity exposure have not been investigated.

Methods

Study population

In the context of the European Space Agency bed rest strategy, an only-male population composed by 20 volunteers in the 20-45 years age range (median (25^{th};75^{th} percentiles), 36 (28;41) years old), body mass index 23 (23;25) kg/m2, maximal oxygen uptake 39 (37;44) ml/min/kg, was enrolled at the Institut de Médecine et de Physiologie Spatiales - MEDES in Toulouse (France). Subjects had no history of cardiovascular disease and were not taking medications of any kind.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The whole bed rest experimental protocol, including sixteen scientific protocols running in parallel by several research teams, assessing changes in the cardiovascular, metabolism, muscle, bone, neuro sensorial, hematological and immunology systems, was approved (Clinical Trial.gov database under the number NCT03594799) by the Institutional Review Board of the "Comité de Protection des Personnes Sud Ouest et Outre Mer I" and by the French Drug Agency (Agence Française de Sécurité Sanitaire pour le Produits de Santé), as well as from the local ethical committee, to which each subject provided written, informed consent.

Study design

Subjects were enrolled in a two-group study design, and randomly assigned to a control (N=10, CTRL) group, undergoing sedentary HDT bed rest, or to a treatment (N=10, CM) group, receiving during HDT a daily nutritional countermeasure consisting in a cocktail of anti-oxidants and vitamins (daily, 741 mg of polyfenol, 168 mg of vitamin E, 80 µg of Selenium-Solgar®, and 2.1 g of Omega-3 – Omacor®). The study was conducted by two campaigns with 10 participants each. The first campaign started in January 2017, the second campaign in September 2017. Each campaign consisted of 15 days of baseline data collection (BDC-15 through BDC-1), 60 days of (HDT) bed rest (HDT1 through HDT60) and 15 days of recovery (R+0 through R+14), where R+0 is the day that started with the end of the HDT period coinciding with an orthostatic tolerance test. All subjects adhered to a monitored, strict 6° negative HDT 24h a day, whereas before and after this phase lying on bed during the day was not allowed. Subjects were exposed to natural light through a window in their room, and day and night cycle was fixed and imposed for the entire duration of the experiment: awakening time at 7:00 AM, and sleeping time at 11:00 PM each day. No napping was allowed during the day.

ECG data acquisition and pre-processing

For each subject, 12-lead, 24-hour Holter ECGs (1000 Hz, H12+, Mortara Instrument Inc.) were acquired at specific epochs, schematized in Figure 1: 9 days before HDT (BDC-9), the 5^{th} (HDT5), 21^{st} (HDT21) and 58^{th} (HDT58) day of HDT, and the first (R+0) and 8^{th} (R+7) day after HDT conclusion.

Fig. 1 Schematization of the phases of the bed rest campaign. Arrows indicate the scheduling of 24-hours Holter ECG collection

Using the research software SuperECG (Mortara Instrument Inc.), the fiducial points corresponding to the Q, R, and end of T wave were detected and used to compute beat-by-beat RR and QTend interval series (ms) from each recording. The extracted variability series were pre-processed in order to exclude outliers or artefacts due to acquisition problems (i.e., electrode detaching, cable interference, others). Then, each series was subdivided into consecutive non-

overlapping 15-minutes segments. For each segment, median and mean values of RR and QTend were computed: median values were used for the Cosinor analysis, while mean values were used for the day-night time series analysis.

Circadianity evaluation by Cosinor analysis

The Cosinor analysis is a widely used method in chronobiology: it is based on a model consisting of cosine curves with known periods fitted by least squares to the data, providing an estimate of the pattern of the rhythm (Refinetti et al. 2007). In this study, it is possible to assume that the period is known, being synchronized to the externally imposed 24-hour day-night cycle. The regression model for a single component can be defined according to the equation:

$$Y(t) = MESOR + OA * cos\left(\frac{2\pi}{\tau} + \varphi\right) + e(t)$$

where MESOR (Midline Statistic of Rhythm) represents a rhythm-adjusted mean, OA is the oscillation amplitude, measuring half variation within a night-day cycle, φ is the acrophase, that is the temporal value at which the amplitude of the fitting sinusoid reaches its maximum value, τ is the period representing the duration of one cycle, and e(t) is the fitting error term. In addition, for each computed RR and QTend series, the maximum and minimum values were computed, and prominent circadian rhythm (24-h cycle) was evaluated by single component Cosinor analysis, resulting in a value of MESOR, OA and acrophase for each subject at each epoch. Additionally, for each subject at each epoch, the difference between RR and QTend acrophases (Δ_φ) was computed as follows:

$$\Delta_\varphi = \varphi_{QTend} - \varphi_{RR}$$

Statistical analysis

Statistical analysis was applied on Cosinor Analysis' parameters, separately in CTRL and CM groups, in order to: 1) test the effects of HDT at each epoch versus BDC-9 (non-parametric Wilcoxon signed rank test, $p<0.05$); 2) to assess post-HDT recovery compared to BDC-9 values (Wilcoxon signed rank test, $p<0.05$). The same analyses were performed on the two groups combined. Additionally, the non-parametric Mann-Whitney test ($p<0.05$) was used to compare CTRL and CM groups at each epoch.

To verify possible changes in QT-RR relation expressed as their Δ_φ, Wilcoxon signed rank test was applied ($p<0.05$, at each epoch vs BDC-9).

To evaluate changes due to HDT in mean RR and QTend, separately for day and night periods, Wilcoxon signed rank test ($p<0.05$ vs BDC-9) for CTRL and CM groups was performed. Moreover, the presence of a day-night difference in RR and QTend mean values was assessed within each epoch by Wilcoxon test. In addition, the Zero-Amplitude Test ($p<0.05$) was performed together with the Cosinor Analysis, to confirm the presence of circadian rhythmicity.

Results

For technical reasons during acquisition, the recording of one subject of the CM group at R+7 was missing. Accordingly, data from this subject were removed from the analyses only in paired comparison involving R+7 epoch.

An example of the Cosinor analysis applied to the RR variability series obtained along the bed rest in one representative subject is shown in Figure 2, in which median values of RR time series at BDC9, HDT5 and R+0 are represented together with the cosine fitting curve derived from the Cosinor model, and relevant values of MESOR, OA and φ are highlighted. This example illustrates the MESOR increase, the reduction in OA and the slight φ anticipation at HDT5, as well as the MESOR increase and φ backward-shift at R+0. These observations were confirmed for both CM and CTRL groups, as summarized in Table 1. At the beginning of HDT, RR and QTend MESOR values increased, and gradually recovered to baseline values towards the end of HDT. The minimum values of RR and QTend as well as the maximum of RR, increased at HDT5, and remained higher than BDC-9 also at HDT21 and HDT58, showing only a slight trend towards baseline. As a result, the OA of RR and QTend was reduced during HDT, reaching the minimum at HDT5 for CTRL, and at HDT21 for CM group.

After HDT conclusion, the opposite changes were elicited: at R+0, RR and QTend MESOR decreased to values lower than BDC-9, with a slight trend of recovery after 8 days. This alteration was confirmed by a simultaneous decrease in both maximum and minimum values, which allowed the OA to restore to baseline values for RR, both in CTRL and CM, and for QTend in CM, but not for QTend in CTRL group at R+0 which showed a larger OA. During HDT an anticipation of the acrophase compared to BDC-9 was visible, though not significant, while it was postponed at R+0, with an opposite trend of anticipation at R+7.

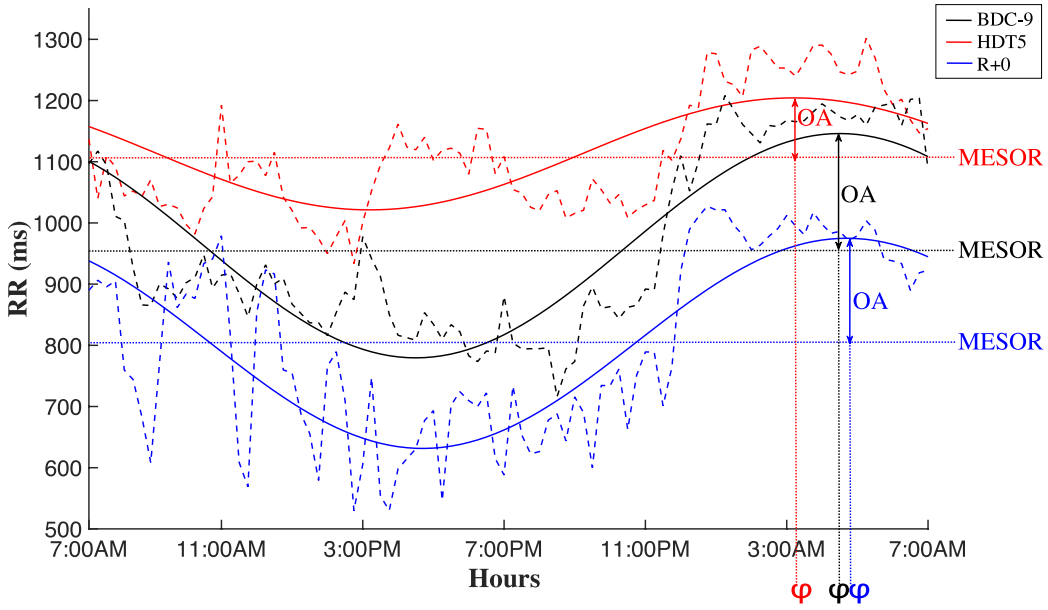

Fig. 2 Example of the Cosinor Analysis applied to RR variability series in one subject of the CM group, before HDT (in black), at HDT5 (in red), and at R+0 (in blue). Dashed lines represent the median RR computed each 15 minutes, while the solid lines are the cosine fitting curves. MESOR, OA and φ of the two series are reported. In this example, at BDC-9 the MESOR is 963 ms, OA is 183 ms, and acrophase is at 4:23AM. At HDT5, MESOR is 1113 ms, OA is 92 ms and acrophase is at 3:14AM. At R+0, MESOR is 803 ms, OA is 171 ms and φ is at 4:42AM

Table 1. Results of Cosinor analysis expressed as median [25th;75th percentiles] of the MAX, MIN, MESOR, OA and φ for the control (CTRL) and countermeasure (CM) groups

			BDC-9	HDT5	HDT21	HDT58	R+0	R+7
MAX (ms)	**RR**	**CTRL**	1189 [1063;1268]	1263 [1189;1388]#	1223 [1118;1299]	1146 [1052;1221]	985 [905;1020]#	1057 [1001;1133]#
		CM	1157 [1038;1304]	1238 [1169;1403]#	1178 [1121;1241]	1066 [1041;1189]	904 [846;1018]#	1045 [940;1119]
	QT_{end}	**CTRL**	430[423;444]	434[415;446]	422[418;443]	416[399;441]	404[382;422]#	417[397;425]
		CM	431[426;445]	428[420;452]	417[413;444]	426[407;442]	401[376;415]#	419[413;428]
MIN (ms)	**RR**	**CTRL**	623[582;640]	799[742;844]#	764[709;820]#	635[602;701]	427[372;447]#	474[453;501]#
		CM	587[535;652]	824[779;913]#	734[706;840]#	749[674;767]#	448[402;480]#	520[465;567]
	QT_{end}	**CTRL**	339[327;351]	367[351;384]#	361[355;374]#	351[339;363]#	298[279;301]#	307[293;315]#
		CM	353[334;359]	379[377;386]#	367[360;375]#	367[355;373]#	303[294;326]#	326[321;348]*
MESOR (ms)	**RR**	**CTRL**	885 [834;922]	1039 [976;1119]#	986 [900;1066]#	883 [863;936]	696 [688;713]#	805 [768;838]#
		CM	893 [831;959]	1042 [1003;1125]#	969 [879;988]	874 [860;981]	652 [647;766]#	829 [760;859]
	QT_{end}	**CTRL**	386 [376;392]	399 [382;412]#	390 [383;405]	381 [368;395]	351 [332;359]#	365 [356;380]
		CM	395 [388;400]	402 [396;417]#	391 [386;403]	390 [382;403]	352 [337;374]#	384 [376;391]
OA (ms)	**RR**	**CTRL**	174 [152;203]	110[104;140]#	124[109;137]#	117 [99;160]#	173 [138;214]	155 [134;173]
		CM	158 [134;180]	117 [85;172]	89 [69;115]#*	98 [73;129#	151 [122;169]	146 [122;170]
	QT_{end}	**CTRL**	28 [26;32]	17 [16;20]#	19 [18;22]#	19 [16;22]#	38 [30;43]#	31 [23;33]
		CM	26 [22;30]	18 [13;20]#	15 [10;19]#	13 [12;20]#	26 [23;32]*	25 [22;37]
φ (hh:mm)	**RR**	**CTRL**	4:00AM [3:30;4:12]	3:19AM [2:42;3:30]	3:44AM [3:12;4:00]	3:20AM [2:42;3:30]	4:19AM [4:06;4:42]#	3:44AM [3:24;4:06]
		CM	3:00AM [3:42;4:16]	2:24AM [2:06;3:00]	3:14AM [2:30;4:36]	2:37AM [2:18;3:30]	4:29AM [3:30;4:42]	3:38AM [3:30;4:06]
	QT_{end}	**CTRL**	4:12AM [3:42;4:30]	3:07AM [2:48;3:24]	3:45AM [3:18;4:12]	3:29AM [3:12;3:30]	4:36AM [4:12;4:54]#	3:48AM [3:18;4:42]
		CM	3:26AM [3:48;4:36]	2:22AM [1:30;3:00]	2:30AM [00:48;3:08]*	2:56AM [2:18;3:36]	4:47AM [4:06;5:18]	4:00AM [3:36;4:06]

*#: p<.05 vs BDC-9, Wilcoxon Signed Rank. *: p<.05 CTRL vs CM, Mann-Whitney*

Both RR and QTend time series exhibited circadian rhythms, maintained at all epochs, as visible in Figure 3, in which for CTRL (panels A and C) and CM groups (panels B and D) the RR (top panels) and QTend (bottom panels) mean values and standard deviation computed every 15 minutes are reported: these graphs confirmed the previously observed changes in the computed parameters, with the same trend of variation in both groups during the experiment. Indeed, major changes were visible in correspondence to HDT5, with an increase in QTend and, particularly, RR mean values, and even more marked at R+0, when the decrease of both RR and QTend mean was particularly visible during the day. The mean ± standard deviation for RR and QTend computed separately for the day and night periods are reported in Table 2: in both groups, a decrease in day/night difference, reflecting the reduction in OA, was noticeable during HDT, compared to BCD-9. In particular, RR and QTend intervals increased at HDT5, both during day and night, with a tendency to return to baseline values towards the HDT conclusion. At R+0, RR dramatically decreased and, simultaneously, QTend decreased, while at R+7 a slight tendency to recover towards pre HDT values was visible.

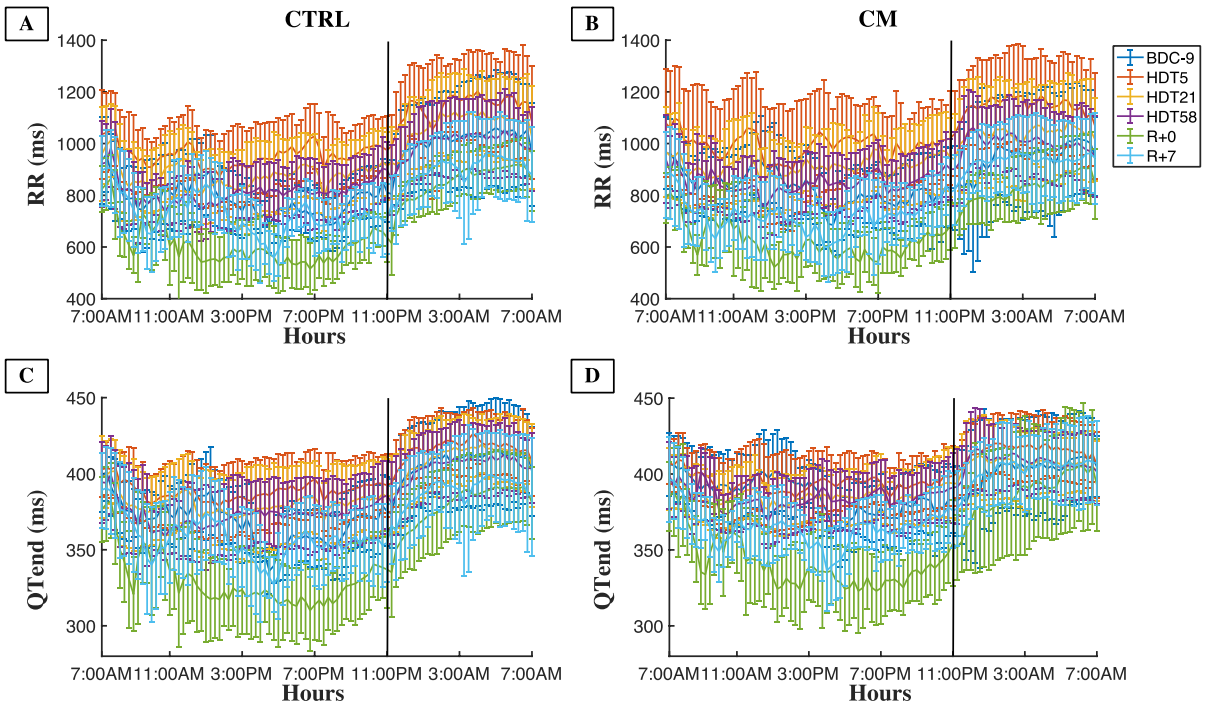

Fig. 3 Mean and standard deviation values of RR (top panels) and QTend (bottom panels) computed every 15 minutes at different recording epochs, for both CTRL (panels A, C) and CM (panels B,D) groups. The x-axis represents the 24h hours of Holter recording, aligned at 7:00AM. The vertical black line represents the beginning of the night period (h 11:00PM).

Table 2 Mean±std values of RR and QTend for the control (CTRL) and countermeasure (CM) groups during the day and night period and night-day difference

			BDC-9	HDT5	HDT21	HDT58	R+0	R+7
RR (ms)	**CTRL**	**Day**	774±68	968±85#	893±110#	819±95#	600±69#	718±82#
		Night	1089±171	1151±117#	1123±129#	1075±119#	903±71#	893±120#
		Night-day	306±128*	191±75*	196±65*	186±71*	251±72*	227±141*
	CM	**Day**	847±74	1038±188#	933±129#	837±93#	586±70#	758±73#
		Night	1115±216	1158±145#	1013±181	1001±116#	829±157#	988±123#
		Night-day	272±214*	186±131*	108±94*	164±79*	211±124*	248±90*
QTend (ms)	**CTRL**	**Day**	373±18	382±23#	378±26#	375±24#	335±24#	354±25#
		Night	417±32	425±21#	414±22#	410±23#	388±20#	392±29#
		Night-day	45±19*	29±7*	33±8*	32±8*	55±14*	42±25*
	CM	**Day**	386±19	396±20#	384±15	379±18	334±39#	373±16#
		Night	420±31	418±20#	398±21#	401±21#	375±47#	407±26#
		Night-day	38±34*	25±16*	15±12*	21±10*	37±23*	40±15*

*#: p<.05 vs BDC-9, Wilcoxon Signed Rank. *: p<.05 day vs night, Wilcoxon Signed Rank*

As the effects of CM were negligible on the observed variables, results of CTRL and CM groups were then pooled together to verify the persistence of the observed variations while increasing sample size. Indeed, the changes previously observed separately in the two groups were also reflected in the entire population (Figure 4). The RR and QTend MESOR values increased at HDT5, then recovered at HDT58 for RR and already at HDT21 for QTend, while the minimum values resulted increased during the entire HDT, with a peak at HDT5. The maximum values, after an initial growth in maximum RR at HDT5, showed an inverse trend of decrease up to HDT58, when both RR and QTend maxima resulted lower than at BDC. At R+0, an abrupt reduction in both MESOR, maximum and minimum values below the baseline was visible, with only a partial recovery at R+7.

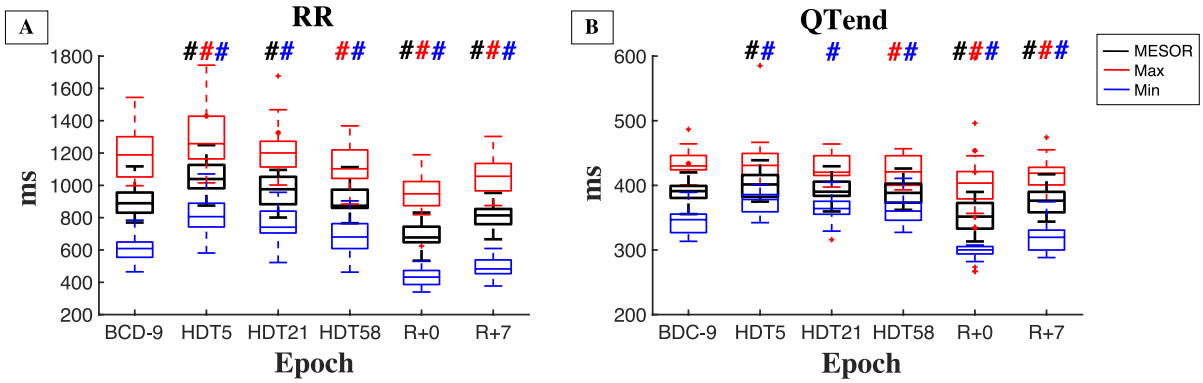

Fig. 4 Box-and-whisker plot of MESOR (black), maximum (red) and minimum (blue) values of RR (Panel A) and QTend (Panel B) variability series of the entire population. #: p<.05 vs BDC-9 (Wilcoxon Signed Rank)

The flattening of the RR and QTend day/night oscillation amplitude observed in both groups is well reflected in the pooled results displayed in Figure 5: at R+0 following HDT conclusion, the OA of RR (panel A) immediately restored to baseline values, while that of QTend (panel B) resulted increased compared to BDC-9, and then recovered at R+7. Also, shifts in acrophases were visible (Fig. 5, panel C and D): a trend of anticipation compared to BDC-9 during the HDT, particularly evident at HDT5 in both φ_{RR} (2:52[2:18;3:25] vs 3:54[2:21;4:18]) and φ_{QTend} (2:54[2:13;3:12] vs 4:10[2:49;4:37]), was visible, with a significant forward shift when vertical position was restored at R+0 (RR 4:22[4:02;4:43]; QTend 4:39[4:10;5.10]). Interestingly, the Δ_φ computed between RR and QTend showed a phase inversion at HDT5 compared to BDC-9 (-7[-13.25;2.5]min vs 11[-1.5;24.5]min), with φ_{QTend} anticipating φ_{RR}. Hereafter, Δ_φ was trending towards BDC-9 values, but it appeared again different at R+7 (0[-5;10]min).

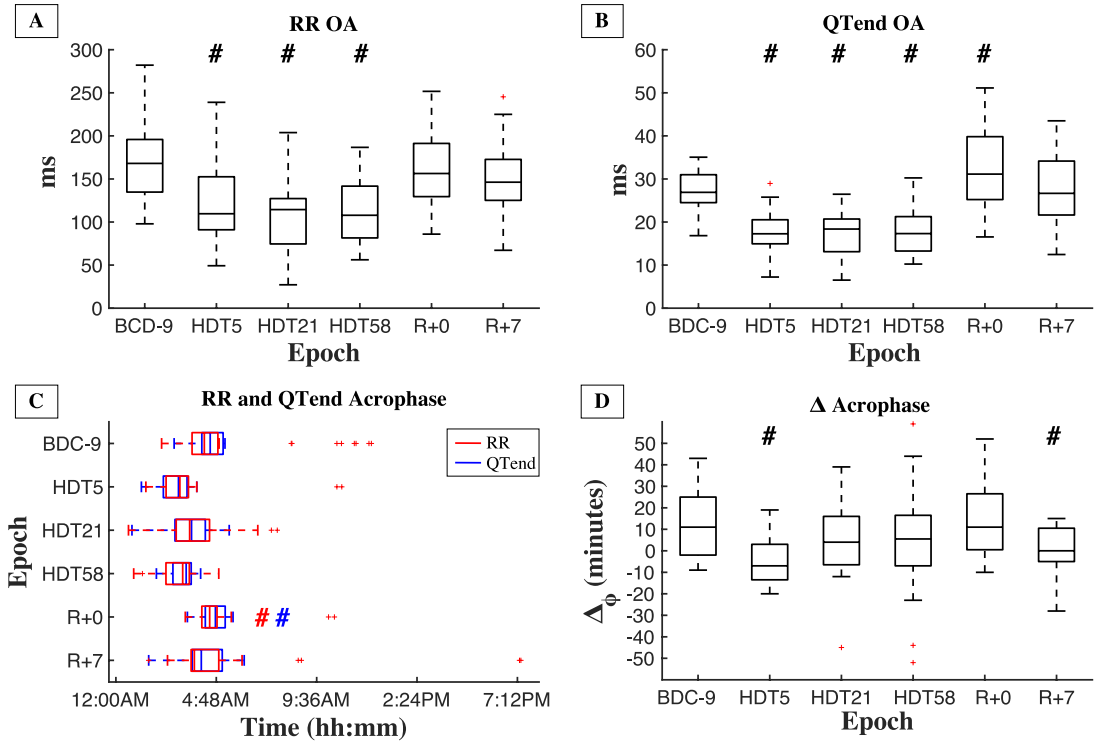

Fig. 5 Box-and-whisker plots of RR and QTend oscillation amplitude of the entire population (Panel A and Panel B, respectively), as well as of their acrophases (Panel C) and relevant Δφ (Panel D). #: p<.05 vs BDC-9 (Wilcoxon Signed Rank)

Discussion

In this study, 24h RR and QTend beat-to-beat variability series extracted from Holter ECG acquired from 20 subjects (10 as CTRL, 10 as CM) at several epochs (i.e., before, during and after 60-day HDT bed rest) were analyzed, aiming at evaluating possible changes in relevant circadian rhythms, together with effectiveness of the applied nutritional countermeasure. Our results obtained using Cosinor analysis showed that both RR and QTend circadian rhythms were affected by the HDT, with the midline value increasing at its beginning, and decreasing during recovery. Moreover, the day-night oscillation amplitude was reduced during the entire HDT, and the acrophase was slightly anticipated in HDT and postponed at R+0, with no significant effects relevant to the intake of the nutritional supplementation countermeasure. To the knowledge of the authors, this is the first time that circadian analysis was applied to ventricular repolarization duration in the setting of bed rest experiments, or even of human space flight. Also, the nutritional CM was novel, and not tested previously in this context.

Effects of HDT bed rest on MESOR

A first morphological characterization of a circadian rhythm is given by its midline, maximum and minimum values. Similarly to what reported in eight healthy men performing a 45-day HDT bed rest (Liang et al. 2014), where the RR interval was significantly increased during HDT bed rest, with the main HR decrease visible at the very beginning, also in this work the RR midline value was increased at HDT5, as well as the maximum and minimum values. This observation well reflects the process of physiological changes to which the subjects underwent in the first days of the HDT, induced by the circulatory unloading and decreased daily activity resulting from tilting position and immobilization. Different authors showed that short duration spaceflight elicits inflight HR reduction: Fritsch-Yelle et al. (Fritsch-Yelle et al. 1996) evidenced in Shuttle astronauts that the mean HR significantly reduced during a 5 to 10 days space mission; a significant inflight increase (the 5th and 8th day of spaceflight) in mean RR interval was reported as well by Beckers et al. (Beckers et al. 2003) in 3 astronauts involved in the Belgian Taxi Flight.

With the persistence of HDT, an inversion of this trend was observed: the RR MESOR was gradually restored towards baseline values, where at HDT58 the maximum was even lower than BDC-9, while the RR minima remained increased during the entire HDT. These results were also in agreement with Liang et al., where the minimum HR after 35 days of HDT was higher than control, whereas the maximum HR during HDT was lower than control. In their study, Liang et al. also highlighted that the variations in HR noticed during bed rest were not dependent from the level of activity, recorded with a wrist accelerometer, which was found lower as expected in HDT compared to baseline, but constant along HDT. For this reason, the HR adaptation in long duration HDT might be related to the chronic head-down body position. This behaviour was also confirmed in other studies: an increased resting HR after the second half of 90 days HDT bed rest was described in the European Space Agency, French Space Agency, and National Space Development Agency of Japan clinical report (Pavy-Le Traon et al. 2007), as well as in another 60-day bed rest study in which HR resulted increased already at HDT41 (Liu et al. 2015). This progressive adjustment and adaptation to the HDT condition is in agreement with the results obtained during ISS expeditions, where in 5 out of 7 astronauts examined, after an initial increase one month after launch in the mean RR interval (by 24h Holter ECG), and a less marked circadian period, a progressive recovery of the circadian rhythm was observed (Yamamoto et al. 2015). However, this process appeared to be subject-specific, with three astronauts developing high bradycardia, two mild bradycardia, and two tachycardia, while in our results the majority (18 out of 20) of the experimental subjects presented the same trend of adaptation to the new condition, with a decreased RR interval at HDT58.

Bradycardia related to long-duration spaceflights could result in QT interval prolongation (Anzai et al. 2014), possibly increasing the risk of cardiac electrical instability. This condition can also be associated with an increased risk of Torsades de Pointes, which is a myocardial repolarization disorder (Anzai et al. 2014), thus indicating that the repolarization phase could be affected by the effects of chronic weightlessness exposure. In this study, the MESOR and minimum values of the QT interval of both CM and CTRL increased especially in the first half of HDT, besides not reaching pathological range values, while the maximum QTend never exceeded baseline values, differently from RR, being further decreased at HDT58.

The sudden return to normal gravity condition after a period of permanence in microgravity, real (Beckers et al. 2003) or simulated (Liang et al. 2014), has been reported to generate an abrupt HR increase. In our study, RR intervals abruptly decreased, in terms of MESOR, minimum and maximum values, immediately after HDT conclusion, and the QTend interval was significantly reduced as well. These variations were elicited by the induced cardiac deconditioning that prevented adaptation to the new hydrostatic pressure gradient and body fluid distribution. The differences compared to BDC-9 were visible also during the night period, when possible interferences due to simultaneous experiments performed daily was limited, and where the subjects were in horizontal position, as before HDT. This indicates a possible impairment in the autonomic regulation of HR, as confirmed by several studies conducted during long-duration spaceflight (Fritsch-Yelle et al. 1994; Baevsky et al. 2007). Eight days after HDT conclusion, an only partial return to baseline was observed, thus indicating the reversibility of the process but the need for a longer period of time to reach a complete recovery, in line with results from both short duration (10-14 days) spaceflight, after which a period between 5 (Beckers et al. 2009) to 25 days (Verheyden et al. 2007) was needed to achieve it, and long duration (45 days) HDT bed rest (Liang et al. 2014), where maximum and minimum HR values remained increased for 10-12

days after HDT conclusion. Additionally, the similar and simultaneous variations in RR and QTend MESOR values over the 24h, as well as their diurnal and nocturnal mean values during and after HDT, provide evidence that the relation between RR and cardiac repolarization was generally maintained.

Effects of HDT bed rest on Oscillation Amplitude

Another important characteristic of circadian rhythms is the amplitude of the 24h oscillation. During a 45-day bed rest, Liang and colleagues (Liang et al. 2014) showed that the circadian oscillation amplitude of HR was significantly reduced compared to both baseline and post HDT. Similarly, in our study the OA of both RR and QTend intervals was found reduced during the entire 60-day HDT, thus resulting in a less marked difference between day and night. This alteration could be due to the combination of reduction in the amplitude of the physical activity/rest cycle, the elimination of the upright/supine cycle and chronic circulatory unloading, and linked to the changes towards cardiac deconditioning that the subjects underwent during the experiment. A loss of amplitude may cause a decreased capacity of adaptation of the physiological function to new stimuli. For example, McKenna et al reported that in healthy subjects the 24h HR oscillation amplitude varied in a range of 5-25 beats per minute (McKenna et al. 2017), while a reduction in its variability was considered a poor prognostic factor for critical illnesses.

After HDT conclusion, the RR OA was immediately restored, while QTend OA significantly rose over baseline values, a condition that might lead to an increased risk of developing cardiac arrhythmia. Indeed, a recent study showed that the 24h QT oscillation amplitude in patients with proven or potential (Solatol-induced) long QT was higher than in normal subjects, and up to two-fold higher in systolic hearth failure patients with history of ventricular arrhythmia compared to those with no history of arrhythmia (Du Pre et al. 2017). In our study, this sudden increase in QTend OA was visible both in the CTRL group (+35.7%), and when both groups were pooled together, with six subjects (five of which belonging to the CTRL) with an increase over 40% compared to baseline values. As episodes of prolonged QT (D'Aunno et al. 2003) and cardiac arrhythmia (Anzai et al. 2014) during spaceflight have been previously reported, the oscillation amplitude of the QTend interval could represent a significant parameter to be derived from 24h Holter ECG capable to monitor a trend towards possible increased cardiac risk.

Effects of HDT bed rest on Acrophase φ

The φ_{RR} and φ_{QTend} circadian rhythm appeared affected by the bed rest, with a backward shift in the circadian pattern noticeable during the HDT, and an opposite significant variation at R+0. The same variations were reported in the circadian phase of HR during a 45-day bed rest (Liang et al. 2014), suggesting that cardiac circadian rhythmicity is altered also in its phase by the bed rest condition. For the first time in our knowledge, the difference (Δφ) between φ_{QTend} and φ_{RR}, as an indicator of the phase in their coupling, was measured: at baseline, φ_{RR} anticipated φ_{QTend}, while an inversion of this relationship was observed at the beginning of the HDT and 8 days after its conclusion. The acrophase identifies the time of the day at which the series reaches its maximum, which for RR series corresponds to the lowest HR during the night period. An abnormal phase can be caused by the altered characteristics of the external synchronizers, in terms of strength, amplitude and timing, as well as in presence of conflicting external synchronizers (Roenneberg et al. 2016). For the entire duration of the experiment, lighting and feeding were fixed, while the activity/rest and the upright/supine cycles were reduced or eliminated, thus possibly contributing to the observed alteration in φ. The observed changes in Δφ could highlight a transient weakening of the QT-RR relation, where circadian changes in QT were preceding instead than following the respective circadian changes in RR. More additional data will be needed to better clarify this novel behaviour.

Effects of the nutritional countermeasure

The nutritional vitamin and antioxidant cocktail tested as potential countermeasure in this bed rest study contained a supplementation of 2.1 g of Omega-3, assumed daily during HDT. It is known that Omega-3 fatty acids are beneficial for cardiovascular health, such as in regulating blood pressure and reducing atherogenesis, inflammation and arrhythmia (Balk et al. 2004), lowering resting HR and decreasing the likelihood of prolonged QT (Mozaffarian et al. 2006) by modulating the sodium, potassium and calcium channels (Kang et al. 1996). However, the effects of Omega-3 consumption on the cardiovascular system depend on many variables, such as age and cardiovascular health status of the subject (Geleijnse et al. 2002), as well as on the quantity, duration, and source or type of fatty acids intake: Omega-3 in form of supplementation was reported to have beneficial influence in improving heart rate variability in myocardial infarction patients, while in healthy subjects the beneficial effect was achieved only by dietary fish consumption and not by Omega-3 supplementation (Balk et al. 2004). In our study, the ineffectiveness of the applied nutritional CM in preventing or attenuating the HDT-related changes in the circadian parameters of RR and QTend intervals could be linked to the source of nutrients intake, here delivered in form of supplementation. At HDT conclusion, a significant increase in the QTend OA was observed, a condition that could lead to an increased risk of arrhythmia. However, this was visible and more prevalent in the CTRL group, thus not excluding in the CM group the possibility of a possible link with Omega-3 fatty acids intake beneficial effects.

Conclusion

This study revealed that 60-day HDT bed rest affected the circadian rhythm of RR and QTend intervals, in terms of midline value, oscillation amplitude and acrophase, even when pre-scheduled day-night period and feeding times were maintained, thus inferring the role of changes in the gravitational stimulus in determining these variations. Major changes were visible at the beginning of the bed rest (HDT5) and at the restoration of the normal head-to-foot gravitational stimulus (R+0). The presence of circadian rhythmicity was maintained in all epochs, with the observed changes remaining within physiological limits, and appearing reversible within 8 days after HDT conclusion. The applied nutritional countermeasure did not show a clear effectiveness in preventing such changes.

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Author Contribution Statement

EC and PV conceived and designed the research. FL conducted experiments. AM contributed in the development of analytical tools. SS and MT analysed data. LC contributed with medical interpretation of the results. SS and EC wrote the manuscript. All authors read and approved the manuscript.