INVESTIGATION OF THE INTER-INDIVIDUAL VARIABILITY OF PHYSIOLOGICAL RESPONSES TO CHANGES IN ACTIVITY LEVELS-, GRAVITY LOADING-, NUTRITIONAL STATUS, PHARMACEUTICALS AND EXPOSURE TO RADIATION

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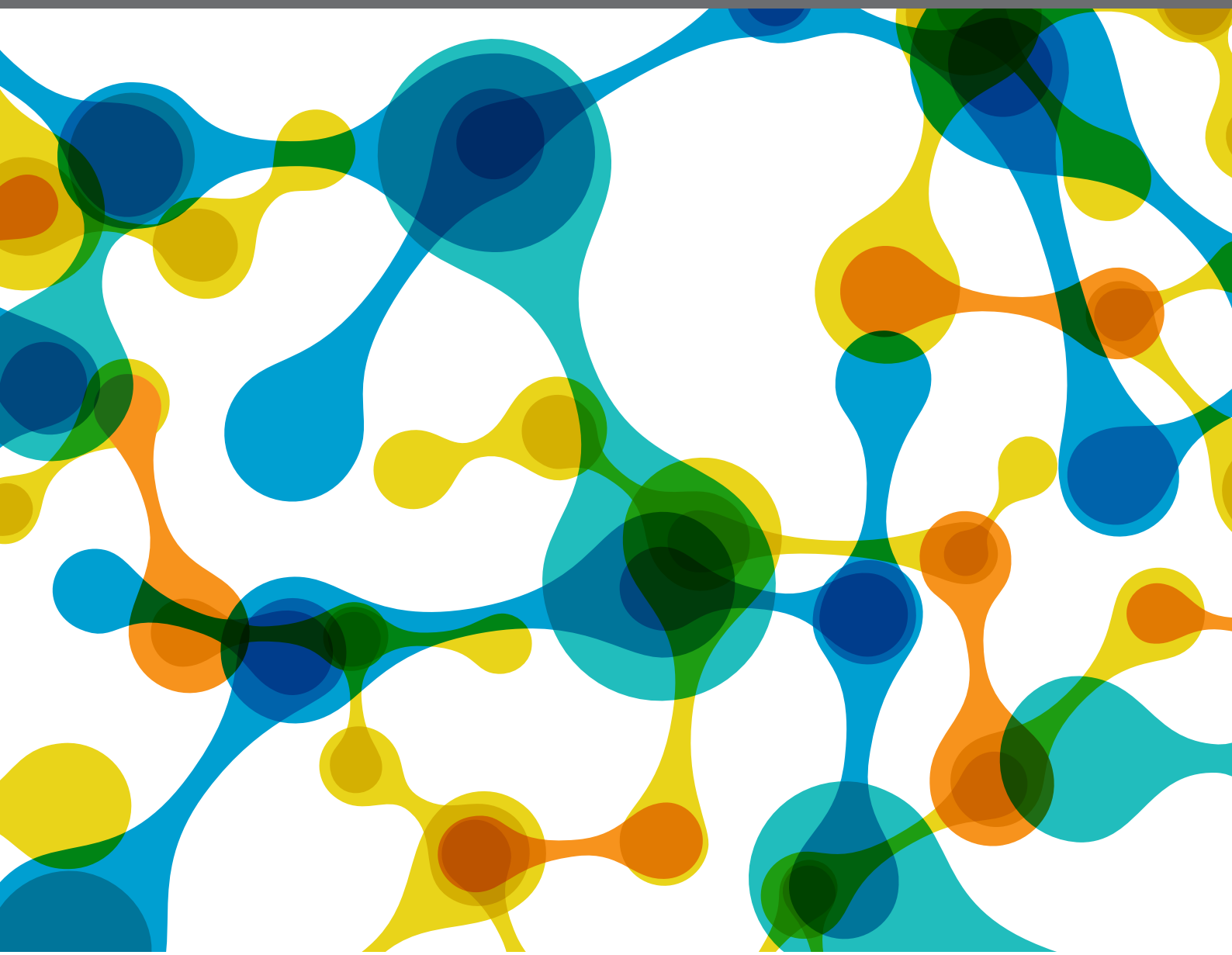

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Table of Contents

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Fetuin-A as a Potential Biomarker of Metabolic Variability Following 60 Days of Bed Rest

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Background: Fetuin-A is a hepatokine linked to the development of insulin resistance. The purpose of this study was to determine if 60 days head-down-tilt (HDT) bed rest increased circulating fetuin-A and if it was linked to whole body insulin sensitivity (IS). Additionally, we examined whether reactive jump training (RJT) could alleviate the metabolic changes associated with bed rest.

Methods: 23 young men (29 ± 6 years, 181 ± 6 cm, 77 ± 7 kg) were randomized to a control (CTRL, $n = 11$) or RJT group (JUMP, $n = 12$) and exposed to 60 days of bed rest. Before and after bed rest, body composition and $\dot{V}O_{2peak}$ were measured and an oral glucose tolerance test was performed to estimate IS. Circulating lipids and fetuin-A were measured in fasting serum.

Results: Body weight, lean mass, and $\dot{V}O_{2peak}$ decreased in both groups following bed rest, with greater reductions in CTRL ($p < 0.05$). There was a main effect of time, but not the RJT intervention, for the increase in fetuin-A, triglycerides (TG), area under the curve for glucose (AUCG) and insulin (AUCI), and the decrease in Matsuda and tissue-specific IS ($p < 0.05$). Fetuin-A increased in participants who became less insulin sensitive ($p = 0.019$). In this subgroup, liver IS and adipose IS decreased ($p < 0.05$), while muscle IS was unchanged. In a subgroup, where IS did not decrease, fetuin-A did not change. Liver IS increased ($p = 0.012$), while muscle and adipose tissue IS remained unchanged.

Conclusions: In this study, we report an increase in circulating fetuin-A following 60 days of bed rest, concomitant with reduced IS, which could not be mitigated by RJT. The amount of fetuin-A released from the liver may be an important determinant of changes in whole body IS. In this regard, it may also be a useful biomarker of individual variation due to inactivity or lifestyle interventions.

Keywords: bed rest, fetuin-A, hepatokine, insulin sensitivity, liver, metabolism

INTRODUCTION

Physical inactivity and exposure to microgravity induce aging-like phenotypic changes that are associated with the etiology of many chronic diseases (Bergouignan et al., 2011; Hart and Zernicke, 2020). The physiological changes in space and during head-down-tilt (HDT) bed rest, the Earth-based analogue of microgravity, have been well-described and include altered cardiovascular capacity, bone loss, muscle atrophy, impaired functional capacity, and metabolic dysregulation, among others (Bergouignan et al., 2011; Narici and de Boer, 2011; Ade et al., 2015; Vico and Hargens, 2018; Konda et al., 2019). There is evidence that underlying mechanisms, such as insulin resistance, play an important role in the regulation of whole body metabolic changes (Gratas-Delamarche et al., 2014). Insulin resistance is a multi-faceted disruption of the action of insulin in skeletal muscle, adipose tissue, vasculature, brain, and the liver, leading to hyperinsulinemia and reduced glucose disposal (Cox-York and Pereira, 2020). It is also associated with impaired oxidative capacity, increased circulating and deposition of lipids, and metabolic inflexibility. These whole body and cellular changes have been observed following bed rest studies, even when energy balance is maintained (Bergouignan et al., 2006, 2009, 2011; Kenny et al., 2017; Rudwill et al., 2018). The severity of insulin resistance has been found to vary considerably between individuals and between the key target organs (Unnikrishnan, 2004; Abdul-Ghani et al., 2007). Therefore, understanding the individual variability in insulin resistance could provide personalized information on disease etiology and individualized interventions to maintain health.

A key strategy for monitoring metabolic homeostasis is communication between peripheral tissues *via* secreted proteins, which perform autocrine, paracrine, and endocrine actions. Disruption of protein production and target-tissue action underpin the development of metabolic dysfunction including insulin resistance (Priest and Tontonoz, 2019). The quantitative measurement of circulating protein biomarkers is a relatively easy and minimally-invasive means of identifying and understanding the etiology of insulin resistance.

The liver is a major regulator of systemic glucose metabolism. Liver-derived proteins, known as hepatokines, are released into circulation and some of them are known to enhance or attenuate insulin sensitivity (Stefan and Häring, 2013; Iroz et al., 2015; Choi, 2016). Fetuin-A is a novel hepatokine, encoded by the alpha-2-HS-glycoprotein (AHSG) gene in humans and is a known regulator of metabolism (Stefan and Häring, 2013). In particular, an increase in fetuin-A is associated with the development of insulin resistance and the pathophysiology of type 2 diabetes mellitus (T2DM). Fetuin-A impairs the insulin signaling cascade by binding to the tandem fibronectin type 3 domains present on the extracellular portion of the transmembrane β-subunit of the insulin receptor, attenuating tyrosine kinase signaling and leading to reduced glucose uptake (Goustin et al., 2013; Ochieng et al., 2018). Additionally, fetuin-A has been proposed to inhibit the production of the insulin-sensitizing hormone adiponectin in adipocytes, indirectly leading to a decrease in insulin sensitivity (Hennige et al., 2008). Finally, fetuin-A is implicated in lipid-induced insulin resistance by acting as an intermediary between palmitate and toll-like receptor 4 (TLR4) leading to adipose tissue inflammation and insulin resistance (Pal et al., 2012).

Exercise training, using a wide-range of modalities, is known to promote glucose control and improve insulin sensitivity in healthy and clinical populations (Ennequin et al., 2019). One of the mechanisms that contributes to improved metabolic health is training-induced alterations in the production and secretion of pro-inflammatory and anti-inflammatory cytokines from tissues of metabolic importance. Numerous studies have reported that exercise training decreases the secretion of fetuin-A from the liver concomitant with improvements in whole body and liver insulin sensitivity in patients with metabolic disease (Malin et al., 2013, 2014; Lee et al., 2017; Ennequin et al., 2019).

The purpose of this study was to determine if 60 days of extreme physical inactivity increased circulating fetuin-A and if those changes correlated with whole body insulin sensitivity. We used the European Space Agency 60 day bed rest model, where subjects were maintained in energy balance, despite a decrease in physical activity. This unique approach means fat accumulation is not a confounding factor on the metabolic outcomes. In addition, we examined whether reactive jump training (RJT), a countermeasure used to maintain skeletal muscle mass, was able to attenuate the deterioration in metabolic health that occurs with prolonged inactivity.

MATERIALS AND METHODS

General Study Information

This research was conducted as part of the "Reactive jumps in a sledge jump system as a countermeasure during long-term bed rest" (RSL) study funded by the European Space Agency, which ran as two separate bed rest campaigns, commencing in August 2015 and January 2016, respectively. This parallel-design randomized controlled training study was conducted at the "envihab" facility at the German Aerospace Center (DLR). A detailed description of the subject recruitment procedures, experimental conditions, diet, countermeasure, and training protocol have been published previously (Kramer et al., 2017b).

In brief, the study was split into three phases: a 15-day baseline data collection phase (BDC-15 to BDC-1), 60 days of strict 6° head-down-tilt bed rest (HDT1 to HDT60), followed by a post-intervention testing phase (R + 0 to R + 14), with a total duration of 90 days. Subjects were randomly assigned to a control group (CTRL) or intervention group involving

Abbreviations: Adipose IR, Adipose tissue insulin resistance; AUCG, Area under the curve for glucose; AUCI, Area under the curve for insulin; BDC, Baseline data collection; BMI, Body mass index; CTRL, Control group; ELISA, Enzyme-linked immunosorbent assay; HDT, Head-down-tilt; IS, Insulin sensitivity; JUMP, Jump countermeasure group; Liver IS, Liver insulin sensitivity; Muscle IS, Muscle insulin sensitivity; OGTT, Oral glucose tolerance test; RJT, Reactive jump training; PV, Plasma volume; T2DM, Type 2 diabetes mellitus; $\dot{V}O2peak$, Peak aerobic capacity.

reactive jump exercise (JUMP). For the duration of the bed rest period, subjects remained at the 6° HDT angle for 24 h/day.

The inclusion criteria have been described previously (Kramer et al., 2017b) but included men, aged 20–45 years, body mass index (BMI) 20–26 kg/m^2, non-smoker, no medication, no history of bone fractures, non-competitive athlete, and no medical conditions. Initially, 24 male volunteers were enrolled for the study, however one subject discontinued during baseline data collection due to medical reasons unrelated to the study. In addition, two subjects were ambulated after 49-days HDT (CTRL) and 50-days HDT (JUMP) due to medical reasons but all post-bed rest data were collected except for $\dot{V}O_{2peak}$.

On HDT1, participants were randomly assigned to either the control group (CTRL, n = 11, age 28 ± 6 years, BMI 23.3 ± 2 kg/m^2) or the countermeasure group (JUMP, n = 12, age 30 ± 7 years, BMI 23.8 ± 2 kg/m^2), which performed RJT 5–6 days per week in a horizontal sledge jump system. A total of 48 training sessions were completed during the HDT bed rest period. Each training session involved a varying amount of repetitive hops and countermovement jumps with an average load equal to or exceeding 80% of the individual's body weight. The maximal workload in one session excluding breaks did not exceed 4 min. During the entire study, the subjects received a strictly controlled and individualized diet, which was tailored to maintain energy balance. The study protocols were approved by the Ethics Committee of the North Rhine Medical Association (Ärztekammer Nordrhein) in Düsseldorf, Germany, as well as the Federal Office for Radiation Protection (Bundesamt für Strahlenschutz). All subjects gave their written informed consent before commencing the study in accordance with the Declaration of Helsinki. This study was registered with the German Clinical Trial Registry (#DRKS00012946, 18th September 2017).

Body Weight and Body Composition

Measurements of body weight and body composition were taken on numerous days before and after bed rest. This is a large scale bed rest study and data are reported by different research groups. The core data have been published elsewhere (Kramer et al., 2017b), however, this study has compared measurements of body weight and body composition recorded on BDC-3 and HDT60. Body weight was measured daily following the first urine void of the day (DVM 5703, Sartorius, Göttingen, Germany). Body composition was examined with dual-energy X-ray absorptiometry (DEXA), using the whole body scan feature on the Prodigy Full Pro (GE Healthcare GmbH, Solingen, Germany) and the manufacturer's enCORE software (version 16.10.151) was used to generate automated reports of total lean mass, fat mass, and bone mineral content.

Peak Oxygen Consumption Test

Peak aerobic capacity ($\dot{V}O_{2peak}$) was measured on BDC-8 and R + 1 using cycle ergometry (Lode, Groningen, The Netherlands) as previously described (Kramer et al., 2017a). In brief, after an initial 5 min of seated rest, subjects were instructed to start pedaling and to maintain a cadence of 75 revolutions per minute (rpm). The warm-up consisted of 3 min cycling at 50 W, followed by 1 min stages, in which the load was increased by 25 W per stage until volitional exhaustion, despite strong verbal encouragement. If the peak respiratory exchange ratio (RER) did not exceed 1.10, the trial was deemed not exhaustive and not considered for further analyses. Due to the absence of post-bed rest data, two subjects were removed from our analysis of $\dot{V}O_{2peak.}$

Oral Glucose Tolerance Test

An oral glucose tolerance test (OGTT) was performed after a 12-h overnight fast, in the morning of BDC-5 and HDT59. A catheter was placed in the antecubital vein and blood samples were drawn before and at 30 min intervals (30, 60, 90, and 120 min) after the ingestion of a 75 g glucose equivalent (ACCU Chek® Dextro OGT, Roche Diagnostics Deutschland GmbH, Mannheim) dissolved in 300 ml water. Following sample extraction, serum was left to coagulate at room temperature for 30 min before centrifugation, while vacutainers containing fluoride and EDTA were centrifuged immediately (184 × g, 4°C, 10 min). Serum and plasma were then aliquoted and stored at −80°C until analysis.

Plasma Volume Correction

As the 6° HDT angle induces a fluid shift and consequent loss of plasma volume (PV), the change in PV was calculated and the concentrations of biochemical parameters following HDT bed rest were corrected for changes in hemoconcentration (Dill and Costill, 1974; Alis et al., 2016). The change in PV (Δ%PV) was calculated as follows: $\Delta\%PV = 100^{*}((Hb_{pre}/Hb_{post})^{*}(100-Htc_{post})/(100-Htc_{pre})-1)$, where hemoglobin (Hb) is given in g/dL and hematocrit (Hct) is expressed as a percentage (%). To correct measured parameters for changes in PV, the following calculation was used: [parameter]c = [parameter] u*(1 + ΔPV(%)/100), where the c and u indices represent corrected and uncorrected concentrations, respectively.

Biochemical Analysis and Assays

Concentrations of glucose, non-esterified fatty acids (NEFA), total cholesterol, LDL-cholesterol (LDL), HDL-cholesterol (HDL), and triglycerides (TG) were measured using colorimetric assay kits on the Randox RX Daytona™ (Crumlin, United Kingdom). Serum insulin was quantified using an immunoassay method on the Cobas® 8000 modular analyser (module e602, Roche Diagnostics, North America). Area under the curve for glucose (AUCG) and insulin (AUCI) were calculated according to the trapezoidal rule. Indexes of insulin resistance and insulin sensitivity including the Matsuda index, liver insulin sensitivity (liver IS), muscle insulin sensitivity (muscle IS), and adipose tissue insulin resistance (adipose IR) were calculated using previously reported equations (**Supplementary Material**; Matsuda and DeFronzo, 1999; Abdul-Ghani et al., 2007; Lomonaco et al., 2012). Using fasting serum samples from the OGTT, concentrations of fetuin-A were assayed in duplicate using the human fetuin-A quantikine ELISA kit, according to the

manufacturer's instructions (Cat No: DFTA00. R&D Systems Inc. Minneapolis, United States). The intra-assay coefficient of variance (%CV) was 11%.

Statistical Analysis

All experimental data are presented as mean ± SD. Normality of distribution for each variable was evaluated using the Shapiro-Wilk test and data violating the assumption of normality was transformed. Differences in baseline characteristics between the two experimental groups were assessed using independent samples *t*-tests or the non-parametric Mann-Whitney U test. Physical and metabolic changes in response to bed rest were analyzed using a mixed between-within factorial analysis of variance using time as the within-group factor and experimental group as the between-group factor (CTRL and JUMP). When a statistically significant interaction was found, simple main effects are reported as mean (M) and standard error (SE). Statistical analysis was performed in SPSS 26.0 (IBM Corp., Armonk, NY, United States) considering a two-sided 0.05 significance level.

In-Depth Data Analysis

An association between insulin sensitivity and fetuin-A has been well-established within the literature (Ochieng et al., 2018; Bourebaba and Marycz, 2019). In the current study, pre- to post-differences in insulin sensitivity and fetuin-A exhibited significant effects of time only, so to further explore the relationship between both variables additional subanalysis was conducted. The participant data from the two experimental groups were pooled and then divided into two subgroups based on participants who improved (↑ Matsuda, n = 6, CTRL n = 4, and JUMP n = 2) or reduced (↓ Matsuda, n = 17, CTRL n = 7, and JUMP n = 10) insulin sensitivity post-HDT bed rest. Paired sample *t*-tests were used to assess the significance of pre- to post-bed rest changes in physical and metabolic parameters in both subgroups.

RESULTS

Physical Characteristics: Body Weight, Body Composition, and $\dot{V}O_{2peak}$

Anthropometric measurements were obtained on BDC-3 and HDT60 (**Table 1**). No significant between-group differences in physical characteristics were identified at baseline. Following 60 days of HDT bed rest, body weight decreased significantly in both groups, with a larger reduction observed in the CTRL group (M = −3.63 kg, SE = 0.54 kg, $p < 0.001$) compared to the JUMP group (M = −2.23 kg, SE = 0.28 kg, $p < 0.001$). Similarly, bed rest significantly reduced lean mass, with a higher decline noticeable in the CTRL group (M = −3.91 kg, SE = 0.70 kg, $p < 0.001$) in comparison to the JUMP group (M = −1.34 kg, SE = 0.32 kg, p = 0.002). Fat mass decreased significantly in the JUMP group (M = −0.87 kg, SE = 0.23 kg, p = 0.003) but did not change significantly in the CTRL group (M = 0.10 kg, SE = 0.31 kg, p = 0.757) following HDT bed rest. Bone mineral content did not change significantly. Absolute $\dot{V}O_{2peak}$, measured on BDC-8 and R + 1, decreased significantly in both groups after HDT bed rest, with a greater loss identified in the CTRL group (M = −1.28 L/min, SE = 0.17 L/min, $p < 0.001$) in comparison to the JUMP group (M = −0.33 L/min, SE = 0.15 L/min, p = 0.049). $\dot{V}O_{2peak}$, when normalized for changes in lean mass, decreased significantly in the CTRL group (M = −18.82 ml/kgLW/min, SE = 2.26 ml/kgLM/min, $p < 0.001$) but did not change in the JUMP group (M = −4.58, SE = 2.17, p = 0.063) after HDT bed rest.

Metabolic Characteristics: Glucose Tolerance, Insulin Sensitivity, Lipid Metabolism, and Fetuin-A

The changes in metabolic parameters are presented in **Figure 1** and **Table 2**. No significant between-group differences in metabolic characteristics were found at baseline. Metabolic characteristics were corrected for changes in hemoconcentration

TABLE 1 | Effects of 60 days HDT bed rest on measures of anthropometry and cardiorespiratory capacity.

Measurement	CTRL (n = 11)		JUMP (n = 12)		Statistics		
	Pre	Post	Pre	Post	Time	Int	T*Int
Age (years)	28 ± 6		30 ± 7				
Height (cm)	181 ± 5		181 ± 7				
BMI (kg/m^2)	23.33 ± 2.03	22.22 ± 1.67*	23.75 ± 1.80	23.07 ± 1.81*	<**0.001**	0.410	**0.021**
BW (kg)	76.10 ± 8.06	72.47 ± 6.76*	77.85 ± 6.55	75.63 ± 6.39*	<**0.001**	0.405	**0.027**
LM (kg)	56.94 ± 6.57	53.03 ± 5.11*	56.41 ± 5.18	55.08 ± 4.29*	<**0.001**	0.731	**0.002**
FM (kg)	16.91 ± 3.95	17.00 ± 3.41	19.21 ± 6.42	18.34 ± 6.18*	0.055	0.412	**0.018**
BMC (kg)	3.14 ± 0.36	3.14 ± 0.37	3.00 ± 0.32	3.00 ± 0.32	0.605	0.355	0.800
Measurement	**CTRL (n = 10)**		**JUMP (n = 10)**		**Statistics**		
	Pre	**Post**	**Pre**	**Post**	**Time**	**Int**	**T*Int**
$\dot{V}O_{2peak}$ (L/min)	3.85 ± 0.68	2.57 ± 0.48*	3.32 ± 0.76	2.99 ± 0.53*	<**0.001**	0.848	**0.001**
$\dot{V}O_{2peak}$ (ml/kgLM/min)	67.55 ± 8.42	48.74 ± 9.35*	58.72 ± 10.95	54.14 ± 8.03	<**0.001**	0.660	<**0.001**

*Data are presented as mean ± standard deviation (SD). Significant p < 0.05 are indicated in bold. Anthropometric measurements were taken on BDC-3 and HDT60. $\dot{V}O_{2peak}$ was measured on BDC-8 and R + 1. When a significant interaction effect was found, an asterisk (*) denotes a significant difference from pre in each intervention group. CTRL, control group; JUMP, jumping countermeasure group; Time, main effect of time; Int, main effect of intervention; T*Int, time*intervention interaction effect; BMI, body mass index; BW, body weight; LM, lean mass; FM, fat mass; BMC, bone mineral content; $\dot{V}O_{2peak}$, peak aerobic capacity.*

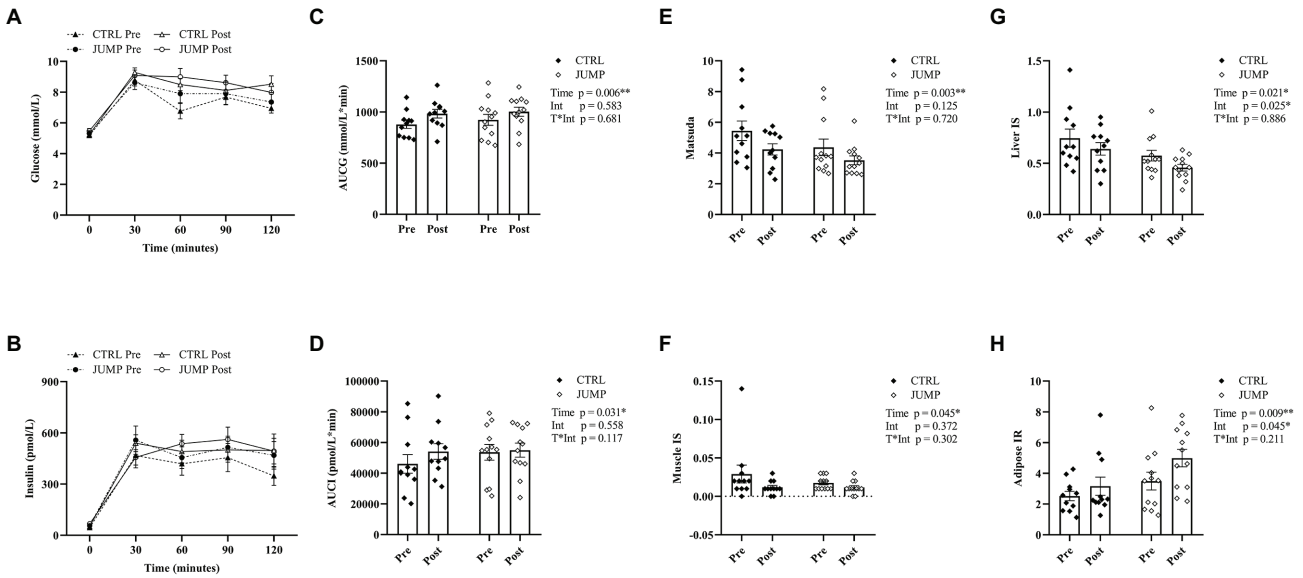

FIGURE 1 | The effects of 60 days of HDT bed rest on metabolic variables measured on BDC-5 (pre) and HDT59 (post). Data are presented as mean ± standard error of mean (SEM). The glucose and insulin response curve to the OGTT are displayed in **(A)** and **(B)**, respectively. The 120 min area under the curve totals for glucose are shown in **(C)** and for insulin in **(D)**. The pre to post changes in OGTT derived indexes of Matsuda, liver IS, muscle IS, and adipose IR are presented in **(E–H)**. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; AUCG, area under the curve for glucose for 120 min; AUCI, area under the curve for insulin for 120 min; IS, insulin sensitivity; IR, insulin resistance; Time, main effect of time; Int, main effect of intervention; T*Int, time*intervention interaction effect. $^{*}p \leq 0.05$ and $^{**}p \leq 0.010$.

TABLE 2 | The effects of 60 days HDT bed rest on metabolic characteristics.

Measurement	CTRL (n = 11)		JUMP (n = 12)		Statistics		
	Pre	Post	Pre	Post	Time	Int	T*Int
$Glucose_0$ (mmol/L)	5.18 ± 0.40	5.24 ± 0.51	5.32 ± 0.64	5.47 ± 0.59	0.447	0.311	0.732
$Glucose_{120}$ (mmol/L)	6.95 ± 1.01	8.51 ± 1.80	7.36 ± 1.82	7.98 ± 1.98	**0.010**	0.919	0.234
$Insulin_0$ (pmol/L)	45.00 ± 13.09	51.48 ± 16.49	55.55 ± 14.92	67.22 ± 19.14	**0.012**	**0.036**	0.437
$Insulin_{120}^{\dagger}$ (pmol/L)	347.16 ± 184.12	496.38 ± 321.36	468.39 ± 281.21	492.68 ± 260.41	0.054	0.587	0.264
NEFA (mmol/L)	0.40 ± 0.13	0.41 ± 0.16	0.41 ± 0.14	0.52 ± 0.17	0.124	0.232	0.215
TG (mmol/L)	0.87 ± 0.26	1.00 ± 0.26	1.16 ± 0.45	1.21 ± 0.41	**0.013**	0.102	0.245
CHOL (mmol/L)	4.09 ± 0.72	4.27 ± 0.86	4.11 ± 0.57	4.06 ± 0.57	0.619	0.710	0.381
HDL (mmol/L)	1.16 ± 0.17	0.99 ± 0.19	1.08 ± 0.27	0.91 ± 0.17	<**0.001**	0.304	0.955
LDL (mmol/L)	2.76 ± 0.71	3.10 ± 0.71	2.73 ± 0.51	2.99 ± 0.56	**0.004**	0.780	0.647

*Data are presented as mean ± standard deviation (SD). Significant $p < 0.05$ are indicated in bold. †Denotes that data were transformed for statistical analysis. Metabolic characteristics were measured on BDC-5 and HDT59. CTRL, control group; JUMP, jumping countermeasure group; Time, main effect of time; Int, main effect of intervention; T*Int, time*intervention interaction effect; $Glucose_0$, fasting glucose; $Glucose_{120}$, glucose concentrations 120 min after the glucose load; $Insulin_0$, fasting insulin; $Insulin_{120}$, insulin concentrations 120 min after the glucose load; NEFA, non-esterified fatty acids; TG, triglycerides; CHOL, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol.*

following HDT bed rest (mean ΔPV% CTRL 0.88%, JUMP -2.13%). NEFA, total cholesterol, fasting glucose ($glucose_0$), and 2-h insulin ($insulin_{120}$) did not change significantly following HDT bed rest. There was a significant effect of time, but not intervention, for the increase in 2-h glucose ($glucose_{120}$; $p = 0.010$), TG ($p = 0.013$), and LDL ($p = 0.004$) and decrease in HDL ($p < 0.001$) after HDT bed rest (**Table 2**). A significant effect of time and intervention was identified for fasting insulin ($insulin_0$), which increased significantly following HDT bed rest and was significantly higher overall in the JUMP group (**Table 2**). There was a significant effect of time, but not intervention, for the increase in AUCG and AUCI post-HDT bed rest (**Figures 1A–D**). A significant main effect of time only was also identified for the decrease in Matsuda and muscle IS following HDT bed rest (**Figures 1E–F**). The main effect of time and intervention were statistically significant for the change in liver IS and adipose IR following HDT bed rest (**Figures 1G–H**). Liver IS decreased significantly after HDT bed rest and was significantly higher overall in the CTRL group. In contrast, adipose IR increased significantly following HDT bed rest and was found to be significantly higher overall in the JUMP group. A significant main effect of time, but not intervention, was found for the increase in circulating fetuin-A (**Figure 2A**). Fetuin-A increased from

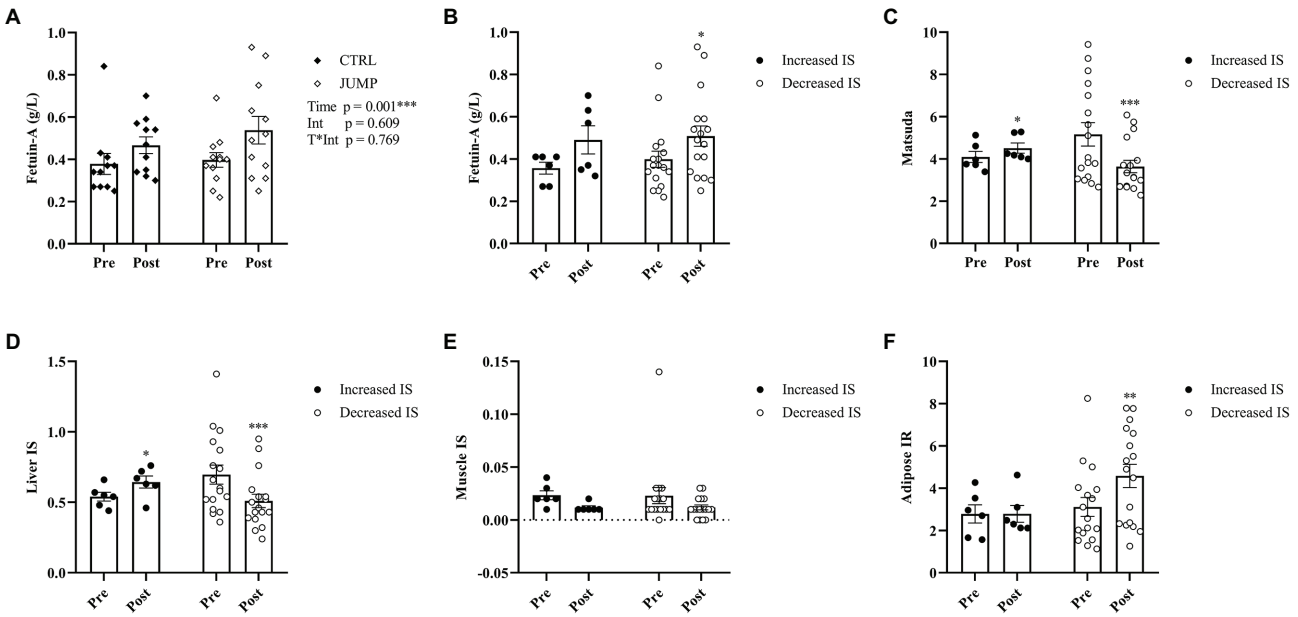

FIGURE 2 | (A) Circulating fetuin-A in the CTRL and JUMP groups before and after 60 days of HDT bed rest. **(B–F)** The effect of HDT bed rest on fetuin-A and OGTT derived indexes of insulin sensitivity and insulin resistance when subjects were divided into two subgroups based on an increase (n = 6) or decrease (n = 17) in insulin sensitivity (Matsuda) post-HDT bed rest. Data are presented as mean ± SEM. Abbreviations: CTRL, control group; JUMP, jumping countermeasure group; IS, insulin sensitivity; IR, insulin resistance; Time, main effect of time; Int, main effect of intervention; T*Int, time*intervention interaction effect. $^{*}p \leq 0.05$, $^{**}p \leq 0.010$, and $^{***}p \leq 0.001$.

0.38 ± 0.16 to 0.47 ± 0.13 g/L in the CTRL group and from 0.40 ± 0.12 to 0.54 ± 0.23 g/L in the JUMP group after HDT bed rest.

Subanalysis Exploring the Relationship Between Insulin Sensitivity and Fetuin-A

Changes in the physical and metabolic parameters in subgroups with decreased and increased insulin sensitivity following HDT bed rest are presented in **Figures 2B–F** and **Supplementary Tables 1** and **2**.

In the subgroup with improved insulin sensitivity following HDT bed rest, the pre- to post-increase in liver IS was statistically significant. Despite this, muscle IS and adipose IR did not change significantly after HDT bed rest. In subjects who became more insulin-sensitive following HDT bed rest, the change in fetuin-A was not statistically significant.

In the opposing subgroup, with reduced insulin sensitivity after HDT bed rest, the pre- to post-decrease in liver IS was statistically significant. Adipose IR increased significantly following HDT bed rest. The change in muscle IS was not statistically significant. Interestingly, in those who became less insulin-sensitive during HDT bed rest, circulating concentrations of fetuin-A significantly increased.

DISCUSSION

The main findings of the current study demonstrate that 60 days of HDT bed rest elicited a significant increase in fetuin-A concomitant with reduced insulin sensitivity, which could not be mitigated by RJT. As considerable individual differences have been found in the responsiveness to lifestyle interventions, we compared changes in metabolic variables in subgroups with decreased and increased insulin sensitivity following HDT bed rest. Our results suggest that fetuin-A may have a role in the regulation of peripheral insulin sensitivity during bed rest and physical inactivity. In addition, fetuin-A has potential as a biomarker to track individual changes in metabolic homeostasis.

Exercise and diet countermeasures have been widely implemented to abrogate deconditioning during bed rest. The RJT protocol combined plyometric movements with high rates of force development with the aim of preserving musculoskeletal mass and strength (Kramer et al., 2017a). The results, from previous publications, show that this time-efficient countermeasure attenuated the loss of whole body lean mass, leg lean mass, $\dot{V}O_{2peak}$ (Kramer et al., 2017a,b), and myofiber size and phenotype (Blottner et al., 2019). While RJT had protective effects for muscle function, it could not prevent the dysregulation of glucose and lipid metabolism. Similar results were reported following flywheel exercise (Bergouignan et al., 2006) and the authors, in this case, suggested that insufficient energy expenditure during the training sessions may be the key factor. While the RJT is a form of high intensity interval training consisting of 48 jumps and 30 hops, the overall workload may not be sufficient to improve insulin sensitivity. There was a decrease in fat mass (0.9 kg), indicating a negative energy balance in the JUMP group but this may be due to the challenges of estimating energy expenditure of the exercise protocol.

Another challenge for countermeasure design and implementation is the individual variation in response to lifestyle interventions (Bouchard and Rankinen, 2001; Solomon, 2018; O'Donoghue et al., 2019). While a specific exercise program may, on average, be effective there can be a broad range in the individual response. This is particularly important for long-term missions in microgravity, where a standard countermeasure program may not confer the same benefit to all the astronauts. It will be important to have individualized countermeasure programs that can be monitored and adjusted depending on the response. While changes in muscle mass can be observed with relative ease, monitoring changes in metabolism is more challenging in microgravity. The role of circulating biomarkers may serve as a simple and effective strategy to track metabolic changes on the health continuum and guide countermeasure recommendations. Previous research has reported that the beneficial and detrimental effects of physical activity and inactivity, respectively, are linked with changes in circulating biomarkers, which are key messengers for inter-organ communication (Pedersen and Febbraio, 2012; Ennequin et al., 2019; Mika et al., 2019).

Fetuin-A is a multifunctional glycoprotein that is predominately synthesized and secreted by hepatocytes (Bourebaba and Marycz, 2019) and associated with insulin action (Ochieng et al., 2018). To the best of our knowledge, fetuin-A has not been previously reported following bed rest and we report a significant increase in circulating fetuin-A following 60 days of HDT bed rest, irrespective of the experimental group. The regulation of hepatic fetuin-A secretion is incompletely understood (Haukeland et al., 2012). Previous research has reported that fetuin-A mRNA expression in the liver correlated positively with hepatic triglyceride content and homeostatic model of insulin resistance (HOMA-IR) and these associations remained significant after adjustment for BMI (Peter et al., 2018). These findings were supported by a cross-sectional study reporting higher fetuin-A in subjects with elevated liver fat content (Stefan et al., 2006). Analysis of longitudinal data following a lifestyle intervention found that the changes in fetuin-A paralleled the changes in liver fat (Stefan et al., 2006). Collectively, this evidence presents fetuin-A as a possible biological and predictive marker of metabolic disease.

Fetuin-A is also responsive to exercise training and a number of studies have reported a decrease in circulating levels with accompanying improvements in body composition, liver fat, and insulin sensitivity (Malin et al., 2013, 2014). Reductions in fetuin-A following aerobic exercise training have been attributable to decreases in waist circumference and body weight and increases in adiponectin, an insulin sensitizing hormone (Zhang et al., 2018). Additionally, favorable changes in fetuin-A, liver fat and insulin sensitivity have also been reported following long-term (12 weeks) aerobic and resistance exercise training (Lee et al., 2017). A recent meta-analysis reported that both aerobic and resistance training significantly reduced fetuin-A in dysglycemic and overweight/obese individuals when performed at a moderate or vigorous intensity, with a volume of 60 min per session and a minimum frequency of 4–7 sessions per week (Ramírez-Vélez et al., 2019).

In this study, we found that circulating concentrations of fetuin-A and insulin sensitivity were not significantly affected by RJT performed during HDT bed rest. This may be due to the type, intensity, and duration of the exercise protocol, which was primarily designed to preserve muscle mass and function. In addition, the physical inactivity of bed rest is the primary intervention and the exercise countermeasure in this case is designed to maintain rather than enhance physiological function. We observed a significant reduction in whole body insulin sensitivity, concomitant with an increase in circulating TG after 60 days HDT bed rest in energy-balanced conditions. These findings are in agreement with other bed rest studies (Bergouignan et al., 2009; Rudwill et al., 2015; Kenny et al., 2017), which support links between the decrease in muscle contraction and elevated TG (Bergouignan et al., 2011), decreases in the amount and activity of key proteins associated with muscle glucose uptake (Biensø et al., 2012), and altered mitochondrial function (Kenny et al., 2017). The observation that whole body and liver insulin sensitivity improved in a subgroup of bed rest participants is intriguing and supports the current emphasis on personalized medicine approaches to disease treatment. Interestingly, the improvement in insulin sensitivity was greater in the CTRL group than in the JUMP group. It has previously been highlighted that there is substantial inter-individual variability in physiological responses following lifestyle interventions (Bouchard and Rankinen, 2001; Solomon, 2018; O'Donoghue et al., 2019). In addition, the genes and pathways underlying the response to exercise training and physical inactivity differ (Booth et al., 2012; Pillon et al., 2020). Therefore, it is important to learn more about the possible mediators of the variation in insulin sensitivity following bed rest.

Our analysis identified six participants (26%) that improved insulin sensitivity following HDT bed rest. In addition, there were no significant changes in fetuin-A, fasting glucose, or TG in this subgroup. One possibility is that the improvement in whole body insulin sensitivity may be an indirect effect of liver metabolism. The amount of intra-hepatic lipid (IHL) is strongly linked to liver and whole body insulin resistance (Mu et al., 2018; Trouwborst et al., 2018). If liver insulin sensitivity does not decrease, it is possible that the release of fetuin-A would be attenuated and the negative effect on peripheral tissues would be mitigated (**Figure 3**). In support of this hypothesis, fetuin-A levels were found to be significantly higher in metabolically-unhealthy compared with metabolically-healthy obese subjects (Khadir et al., 2018). Fetuin-A knockout (KO) mice display enhanced insulin sensitivity, improved glucose tolerance, and lowered plasma lipid content (Mathews et al., 2002). When fed a high fat diet, fetuin-A KO mice remain insulin-sensitive, are resistant to weight gain and have less adiposity than wild-type controls. As there is inter-individual variation in IHL following lifestyle interventions (Winn et al., 2018), it is possible that fetuin-A could be a driver of peripheral insulin resistance and a biomarker to track the physiological responsiveness to bed rest and physical inactivity.

Despite the highly controlled nature of this bed rest study, there are some limitations to acknowledge. Firstly, the sample size for parallel-designed bed rest studies is generally low, in the order of 8–12 participants per group (Bergouignan et al., 2006, 2009; Rudwill et al., 2015, 2018; Kenny et al., 2017).

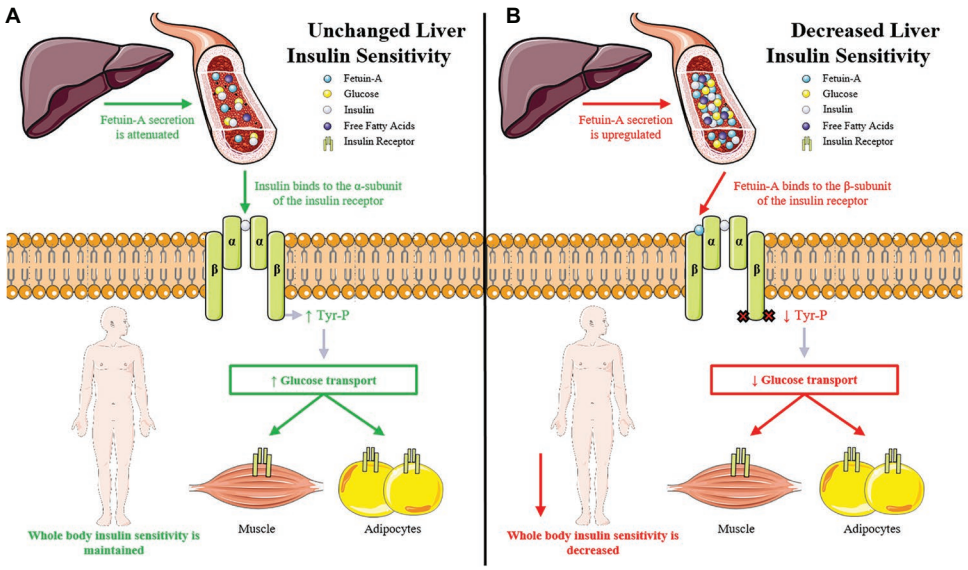

FIGURE 3 | The impact of liver insulin sensitivity and fetuin-A secretion on whole body insulin sensitivity. **(A)** Unchanged liver insulin sensitivity: if liver insulin sensitivity is unchanged then the release of fetuin-A from the liver is attenuated. This allows insulin to bind to the α-subunit of the insulin receptor, which promotes the auto-phosphorylation of the insulin receptor and subsequent tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins. These actions initiate a cascade of events leading to increased glucose uptake in peripheral tissues and an improvement in whole body insulin sensitivity. **(B)** Decreased liver insulin sensitivity: when liver insulin sensitivity is decreased, the release of fetuin-A from the liver is upregulated. Fetuin-A binds to the extracellular portion of the β-subunit of the insulin receptor. Fetuin-A inhibits insulin receptor auto-phosphorylation and tyrosine kinase activity leading to decreased glucose uptake in peripheral tissues and a reduction in whole body insulin sensitivity.

It is possible that the small sample size in the subgroup with improved insulin sensitivity is too small for a definite conclusion, and therefore, we suggest that further studies are required to investigate the potential role of fetuin-A in the regulation of whole body insulin sensitivity following HDT bed rest. Secondly, although it was not the objective of this study, we did not obtain any measurements of liver fat precluding our ability to fully explore the association between fetuin-A and hepatic insulin resistance in response to physical inactivity; this requires further investigation. Thirdly, an OGTT was used to estimate insulin sensitivity. While a euglycaemic-clamp is the gold standard measure, it was not possible in the current study. However, the OGTT sufficiently reflects changes in glucose tolerance and the measurement of whole body insulin sensitivity using the OGTT has been validated previously in healthy non-overweight adults (Trikudanathan et al., 2013). Finally, the results of this study have been obtained from healthy, lean adult males and similar investigations will need to be extended to other populations (e.g., women, elderly, and metabolically unhealthy) to determine the specific links with disease etiology.

In conclusion, we report that 60 days of HDT bed rest led to a significant increase in circulating fetuin-A and decreased insulin sensitivity, which could not be ameliorated by RJT. Exploring individual responses to lifestyle interventions is a growing area of interest in personalized medicine. While HDT bed rest reduced insulin sensitivity at the group level, there was considerable individual responses, including a subgroup for which insulin sensitivity improved. We propose that the regulation of insulin sensitivity is related to circulating fetuin-A, which is attenuated when liver metabolism is maintained. Collectively, our results show that fetuin-A is a candidate biomarker to assess the physiological responses to bed rest and physical inactivity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the North Rhine Medical Association (Ärztekammer Nordrhein) in Düsseldorf, Germany, as well as the Federal Office for Radiation Protection (Bundesamt für Strahlenschutz). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KW, DC, and DO'G had full access to the data used in this study and take responsibility for the integrity of the data and the accuracy of the data analysis. The study was designed by KW, DC, DO'G, EM, and PF-M. EM was the project scientist and PF-M leads the work package "biological samples" and "nutrition" for the ESA RSL study. KW, DC, and DO'G conducted

the biological sample analysis and were responsible for the formal analysis and interpretation of the data. KW, DC, and DO'G drafted the original manuscript and all authors were involved in the critical review for important intellectual content. Approval of the final manuscript was given by all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.573581/full#supplementary-material

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BRIEF RESEARCH REPORT
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Inter-Individual Different Responses to Continuous and Interval Training in Recreational Middle-Aged Women Runners

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A crucial subject in sports is identifying the inter-individual variation in response to training, which would allow creating individualized pre-training schedules, improving runner's performance. We aimed to analyze heterogeneity in individual responses to two half-marathon training programs differing in running volume and intensity in middle-aged recreational women. 20 women (40 ± 7 years, 61 ± 7 kg, 167 ± 6 cm, and VO_2max = 48 ± 6 mL·kg^{-1}·min^{-1}) underwent either moderate-intensity continuous (MICT) or high-intensity interval (HIIT) 12-week training. They were evaluated *before* and *after* training with maximal incremental tests in the laboratory (VO_2max) and in the field (time to exhaustion, TTE; short interval series and long run). All the women participated in the same half-marathon and their finishing times were compared with their previous times. Although the improvements in the mean finishing times were not significant, MICT elicited a greater reduction (3 min 50 s, $P = 0.298$), with more women (70%) improving on their previous times, than HIIT (reduction of 2 min 34 s, $P = 0.197$, 50% responders). Laboratory tests showed more differences in the HIIT group ($P = 0.008$), while both groups presented homogeneous significant ($P < 0.05$) increases in TTE. Both in the short interval series and in the long run, HIIT induced better individual improvements, with a greater percentage of responders compared to MICT (100% vs 50% in the short series and 78% vs 38% in the long run). In conclusion, variability in inter-individual responses was observed after both MICT and HIIT, with some participants showing improvements (*responders*) while others did not (*non-responders*) in different performance parameters, reinforcing the idea that individualized training prescription is needed to optimize performance.

Keywords: middle-aged women, half-marathon runners, high intensity interval training, exercise and sport, moderate intense endurance exercise training, non-responder, responder

INTRODUCTION

One of the general bases of sports training is the principle of individuality, grounded in the specific adaptive responses shown by individuals to a given training program (Matveyev, 1981). This principle of individuality means that the selection or combination of different performance indicators must be carefully chosen to properly assess the training process, since there could be different responses to the same training program (Kenney et al., 2012). A pioneering study by Prud'homme et al. (1984) found considerable individual differences in the adaptive capacity to training in ten pairs of monozygotic twins participating in a short-term endurance training program, with sensitivity to maximal aerobic power being largely genotype-dependent. Later, although similar adaptations in performance and physiological parameters were reported after training schedules involving different exercise volumes and intensities (Gibala et al., 2006; Burgomaster et al., 2008; Scribbans et al., 2014), several studies demonstrated the existence of inter-individual variability in training responses, both after moderate-intensity continuous training (MICT) and high intensity interval training (HIIT; Astorino and Schubert, 2014; Gurd et al., 2016; Whipple et al., 2018).

Participation in half-marathon races has increased across years, especially for the middle-aged female runners (Knechtle et al., 2014, 2016; Knechtle and Nikolaidis, 2018). For example, data from races held in Switzerland between 1999 and 2014 indicate that more endurance athletes compete in half-marathon than in marathons, and that the finisher's men-to-women ratio decreased significantly throughout the years, meaning an increase in women's participation (Knechtle et al., 2016). It is also interesting to note that, between 2014 and 2016 in the world's largest half-marathon (GöteborgsVarvet), approximately 44% of the women's runners were in the middle-aged group (35 to 50 years; Knechtle and Nikolaidis, 2018). Although amateur running is a leisure activity that has become increasingly popular in recent decades (van Dyck et al., 2017), there are still a few studies focusing on middle-aged non-elite women recreational runners (Machado et al., 2011). Only recently, some studies have been centered on middle-aged female marathon recreational runners, highlighting the effect of sex and age on pacing (Nikolaidis et al., 2018), and reporting new data on anaerobic power and neuromuscular fitness for this population group (Nikolaidis and Knechtle, 2018). These findings are of practical relevance for practitioners and coaches, considering their implications in racing times. Thus, to determine the variability (inter-individual differences) in the responses to different training programs among middle-aged women who normally participate in recreational running would be useful for optimizing strength and pacing times and hence improving health-related physical fitness and race finishing times.

During the last decade, several schedules for preparing half-marathon have been published including different strategies regarding the volume and the intensity of the training. There are programs prioritizing MICT with maximal weekly running volumes of 40 km and long run sessions of 25 km (Galloway and Galloway, 2012), contrasting to those where HIIT and fast pace short distance runs predominate (Lanier et al., 2012). Other schedules alternate high-volume programs with speed and hill training (Hamilton and Sorace, 2018) or with resistance training (Hagerman, 2005). A recently published study from our laboratory (Bonet et al., 2020) was aimed to assess if a mixed program, with HIIT sessions of 40 s to 90 s followed by short recovery periods and interspersed with low-volume endurance sessions, was better for training a half-marathon than a traditional MICT program, based on high-volume endurance sessions of moderate-intensity training. We reported a detailed description of the training schedules, data on the performance and physiological changes elicited by each training program.

Here, we present a preliminary brief research report focused on the analysis of the heterogeneity in individual responses to the training programs. We analyze some parameters that could affect performance and could induce different responses depending on the training program. Our hypothesis was that the HIIT program would result in a more homogeneous response than the MICT program, since HIIT would activate more skeletal muscle metabolic ways and promote greater changes in cardiovascular structure and function. To study the inter-individual variation in response to training is of interest because it has been reported that individuals who fail to respond to an endurance exercise protocol may respond to other training protocols, such as resistance or interval training (Hautala et al., 2006; Bonafiglia et al., 2016). Identifying responders and non-responders to a given training protocol would allow creating individualized pre-training schedules and improve runner's performance. This would be especially interesting in the population group studied here: middle-aged women, who run at a recreational level, normally having difficulties in combining sports practice with daily professional and family life (Macias et al., 2014). As suggested in a recent review (Pickering and Kiely, 2019), more research is needed to identify responders and non-responders to exercise interventions so that alternative training schedules can be developed for non-responders to increase their fitness in an effective manner.

MATERIALS AND METHODS

Subjects

A total of 20 women aged 40 ± 7 years, 61 ± 7 kg, and 167 ± 6 cm with a body mass index of 23 ± 3 (mean ± SD) participated in this study. They were recruited from different running clubs in the city of Barcelona (Spain) after completing a questionnaire and an interview to assess the following inclusion criteria: to be pre-menopausal, non-smokers, to have no injuries and not to take any medication (including oral contraceptives). Moreover, they age range must be between 35 and 45 years and be regular runners, training a minimum of 5 h and 3 days per week and with previous experience in running half-marathons recreationally. The women were randomly divided into two training groups (n = 10 in each): (1) MICT group, which participated in a high-volume and low-intensity training program; and (2) HIIT group, which completed a low-volume and high-intensity interval running program with bodyweight resistance exercises. No significant statistical

differences were observed between randomly selected groups in performance parameters prior to training (**Table 1**). There were no significant differences in the anthropometrical parameters between the groups both at the beginning and at the end of the training protocols. After being informed about the experimental procedures, as well as their risks and benefits, the participants signed an informed consent form and were free to withdraw from the experimental protocol at any time. The study was developed in accordance with the Declaration of Helsinki concerning the ethical principles of human experimentation and was approved by the Institutional Ethics Committee of the University of Barcelona (Institutional Review Board number, IRB00003099).

Training Programs

Both training programs lasted for 12 weeks, involving 3 non-consecutive sessions per week, from September to December after a 1-month rest in August. For a detailed explanation of the training programs, see Bonet et al. (2020). After the training programs, all the women participated in the same half-marathon race held in Vilanova i la Geltrú (Spain), located at sea level on the Mediterranean coast (41°13'27" N, 1°43'33" E). The race had a mostly flat profile with slight ups and downs between kilometer 9th and 10th and from the 20th to the finish line. Weather conditions during the race were sunny and windless with a temperature of 15°C.

Moderate-Intensity Continuous Training

The MICT group followed a training program based on the one described in Galloway and Galloway (2012). This consisted of 2 days of continuous running (40 min and 60 min) and 1 day alternating between long-distance running (from 12 km to 25 km) and 800-meter intervals every week, that were run in approximately 4 min. The total distance trained was approximately 383 km and the overall time invested, calculated as an average among all the participants, was 40 h and 30 min.

High-Intensity Interval Training

The HIIT group participated in a weekly training program that was designed to reduce training volume and increase training intensity. The program consisted of a first session of long-distance running (from 8 km to 16 km), a second day dedicated to interval running (200-m and 400-m series), and a third day that alternated between an uphill run and a fast run with bodyweight resistance exercises. The speed of the long-distance running on the first day was calculated for each subject based on their percentage of VO_2max. The 200-m or 400-m series on the second day were grouped into 1, 2, or 3 blocks, with recoveries between series of the same block ranging from 30 s to 1 min and the recoveries between blocks lasting 3 min. The uphill run on the third day of the week consisted of climbing 100-m 10% to 12% slopes at an intensity of 85% VO_2max, which was followed by running downhill at a slow pace and then a 10-min fast run at 80% VO_2max. This was combined with a circuit of 12 stations of weight resistance exercises performed at maximum intensity, based on the training described in Klika and Jordan (2013). When the circuit was completed, 4 50-m sprints and 3 30-s sets of Bosco's countermovement jump tests (Bosco et al., 1983) were performed in order to improve anaerobic power and increase the efficiency of using elastic energy. The total distance trained by the HIIT group was approximately 301 km and the overall time invested, calculated as an average among all the participants, was 33 h and 26 min. Thus, the women in the HIIT program covered 21% less distance and invested 17% less time than those in the MICT group.

TABLE 1 | Finishing times for the half-marathon and results from the maximal incremental tests performed in the laboratory (VO_2max) and on the athletic track (field time to exhaustion, TTE) *before* and *after* the training programs.

	Whole group			**MICT**			**HIIT**			
Finishing time (h:min:s)	**Mean**	**Range**	**CV (%)**	**Mean**	**Range**	**CV (%)**	**Mean**	**Range**	**CV (%)**	***P*-value MICT *vs* HIIT**
Before	1:59:36	0:45:33	9.4	2:02:27	0:32:51	7.1	1:56:45	0:36:23	11.3	0.268
After	1:56:26	0:36:54	8.4	1:58:37	0:23:40	7.0	1:54:16	0:36:54	9.6	
P-value	0.085			0.298			0.197			
VO_2max ($mL \cdot kg^{-1} \cdot min^{-1}$)	**Mean**	**Range**	**CV (%)**	**Mean**	**Range**	**CV (%)**	**Mean**	**Range**	**CV (%)**	***P*-value MICT *vs* HIIT**
Before	47.9	18.3	11.6	46.1	14.6	10.5	49.7	16.2	11.9	0.153
After	46.0	18.7	11.3	45.8	13.7	9.8	46.1	18.7	13.2	
P-value	0.05			0.833			0.008			
TTE (h:min:s)	**Mean**	**Range**	**CV (%)**	**Mean**	**Range**	**CV (%)**	**Mean**	**Range**	**CV (%)**	***P*-value MICT *vs* HIIT**
Before	0:15:59	0:09:17	14.2	0:16:19	0:06:26	12.7	0:15:38	0:09:11	15.8	0.508
After	0:16:36	0:09:52	13.3	0:17:06	0:07:16	13.4	0:16:06	0:07:27	13.1	
P-value	0.002			0.035			0.029			

Results are shown for the whole group consisting of all the women participating in the study, as well as separately for the moderate-intensity continuous training (MICT) and high-intensity interval training (HIIT) groups. Data are given as the mean, range and coefficient of variation (CV). P-values in the rows show the statistical significance of the differences between before and after conditions, as determined using paired Student's t-test. P-values in the last column show the absence of statistical significance between randomly assigned participants to MICT and HIIT programs prior to the training.

Maximal Incremental Tests

Laboratory

All subjects performed two University of Montreal Track Tests (Léger and Boucher, 1980) in the laboratory (L-UMTT) on a treadmill (Quasar h/p Cosmos® Sports & Medical GmbH, Nussdorf-Traunstein, Germany) to determine the maximal oxygen uptake (VO_2max). The first test was performed during the first week, prior to the beginning of the training programs; and the second during the last week, prior to the half-marathon race. Briefly, the L-UMTT consisted of: (1) an initial 4-min warm-up period at a speed of 6 km·h^{-1} and a slope of 0.6°; (2) an incremental phase with increases of 1 km·h^{-1} every 2 min until exhaustion; and (3) a 6-min recovery period that involved sitting down to rest. This test was preceded by a resting electrocardiogram (ECG; CardioScan v. 4.0, DM Software, Stateline, NV, United States) and a 5-min standing rest to determine the baseline VO_2 with an automated gas analysis system (TR-plus Metasys, Brainware SA, La Valette, France) equipped with a two-way mask (Hans Rudolph, Kansas, United States).

Field

To provide a follow-up to the training process and adjust the training loads, two field UMTTs (F-UTTM) were conducted on an official athletics track. Field time to exhaustion (TTE) was recorded at the end of each test. The first test was performed prior to the beginning of the training programs, separated by at least 72 h from the first L-UMTT to avoid the effects of residual fatigue on performance. The second F-UMTT was conducted at the end of the 8th week of training. Cones were set at 50-m intervals along the track and the speed of each stage was controlled by an examiner equipped with a whistle and a chronometer, who emitted sounds when the subjects had to pass a cone in order to maintain a constant speed for each stage of the test. The test finished when the participant was either behind a cone on three consecutive occasions or when she could no longer keep the pace required to pass the cones and decided to stop the exercise.

Two field tests, at the beginning and at the end of the training periods, were performed to assess power performance in short-run series (200 m and 400 m for HIIT and 800 m for MICT) and the long run pace (8 km for both training programs). The first short-run series test (*before*) was performed during the first week (200-m and 800-m series) and second week (400-m series) of training, whereas the second test (*after*) was conducted during the tenth (200-m and 800-m) and eleventh (400-m series) week. The long run tests were performed for both training programs during the first (*before*) and tenth (*after*) week of training.

Statistical Analysis

We powered the sample size on the variable finishing time to fit the power parameters of $\alpha = 0.05$ and $\beta = 0.20$, estimating both the size of the change to be detected and the size of the standard deviation change as 0.05. A minimum sample size of $n = 10$ was required for paired *t*-tests (comparing *before* vs *after* parameters for each training program). Since an initial sample of twenty-two participants was selected during the recruitment procedure, the study began with eleven participants in each group. However, one woman from each group failed to follow the complete training schedule. Thus, the final sample contained 10 women per group. After checking normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene test), to evaluate changes in the performance indicators, we used paired Student's *t*-tests (comparisons: *before* vs *after*). One-way ANOVA was run to evaluate intragroup differences in the finishing times between the different types of responders. *P*-values are given throughout the text, tables and figures, considering significant statistical differences at $P < 0.05$. To evaluate variability in the responses to the training programs, we used the coefficient of variation (CV), i.e., the ratio between the standard deviation and the mean (expressed as %). This parameter is normally used to assess variability in a group's response to a stimulus such as a training program. All data were statistically analyzed using SigmaPlot 11 (Systat Software, Inc., San Jose, CA, United States, 2008–2009).

RESULTS

Half-Marathon Finishing Times

After completing the training period, the mean finishing time obtained in the half-marathon by the whole group (i.e., irrespective of the training schedule) showed a non-significant ($P = 0.085$) improvement (**Table 1**). The previous finishing times were obtained in the half-marathon held 10 months before in Granollers (Spain, 41°36'30" N, 2°17'20" E), with a similar profile, 145 m of elevation and similar weather conditions. This improvement consisted of a 2.4% reduction compared to the mean of previous finishing times, indicating a decrease of 3 min and 10 s compared to the mean of previous finishing times. However, when the data were analyzed by considering the training programs separately, the MICT and HIIT groups behaved differently.

The MICT group showed a non-significant ($P = 0.298$) 2.9% reduction compared to its mean of previous finishing times (**Table 1**), indicating that it took them 3 min 50 s less to complete the half-marathon compared to previous times. The times before and after the training program showed a similar intragroup variability (CV∼7%), with more than a 9-min difference in the range of the times obtained in the half-marathon after the training protocol. Among the women that followed this training program, three did not improve on their previous finishing times, while another three achieved a time reduction greater than 10% (**Figure 1A**). Thus, regarding their response to the MICT program, the participants were classified into three categories showing significant differences ($F = 159.9$, $P < 0.001$) in their mean finishing times: (i) three subjects were *high responders*, with a mean time reduction of 15 min; (ii) four subjects could be considered *normal responders*, achieving a mean decrease in their finishing time of 2 min 26 s; and (iii) three subjects were *non-responders*, increasing on their mean previous finishing time by 5 min 31 s.

The HIIT group showed a non-significant ($P = 0.197$) 1.8% decrease compared to its mean of previous finishing times (**Table 1**), which meant that it took them 2 min 34 s less to complete the half-marathon compared to their previous finishing

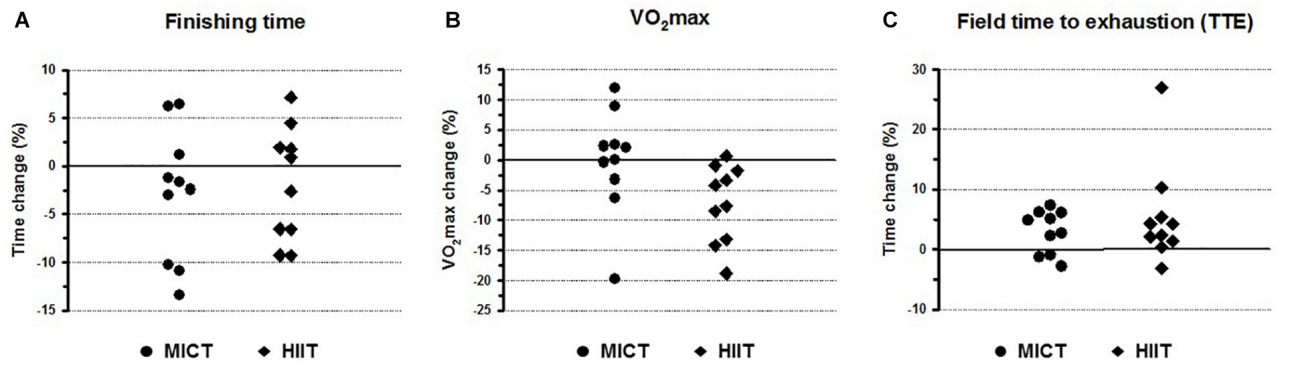

FIGURE 1 | Individual percentage changes *before* and *after* the training program in women participating in moderate-intensity continuous training (MICT) and high-intensity interval training (HIIT). **(A)** percentage changes in time comparing previous (*before*) finishing times and the finishing times registered in the half-marathon race *after* the training programs. **(B)** percentage changes in VO_2max obtained in a laboratory maximal incremental test *before* and *after* the training programs. **(C)** percentage changes in time to exhaustion (TTE) obtained in a field maximal incremental test *before* and *after* the training programs.

times. The times obtained *after* the training program showed a slight reduction in intragroup variability (CV = 9.6%) compared to the times obtained *before* the training (CV = 11.3%), but with a similar range difference of ∼36 min in both cases. The women in the HIIT group were classified into two categories (**Figure 1A**) showing significant differences (F = 38.2, $P < 0.001$) in their mean finishing times: (i) five were *responders*, with a mean time reduction of 7 min 54 s compared to the mean of their previous finishing times; and (ii) five were *non-responders*, increasing on their mean previous finishing time by 3 min 37 s.

Maximal Incremental Tests

When considering the whole group, the VO_2max recordings ($mL \cdot kg^{-1} \cdot min^{-1}$) obtained in the laboratory maximal incremental tests (L-UMTT) after the training protocols showed a significant decrease of 3.7% (P = 0.05) compared to the values obtained before the training started (**Table 1**). However, when the training groups were analyzed separately, VO_2max did not show significant differences (P = 0.833) *before* and *after* the training program in the MICT group, whilst a significant decrease of 7.2% (P = 0.008) was observed in the HIIT group. Moreover, there was greater variability in VO_2max after the training program in the HIIT group, which presented a larger CV (13.2%) and range (18.7 mL $O_2 \cdot kg^{-1} \cdot min^{-1}$) than the MICT group (CV = 9.8%; range = 13.7 mL $O_2 \cdot kg^{-1} \cdot min^{-1}$). **Figure 1B** shows that half the women in the MICT group increased their VO_2max, while the other half of the group decreased their VO_2max. By contrast, almost everyone (9 out of 10) in the HIIT group decreased their VO_2max.

Regarding the maximal incremental test performed in the field (F-UTTM), the whole group significantly increased (P = 0.002) the TTE in the 8th week of training compared to that recorded in the F-UMTT prior to the start of the training programs (**Table 1**). When the training programs were considered separately, the TTE significantly increased in both the MICT (4.7% increase) and HIIT (3.0% increase) groups (**Table 1**). Variability in this performance indicator after the training programs was similar in both groups, with almost the same CV (∼13%) and range (∼7 min). However, there were some differences between the groups in the responses, since three participants in the MICT group failed to improve their times, whilst only one from the HIIT group worsened on her previous times (**Figure 1C**).

Field Training Tests

Figure 2 shows the percentage variations in individual field tests performed to assess (i) the interval training power output in the 800-m series (MICT program) and in the 200-m and 400-m series (HIIT program; **Figure 2A**), and (ii) the long run pace over 8 km (**Figure 2B**).

The women in the MICT group showed varying improvements in their power output in the 800-m interval series. Approximately half of them increased their power output after the training program, while the other half did not increase or even decreased it. This was in contrast to the clear improvement in all the women in the HIIT group for both the 200-m and 400-m series, with mean increases of 9% and 20%, respectively.

The mean long run pace over 8 km showed a 2% to 3% non-significant decrease, i.e., less time was invested per km. Moreover, the analysis of individual results for this field test (**Figure 2B**) indicated that, overall, the women in the HIIT group showed a better response to training than those in the MICT group (78% vs 38% improved their pace).

DISCUSSION

Our study examined responses to two training programs involving different intensities and running volumes, both performed 3 days per week over 12 weeks. A global improvement in field performance was observed at the end of the training period for the two training groups. However, there were different inter-individual responses after the training period, with some participants showing improvements in performance indicators (*responders*), while others failed to exhibit positive changes (*non-responders*).

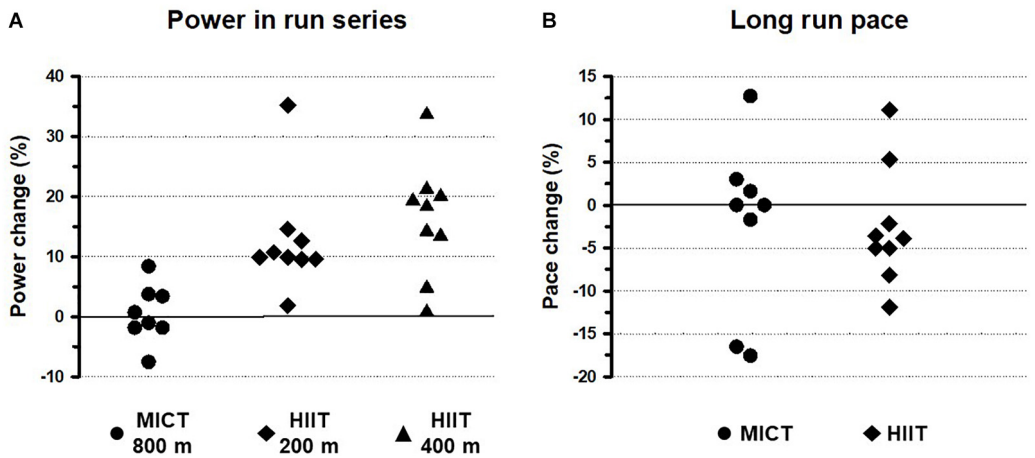

FIGURE 2 | Individual percentage changes *before* and *after* the training program in women participating in moderate-intensity continuous training (MICT) and high-intensity interval training (HIIT). **(A)** Percentage changes in power obtained in interval run series in the field *before* and *after* the training programs. **(B)** Percentage changes in the long run pace (8 km) in the field *before* and *after* the training programs.

Regarding the finishing times in the half-marathon, the MICT group exhibited a wider range in their responses, with more responders (70%) in this group than in the HIIT group (50%; **Table 1**). The fact that there were responders and non-responders in both groups (**Figure 1A**) indicates that the adaptive response to the training programs varied individually, even if the participants followed a standardized and supervised endurance training program with prescribed intensities based on their percentage of VO_2max. This finding in middle-aged women is in accordance with those of studies on young women (McPhee et al., 2010) and men (Vollaard et al., 2009; Scharhag-Rosenberger et al., 2010). These studies concluded that it is advisable to standardize exercise intensity using other measures related to performance power output, such as blood lactate, rather than the percentage of VO_2max, due to the inhomogeneous blood lactate responses obtained after prolonged endurance exercise at given percentages of VO_2max. This is especially relevant in advanced age, as has been reviewed by Lepers and Stapley (2016), who reported that VO_2max seems to be the parameter most altered by age, contrasting to exercise economy and the lactate threshold, which decline to a lesser extent with advancing age. Taking into consideration this variability in the responses, an individualized exercise prescription might be needed instead of standardized methods. Some proposals have been made, such as those based on kilocalories expenditure per week in relation to body mass (Weatherwax et al., 2016).

Regarding VO_2max, it must be noted that we found a decrease at the end of the training programs, which was significant in the HIIT group (**Table 1**). Moreover, all the participants in the HIIT group were *non-responders* for this performance indicator (**Figure 1B**), suggesting a worsening in performance. However, this could not be the case, especially at high loads, if other performance variables, such as running economy, were improved. Silva et al. (2017) reported that a HIIT program mediates a reduction in the energetic cost of running, allowing runners to achieve higher speeds at the end of the maximal incremental treadmill test without significant increases in the VO_2max. There is also some evidence that improvements in high-intensity aerobic performance are not strongly associated with improvements in VO_2max since low responders for one parameter are not necessarily low responders for another (Vollaard et al., 2009). Moreover, greater efficiency at moderate and high loads in a maximal progressive short effort could indicate the effectiveness of training, but its translation to a specific performance (e.g., half-marathon) is of less importance, since the speeds of running a half-marathon are lower than those at which the improvement has occurred.

Our results also indicated that focusing on other specific tests in the field could be a better tool in assessing the improvement in endurance elicited by training programs. Thus, in both groups, maximal incremental tests in the field showed that after the 12-week training period, most of the participants increased their TTE, exhibiting a homogeneous response (**Figure 1C**) and significantly higher mean times (**Table 1**). However, the two groups showed different behaviors in their responses for the interval series (power) and long run (pace) field tests (**Figure 2**). For both tests, there was a greater number of responders among the women in the HIIT group than in the MICT group. This could be due to the fact that the HIIT sessions of short-burst intervals interspersed with low-intensity recovery periods lead to a strong engagement of neuromuscular and musculoskeletal systems (improving power in 200-m and 400-m series), allowing individuals to run at high intensities with low lactate levels (increasing pace during long runs; see for review, Buchheit and Laursen, 2013). The heterogeneity in the responses and the different behaviors in the MICT and HIIT groups reported here for middle-aged women are consistent with those recently found for young males and females (Bonafiglia et al., 2016) and also with the findings of a multi-center comparison study of trainability by Williams et al. (2019) involving a big sample size with different ages and conditions.

The different individual responses to the training interventions, with some subjects as *responders* and others as *non-responders*, may be a consequence of a combination of two factors. First, all the women participating in this study were amateur runners with some level of previous training experience. This previous high or near maximal physical fitness could have affected the changes in the performance indicators analyzed in some participants, since diminished improvements in performance indicators such as VO_2max have been described in trained subjects enrolled in training programs, in contrast to the rapid increases observed in untrained individuals (Wenger and Bell, 1986). Second, responses to training might be highly individual, as has been recently described for HIIT by Astorino et al. (2018), who found that some participants showed meaningful increases in some performance variables, whereas others showed no changes in their previous values. Several anatomical, biochemical and physiological systems interact to influence sports performance and could account for this inter-individual variability to training. The individual differences in metabolic pathways could quantitatively increase or decrease the measured parameters and induce synergistic or antagonistic effects depending on the training protocol. For example, it is well known the human individual variation in skeletal muscle fiber-type proportion (Simoneau and Bouchard, 1989) which is highly correlated with sports performance (Costill et al., 1976). Other factor involved in the variability of the responses to training could be the metabolic and biomechanical specificity of each training program (Kenney et al., 2012). The different constraints imposed by MICT or HIIT would result in more or less evident changes in performance depending on the characteristics of each training schedule and the parameters assessed. This could explain finding more responders in the HIIT group in the interval run series (**Figure 2A**).

Our results provide further support for individualized exercise prescription to optimize workouts. Furthermore, our findings suggest that several performance indicators can be used to assess training programs. For instance, the analysis of continuous variables throughout a training process beyond maximal values could provide more sensitive information to determine specific adaptations in different training programs. This has been recently demonstrated by Garcia-Retortillo et al. (2019), who compared HIIT and MICT programs and observed that, despite improving markers of aerobic fitness to a similar extent, changes in cardiorespiratory coordination were specific for each training intervention. For endurance recreational runners, moderate continuous training with extensive aerobic loads is the most frequently used type of training (see for example, Fokkema et al., 2020). However, a huge amount of time is invested in training and the protocols are repetitive, increasing the risk of musculoskeletal injuries due to overuse (Dias-Lopes et al., 2012). In fact, one of the initial participants following MICT abandoned the study suffering from an injury. Conversely, high intensity training with low repetition not only reduces training time, but also decreases the risk of injuries due to overuse, as has been recently reported by Mallol et al. (2020). In the present work, we did not report any injuries in HIIT ruling out the possible increased risk of acute injury due to the greater intensity of the HIIT program.

The main strength of this study was the homogeneity of the group studied, i.e., middle aged women with previous amateur experience in running events. This population group is poorly studied and has become increasingly involved in amateur running events in recent years (van Dyck et al., 2017). The main limitation of the study was the small sample size of $n = 20$, with $n = 10$ in each training group. It would have been better to have a greater sample size, which, undoubtedly, would have rendered higher power to the conclusions and provided more sensitivity to detect significant differences in the eventual changes. Similar studies could be performed in the future in population groups with different genders and/or ages, as well as using participants with different levels of fitness (from sedentary to elite athletes). Our idea for future studies is to design experimental work that can identify in advance responders and non-responders with the aim of creating a pre-training schedule that can be modified to help non-responders improve their performance. In this sense, incorporating complex system approaches, such as those reported by Balagué et al. (2016) and Garcia-Retortillo et al. (2017) on cardiorespiratory coordination, will be of great value in assessing a strategic research framework for individual training prescriptions.

CONCLUSION

Two different training programs for a half-marathon, one based on high intensity and moderate training volume (HIIT) and the other involving moderate intensity and greater training distances and times (MICT), were compared in a group of amateur middle-aged women runners. A global improvement in the mean finishing time for the half-marathon and improvements in field performance indicators at the end of the training period, such as TTE, power in short-run series and long run pace, were observed in the two training groups. However, there were different inter-individual responses after the training period, with some participants showing improved performance (*responders*) and others failing to exhibit positive changes (*non-responders*). These different responses depended on the training group, with more heterogeneous results in HIIT group. As a future perspective, these findings could help running coaches and amateur running practitioners to modify workloads to optimize performance. Compiling data on individual measurements (such as anthropometrical, epidemiological, physiological, and those regarding to performance), a predictive model could be constructed with the goal of deciding the suitability of the training protocol to be applied. Depending on the runner's vital status, a MICT, HIIT or a mixed training model could be prescribed, optimizing runner's effort and time dedicated to training, which would decrease injury risk factors and improve training adherence.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Ethics Committee of the University of Barcelona (Institutional Review Board number, IRB00003099). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CJ and JT conceived and designed the study. JB and CJ conducted the experiments. JB, CJ, and JT analyzed the data and wrote the draft manuscript. JV, JM, GV, and TP corrected the draft manuscript and contributed the analytical tools. All authors read and approved the manuscript.

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PERSPECTIVE
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The Role of Long-Term Head-Down Bed Rest in Understanding Inter-Individual Variation in Response to the Spaceflight Environment: A Perspective Review

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Exposure to the spaceflight environment results in profound multi-system physiological adaptations in which there appears to be substantial inter-individual variability (IV) between crewmembers. However, performance of countermeasure exercise renders it impossible to separate the effects of the spaceflight environment *alone* from those associated with exercise, whilst differences in exercise programs, spaceflight operations constraints, and environmental factors further complicate the interpretation of IV. In contrast, long-term head-down bed rest (HDBR) studies isolate (by means of a control group) the effects of mechanical unloading from those associated with countermeasures and control many of the factors that may contribute to IV. In this perspective, we review the available evidence of IV in response to the spaceflight environment and discuss factors that complicate its interpretation. We present individual data from two 60-d HDBR studies that demonstrate that, despite the highly standardized experimental conditions, marked quantitative differences still exist in the response of the cardiorespiratory and musculoskeletal systems between individuals. We also discuss the statistical concept of "true" and "false" individual differences and its potential application to HDBR data. We contend that it is currently not possible to evaluate IV in response to the spaceflight environment and countermeasure exercise. However, with highly standardized experimental conditions and the presence of a control group, HDBR is suitable for the investigation of IV in the physiological responses to gravitational unloading and countermeasures. Such investigations may provide valuable insights into the potential role of IV in adaptations to the spaceflight environment and the effectiveness of current and future countermeasures.

Keywords: microgravity, countermeasure exercise, spaceflight, inter-individual variability, bed rest, musculoskeletal, cardiorespiratory

INTRODUCTION

Inter-individual variation (IV), where participants display markedly different responses to a standardized intervention, is a recognized phenomenon in clinical and basic research studies (Bouchard and Rankinen, 2001; Timmons, 2010). This variation in responses led, initially, to the adoption of terminology such as "responders" and "non-responders" (Timmons, 2010), which has subsequently evolved into more precise definitions including individuals that "did not respond" (Pickering and Kiely, 2019) or have "low sensitivity" (Booth and Laye, 2010). The careful identification and quantification of IV has important implications, not only the optimization of health interventions, but also determination of pathophysiological processes that can underpin the provision of personalized medicine. Should such IV also exist in response to the spaceflight environment, known to induce multi-system physiological adaptation (Demontis et al., 2017), and the performance of countermeasure (CM) exercise in an attempt to mitigate these adaptations (Loehr et al., 2015), this could have important implications for astronaut health management, particularly during future exploration missions where the operational constraints will be more severe (Scott et al., 2019).

In this Perspective, we review the available evidence of IV in response to the spaceflight environment and discuss biological, operational, and environmental factors that may contribute to it, and thus complicate its interpretation. We also present individual data from two 60-d head-down bed rest (HDBR) studies to evaluate the existence of IV, as HDBR is considered the most appropriate ground-based analog (Hargens and Vico, 2016) of cardiovascular and musculoskeletal deconditioning associated with spaceflight (Pavy-Le Traon et al., 2007).

EVIDENCE OF IV IN HUMAN SPACEFLIGHT DATA

A significant barrier in understanding IV in response to spaceflight is the manner in which data are published. The primary goal of most experiments in space is to compare data points (e.g., pre- to post-flight) or conditions/groups (e.g., crew following of two different diets) (Zwart et al., 2018) and, as such, data are typically presented only as group means with standard deviations/errors, although there are exceptions (Moore et al., 2014, Rittweger et al., 2018; McNamara et al., 2019). In addition to selected scientific experiments, a standard set of physiological measurements is performed before, during, and after flight as part of medical monitoring by the Space Agencies, and thus this data set comprises of data from astronauts that have flown on different space missions [e.g., NASA's Shuttle and International Space Station (ISS)] with varying crew compositions and a wide range of durations (Smith et al., 2020). As these measurements are specifically for medical monitoring, they are not, by default, analyzed and published. However, some of these data have been published, both as group means and individual data (Spector et al., 2009; English et al., 2015; Sibonga et al., 2015). Data from spaceflight studies where individual data has been provided suggest that marked IV exists in the response of the musculoskeletal and cardiovascular systems during human space missions (**Figure 1**). However, the complex nature of human spaceflight missions means that this apparent IV must be interpreted with care.

INTERPRETATION OF IV FROM SPACEFLIGHT DATA

A major issue in understanding IV in response to the spaceflight environment – comprising of a number of factors such as microgravity, radiation, and space-specific nutrition – is the absence of astronauts who have performed no CM exercise (Scott et al., 2019). As many microgravity-induced physiological adaptations appeared to reflect those that occur with prolonged inactivity, physical exercise was identified as a key adaptation management strategy (Berry et al., 1962; Moore et al., 2010). As a consequence, CM exercise has been used in some form in almost all space missions. On ISS, exercise is the cornerstone of the CM program for long-duration missions (LDM) (Loehr et al., 2015) and Flight Rules dictate that all LDM crewmembers must perform CM exercise, which precludes abstinence, or intervention studies with a "no exercise" control group. As a result, all recent physiological data collected from space missions reflects the physiological responses to both the spaceflight environment *and* CM exercise.

Interpretation of spaceflight data with respect to IV is further complicated by the lack of standardization of the CM exercise program between agencies and individuals within a program, and thus can vary markedly between crewmembers (Rittweger et al., 2018). This is a result of several factors:

- Evolving CM exercise device technology. CM exercise devices have evolved over time and, in general, increased in their capacity to provide training stimuli. As a result, in the past, stronger, fitter crew may have been significantly limited by device capacity and thus received a sub-optimal exercise stimulus (Korth, 2015). It is possible that, only recently on ISS, have exercise programs not, in some way, been limited by device capacity. Even during the lifetime of ISS, the complement of exercise devices has changed, with more recent (post-2009-2010) crew having access to upgraded aerobic and resistance devices (Korth, 2015). As such, even when a study reports data from the same mission (e.g., ISS), if individual astronaut flights included in a study span a prolonged time period (e.g., English et al., 2015), it is possible that not all crewmembers had access to the same exercise devices.
- Increase in volume available for devices. With the advent of space "stations" (e.g., MIR and ISS), the volume available for exercise devices has increased and, as a result, so has the number and variety. As such, whereas previously crew had access to only a single, uni-modal (e.g., cycle ergometer) device, ISS crew are now able to perform both aerobic

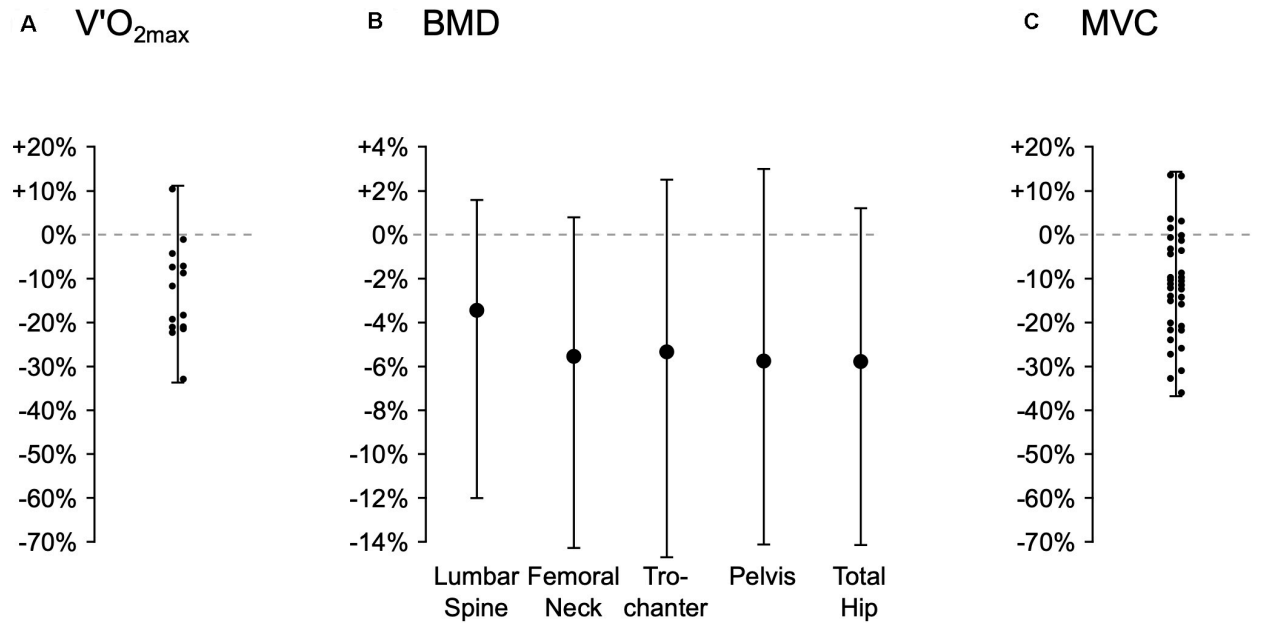

FIGURE 1 | Range of individual changes (percentage change from pre- to post-flight) in key physiological parameters from spaceflight studies. The figure shows derived estimates of changes in maximal rate of oxygen uptake (VO_{2max}, Panel **A**, extracted from Figure 3; Moore et al., 2014), bone mineral density (BMD, Panel **B**, extracted from Figure 2; Sibonga et al., 2015) and maximal force production of the knee extensors during a maximal voluntary contraction (MVC, Panel **C**, extracted from Figure 6; English et al., 2015). Minima, mean, and maxima data, and where possible (i.e., no overlap of data points) individual data points, were extracted from published figures using WebPlotDigitizer (United States). Thus, for Figure 2 of Sibonga et al. (2015), only the minima, mean and maxima data are presented.

and resistance exercise, and choose between mechanically-loaded (treadmill running) and unloaded (cycle ergometry) aerobic exercise (Korth, 2015);

- Non-availability of devices within a mission. Sometimes exercise devices may not be available or their use constrained. For instance, on ISS between 2001 and 2004 on several occasions, one or more of the three exercise devices (CEVIS, T2, and iRED) were not operating nominally or unavailable (Hayes, 2015). As a result, even when a study reports data from crew who flew close to each other chronologically, it is possible that their use of devices differed;
- Operational factors and priorities. As important as CM exercise is considered by spaceflight operations, on occasions, it must be canceled due to activities related to arriving and departing vehicles, external "space walks," science experiments and internal engineering/maintenance activities. Thus, through no fault of their own, an individual crewmember's exercise program may be interrupted or constrained. In addition, both before and after missions, operational factors, and logistics can result in variation in the timing of pre- and post-flight measurements, and on occasions even cancellation at the request of the astronaut or attending Flight Surgeon.
- Adherence to the CM exercise program. Finally, astronauts' attitudes to, and motivation for, exercise vary. Space Agencies provide crew with exercise devices, with exercise CM programs to follow, time to perform them, and education regarding their importance. However, the performance of CM exercise is an individual crewmember's choice and thus engagement and adherence vary, including both the number, and intensity and duration, of sessions.

In summary, although the data in **Figure 1** suggests marked IV in the biological response to the *combined* effects of the spaceflight environment and CM exercise, it is impossible to exclude the influence of factors including those outlined above. Indeed, some published data suggest that the quantity and "quality" of CM exercise may be a significant factor in the magnitude of spaceflight adaptation (Moore et al., 2014; Lee et al., 2015; Rittweger et al., 2018). However, as exercise performance metrics are considered private medical data by the Space Agencies, this data is rarely published or accounted for in the analysis.

There are also a number of individual biological effects related to exercise that may, in part, contribute to IV in spaceflight studies:

- Age. Astronauts have careers lasting several decades with the average age tending to increase (Smith et al., 2020). Age may influence bone's adaptation to both mechanical loading (Rubin et al., 1992; Turner et al., 1995) and unloading (Perrien et al., 2007), as well as re-loading following unloading (Cunningham et al., 2018), although the effect upon the responsiveness of muscle (Cartee, 1994) or cardiorespiratory function (Mazzeo et al., 1984; Rossiter et al., 2005) is less clear. Thus, the importance of age across

the typical astronaut career range (30–60 years old) is not well understood.

- Pre-flight physiological status. There is considerable variation between individual astronauts in the pre-flight values of parameters such as bone mineral density, muscle force production capacity, and maximal rate of oxygen uptake (VO_{2max}) (Orwoll et al., 2013; Moore et al., 2014; English et al., 2015; Sibonga et al., 2015). To what extent this reflects genetic differences and/or the effects of physical activity (i.e., adaptation) is unknown, but they may reflect differences in training history and thus training status. If so, the transition from Earth's gravity (normal mechanical loading) to space (no mechanical loading), may represent markedly different adaptive stimuli for different crewmembers. This may also be true for the transition from pre-flight exercise habits – which are likely highly variable between crewmembers and may also vary within an individual in the intensive pre-launch period – to the high volume ISS CM exercise program (Korth, 2015), as prior capacity may influence the response to an aerobic exercise intervention (Milanović et al., 2015) and training history to a strength intervention (Mangine et al., 2018). Some bed rest data does suggest the reduction in VO_{2max} is dependent on the initial level of aerobic fitness (Convertino, 1997) consistent with a greater magnitude of spaceflight adaptation (loss of aerobic fitness or muscle strength) in crewmembers who have higher pre-flight values (Moore et al., 2014; English et al., 2015).
- Responders vs. non-responders. Differences in pre-study status may, in part, explain the apparent IV in response to terrestrial exercise interventions. Terrestrial studies demonstrate IV in post-exercise training adaptations, with some subjects exhibiting no meaningful improvements (Bouchard and Rankinen, 2001; Timmons, 2010) or even a decrease in capacity (Bouchard et al., 2012). Recent evidence (Pickering and Kiely, 2019), however, suggests that it is unlikely that global non-responders to exercise exist, that the "non-response" can be mitigated by changes in training variables, and that individual responses to an intervention should be considered specific to that intervention, at that time, and with the selected outcome measures. Due to the high number of factors that might influence the pre- to post-flight change in the physiological variables measured from astronauts, to what extent this is the case with the performance of in-flight CM exercise is difficult to elucidate.

Spaceflight also exposes crewmembers to several unique factors that may contribute to spaceflight-induced adaptation and the response to CMs. Radiation exposure in space is markedly different compared to that on Earth, which is associated with a range of biological effects that can differ between tissues and systems (Chancellor et al., 2014). Moreover, radiation may also influence the effects of microgravity (Yatagai et al., 2019). As such, combined with the fact that there is significant IV in the sensitivity to radiation (Cucinotta, 2001), radiation exposure could be a contributor to astronaut physiological IV. Furthermore, although ISS crew are limited to the (largely pre-packaged) on-board food supply and receive nutritional guidance on the optimal quantity and combination of foodstuffs to consume, they are free to choose their own food from the pantry. As a result, nutritional intake varies between individuals and, possibly combined with space-specific issues such as motion sickness, loss of appetite, and difficulties in metabolizing food in microgravity (Laurens et al., 2019), may result in different energy intakes. Energy intake required for energy balance varies with body size and the level of physical activity (including CM exercise) (Scott et al., 2020) and there is evidence of a negative energy balance (Stein, 2000) and the loss of body mass (Wade et al., 2002; Matsumoto et al., 2011) in-flight. Indeed, CM exercise itself may be a key factor in generating this imbalance (Laurens et al., 2019). The loss of body mass in space is associated with decreased muscle mass and functionality, incidence of cardiovascular issues, and even oxidative stress (Stein, 2002; Smith and Zwart, 2008). Terrestrial studies of an energy deficit demonstrate comparable effects (Bergouignan et al., 2016), whilst the deleterious consequences of an energy deficit on health, and the adaptive response to physical activity are well documented (Ihle and Loucks, 2004; Bergouignan et al., 2016; Murphy and Koehler, 2020), including in both athletic (Sale and Elliott-Sale, 2019) and military (Murphy et al., 2018) populations. Thus, in-flight energy balance differences (Bergouignan et al., 2016) may contribute to IV in the physiological adaptive responses to spaceflight. Finally, crewmembers complete a fluid loading protocol in the hours before landing to reduce the risk of orthostatic intolerance (Bungo et al., 1985) and may also be administered a saline infusion on landing, the volume of which is determined by medical personnel as clinically indicated. The effects of both of these treatments (if administered and in what quantity) may have individual effects on fluid volumes and associated cardiovascular function and performance.

Even if all of the factors described above could be controlled or eliminated, an additional consideration is to what extent the observed IV is "true" biological IV in response to the spaceflight environment. Atkinson and Batterham (2015) argue that, because both technical error and random within-subject variation are inherent within any measurement, IV cannot be confirmed from the pre- and post-intervention (or exposure) measurements alone. Thus, without an appropriate control group, there is a risk of identifying "false" IV (Williamson et al., 2017). Published data from spaceflight do not include a control group, either one that performs no exercise (to investigate the effects of CM exercise), or a ground-based group (to compare to those in space). Thus, confirmation of the presence of "true" IV is not possible, either in response to the spaceflight environment or the use of CM exercise.

ROLE OF HEAD-DOWN BED REST IN UNDERSTANDING IV IN SPACEFLIGHT

Long-term HDBR is the pre-eminent ground-based experimental approach to study the effects of prolonged gravitational unloading and disuse (Hargens and Vico, 2016). Like all

Earth-based analogs, HDBR is confounded by the presence of gravity and the absence of space radiation exposure rendering it unsuitable as a model for spaceflight-induced adaptation in all physiological systems. Specifically in relation to the presence of gravity, unlike spaceflight, HDBR may not affect signaling from the semicircular canals or the otoliths (Dupui et al., 1992), with only the somatosensory system being affected. This may result in differential effects in outcome measures that directly test these systems (e.g., postural stability), those that may be influenced by them, such as blood pressure (Hallgren et al., 2015), and performance in functional tasks to which they may contribute (Miller et al., 2018; Mulavara et al., 2018). Despite these limitations, however, HDBR is still considered a valid analog for the musculoskeletal and cardiovascular systems (Pavy-Le Traon et al., 2007) and, as such, may be a valuable tool for the determination of the presence of, and factors determining,

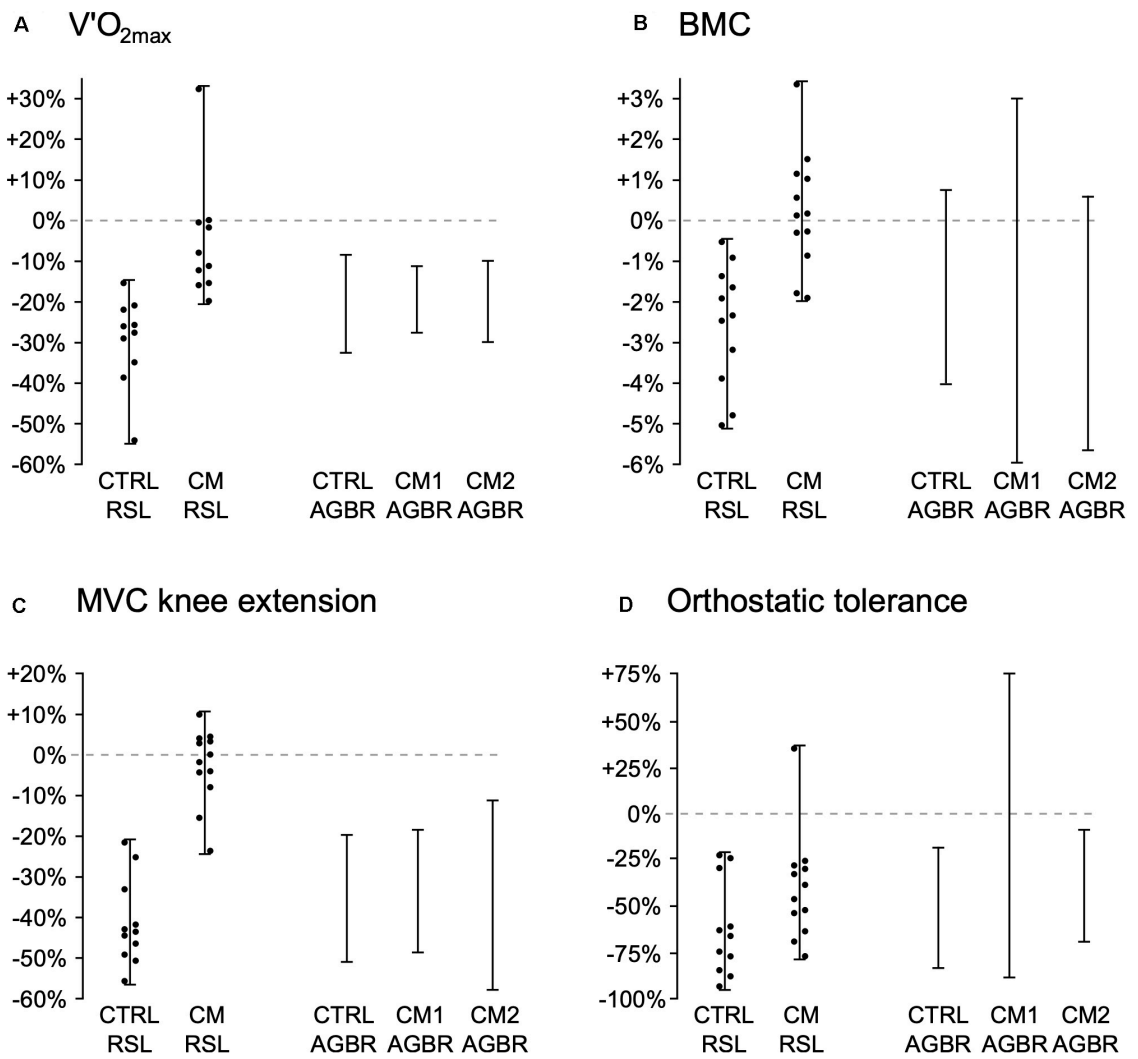

FIGURE 2 | Range of individual changes (percentage change from pre- to post-study) in the control (CTRL) and countermeasure (CM) groups from two European Space Agency (ESA) 60-d, -6° head-down bed rest (HDBR) studies: ESA's "*Reactive jumps in a Sledge jump system as countermeasure during Long-term bed rest*" (RSL) study, in which HDBR only (CTRL RSL) was compared to 48 training sessions of "reactive jumps," consisting of 4 × 12 countermovement jumps and 2 × 15 repetitive hops (CM RSL; see Kramer et al., 2017a for full description of protocol) and from ESA's "*Artificial Gravity Bed Rest – European Space Agency*" (AGBRESA) study, in which HDBR only (CTRL AGBR) was compared to two different artificial gravity protocols, 1 × 30-min session/day of supine centrifugation at +1Gz at the center of mass (AGBR CM1; see Laing et al., 2020 for full description of protocol), and 6 × 5-min/day (all separated by 5-min rest breaks) supine centrifugation at +1Gz at the center of mass (AGBR CM2; see Laing et al., 2020). The figure shows changes in maximal rate of oxygen uptake (VO_{2max}, Panel **A**), bone mineral content (BMC) as assessed by pQCT of the tibia at 98% of tibial length (Panel **B**), force production during a maximal voluntary contraction (MVC) of the knee extensors (Panel **C**), and orthostatic tolerance (OT) time during a tilt-table test (Panel **D**). Individual values from the RSL study come from the data (mean and standard deviations) sets published in Kramer et al. (2017b), where the full study protocol is also described, but individual values were not published. The range of individual values (individual maximum and minimum changes) from the AGBRESA study was provided courtesy of ESA's Human Research Office. Individual values from the AGBRESA study could not be provided as these data have not yet been published and will be included in future publications assessing the efficacy of the artificial gravity countermeasures.

IV in the response to gravitational unloading and performance of CMs. Although the typical duration of current ISS LDMs (∼6-months) exceeds the duration of even the longest HDBR studies (90-d) and it remains unknown if the duration of exposure to microgravity or axial unloading leads to increased or decreased IV, HDBR is free of many of the factors that potentially confound the interpretation of spaceflight data:

- The majority of studies include a group of subjects whom are exposed *only* to HDBR. Where the primary goal is to test a CM, this group serves as the control group against which the CM is compared, allowing the effects of HDBR alone to be isolated. The inclusion of a control group has the added advantage of allowing "true" IV in response to the CM (but not HDBR alone as there is no ambulatory control condition) to be detected by comparing the standard deviations of the two groups (Atkinson and Batterham, 2015)];
- When a CM is applied, it is applied in a systematic and rigorous manner, and any deviations accurately recorded.
- Experimental conditions are tightly controlled, thus reducing the potential impact of "non-exercise" biological factors (e.g., nutrition) and eliminating the "operational" factors (e.g., vehicle visits, spacewalks, and engineering) present in spaceflight.

However, despite the potential value of HDBR in understanding IV in the response to gravitational unloading and CMs, as the majority of published HDBR data are from science experiments (most typically comparing the CM and control groups), there is again a scarcity of individual data from which to assess IV in response to HDBR alone and to CMs. **Figure 2** shows individual data from two recent European Space Agency (ESA) 60-d HDBR studies, the "*Reactive jumps in a Sledge jump system as countermeasure during Long-term bed rest*" (RSL) and "*Artificial Gravity Bed Rest – European Space Agency*" (AGBRESA) studies, both including control and CM (RSL: reactive jumps; AGBRESA: artificial gravity) groups. These data suggest that, despite the high degree of standardization, and the control of many (but not all) of the factors that could influence the response to the spaceflight environment and CM exercise, there appears to be substantial IV in both the cardiorespiratory and musculoskeletal response to HDBR, and in each of the three different CM regimes. Specifically, after 60-d of HDBR *alone*, individual changes in VO_{2max}, tibial bone mineral content as assessed by pQCT, knee extensor maximal force production, and orthostatic tolerance, ranged from −54% to −9%, −5% to +1%, −56% to −20%, and −94% to −19%. Changes with the RSL's jumping intervention for these outcome measures ranged from −20% to +32%, −2% to +4%, −24% to +10%, and −78% to +35%, and with the AGBRESA's artificial gravity interventions from −29% to −11%, −6% to +3%, −58% to −9% and −87% to +76%.

A further advantage of the HDBR model in investigating IV is the attempt to standardize conditions between studies. This standardization has resulted in two distinct, but equally important outcomes: standardization of conditions between studies and a standard set of "core" measurements from every study (Sundblad et al., 2016). As a result of their complexity and expense, bed rest studies are, and will likely continue to be, small, with typically only 8–12 subjects in each group. However, standardization of conditions and outcome measures between studies means that, in principle, results of different studies, in particular the control groups, can now be not only compared, but also potentially combined. Specifically, in relation to IV, data from comparable studies (e.g., 60-d of -6° HDBR only) could be pooled. Thus, the more studies that adhere to the standardization guidelines, the larger this pool will become.

DISCUSSION

Whilst the spaceflight data presented in this Perspective suggests a marked degree of IV in response to long-term spaceflight, it is clear that numerous biological, operational, and environmental factors may contribute to this, and thus complicate its interpretation. As such, we conclude that it is currently not possible to evaluate IV in response to the spaceflight environment, and/or the use of CM exercise. In contrast, despite highly standardized experimental conditions, IV is also evident in response to long-duration HDBR. Thus, we propose that HDBR is suitable for the investigation of IV in the physiological response to gravitational unloading and CMs. Such analysis could represent the first critical step in understanding the existence of IV in spaceflight adaptation. Should "true" IV be confirmed, investigation of possible mediators will be warranted. In turn, this may provide insights into the potential role of IV in the apparent effectiveness of current and future CMs. In the longer-term, characterization of IV may even aid the selection of individuals for specific exploration missions, where a comprehensive understanding of the effects of the spaceflight environment and the effectiveness of CMs will be critical to the successful execution of mission objectives and the safe return of crews to Earth.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Data from the European Space Agency (ESA)'s "AGBRESA" study are currently owned by ESA and were provided courtesy of ESA's Human Research Office. As such, they are not currently publicly available. The re-analysis of spaceflight data was performed directly from previously published figures and we do not know if the datasets are publicly available. Data from the ESA "RSL" study are owned by the authors and can be made available. Requests to access these datasets should be directed to AK, andreas.kramer@uni-konstanz.de.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Northern Rhine Medical Association (Ärztekammer Nordrhein)

in Duesseldorf, Germany, and Federal Office for Radiation Protection (Bundesamt für Strahlenschutz). The patients/participants provided their written informed consent to participate in these studies.

AUTHOR CONTRIBUTIONS

JS wrote the first draft of the manuscript. All authors commented on previous versions of the manuscript, and read and approved the final manuscript.

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Limited Effect of 60-Days Strict Head Down Tilt Bed Rest on Vascular Aging

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Background: Cardiovascular risk may be increased in astronauts after long term space flights based on biomarkers indicating premature vascular aging. We tested the hypothesis that 60 days of strict 6° head down tilt bed rest (HDTBR), an established space analog, promotes vascular stiffening and that artificial gravity training ameliorates the response.

Methods: We studied 24 healthy participants (8 women, 24–55 years, BMI = 24.3 ± 2.1 kg/m^2) before and at the end of 60 days HDTBR. 16 subjects were assigned to daily artificial gravity. We applied echocardiography to measure stroke volume and isovolumetric contraction time (ICT), calculated aortic compliance (stroke volume/aortic pulse pressure), and assessed aortic distensibility by MRI. Furthermore, we measured brachial-femoral pulse wave velocity ($_{bf}$PWV) and pulse wave arrival times (PAT) in different vascular beds by blood pressure cuffs and photoplethysmography. We corrected PAT for ICT (cPAT).

Results: In the pooled sample, diastolic blood pressure (+8 ± 7 mmHg, $p < 0.001$), heart rate (+7 ± 9 bpm, $p = 0.002$) and ICT (+8 ± 13 ms, $p = 0.036$) increased during HDTBR. Stroke volume decreased by 14 ± 15 ml ($p = 0.001$). $_{bf}$PWV, aortic compliance, aortic distensibility and all cPAT remained unchanged. Aortic area tended to increase ($p = 0.05$). None of the parameters showed significant interaction between HDTBR and artificial gravity training.

Conclusion: 60 days HDTBR, while producing cardiovascular deconditioning and cephalad fluid shifts akin to weightlessness, did not worsen vascular stiffness. Artificial gravity training did not modulate the response.

Keywords: pre-ejection period, pulse wave arrival time, pulse wave velocity, artificial gravity, AGBRESA, arterial stiffness, aortic distensibility, isovolumetric contraction time

INTRODUCTION

Vascular biomarker studies suggest that the harsh environmental conditions in space including microgravity, galactic cosmic radiation, and perturbed circadian rhythms may promote vascular aging. Premature vascular aging could pose risks for astronauts' performance and cardiovascular health during missions to the Moon and from there to Mars. Indeed, decreased carotid distensibility (Hughson et al., 2016; Arbeille et al., 2017), increased carotid and femoral intima media thickness (Arbeille et al., 2016), and decreased pulse wave arrival times (PAT) at the finger (Baevsky et al., 2007; Hughson et al., 2016) have been reported following long-term space missions. PAT relates to pulse wave velocity (PWV), an established cardiovascular risk marker (Williams et al., 2018), provided that isovolumetric contraction time (ICT) and vessel length do not change.

Beside aging, physical inactivity during bed rest rapidly evokes blood vessel remodeling (Thijssen et al., 2011). Sixty days head down tilt bed rest (HDTBR), which additionally models cardiovascular deconditioning and cephalad fluid shifts in space, also increased carotid and femoral intima media thickness (van Duijnhoven et al., 2010) and aortic PWV (Fayol et al., 2019). In another 35 days HDTBR study, the same vascular stiffness markers did not change (Palombo et al., 2015). Physical exercise reduced intima media thickening during HDTBR (van Duijnhoven et al., 2010). Artificial gravity through daily short-arm centrifugation, which partially attenuated cardiovascular deconditioning during 21 days HDTBR (Stenger et al., 2012), also holds promise in this regard.

Therefore, we hypothesized that 60 days HDTBR would increase aortic PWV and that artificial gravity would attenuate the response. Moreover, we recorded PAT at different sites to test for regional differences in vascular adaptation. Finally, unlike previous studies, we assessed distensibility and compliance of the aortic arch with magnetic resonance imaging and echocardiography, respectively.

MATERIALS AND METHODS

Participants

After providing written informed consent, 8 women and 16 men aged between 24 and 55 years (33.4 ± 9.3 years, 24.3 ± 2.1 kg/m^2) participated in the Artificial Gravity Bed Rest Study (AGBRESA). AGBRESA was a joint project between the National Aeronautics and Space Administration (NASA), the European Space Agency (ESA), and the German Aerospace Center (DLR). All participants had no history of cardiovascular disease, took no medication, and were non-smokers for at least 6 months prior to enrollment. The study was conducted in accordance with the declaration of Helsinki and was registered at the German Clinical Trials Register under number DRKS00015677. The protocol was approved by the ethics commissions of the Medical Association North Rhine (number 2018143) and NASA (Johnson Space Center, Houston, United States).

Study Design

The overreaching goal of AGBRESA was to assess the efficacy of artificial gravity as countermeasure for physiological adaptations evoked by 60 days strict HDTBR. Strict HDTBR means that no pillows were allowed except for a thin cushion when participants lay on the side, one shoulder always had to touch the mattress, and all daily activities including personal hygiene were done in the −6° position. Including the pre and post bed rest phases, the participants spent 87 days at the Envihab research facility in Cologne, Germany. All participants ingested standardized isocaloric diets with controlled fluid intake and were subjected to regulated bed times. Caffeine or alcohol containing beverages were not allowed. Participants did not exercise at least 24 h before we obtained our recordings. After the 14-day base line data collection (BDC), participants were distributed to three groups subjected to no artificial gravity (control, Ctr), continuous artificial gravity (cAG), or intermittent artificial gravity (iAG) (**Figure 1**). The bed rest period was followed by 14 days of recovery.

Artificial Gravity Countermeasure

Artificial gravity was generated by a short arm centrifuge with participants in the supine position and the head toward the center of rotation. Distance to the center of rotation and angular velocity were adjusted to body height to reach 1 g at the center of body mass, and approximately 2 g at the feet. Daily artificial gravity was applied continuously for 30 min (cAG) or intermittently for 6 × 5 min (iAG) with 3-min breaks in between. Rotation direction was changed daily. A more detailed description of the artificial gravity countermeasure can be found elsewhere (Kramer et al., 2020).

Instrumentation and Experimental Protocol

We obtained recordings 6 days before (BDC-6, PRE) and on the last day of HDTBR (HDT60, HDT) following a complete clinical echocardiographic examination (Vivid-IQ with M5SC-RS sector-probe, GE Healthcare, Buckinghamshire, Great Britain) according to current guidelines (Galderisi et al., 2017). We assessed ICT as the interval between ventricular contraction onset, indicated by the electrocardiogram R-peak, and aortic valve opening, as indicated by flow onset in the left ventricular outflow tract (**Figure 2**). We calculated stroke volume by multiplying velocity time integral with left ventricular outflow tract area. For the PRE-sessions, participants were in the horizontal supine position. At HDT60, the echocardiography table was tilted to −6° in order to maintain strict HDTBR.

After the echocardiographic examination, we instrumented participants as shown in **Figure 2**. We placed a finger blood pressure cuff (Finometer MIDI, Finapres Medical Systems, Enschede, Netherlands) around the left ring or middle finger and recorded its raw analog signals at 2000 Hz (BIOPAC MP150, Biopac Systems Inc., Goleta, United States). We also placed blood pressure cuffs at the right upper arm and right thigh. During the PRE-sessions, we measured the distance between these cuffs to exactly reposition them at HDT60

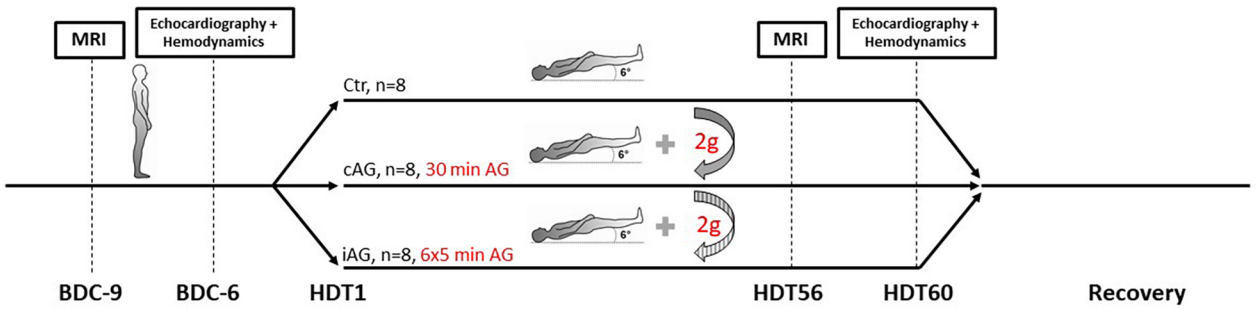

FIGURE 1 | Illustration of the study design. During 14-day baseline data collection (BDC), subjects were ambulatory but supine for measurements. Shortly before the onset of the 60-day head down tilt period (HDT), subjects were assigned to three different intervention groups named control (Ctr, no artificial gravity), continuous artificial gravity (cAG), and intermittent artificial gravity (iAG). We obtained magnetic resonance imaging (MRI) measurements 9 days before (BDC-9), and on the 56th day of HDT (HDT56). We conducted echocardiography and assessed hemodynamics (blood pressure, pulse wave velocity, etc.) on BDC-6 and on HDT60.

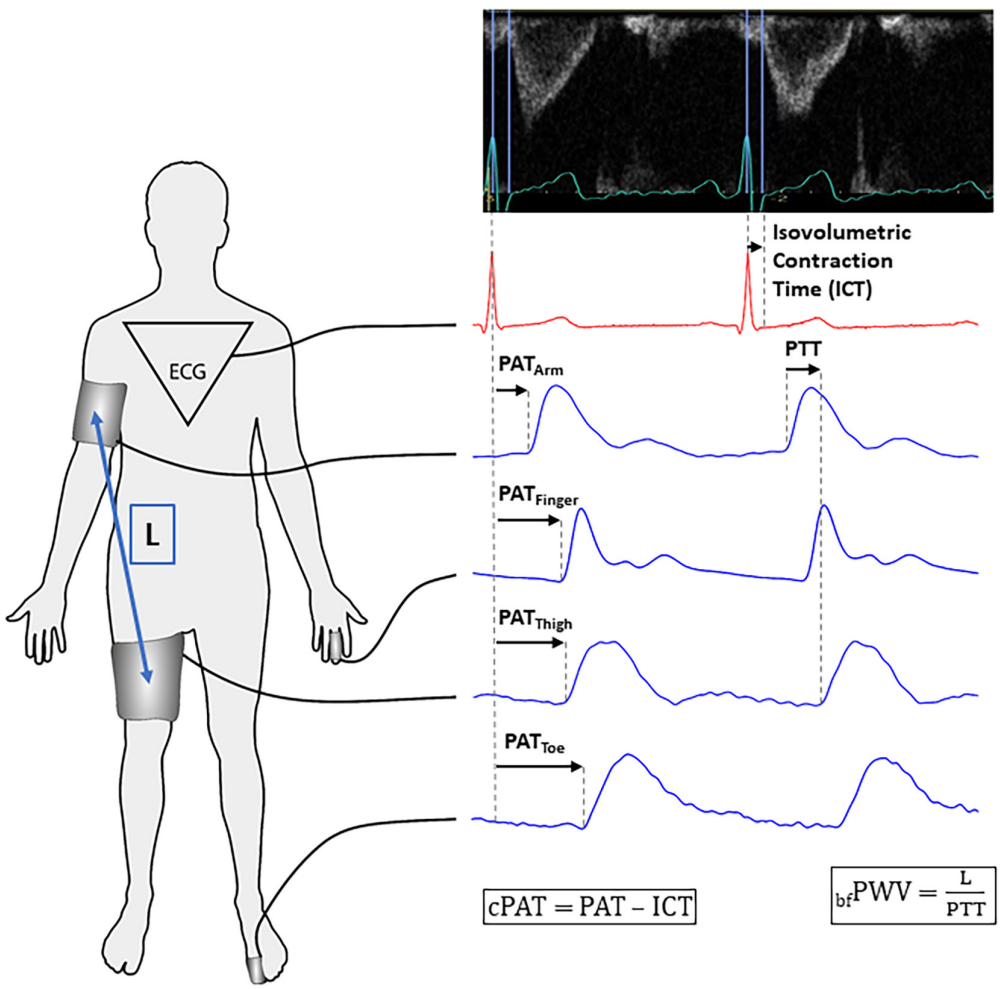

FIGURE 2 | Data acquisition for vascular measurements. **Left panel:** Recording sites and length (L) determination for brachial-femoral pulse wave velocity ($_{bf}$PWV) assessment. **Right panel – top:** ECG and Doppler flow curves in the left ventricular outflow tract. We measured isovolumetric contraction time (ICT) from the R-peak to aortic valve opening indicated by flow onset. **Right panel – bottom:** Pulse wave arrival time (PAT) is defined as the time from the ECG R-Peak to pulse wave arrival at a certain landmark. By dividing L by pulse transit time (PTT) between arm and thigh cuffs, we obtained $_{bf}$PWV. We obtained measurements from every heartbeat recorded during a 60-s period. For the sake of clarity, PAT and PTT are shown for two consecutive heart beats. We calculated corrected PAT (cPAT) by subtracting ICT from PAT.

TABLE 1 | All recorded parameters sorted by recordings before (PRE) and at the end of head down tilt bed rest (HDT).

Parameter	Pooled analysis (n = 24)			p-value
	PRE	Δ (HDT-PRE)	HDT	
Aortic SBP (mmHg)	108 ± 12	0 ± 11	108 ± 8	0.935
Aortic DBP (mmHg)	72 ± 7	+9 ± 7	81 ± 7	**<0.001**
SBP (mmHg)	125 ± 11	−1 ± 10	124 ± 9	0.652
DBP (mmHg)	70 ± 7	+8 ± 7	78 ± 7	**<0.001**
Pulse pressure (mmHg)	55 ± 13	−9 ± 9	46 ± 9	**<0.001**
Heart rate (bpm)	62 ± 9	+7 ± 9	69 ± 11	**0.002**
Stroke volume (ml)	97 ± 20	−14 ± 15	83 ± 15	**0.001**
Cardiac output (l/min)	5.9 ± 0.9	−0.3 ± 0.8	5.6 ± 1.1	0.265
PAT_{Arm} (ms)	154 ± 15	+9 ± 12	163 ± 17	**0.002**
PAT_{Finger} (ms)	205 ± 17	−1 ± 20	204 ± 18	0.898
PAT_{Thigh} (ms)	209 ± 19	+8 ± 15	217 ± 19	**0.022**
PAT_{Toe} (ms)	353 ± 22	−3 ± 27	350 ± 24	0.957
ICT (ms)	49 ± 8	+8 ± 13	57 ± 11	**0.036**
$cPAT_{Arm}$ (ms)	105 ± 14	+2 ± 15	107 ± 15	0.354
$cPAT_{Finger}$ (ms)	156 ± 17	−8 ± 19	148 ± 13	0.058
$cPAT_{Thigh}$ (ms)	160 ± 19	0 ± 16	160 ± 17	0.561
$cPAT_{Toe}$ (ms)	304 ± 18	+11 ± 24	293 ± 22	0.188
$_{bf}$PWV (m/s)	9.0 ± 1.2	+0.2 ± 1.0	9.2 ± 1.3	0.864
AOC (ml/mmHg)	2.7 ± 0.6	+0.4 ± 0.8	3.1 ± 0.6	0.094
AOD (10^{-3} mm Hg^{-1})	4.8 ± 2.0	−0.5 ± 2.4	4.3 ± 2.2	0.364
AOA_{min} (mm^2)	481 ± 88	+24 ± 53	505 ± 102	0.050

Aortic SBP and aortic DBP, aortic systolic and diastolic blood pressure; SBP and DBP, brachial systolic and diastolic blood pressure; PAT, pulse wave arrival time; ICT, isovolumetric contraction time; cPAT, PAT corrected for ICT; $_{bf}$PWV, brachial-femoral pulse wave velocity; AOC, aortic compliance; AOD, aortic distensibility; AOA_{min}, minimum aortic area. Results are represented as mean values ± standard deviation from 24 subjects and statistical significance ($p < 0.05$) is indicated by p-values in bold.

(**Figure 2**). These cuffs were connected to a research device for non-invasive hemodynamic analysis (CardioCube, AIT Austrian Institute of Technology, Vienna, Austria). Beside the option to simultaneously operate two blood pressure cuffs, the device features a 3-lead-electrocardiogram module and two analog input channels recording signals at 250 Hz. The first channel recorded the pulse curve obtained with a photoplethysmography sensor (Blood Volume Pulse, PLUX wireless biosignals, Arruda dos Vinhos, Portugal) attached to the left long toe. The second channel recorded a trigger signal synchronizing CardioCube with Biopac. After instrumentation, we obtained three oscillometric blood pressure measurements using the upper arm cuff to record heart rate and brachial systolic and diastolic blood pressure (SBP and DBP). The CardioCube also calculated aortic systolic and diastolic blood pressure using the ARCSolver algorithm (Weber et al., 2011). Then, the cuffs were inflated to the previously determined DBP to record pulse curves and electrocardiogram for 1 min (**Figure 2**).

Signal and Data Analysis

We determined PAT as the time between electrocardiogram R-Peak and pulse wave arrival at the upper arm (PAT_{Arm}), finger (PAT_{Finger}), thigh (PAT_{Thigh}), and toe (PAT_{Toe}) (**Figure 2**). For precise determination of pulse wave upstroke onset, we applied the so-called "diastole-patching method" to find the pairwise maximum cross correlation (implemented with MATLAB 2018, The MathWorks Inc., Natick, United States) (Bachler et al., 2013).

Because PAT could be confounded by ICT, we subtracted ICT from PAT values to obtain corrected measurements $cPAT_{Arm}$, $cPAT_{Finger}$, $cPAT_{Thigh}$, and $cPAT_{Toe}$. As aortic PWV surrogate, we determined brachial-femoral PWV ($_{bf}$PWV) by dividing the distance between arm and thigh cuffs by pulse transit time between these cuffs (Baier et al., 2018). Cardiac output was calculated by multiplying stroke volume with heart rate. Brachial pulse pressure was calculated by subtracting DBP from SBP. Aortic compliance (AOC) was obtained by dividing stroke volume by aortic pulse pressure (aortic SBP – aortic DBP).

Magnetic Resonance Imaging

We obtained aortic distensibility (AOD) using magnetic resonance imaging at BDC-9 (PRE) and HDT56 (HDT). To measure aortic cross-sectional area changes during one heart cycle, we applied two-dimensional steady-state-free-precession (SSFP) cine imaging (3 Tesla mMR Biograph PET/MRI scanner, Siemens Healthineers, Erlangen, Germany). We also measured brachial blood pressure at the right arm (Expression MR400, Philips Healthcare, Eindhoven, Netherlands) to obtain pulse pressure during the magnetic resonance imaging session. Then, we calculated AOD as relation between cross-sectional area changes of the ascending aorta and brachial pulse pressure (Voges et al., 2012). We also report the end diastolic minimum of the

TABLE 2 | All parameters recorded before (PRE) and at the end of head down tilt bed rest (HDT), sorted by intervention: control (Ctr), continuous artificial gravity (cAG) and intermittent artificial gravity (iAG).

Parameter	Ctr (n = 8, 2 women)		cAG (n = 8, 3 women)		iAG (n = 8, 3 women)		p-values	
	PRE	HDT	PRE	HDT	PRE	HDT	Main effect	Interaction
Aortic SBP (mmHg)	112 ± 10	113 ± 8	109 ± 17	108 ± 7	104 ± 7	105 ± 5	0.116	0.463
Aortic DBP (mmHg)	73 ± 9	82 ± 10	72 ± 7	81 ± 5	70 ± 7	79 ± 3	0.680	0.803
SBP (mmHg)	125 ± 8	129 ± 8	127 ± 15	124 ± 11	123 ± 10	120 ± 6	0.542	0.050
DBP (mmHg)	71 ± 8	80 ± 10	70 ± 6	79 ± 5	68 ± 8	76 ± 3	0.532	0.921
Pulse pressure (mmHg)	54 ± 8	49 ± 10	57 ± 15	45 ± 9	55 ± 15	44 ± 7	0.773	0.112
Heart rate (bpm)	62 ± 8	70 ± 10	63 ± 12	71 ± 15	62 ± 7	65 ± 10	0.710	0.162
Stroke volume (ml)	99 ± 27	84 ± 12	94 ± 21	78 ± 11	99 ± 14	87 ± 20	0.683	0.712
Cardiac output (l/min)	6.0 ± 1.3	5.9 ± 0.9	5.7 ± 0.7	5.5 ± 1.0	6.0 ± 0.8	5.6 ± 1.5	0.859	0.706
PAT_{Arm} (ms)	160 ± 14	173 ± 19	153 ± 14	160 ± 14	149 ± 16	156 ± 15	0.125	0.993
PAT_{Finger} (ms)	209 ± 15	214 ± 17	208 ± 22	198 ± 19	199 ± 15	201 ± 13	0.390	0.582
PAT_{Thigh} (ms)	215 ± 14	222 ± 17	205 ± 19	208 ± 18	208 ± 24	221 ± 22	0.188	0.376
PAT_{Toe} (ms)	349 ± 17	354 ± 29	360 ± 26	347 ± 26	349 ± 23	350 ± 21	0.716	0.443
ICT (ms)	50 ± 10	63 ± 12	50 ± 7	54 ± 11	47 ± 6	54 ± 9	0.307	0.445
$cPAT_{Arm}$ (ms)	110 ± 14	110 ± 18	103 ± 15	106 ± 14	101 ± 14	103 ± 12	0.218	0.410
$cPAT_{Finger}$ (ms)	159 ± 16	151 ± 11	158 ± 22	145 ± 18	151 ± 10	147 ± 9	0.811	0.447
$cPAT_{Thigh}$ (ms)	165 ± 16	160 ± 11	155 ± 20	154 ± 16	161 ± 22	167 ± 22	0.343	0.063
$cPAT_{Toe}$ (ms)	300 ± 12	291 ± 25	310 ± 21	290 ± 23	301 ± 21	297 ± 22	0.555	0.405
$_{bf}$PWV (m/s)	9.0 ± 1.1	9.2 ± 1.5	9.1 ± 1.0	9.5 ± 0.8	9.0 ± 1.5	9.0 ± 1.6	0.664	0.875
AOC (ml/mmHg)	2.6 ± 0.6	2.8 ± 0.2	2.8 ± 0.7	3.0 ± 0.5	2.9 ± 0.3	3.4 ± 0.7	0.176	0.386
AOD (10^{-3} mm Hg^{-1})	5.2 ± 2.4	4.4 ± 2.9	4.7 ± 2.1	4.5 ± 1.9	4.6 ± 1.7	4.1 ± 1.9	0.465	0.865
AOA_{min} (mm^2)	496 ± 70	504 ± 107	456 ± 79	487 ± 52	492 ± 116	522 ± 138	0.630	0.317

Aortic SBP and aortic DBP, aortic systolic and diastolic blood pressure; SBP and DBP, brachial systolic and diastolic blood pressure; PAT, pulse wave arrival time; ICT, isovolumetric contraction time; cPAT, PAT corrected for ICT; $_{bf}$PWV, brachial-femoral pulse wave velocity; AOC, aortic compliance; AOD, aortic distensibility; AOA_{min}, minimum aortic area. Each parameter is represented as mean value ± standard deviation together with p-values for main effects (intervention) and interaction (intervention vs. bed rest).

aortic area (AOA_{min}) since aortic dilation may influence stiffness (Guala et al., 2019).

Statistical Analysis

Linear mixed effects (LME) model (IBM SPSS, Version 21) was used for statistical analysis. Given the strong effect of age on vascular stiffness, we conducted an exploratory analysis by stratifying participants in age tertiles, which resulted in three different age groups: age group 1, 24–27 years, n = 9; age group 2, 29–36 years, n = 8; and age group 3, 37–55, n = 7. Bed rest (PRE vs. HDT) and its interaction with age group, sex, and intervention (Ctr, cAG, and iAG) were defined as fixed effect whereas the subjects were defined as random effects. In case of significant interaction, we identified affected groups using Bonferroni *post-hoc* correction for the multiple pairwise comparisons of PRE vs. HDT. A p-value < 0.05 indicated statistical significance. Results are reported as measured mean values ± standard deviation. We used Kolmogorov–Smirnov test for checking the distribution of residuals calculated by LME analysis.

RESULTS

Hemodynamic and Vascular Response

Because baseline characteristic and cardiovascular responses did not differ between interventions, we conducted a pooled analysis (**Table 1**). All results sorted by intervention are provided in **Table 2**. Over all subjects, SBP remained unchanged after 60 days HDTBR (p = 0.652), whereas DBP increased from 70 ± 7 mmHg to 78 ± 7 ($p < 0.001$), such that pulse pressure decreased by 9 ± 9 mmHg ($p < 0.001$). On the fourth day of recovery, DBP had returned to baseline values at 70 ± 6 mmHg. To verify these findings, we also analyzed safety blood pressure measurements at PRE and HDT59 obtained through an oscillometric device (IntelliVue X2, Philips Healthcare, Eindhoven, The Netherlands). These measurements were obtained in duplicate immediately after awakening. At HDT59, DBP was increased by 7 ± 7 mmHg ($p < 0.001$). Aortic blood pressure followed brachial blood pressure with an unchanged aortic SBP (p = 0.935) and an increased aortic DBP (9 ± 7 mmHg, $p < 0.001$). A 14 ± 15 ml (p = 0.001) stroke volume reduction was partly compensated by a heart rate increase (7 ± 9 bpm, p = 0.002) such that cardiac output remained stable (5.9 ± 0.9 vs. 5.6 ± 1.1 l/min, p = 0.265). AOC remained unchanged following HDTBR (p = 0.094). While AOA_{min} tended to increase at the end of HDTBR (**Table 1**), we observed no changes in AOD (p = 0.364).

Pulse Wave Velocity and Arrival Times

Averaged over all subjects, $_{bf}$PWV remained unchanged between PRE and HDT (p = 0.864, **Figure 3**). In contrast, the uncorrected arrival times at brachial and femoral artery, which include the ICT, increased by 9 ± 13 ms (PAT_{Arm}, p = 0.002) and

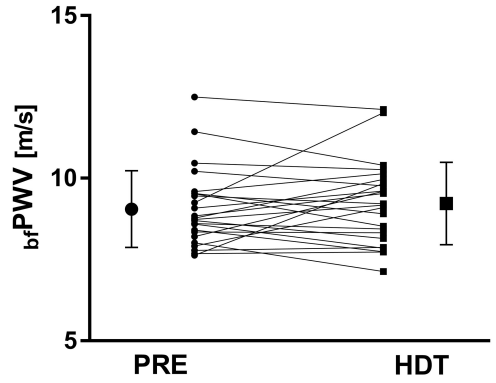

FIGURE 3 | Line graph for individual brachial-femoral pulse wave velocities ($_{bf}$PWV) 6 days before (PRE, dot symbol) and after 60 days HDTBR (HDT, square symbol). Scaled-up single symbols to the left and right represent corresponding mean values and error bars indicate the first standard deviation. $_{bf}$PWV did not change with 60 days HDTBR (9.0 ± 1.2 vs. 9.2 ± 1.3 m/s, p = 0.864).

8 ± 15 ms (PAT_{Thigh}, p = 0.022), respectively. PAT_{Finger} and PAT_{Toe} remained unchanged (p = 0.898 and 0.957). However, ICT increased by 8 ± 13 ms (p = 0.036) after 60 days in HDTBR and the corrected PAT values (see **Figure 4**) for the femoral and brachial artery ($cPAT_{Thigh}$ and $cPAT_{Arm}$) were unaffected by HDTBR. In contrast, $cPAT_{Finger}$ and $cPAT_{Toe}$ tended to decrease by 8 ± 5 and 11 ± 13 ms, without reaching significance after correction for the increased ICT (see **Table 1**).

Potential Age and Sex Influences

In general, AOA_{min} and $_{bf}$PWV increased from the youngest to the oldest tertile (p = 0.013 and p = 0.027). For some of the measurements, we observed a significant interaction between age and HDTBR. These results are listed in **Table 3** and an exemplary selection is displayed in **Figure 5**. We did not observe a qualitative difference in the response to HDTBR between women and men (**Table 4**).

DISCUSSION

The important finding of our study is that 60 days strict HDTBR did not produce clinically relevant changes in $_{bf}$PWV or cPAT in healthy persons. In fact, $_{bf}$PWV and cPAT at different vascular beds were almost identical before and after HDTBR. However, we observed an unexpected albeit modest transient increase in aortic area following strict HDTBR, which has not been previously described.

Strict HDTBR models some aspects of space travel, particularly cardiovascular deconditioning and cephalad fluid shifts. Both responses could affect vascular aging biomarkers including PAT, PWV, or AOD. The increase in heart rate with reductions in pulse pressure and stroke volume in our study is consistent with cardiovascular deconditioning. Similar stroke volume reductions indicating cardiac deconditioning and atrophy during HDTBR have been previously described (Levine et al., 1997; Iwasaki et al., 2000; Perhonen et al., 2001; Stenger et al., 2012; Palombo et al., 2015). HDTBR also reduces cardiopulmonary fitness (Saltin et al., 1968; Wagner, 2015) and orthostatic tolerance (Guinet et al., 2009). Strict HDTBR results in significant cephalad fluid shifts indicated by retinal papillary edema (Laurie et al., 2019) and mastoid effusions (Lecheler et al., 2021). Similar changes have been reported in astronauts returning from longer duration space missions (Inglesby et al., 2020).

An increase in heart rate is commonly associated with decreased pre-ejection period, the interval between electrocardiogram Q-wave and aortic valve opening (Weissler et al., 1968). If so, uncorrected PAT could overestimate vascular stiffness. In contrast, we observed concomitant increases in HR and ICT following strict HDTBR. The phenomenon has been previously observed with HDTBR (Hodges et al., 2010) and may result from an increased cardiac afterload (Hassan and Turner, 1983), as indicated by the observed increase in aortic DBP. Thus, uncorrected PAT_{Arm} and PAT_{Thigh} underestimated aortic stiffness in our study whereas

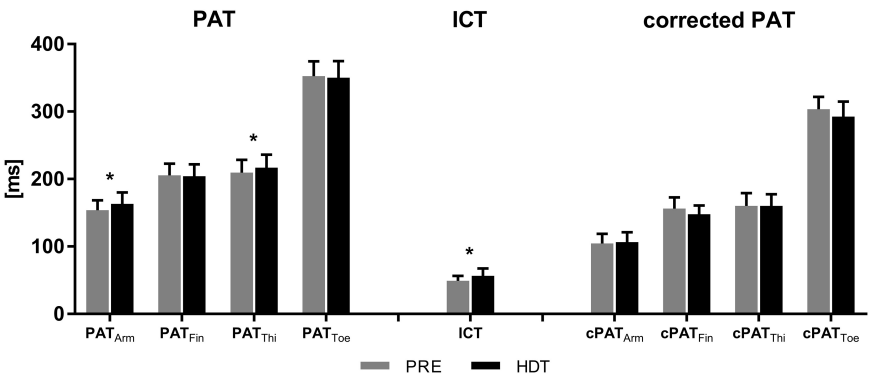

FIGURE 4 | Pulse wave arrival times (PAT) increase with greater topological distance from the heart to arm, finger, thigh, and toe, all indicated by subscripted acronyms. PAT_{Arm} and PAT_{Thigh} significantly increased after 60 days HDTBR (black bars) compared to baseline recordings 6 days before HDTBR (gray bars). Isovolumetric contraction time (ICT) also increased with HDTBR. Corrected PAT did not change with HDTBR. Results are represented as mean values from 24 subjects with error bars indicating the first standard deviation; * < 0.05.

TABLE 3 | All parameters recorded before (PRE) and at the end of head down tilt bed rest (HDT).

	AgeGr. 1 (*n* = 9, 2 Women)		AgeGr. 2 (*n* = 8, 4 Women)		AgeGr. 3 (*n* = 7, 2 Women)		*p*-values	
	PRE	HDT	PRE	HDT	PRE	HDT	Main effect	Interaction
Aortic SBP (mmHg)	104 ± 7	110 ± 9	104 ± 8	103 ± 4	119 ± 14	113 ± 5	**0.004**	0.070
Aortic DBP (mmHg)	69 ± 6	82 ± 8	72 ± 5	79 ± 4	75 ± 10	82 ± 7	0.538	0.096
SBP (mmHg)	122 ± 12†	127 ± 10	122 ± 8	118 ± 7	133 ± 11†	127 ± 7	0.071	**0.009**
DBP (mmHg)	67 ± 6	80 ± 9	70 ± 4	76 ± 4	73 ± 11	79 ± 8	0.569	0.144
Pulse pressure (mmHg)	55 ± 13	48 ± 9	52 ± 10	42 ± 5	60 ± 15	48 ± 11	0.527	0.217
Heart rate (bpm)	59 ± 6‡	70 ± 10	68 ± 11	70 ± 14	60 ± 7	65 ± 11	0.665	**0.047**
Stroke volume (ml)	108 ± 21	84 ± 17	83 ± 19	74 ± 11	100 ± 11	91 ± 13	0.129	0.093
Cardiac output (l/min)	6.3 ± 1.2	5.9 ± 1.4	5.5 ± 0.7	5.2 ± 1.0	5.9 ± 0.3	5.9 ± 0.7	0.572	0.830
PAT_{Arm} (ms)	161 ± 15	163 ± 18	154 ± 10†	162 ± 11	144 ± 15†	164 ± 23	0.441	**0.014**
PAT_{Finger} (ms)	212 ± 16	201 ± 20	205 ± 21	202 ± 15	198 ± 14	211 ± 18	0.978	0.095
PAT_{Thigh} (ms)	220 ± 18	222 ± 21	208 ± 15	212 ± 16	198 ± 19	216 ± 22	0.158	0.086
PAT_{Toe} (ms)	351 ± 21	342 ± 22	357 ± 18	359 ± 17	349 ± 29	353 ± 32	0.167	0.567
ICT (ms)	46 ± 7	59 ± 10	52 ± 8	52 ± 13	50 ± 7	59 ± 9	0.849	0.187
$cPAT_{Arm}$ (ms)	115 ± 13†	104 ± 11	102 ± 10	110 ± 13	94 ± 11†	106 ± 21	0.203	**0.001**
$cPAT_{Finger}$ (ms)	166 ± 15‡	142 ± 15	153 ± 20	150 ± 13	148 ± 9	153 ± 8	0.828	**0.004**
$cPAT_{Thigh}$ (ms)	173 ± 16†	163 ± 18	157 ± 15	161 ± 19	148 ± 18†	157 ± 17	0.057	**0.006**
$cPAT_{Toe}$ (ms)	305 ± 19	282 ± 19	305 ± 14	304 ± 19	300 ± 24	294 ± 26	0.192	0.105
$_{bf}$PWV (m/s)	8.5 ± 0.5	8.7 ± 0.8	9.1 ± 1.0	9.6 ± 1.5	9.7 ± 1.7	9.4 ± 1.5	**0.013**	0.219
AOC (ml/mmHg)	3.1 ± 0.5	3.1 ± 0.8	2.6 ± 0.5	3.1 ± 0.5	2.3 ± 0.4	2.9 ± 0.3	0.448	0.163
AOD (10^{-3} mm Hg^{-1})	5.3 ± 1.6	4.6 ± 2.6	5.4 ± 2.5	5.1 ± 2.2	3.6 ± 1.3	3.1 ± 1.0	0.053	0.937
AOA_{min} (mm^2)	417 ± 46	441 ± 69	497 ± 62	497 ± 65	547 ± 105	595 ± 113	**0.027**	0.139

Results are sorted by three different age groups (AgeGr): AgeGr. 1, 24–27 years; AgeGr. 2, 29–36 years and AgeGr. 3, 37–55 years. Aortic SBP and aortic DBP, aortic systolic and diastolic blood pressure; SBP and DBP, brachial systolic and diastolic blood pressure; PAT, pulse wave arrival time; ICT, isovolumetric contraction time; cPAT, PAT corrected for ICT; $_{bf}$PWV, brachial-femoral pulse wave velocity; AOC, aortic compliance; AOD, aortic distensibility; AOA_{min}, minimum aortic area. Results are represented as mean values ± standard deviation. Statistical significance ($p < 0.05$) for main effect (AgeGr.) and interaction (AgeGr. vs. bed rest) is indicated by p-values in bold. Significant difference between PRE and HDT is indicated by $^{\dagger}(p < 0.05)$ and $^{\ddagger}(p < 0.001)$.

corresponding cPAT remained unchanged. Furthermore, $_{bf}$PWV, which is an ICT independent aortic stiffness parameter, also remained unchanged. However, most PWV measurements have the potential disadvantage of being blind to vascular stiffness changes in the aortic arch (Mitchell, 2009). Yet, AOD of the ascending aorta, which is an accepted vascular stiffness marker (Redheuil et al., 2010; Dogui et al., 2011; Voges et al., 2012), was also unchanged following HDTBR. We observed a trend for aortic area to increase with HDTBR likely indicating increased vascular filling. The observation that aortic area had almost returned to baseline after fourth day recovery is consistent with changes in vascular filling rather than remodeling. Increased vascular filling tends to increase vascular stiffness measurements (Guala et al., 2019). It is, therefore, unlikely that PWV changes were masked by altered vascular loading.

The finding that PWV does not change despite 60 days bed rest deconditioning is somewhat counterintuitive because physical inactivity is associated with increased arterial stiffness (Thijssen et al., 2011), whereas mild physical exercise appears to ameliorate aortic PWV (Havlik et al., 2005). In one previous HDTBR study, which has only been published as abstract, carotid-femoral PWV increased in every participant (Fayol et al., 2019). In another study, carotid-femoral PWV was 6.9 m/s before and 6.9 m/s after 35 days HDTBR (Palombo et al., 2015). Overall, HDTBR for up to 60 days may not elicit a consistent, clinically relevant change in PWV. Among others, changes in the collagen-to-elastin ratio, collagen crosslinks (Schellinger et al., 2019), and changes in the expression of endothelial and inducible nitric oxide synthases (Cau et al., 2012) have been implicated in vascular aging. Possibly, HDTBR alone is not sufficient to drive vascular (aortic) aging in the absence of additional risk factors. Age, which affects metabolic and muscular adaptation to bed rest (Pišot et al., 2016), is a prime suspect. In contrast to our expectation, an exploratory analysis did not provide evidence that older age exacerbates aortic stiffening during HDTBR. In fact, we observed the opposite response. Another potential explanation is that aortic remodeling sufficient to cause consistently measurable PWV changes in a smaller scale study requires more than 60 days HDTBR.

An important limitation of our study is the relatively small number of participants in each intervention group limiting the statistical power to detect potential protective actions of artificial gravity. At this point we would like to point out that we had no influence onto the overall study design. Since vascular stiffness biomarkers did not deteriorate with HDTBR, artificial gravity cannot improve the outcome. Another potential limitation is that, while covering a relatively large age range, the study is too small for a detailed analysis relating HDTBR responses to age. The same is true for a sex-specific analysis. It is reassuring that we

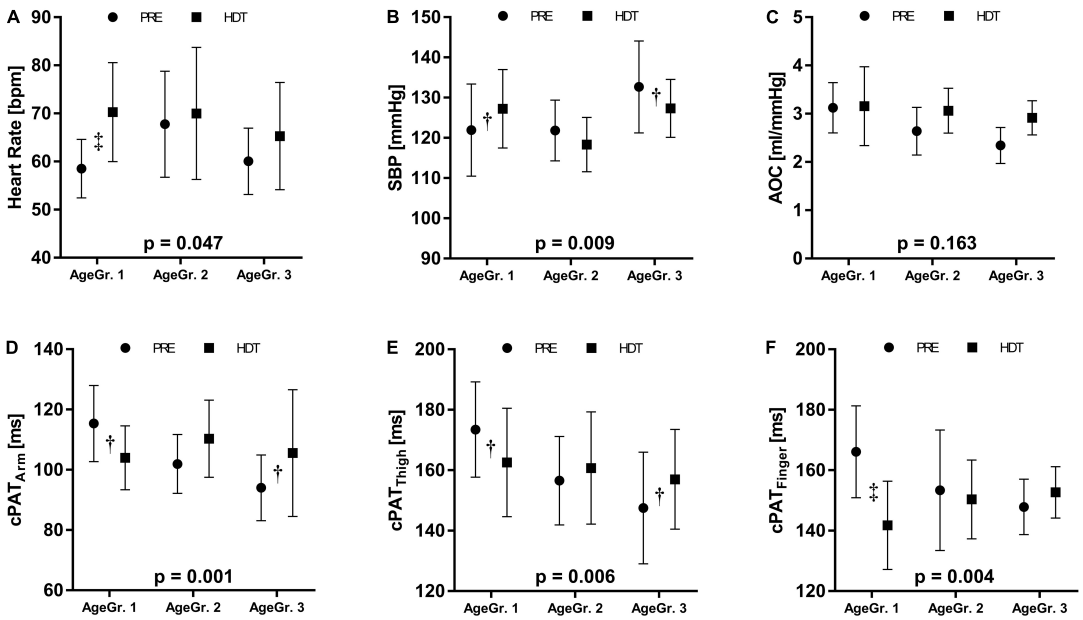

FIGURE 5 | Exploratory analysis relating the HDTBR response to age. We stratified participants in three age groups (AgeGr.): AgeGr. 1 = 24–27 years; AgeGr. 2 = 29–36 years, and AgeGr. 3 = 37–55 years. **(A)** heart rate, **(B)** systolic blood pressure (SBP), **(C)** aortic compliance (AOC), and corrected pulse wave arrival times to the **(D)** arm (cPAT$_{Arm}$), **(E)** thigh (cPAT$_{Thigh}$), and **(F)** finger (cPAT$_{Finger}$). The *p*-values indicate the interaction between AgeGr. and bed rest. Results are represented as mean values ± standard deviation from base line recordings (PRE, dot symbol) and HDT (square symbol). Significant difference between these time points is indicated by †($p < 0.05$) and ‡($p < 0.001$).

TABLE 4 | All parameters recorded before (PRE) and at the end of head down tilt bed rest (HDT).

Parameter	Men (n = 16)		Women (n = 8)		p-values	
	PRE	HDT	PRE	HDT	Main effect	Interaction
Aortic SBP (mmHg)	111 ± 12	111 ± 7	102 ± 8	103 ± 5	**0.017**	0.439
Aortic DBP (mmHg)	70 ± 8	81 ± 8	75 ± 5	80 ± 4	0.394	0.109
SBP (mmHg)	129 ± 10	128 ± 7	117 ± 8	116 ± 6	**0.001**	0.389
DBP (mmHg)	68 ± 8	79 ± 8	73 ± 5	78 ± 4	0.278	0.157
Pulse pressure (mmHg)	61 ± 11	50 ± 8	44 ± 4	38 ± 3	**<0.001**	0.071
Heart rate (bpm)	58 ± 5	66 ± 11	70 ± 9	74 ± 12	**0.022**	0.614
Stroke volume (ml)	107 ± 17	89 ± 13	78 ± 12	71 ± 11	**0.001**	0.289
Cardiac output (l/min)	6.1 ± 1.0	5.9 ± 1.1	5.4 ± 0.5	5.2 ± 1.0	0.243	0.726
PAT$_{Arm}$ (ms)	155 ± 17	167 ± 19	15 ± 19	155 ± 10	0.274	0.090
PAT$_{Finger}$ (ms)	209 ± 20	208 ± 18	199 ± 11	198 ± 15	0.257	0.981
PAT$_{Thigh}$ (ms)	215 ± 20	223 ± 19	199 ± 12	205 ± 17	0.050	0.732
PAT$_{Toe}$ (ms)	353 ± 23	352 ± 25	350 ± 22	346 ± 24	0.181	0.910
ICT (ms)	48 ± 7	58 ± 11	52 ± 7	54 ± 12	0.958	0.328
cPAT$_{Arm}$ (ms)	108 ± 16	109 ± 17	98 ± 7	101 ± 7	0.177	0.588
cPAT$_{Finger}$ (ms)	161 ± 18	150 ± 14	147 ± 11	144 ± 9	0.131	0.438
cPAT$_{Thigh}$ (ms)	167 ± 19	164 ± 17	147 ± 12	152 ± 16	**0.024**	0.567
cPAT$_{Toe}$ (ms)	306 ± 18	294 ± 24	298 ± 20	290 ± 20	0.115	0.405
$_{bf}$PWV (m/s)	8.6 ± 0.6	9.0 ± 1.2	9.9 ± 1.6	9.7 ± 1.4	**0.016**	0.161
AOC (ml/mmHg)	2.7 ± 0.6	3.1 ± 0.6	2.9 ± 0.4	3.0 ± 0.4	0.588	0.304
AOD (10^{-3} mm Hg^{-1})	4.8 ± 2.0	4.4 ± 2.3	4.8 ± 2.1	4.1 ± 2.1	0.705	0.740
AOA$_{min}$ (mm^2)	486 ± 97	512 ± 112	471 ± 73	490 ± 82	0.384	0.879

Aortic SBP and aortic DBP, aortic systolic and diastolic blood pressure; SBP and DBP, brachial systolic and diastolic blood pressure; PAT, pulse wave arrival time; ICT, isovolumetric contraction time; cPAT, PAT corrected for ICT; $_{bf}$PWV, brachial-femoral pulse wave velocity; AOC, aortic compliance; AOD, aortic distensibility; AOA$_{min}$, minimum aortic area. Results are sorted by sex and represented as mean values ± standard deviation. Statistical significance ($p < 0.05$) for main effect (sex) and interaction (sex vs. bed rest) is indicated by bold p-values.

did not observe an obvious qualitative difference in the HDTBR response between women and men. Another potential limitation of the study is that we did not assess the intima media thickness of different arteries. We also did not measure pulse wave arrival time to carotid artery or its therefrom derived carotid-femoral PWV, which is considered to be the non-invasive gold standard.

Despite these limitations we suggest that 60 days HDTBR, while producing cardiovascular deconditioning and cephalad fluid shifts, do not elicit clinically relevant changes in vascular stiffness biomarkers. Furthermore, the cardiovascular adaptation to HDTBR was not affected by daily artificial gravity training. Other analyses from AGBRESA also report no relevant influences of the artificial gravity intervention on physiological outcomes (Attias et al., 2020; Hoffmann et al., 2020; Kramer et al., 2020; Ganse et al., 2021; Lecheler et al., 2021). Combining artificial gravity with exercise holds promise for future bed rest studies (Diaz-Artiles et al., 2018).

Clearly, our findings cannot be simply extrapolated to real space conditions. In fact, current missions in low Earth orbit and future missions to Mars are substantially longer. Moreover, astronauts are exposed to additional risks that could exacerbate vascular aging, particularly radiation (Hughson et al., 2018). Our findings may also help interpreting previous vascular function data obtained in space and guide technology development for future missions. For example, previous studies in astronauts reported reduced PAT without measuring ICT (Baevsky et al., 2007; Hughson et al., 2016). Cardiac deconditioning in space (Hughson et al., 2018) could conceivably affect ICT. In fact, prolonged pre-ejection periods were reported in three Skylab astronauts after 59 days in space (Bergman et al., 1976). Combined with our findings from HDTBR, we conclude that only ICT corrected PAT values should be used as vascular biomarkers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Commissions of the Medical Association North Rhine (number 2018143) and NASA (Johnson Space Center, Houston, United States). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SM, JT, MB, and BH designed the experiment. MB provided technical support. SM, FH, and JR performed data acquisition. SM, FH, and SO were in charge of data analysis. BJ did statistical analysis. SM was primarily in charge of drafting the manuscript. EM, JT, and JJ primarily revised the manuscript. All authors contributed and accepted the final version.

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Impaired DNA Repair Fidelity in a Breast Cancer Patient With Adverse Reactions to Radiotherapy

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We tested the hypothesis that differences in DNA double-strand break (DSB) repair fidelity underlies differences in individual radiosensitivity and, consequently, normal tissue reactions to radiotherapy. Fibroblast cultures derived from a radio-sensitive (RS) breast cancer patient with grade 3 adverse reactions to radiotherapy were compared with normal control (NC) and hyper-radiosensitive ataxia-telangiectasia mutated (ATM) cells. DSB repair and repair fidelity were studied by Southern blotting and hybridization to *Alu* repetitive sequence and to a specific 3.2-Mbp *Not*I restriction fragment on chromosome 21, respectively. Results for DNA repair kinetics using the *Not*I fidelity assay showed significant differences ($P < 0.001$) with higher levels of misrepaired (misrejoined and unrejoined) DSBs in RS and ATM compared with NC. At 24-h postradiation, the relative fractions of misrepaired DSBs were 10.64, 23.08, and 44.70% for NC, RS, and ATM, respectively. The *Alu* assay showed significant ($P < 0.05$) differences in unrepaired DSBs only between the ATM and both NC and RS at the time points of 12 and 24 h. At 24 h, the relative percentages of DSBs unrepaired were 1.33, 3.43, and 12.13% for NC, RS, and ATM, respectively. The comparison between the two assays indicated an average of 5-fold higher fractions of misrepaired (*Not*I assay) than unrepaired (*Alu* assay) DSBs. In conclusion, this patient with increased radiotoxicity displayed more prominent misrepaired than unrepaired DSBs, suggesting that DNA repair fidelity is a potential marker for the adverse reactions to radiotherapy. More studies are required to confirm these results and further develop DSB repair fidelity as a hallmark biomarker for interindividual differences in radiosensitivity.

Keywords: DNA double-strand breaks, misrepair, *Not*I fragment, *Alu* sequence, radiosensitivity, adverse reactions to radiotherapy, repair fidelity, pulsed field gel electrophoresis

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INTRODUCTION

Compelling evidence suggests that adverse reactions to radiotherapy are associated with increased patient sensitivity to ionizing radiation (1). Likewise, individual variation in radiosensitivity is well-recognized and at least partly determined by genetic factors, as clinical, epidemiologic, and laboratory data have indicated (2–6). Initial evidence for

the heritability of radiosensitivity originated from the studies of rare genetic disorders such as ataxia-telangiectasia (A-T), Nijmegen breakage syndrome, Nijmegen breakage syndrome-like disorder (RAD50 deficiency), ligase IV deficiency, A-T-like disorder, and Fanconi's anemia (7, 8). Although each syndrome has its own phenotypical characteristics, cells derived from those patients demonstrate spontaneous chromosomal instability and hypersensitivity to ionizing radiation due to mutations affecting DNA strand breaks signaling, recognition, and repair capability (9).

Between the multiple damages produced by ionizing radiation, DNA double-strand breaks (DSBs) are the main critical lesions and are highly consequential for genome integrity (10). Repair is a fundamental inherent mechanism of genome protection, and the ability to rejoin DSBs with appropriate fidelity determines cell fate, recovery, death, or mutagenesis (11). Unrejoined and misrejoined DSBs are important lesions for radiation-induced cell killing, although the relationship between DNA repair, misrepair, and cell survival is not fully understood (12). Misrepaired (unrejoined or misrejoined) DSBs can lead to chromosome aberrations and micronucleus formation, and both endpoints generally correlate with the degree of cell killing (13). However, cellular death mechanisms, cell cycle kinetics, and the various underlying genetic defects influence the expression and detection of chromosome damage, thus making qualitative cytogenetic approaches less precise as quantitative measures of cellular radiosensitivity (14). Another method for examining the fidelity of DNA repair is to measure the ability of cells or cell extracts to reactivate plasmids containing damaged reporter genes. This approach has proven useful for examining DNA repair in some radiosensitive cell lines (15); however, it is also not particularly quantitative, as there was little difference in this measure of DSB repair fidelity between some cell lines with wide differences in radiosensitivity (16).

Another appealing DNA repair fidelity technique that assesses DSB misrepair has been described (17, 18). The procedure relies on the use of endonucleases to cleave out specific DNA fragments that are subjected to pulsed-field gel electrophoresis and detected by Southern hybridization to a known probe. Although it is not a widely used technique, the few data obtained using this method were scientifically interesting, as it allowed the detection of differences in the proportion of correctly rejoined DSBs produced by irradiations of different LETs (19) and in the fraction of unrejoined DSBs in cell lines of different origins (20). We have previously used this technique to detect misrepair of radiation-induced DSBs in a patient with unidentified chromosomal fragility syndrome and a family history of radiosensitivity (21). Here, we extend this basic research work to examine the ability of this technique to detect differences in DSB repair fidelity in a fibroblast cell line derived from a breast cancer patient who developed marked late adverse reactions to radiotherapy. The results were compared with a cell line of a patient with no radiotherapy tissue reactions and an extremely radiosensitive A-T mutated (ATM) cell line.

MATERIALS AND METHODS

Cell Cultures, Patients, and Ethical Considerations

Three primary non-transformed human skin fibroblast cultures, normal control (NC), radiosensitive (RS), and ATM were used. The ethics committee of the institutional review board approved the study (CA-06294/16672/50192; 1990). The ATM cell line (GM01588A) was purchased from the American Type Culture Collection (Manassas, VA, USA). The NC and RS were derived from two breast cancer patients, as described previously (22). Briefly, the NC patient did not develop any discernable (grade 0; Radiation Therapy Oncology Group and the European Organization for Research and Treatment of Cancer grading system) adverse effects, whereas the RS patient developed marked (grade 3) skin atrophy and telangiectasia. Both patients were treated by definitive radiotherapy (50 Gy in 2 Gy fractions). Compared with NC, RS was considered to have excessive late reactions for the dose received, which was verified from their treatment and dosimetry records. The cumulative total dose delivered to different normal tissues was estimated from computed tomography treatment plans. Late effects were documented from patient records. The median follow-up was 19 months (range: 13 to 25) at the time of data collection. The *in vitro* radiosensitivity characterization of the cell strains using clonogenic survival curves was published previously (23, 24). Briefly, the surviving fraction at 2 Gy radiation dose was 0.34 [95% confidence interval (CI) = 0.31–0.37], 0.18 (95% CI = 0.13–0.25), and 0.03 (95% CI = 0.02–0.04) for NC, RS, and ATM cell strains, respectively. The three cell lines were maintained in alpha minimal essential medium supplemented with 15% fetal bovine serum. Experiments were performed with contact-inhibited cultures to minimize cell cycle-dependent variations in DNA repair. All incubations were performed at 37°C in a humidified atmosphere of 5% carbon dioxide.

Irradiation

Cells were irradiated on ice in 150-mm Petri dishes using a ^{137}Cs source at a dose rate of 3.65 Gy/min.

*Not*I and *Alu* DNA Double-Strand Breaks Repair Assays

The *Not*I repair fidelity assay involves the use of endonuclease to cleave out a specific DNA fragment that can be detected by Southern hybridization to a known probe. Using the *Not*I rare cutting restriction enzyme, a unique 3.2-Mbp restriction fragment is cleaved out of the long arm of chromosome 21. After subjecting DNA to pulsed-field gel electrophoresis, the *Not*I 3.2-Mbp fragment migrates as a single band, which is detected and quantified by hybridization to the D21S1 single copy probe. Unrejoined and misrejoined *Not*I fragments induced by irradiation migrate separately from the *Not*I band. The extent of incomplete restoration of the 3.2-Mbp *Not*I band is taken as a quantitative measure of misrepair in this specific region of the genome. The *Alu* assay follows the same principal except for the

endonuclease use; being a highly repetitive sequence, it assesses unrepaired DSBs in the whole genome.

The Southern blot procedures for *Not*I and *Alu* DNA repair assays were published in detail previously (21, 25). Briefly, confluent cells were irradiated with either 30 Gy (for *Alu* genomic probe) or 80 Gy (for *Not*I specific fragment probe) and incubated at 37°C for up to 24 h. The choice of radiation doses was determined in preliminary experiments and optimized to induce DSBs in approximately 80% of the target DNA in each of the *Alu* and *Not*I fragments (21). The cells were trypsinized and centrifuged. The pellet was resuspended at a concentration of 2–4 $\times$ 10^7 cells/mL, for *Not*I DSBs repair fidelity, or 10^5 cells/mL for *Alu* total genomic DSB repair assays. The cell suspension was mixed with 1% low-melting point agarose (InCert, FMC BioProducts) and poured into plastic molds. Solidified plugs were lysed [0.5-M ethylenediaminetetracetic acid disodium (Na_2EDTA), 1% sodium lauroyl sarcosine, 1 mg/mL proteinase K, pH 8] at 50°C for 2–3 days, washed and stored in 0.5-M Na_2EDTA (pH 8) at 4°C. For restriction enzyme digestion, DNA in a half-plug was digested with 25 units of *Not*I restriction enzyme (Promega, Madison, WI, USA) at 37°C overnight and inactivated at 50°C for 2 h. As for genomic DNA repair (*Alu*), there is no need for a restriction enzyme treatment.

DNA DSBs were separated by pulsed-field gel electrophoresis, using a CHEF Mapper or CHEF-DR II electrophoresis system (Bio-Rad), in a 0.5× Tris/borate/EDTA buffer (45-mM Tris-base, 45-mM boric acid, 1-mM Na_2EDTA; pH 8). The field strength was 1.5 V/cm with pulse times linearly increasing from 50 to 5,000 s. Electrophoresis was carried out for 18 h at 24°C for total genomic DNA (*Alu* assay) and at 12°C for 140 h for DNA repair fidelity (*Not*I assay). Gels were stained with ethidium bromide for 15 min, destained for 1 h, and photographed with a digital camera system under ultraviolet transillumination. *Schizosaccharomyces pombe* and *Hansenula wingei* chromosomes served as size markers. For DNA transfer, gels were exposed to a germicidal ultraviolet lamp and soaked in the alkaline transfer solution (0.4-M NaOH; 0.6-M NaCl) for 30 min, and the DNA was transferred by capillary action to nylon membranes (GeneScreen Plus, Du Pont, NEN Research Products, Boston, MA, USA) over 2 days and air-dried.

For hybridization to the 3.2-Mbp *Not*I restriction fragment, the plasmid containing the D21S1 probe (pPW228C) was isolated from the host bacteria; the insert was cut out with *Eco*RI and gel-purified by standard procedures. Radioactively labeled probe was produced *via* random priming kit (Boehringer Mannheim, Gaithersburg, MD, USA) using [α-^{32}P]dCTP (222 TBq/mmol, NEN Life Science Products, Boston, USA). The membranes were hybridized for 2–2.5 days at 45°C in Hybrisol I (Intergen, Burlington, MA, USA) and heat-denatured ^{32}P-labeled DNA probe (0.3–1 $\times$ 10^8 cpm/membrane). The membranes were washed and exposed to storage phosphor screens for 1 to 6 days, depending on the signal intensity of each membrane. Likewise, the *Alu* probe was prepared and processed in a similar way except that it was cut out of hosting plasmid (BLUR8) using *Bamh1* restriction enzyme, and the labeled probe was hybridized overnight. At least two experiments were carried out for each cell line, two samples from each experiment were run on separate gels, and the results were pooled.

Data Analysis

The quantitative analysis of the Southern blot data was described previously (21). Briefly, the total intensity (I_0) was calculated as the integral of the signal in the whole lane after baseline adjustment. For DNA repair in the whole genome (*Alu* assay), the intensity of the migrating DSBs in the *Alu* sequence (I_1) was quantified by the integral of the signal below the well. The relative amount of DNA released from the plugs (corresponding to DSBs $\leq$ 10–12 Mbp) was calculated by dividing the signal intensity of the migrating DNA (I_1) by that of the whole lane, including the wells (I_0). For DSB repair fidelity (*Not*I assay), the intensity of the full-length *Not*I fragments (I) was quantified by the integral of the signal in the 3.2-Mbp band. The signal intensity of misrepaired (misrejoined and unrejoined) DSBs (I_1) was calculated by subtracting I from I_0. The relative amount of misrepaired 3.2-Mbp *Not*I restriction fragment in each lane was calculated by dividing I_1 by I_0. For both assays, the kinetics of DSB repair, after various repair times, was presented as a fraction (%) of DSBs remaining unrepaired or misrepaired (also known as the fraction of "radiation" activity released). The DSBs remaining unrepaired (Alu assay) or misrepaired (NotI assay) were calculated by dividing the relative amount by that induced before any repair (0 h repair time) after subtraction of background (0 Gy) for each. Testing for statistically significant differences in the kinetics of DSBs misrepaired or remaining unrepaired between the cell strains was carried out using the one-way repeated measures analysis of variance test. This test compares differences in the mean values, computed over all the repair time points, among the cell strains. Comparing between the cell strains at each time point of repair was carried out using the one-way analysis of variance test. Testing for the difference from baseline zero-level was conducted using a one-tailed *t*-test. SigmaPlot software (versions 12.5 and 13; Systat Software Inc., San Jose, CA, USA) was used for statistical analysis. $P < 0.05$ was considered significant.

RESULTS

Figure 1 shows representative examples of ethidium bromide-stained gels (left panels) and the corresponding membranes hybridized to the *Alu* (BLUR8) and *Not*I (D21S1) probes (right panels). On the gel photographs, we can distinguish the wells containing non-migrating high molecular weight DNA and a smear of migrating DSBs of approximately 5.7 Mbp or less. On the membranes, we only see the DNA hybridized with the probes. The *Alu* genomic probe hybridizes to the widespread *Alu* sequence in the human genome. The signal below the wells (plugs) represents the amount of DSBs in the whole genome. The D21S1 probe hybridizes to a specific 3.2-Mbp *Not*I fragment on chromosome 21. Full-length *Not*I fragments are located in a band at 3.2 Mbp. Irradiation breaks down the *Not*I fragments leading to a smear seen below the *Not*I band. When the time for repair increases from 0 to 24 h, the broken DSBs are repaired, resulting

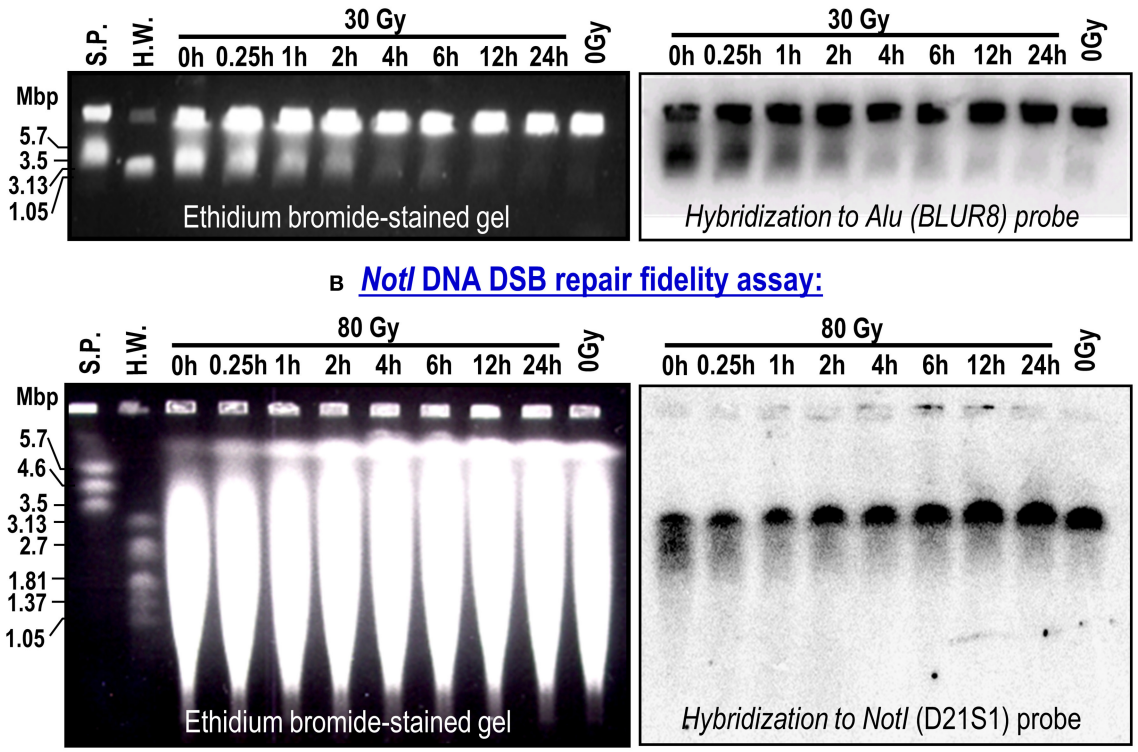

FIGURE 1 | Representative ethidium bromide-stained gels and corresponding membranes hybridized to *Alu* repetitive sequence in whole genome (BLURB probe; **A**) or specific *Not*I fragments located on chromosome 21 (D21S1 probe; **B**). Bulk rejoining of DSBs in genomic DNA and DSB repair fidelity in a 3.2-Mbp DNA fragment. Data from NC cell strain. S.P. (*S. pombe*) and H.W. (*H. wingei*) are DNA size standards.

in the gradual diminishing of the *Alu* signal below the wells and restitution of the full-length *Not*I fragments.

Illustrative comparison of *Alu* and *Not*I hybridized membranes in the three cell lines are shown in the upper panels of **Figure 2**. With increasing time for repair, the hybridization signals were different between the cell lines. The control cell line showed the highest restitution of DSBs compared with the radiosensitive patient and particularly the extremely sensitive ATM cell strain that showed a comparatively high level of unrepaired DSBs. Although the average background level of DSBs (0 Gy) was 7.31% [range: 6.48–9.36%; standard deviation (SD) = 1.79] in the *Alu* assay, it was 27.47% (range: 21.76–35.28%; SD = 6.99) in the *Not*I assay. In the *Alu* whole-genome assay, the fraction of induced DSBs, without repair, after a dose of 30 Gy was 0.84 (SD = 0.02), 0.87 (SD = 0.02), and 0.83 (SD = 0.03) for NC, RS, and ATM, respectively. Similarly, in the *Not*I fragments, a dose of 80 Gy induced comparable fractions in the cell strains [NC: 0.79 (SD = 0.04), RS: 0.83 (SD = 0.05), and ATM: 0.84 (SD = 0.03)].

The results for DSBs remaining unrepaired or misrepaired after subtraction of 0 Gy and normalizing to the induction level (0 h) are presented in the lower panels of **Figure 2**. The repair kinetics indicated differences not only between the three cell strains but also between genomic *Alu* and specific *Not*I repair assays. Although it is obvious that these cell lines display a wide range of radiosensitivity exhibited by different capacities to restitute broken DNA, the *Not*I repair fidelity assay showed higher levels of misrepaired DSBs. For example, at 24 h of repair, where the largest differences are seen, the average values for residual DSBs were 5.60% (SD = 5.73) for *Alu* and 26.13% (SD = 17.25) for *Not*I assays. This is an average of a 5-fold increase in residual DSBs detection in the DNA repair fidelity assay compared with general or bulk DNA repair. In addition, using a one-tailed *t*-test to examine the divergence of the 24-h residual DSBs in the three cell strains from the baseline (0%) level, the differences for the *Not*I assay were statistically significant ($P = 0.03$), although not so for the *Alu* assay ($P = 0.08$).

The percentage of DSBs misrepaired or remaining unrepaired in the *Not*I fragments after 24 h of repair were 10.64, 23.08, and 44.70% for NC, RS, and ATM, respectively. This indicates a 2.2- and 4.2-fold increase in misrepair in RS and ATM as compared with NC, respectively. Statistical analysis of *Not*I DNA repair kinetics showed a significant difference in DSBs misrepaired between the three cell strains (one-way repeated measures analysis of variance, $P < 0.001$). In comparison, the *Alu* assay showed lower levels of DSBs remaining unrepaired. The NC and RS showed similar repair kinetics, whereas ATM displayed a relatively higher level of unrepaired DSBs. The percentages of

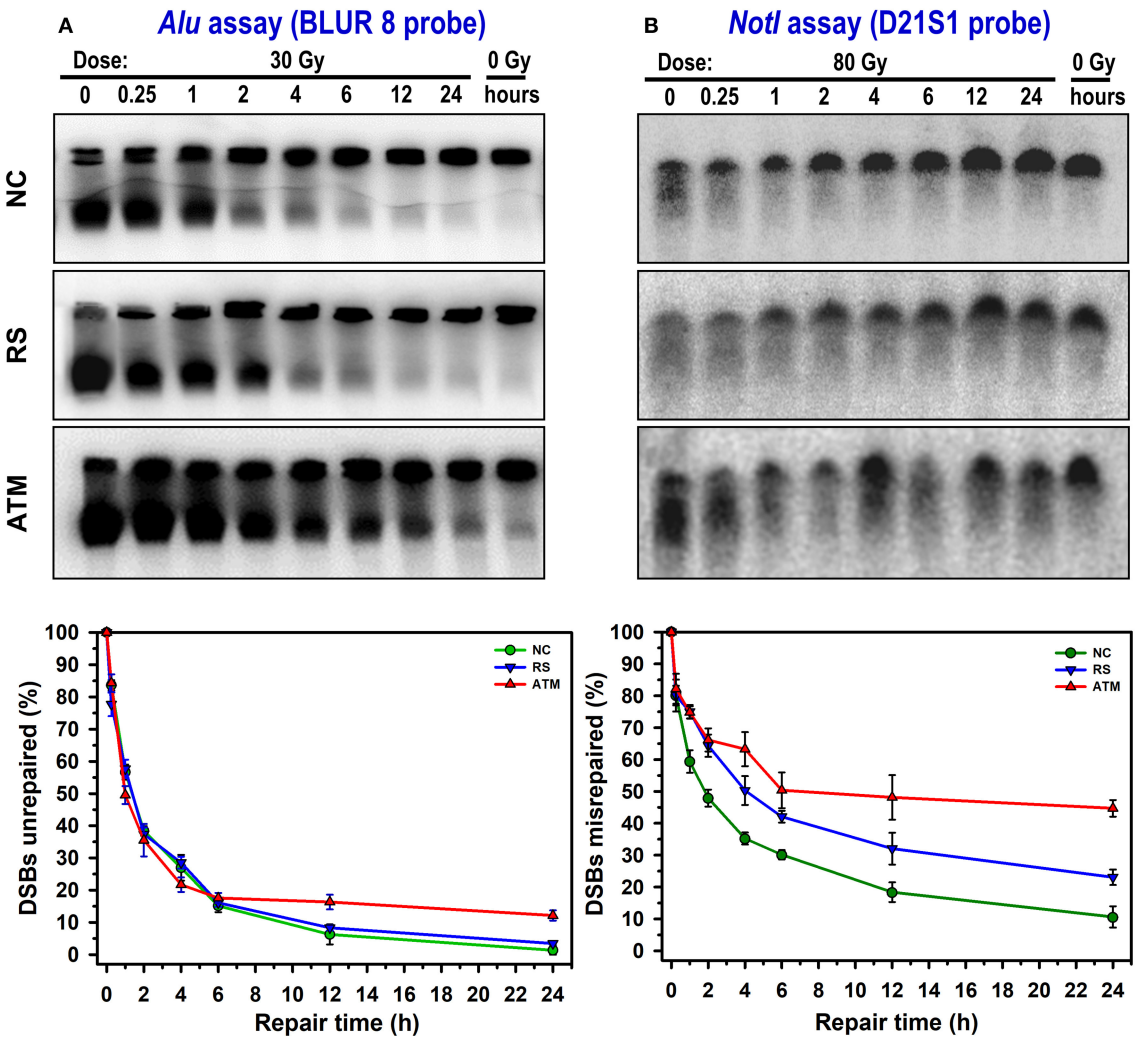

FIGURE 2 | Representative examples of DSB repair and repair fidelity in NC, RS, and ATM cell strains. Membranes were hybridized to *Alu* repetitive sequence (whole-genome DSBs unrepaired; **A**) and specific *Not*I fragments (DSBs misrepaired; **B**). Curves show corresponding kinetics of DSB repair after subtraction of membrane background and normalizing to total amount of DSBs induced immediately after irradiation (0-h repair time). Data points represent mean, and error bars represent standard error of mean. Results of statistical analysis (one-way repeated measures analysis of variance) are as follows: *Not*I assay, there is an overall significant difference between cell lines ($P < 0.001$). Pairwise comparison: ATM *vs*. NC: $P < 0.001$; RS *vs*. NC: $P = 0.014$; ATM *vs*. RS: $P = 0.034$. For *Alu* assay, no overall significant differences between cell lines ($P = 0.82$). However, significant differences (one-way analysis of variance) between cell lines ($P < 0.05$) were observed at 12 and 24 h. Pairwise comparison at 12 h: ATM *vs*. NC: $P = 0.024$; ATM *vs*. RS: $P = 0.054$; RS *vs*. NC: $P = 0.548$. Pairwise comparison at 24 h: ATM *vs*. NC: $P < 0.001$; ATM *vs*. RS: $P < 0.001$; RS *vs*. NC: $P = 0.287$.

the DSBs unrepaired at 24 h in the *Alu* whole-genome assay were 1.33, 3.43, and 12.13% for NC, RS, and ATM, respectively. This indicates a 2.6- and 9.3-fold increase in DSBs unrepaired in RS and ATM as compared with NC, respectively. Nevertheless, there was no statistically significant difference in unrepaired DSBs between the three cell strains when all time points are considered together (one-way repeated measures analysis of variance, $P = 0.82$). However, testing for dissimilarities at each time point of repair kinetics revealed statistically significant differences at 12 and 24 h between ATM and both NC and RS (one-way analysis of variance, $P < 0.05$); meanwhile, no difference was observed between NC and RS (**Figure 2**).

DISCUSSION

In this study, we examined the possibility of detecting differences in DSB misrejoining (unrepaired and misrepaired) in a primary human fibroblast strain derived from a clinically radiosensitive patient with marked (grade 3) adverse effects to radiotherapy. The results were compared with a cell line from a patient who exhibited no adverse effects (grade 0) and a cell line derived from an ATM patient who is known for their extreme radiosensitivity (patients typically develop grade 4 adverse reactions). In the three cell lines, a dose of 30 Gy led to the induction (before any repair) of comparable amounts of DSBs in the entire genome (*Alu* probe,

0.85, SD = 0.02). Similarly, a dose of 80 Gy produced comparable amounts of DSBs in the *Not*I fragments (0.81, SD = 0.02). These results are in line with results obtained by other investigators (26). However, when irradiated cells were allowed to repair, these different cell lines displayed a distinguishable capacity to join DSB ends (**Figure 2**). The repair kinetics displayed an exponential decay shape with an initial rapid decrease in the fraction of DSBs misrepaired or unrepaired that extends to approximately 4 h, followed by a slight decrease after that. This shape is quite common for DSBs repair kinetics, which can be mathematically fitted by first-order or biphasic exponential decay equations (27, 28). Although such a fitting may facilitate analysis and contribute to the characterization of hypothetical biological processes, it is not required to derive conclusions from the results presented (29).

The three cell strains showed comparable initial repair kinetics for the entire genome (*Alu* assay), whereas differences were mainly detectable in the slow distal time points from 12 to 24 h (**Figure 2**). The fraction of DSBs remaining unrepaired was between 1 and 12% of the initial damage (i.e., 88 to 99% were rejoined). However, this level does not seem to significantly diverge from the baseline (0%) level ($P = 0.08$). Note that the NC and the RS cell lines showed similar kinetics when compared with ATM, which seemed to have a slightly faster repair up to 4 h, followed by slower repair after that. Although there were no overall significant differences between the three cell strains ($P = 0.82$), the ATM showed, as expected, significantly ($P < 0.05$) higher unrepaired DSBs at 12 and 24 h postradiation. In contrast, for the repair fidelity assay, the three cell strains showed distinguishable kinetic curves of statistical significance ($P < 0.001$) at much earlier repair times, which continued to 24 h. On average, the *Not*I assay showed a 5-fold higher level of residual DSBs than the *Alu* assay. Therefore, the repair fidelity assay was able to detect differences in DSB repair between patients with and without radiotoxicities and the extreme radiosensitivity of ATM. This is consistent with a previous observation of normal, ATM, and AT-heterozygous cell types (26). The NC cell line exhibited the lowest rate of misrepair (highest repair fidelity) in the 3.2-Mbp *Not*I restriction fragment with a level of misrejoining and unrejoining at 24 h of 11% of the initial damage, i.e., 89% of ends were rejoined. The ATM cell line showed a relatively high level of misrepair, 45% (55% of rejoining), whereas the RS cell line was intermediate with 23% (77% rejoining) misrepair. The latter is a relatively lower misrepair in comparison with a previously reported radiosensitive cell strain, which showed a *Not*I DSBs misrepair level at 24 h of 37% (21). Furthermore, these levels of DSB misrepair at 24 h seem to significantly diverge from the baseline (0%) level ($P = 0.03$).

The results suggest that, for the radiosensitive patient, misrepair of broken DNA ends in non-transformed fibroblasts is associated with late normal tissue reactions after radiotherapy. In view of the results with a normally sensitive patient, a radiosensitive patient who developed late adverse complications, and an ATM patient (GM01588), who will invariably develop severe and even fatal complications if treated with a standard radiotherapy regimen, it is tempting to speculate that the misrepair assay could provide a better resolution and more accurate measure of radiosensitivity than DNA repair within the entire genome. Nevertheless, a previous work using the *Not*I assay identified differences in misrepair only after fractionated irradiation (26). Using normal (derived from a healthy volunteer with no radiosensitivity data), ATM, and AT heterozygous cell lines, differences were observed after low dose per fraction (5 and 10 Gy) and not with a high (20 Gy) or at the total 80 Gy given as a single dose. In our study, the variation was observed after a single 80-Gy dose. Other doses were not examined. The apparent discrepancy may lay in the levels of misrejoined DSB observed in the normal cell strains. However, the dissimilarities in the methodological details between the two studies preclude a precise comparison.

Although this study is not designed to delve into the particular radiosensitivity of the ATM cell strain, it can be speculated from the data that radiosensitivity is associated with an increased level of misrepaired DSBs. This is in line with the observation that it displayed increased levels of unrepaired DSBs (30). The present demonstration of a higher level of misrejoining observed in the *Not*I repair fidelity assay (**Figure 2**) supports a mechanistic answer for the increased rate of chromosomal aberrations in ATM and other chromosomal fragility syndromes (24). Genome editing technology demonstrated that artificially introduced heterozygous mutations of the ATM gene increased the number of chromosomal aberrations after irradiation and shed light on the genetic basis underlying individual differences in radiosensitivity within the human population (31). In addition, researchers described ATM cells as having higher initial repair speed followed by a stagnant slow repair leading to higher DSBs unrepaired in the whole genome (30), which can be perceived in this study (**Figure 2**, left panels). However, this is not the case in the repair fidelity assay, which suggests slower initial repair kinetics than that observed with NC and RS cell strains (**Figure 2**, right panels).

Finally, this study contributes to the ever-expanding experimental evidence for the increased radiosensitivity of a small percentage of radiotherapy patients treated with standard regimens. Those patients develop adverse radiation sequelae that cannot be attributed to dose distribution or volume irradiation (22). Many reports about cohorts and individual cases of radiosensitivity have been published where clinical radiosensitivity was associated with certain *in vitro* experimental endpoints with variable results (14). Between the various mechanistic pathways investigated, the radiation-induced DNA damage response remains the most well-characterized (32, 33). However, some other mechanisms and pathways were suggested to be involved in patient radiosensitivity, including oxidative stress, stem cell response, activation of inflammation pathways with the secretion of cytokines, genetics, non-coding RNA, and potentially epigenetic factors that can be studied using a large number of functional assays (3). For instance, individual variations in radiosensitivity have been attributed to a dissimilar genetic makeup affecting various cellular processes (2). However, besides a few syndromes with identified genetic mutations, the culprit molecular pathway affecting the radiosensitivity of phenotypically normal patients remains elusive (34). For mildly and moderate overreacting patients, polymorphic

genetic variations in DNA repair and other processes are believed to be related to the interindividual reactions to radiotherapy (35, 36).

The genomic basis of radiosensitivity is important both in cancer therapy, where normal and tumor cells differ in their response to treatment (37), and in neoplastic transformation, where exposure to radiation (as in occupational and diagnostic radiology) and environmental hazards may have different carcinogenic susceptibilities in the population (38). In fact, DSB repair fidelity is believed to be an important mechanism for radiation-induced cancer and a potential marker for radiation susceptibility. However, both NC and RS are breast cancer patients, and ATM is known to predispose to a certain type of malignancies. Thus, the lowest residual level of DSB misrepaired observed in the NC cells could reflect cancer susceptibility in this breast cancer patient with normal radiosensitivity. Furthermore, the striking observation of the larger amount of misrepaired DSBs in the *Not*I repair fidelity assay compared with the modest amount of unrepaired DSBs in the whole genome (*Alu* assay) suggests that DNA repair fidelity is also a mechanism involved in radiation sensitivity. Therefore, it can be speculated that assessing DNA repair fidelity in cancer patients may be a useful indicator as a prognostic or predictive marker for the likelihood of developing radiotoxicity after radiation treatment or exposure to other clastogenic agents.

CONCLUSIONS

This study demonstrated that in the cells derived from this patient who had severe adverse reactions to radiotherapy, DNA DSBs were more likely to be misrepaired rather than unrepaired. This may imply that DNA repair fidelity is a mechanism leading to adverse reactions to radiotherapy. More studies with large patients' cohort are required to confirm these results. The DNA repair fidelity assay may be an important endpoint to be further perused and developed as a hallmark for radiosensitivity.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Institutional Review Board (CA-06294/16672/50192). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS and GA: conceptualization and funding acquisition. GA and SI: methodology. GA, SI, and NA-H: investigation. GA: formal analysis. MS and GA: validation. GA: writing the original draft. All authors: review and editing.

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Substantial and Reproducible Individual Variability in Skeletal Muscle Outcomes in the Cross-Over Designed Planica Bed Rest Program

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To evaluate the individual responses in skeletal muscle outcomes following bed rest, data from three studies (21-day PlanHab; 10-day FemHab and LunHab) were combined. Subjects (n = 35) participated in three cross-over campaigns within each study: normoxic (NBR) and hypoxic bed rest (HBR), and hypoxic ambulation (HAMB; used as control). Individual variability (SD_{IR}) was investigated as $\sqrt{(SD^2_{Exp}–SD^2_{Con})}$, where SD_{Exp} and SD_{Con} are the standard deviations of the change score (i.e., post – pre) in the experimental (NBR and HBR) and the control (HAMB) groups, respectively. Repeatability and moderators of the individual variability were explored. Significant SD_{IR} was detected for knee extension torque, and thigh and calf muscle area, which translated into an individual response ranging from 3 to −17% for knee extension torque, −2 to −12% for calf muscle area, and −1 to −8% for thigh muscle area. Strong correlations were found for changes in NBR vs. HBR (i.e., repeatability) in thigh and calf muscle area (r = 0.65–0.75, $P < 0.0001$). Change-scores in knee extension torque, and thigh and calf muscle area strongly correlated with baseline values ($P < 0.001$; r between −0.5 and −0.9). Orthogonal partial least squares regression analysis explored if changes in the investigated variables could predict calf muscle area alterations. This analysis indicated that 43% of the variance in calf muscle area could be attributed to changes in all of the other variables. This is the first study using a validated methodology to report clinically relevant individual variability after bed rest in knee extension torque, calf muscle area, and (to a lower extent) thigh muscle area. Baseline values emerged as a moderator of the individual response, and a global bed rest signature served as a moderately strong predictor of the individual variation in calf muscle area alterations.

Keywords: FemHab, LunHab, PlanHab, skeletal muscle atrophy, microgravity

INTRODUCTION

Interindividual differences in the physiological responses to an intervention (e.g., exercise or drugs) have received great research attention in the last decades with the aim to identify "responders" and "non-responders," to explore the mechanisms that influence the individual responsiveness, and to promote "personalized medicine" (Mann et al., 2014; Hecksteden et al., 2015; Ross et al., 2019). However, some of the approaches used to analyze individual variability have not taken into account the variability explained by technical and/or random errors, and thus have not reported biological variability alone (Atkinson and Batterham, 2015). Hence, controlling for these factors is essential for the accurate determination of the true individual response to an intervention (Atkinson and Batterham, 2015; Atkinson et al., 2019). To tackle these limitations, the inclusion of a control group and a sufficiently large sample size to analyze variance rather than effect-sizes is crucial (Atkinson and Batterham, 2015; Hopkins, 2015). In addition, the use of cross-over designs with complete wash-out periods between intervention/control stages can be very helpful, since they offer the advantage of controlling for genetic factors influencing the individual response. It follows that only when an individual response is confirmed, potential factors (i.e., moderators or mediators) that may influence the observed individual response can be explored.

Bed rest is the gold-standard spaceflight analog to investigate skeletal muscle alterations induced by unloading, to test countermeasures designed to combat unloading-induced changes, and to explore the molecular processes underpinning inactivity-induced muscle atrophy (Rullman et al., 2018; Fernandez-Gonzalo et al., 2020). Despite the numerous advantages of this model, performing bed rest studies of long duration is extremely challenging due to staff- and economic-related constraints. Only large national or international space agencies can afford to run long-duration bed rest campaigns, which include a rather limited number of subjects per intervention group. The small sample size is indeed a significant constraint when exploring individual variability in bed rest studies (Scott et al., 2021). Furthermore, the lack of a genuine control group in bed rest studies, i.e., an ambulatory group, has limited the validity of any past attempt to detect individual responses to bed rest interventions (Atkinson and Batterham, 2015; Scott et al., 2021). Overcoming these limitations could translate into improved health management of astronauts and optimized individual programs to counteract the negative effects of unloading both during space missions and on Earth (Scott et al., 2021).

A unique opportunity to address individual variability upon bed rest responses is the Planica bed rest program, where three studies lasting 10 or 21 days have been performed using identical pre- and post-bed rest tests under strictly-controlled conditions (Keramidas et al., 2016; McDonnell et al., 2019, 2020). In the three studies, each participant completed three interventions [i.e., hypoxic ambulation (HAMB), normoxic bed rest (NBR), and hypoxic bed rest (HBR)] in a randomized, cross-over design. Combining the three studies offers one of the largest reported bed rest datasets to date, increasing the statistical power to analyze the individual variability in skeletal muscle outcomes after bed rest.

In the current report, we combined the results from three bed rest studies performed at the Planica facility to evaluate changes in skeletal muscle mass and function induced by 10 and 21 days of bed rest with/without a hypoxic environment in a cohort of 35 participants. Then, we investigated the individual response in the variables showing robust changes to bed rest and assessed the potential moderators that may explain the variability across individuals. Given the current knowledge in bed rest-induced muscle alterations, we hypothesized that the loss of knee extension strength and reduction in muscle mass in both the thigh and the calf would be the muscle features most markedly affected by bed rest. We further hypothesized that changes in knee extension strength and muscle areas would show clinically relevant individual variability that is influenced by baseline levels and energy intake during the intervention.

MATERIALS AND METHODS

General Study Design

Data were collected from three studies during the following periods: LunHab (from March to September 2011), PlanHab (from September 2012 to October 2013), and FemHab (from November 2013 to May 2014). These three bed rest studies were conducted at the Planica facility in the Olympic Sport Centre Planica, Ratece, Slovenia. In each study, subjects participated in three experimental campaigns in a counterbalanced randomized, cross-over design: NBR, HBR, and HAMB (the hypoxic conditions corresponded to an altitude of 4,000 m). The ambient conditions for each study are detailed in **Supplementary Table 1**. In both FemHab and LunHab each intervention lasted 10 days, while in the PlanHab study, the interventions had a duration of 21 days. The detailed study protocols have been described elsewhere (Debevec et al., 2014; McDonnell et al., 2019, 2020). In the current study, data related to muscle function, muscle area, body composition, and caloric intake from the three individual studies were merged into a single database, where subjects were grouped according to the particular intervention, irrespective of the study of origin. To be included in the final database, a participant had to complete at least two of the three campaigns. Then, data were analyzed for each variable as described below, and individual response was calculated. Finally, we analyzed potential moderators that could have influenced the subjects' individual response.

Participants

Inclusion and exclusion criteria for PlanHab, LunHab, and FemHab have been described in detail elsewhere (Debevec et al., 2014; McDonnell et al., 2019, 2020), and those criteria followed the European Space Agency (ESA) guidelines (Heer et al., 2009). Briefly, following a reply to a national advertisement, participants were provided with a detailed document outlining the specifics of the study. If they were interested and understood the protocol, they were then invited to a panel interview with at least 3 experienced researchers. From the initial pool of participants, a

minimum of 20 were chosen in each study, and took part in a familiarization weekend at the Planica facility. This weekend provided an excellent opportunity to observe the participants interact, take part in experiments and deal with researchers. The final inclusion of the successful participants was based on the ESA guidelines, their initial interview and their performance during the familiarization. Once the participants were selected, they signed a written informed consent to participate in the particular study. The three studies conformed to the standards set by the Declaration of Helsinki. The procedures were approved by the Committee for Medical Ethics at the Ministry of Health (Republic of Slovenia; approval numbers: 205/2/11 and 88/04/12).

Intervention Procedures

With the exception of the length of intervention (10-day interventions in the LunHab and FemHab studies; 21 day interventions in the PlanHab study) and participant sex (males in the LunHab and PlanHab studies, and females in the FemHab study), the protocol of the three interventions (HAMB, HBR, and NBR) was similar in all three studies. The studies were designed as cross-over repeated measures, such that each subject participated in all three interventions. Each study comprised three research campaigns, during which all subjects were confined to the Olympic Sport Centre. In the first campaign, the participants were randomly assigned to an intervention, and in the following two campaigns, they were then exposed to the remaining interventions. In any given research campaign (in all three studies), all three interventions were conducted simultaneously. The washout period between interventions was a minimum of 1-month for the 10-day interventions (LunHab and FemHab studies), and a minimum of 4 months for the 21-day interventions (PlanHab study). This ensured recovery of the participants taking part in more than one intervention (Convertino et al., 1985; Sandler et al., 1988). Briefly, during an ambulatory pre-intervention period of 5 (LunHab, FemHab) or 7 (PlanHab) days, subjects acclimated to the regime requested during the studies (i.e., sleep/wake cycle, nutrition, etc.), and baseline experimental measurements were obtained. This was followed by the intervention period, i.e., NBR, HBR, or HAMB. Finally, upon completion of the intervention, participants were requested to remain at the facility for an additional 4 to 7 days, so that post-intervention measurements could be obtained. For those who had completed a bed rest intervention, this also allowed safe re-ambulation. During the interventions, participants adhered to a strict daily schedule. They were awakened at 07:00 AM, with lights out at 11:00 PM. Napping during the day was not allowed, and participants in the HAMB interventions had to maintain a seated upright or standing position during the day (i.e., feet had to be in contact with the floor at all times). During the bed rest interventions (NBR and HBR), the participants had to maintain a strict horizontal bed rest. All daily activities were carried out in the horizontal position. The participants could use one pillow for head support, and were allowed to support themselves on an elbow during meals. Physical activity, apart from changing positions from supine to prone or lateral, was not permitted during the bed rest interventions. To ensure compliance to the bed rest protocol, participants were monitored at all times using continuous closed-circuit television, and by the medical staff. During the HAMB intervention the participants performed two daily low-intensity physical activity sessions to mimic their previous habitual daily activity. In LunHab, the participants performed 30 min of a stepping exercise in the morning (heart rate, HR: 115.9 $\pm$ 3.3) and afternoon (HR: 112 $\pm$ 2.7; McDonnell et al., 2014). The exercise mode was varied for PlanHab and FemHab to offer variety (stepping, cycling, or dancing), and it was rotated in the afternoon to avoid monotony. The participants always took part in a stepping exercise in the morning and chose their preferred activity for the afternoon. The average HR for both morning and afternoon sessions in PlanHab was 124 $\pm$ 9 (Keramidas et al., 2016). The FemHab participants HR response to the activity was 131 $\pm$ 10 (Debevec et al., 2016). The target heart rate for each of these exercise sessions was equivalent to that attained at 50% of the parpitants peak power out during a hypoxic (4,000 m) cycle ergormetry test to exhaustion.

Oxygen Depleted Gas

During the HBR and HAMB interventions, normobaric hypoxia was maintained with a vacuum pressure swing adsorption system (VPSA: b-Cat, Tiel, Netherlands), which delivered oxygen-depleted gas to the hypoxic area of the Planica facility. The oxygen content of each room in the facility was assessed by the VPSA system at 15 min intervals throughout the interventions. If the concentration measured in the rooms was above the target fraction of ambient O_2 (F_IO_2: 0.142) a hypoxic gas mixture generated by the VPSA system was pumped to the desired room. However, if the ambient O_2 was below the target, the delivery of external normoxic air was iniated to that area. As a safety precaution, the participants also carried a personal O_2 analyzer (PGM-1100; Rae Systems, San Jose, CA, United States), which would provide immediate feedback of the ambient F_IO_2.

Muscle Function

The Biodex S4 Pro isometric dynamometer (Biodex Medical Systems, System Pro 4, Shirley, New York, United States) was used to assess muscle function through a maximal isometric voluntary contraction before and after each campaign. The following joints and angles of assessment were measured unilaterally: ankle: 15° plantar flexion; knee: 60° and the elbow 60°. The dynamometer was calibrated prior to any testing. The joint center of rotation was aligned with the axis of rotation of the dynamometer and the participants were requested to conduct a maximal-effort muscle action. The protocol was standardized according to the ESA standard operating procedures and consisted of a 5-s isometric contraction of the agonist followed by a 5-s recovery, then a 5-s isometric contraction of the antagonist followed by a 5-s recovery. This pattern was repeated until 5 contractions of each muscle group were completed. The maximal torque was described as the peak isometric force attained during 50 ms epochs in any of the five 5-s contractions.

Muscle and Fat Area

The muscle cross sectional area (CSA) and fat area in the thigh and calf were assessed using peripheral quantitative

computer tomography (pQCT, XCT3000 Stratec Medizintechnik, Pforzheim, Germany). The participants' non-dominant leg was fully extended and positioned within the device while they lay supine on an adjacent medical bed. The foot and thigh were fixed to the supporting structures of the pQCT device. Muscle CSA and fat area were assessed before the start of each intervention and on the final day (day 10 in LunHab and FemHab; day 21 in PlanHab). pQCT scans were obtained at 66% of the tibial length (from the ankle) and at 33% of the femoral length (from the knee) and the resultant images were analyzed with the manufacturers' software (XCT3000 version 5.4) and data stored for subsequent analysis.

Body Composition

Whole body and regional fat and lean mass were assessed with dual-energy X-ray absorptiometry (DEXA) before and immediately after each campaign using a Hologic fan-beam densitometer (Discovery W-QDR series, Hologic, Bedford, United States). DEXA scans were analyzed with Hologic DOS software (Hologic APEX System Software, version 3.1.2). During the DEXA scans, participants were dressed in minimal clothing (i.e., *t*-shirt and underwear). Daily calibration was conducted with the soft tissue calibration modules provided by the manufacturer. Participants were scanned supine in a fasted state and well rested. The same researcher analyzed all scans. The regions of interest used for the analysis were: (a) upper arm (elbow joint center–gleno-humeral joint center; lower–upper boundary); (b) thigh (knee joint center–acetabulo-femoral joint center); and (c) lower leg: (ankle joint center–knee joint center).

Diet and Energy Intake

A strictly controlled, standardized diet was adapted to the individual participant's requirements to facilitate energy balance during the study (Debevec et al., 2014). Daily resting energy expenditure was estimated for each participant using the modified Harris–Benedict equation (Roza and Shizgal, 1984) and multiplied by a physical activity level factor (PAL) to calculate daily dietary energy requirement according to the study phase. During the ambulatory phases (Pre and Post intervention and HAMB confinement) a PAL of 1.4 was used, with a PAL of 1.2 applied for the bed rest phases (NBR and HBR confinement), according to standard guidelines (Sundblad and Orlov, 2014). The target macronutrient composition of the diet, expressed as a proportion of total dietary energy intake, was 55% carbohydrate, 30% fat, and 15% protein. Additionally, the diet aimed to provide 1.2 g of protein/kg body weight and a sodium intake of $<$3,500 mg per day, and was supplemented with vitamin D3 (1,000 IU/day).

The standard menu was devised using a web-based application "Open Platform for Clinical Nutrition" (www.opkp.si, Jozef Stefan Institute, Ljubljana, Slovenia), and repeated during each subsequent campaign to ensure the consumption of identical meals on the same days of each respective campaign. Five meals (breakfast, morning snack, lunch, afternoon snack, and dinner) were served daily and at the same time of the day throughout the campaigns. Meals were based on the standard Slovene diet and participants were encouraged to eat all of the food provided. Moreover, a daily intake of $>$28.5 ml of fluid per kg body weight was encouraged through *ad libitum* consumption of water and unsweetened fruit tea. No additional food or drink (outside of the provided menu) were allowed, including consumption of alcohol or caffeine-containing beverages, but participants could choose to consume less food than was provided. During plating of the meals, each food item was weighed on a precision ($\pm$0.1 g) scale (TPT 6C, Libela ELSI, Celje, Slovenia) connected to a custom-developed, computer-based food recording and analysis system (Piki 2.0, Faculty of Computer science, University of Ljubljana, Ljubljana, Slovenia), with unconsumed food items being re-weighed and the value deducted from the initial weight to provide actual food intake.

The daily difference between actual and targeted energy intake was calculated for each participant and averaged across each intervention. In addition, the coefficient of variation (CV; standard deviation divided by the mean of the daily differences in the actual vs. targeted energy intake) was calculated for each participant and intervention.

Data Analysis

Potential differences in pre-to-post for all outcome measures in the three campaigns (HAMB, NBR, and HBR), were analyzed parametrically in a pairwise fashion and standardized effect sizes were calculated, where values of $<$0.1, 0.1–0.3, 0.3–0.5, and $>$0.5 were deemed as trivial, small, moderate, and large, respectively, (Hopkins, 2006). In addition, a mixed-effects model with study (i.e., PlanHab, FemHab, and LunHab) and intervention (i.e., HAMB, NBR, and HBR) as fixed-factors and subject as a random factor, was performed using the change scores (i.e., difference post – pre) of all available variables. Significant interactions were followed by Tukey *post-hoc* tests to correct for multiple comparisons. Individual variability was investigated by using the definition suggested by Hopkins (2015) and Atkinson and Batterham (2015), that is; $SD_{IR} = \sqrt{(SD^2_{Exp} - SD^2_{Con})}$, where SD_{IR} is the standard deviation for the individual response and SD_{Exp} and SD_{Con} are the standard deviations of the change score in the experimental (NBR or HBR) and the control (HAMB) groups, respectively. In the event of a greater SD_{Con} vs. SD_{Exp}, and given that it is not possible to calculate the square root of a negative number, the sign was changed to perform the square root but the final result was considered negative (Hopkins, 2015). The typical overall effect of NBR and HBR on an individual was calculated as the mean intervention effect (vs. HAMB) $\pm$ SD_{IR}. Pearson's correlation coefficient (r) was used to investigate the repeatability of responses across campaigns (i.e., during NBR and HBR) and to follow up any variable showing individual response. These statistical analyses were performed using Prism 7 for Mac OS X (GraphPad Software Inc, San Diego, CA, United States). Principal component analysis (PCA) and Orthogonal Partial Least Squares (OPLS) regression on normalized change scores were performed on R version 3.5.3 – "Great Truth," using the factominer and rOPLS libraries (Thévenot et al., 2015). Data series from participants with $>$75% valid outcome measures across the campaigns were used. For the OPLS analysis, singular missing values were imputed using a weighted average of the 5 nearest neighbors (k-nearest neighbors algorithm; kNN; Torgo, 2016), which was used for only two data points. Confidence

intervals and *p*-values were generated numerically through bootstrapped cross-validation. The level of significance was set at 5% ($P < 0.05$). Data are presented as means ± standard deviation (SD) or as relative changes in % units.

RESULTS

After filtering for inclusion criteria (i.e., at least two interventions performed within one study), the number of participants included in the database was 35, with 9 in FemHab, 12 in LunHab, and 14 in PlanHab. Participants' baseline characteristics are displayed in **Table 1**. The combination of available PlanHab, LunHab and FemHab body composition and skeletal muscle data translated into a database with 19 variables with results from the three studies, with the exception of fat area in thigh and calf during PlanHab (**Supplementary Table 2**). In addition, information regarding nutritional status (i.e., change score and CV of actual vs. targeted energy intake; **Supplementary Table 2**) was collected for the three studies. Results for some of these outcomes from each individual study have been published elsewhere (Debevec et al., 2014; McDonnell et al., 2019, 2020; Mekjavic et al., 2020).

Changes Induced by the Three Interventions

The first aim of this analysis was to establish combined effect sizes in each of the three interventions performed. Thus, we ran a comparison of pre vs. post values for HAMB, NBR, and HBR independently of the study of origin (**Supplementary Table 3**). Many of the strength-related measurements showed a significant pre-to-post decrease after bed rest. The magnitude of the standardized effect sizes (ES) was small in most cases, except for knee extension (KE) torque (NBR; −8.8%, large ES, HBR; −7.0%, moderate ES), knee flexor torque (NBR; −6.2%, HBR; −7.1%, both moderate ES), and dorsiflexor (HBR; −10.5%, moderate ES; **Supplementary Table 3**). The results also indicated significant muscle area decrements for thigh and calf muscles after the three interventions, although the effect sizes for HAMB were small (change lower than −2.9%), while the effect sizes for NBR and HBR for the same outcomes were between 2 and 4 times greater (change between −4.7 and −10.3%; **Supplementary Table 3**). Several body composition outcomes were significantly reduced after the three interventions with effect sizes of low magnitude (trivial-small), and comparable across HAMB, NBR, and HBR (**Supplementary Table 3**).

TABLE 1 | Descriptive characteristics of the pariticpants included in the analysis.

	n	Age (year)	Height (cm)	Weight (kg)
PlanHab	14	26.4 ± 5.2	179.6 ± 5.1	76.9 ± 10.8
FemHab	9	26.7 ± 3.7	167.5 ± 6.1	60.0 ± 7.3
LunHab	12	24.1 ± 2.2	180.2 ± 6.7	72.4 ± 11.6
All combined	35	25.7 ± 4.1	176.7 ± 8.0	71.0 ± 12.1

PlanHab, Planetary Habitat Simulation; FemHab, Female Habitat Simulation; and LunHab, Lunar Habitat Simulation.

Thereafter, we sought to identify variables significantly and consistently affected by the two bed rest campaigns (i.e., NBR and HBR) in relation to HAMB. Although the HAMB group may not be seen as a regular control group due to the hypoxic condition, it allowed us to compare the data controlling not only for genetic factors (each subject was his/her own control), but also for intervention time and energy intake, traits that can be important for the outcomes under study. It should be acknowledged that by employing HAMB as the control group we used a rather conservative approach, since the majority of the detected changes in HAMB went in the same direction (i.e., reduction) as the changes during the bed rest campaigns (**Supplementary Table 3**), and therefore may have masked some of the effects of bed rest. Keeping this in consideration, our investigation indicated that the change-scores significantly differed in several muscle and body composition outcomes (**Figures 1A–D**). However, there were only three variables where the difference between HAMB vs. NBR/HBR was true for both bed rest interventions, i.e., knee extension torque (KE; $P < 0.02$), thigh muscle area ($P < 0.014$), and calf muscle area ($P < 0.0001$), indicating reduced force and muscle mass with unloading.

Since FemHab, LunHab, and PlanHab differed in terms of campaign length and participant sex, we further analyzed the effect of study on the three outcomes highlighted by our previous analysis, i.e., KE torque, and thigh and calf muscle area. To this end, we conducted a mixed-effects model (see section "Materials and Methods"). KE torque decrements were larger in men (i.e., PlanHab and LunHab) than women (FemHab; main effect of study, $P = 0.0019$; $F = 6.7$). Also, both bed rest interventions (i.e., NBR and HBR) triggered greater losses on KE torque than HAMB (main effect of intervention, $P = 0.0097$; $F = 4.9$). Regarding thigh muscle area, longer bed rest periods exacerbated muscle loss, as indicated by a main effect of study ($P < 0.0001$; $F = 14.6$), with PlanHab subjects suffering an overall greater thigh muscle atrophy than LunHab and FemHab participants. Both bed rest campaigns induced greater overall thigh muscle atrophy than HAMB (main effect of intervention, $P = 0.0001$; $F = 12.9$). Calf muscle area results showed a significant interaction study × intervention ($P < 0.0001$; $F = 9.8$) where *post-hoc* analyses revealed that the longer (21 days) PlanHab bed rest interventions induced greater calf muscle loss than those in LunHab and FemHab (10 days; $P < 0.0001$). In addition, the magnitude of calf muscle loss during HAMB was significantly lower than NBR and HBR in LunHab and PlanHab ($P < 0.008$), and tended to be lower than HBR in FemHab ($P = 0.057$). The effect of NBR and HBR was not significantly different in any study. The small effect sizes of HAMB were very similar across studies.

Individual Variability

As hypothesized, our analyses demonstrated that muscle mass and force are the variables more heavily affected by bed rest. Consequently, we investigated individual variability in the response to bed rest in KE torque, and thigh and calf muscle area by calculating the individual response by means of SD_{IR} (see section "Materials and Methods"). The SD_{IR} for KE torque was 18.2 and 22.1 for NBR and HBR, respectively.

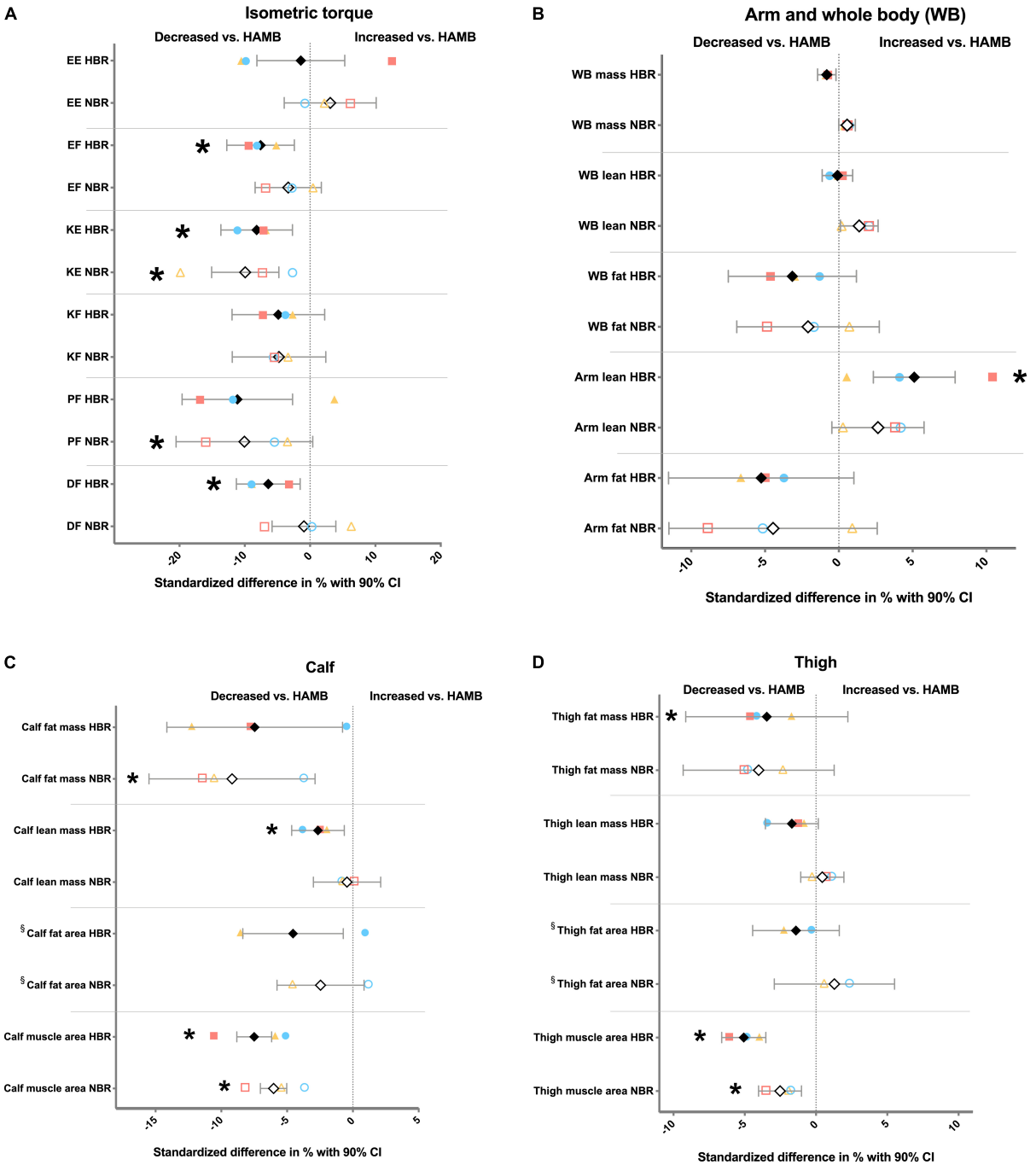

FIGURE 1 | Differences in change scores expressed as relative values (i.e., standardized difference) between hypoxic ambulation (HAMB) and normoxic or hypoxic bed rest (NBR and HBR, respectively) for **(A)** force variables; **(B)** arm and whole-body (WB) variables; **(C)** calf-related variables; and **(D)** thigh-related variables. Open symbols; % difference between NBR vs. HAMB. Full symbols; % difference between HBR vs. HAMB. Black diamonds, all subjects from FemHab, LunHab and PlanHab combined. Blue circles; FemHab. Yellow triangles; LunHab. Red squares; PlanHab. CI, confidence interval; EE, elbow extension; EF, elbow flexion; KE, knee extension; KF, knee flexion; PF, plantar flexion; and DF, dorsiflexion. * denotes significant difference ($P < 0.05$) in change score in absolute values vs. HAMB. § no PlanHab data available for this variable.

This translates into a typical overall effect ranging from −2.0 to −38.3 Nm (−0.9 to −17.8%) for NBR, and from 6.0 to −38.2 Nm (2.8 to −17.9%) for HBR. For calf muscle area, the SD_{IR} was 344.6 and 332.1 for NBR and HBR, respectively, which would indicate that the typical overall effect ranges from −142.8 to −831.9 mm^2 (−1.9 to −11.3%) for NBR, and from −238.2 to −902.3 mm^2 (−3.3 to −12.4%) for HBR. SD_{IR} values for thigh muscle area were −147.9 for NBR

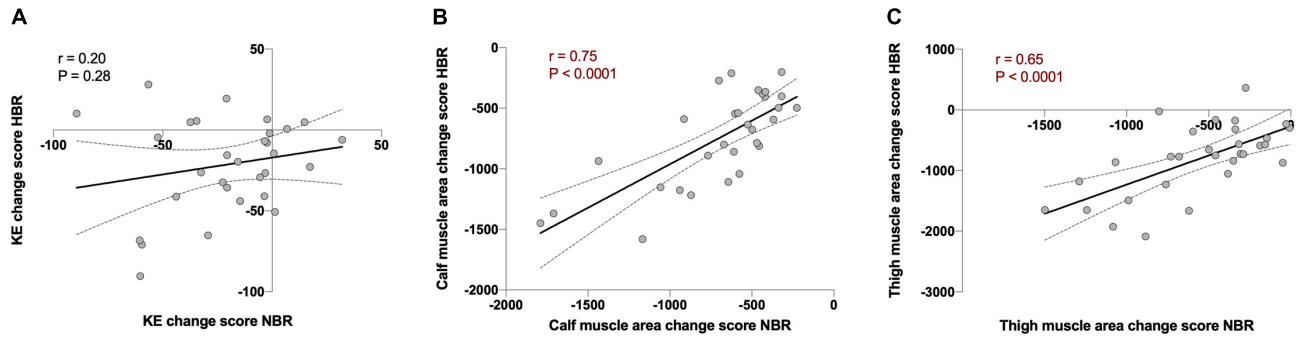

FIGURE 2 | Repeatability analysis using Pearson's correlations between the change score of normoxic bed rest (NBR) vs. hypoxic bed rest (HBR) in **(A)** knee extension torque (KE; in Nm, $r = 0.20$, and $P = 0.28$), **(B)** calf muscle area (in mm^2, $r = 0.75$, and $P < 0.0001$), and **(C)** thigh muscle area (in mm^2, $r = 0.65$, and $P < 0.0001$) in FemHab, LunHab, and PlanHab bed rest studies combined. Dotted lines represent 95% confident intervals.

(note the negative value of SD_{IR}) and 371.2 for HBR, which translates into a typical overall effect ranging from −150.1 to −445.8 mm^2 (−1.4 to −4.2%) for NBR, and from −211.9 and −954.2 mm^2 (−2.0 to −8.9%) for HBR. The SD_{IR} results for all variables included in this study are shown in **Supplementary Table 4**. Overall, and considering previous reports (Fearon et al., 2011), these results indicate that the individual response for KE torque and calf muscle area are indeed clinically relevant (i.e., range of the typical overall effect greater than 5%), while the magnitude of individual response expected for thigh muscle area is not assessed as being as clinically important, especially after NBR.

Repeatability

Once an individual response was identified, we investigated the repeatability of such a response by analyzing the magnitude of alterations each participant underwent during both bed rest interventions. The individual response during NBR and HBR (i.e., repeatability) correlated highly to each other for calf ($r = 0.75$, $P < 0.0001$) and thigh ($r = 0.65$, $P < 0.0001$) muscle area, but not for KE torque (**Figures 2**, **3**). These data suggest that the magnitude of muscle atrophy for a particular individual after a bed rest intervention could be predicted quite accurately by the muscle mass lost in a previous unloading intervention. However, this type of prediction would not be possible for maximal torque production.

Moderators of the Individual Variability

The next step we took was to analyze potential factors, or moderators, that could influence the individual response. Our first candidate was baseline values. As hypothesized, the change score in KE torque, and thigh and calf muscle area showed strong, negative and significant correlations with baseline values (r between −0.5 and −0.9; $P < 0.001$). This was true for both NBR and HBR combined (**Figures 4A–C**) and when analyzed separately (**Supplementary Figure 1**). When the analyses were performed considering the relative change (% from PRE) instead of the change score, the significant correlations remained (r between 0.45 and 0.47; $P < 0.0005$). This clearly points out that the stronger and/or bigger (in terms of muscle mass) an individual is, the more force and muscle mass would be lost under microgravity conditions.

The next factor analyzed was dietary energy intake, investigated as actual intake in relation to targeted intake in terms of both change score and CV across days. No diet variable correlated with the change scores of thigh muscle area or KE torque, and only the deviations in energy intake in relative terms showed a weak significant correlation with the relative loss of calf muscle area decrements ($r = -0.258$; $P = 0.048$). The change score of targeted vs. actual energy intake correlated in a positive fashion with whole-body (WB) mass in both relative ($r = 0.322$; $P = 0.013$) and absolute values ($r = 0.317$; $P = 0.014$). Deviation in energy intake in relative values also correlated with whole body mass ($r = 0.339$; $P = 0.008$, WB mass in absolute values, and $r = 0.349$; $P = 0.006$, WB mass in relative values). The relative change in whole-body fat mass after the bed rest interventions correlated with the absolute ($r = 0.316$; $P = 0.015$) and relative ($r = 0.309$; $P = 0.016$) deviations in energy intake. Taken together, these results indicate that while deviations from the targeted energy intake seem to have a role in the degree of weight change and fat accumulation, their role in muscle atrophy and loss of force during bed rest is very limited.

Finally, we employed a global, exploratory approach to assess the changes in calf muscle area in the Planica bed rest studies. This was based on our observation indicating that calf muscle area is the most consistently altered outcome measure across the studies and bed rest campaigns, and also one of the outcomes with the most significant inter-individual variability. To this end, we first analyzed the degree of covariance amongst the change scores across the campaigns and studies using PCA (**Supplementary Figure 2A**). This revealed a modest degree of covariance in the change scores with 21.9 and 16.6% captured by the first two principal components and with little variable clustering. In line with this, calf muscle area contributed equally to these components (coefficient of regression 0.47 and 0.40, respectively). Next, we utilized the supervised machine learning method OPLS regression to explore if the global change scores could be used to model the change in calf muscle area. Based on calf area being equally correlated to both PC1 and PC2 we chose to retain all variables from the PCA to test in the OPLS-model.

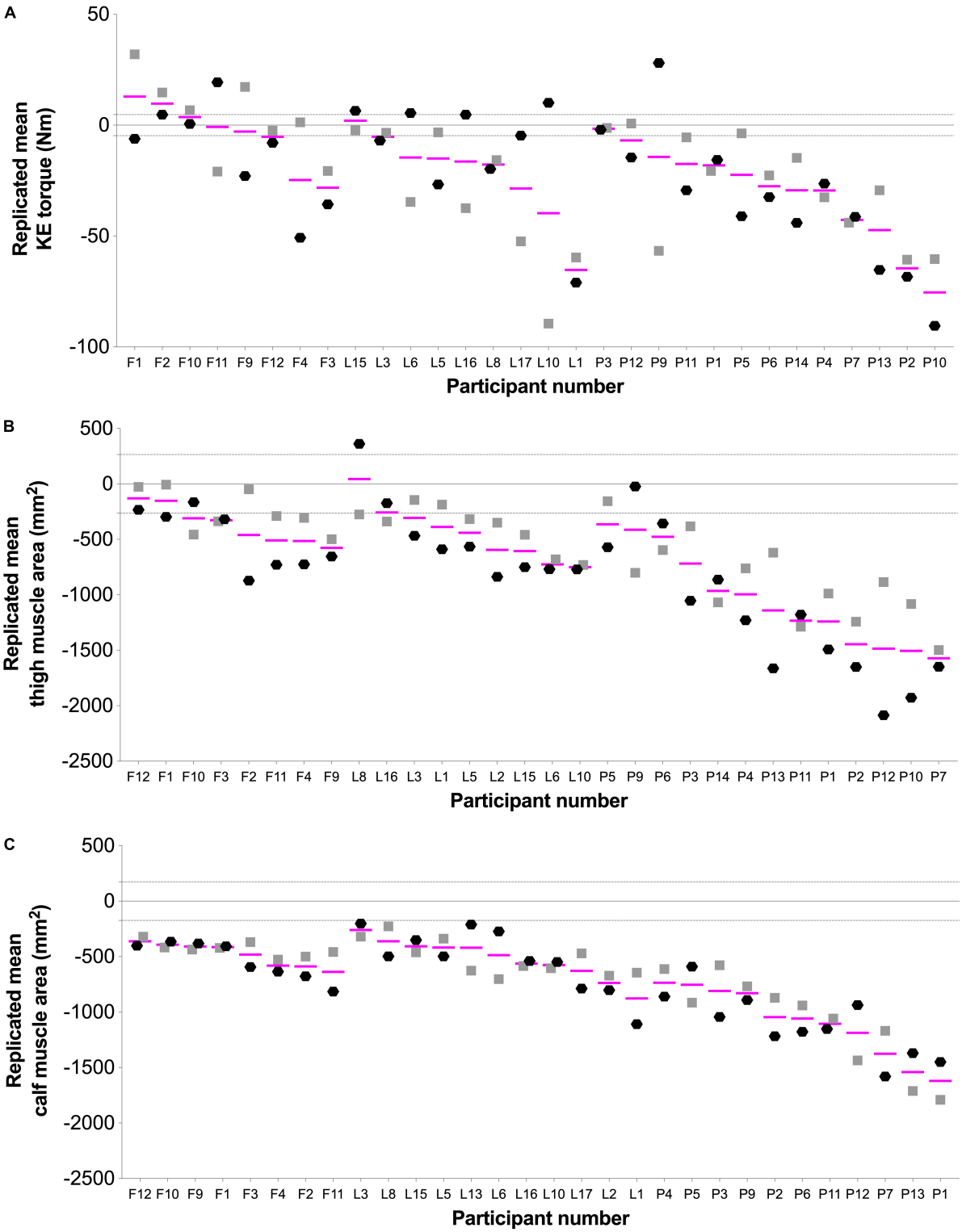

FIGURE 3 | Individual changes showing the repeatability response in **(A)** knee extension (KE) torque, **(B)** thigh muscle area, and **(C)** calf muscle area. Black hexagons (⬣; hypoxic) and gray squares (■; normoxic) indicate change scores (post minus pre) for the responses to bed rest interventions. Pink lines (—) represent each participant's replicated mean. Gray-dashed lines represent the standardized minimally clinically important difference, which was calculated by multiplying 0.1 by the baseline between-subject standard deviation (Atkinson and Batterham, 2015; Goltz et al., 2018, 2019). Letters in X axis indicate FemHab (F), LunHab (L), or PlanHab (P) study subjects, and are ordered from lower to higher average loss after bed rest within each study for each particular variable.

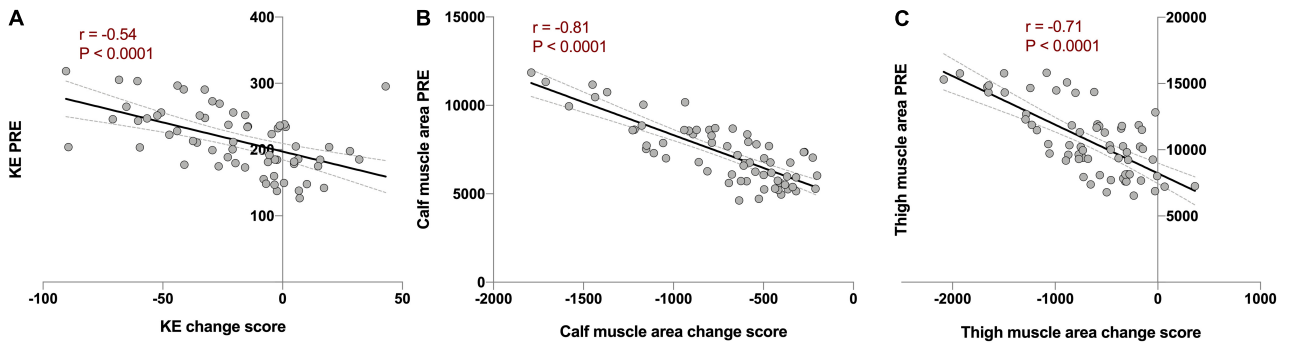

FIGURE 4 | Analysis of baseline value as a moderator of individual variability using Pearson's correlations between combined change scores in normoxic (NBR) and hypoxic (HBR) bed rest campaigns vs. PRE values before the corresponding intervention in **(A)** knee extension torque (KE; in Nm. $r = -0.54$, $P < 0.0001$), **(B)** calf muscle area (in mm^2, $r = -0.81$, $P < 0.0001$), and **(C)** thigh muscle area (in mm^2, $r = -0.71$, $P < 0.0001$) in FemHab, LunHab, and PlanHab bed rest studies combined. Dotted lines represent 95% confident intervals.

After 10,000-fold cross validation, a successful OPLS regression ($P < 0.01$) with 1 predictive and 1 orthogonal component with a predicted r^2-value of 0.43 for calf muscle area was generated (**Figure 5A** and **Supplementary Figure 2B**). Variables contributing most to the successful modeling were "Thigh muscle area," "KE torque," and "whole-body mass," based on Variable Importance in Projection (**Figure 5B**). This was corroborated by direct univariate correlation analysis between change scores of calf muscle area vs. thigh muscle area, KE torque and whole-body mass, rendering correlation coefficients of 0.593, 0.285, and 0.262, respectively. Comparable correlation coefficients were obtained when the different campaigns were analyzed separately.

DISCUSSION

By combining individual data from three studies, we generated the largest muscle and body composition bed rest dataset to date. Of the 19 variables examined, KE isometric torque, and calf and thigh muscle area were affected by bed rest the most. The main finding of this study is that there was clinically relevant individual variability in KE torque, and calf and thigh muscle area, and this individual response was repeatable across bed rest interventions, at least for muscle mass readouts. The results also showed that baseline values, but not deviations in the tailored energy intake, seemed to be a moderator of the variability. Another moderator of the individual response to calf muscle area loss with bed rest, was a global bed rest campaign response including all of the other variables studied, where muscle mass and function changes contributed the most.

The current analysis translated into approximately a 3-times greater number of observations compared to what is commonly found in bed rest studies (Alkner and Tesch, 2004; Trappe et al., 2007a; Blottner et al., 2020). The analyses performed indicated that the mass of the muscles involved in posture and locomotion (i.e., thigh and calf) and the function of the knee extensor muscles are the outcomes more robustly affected by bed rest, supporting previous results (Pavy-Le Traon et al., 2007). However, other outcome measures that have been used to assess the effects of bed rest on skeletal muscle showed effect sizes of small magnitude and substantially lower reproducibility both across trials and within individual subjects [e.g., lean whole-body/leg mass (Pavy-Le Traon et al., 2007; English et al., 2016)]. This indicates that these variables are more influenced by the study design, method limitations, or other external factors, rather than by unloading *per se*, and therefore should be used and interpreted with caution when examining the consequences of bed rest in skeletal muscle.

Anecdotal evidence has suggested that there is substantial individual variability in physiological variables following bed rest, including losses in muscle mass and function (Akima et al., 1997; Downs et al., 2016; Scott et al., 2021). However, the limited number of participants included in previous studies and the lack of an ambulatory control group prevented any firm conclusion. In contrast, the current study provided a framework including a control group and around 30 observations per variable, which together offered a unique opportunity to study individual variability in response to bed rest. This strategy allowed us to control for heritable factors, intervention time and energy intake, and to use a conservative control-arm for each subject in the form of HAMB under the same experimental set-up/environment as the bed rest interventions (see "Results" section for details). Thus, a methodology described by experts in the field (Atkinson and Batterham, 2015; Hopkins, 2015), and used in other physiological settings to investigate individual variability to an intervention (Goltz et al., 2018, 2019), was employed for the three variables showing more robust changes to bed rest. The data showed that if a random individual would undergo bed rest in similar conditions as the ones used in the Planica studies, a loss ranging from 0 to 17% and from 2 to 12% could be expected for KE torque and calf muscle area, respectively, which could be interpreted as clinically relevant (Fearon et al., 2011). When it comes to thigh muscle area, the negative SD_{IR} for NBR indicated that there was more variability in response to HAMB than NBR; yet, the individual response in thigh muscle area losses after HBR would be close to a clinically relevant threshold. A factor to consider is that during bed rest, there is a complete standardization (i.e., absence) of the mechanical load, while in HAMB, despite potential drops in physical activity induced by

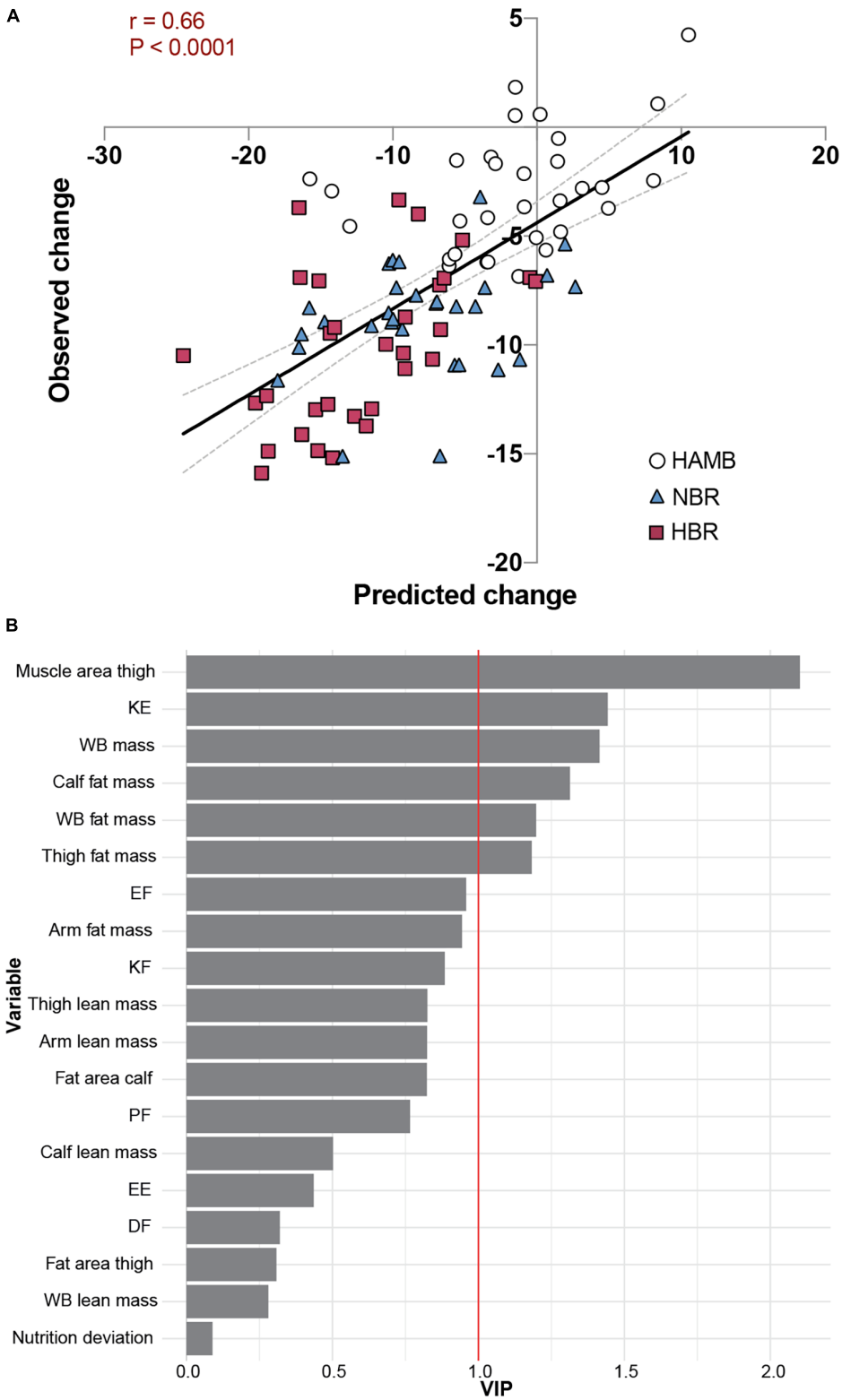

FIGURE 5 | Global, exploratory approach to the changes in calf muscle area in Planica bed rest studies. HAMB; hypoxic ambulation, HBR; hypoxic bed rest, and NBR; normoxic bed rest. **(A)** Scatter plot of predicted and observed changes in calf muscle area using our OPLS regression ($r = 0.66$, $P < 0.001$). Dotted lines represent 95% confident intervals. **(B)** Variable Importance in Projection (VIP) showing the rank of contributing variables to the successful modelling (KE, knee extension torque, WB; whole body, EF; elbow flexion torque, KF; knee flexion torque, PF; plantar flexion torque, EE; elbow extension torque, and DF; dorsiflexion torque).

the confinement, and thus mechanical load, there was a retained behavioral variance, i.e., some participants would move around more than others, augmenting the mechanical load variability across individuals.

The differences in the standardization of the mechanical load during NBR vs. HAMB could partly explain the negative SD_{IR} noted following NBR, since a greater regression to the mean phenomenon could be induced by NBR than by HAMB. Yet, if this phenomenon was completely true, it would have appeared for the HBR intervention as well. An explanation for this could be the differences in the magnitude of the effect size alterations for thigh muscle area during NBR and HBR, with higher values (almost double) for HBR. Altogether, these results highlight that individual variability estimations are mainly relevant in situations where the effect size of the intervention arm is substantially larger than in the control group, as well as the undisputable necessity to include a control arm in the experiments (i.e., ambulatory group in bed rest studies).

Repeatability was investigated by comparing the change scores in KE torque, and calf and thigh muscle area in NBR vs. HBR. Despite the trivial/small impact of hypoxia in bed rest-induced alterations, the current strategy to investigate repeatability allowed us to address whether the individual variability to a bed rest intervention was mainly explained by inter-campaign/tests effects, i.e., external, random factors (i.e., different individual response in NBR vs. HBR), or if the variability could be explained by intra-subject factors, and therefore could be considered as real individual variability (i.e., similar individual response in NBR and HBR). We report for the first time a high degree of repeatability for calf and thigh muscle area change scores, but not for KE torque, after bed rest. Therefore, while the individual variability reported above for bed rest-induced muscle atrophy can be considered as real intrinsic intra-individual variation in response to microgravity, other random/extrinsic elements (e.g., learning effects) seemed to have affected the losses in KE torque. Apart from any potential inter-test effect, the mismatch in repeatability between muscle mass and force losses could be partly explained by periodic variations in maximal force production (Ahtiainen et al., 2016). Such variations may be influenced by day-to-day differences in neuromuscular performance, psychological confounders or, to a lower degree in the current study design, variations in daily physical activity (Ahtiainen et al., 2016).

Once clinically-relevant individual variability was identified in specific outcomes, and the reproducibility confirmed across bed rest studies, factors that could influence the individual response were investigated, i.e., potential moderators of the individual response (Atkinson and Batterham, 2015). Given the available data, baseline values and deviations in energy intake from the targeted diet were examined. A strong, negative correlation between baseline values and loss of muscle force, and calf and thigh muscle area was found, suggesting that initial levels of force and muscle mass are important to explain the individual variability after bed rest. Although such relationships might be seen as a "regression to the mean" phenomenon (Bland and Altman, 1994; Linden, 2013), the physiological relevance of the current results should not be overlooked, more so when the correlations were still present after controlling for baseline levels. Indeed, the fact that the correlation was not found in the HAMB group does not support the proposal of a potential regression to the mean effect caused by sampling or methodological reasons. To interpret these data in relation to spaceflight, researchers should consider that while bigger and stronger individuals could lose more muscle mass and force during space missions, they would still have bigger safety margins to overcome the consequences of those space-induced alterations, as inferred in the past (Winnard et al., 2019).

The second potential moderator investigated was the deviation in energy intake from the targeted, individualized diet. The results showed that, in the context of a strictly controlled diet, there was practically no relationship between energy-intake deviations and muscle outcomes. Yet, deviations in the diet correlated with bed rest-induced changes in whole-body mass and whole-body fat mass. This is not surprising given that fat mass and body weight are heavily dependent on the overall energy intake, while muscle mass is regulated by protein intake and contractile activity (Longland et al., 2016). In the context of microgravity, muscle contractile activity seems to be the critical factor governing muscle mass during bed rest (Trappe et al., 2007b). The current data highlight that the results presented herein were not a consequence of any methodological artifact in diet registration or body composition and muscle mass/function testing, but of real biological origin.

The classic technique to analyze moderators of individual variability has been, as explained above for baseline values and diet deviations, to investigate the influence of one single factor on a particular outcome. However, given the current development of biostatistical and data-integration approaches, it could be more useful to investigate the overall signature of an intervention on the individual variability of a selected variable. For this approach to be valid, the intervention should be conducted under extremely controlled conditions, such that most of the environmental factors are accounted for. Thus, bed rest studies offer a unique opportunity to test and develop such moderator models. With this in mind, an exploratory approach to the changes in calf muscle area in the Planica bed rest studies was carried out to test the idea that the summatory effects of changes during bed rest (independently of their magnitude) in a considerable number of variables, could explain the change in calf muscle area. The calf muscle area was chosen for this set of analyses because (i) it is the most consistently altered outcome measure across the studies and bed rest interventions, (ii) it is one of the outcomes with the most significant inter-individual variability, and (iii) it presents a high degree of repeatability across bed rest interventions. In addition to the observations from the current experiments, calf muscle mass is one of the most investigated outcomes in the context of bed rest (Alkner and Tesch, 2004; Trappe et al., 2007b; Salanova et al., 2015; Blottner et al., 2020). Our analyses indicated that the variability in calf muscle area changes induced by bed rest could be moderately explained by the summatory effects of all of the other variables included in the database, with "thigh muscle area," "KE torque,"

and "whole-body mass" as the three top-ranked variables in the model. These results indicate that, despite minor or residual moderator role of the other variables when analyzed one-by-one, when they were merged, they accounted for ∼43% of the variance in calf muscle area changes induced by bed rest. Thus, the interindividual variance to bed rest is to an extent a global event where interindividual traits are shared across different physiological outcomes.

To fully interpret the results of the present study, there are some considerations that need to be taken into account. While we had access to individual traits for each individual participant, the analyses performed pooled studies with different bed rest duration (10 and 21 days) and sex. Although this could be seen as a limitation, the database introduced in this study is one of the biggest aggregated datasets, i.e., greatest number of observations on bed rest to date. Another factor to consider is the selection of HAMB as the control group. We acknowledge that HAMB is not the classic control group due to the soft intervention with hypoxia. Yet, the changes in most variables went in the same direction as those after bed rest, suggesting that this was a rather conservative approach, decreasing the risk of false-positive results to negligible levels. If anything, the current strategy might have masked some of the effects of bed rest. Despite these issues, this is the only set of bed rest studies using an ambulatory group for comparisons, which is paramount when investigating individual variability to an intervention (Atkinson and Batterham, 2015).

CONCLUSION

Using the three studies performed at the Planica bed rest facility, clinically relevant individual variability was identified in changes in muscle force and mass. This individual variability was repeatable across bed rest interventions, at least for muscle mass alterations, and partly dependent on baseline values. In addition, the summatory effects of all of the variables analyzed became a fairly strong moderator of the variance in the calf muscle area changes after bed rest. The current results indicate clinically relevant individual variability in muscle responses to unloading/inactivity in the Planica bed rest campaigns. These data may serve as one of the cornerstones to develop (bio)markers of the individual response, which would offer new tools to improve health management of astronauts and to optimize individual programs to counteract the negative effects of unloading both during space missions and here on Earth.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Committee for Medical Ethics at the Ministry of Health (Republic of Slovenia). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RF-G, AM, ER, and IBM conceived and designed the work. AM, IBM, ES, and IAM acquired the data. RF-G and ER analyzed and interpreted the data of the work and drafted the manuscript. RF-G, AM, ES, IAM, ER, and IBM critically revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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BRIEF RESEARCH REPORT
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Effects of High-Intensity Interval Training With Specific Techniques on Jumping Ability and Change of Direction Speed in Karate Athletes: An Inter-individual Analysis

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This study investigated the effect of 4 weeks of high-intensity interval training (HIIT) with specific techniques and analyzed inter-individual variability [classified in responders (Rs) and non-responders (NRs)] on jumping ability and change of direction speed (CODS) in youth karate athletes. Athletes of both genders ($n = 10$) were randomly assigned into experimental group (EG; $n = 5$) and the control group (CG; $n = 5$). The EG trained 2–3 days per week applying HIIT (three rounds [15 sets of 4 s all-out specific efforts with 8 s of dynamical pauses] with 3 min of recovery between rounds) during their usual training during 4 weeks. Assessments included squat jump (SJ) and countermovement jump (CMJ) and CODS by T-test. No significant interaction effect group by time was found. Although, in percentage and effect size (ES) terms increases were reported in both groups for SJ (EG: 15.2%, ES = 0.91 vs. CG: 12.4%, ES = 0.02) and only in EG for the T-test (−1.7%; ES = −0.35). In turn, a trend toward a higher proportion of Rs was observed in the EG (40% Rs) vs. CG (20% Rs) for SJ and CODS, respectively. In conclusion, the addition to regular training of a HIIT with specific techniques and based on the temporal combat structure after 4 weeks was not a sufficient stimulus to increase jumping ability and CODS in karate athletes.

Keywords: combat sports, martial arts, athletes, physical fitness, strength and conditioning

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INTRODUCTION

Karate is a popular combat sport that officially debuted at the Tokyo 2020 Olympic Games and whose performance requires athletes to possess a specific physical and physiological profile and technical expertise of the discipline (Chaabene et al., 2012). The "kumite" or combat modality is described as an intermittent nature (average effort/pause ratio 10:16.2 s or 1:1.5–1:2; Chaabene, 2015; Tabben et al., 2018) and high-intensity activity (>90%HRmax; $La^{-1} > 7.7 \pm 1.9$ mmol/L). In terms of physical performance, during combat, the athletes must strike and/or kick applying force quickly and explosively to score (Tabben et al., 2018). Among the most commonly used techniques include punching techniques with upper (in form of straight attacks) and lower limbs (using e.g., circular kicks or "mawashi geri"; Chaabène et al., 2014; Chaabene, 2015; Tabben et al., 2018). In addition, they must move in multiple directions to evade and/or counterattack (Chaabène et al., 2014; Chaabene, 2015; Tabben et al., 2018).

Based on the above approach, coaches should incorporate effective training strategies to develop sport-related fitness. Among other physical abilities, include the dynamic strength characteristics such as muscle power and efficient use of the stretch-shortening cycle (Chaabene et al., 2012; Loturco et al., 2014; Quinzi et al., 2020). Particularly, the dynamic strength characteristics of lower limbs are assessed using different technologies (e.g., contact platform, smartphone, force platform, and isokinetic device) and metrics (e.g., rate of force development, muscle power, and one-repetition maximum Loturco et al., 2014; Margaritopoulos et al., 2015; Kavvoura et al., 2018; Kostikiadis et al., 2018; Quinzi et al., 2020). In addition, in karate, a specific systematic review (Chaabene et al., 2012) and correlational and explanatory studies use the squat jump (SJ) and countermovement jump (CMJ; Chaabene et al., 2012; Loturco et al., 2014; Chaabene, 2015). In this sense, international athletes exhibit higher SJ and CMJ height performance than amateur athletes (Chaabene et al., 2012). Furthermore, this ability has been shown to significantly influence the speed and acceleration of punching execution (Loturco et al., 2014; Quinzi et al., 2020). In turn, agility including change of direction speed (CODS) is proposed as another important physical ability in this sport (Chaabene et al., 2012; Herrera-Valenzuela et al., 2020). In this regard, recent evidence shows a significant relationship between CODS with jumping ability in junior and cadet elite level karate athletes (Herrera-Valenzuela et al., 2020), as well as being a predictor of competitive success (i.e., medalists in European championships) in female karate athletes (de Quel et al., 2020).

In this context, high-intensity interval training (HIIT) according to recent systematic reviews in combat sports reports shows improvements in athletes' fitness (Franchini et al., 2019; Vasconcelos et al., 2020). In karate, HIIT studies include protocols based on repeated-CMJ (Ojeda-Aravena et al., 2019) and repeated-sprints (Ravier et al., 2009) after 6–7 weeks on jumping ability, CODS (Ojeda-Aravena et al., 2019), aerobic (Ojeda-Aravena et al., 2019), and anaerobic (Ravier et al., 2009) components. In addition, recent reports have incorporated the inclusion of HIIT using specific techniques in combat sports such as taekwondo (Aravena et al., 2020; Ouergui et al., 2020, 2021; Ojeda-Aravena et al., 2021a) and boxing (Kamandulis et al., 2018; Herrera-Valenzuela et al., 2021). Among the relevant results, significant inconsistent increases in jump height and CODS performance are reported (Ouergui et al., 2020, 2021; Herrera-Valenzuela et al., 2021; Ojeda-Aravena et al., 2021b).

In addition to the above, it is relevant to indicate that studies usually report the outcomes in group form (i.e., the mean change within a training group), without considering the athletes inter-individual variability of the athletes after training. In this sense, this research topic has been the subject of study since the 1980s in precision medicine to find responders (Rs) and non-responders (NRs) to physical exercise treatment applied to sedentary and/or comorbid obese individuals and recently in the field of applied sports science to understand athlete responses (Bonafiglia et al., 2016; Güllich, 2018; Ramirez-Campillo et al., 2018; Pickering and Kiely, 2019; Schulhauser et al., 2020; Talsnes et al., 2020). Furthermore, in combat sports, to date, some reports include taekwondo (Ojeda-Aravena et al., 2021b) and boxing (Herrera-Valenzuela et al., 2021).

Consequently, the potential efficacy of HIIT with specific techniques on the group and inter-individual response on jumping ability and CODS performance in karate athletes could be useful to provide relevant information to coaches on training adaptation mechanisms and individualization in sports training programming. Therefore, this study investigated the effect of 4 weeks of HIIT with specific techniques and analyzed inter-individual variability (classified in Rs and NRs) on jumping ability and CODS in youth karate athletes. The rationale for the hypothesis is based on the notion that the ecological specificity of HIIT (i.e., using a sport-specific time structure and modality) could develop greater adaptations than usual training.

MATERIALS AND METHODS

Participants

Ten cadet karate athletes (age 15.2 ± 1.6 years; height 164.8 ± 7.7 cm; body mass 64.0 ± 14.5 kg) who compete annually in national and international level tournaments completed this study. They were invited to participate in the study during the annual planning transition period (January 2020) and randomly assigned into experimental group (EG; $n = 5$; age 16.1 ± 1.12 years; height 168.8 ± 7.6 cm; body mass 68.5 ± 20.9 kg) and control group (CG; $n = 5$; age 14.5 ± 2.0 years; height 160.8 ± 7.1 cm; body mass 59.6 ± 6.5 kg). Each group consisted of two females and three males (for details see **Figure 1**). To participate in the study, all athletes had to meet the following inclusion criteria (i) three years or more of karate experience; (ii) no history of disease and medication; (iii) no injuries or fractures during at least the last 6 months; (iv) consistently training at least three times per week for at least six h per week; (v) membership in the National Karate Federation; (vi) not undergoing a period of body mass reduction; and (vii) participation in at least 85% of the intervention sessions. All

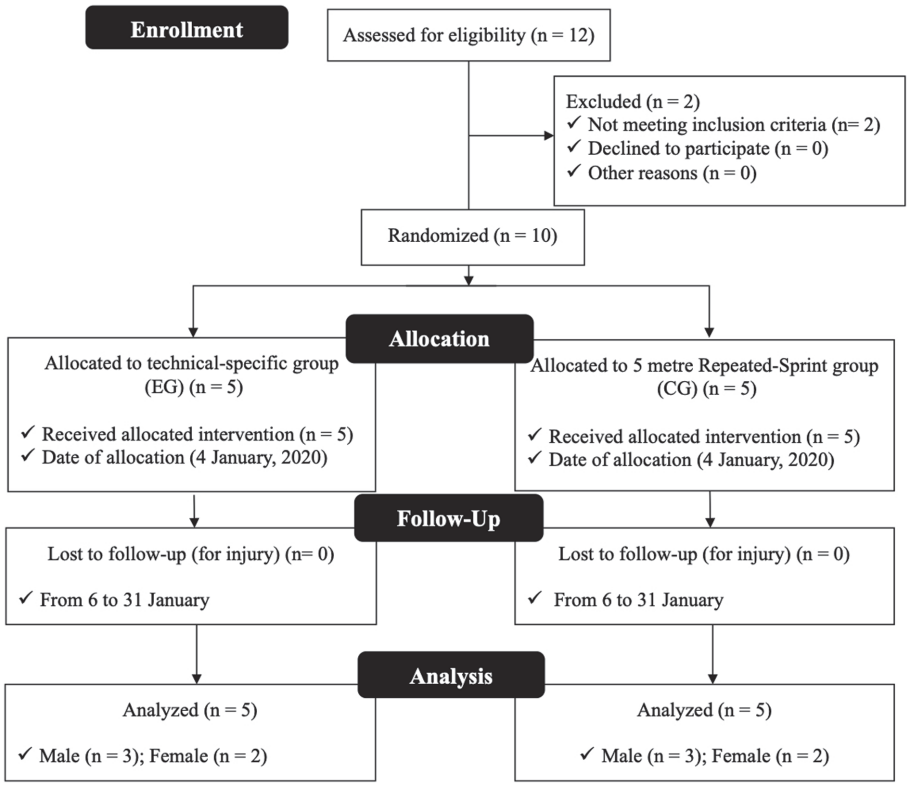

FIGURE 1 | Flow chart of the process followed in the study.

athletes and/or family members of athletes under 18 years of age were previously informed of the study purposes, associated benefits, experimental procedures, and potential by informed consent or informed assent before the assessments and training sessions. The study was conducted in compliance with the ethical standards for sport science studies (Harriss and Atkinson, 2015) and implemented after approval by the university ethics committee Autónoma university following the Helsinki declaration for work with humans (General Assembly of the World Medical Association, 2014).

Assessments

Jumping Ability

Jumping ability was assessed by the SJ to assess concentric muscle actions and the CMJ to assess the slow stretch-shortening cycle or SSC through the maximum height reached (cm) using an electronic contact platform (Ergojump; Globus, Codogne, Italy; accuracy: 0.01 m). For the SJ test, each athlete was previously instructed to place hands on hips, feet, and shoulders wide apart, and adopt a flexed-knee position (approximately 90°) for three s, and then perform a maximal effort vertical jump. Meanwhile, for the CMJ test, each athlete was previously instructed to rest hands on hips, feet, and shoulders well apart, and perform a downward movement (no restriction was placed on the knee angle achieved) followed by a vertical maximal effort (Ramirez-Campillo et al., 2013; Groeber et al., 2020). Intra-class correlation or ICC SJ pre = 0.91 (CI 95% 0.80–0.96); ICC SJ post = 0.90 (CI 95% 0.80–0.90), ICC CMJ pre = 0.93 (CI 95% 0.90–0.98); and ICC CMJ post = 0.95 (CI 95% 0.90–0.98).

Change of Direction Speed

The T-test was used to assess CODS during multidirectional movement (i.e., forward, lateral, and backward; Seo et al., 2019). For which four cones were set up in a "T" shape. Where the athlete started at a sound signal to run in a straight line to cone A, then ran at maximum speed to cone B (A – B: 5 m) touching the top of the cone with the right hand; then, he turned left and ran away as fast as possible with lateral steps to cone C (B – C: 5 m) until he touched the top of the cone. Then, he reversed directions and moved away using lateral steps to meet cone D (C – D: 10 m) and touched the top of the cone. After that, he laterally stepped backward to touch cone B (D – B: 5 m) and finally ran backward to cone A (B – A: 5 m). Speed was recorded by an automatic timing system using electronic photocells (Brower Timing System, Salt Lake City, UT) accurate to 0.001 s. The gates were positioned 1-m above the ground. ICC pre = 0.90 (CI 95% 0.87 a 0.92) and ICC post = 0.92 (CI 95% 0.90 a 0.96).

Training Program

The training program had a duration of 10 sessions (4 weeks) of 90 min each session and was applied on 3 non-consecutive days (Monday, Wednesday, and Friday). The training load distribution was oriented to technical-tactical development with

the coach's permanent intervention during the training sessions. The HIIT was performed in front of a partner who did not participate in the study. Specifically, the protocol mimicked the official combat duration (3 min). In addition, the HIIT intervals were based on the documented temporal structure for this sport (1:2; Tabben et al., 2018). Previously, both groups were instructed to use the rating of perceived exertion scale (RPE 0–10) to internal load control (Tabben et al., 2015; Slimani et al., 2017; Ouergui et al., 2020; Ojeda-Aravena et al., 2021a). The All-out HIIT format was used (Laursen and Buchheit, 2018). The training load was increased by decreasing the density volume during the last week, without modifying the high-intensity time. Briefly, the first 2 weeks the athletes recovered in 3 min between rounds and performed HIIT at a frequency of twice a week. In the last 2 weeks, the density decreased to 2 min of recovery and performed three times per week. Specifically, each training session started with a standardized 15-min warm-up group consisting of circle jogging (5 min) and lower and upper body dynamic stretching (10 min). Subsequently, the EG group was separated from the total group of athletes to execute the HIIT with specific techniques (∼20 min; Laursen and Buchheit, 2018; Franchini, 2020). Particularly, athletes executed three rounds of 15 sets of 4 s all-out efforts of straight punch and circular kick combinations in front of a partner followed by 8 s of low intensity by performing a combat stance (imitating the combat stance). The striking sequence included an initial straight punch with the front hand or "oi tsuki," followed by a circular kick "mawashi geri" with the back leg or "giaku mawashi geri," a straight punch with the backhand or "giaku tsuki" and a kick with the front leg "oi mawashi geri" (Chaabene et al., 2015). In parallel, the CG continued with their usual training.

Subsequently, all athletes participating in the study were reintegrated to the usual training by continuing with three blocks of dynamic tasks of exercises for 40 min with an RPE of 5–6. Specifically, the first block (15 min) consisted of the application of attack techniques with hands (four sets of 20 repetitions of attack techniques with straight punches with the front hand, and later with the backhand with a recovery of 3 min between series). The second block (15 min), consisted of the application of attack techniques with kicks (four sets of 20 "mawashi geri" with the front leg, and then with the back leg with a recovery of 3 min between sets). The third block consisted of free combats (15 min) with the permanent intervention of the coach to point out technical and tactical aspects. The training sessions finished with stretching (10 min; **Figure 2**).

Procedures

A week prior the investigation began the athletes completed a familiarization session and EG practiced the HIIT protocol to reduce the learning effect. In addition, both the coach and athletes received an induction on the RPE 0–10 scale. All assessments were scheduled between 9:00 and 11:00 AM, completed in the same order, at the same location (gymnasium with wooden floor), with the same sports clothing, and by the same sports science professional before and after the intervention, previously blinded to the intervention. Previously, all participants were instructed to (i) sleep 8 h between each assessment session, (ii) not to modify their usual eating and hydration habits during the days before the assessments, and (iii) not to consume caffeinated beverages. The tests were assessed according to exercise intensity in the following order: SJ, CMJ, and T-test. Before the execution of the tests, a general warm-up of ∼10 min (e.g., submaximal running with a change of direction, 10 vertical and 10 horizontal submaximal jumps) was performed. This was followed by a specific warm-up with potentiation exercises, including stretching, and two submaximal jumping attempts (∼5 min). The best of the two attempts was considered for performance for each assessment. A 2-min rest interval was performed between each trial, and a 5–10-min rest interval was applied between each test to reduce the effects of fatigue.

Statistical Analysis

Data analysis was performed with SPSS version 26 for Mac (SPSS Institute, Chicago, IL, United States). Data are presented as mean ± SD. Homoscedasticity of variance and normality was checked by Levene's test and the Shapiro-Wilk test, respectively. The unpaired *t*-test was used to examine for possible gender biases. The interaction of group (inter-subject factor) EG vs. CG and time (intra-subject factor) pre-intervention vs. post-intervention was analyzed by a repeated-measures mixed ANOVA. If significant effects or interactions were observed, the Bonferroni *post hoc* test was applied to adjust for differences between the means of the two groups. For ANOVA outcomes, effect sizes (ES) were calculated using partial eta squared (η^2_p). Complementarily, post-intervention changes within and between groups were calculated using Cohen's d following the classification proposed by Rhea for recreationally trained participants (individuals training consistently for 1–5 years; trivial <0.25; small 0.25–0.50; moderate 0.50–1.0; large >1.0; Rhea, 2004). Subsequently, the sample was classified into Rs and NRs using the two-technical error (TE) criterion according to a previously established equation (Bonafiglia et al., 2016). NRs were identified and defined as individuals who were unable to demonstrate an increase or decrease (in favor of beneficial changes) in sport-related fitness that was greater than twice the TE away from zero (Ramirez-Campillo et al., 2018). For the current study, two replicates of all outcomes analyzed were used to calculate TE. A change beyond twice the TE was representative of a high probability (i.e., 12–1 odds) that the observed response was a true physiological adaptation beyond what might be expected as a result of technical and/or biological variability (Ramirez-Campillo et al., 2018). Therefore, the TEs were as follows: [SJ; 3.10 (cm) × 2; CMJ, 3.32 (cm) × 2; T-test, 0.28 (s) × 2]. All assessments showed acceptable reliability coefficient of variation or CV < 5% and intraclass correlation or ICC > 0.90 (Hopkins, 2000). The level of statistical significance used was set at $p < 0.05$.

RESULTS

No significant differences were reported between both genders in chronological age ($t = -0.22$; $p = 0.08$), body mass ($t = -0.76$;

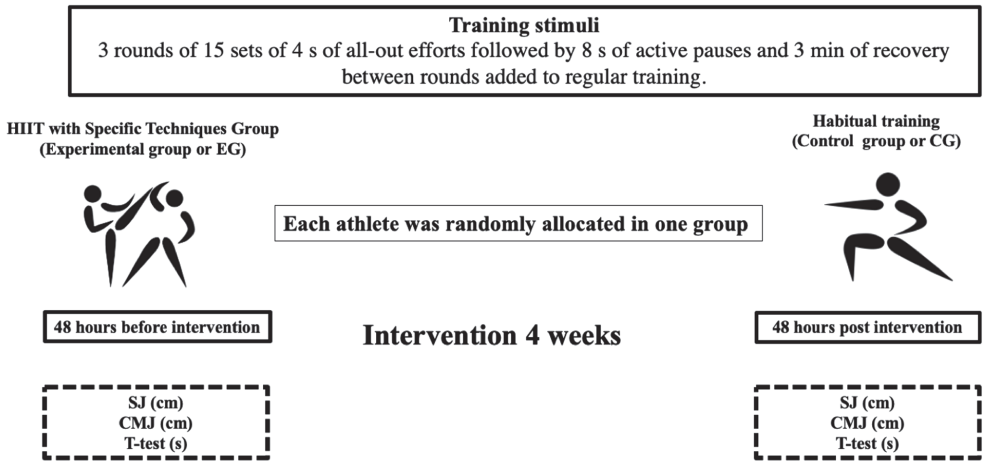

FIGURE 2 | Graphic representation of the experimental design of the study. SJ, squat jump and CMJ, countermovement jump.

$p = 0.46$), and stature ($t = -1.66$; $p = 0.13$), SJ ($t = 0.29$; $p = 0.77$), CMJ ($t = 0.81$; $p = 0.42$), and CODS ($t = 2.20$; $p = 0.06$).

Effect and Interaction of the Factors Analyzed

Table 1 presents the summary of the time factor analysis independently in each group and group-by-time interaction for SJ, CMJ, and CODS. Specifically, for SJ no significant effect was reported in the group factor ($F_{1,8} = 1.03$; $p = 0.33$; $\eta^2_p = 0.11$) and time factor ($F_{1,8} = 4.53$; $p = 0.06$; $\eta^2_p = 0.36$). Neither for CMJ in the group factor ($F_{1,8} = 0.15$; $p = 0.70$; $\eta^2_p = 0.01$) and time factor ($F_{1,8} = 0.19$; $p = 0.67$; $\eta^2_p = 0.02$). In addition, the CODS showed no significant effect in the group factor ($F_{1,8} = 0.65$; $p = 0.44$; $\eta^2_p = 0.07$) and time factor (F1,8 = 0.45; $p = 0.51$; $\eta^2_p = 0.05$).

Magnitude of Change Based on Inference

Table 1 presents the changes based on inference after the intervention. Particularly, in EG increases in jumping ability were reported for SJ with a *moderate* increase (15.2%; ES = 0.91). In contrast, in CG a *trivial* increase in this outcome (16.2%; ES = 0.28). For CODS, an increase *small* in performance was reported in EG (−1.7%; ES = −0.35). On the other hand, a *trivial* decreased performance in CG (0.48%; ES = 0.03).

On the other hand, for CMJ performance a decrease was reported *trivially* in EG (1.7%% ES = 0.35) and CG (0.48% ES = 0.03).

Inter-individual Variability in Response to the HIIT Program

Figure 3 and **Table 1** show the inter-individual variability analysis of jumping ability and CODS in athletes from both groups analyzed. In particular, in EG athlete Rs were reported for SJ and T-test ($n = 2$; 40%). Additionaly, for CMJ in the CG ($n = 1$; 20%).

On the other hand, in EG for CMJ, 100% of the athletes were classified as NRs.

DISCUSSION

This study investigated the effect of 4 weeks of HIIT with specific techniques and analyzed inter-individual variability (classified in Rs and NRs) on jumping ability and CODS in youth karate athletes. Among the main results, no significant group-by-time interaction effect was found. However, increases in performance in percentage terms and ES in EG were found for CODS and both groups for SJ. At the same time, a trend of higher percentage of Rs athletes in EG vs. CG was observed for SJ and CODS. Consequently, the stated hypothesis was not fulfilled. Indicating that the addition to regular training of a HIIT with specific techniques and based on the temporal structure of combat after 4 weeks was not a sufficient stimulus to increase jumping ability and change of direction speed in karate athletes.

Jumping Ability

Current evidence shows inconsistencies to the effect of HIIT with specific techniques on this physical ability in karate athletes. In this regard, a previous study on HIIT in karate did not document significant effects for SJ and CMJ after comparing a HIIT based on repeated-CMJ vs. repeated-sprints incorporated during the usual training session after 6 weeks (Ojeda-Aravena et al., 2019). Although, the authors reported percentage and ES increases for SJ in the repeated-CMJ group (5%; ES = 0.30) and repeated-sprints group (8.3%; ES = 0.82). Additionally, in another combat sport such as taekwondo, not reported significant increases in CMJ after adding two sessions of simulated combat in different areas sizes (4 × 4 m; 6 × 6 m; and 8 × 8 m) to regular training after 8 weeks (Ouergui et al., 2021). On the other hand, in the same sport, Ouergui et al. (2020) documented significant increases for CMJ height in both groups independently after comparing a HIIT with repeated-sprints vs. technical-specific efforts (three sets of 10 repetitions of 6 s of repeated kicks with 10 s of rest between repetitions and 3 min of recovery between sets) after 4 weeks (Ouergui et al., 2020).

TABLE 1 | Effects and response rate of high-intensity interval training (HIIT) with specific techniques vs. usual training ($n = 10$).

	EG ($n = 5$)						CG ($n = 5$)				EG vs. CG
	Pre intervention	Post intervention	$F_{1,8}$; p; η^2_p	% change ± SD	ES	Rs; %	Pre intervention	Post intervention	$F_{1,8}$; p; η^2_p	% Change ± SD	ES	Rs; %	$F_{1,8}$; p; η^2_p
Outcomes													
SJ (cm)	26.9 ± 4.5	31 ± 5	3.86; 0.85; 0.32	15.2 ± 11.9	0.91 Moderate	2 (40)	24.8 ± 7.3	26.9 ± 3.1	1.09; 0.32; 0.12	16.2 ± 36.6	0.28 Trivial	1 (20)	0.42; 0.53; 0.05
CMJ (cm)	29.0 ± 5.6	28.1 ± 4.1	0.14; 0.71; 0.17	−1.8 ± 10.1	−0.16 Trivial	0 (0)	27.6 ± 3.7	27.0 ± 7.9	0.59; 0.81; 0.00	2.9 ± 24.2	−0.16 Trivial	1 (20)	0.09; 0.92; 0.01
CODS (s)	12.28 ± 0.68	12.04 ± 0.80	1.60; 0.24; 0.16	−1.7 ± 3.8	−0.35 Small	2 (40)	12.80 ± 1.69	12.86 ± 1.65	0.09; 0.76; 0.01	0.48 ± 2.45	0.03 Trivial	0 (0)	1.23; 0.29; 0.13

EG, experimental group; CG, control group; % change, changes in means with 90% CI; SD, standard deviation; ES, effect size with 90% CI; Rs, responders; F, value of F; p, value of p; η^2_p, partial Eta squared; SJ, squat jump; and CMJ, countermovement jump.

According to the above, through the results analyzed and given the heterogeneity of the HIIT protocols applied, it is still not possible to be conclusive about the increase in performance in this physical ability in karate. In this sense, the lack of volume of training load applied may have influenced the results. In this regard, studies in combat sports such Taekwondo, that report significant increases in jumping ability add independent training sessions (Ouergui et al., 2020, 2021). Furthermore, the different motor patterns used during HIIT (running, jumping, and specific techniques) involve different muscle tension for the lower limbs and potentially different adaptations. The above, considering the growing evidence in other collective sports such as ice hockey (Kinnunen et al., 2019), highly trained running athletes (Kohn et al., 2011), and recreationally active individuals (Schaun et al., 2019; Moghaddam et al., 2020) who primarily use HIIT based on running. In these studies, neuromuscular (improvement of lower body muscular power, increase of the force-velocity curve and decreased maximal voluntary contraction times), histological (increases in type 2 fiber pool, increases in muscle cross-sectional area), and biochemical (increases in lactate dehydrogenase and decreased lactate levels; Kohn et al., 2011; Kinnunen et al., 2019; Schaun et al., 2019; Moghaddam et al., 2020) adaptations are reported.

Based on the current background, it would be necessary to increase the weekly training volume, either with a higher number of rounds during the training session, by adding independent HIIT sessions or by increasing the training weeks. In addition to HIIT, other specific training strategies such as high-intensity functional training, plyometric training or speed-based training could be applied in order to optimize the dynamic strength components (Loturco et al., 2014; Neto and Kennedy, 2019; Franchini, 2020; Quinzi et al., 2020).

Change of Direction Speed

According to the CODS results, an increase in percentage and ES (−1.7%; −0.35, respectively) was observed post-intervention. About this, the evidence regarding the improvement of CODS using HIIT in karate athletes is still controversial. In this regard, for example, previously in this sport, percentage and ES increases (−11.6%; ES = 1.20) are observed, although without significant decreases in the performance of this ability after HIIT intervention based on repeated-CMJ vs. repeated-sprints (Ojeda-Aravena et al., 2019). On the other hand, in taekwondo, significant increases ($p = 0.04$) in this ability are documented in favor of the group that performed simulated bouts in the 4 × 4 m vs. 6 × 6 m and 8 × 8 m area size after 8 weeks (Ouergui et al., 2021). In addition, this same group of researchers in youth taekwondo athletes reported significantly greater performance in the HIIT with specific-techniques vs. HIIT with repeated-sprints ($p < 0.01$) after 4 weeks of training (Ouergui et al., 2020).

However, despite the growing positive evidence of HIIT on this physical ability, it is still not possible to state this with certainty, considering the disparity of protocols and the number of athletes analyzed. Also, the lack of specific neuromuscular stress between HIIT and CODS has likely influenced the results obtained. In this sense, it may be that the lack of accelerations and decelerations in the motor patterns used influenced the observed response. In this regard, it is important to emphasize

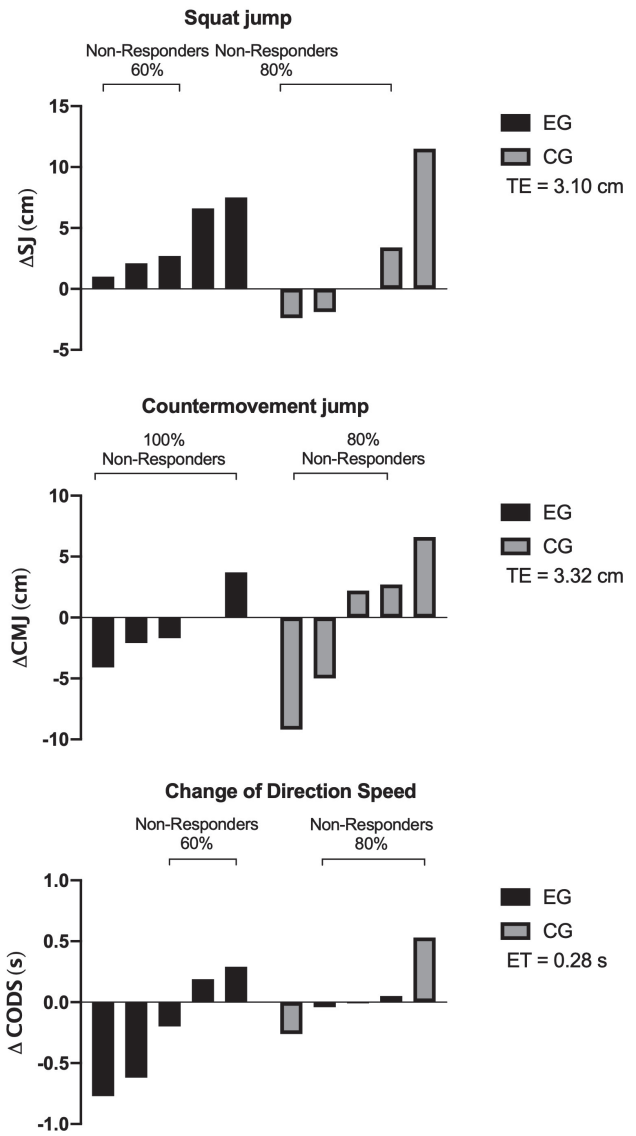

FIGURE 3 | Inter-individual variability analysis of the outcomes analyzed ($n = 10$). NRs, non-responders; TE, technical error; Δ, difference post-pre intervention expressed as delta; EG, experimental group; CG, control group; SJ, squat jump; CMJ, countermovement jump; and CODS, change of direction speed.

that in acute terms the evidence shows that muscle power expressed indirectly through jumping ability and including indirect eccentric indexes, in this sport is significantly related to and influences CODS performance (Herrera-Valenzuela et al., 2020; Ojeda-Aravena et al., 2021a). Furthermore, these results are consistent when examining the relationship between jumping ability and specific CODS in male youth karate athletes (Herrera-Valenzuela et al., 2020).

Another aspect that may have affected the analyzed results is the phenomenon of interference about muscle hypertrophy and/or power or force rate development adaptations resulting from concurrent training (strength and endurance) performed during the same session or as part of the training program (Wilson et al., 2012; Coffey and Hawley, 2017; Fyfe and Loenneke, 2018; Neto and Kennedy, 2019). However, this phenomenon is currently debated and associated factors (including exercise volume, intensity, and nutritional status, among others) must be taken into account (Neto and Kennedy, 2019). Evidence points out that such an effect could also depend on the participants' general fitness level, their training experience, and the frequency of sessions in a week (Fyfe and Loenneke, 2018). On the other hand, HIIT has been shown to reduce the phenomenon of concurrent training interference (Methenitis, 2018). Additionaly, it is important to mention that this study did not apply strength training with external loads, therefore, it is pertinent to question whether bodyweight training could be sufficient to generate this phenomenon or whether it is due to the aforementioned factors.

Inter-individual Variability in Response to the HIIT Program

Another purpose was to analyze the athletes inter-individual variability. Among the main results, Rs were reported for the two groups in SJ and only for the T-test in EG. Meanwhile, athletes' NRs were reported for all the analyzed outcomes. These results are similar to those reported recently in taekwondo athletes after 4 weeks of HIIT with specific-techniques (Ojeda-Aravena et al., 2021b). In this study, the authors documented Rs for SJ ($n = 2$) and CODS ($n = 3$; Ojeda-Aravena et al., 2021a). In another combat sport such as boxing, recently the authors Herrera-Valenzuela et al. (2021) interestingly documented after the application of a HIIT with specific-techniques after 4 weeks a higher proportion of athletes Rs in outcomes related to specific actions and performance of bipodal and unipodal CMJ of both limbs (Herrera-Valenzuela et al., 2021).

Accordingly, the inter-individual variability of observed responses to training, including HIIT, according to Walsh et al. (2020) is a combination of (i) individual responses to perseverative exercise training (subject-training interaction), (ii) day-to-day biological variation, and technical error (random variation), and (iii) physiological responses associated with behavioral/maturational changes not attributable to exercise (e.g. within-person variability; Walsh et al., 2020). This includes genetic (Mann et al., 2014; Sparks, 2017; Bonafiglia et al., 2020; Del Coso et al., 2020), climatic (Corbett et al., 2018), cognitive (Atkinson and Batterham, 2015), stress and sleep status (Mann et al., 2014), gender, age, time of day variation (Mann et al., 2014; Sparks, 2017), training status (Pickering and Kiely, 2019), physiological (Williamson et al., 2017; Atkinson et al., 2019), and statistical (Swinton et al., 2018; Chrzanowski-Smith et al., 2020).

Limitations

However, it is important to mention that the results should be analyzed for their merit, as they could be influenced by (i) the small sample size, (ii) the menstrual cycle of females (Schmitz et al., 2020); (iii) the lack of neuromuscular stress applied; and (iv) the homogeneity of the athletes according to their biological age. Nevertheless, considering the above, the incorporation of HIIT with specific-techniques in combat sports fitness is an early-stage research topic in applied sports science reflected in growing evidence (Franchini et al., 2016, 2017; Kamandulis et al., 2018; Ouergui et al., 2020, 2021; Herrera-Valenzuela et al., 2021;

Ojeda-Aravena et al., 2021a). In this sense, future research could use a greater number and experience level of athletes and verify the results by gender. Also, could verify the physiological and neuromuscular effect of HIIT protocols with specific-techniques, in addition to verifying the efficient interval for this sport.

Highlights

Although it requires further study, the incorporation of HIIT protocols with specific-techniques and using the time structure of combat could be an alternative as part of the training session during inter-competitive periods (e.g., during a shock microcycle) due to the limited time available to athletes to cope with the demands of this period. In addition, these HIIT protocols can be performed in reduced places. In turn, coaches could use inter-individual response analysis as a practical monitoring tool to follow the training progress of each athlete.

CONCLUSION

In conclusion, the addition to regular training of a HIIT protocol with specific techniques and based on the temporal structure of combat after 4 weeks was not a sufficient stimulus to increase jumping ability and change of direction speed in karate athletes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Universidad Autónoma (Code: 080–18). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Heterogeneity of Hematological Response to Hypoxia and Short-Term or Medium-Term Bed Rest

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Hematological changes are commonly observed following prolonged exposure to hypoxia and bed rest. Typically, such responses have been reported as means and standard deviations, however, investigation into the responses of individuals is insufficient. Therefore, the present study retrospectively assessed individual variation in the hematological responses to severe inactivity (bed rest) and hypoxia. The data were derived from three-bed rest projects: two 10-d (LunHab project: 8 males; FemHab project: 12 females), and one 21-d (PlanHab project: 11 males). Each project comprised a normoxic bed rest (NBR; $P_IO_2 = 133$ mmHg) and hypoxic bed rest (HBR; $P_IO_2 = 91$ mmHg) intervention, where the subjects were confined in the Planica facility (Rateče, Slovenia). During the HBR intervention, subjects were exposed to normobaric hypoxia equivalent to an altitude of 4,000 m. NBR and HBR interventions were conducted in a random order and separated by a washout period. Blood was drawn prior to (Pre), during, and post bed rest (R1, R2, R4) to analyze the individual variation in the responses of red blood cells (RBC), erythropoietin (EPO), and reticulocytes (Rct) to bed rest and hypoxia. No significant differences were found in the mean Δ(Pre-Post) values of EPO across projects (LunHab, FemHab, and PlanHab; $p > 0.05$), however, female EPO responses to NBR (Range - 17.39, IQR – 12.97 mIU·ml^{-1}) and HBR (Range – 49.00, IQR – 10.91 mIU·ml^{-1}) were larger than males (LunHab NBR Range – 4.60, IQR – 2.03; HBR Range – 7.10, IQR – 2.78; PlanHab NBR Range – 7.23, IQR – 1.37; HBR Range – 9.72, IQR – 4.91 mIU·ml^{-1}). Bed rest duration had no impact on the heterogeneity of EPO, Rct, and RBC responses (10-d v 21-d). The resultant hematological changes that occur during NBR and HBR are not proportional to the acute EPO response. The following cascade of hematological responses to NBR and HBR suggests that the source of variability in the present data is due to mechanisms related to hypoxia as opposed to inactivity alone. Studies investigating hematological changes should structure their study design to explore these mechanistic responses and elucidate the discord between the EPO response and hematological cascade to fully assess heterogeneity.

Keywords: bed rest, hypoxia, hematology – red cells, variability – individual, inactivity

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INTRODUCTION

Due to the likelihood that future space vehicles and habitats, for logistical reasons, will be hypoxic (Bodkin et al., 2006; Norcross et al., 2013), our program of research (Keramidas et al., 2016b, 2017; McDonnell et al., 2019a; Mekjavic et al., 2020) investigated the effects of hypoxia on the known adaptation of physiological systems to short- and medium-term bed rest (i.e., ground-based experiments simulating the inactivity and unloading of the weight-bearing limbs).

The hematological changes that occur with severe inactivity were first reported by Taylor et al. (1945), who observed a 9.3% loss of blood volume, concomitant with a 15.5% contraction of plasma volume (PV) in healthy young males as a consequence of three-weeks of bed rest. Their results also revealed significant individual variability in the hematological responses to bed rest but were not explored. The observed normoxic bed rest (NBR)-induced hypovolemia was attributed to the prolonged cephalad fluid shift (CFS) that stimulates central volume carotid, aortic and cardiac receptors, releasing atrial natriuretic peptide (ANP) in turn causing diuresis and natriuresis and a resultant decrease in PV (Fortney et al., 2010). Renal release of the hormone erythropoietin (EPO) is inhibited by the resultant increase in central venous pressure during CFS bed rest (Kirsch et al., 1984; De Santo et al., 2005). Gunga et al. (1996) reported a rapid decline of EPO in the initial 24 h ($p < 0.01$) due to the initial increase in central venous pressure. Thereafter it returns gradually to pre-bed rest levels. Additionally, despite the EPO suppression found in the first 24 h of NBR, some individuals experience concomitant increases in the concentrations of reticulocytes (Rct) and red blood cells (RBC; Ryan et al., 2016).

Reduced oxygen (O_2) availability in the tissues resulting from a lower partial pressure of O_2 in the ambient air stimulates the renal release of EPO (Scholz et al., 1990), which in turn promotes erythropoiesis in the bone marrow. When a red blood corpuscle has matured to a Rct, it is released into the circulating blood. The fraction of Rcts is typically 0.5–2.5% of the total RBCs circulating in the blood (Koepke and Koepke, 1986; Banfi et al., 2006). Once matured to RBC, the blood's O_2 carrying capacity is increased due to the rise in total RBC volume. PV decreases during hypoxic acclimation; however, studies that report this variable tend to report a large degree of interstudy variability (Sawka et al., 1996; Heinicke et al., 2003). Theoretically, as PV reduction is seen in both prolonged hypoxia and bed rest *via* different mechanisms, hypoxic bed rest (HBR) should produce compounded PV loss compared to hypoxia or bed rest alone (Loeppky et al., 1993; Keramidas et al., 2016a).

The concept of individuals being either responders or non-responders in response to an intervention is commonplace in physiology. Categorization of individuals into these groups is based on observing a response that exceeds the typical error of the measurement (Montero and Lundby, 2017). Ge et al. (2002), reported substantial individual variability in the EPO responses after several 24-h hypoxic exposures at a range of simulated altitudes. The authors also commented that individuals that had the largest responses to lower simulated elevations also had the largest responses to higher altitudes. Similar mean increases and a significant correlation between individual's hemoglobin mass responses after normobaric and hypobaric hypoxic live high train low interventions have been reported (Hauser et al., 2017). A moderate altitude (~2100 m) training camp attended by 12 Australian-Football players on 2 consecutive years found that individuals' EPO responses to the same stimuli were not consistent from one year to the next (McLean et al., 2013). Of note, this finding was also present in the variability in the hemoglobin mass response of Finnish endurance athletes (Nummela et al., 2020), and German elite swimmers (Wachsmuth et al., 2013), both attributing this intraindividual variability to the lack of consistency and monitoring of athletes prior to each altitude exposure. Therefore, categorization of individuals into "responders" or "non-responders" after a single intervention is unreasoned and should not be considered an unchanging and distinguishable trait. Each of these studies' authors stress the importance of individual evaluation of hematological variables in response to hypoxic exposures.

Levels of estrogen and progesterone change over the course of the menstrual cycle. Estrogen typically peaks during the late follicular phase, close to the start of ovulation, driving plasma volume expansion (Øian et al., 1987). However, in the late luteal phase, levels of progesterone increase, causing natriuresis and resulting in body fluid loss (Maffei et al., 1999). Reports investigating hematological changes during the menstrual cycle have described reductions (Vellar, 1974; Javaid et al., 2007; Ofojekwu et al., 2013), or no change (Kim et al., 1993; Lebrun et al., 1995) in Hb concentration during the early follicular compared to the luteal phase. However, it should be noted that Hb concentration's stability is dependent upon the intra-extracellular fluid movements. Fortney et al. (1994), in a review of their previous series of studies, reported large fluctuations of PV and red cell mass within the menstrual cycle. The absolute PV and red cell mass were measured using a technetium radioisotope technique during the follicular and luteal phases of each woman's menstrual cycle. A peak increase in PV was observed within two days of the estimated ovulation day, preceded by a decreased PV lasting 1 to 3 days. Fortney et al. (1994) also reported that in both sexes, PV was significantly reduced post bed rest compared to pre; however, a greater degree of blood volume and PV loss was noted in males than females. While the menstrual cycle has a varying effect on PV, previous studies have reported no effect on red blood cell volume or hemoglobin mass (Chapman et al., 1997; Reeves et al., 2001; Malipatil and Patil, 2013; Aguree et al., 2020). Keller and colleagues (Keller et al., 2020) identified that although there was no significant change in hemoglobin mass across the menstrual cycle, the coefficient of variation (CoV) for hemoglobin mass over the duration of a single menstrual cycle was 4.1%, which is above the typical CoV commonly reported when using the carbon monoxide rebreathing technique (2.2%) and thus may be under-reported.

Common to the aforementioned studies is that individual variability is noted by presenting the standard deviation in the hematological changes observed in response to the bed

rest, however, it is not discussed or expounded. Due to the potential for large individual variation in the responses to hypoxia and/or simulated microgravity, the recruitment of astronauts for future deep-space missions should consider individuals' physiological systems' responses to the adaptations to microgravity. Therefore, in conjunction with the European Astronaut Centre, a retrospective analysis was initiated on all hematological variables collected under the umbrella of the Slovene bed rest program conducted in the Planica facility. The present study aimed to assess the individual variability in the cascade of hematological responses to normoxic bed rest and HBR. We hypothesized that the process of acclimation as reflected in the hematological changes observed in subjects exposed to bed rest alone or in combination with hypoxia would not be the same for all participants.

METHODOLOGY

Ethical Approval

Subjects' written informed consent was obtained prior to each project, and they were informed that they were free to withdraw their consent at any time. The procedures were approved by the Committee for Medical Ethics at the Ministry of Health (Republic of Slovenia; approval numbers: 205/2/11 and 88/04/12) conformed to the standards set by the Declaration of Helsinki (PlanHab: NCT02293772), except for the registration of the LunHab and FemHab projects in a database.

Study Design

Data used for these analyses were derived from 3-bed rest projects: two 10-day bed rest projects (LunHab: male subjects; FemHab: female subjects) and one 21-day bed rest project (PlanHab: male subjects). Each project comprised two experimental interventions: normobaric normoxic bed rest (NBR) and normobaric HBR (simulated altitude of 4,000 m, $P_IO_2 \approx 91$ mmHg). In each intervention, subjects were confined to one floor of the Olympic Sports Centre Planica (Rateče, Slovenia) situated at an altitude of 940 m (partial pressure of inspired oxygen; $P_IO_2 = 133$ mmHg). A horizontal position was maintained throughout all interventions, and subjects could only use one pillow for head support. Additionally, all activities were conducted in the horizontal position (i.e., hygiene, toilet, etc.), with the exception that during meals, they were allowed to rest on an elbow. Inclusion and exclusion criteria for PlanHab, LunHab, and FemHab have previously been described in detail (McDonnell et al., 2019a, 2020; Mekjavic et al., 2020) and followed the guidelines recommended by the European Space Agency (Heer et al., 2009). Concerning prior altitude exposure, subjects were excluded if they had been to altitudes above 2,000 m within two months of the start of an intervention. The subjects' physical characteristics are presented in **Table 1**. The detailed methodologies for each of these projects have been reported previously (Ciuha et al., 2015; Salvadego et al., 2016; Keramidas et al., 2016b; McDonnell et al., 2019a; Mekjavic et al., 2020).

TABLE 1 | Physical characteristics of subjects that completed both NBR and HBR interventions in the 10-d LunHab, FemHab projects, and the 21-d PlanHab project.

Study	*n*	Sex	Age (yrs)	Height (*m*)	Weight (kg)
LunHab	8	M	23.4 (SD 1.7)	1.78 (SD 0.07)	74.1 (SD 14.1)
FemHab	12	F	26.1 (SD 3.7)	1.69 (SD 0.06)	59.5 (SD 8.8)
PlanHab	11	M	25.4 (SD 3.6)	1.80 (SD 0.04)	79.9 (SD 13.6)

The fraction of O_2 in the Planica facility was maintained using a Vacuum-Pressure Swing Absorption system (VSPA, B-Cat, Tiel, The Netherlands). Samples of air from within each of the hypoxic rooms and common areas were analyzed at 15-min intervals for O_2 and carbon dioxide content throughout the interventions. Should the O_2 levels be above the target level, the introduction of a hypoxic gas mixture was initiated. In contrast, should the O_2 levels decrease below the pre-set value, the system would terminate further delivery of hypoxic gas to that room. If the O_2 did not return to the required level, delivery of external ambient (normoxic) air would be initiated, concomitant with the triggering of an audible alarm. In addition, each subject wore a personal portable (clip-on type) O_2 analyzer (PGM-1100; Rae Systems, San Jose, California), providing immediate feedback of the F_IO_2 of the surrounding air and with an alarm alerting the user to the lower than anticipated O_2 fraction. As a result of the VPSA monitoring system, the F_IO_2 was tightly controlled throughout all hypoxic interventions (LunHab: 0.144 SD 0.001, PlanHab: 0.141 SD 0 0.004, FemHab: 0.142 SD 0.001). As a result, the partial pressure of O_2 in each project was the following: LunHab: 91.6 SD 0.14 mmHg; PlanHab: 89.6 SD 0.4; FemHab: 90.4 SD 0.4 mmHg).

Measurements

Peripheral O_2 saturation (SpO_2) was measured daily in the morning after waking (07:00) in all interventions using a finger pulse oximeter (3,100 WristOx, Nonin Medicals, Minnesota, United States).

Venous blood was drawn from an antecubital vein at specific time points during each bed rest intervention; details of the exact blood sample timings may be found below in the Data Processing section. Blood samples were collected just after waking and prior to ambulation (relevant to the Pre and Post bed rest data collection) following an overnight fast. Approximately 200 ml of blood was collected per participant in LunHab and FemHab, with 516.5 ml of blood drawn per participant in PlanHab.

Blood samples for EPO analysis when collected (EDTA vacutainers) were allowed to coagulate for 20 min, then centrifuged, and subsequently, aliquoted serum was frozen at −80°C for future analyses. EPO concentration was determined by sandwich enzyme-linked immunoassay (Quantikine IVD EPO ELISA; R&D Systems, Minneapolis, MN) using 100 µl of serum. Optical density was quantified on a SPECTRAmaxTM PLUS384 microplate spectrophotometer (Molecular Devices Corporation, 1,311 Orleans Drive, Sunnyvale, California) set

at 450 nm and corrected at 600 nm. The estimated CoV of the analysis was 2.2%.

Hb, Hct, RBCs, and Rct counts were analyzed with an automated laser-based hematology analyzer (Advia 120; Siemens, Munich, Germany) within 8 h of blood sampling using clinical laboratory standards. All hematological variables were determined in duplicate by researchers blinded to the nature of the interventions.

Changes in PV were estimated from the Dill and Costill equation using Hct and Hb values (Dill and Costill, 1974). This approach was deemed appropriate for qualitative uses for this manuscript due to the concomitant changes in plasma renin concentration highlighted during a previous analysis of the PlanHab data (Keramidas et al., 2016b). Thus, any differences in PV between HBR and NBR in the three projects indicate qualitative variations in the response and do not permit us to draw firm conclusions regarding PV changes' exact magnitude.

Data Processing

The current study is an amalgamation of the results from three research projects designed to assess the separate and combined effects of hypoxia and bed rest on multiple physiological systems and the participants' psychological status. Therefore, the data analysis was not included in the original design of the studies, namely, to assess individual variation and the chronological changes in the hematological variables. The three projects were similar in design and protocol. The experimental schedules have been reported previously for the LunHab (McDonnell et al., 2019b) and FemHab (McDonnell et al., 2020) projects. Due to minor changes in the experimental schedules the hematological sampling frequency is not consistent across the three projects.

Each of the three projects consisted of three interventions where subjects would experience one of the three conditions (NBR, HBR, and Hypoxic Ambulatory). Due either to subject dropouts, methodical error or human error, the Hypoxic Ambulatory data were too incomplete to compare with the NBR and HBR interventions. A minimum washout period of one month and three months was instituted between interventions for the 10-d (LunHab and FemHab) and 21-d (PlanHab) projects, respectively. Due to the sample sizes involved and the possibility of carryover at some physiological level between interventions despite the washout periods, the current data set was considered inappropriate for quantifying true individual response (SD_{IR}; Williamson et al., 2017). As a result, the data presented in this study does not allow us to make conclusions as to the source of the variability; however, the current study highlights the importance of providing measures of individual variability when presenting results. As a result, the primary purpose of the current manuscript was to investigate the variability in the presented data and highlight the importance of providing measures of individual variability in the hemopoietic cascade of adaptation to bed rest.

The blood sampling draws for each project were:

- LunHab: Pre (Day-1) and Post (Day R1).
- FemHab: Pre (Day-2), During (Days 2 and 6), and Post (Days R1 and R2).
- PlanHab: Pre (Day-2), During (Days 2, 5, 14, and 21), and Post (Days R2 and R4).

On Day 1 of each intervention, subjects woke at 07:00, and continued with their assigned daily routine, following which they entered into the intervention, either HBR or NBR at 09:00. The subjects then conducted 10 (240 h: LunHab & FemHab) or 21 (504 h: PlanHab) days in that intervention. Thus, upon waking on the morning of R1, prior to reambulation at 09:00, the subjects were still in their designated intervention when a blood sample was collected at 07:00. Therefore, as there was no R1 data collection point for PlanHab, in order to ensure all Post blood draws were collected before reambulation, the Post values were collected on R1 for both LunHab and FemHab (R1) and Day 21 for PlanHab.

Statistical Analyses

Data are expressed as individual responses, mean and SD, or as ranges and interquartile values. Statistical analyses were undertaken using SPSS (Version. 25, IBM, New York, United States) with significance set as $p \leq 0.05$. To assess whether significant statistical change had occurred in the pre- to post-intervention hematological values, a paired samples *t*-test was applied to the means. In the current analyses, subjects who completed both NBR and HBR interventions were included and paired-samples *t*-tests were used to distinguish differences between Δ(Pre-Post) values.

A One-way ANOVA was used to assess for significance between the NBR and HBR Δ(Pre-Post) values between projects (e.g., LunHab NBR vs. FemHab NBR vs. PlanHab NBR). The between variable relationship strength was calculated using Pearson's or Spearman's correlation analysis. Correlation analysis was used to assess potential relationships between SpO_2 and EPO throughout the intervention. In all studies, potential relationships between pre-intervention hematological values and both absolute and relative degrees of change to post-intervention were investigated. Correlation coefficients were applied as recommended (Cohen, 2013; strong $\geq$0.60; moderate $\geq$0.40 $-<$0.59; weak $\geq$0.20 $-<$0.39).

A two-way repeated measures ANOVA was employed to assess the effect of time (Pre– vs. Post-bed rest) and condition (normoxia and hypoxia) within each bed rest project (LunHab, FemHab, and PlanHab). A two-way mixed-model ANOVA was employed to determine whether differences in the hematological markers existed due to the duration of comparable interventions (FemHab and PlanHab). In addition, post-hoc analyses using a Bonferroni corrected independent (between studies) and paired *t*-tests (within studies) were performed and reported where appropriate.

RESULTS

The Δ(Pre-Post) values [mean, (SD), overall range, and interquartile range] of EPO, Rct, RBC, and PV from each

intervention for LunHab, PlanHab, and FemHab are presented in **Table 2**. The duration of bed rest did not have a statistical effect on any of the hematological variables, nor did subject sex ($p > 0.05$). No correlations were found between the Δ(Pre-Post) bed rest hematological responses to NBR and HBR in EPO, Rct, or RBC (**Figure 1**).

SpO_2 and EPO Response Relationship

Correlation between SpO_2 and EPO values throughout both the NBR and HBR interventions in FemHab and PlanHab were assessed (**Figure 2**). SpO_2 presented in **Figure 2** was collected on the same day as the corresponding blood draw. In PlanHab HBR, a significant moderate negative correlation was identified ($r = -0.561$, $p < 0.001$). No significant relationship was discovered in either FemHab intervention (HBR: $r = -0.252$, $p = 0.117$; NBR: $r = 0.200$, $p = 0.271$) or in PlanHab NBR ($r = 0.143$, $p = 0.253$).

Changes in Erythropoietin

While mean EPO peaked in FemHab and PlanHab on Day 2 of HBR, in FemHab (**Figure 3A**), only six of the twelve subjects peaked on Day 2. Four of FemHab subjects' peak EPO values did not increase above baseline for the HBR. All but one of FemHab's subjects' EPO values reduced to lower than baseline on Day R2.

The results of the PlanHab project (**Figure 3D**) indicate that eleven of the twelve subjects EPO peaked on Day 2. All subjects' EPO reduced to lower than pre-HBR levels on Day R2. At the group level, significance was detected between Δ(Pre-Post) EPO values in LunHab HBR ($p = 0.028$), FemHab NBR ($p = 0.040$) and PlanHab NBR ($p = 0.014$). The within-project ranges for EPO Δ(Pre-Post) during HBR were −0.5 to 6.6 $mIU \cdot ml^{-1}$ (LunHab), −25.34 to 23.66 $mIU \cdot ml^{-1}$ (FemHab) and −7.15 to 2.57 $mIU \cdot ml^{-1}$ (PlanHab). The within-project ranges for EPO Δ(Pre-Post) during NBR were −2.2 to 2.4 $mIU \cdot ml^{-1}$ (LunHab), −16.41 to 0.98 $mIU \cdot ml^{-1}$ (FemHab) and −4.47 to 1.77 $mIU \cdot ml^{-1}$ (PlanHab). Pearson's correlation analyses showed that there were no correlations between NBR and HBR Δ(Pre-Post) EPO (LunHab: $r = -0.162$, $p = 0.702$; FemHab: $r = 0.469$, $p = 0.241$; PlanHab: $r = -0.229$, $p = 0.050$).

Changes in Reticulocytes

Group mean Rct peaked in FemHab on Day 6 and on Day 5 in PlanHab (**Figures 3B,E**) due to the sampling timeline. In FemHab, 2 of the subjects' Rct values peaked on Day R2. However, in PlanHab, 4 subjects did not reach their maximum Rct concentration on the same day as the group mean peak value. After the Rct peak, these values gradually reduced to baseline levels in both projects.

At the intervention level, significance was discovered between pre and post Rct values in FemHab HBR ($p = 0.001$), PlanHab NBR ($p = 0.038$) and PlanHab HBR ($p = 0.009$). From HBR Δ(Pre-Post), the range for each data set were 6.70 to $42.90 \times 10^9 \cdot L^{-1}$ (FemHab) and −8.2 to $65.9 \times 10^9 \cdot L^{-1}$ (PlanHab). No significance was found in the FemHab NBR intervention ($p = 0.112$).

The inter-subject range for the changes in Rct NBR Δ(Pre-Post) -6.50 to $71.20 \times 10^9 \cdot L^{-1}$ (FemHab) and −8.70 to $27.70 \times 10^9 \cdot L^{-1}$ (PlanHab). Pearson's correlation analyses showed there were no correlations between the Δ(Pre-Post) values in Rct for NBR and HBR (FemHab: $r = -0.280$, $p = 0.501$; PlanHab: $r = -0.218$, $p = 0.520$).

TABLE 2 | Bed rest Δ(Pre-Post) values in EPO concentration (ΔEPO), number of reticulocytes (ΔRct), number of red blood cells (ΔRBC), and plasma volume (ΔPV) during NBR and HBR interventions in all three projects.

		LunHab			FemHab			PlanHab		
		NBR	HBR	$p^{\dagger}$	NBR	HBR	$p^{\dagger}$	NBR	HBR	$p^{\dagger}$
ΔEPO ($mIU \cdot ml^{-1}$)	Mean	−0.59†,‡	2.18*,†	0.036	−6.06*,‡	1.70	0.179	−2.21*,†	−1.28†	0.504
	SD	1.51	2.22		6.79	13.83		2.45	3.11	
	Range	4.60	7.10		17.39	49.00		7.23	9.72	
	IQR	2.03	2.78		12.97	10.91		1.37	4.91	
	p*	0.306	0.028		0.040	0.739		0.014	0.204	
ΔRct ($\times 10^9 \cdot L^{-1}$)	Mean		N/A		16.40	27.38*	0.364	8.94*	22.92*	0.138
	SD				25.53	13.62		12.37	23.30	
	Range				77.70	36.00		36.40	74.10	
	IQR				29.75	26.75		22.50	40.10	
	*p**				0.112	0.001		0.038	0.009	
ΔRBC ($\times 10^{12} \cdot L^{-1}$)	Mean	0.28*,†	0.81*,†	0.016	0.40*,†	0.88*,†	0.002	0.38*,†	0.92*,†	0.001
	SD	0.20	0.33		0.16	0.28		0.32	0.33	
	Range	0.48	0.83		0.44	0.67		1.03	1.02	
	IQR	0.41	0.64		0.29	0.56		0.51	0.56	
	*p**	0.018	0.018		<0.001	<0.001		0.003	0.009	
ΔPV (%)	Mean	−5.08	−13.30		−8.01	−15.86		−6.18	−14.51	
	SD	3.73	4.22		3.85	4.42		5.27	4.34	
	Range	8.86	11.18		10.34	12.50		15.72	14.86	
	IQR	7.65	7.80		7.25	7.30		7.49	6.81	

*LunHab - 6 males (8 for EPO), FemHab - 8 females, PlanHab - 11 males; IQR – Interquartile Range. *denotes significance between Δ(Pre-Post) values for condition ($p \leq 0.05$).*
†denotes significance in Δ(Pre-Post) values within study between normoxic and hypoxic gases ($p \leq 0.05$).
‡denotes significance in the Δ(Pre-Post) EPO response in NBR between LunHab and FemHab.

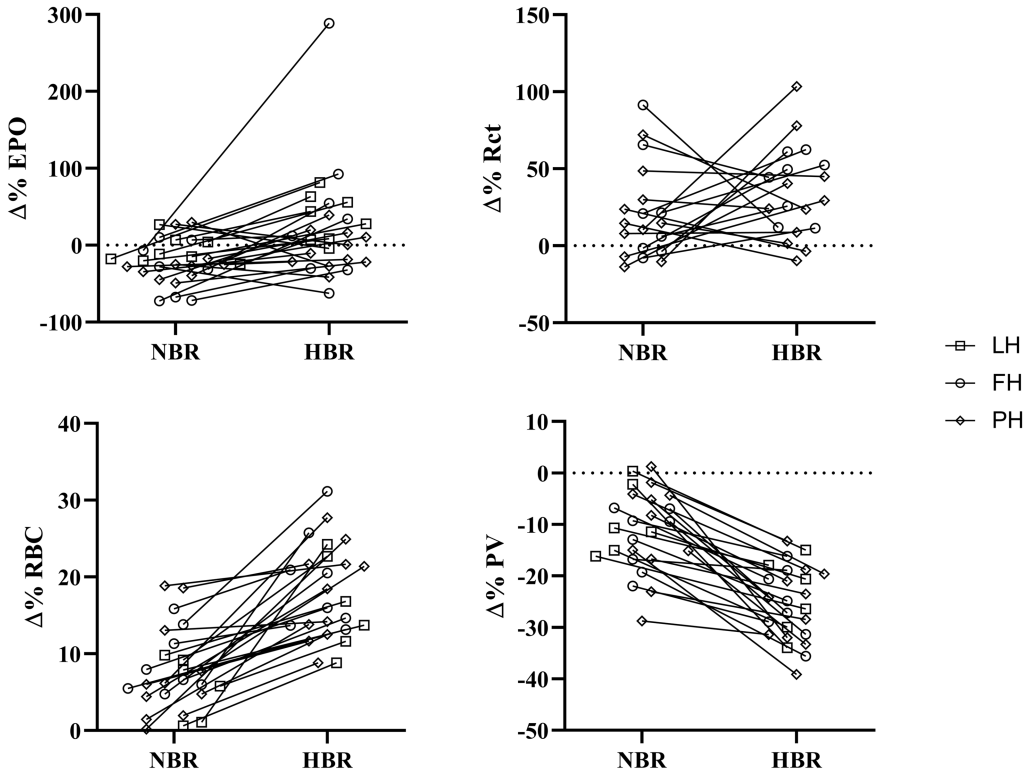

FIGURE 1 | Individual relative (Δ(Pre-Post)) responses of erythropoietin (Δ%EPO), reticulocytes (Δ%Rct), red blood cells (Δ%RBC), and plasma volume (Δ%PV) for NBR and HBR trials. Squares: LunHab; Circles: FemHab and Diamonds: PlanHab.

Changes in Red Blood Cell Volume

RBC peaked in FemHab HBR on Day R1 despite half of the subjects' individual peak scores occurring on Day 6 (**Figure 3C**). In PlanHab HBR, the peak in RBC was on Day 14 of the intervention, although 5 of the 11 subjects' individual peaks were on a day other than Day 14 (**Figure 3F**). In both studies, after the group peak in RBC, values dropped to around that observed at baseline. At the group level, significance was discovered between Pre- vs. Post-bedrest RBC values in all data sets (LunHab, FemHab, and PlanHab) and conditions (NBR and HBR; $p < 0.05$). NBR 0.03 to $0.51 \times 10^{12} \cdot L^{-1}$ (LunHab), 0.24 to $68 \times 10^{12} \cdot L^{-1}$ (FemHab) and 0.01 to $1.04 \times 10^{12} \cdot L^{-1}$ (PlanHab). In HBR the Δ(Pre-Post) ranges for each data set were -0.11 to $1.27 \times 10^{12} \cdot L^{-1}$ (LunHab), 0.62 to $1.29 \times 10^{12} \cdot L^{-1}$ (FemHab) and 0.48 to $1.33 \times 10^{12} \cdot L^{-1}$ (PlanHab). Pearson's correlation analyses showed there were no correlations between the Δ(Pre-Post) changes in RBC for NBR and HBR (LunHab: $r = 0.161$, $p = 0.761$; FemHab: $r = 0.355$, $p = 0.388$; PlanHab: $r = 0.247$, $p = 0.464$).

Baseline Corrected Responses

The only correlation between mean peaks in the measured hematological variables during Pearson's correlation analysis was a significant positive moderate correlation between FemHab HBR Rct and RBC ($r = 0.597$, $p = 0.040$). No other correlation existed between the mean peaks in the measured hematological variables as either absolute or relative changes from baseline ($p > 0.05$; **Figure 4**).

EPO, Rct and RBC significantly changed over the duration of the HBR in both FemHab and PlanHab (FemHab EPO: F (2.106, 23.166) = 3.037, $p = 0.027$, $\eta^2 = 0.216$; FemHab Rct: F (2.007, 22.074) = 18.823, $p < 0.001$, $\eta^2 = 0.631$; FemHab RBC: F (4, 44) = 37.919, $p < 0.001$, $\eta^2 = 0.775$; PlanHab EPO: F (2.007, 20.068) = 30.176, $p < 0.001$, $\eta^2 = 0.751$; PlanHab Rct: F (6, 60) = 13.802, $p < 0.001$, $\eta^2 = 0.580$; PlanHab RBC: F (2.485, 24.850) = 30.243, $p < 0.001$, $\eta^2 = 0.752$; **Figure 3**).

Changes in Plasma Volume

Any differences found in ΔPV between HBR and NBR in the three projects (LunHab, PlanHab, FemHab: **Table 2**; **Figure 5**) are purely speculative due to the calculation methods employed and do not permit us to draw firm conclusions regarding the exact magnitude of PV changes. Pearson's correlation analyses revealed no correlations in the ΔPV between NBR and HBR (LunHab: $r = 0.209$, $p = 0.691$; FemHab: $r = 0.385$, $p = 0.346$; PlanHab: $r = 0.489$, $p = 0.127$).

DISCUSSION

It is well documented that both genotypic and phenotypic factors influence the responses of individuals exposed to hypoxia (Moore,

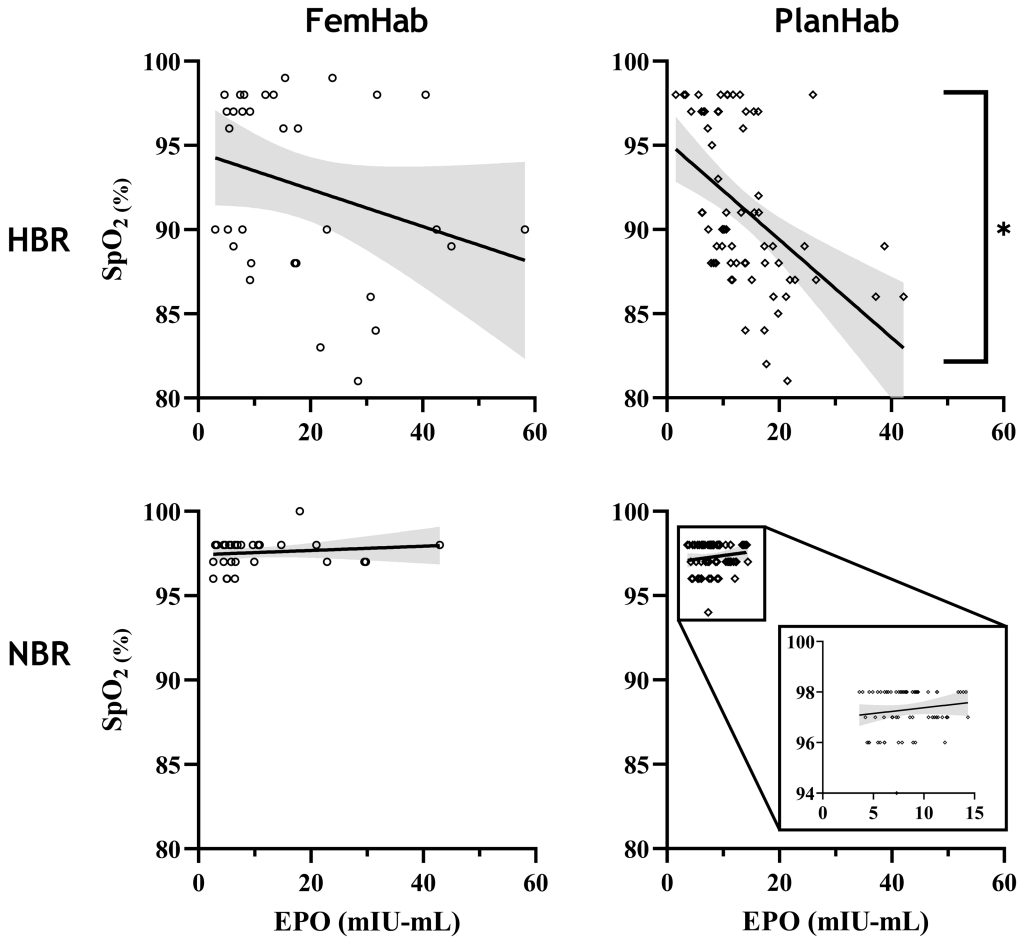

FIGURE 2 | Correlation analysis between EPO and SpO2 values throughout the FemHab and PlanHab studies. SpO_2 values were measured on the same days as EPO values were taken in each respective study. *denotes significant correlation.

2001; Beall, 2014). The principal finding of the present study is that for the same hypoxic stimulus, these factors affect the cascade of hematological responses among inactive subjects differentially. Namely, for a given hypoxic exposure (approximately $P_IO_2 = 90$ mmHg) of 10- and 21-day duration, we observed the resultant responses of arterial O_2 saturation, which placed in motion the cascade of events from the release of EPO to the resultant increased production of Rcts and finally RBCs. The latter being essential for the ability of blood to bind O_2. Hypoxic acclimatization and training protocols strive to achieve an optimal outcome of the last event in this cascade, namely an increase in RBCs. This is considered essential for maintaining the performance of lowlanders at high altitudes (i.e., alpinists), or for improving their sea-level performance (i.e., athletes). Surprisingly, the substantial individual variation observed in the first steps (SpO_2, EPO) of the cascade in HBR, gradually diminishes towards the last step of the cascade (RBC), as evident in **Figure 4**.

SpO_2 and EPO Response Relationship

A significant negative correlation between SpO_2 and EPO only existed in the PlanHab HBR intervention (**Figure 2**). FemHab NBR had a larger variation in EPO response than values at the same SpO2 in PlanHab, with some females eliciting a response equal or greater to that of males and females in HBR. In females, the hypoxic ventilatory response has been stated to be significantly more extensive during the luteal versus the follicular phase (Takano, 1984). As the hypoxic ventilatory response is an indicator of the chemosensitivity to hypoxia, this change in chemosensitivity may affect the EPO response at a given SpO_2. Since no control was in place, and monitoring of females' menstrual phase in the FemHab study was limited, it cannot be verified whether the females with exaggerated EPO responses during NBR, despite no change in SpO_2, were also in the luteal phase at that time. The higher degree of variation in females' EPO response may also be a contributing factor as to why no statistical correlation was observed in HBR during FemHab.

Magnitude of the Hematological Responses

The EPO response to NBR and HBR in females appears to be considerably larger than in males (**Table 2**). Additionally,

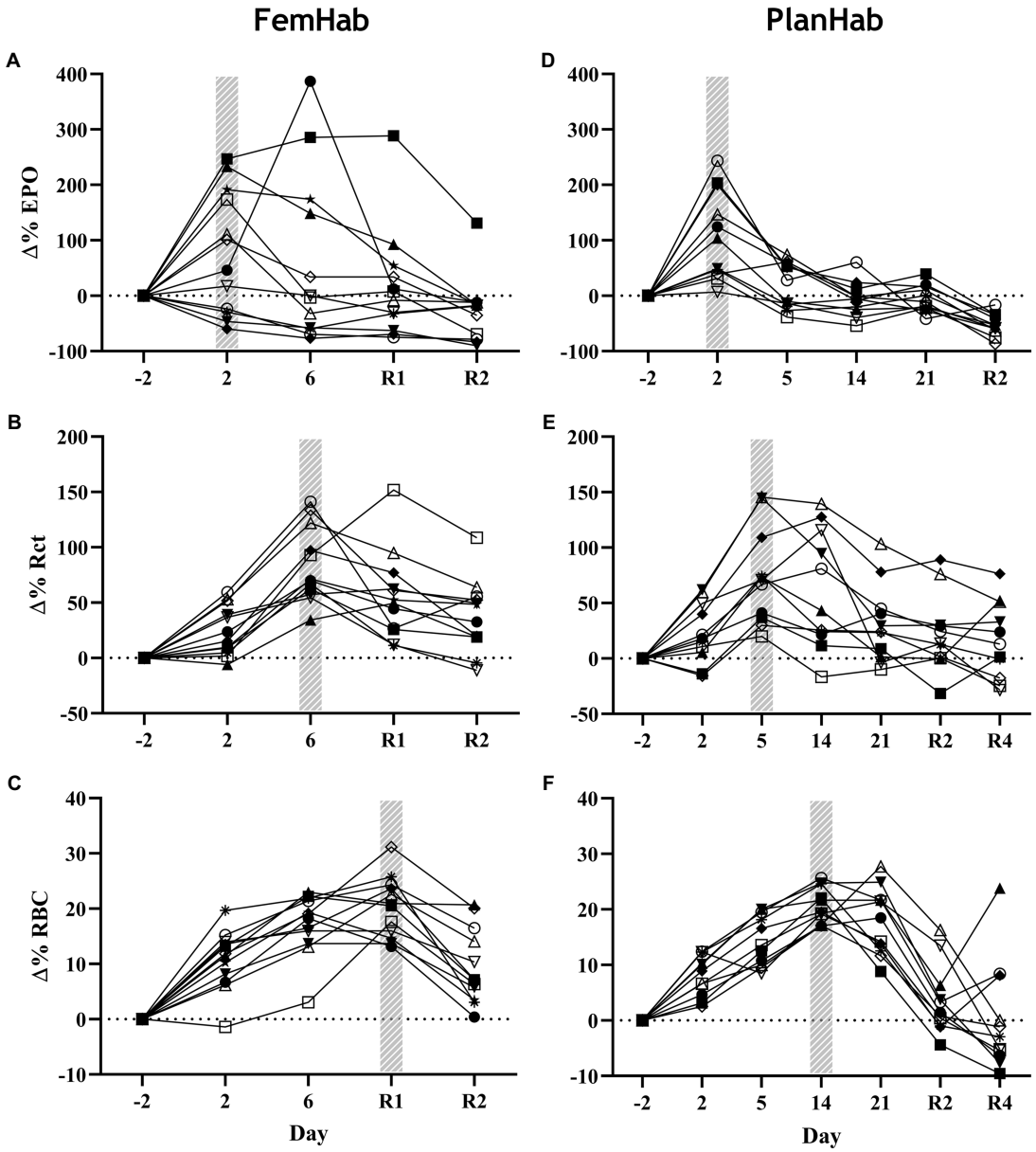

FIGURE 3 | Baseline corrected relative individual changes (Δ%) of erythropoietin (ΔEPO; **A,D)**, number of reticulocytes (ΔRct; **B,E)**, and number of RBCs (ΔRBC; **C,F)** during the hypoxic bed rest (HBR) trials in the FemHab and PlanHab projects, respectively. The hatched columns indicate the highest mean group value for each variable. Each different symbol represents an individual subject that completed the intervention.

bed rest duration appears to have no impact on the heterogeneity of hematological responses (**Table 2**). Our analyses also show that the resultant hematological changes (Rct and RBC) that occur during NBR and HBR are not proportional to the EPO level when individual responses are considered. Considering hypoxia as the stimulus for the hematological changes, we demonstrate the heterogeneity of the cascade of responses to this stimulus, from arterial O_2 saturation (**Figure 2**) to increased EPO release and production of Rct and RBC (**Figure 3**). The increase in EPO concentration (**Figures 3A**,**D**) within the first days of exposure is followed by an increase in Rct concentration (**Figures 3B**,**E**) by Days 5 (FemHab) and 6 (PlanHab), finally resulting in an increase in RBC (**Figures 3C**,**F**) by Days R1 (FemHab) and 14 (PlanHab). Qualitative analysis of the individual responses indicates a large degree of individual variation in these responses' magnitude and kinetics. Finally, these responses need to be considered from the perspective of the plasma volume changes, which is largely affected by individual variation itself. Intriguingly, despite the large range of EPO responses observed in both studies, the range of RBC concentration, the last step in this cascade, is substantially lower. These issues are discussed in further detail below.

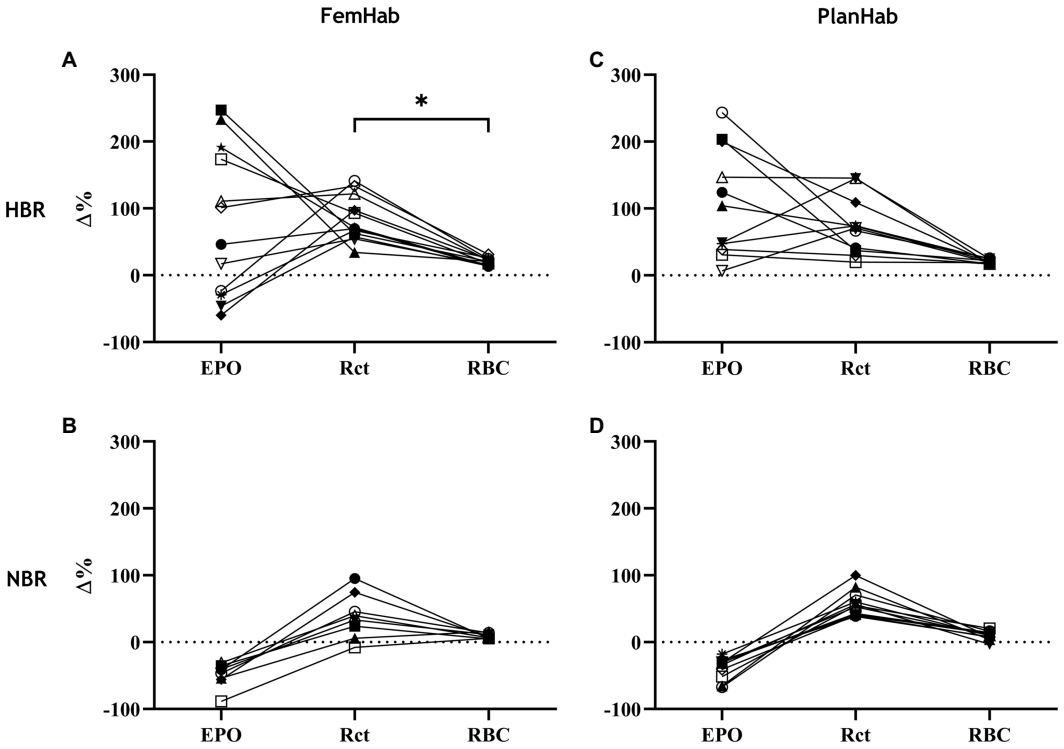

FIGURE 4 | represents the relationships between individuals' ΔEPO, ΔRct, and ΔRBC in HBR **(A,C)** and NBR **(B,D)** in FemHab and PlanHab on the days of the highest mean group change (Δ%) for each variable during the HBR confinement. * denotes significant correlation between relative responses in FemHab HBR Δ(Pre-Post) Rct and RBC ($p \leq 0.05$). Each different symbol represents an individual subject that completed the intervention.

The magnitude of an individual's relative EPO or Rct response is not necessarily indicative of the size of their relative Rct or RBC response, respectively (**Figure 4**). The reason the relative increases across the hematological variables are not consistent, could be due to scale relativity; however, the increases in these hematological variables are most likely primarily attributable to increases in hemoconcentration from hypovolemia. Rct fraction of total RBC volume is typically 0.5–2.5% (Koepke and Koepke, 1986; Banfi et al., 2006); therefore, an increase of 100% in Rct concentration would hypothetically only account for a consequential RBC increase of 1–5%. Reductions in RBC volume seen in Days R2 (FemHab), 21, and R2 (PlanHab) initially seem implausible due to the typical lifespan of circulating RBC and are most likely due to the sudden rise in PV seen in the latter half of the interventions (**Figure 5**).

The magnitude of EPO production is largely dependent on the level of an individual's hypoxic stress (Chapman et al., 2014b); however, the increase in EPO level during a fixed hypoxic stimulus between subjects varies considerably (Ploszczyca et al., 2018), as demonstrated in **Figures 2**, **4**. The differences in the spread of individual responses to NBR and HBR shown in **Figure 4** indicate that the majority of variability seen in the hematological variables is due to the mechanisms responding to hypoxic acclimation rather than bed rest. Inter-individual variation in EPO response has previously been observed by Klausen et al. (1996), with some subjects having a serum EPO response almost 10x greater than others after 42-h at an altitude of 4,350 m. Chapman et al., (2010) also found large variation in the EPO response when taking 26 elite distance runners to 2,500 m elevation for 20 h (ΔEPO – 2.9 ng ml^{-1} to 20.5 ng ml^{-1}; −19.9 to 415.4%). Variation amongst subjects in the magnitude of the EPO response to a fixed hypoxic stimulus could potentially be influenced by a multitude of factors. Disparities in subjects' carotid body chemosensitivity, hypoxic ventilatory drive, hemoconcentration, or renal blood flow at the moment of renal EPO release, and factors that are potentially hereditary traits, may explain why inter-individual variation is often more common than intra-individual variation (Collins et al., 1978; Scoggin et al., 1978).

Hematological Kinetic Response

The timing of the mean group peaks in EPO, Rct, and RBC in **Figure 3** concur with previous reports (Scholz et al., 1990; Banfi et al., 2006). A reduction in EPO level after the initial peak is apparent in both FemHab and PlanHab studies (**Figures 3A**,**D**), a finding that is in line with other altitude studies (Ploszczyca et al., 2018). However, the underlying mechanisms for this reduction are still not entirely clear. Lundby et al. (2009) noted that hypoxic inducible factor-1 (HIF-1) peaks within the first hours of hypoxic exposure and then reduces gradually to pre-hypoxic exposure levels. The authors

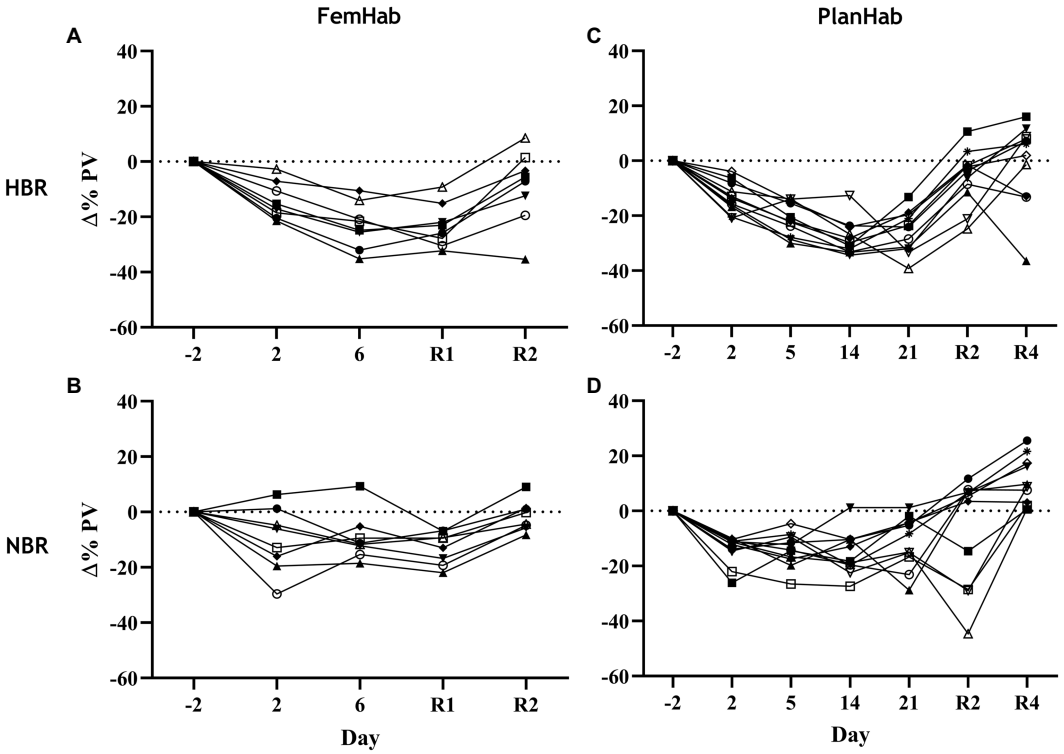

FIGURE 5 | Individual changes in PV relative to pre-intervention baseline values during NBR and HBR interventions in FemHab **(A,B)** and PlanHab **(C,D)** studies. Each different symbol represents an individual subject that completed the intervention.

speculated that the response to the initial hypoxic stimulus might diminish the degree of cellular hypoxia. Ploszczyca et al. (2018) have also suggested that the gradual reduction in EPO over prolonged hypoxic exposure is likely associated with the reduction in HIF-1. However, the rate of decrease in EPO is not equivalent to the lesser reduction in circulating Rcts (Rusko et al., 2004). Therefore, the authors suggested that the reduction in serum EPO after the initial peak is due to the establishment of an equilibrium between EPO production and consumption for Rct creation.

Sex

Absolute and relative EPO responses had considerably greater inter-individual variability (**Table 2**; **Figures 3, 4**) in the female bed rest study than the two male bed rest studies, and no statistical mean differences in the relative responses. Distribution values (SD, range and IQR; **Table 2**) in the FemHab study are all larger in both NBR and HBR interventions compared to their male counterparts. The relative EPO responses of females to hypoxia in comparison to LunHab and PlanHab studies appear to have large variability, which diminishes when compared to previous studies with prolonged hypoxic stimuli (Ploszczyca et al., 2018). Females tend to have lower [Hb] than males, meaning their concentration of arterial O_2 at any given O_2 saturation is usually lower (Murphy et al., 2010). Chapman et al. (2010) speculated that this hypothetically means females have a larger EPO response to hypoxia than males. Although in the present study no differences were found in EPO between males and females at the group level, the variability in EPO response found in FemHab could be due to individuals' being at different phases of their menstrual cycle as no control was implemented on menstrual cycle phase during the bed rest interventions. Stachenfeld (2008) suggested that estrogens play an important role in stabilizing body fluid volume. During a normal menstrual cycle, estrogens potentially function to counter the fluid loss effects of progestins, maintaining PV. In response to long-duration stress, such as a prolonged bed rest (greater than 5–7 days) or weightlessness, estrogens are speculated to stabilize the vascular compartment (Fortney et al., 1988). A manner to potentially reduce EPO variability in females would be through control of estradiol and progesterone. Contraception has been shown to control levels of estrogen but not progesterone so is not ideal. The use of a gonadotropin releasing hormone agonist or antagonist for reproductive function suppression with a controlled administration or estradiol and progesterone would negate hormonal fluctuations (Stachenfeld, 2008), and in turn potentially reduce variability. Tracking the menstrual cycle and hormonal changes throughout the intervention would not change the level of variability however it would allow researchers to identify if larger EPO responses were concurrent with changes in either estradiol or progesterone.

Until the age of puberty, males and females have similar increases in Hbmass during their development. After puberty has begun, Hbmass increases exponentially in males, whilst

remaining at a rate similar to pre-puberty in females (Prommer et al., 2018). It is believed the source of this change of rate is due to the role of androgens in males' puberty (Hero et al., 2005). The introduction of testosterone causes a rightward shift in the EPO-Hb relationship curve as well as a new physiological "set point" (Bachman et al., 2014). Mancera-Soto et al. (2021), claims that during the most sensible phase of puberty, an increase in testosterone plasma of 1 ng/ml is correlated to an increase in hemoglobin mass of ~65 g. The effects of testosterone on individual variability in men compared to women; however, is currently unknown and would require further examination. Furthermore, Goodrich et al. (2020) demonstrated that variations in hemoglobin mass across groups of varying athleticism and sex were more closely related to lean body mass than whole body mass. Once normalized for lean mass, seen previously variations were greatly diminished. In addition, they also pointed to deficiency as a strong determinator of lower hemoglobin mass. Further, iron deficiency anemia was identified to be in far higher prevalence in women of childbearing age due to blood and iron loss during the menstrual cycle (Fernandez-Jimenez et al., 2020). Both of these mechanisms may explain to a certain extent the potential sex difference noted in the current manuscript.

Plasma Volume Changes

Prolonged bed rest and hypoxia independently both cause reductions in PV (**Table 2**; **Figure 5**). Bed rest duration or subject sex do not appear to contribute to the inter-individual variability of the PV response. Bed rest's horizontal positioning stimulates receptors in the upper body after the CFS, which, in turn, results in the release of ANP, causing diuresis and natriuresis. PV changes during hypoxic acclimation have considerable variability and appear to be mediated primarily by changes in oncotic pressure (Siebenmann et al., 2017b). The majority of the variability in the PV response to hypoxic acclimation is attributed to the variability in the concomitant changes in total circulating protein (TCP) during hypoxic acclimation. Young et al. (2019) speculated that this decrease in TCP could be attributed to both reduced plasma protein synthesis and leaking of plasma proteins into the extravascular space. The combination of both of these mechanisms results in approximately twice the reduction of PV in comparison to NBR alone at the intervention level (**Table 2**). The changes in PV seen in NBR have been speculated to modulate the production and release of EPO (Gunga et al., 1996). Keramidas et al. (Keramidas et al., 2016b) concluded that tissue O_2 saturation is a primary mediator for the degree of renal EPO synthesis and that PV is a secondary factor. Bed rest duration appears to have little to no impact upon the amount of individual variation in PV seen in the Δ(Pre-Post) and day-to-day values (**Table 2**).

Limitations

Using hemoglobin mass (Hb_{mass}) as opposed to [Hb] concentration and RBC would eliminate the effects of PV retraction on measuring the variability in changes to blood O_2 carrying capacity. Hemoglobin mass typically has a measurement error of around 2% in well trained research teams (Gough et al., 2011; Steiner and Wehrlin, 2011; Siebenmann et al., 2017a). The Dill and Costill (1974) equation, utilized in this study, is deemed appropriate for calculation of plasma and serum biomarkers; however, not for whole blood biomarkers (Matomäki et al., 2018). Future research should consider using a direct tracer-dilution method to study PV changes and draw firm conclusions as to the exact extent of PV changes.

The debate between hypo- and normobaric hypoxic continues and is sometimes overlooked when discussing hypoxic exposure in general. There are many discrepancies in the responses of physiological systems between normobaric hypoxia and hypobaric hypoxia which have been attributed to the differences in barometric pressure (Millet and Debevec, 2020) and may indeed be worth further study. Despite this, Hauser et al. (2016) found similar Hb_{mass} responses to live high train low interventions in normobaric and hypobaric hypoxia. Wide variability existed in individual responses to both intervention types after the same hypoxic dose and after 18 days post-intervention. The heterogeneity of hematological responses to inactivity and hypobaric hypoxia may differ either in source or in magnitude to that of inactivity and normobaric hypoxia and require further investigation.

Another stipulation worth mentioning is that of the total 562 astronauts that have ventured to space between 1961 and 2020, the average age of astronauts during their first mission is 39.8 years SD 5.28. It should also be noted that astronauts from the 2000s and 2010s are significantly older during their first missions than those before the turn of the century (Smith et al., 2020). The average ages of the participant groups used in this study were in their mid-twenties, and therefore may not be seen as a complete representation of aging astronauts, especially those completing their second and third missions. The effects of aging on serum EPO are known, with increased values with age potentially as a compensation mechanism for increased hemolysis or a rise in EPO resistance (Ershler et al., 2005). Whether these changes would affect the heterogeneity of hematological responses, however, is unknown.

CONCLUSION

A group of individuals' responses to a stimulus or intervention are typically reported as means and standard deviations. The current spike of interest in individual variation noted in recent years demonstrates that this analytical approach may mask the true range of individual responses potential led researchers to draw inappropriate inferences. Acknowledgment of this variability is essential to optimize personal future medical and physiological interventions. The recent pandemic of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has highlighted the substantial heterogeneity observed in the development of systemic hypoxia among infected patients. The degree of hypoxia developed during the illness not only dictates whether hospitalization is required but also whether the induction of coma with supplemental O_2 breathing is required for survival.

Clearly, there is great individual variability in the ability of the body to respond and adapt to the severe hypoxia, which in extreme situations, is essential for survival. The current investigation of the hematological responses of inactive males and females exposed to the same magnitude and duration of a hypoxic stimulus demonstrated the substantial heterogeneity in the cascade of responses from arterial O_2 saturation to RBC production. The individual variability in the EPO response to NBR and HBR in females appears to be considerably larger than in males, and the duration of bed rest appears to have no impact on the heterogeneity of the hematological responses. Our findings suggest that relative EPO responses are not sufficient indicators of the resultant increased production of Rcts and RBCs. The data would suggest that the majority of the variability seen in HBR is due to mechanisms responding to hypoxia rather than bed rest and inactivity. The significance of the current data is the identification and acknowledgment of large individual variability within the mechanistic response to hypoxia, thus creating justification to further investigate the sources and moderating factors of such variability.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Komisija za Medicinsko Etiko. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MK, OE, AM, and IM designed and coordinated the original research. AM, MK, OE, and IM collected the data. JR analyzed data. JR drafted the manuscript. All authors critically read, edited, and approved the final manuscript.

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Individual Variation Exists Within the Psychological Response to Hypoxic Bed Rest: A Retrospective Analysis

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Individual variation is of interest to Space Agency's, which cannot be explored with astronauts due to anonymity. We retrospectively analysed data collected throughout three projects (LunHab: 10-day male, PlanHab: 21-day male, and FemHab: 10-day female) to elucidate the potentially masked individual variation in the psychological responses to bed rest. The Profile of Mood State (POMS) and Positive and Negative Affect Schedule (PANAS) – instruments used to asses psychological state – and Lake Louise Mountain Sickness (LLMS) scores were collected prior to, following and throughout three interventions: 1: normoxic bed rest 2: hypoxic bed rest and 3: hypoxic ambulatory confinement. Total Mood Disturbance (TMD) was calculated from the POMS results, positive affect (PA), and negative affect (NA) from PANAS. The three instruments were included in a latent class mixed model. TMD, NA, and LLMS were included in a four-class model, with each class representing a specific type of response (Class 1: descending, Class 2: flat, Class 3: somewhat flat, Class 4: ascending). Responses for PA were assigned to only two classes (Classes 1 and 2). 54.55% or 24 participants were included in Class 2 (TMD, NA, and LLMS), where the responses did not change and neither hypoxia or activity level had a significant effect on emotional state. The remaining participants were allotted to Class 1, 3, or 4, where hypoxia was a significant covariate, while activity (bed rest) was significant only for class 3. For PA, 84.09% or 37 participants were assigned to class 2 indicating a significant effect of hypoxia on the participants responses with no effect of physical activity. Class 1 participants (n = 7) were not affected by hypoxia, however, physical activity improved their PA. Participants undergoing confinement, hypoxia and bed rest do not exhibit a uniform emotional response and may be categorised into 2–4 distinct classes. These results indicate significant individual emotional responses, that may be masked and underreported by traditional statistical approaches like means ± SD. The emotional state of our participants is a complex construct likely influenced by past experiences and different coping mechanisms which allowed some to adapt to the experimental environment more readily.

Keywords: individual variability, hypoxia, bed rest, psychology, emotion, psychological trajectory

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INTRODUCTION

Extra vehicular activity (EVA) on the International Space Station (ISS) has taken place on average once every month over the last 20 plus years of flight. While ISS is a phenomenal aerospace and research platform, it has become clear that future long-term space exploration will establish permanent habitats on the Moon and Mars rather than continue a singular reliance on low earth orbit research. One critical difference between the current (ISS) and future (planetary) platforms will be the rate of EVAs, which are expected to be daily or more often. Currently, the pressure inside space suits is about one third of the normobaric (1 atmosphere) pressure inside ISS. Decompression sickness mitigation protocols are utilised in the preparation for EVAs and are not compatible with daily use. If one were to move freely between, for example, a Martian habitat and the external environment (in an EVA or extrahabitat activity suit; EHA) with the current ambient, the risk of decompression sickness would be too great. In order to minimise the astronauts' risk of such, future habitats will likely be hypobaric and hypoxic (Norcross et al., 2005, 2013; Bodkin et al., 2006) allowing for safe and straightforward ingress and egress. While the effect of bed rest – as a reduced gravity analogue – in normoxia has been extensively studied, the addition of hypoxia in combination with bed rest has been a core research idea of our laboratory for more than a decade.

A reduction in oxygen supply to the central nervous system may result in symptoms of altitude sickness, such as headache, nausea, fatigue, and weakness (Matthys, 2011) along with a variety of neuropsychological impairments (Bahrke and Shukitt-Hale, 1993; Davidson, 2001) that includes alterations in cognition, mood, behaviour, and sleep indices (Winget and DeRoshia, 1986; Hornbein, 2001). Additionally, acute hypoxic exposure reduces exercise performance by 18% (Deb et al., 2018) and worsens sleep efficiency and memory performance (de Aquino Lemos et al., 2012). The degree of performance loss varies greatly depending on the measurement item. With regard to personality, undesirable changes may occur, namely increased paranoia and obsessive compulsiveness (Nelson, 1982). Given that emotional stability enhances physical adaptation to altitude in terms of fatigue and acute mountain sickness symptoms (Virues-Ortega et al., 2004), it would be important to clarify the effects of hypoxic exposure on one's psychological state. Further, it may provide useful insights into the smooth operation of communities in enclosed spaces. Additionally, it should be noted that decreased gravitational load and physical activity levels have been shown to affect cognitive and psychomotor functioning (Kanas et al., 2009; Lipnicki and Gunga, 2009; Basner et al., 2021). Some of those studies have reported that the participants' emotions changed negatively (Ishizaki et al., 2002; Liu et al., 2012), while others have reported no change (Zhao et al., 2011). With regard to spaceflight, the conclusion of one review (Strangman et al., 2014) points to small sample sizes and effect sizes that provide weak support for cognitive changes and as such, there is a need to better understand the influence of individual variability on cognitive performance.

More recently, the European Space Agency (ESA) has indicated an interest in the individual variation exhibited in physiological and psychological responses to bed rest, microgravity and space flight. There are several sources of error or variability within an experimental study, particularly in a descriptive or observational experiment compared to a mechanistic intervention. The primary point of variation to be considered is the precision and accuracy of the measurement tool, secondly, inherent random effects of the participants and finally, the observer effect, that which you examine or measure you alter. While the mean or median is useful in describing the expected outcome of the intervention, it does not accurately describe the response of all individuals within the experimental group or take into account these sources of error. Therefore, it becomes important to quantify the given individual response in order to further understand the expected outcome. Individual differences in emotional reactivity and emotional style have been identified as manifesting in the peak amplitude of the response, the rise time to the peak, and the recovery time (Davidson, 1998, 2001). Unfortunately, because hypoxia and inactivity studies require sophisticated research facilities and great effort on the part of both participants and experimenters, the sample size is typically relatively small and individual differences cannot be tested. As such, we sought to combine the results from three near identical research projects to establish the degree of variability in the psychological state of the individuals after the interventions. For the readers interest and specific comparison between the styles of data presentation, one is referred to previous publications from our group (Stavrou et al., 2015, 2018a,b) where some of the data used in the current retrospective analysis has been published as means and standard deviations.

This was the impetus for the current work, which set out to reanalyse the results of the profile of mood state (POMS), Positive and Negative Affect Schedule (PANAS), instruments of emotional state and Lake Louise Mountain Sickness (LLMS) which were collected during the Slovene Bed Rest Programme.

MATERIALS AND METHODS

The European Space Agency (ESA) established a programme of research to collect and reanalysis bed rest data conducted in the Slovene Bed Rest Programme. The current manuscript retrospectively analysed data collected during that programme. The purpose in doing so was to evaluate the extent of individual variability evident in the psychological response to bed rest, confinement and/or hypoxia. The projects included were: LunHab – 10-day male Lunar Habitat Simulation; PlanHab – 21-day male Planetary Habitat Simulation; and FemHab – 10-day female Planetary Habitat Simulation. All experimental

Abbreviation: AMS, Acute Mountain Sickness; AIC, Akaike information criterion; BIC, Bayesian information criterion; ESA, European Space Agency; EHA, Extrahabitat activity; EVA, Extra vehicular activity; FemHab, Female Planetary Habitat Simulation; F_IO_2, Fraction of inspired O_2; HAMB, Hypoxic Ambulatory; HBR, Hypoxic Bed rest; ISS, International Space Station; LLMS, Lake Louise Mountain Sickness; lcmm, Latent classes mixed modelling; LunHab, Lunar Habitat Simulation; NA, Negative Affect; NBR, Normobaric Bed rest; P_IO_2, Partial pressure of inspired oxygen; PlanHab, Planetary Habitat Simulation; PA, Positive Affect; PANAS, Positive and Negative Affect Schedule; POMS, Profile of Mood State; TMD, Total Mood Disturbance.

procedures were conducted according to the ESA bed rest standardisation recommendations (Standardisation of bed rest study conditions 1.5, August 2009) and conformed to the Declaration of Helsinki. The study protocols were approved by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia; approval numbers: 205/2/11 and 88/04/12.

Participants

Inclusion and exclusion criteria were applied according to the standard operating procedures set out by ESA (Heer et al., 2009). Fifteen males, fourteen males and fifteen females took part in the LunHab, PlanHab, and FemHab projects, respectively. Participants were recreationally active lowland Slovene residents (<500 m). The baseline characteristics of the participants are outlined in **Table 1**.

Study Outline

The detailed study protocols have been described elsewhere (McDonnell et al., 2019, 2020). Briefly, 9 study campaigns were conducted in the hypoxic facility at the Olympic Sports Centre Planica (Rateče, Slovenia) situated at an altitude of 940 m under the Slovene Bed Rest Programme. Each experimental campaign comprised baseline and recovery periods – before and after the intervention – so that the participants commitment to the project was 33 days in PlanHab and 18 days in both LunHab and FemHab. Each participant underwent three interventions in a cross-over randomised design manner: normobaric normoxic [fraction of inspired O_2, F_IO_2: 0.209; partial pressure of inspired oxygen (P_IO_2): 133 mmHg] horizontal bed rest (NBR); normobaric hypoxic (F_IO_2: 0.142; P_IO_2: 91 mmHg, target simulated altitude of 4,000 m) horizontal bed rest (HBR); and normobaric hypoxic (F_IO_2: 0.142; P_IO_2: 91 mmHg) ambulatory confinement (HAMB). The interventions were separated by at least 4 weeks for the washout period (4 weeks in LunHab; 2 months in FemHab; 4 months in PlanHab) to allow for the effects of the prior exposure to hypoxia and/or inactivity to be eliminated. With the exception of length of interventions and participant sex, the protocols of the three interventions were similar in all three projects, thus allowing for the comparison of the data. Complete bed rest schedules have been published for LunHab (McDonnell et al., 2019) and FemHab (McDonnell et al., 2020).

TABLE 1 | Baseline characteristics of the participants.

	LunHab	PlanHab	FemHab
N	15	14	15
Age, year	24.1 ± 2.2	26.4 ± 5.0	26.1 ± 3.6
Stature, cm	179.2 ± 7.6	179.5 ± 5.0	168.4 ± 6.0*
Body mass, kg	71.9 ± 10.9	76.9 ± 10.4	59.6 ± 8.2*
BMI, kg/m^2	22.4 ± 2.8	23.8 ± 2.7	21.0 ± 2.3
VO_2 max, mL/kg/min	43.3 ± 5.5	44.3 ± 6.1	41.0 ± 3.8

BMI, Body mass index; $\dot{V}O_2$ max, maximal volume of oxygen uptake.
**Significance between projects.*

During the bed rest interventions (NBR and HBR), no deviations from the lying position, muscle stretching or static contractions were permitted. Participants in the HAMB condition were allowed to move freely within the hypoxic area and engaged in two 30-min bouts of daily aerobic physical activity. The purpose of this physical activity was to mimic the level and amount of daily activity that the participants would normally perform outside of the present project and not to induce a training stimulus. Adherence to the assigned protocol was ensured using continuous closed-circuit television surveillance and constant supervision by the research and medical staff.

Measurements

Profile of Mood States

Participants completed the Profile of Mood States – Short Form (POMS; Shacham, 1983) at regular time points throughout the interventions. POMS consists of a list of 37 adjectives with a 5-point Likert scale. Participants reported their subjective mood states on the questionnaire ranging from 0 "not at all" to 4 "extremely." Total Mood Disturbance (TMD) was calculated based on the results given to the questionnaire by the participants.

Positive and Negative Affect Schedule

The Positive and Negative Affect Schedule is a self-report mood scale that can measure positive affect (PA) and negative affect (NA) separately (Watson et al., 1988). The Instrument consists of two 10-item mood words, and participants rated the extent to which they experienced each of the emotions described in PANAS. Participants answered each item based on a 5-point scale with anchors of "very slightly or not at all" (1) to "very much" (5), with intermediate points of 2 representing "a little," 3 "moderately," and 4 "quite a bit." The total score for each affect factor (PA and NA) ranging from 10 to 50 was calculated by summating the 10-items' score.

Lake Louise Mountain Sickness

The Lake Louise Mountain Sickness self-report questionnaire (Roach et al., 1993) was completed daily to assess for the presence of acute mountain sickness (AMS). The respondents' rate five symptoms (headache, gastrointestinal upset, fatigue/weakness, dizziness/light-headedness, and sleep disturbance) on a scale of 0–3 for severity, with a total score of 3 or higher and the presence of headache considered a diagnosis of AMS. The rating for each symptom is accompanied by a brief description of the severity of the symptom.

The POMS, PANAS, and LLMS data collection schedules are provided in **Table 2** for each of the three projects.

Statistical Analysis

Background

Latent classes mixed modelling (lcmm) for multivariate longitudinal markers (Proust et al., 2006; Proust-Lima et al., 2013) was used to identify trajectories of the participants psychological state during the interventions. Instead of treating all individuals as those that share the same psychological profiles across time, lcmm may identify unmeasured latent classes that represent subgroups of participants with similar

TABLE 2 | Measurement day overview.

	POMS			PANAS			LLMS		
	FemHab	**LunHab**	**PlanHab**	**FemHab**	**LunHab**	**PlanHab**	**FemHab**	**LunHab**	**PlanHab**
PRE	x	x	x	x	x	x	x		x
D1	x		x	x		x	x	x	x
D2							x	x	x
D3							x	x	x
D4							x	x	x
D5	x	x		x	x		x	x	x
D6							x	x	x
D7			x			x	x	x	x
D8							x	x	x
D9							x	x	x
D10	x	x		x	x		x	x	x
D11									x
D12									x
D13									x
D14			x			x			x
D15									x
D16									x
D17									x
D18									x
D19									x
D20									x
D21			x			x			x
POST	x	x	x	x	x	x	x		x

POMS, Profile of Mood States; PANAS, Positive and Negative Affect Schedule; LLMS, Lake Louise mountain sickness.

psychological trajectories. A series of psychological data sets were assessed using the multlcmm function of the lcmm package version 1.7.9 (Proust-Lima et al., 2017) in R, version 3.5.0 (R Core Team, 2020). The model estimation is based on a robust maximum likelihood and can handle longitudinal data series with intermittent data missing at random (Proust and Jacqmin-Gadda, 2005; Proust-Lima et al., 2017). No participant had missing values for any of the covariates (please see below). The linear, quadratic, and cubic models were tested for each day that data was collected in all interventions (HBR, NBR, HAMB) in order to calculate the coefficients of TMD, Negative affect and LLMS. The lcmm that included PA as a dependent variable did not converge, as a result, PA was analysed with a univariate model and thus presented separately. As aforementioned, the data collection days were not identical across the three bed rest projects; to allow the quadratic and cubic terms in the model to adequately process the values and align the time variable labels, the day of the intervention when data was collected was transformed from PRE, D1, D2. . ., POST to whole numbers and then divided by the campaign duration and finally multiplied by 100 to give a percentage value.

Covariates

Sex, F_IO_2 (normoxia or hypoxia), intervention duration (10 or 21 days) and activity level (bed rest or ambulatory) were entered as covariates within the model. Sex and intervention duration were not significant, and as such the model had a poor "goodness of fit" and therefore these variables were excluded as covariates. The significance of each of these factors was assessed with the multivariate Wald test (Wald, 1943). Random effects for intervention day, F_IO_2 and activity level all reduced the models' goodness of fit, thus the intercept was included in the model as a random effect on an individual level.

Procedures

Following the recommended approaches for lcmm (Andruff et al., 2009), a one-class model was performed first, and the number of estimated latent classes was increased sequentially until additional classes no longer improved the models fit. When the best model was identified, each class was named based on its visual pattern of trajectory. A posterior probability was computed for each participant to evaluate their membership to each of the latent classes. The lcmm assigned participants exclusively to that class for which the highest probability was obtained.

A successful classification of the participants was accepted above 0.7 and preferentially the closer the posterior probability was to 1. The best-fit model was selected according to the following criteria: (1) If the Bayesian information criterion (BIC; Schwarz, 1978), Akaike information criterion (AIC; Akaike, 1998) and negative log-likelihood were low; (2) If the mean of the posterior probabilities of the individuals classified in each latent class was above 0.7; and (3) There were no less than 10% of the total number of participants allotted to a single trajectory class.

TABLE 3 | Latent class mixed model (lcmm) results of the model fitting process for TMD, NA, and LLMS.

No. of latent classes	Polynomial degree	Log-Lik	AIC	BIC	Entropy	% Participants per class	Mean posterior probabilities
1	Linear	−6518	13065	13092	–	100	na
1	Quadratic	−6518	13067	13096	–	100	na
1	Cubic	−6511	13057	13087	–	100	na
2	Linear	−6482	13003	13039	0.801	79.5/20.5	0.93/0.97
2	Quadratic	−6475	12994	13034	0.817	63.6/36.4	0.95/0.95
2	Cubic	−6468	12983	13026	0.814	61.4/38.6	0.96/0.92
3	Linear	−6453	12956	13001	0.757	59.1/15.9/25	0.89/0.94/0.87
3	Quadratic	−6449	12954	13004	0.867	20.5/70.5/9.1	0.98/0.93/0.99
3	Cubic	−6436	12933	12989	0.871	27.3/63.6/9.1	0.9/0.95/0.99
4	Linear	−6435	12929	12983	0.831	9.1/18.2/54.5/18.2	0.99/0.91/0.88/0.96
4	Quadratic	−6412	12892	12953	0.878	29.5/9.1/54.5/6.8	0.86/0.98/0.95/0.99
4	**Cubic**	**−6402**	**12881**	**12949**	**0.871**	**13.6/54.5/20.5/11.4**	**1/0.92/0.92/0.94**
5	Linear	−6402	12874	12936	0.887	6.8/15.9/59.1/6.8/11.4	1/0.96/0.92/0.99/0.88
5	Quadratic	−6397	12875	12946	0.880	4.5/25/54.5/9.1/6.8	1/0.88/0.91/0.98/1
5	Cubic	–	–	–	–	Non-convergence	Non-convergence

Data presented are: the number of latent classes considered, the polynomial form of the model, the maximum Log-Likelihood (Log-Lik), Akaike information criterion (AIC), the Bayesian Information Criterion (BIC), entropy, the posterior classification of participants into each class (%), the mean of posterior probabilities in each latent class. The model chosen to categorise the data and make inferences from in the current manuscript is highlighted in red.

TABLE 4 | Latent class mixed models (lcmm) results of model fitting process for PA.

No. of latent classes	Polynomial degree	Log-Lik	AIC	BIC	Entropy	% Participants per class	Mean posterior probabilities
1	Linear	−1795	3602	3613	–	100	na
1	Quadratic	−1782	3577	3590	–	100	na
1	Cubic	−1782	3579	3593	–	100	na
2	Linear	−1782	3586	3606	0.729	84.1/15.9	0.92/0.92
2	**Quadratic**	**−1766**	**3558**	**3581**	**0.761**	**15.9/84.1**	**0.96/0.93**
2	Cubic	−1762	3555	3582	0.859	86.4/13.6	0.97/0.90
3	Linear	−1771	3574	3603	0.734	36.4/15.9/47.7	0.87/0.84/0.89
3	Quadratic	−1747	3532	3566	0.907	84.1/13.6/2.3	0.97/0.89/1
3	Cubic	−1746	3536	3575	0.898	4.5/13.6/81.8	0.96/0.90/0.97

Data presented are: the number of latent classes considered, the polynomial form of the model, the maximum Log-Likelihood (Log-Lik), Akaike information criterion (AIC), the Bayesian Information Criterion (BIC), the entropy, the posterior classification of subjects in each class (%), the mean of posterior probabilities in each latent class. The model chosen to categorise the data and make inferences from in the current manuscript is highlighted in red characters.

All models were run for a maximum of 15 iterations from 30 vectors of initial values to avoid convergence to local maxima.

The benefit of the lcmm is that it links multiple measurements (TMD, PA, NA, and LLMS) within one model, allowing the participants to be classified into latent classes (subpopulations) based on multiple indicators.

RESULTS

The results of the lcmm classifications of the participants responses are displayed above in **Tables 3**, **4**.

Table 3 indicates the process of searching for the best model with lcmm based on TMD, NA, and LLMS (i.e., moving from identifying only 1 class to 2, to 3, etc.). Following the model selection criteria outlined in the section "Statistical Analysis," a cubic model with four classes was accepted because it had a low BIC and no less than 10% of the total number of participants allotted to any single trajectory class. The detailed parameter estimates for each class in the optimal model are given in **Table 5**.

Figure 1 depicts the predicted average trajectories of TMD, PN, and LLMS in each class, as well as the actual measured values of participants classified into those classes.

Class 1 "descending" (13.64%; $n = 6$) was characterised by an increase in the first half of the intervention period, followed by a decrease then by a slight increase during the recovery period. Hypoxia was significant as a covariate ($p < 0.001$), with HBR and HAMB scoring higher than NBR. Activity level did not have a significant effect on this class ($p = 0.090$).

Class 2 "flat" (54.55%; $n = 24$) maintained a flat trajectory with low values and little variation in the scores from pre-intervention values. The majority of participants are included in this class. The

TABLE 5 | The fixed effects in the longitudinal model for TMD, NA, and LLMS.

	Class 1				Class 2				Class 3				Class 4			
	Coefficient	**SE**	**Wald**	***p*-value**	**Coefficient**	**SE**	**Wald**	***p*-value**	**Coefficient**	**SE**	**Wald**	***p*-value**	**Coefficient**	**SE**	**Wald**	***p*-value**
Intercept	0 *(not estimated)				−0.408	0.580	−0.703	0.482	−0.401	0.661	−0.606	0.545	−0.831	0.780	−1.065	0.287
Day	0.821	0.250	3.288	**0.001**	0.075	0.107	0.698	0.485	0.182	0.186	0.978	0.328	0.589	0.248	2.375	**0.018**
Day^2	−2.611	0.655	−3.988	**0.000**	−0.478	0.264	−1.811	0.070	−0.169	0.450	−0.375	0.708	−0.511	0.570	−0.896	0.370
Day^3	0.176	0.044	4.049	**0.000**	0.039	0.018	2.208	**0.027**	0.000	0.030	0.014	0.989	0.011	0.038	0.300	0.764
F_IO_2	1.707	0.344	4.970	**0.000**	0.160	0.105	1.521	0.128	1.253	0.253	4.964	**0.000**	1.621	0.317	5.113	**0.000**
Activity	0.393	0.232	1.696	0.090	0.116	0.100	1.153	0.249	−1.397	0.274	−5.093	**0.000**	0.075	0.243	0.311	0.756

**Not estimated, the mean intercept in the first class is constrained to 0. Statistical significance is indicated in bold.*

effects of hypoxia and activity level were both insignificant (F_IO_2: $p = 0.128$; Activity: $p = 0.249$), as such there were no differences in scores between intervention conditions.

Class 3 is "somewhat flat" like class 2 (20.45%, $n = 9$) and was classified by no significant time variables (Day: $p = 0.328$; Day^2: $p = 0.708$; Day^3: $p = 0.989$). The effects of hypoxia and activity level were significant (F_IO_2: $p < 0.001$; Activity: $p < 0.001$), and according to the coefficients of each variable, hypoxia had the effect of increasing the score while activity level had the effect of decreasing the score. HBR indicated higher scores than the NBR and HAMB conditions.

Class 4 "ascending" (11.36%; $n = 5$) displayed a trajectory in which scores increased substantially early in the intervention and remained high. Hypoxia was significant as a covariate ($p < 0.001$), with HBR and HAMB scoring higher than NBR. The activity level was not significant as a variable ($p = 0.756$).

The search process of the optimal latent class model for PA is presented in **Table 4**. During the model selection process, the results of AIC and BIC were in conflict, thereafter BIC was given priority (Nylund et al., 2007; van de Schoot et al., 2017). According to the model selection criteria, a quadratic model with two classes was accepted. **Table 6** lists the parameter estimates for each class according to the best model. The fact that the participants PA responses could be categorised into two trajectories rather than four (TMD, NA, and LLMS) suggests that there is less individual variation present in positive emotions trajectories. However, there is still considerable variation in the actual response (please see **Figure 2**).

Figure 2 depicts the predicted mean trajectory of the PA and the measured values for the individual participants allotted to each class.

Class 1 "flat" (15.91%; $n = 7$) exhibited a flat slightly descending trajectory with no significant effect of time or hypoxia (Day: $p = 0.240$; Day^2: $p = 0.410$; F_IO_2: $p = 0.513$). However, there was a significant effect of activity level ($p < 0.001$) and higher PA scores were reported in HAMB than in either HBR or NBR.

Class 2 "U-shape" (84.09%; $n = 37$) categorised more than 80% of the total number of participants. The trajectory of PA reported, decreased in the first half of the intervention period and tended to increase in the second half and into the recovery period. While hypoxia significantly decreased PA ($p = 0.002$), activity level did not significantly influence the PA of these participants ($p = 0.163$).

DISCUSSION

The principle finding of the current study is that significant individual variation exists in the emotional or psychological response to hypoxia and bed rest. When the data is analysed as means, this variation or noise may distract from drawing concrete inferences on the data. Additionally, these findings provide an insight to the inconsistent emotional changes reported in previous bed rest studies. The present study analysed the combined data from three bed rest projects and classified the participants based on the trajectories of their psychological mood state into four distinct classes for TMD, NA, and LLMS, and into two classes for PA. The largest number of participants were classified as having a flat trajectory in both negative and positive models. It is noteworthy that more than half of the participants were able to maintain a flat mental state, as negative emotions increase under stressful conditions. A significant effect of hypoxia on negative affect was found in about half of the participants (Class 1, 3, and 4), importantly, for the remaining half (class 2 "flat"), hypoxia was not significant. There are large individual differences in physical adaptation to the hypoxic environment (Chapman et al., 1998; Townsend et al., 2002), and the current study indicates that psychological responses also vary among individuals.

General Effects

Emotional tension resulting from an unfamiliar, adverse or demanding situation may result in stress. The results of such a situation may manifest in a heightened state of arousal (positive outcome) or more negatively, with an increase of anxiety, fear, or hostility (negative outcome). Typical stressors are external, usually environmental (temperature, hypoxia, bed rest, space flight) or internal and psychological (relationships or social encounters). Stress is a normal reaction ubiquitously experienced by humans; however, the resultant emotional profile may differ. During endeavours, such as space flight and mountaineering, particularly with increased elevation, there is undoubtedly a heightened level of stress related to the potential success of the mission and the ever-present threat of an accident. Further, the potential effect of increased levels of hypoxia on the individual and group dynamics is currently unknown. There is an importance to examining the interplay between mood dimensions in hypoxic environments and demonstrated significant interaction between psychomotor

ability, mental efficiency and the profile of mood state (POMS) mood dimensions, including confusion, fatigue and tension (Bolmont et al., 2000) along with how each individual is behaving or adapting to the current stressor. While we previously reported (Stavrou et al., 2015) that activity (HAMB) would counteract the negative aspect of hypoxia, the current results indicate that this was only the case in nine participants or 20% of those enrolled in the programme. The effect of activity on decreasing negative emotions was found only in class 3, with no significant effect in the other 80% of participants. The positive affect model showed similar results, with only 15% of participants indicating an increase in positive affect from the activity (please see class 1,

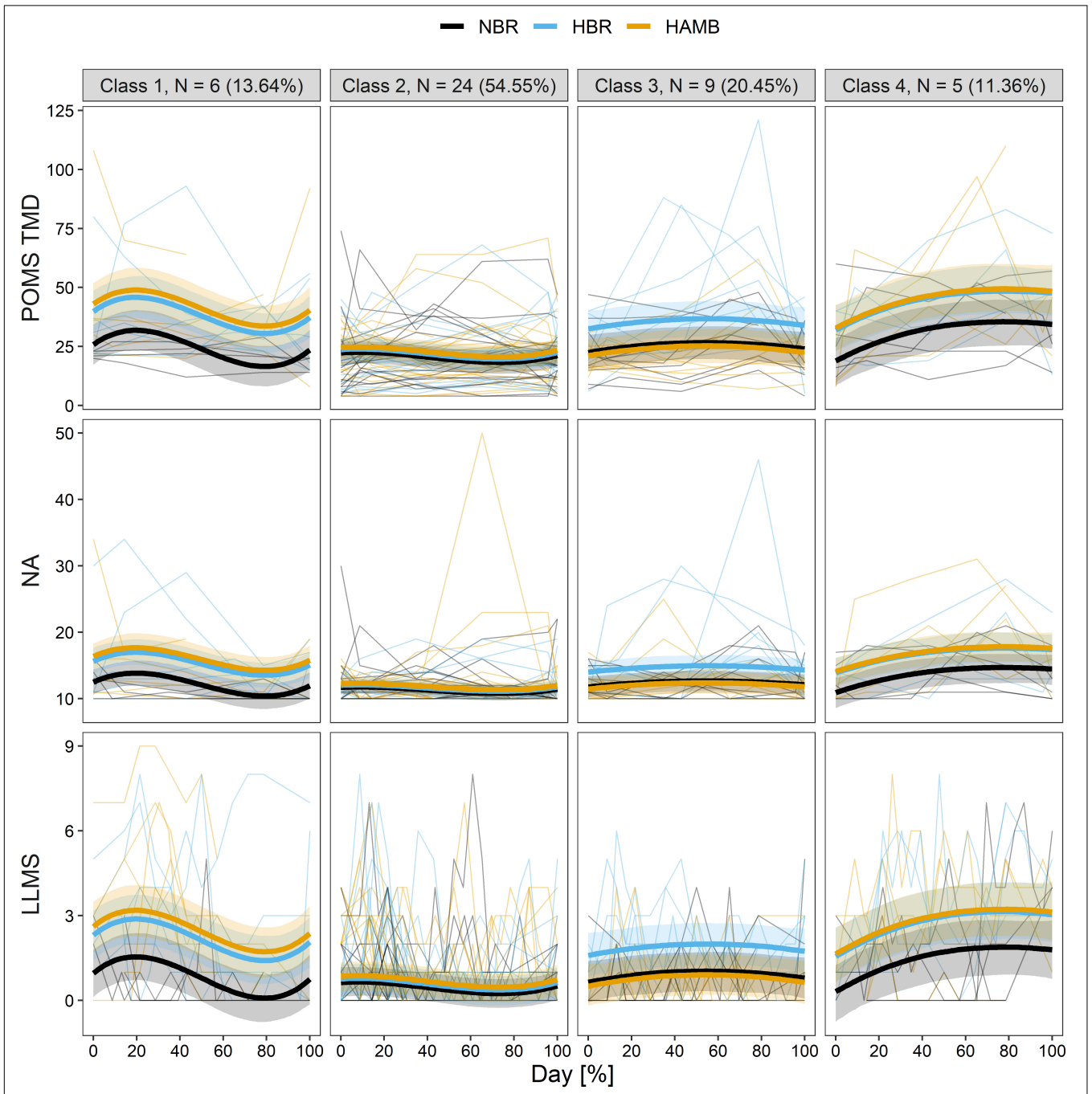

FIGURE 1 | The predicted trajectories of the four distinct classes in the longitudinal total mood disturbance of profile of mood states (POMS TMD; top row), negative affect (NA; middle row), and Lake Louis mountain sickness (LLMS; bottom row) during experiment of each of the interventions (HAMB, hypoxic ambulatory confinement; HBR, hypoxic bed rest; and NBR, normobaric normoxic bed rest). Bold lines show the class-specific mean predicted levels as a function of the percentage of duration, and the ribbons represent the corresponding 95% CI. Thin lines depict individual scores.

TABLE 6 | The fixed effects in the longitudinal model for PA.

	Class 1				Class 2			
	Coefficient	**SE**	**Wald**	***p*-value**	**Coefficient**	**SE**	**Wald**	***p*-value**
Intercept	27.420	3.089	8.876	**0.000**	30.006	1.509	19.882	**0.000**
Day	−0.675	0.575	−1.175	0.240	−1.640	0.295	−5.557	**0.000**
Day^2	0.444	0.539	0.824	0.410	1.573	0.283	5.556	**0.000**
F_IO_2	−0.968	1.479	−0.655	0.513	−2.391	0.789	−3.032	**0.002**
Activity	9.515	1.629	5.842	**0.000**	1.029	0.738	1.395	0.163

Statistical significance is indicated in bold.

Figure 2). Thus, the types of physical activity on offer to the participants may not have been desirable, or the required commitment to a physical activity session was not tolerated well by the majority of the participants. Appropriate exercise prescription beyond a general mimicry of physical activity may provide more emotional relief in hypoxia. However, given the current results, this should be specific and individually tailored.

In most bed rest studies, participants' emotions changed negatively (Ishizaki et al., 2002; Liu et al., 2012, 2015; Stavrou et al., 2015, 2018a), although there are reports that participants' emotions did not change in a 15-day restraint experiment (Zhao et al., 2011). Our results indicate that the 10- and 21-day durations were not significant as covariates predicting differences in psychological state, and that individual variability was a substantial factor in the inconsistency between previous studies. Thus, duration was not a factor in the response which was driven by the intervention and intrinsic factors. Differences in emotional changes between participants may have been due to different emotional regulation strategies. Emotional regulation is the ability to respond throughout the duration of any experience to its ongoing demands (Cole et al., 1994). To worry by focussing on only the potentially negative events in the future, serves to aid in the upregulation of negative emotion (Campbell-Sills and Barlow, 2007). Before taking part in the current experiment, participants of the "flat" class were unlikely to have been anxious about what life would be like during the period of confinement, they coped well with the intervention and were not negatively affected by hypoxia or inactivity. The "ascending" class experienced more stress than they had imagined or could cope with and so their negative emotions increased throughout the intervention.

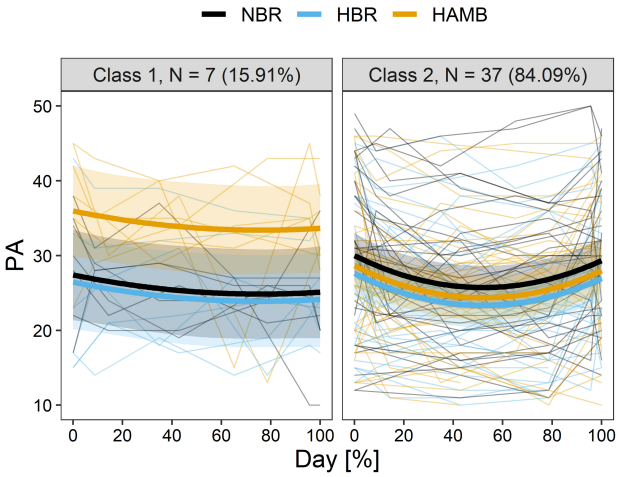

FIGURE 2 | The predicted trajectory of two distinct classes in longitudinal positive affect (PA) during experiment of each of the interventions (HAMB, hypoxic ambulatory confinement; HBR, hypoxic bed rest; and NBR, normobaric normoxic bed rest). Bold lines show class-specific mean predicted levels as a function of percentage of duration, and the ribbons represent the corresponding 95% CI. Thin lines depict individual scores.

It has been reported that confinement experiments often result in a lack of emotional upheaval in the participants. At the Concordia Station in Antarctica, the over wintering participants may enter a state of psychological hibernation as a stress coping mechanism (Sandal et al., 2018). Participants are released from undesirable thoughts and situations by diverting their attention from certain thoughts and mental images to other contents (Campbell-Sills and Barlow, 2007). Although, thought suppression and psychological hibernation can only occur after a long period of confinement, up to several months, this does not apply to the present experiment, which involved up to a few weeks of confinement.

It is well established that as we age, we become more skilful at regulating our emotions, or not allowing innocuous events to overload us emotionally, we may conserve energy for the tasks that really do challenge us. Labouvie-Vief et al. (2010) conclude that middle aged adults experience more positive affect and less negative affect than younger adults. When older adults are exposed to unpleasant stimuli, they are able to regulate their emotional responses in such a way as to avoid negative confrontations (Charles and Carstensen, 2008). Since participants in both 10 days and 21 days confinement were young adults in their twenties, there appears to be no age-related benefits in emotion regulation strategies. However, astronauts are typically 45 years of age and older when they fly, therefore it's likely that these age-related emotional regulation patterns come into play for that demographic, combined with extensive specialty training. It is possible that astronauts would therefore present with a different set of trajectories, albeit still displaying individual variation in their responses.

The Effect of Sex

Existing studies have reported that females have higher anxiety (Feingold, 1994; Kessler et al., 1994; McLean et al., 2011), depression (Hathaway and McKinley, 1989;

Cyranowski et al., 2000; Whissell, 2003; Ford and Erlinger, 2004; Albert, 2015), and fatigue than their male counterparts (Verbrugge, 1985; van Wijk and Kolk, 1997; Miaskowski, 2004). On the other hand, outside of a bed rest study, it is typical for males to score higher than a parallel group of females on both state anger and trait anger (Spielberger, 1999; Whissell, 2003). However, with regard to the present results, the sex variable was not significant as a covariate of lcmm, and there were no sex differences in psychological state. There are potentially several reasons to believe that changes in sleep as a result of bed rest and hypoxia can invalidate sex differences in emotion. Indeed, Rojc et al. (2014) found that in the LunHab study, exposure to both hypoxia and bed rest resulted in greater sleep fragmentation due to more awakenings throughout the night. A separate study at the German Antarctic Station, Neumayer, indicates that confinement experiments may lead to poor sleep quality in women (Steinach et al., 2016). Sleep deprivation is associated with an increased emotional response to negative and stressful stimuli (Walker, 2009). As a result of the deterioration in sleep quality in both women and men, negative emotions may have increased and sex differences in the emotional state may have disappeared.

Source of Variation

There may be several sources of variation seen within the results. This could be the precision and accuracy of the measurement tool, the POMS and PANAS instruments themselves or the Planica environment and how all the staff and participants interact. Due to the nature of the protocol, the participants in the present study were subject to confinement and limited to social interaction within the participant pool. Both of these factors may contribute to the enhancement of negative mood. Results from normoxic bed rest studies, in which participants are inactive and their lower limbs unloaded to induce the musculoskeletal atrophy observed in astronauts during space missions, have shown that cortisol levels remain stable during 14 days, 17 days, or 20 days of bed rest (Ishizaki et al., 1994; Ferrando et al., 1996; Millet et al., 2001). However, others have found that cortisol increased concomitantly with depression after 20 days of bed rest (Ishizaki et al., 2000, 2002). It is possible that with modifications to the social interaction of participants, both mood and cortisol remain unchanged after 20 days of bed rest (Ishizaki et al., 2004). Thus, socialisation is an important factor along with group dynamics; however, whilst it can have beneficial effects, it must also be considered that the social interaction and information provided by others within the social group can result in an increase in the stress response (Ishizaki et al., 2004; Gallagher et al., 2014). It is pertinent to consider that some participants enjoyed and some disliked this setup, along with the potential for differing experiences in different rooms or shared spaces.

Finally, one must consider that the process of physiological adaptation to an environmental stressor also varies among participants. How the variance of those physiological adaptations or mal adaptations combine to affect the psychological response is currently unknown.

CONCLUSION

The use of the latent classes mixed modelling analysis provides clear evidence that presenting the psychological response to bed rest and hypoxia as means and standard deviations is not appropriate. The lcmm approach categorised the participants into four distinct classes based on the trajectory of their responses throughout the interventions. Individual participants exhibit a range of emotional responses to bed rest and hypoxia, which may be influenced by sex, activity level and intervention duration, along with inherent factors as yet unidentified. Lcmm provides an opportunity for clear interpretation of the data by not presenting results as means and disregarding the responses of 50% of the participant group.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Committee for Medical Ethics at the Ministry of Health of the Republic of Slovenia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ACM and IM contributed to the conception and design of the study as well as collected the data. KT performed the statistical analysis and prepared a first draft of the manuscript. KT, IM, and ACM wrote individual sections culminating in the final draft. All authors contributed to the manuscripts critical revision, have read and approved the submitted version.

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frontiers in Physiology

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An Empirical and Subjective Model of Upper Extremity Fatigue Under Hypogravity

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In the context of extra-terrestrial missions, the effects of hypogravity ($0 < G < 1$) on the human body can reduce the well-being of the crew, cause musculoskeletal problems and affect their ability to perform tasks, especially during long-term missions. To date, studies of the effects of hypogravity on human movement are limited to experiments on the lower limbs. Here, we extend the knowledge base to the upper limbs, by conducting experiments to evaluate the effect of hypogravity on upper limb physical fatigue and mental workload in participants. Our hypothesis was that hypogravity would both increase participant productivity, by reducing overall physical fatigue expressed in Endurance Time, and reduce mental workload. Task Intensity-Endurance time curves are developed especially in seated positions, while performing static, dynamic, repetitive tasks. This experiment involved 32 healthy participants without chronic problems of the musculoskeletal system aged 33.59 ± 8.16 years. Using the collected data, fatigue models were constructed for tasks of varying Intensity. In addition, all participants completed the NASA – Task Load Index subjective mental workload assessment, which revealed the level of subjective workload when executing different tasks. We found two trends in the empirical fatigue models associated with the difference between the strength capabilities of males and females. The first is a significant positive ($p = 0.002$) relation between Endurance time and gravity level (⅙ G Moon, ⅓ G Mars, 1G) with negative coefficient for males and females for a static task. And there is marginal relation ($p < 0.1$) between overall mental workload and gravity level with a positive coefficient for males and females for the same task. The same trend was observed for dynamic and repetitive tasks. We concluded that the Task Intensity-Endurance Time model, adapted to hypogravity in combination with subjective mental assessment, is useful to human fatigue investigation. The combination of these methods used for ergonomic analysis and digital human modeling, could improve worker productivity. Finally, this study may help prepare astronauts for long-term missions on the Moon and Mars and improve our understanding of how we can prevent musculoskeletal disorders caused by hazardous manual handling under such extreme environments.

Keywords: reduced gravity, partial gravity, workplace, fatigue models, numerical simulation, sitting posture, biomechanics, underwater

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1. INTRODUCTION

As we return to the Moon and aim for Mars, the effect of hypogravity (HG) on the human body will play a crucial role (Riley et al., 1987; D'Aunno et al., 2003; Horneck et al., 2003; Orwoll et al., 2013; Widjaja et al., 2015; Clément, 2017; Reynolds, 2019; De Martino et al., 2020; Swanenburg et al., 2020) whether running, walking, or sitting. To the best of the authors' knowledge, research on the effects of hypogravity on human movement has only focused on the lower limbs (Hewes and Spady, 1964; Rajulu and Klute, 1992; Newman and Alexander, 1993; Sylos-Labini et al., 2014; Richter et al., 2017; Kang et al., 2019; Weber et al., 2019)with little information about the upper extremities under HG and in particular, during manual handling operations.

In spite of significant automation in industrial environments, muscle strength remains an integral part of many working operations (De Looze et al., 2016). To a large extent, such work involves manual handling of equipment and maintenance work. Manual handling of heavy objects can cause high loads on the musculoskeletal system, potentially leading to accidents (Edlich et al., 2005; Clarke, 2020), lost time and additional costs. There were registered (15.8%) injuries during heavy lifting and 16.9% of accidents associated with lumbar and back injuries (Spengler et al., 1986; Clarke, 2020; Wizner et al., 2021) on Earth. At the same time, such parameters as the type of task, size, shape and weight of the manual tool play an important role in the impact on the spine and upper limbs. According to Hernandez et al. (2019) astronaut injuries associated with spacesuit wearing have been reported. For example, potential risk for shoulder injuries in space can be overhead tasks.

In the context of extra-terrestrial missions under HG, musculoskeletal, as well as cardiovascular, vestibular systems will be affected because of gravity changes (Morey-Holton, 2003). According to Axpe et al. (2020) body mineral density (BMD) is one of the most important components used by ISS crewmembers to monitor bone health. Based on the collected data on board the International Space Station (ISS), Axpe et al. (2020) predicted with non-linear exponential model during 6 months of flight to Mars, and during long stays on Mars and on the Moon, astronauts may lose approximately 32.4–36.8% of the bone mineral density in the femoral neck. If such measurements are completed after 6 months of experimentation, similar results are expected. According to Morey-Holton (2003), bones and muscles are lost only in the lower limbs and upper limbs including the back. This suggests that changes in the musculoskeletal system are local.

Upper limbs have not been extensively researched. The first experiments related to the study of upper limb fatigue in weightlessness were conducted on parabolic flight (Bock, 1996) and on MIR (Gallasch et al., 1996). Upper limb experimentation is gaining in importance as part of missions to the ISS (Pastacaldi et al., 2004). This is due to a change in motor control programs in microgravity conditions, affectings not only the biomechanics of the astronauts, but in general their entire psychophysical state. Finally, another study (Nagatomo et al., 2014) based on parabolic flight study, simulating different levels of gravity (0G - 1.5G) compared the blood flow of the upper and lower extremities of seated participants, finding that blood flow in the upper extremities had a normal levels for microgravity, at the same time blood flow in the lower extremities decreased for 0 G. However, long-duration effects of HG on upper limbs were not analyzed.

During long missions, manual handling and repair work will be performed. The associated physiological changes and possible risks and injuries in the workplace should thus be studied and considered. It is necessary to assess physical fatigue and perform manual control. To the best of our knowledge, no such studies have yet been conducted on the work of the upper limb in conditions of HG.

Handling capacities can be assessed by directly measuring muscle fatigue. Muscle fatigue is designated as a decrease in the capacity to maintain the required level of strength after continuous muscle engagement (Boyas and Guével, 2011). This is a phenomenon that depends on many factors, including the characteristics of the task being performed (Mehta and Parasuraman, 2014). If not enough to recover, muscle fatigue reduces the tissue's ability to withstand stress. This can lead to musculoskeletal disorders (Kumar, 2001). Productivity can also be affected by muscle fatigue, which can then reduce workers' potential to develop different ways of working to achieve production goals without without prejudice to their health (Durand et al., 2009).

To assess physical fatigue, many empirical models can be built. Such models are described by Intensity - Endurance time (ET) curves with exponential or power function (Rohmert, 1960; Monod and Scherrer, 1965; Huijgens, 1981; Rose et al., 2000; Garg et al., 2002; Imbeau et al., 2006; Frey Law and Avin, 2010; Ma et al., 2011) used to calculate maximum holding time for any particular task. These models differ by joint regions (shoulder, elbow, wrist), where maximum strength is exponential due to the amount of energy the body is able to transfer to the muscles. According to Seo et al. (2016), such models can't be used for dynamic tasks studies with out of order pauses. For this, dynamic fatigue models were developed. One of such models was described by Liu et al. (2002) as s set of dynamic equations considering effect of muscle fatigue and recovery. Then Xia and Law (2008) defined a muscle fatigue mathematical model for complex tasks. Model of Ma et al. (2009) can be applied for static and dynamic tasks, with respect to specific body parts. And it estimates muscle ability based on the history of muscle activity of different body parts without fatigue recovery consideration.

To measure physiological parameters for upper extremity physical fatigue investigation, various tools, including the dynamometer (Alizadehkhaiyat et al., 2007; Romero-Franco et al., 2019), electromyogram (Chany et al., 2007; Lalitharatne et al., 2012), electroencephalographic measure (Wang et al., 2021) and electrocardiogram (Redgrave et al., 2018) can be used. The following monitoring approaches can also be applied: posture sway (Davidson et al., 2004), joint kinematics (Riley and Bilodeau, 2002), perceived discomfort/fatigue (Balci and Aghazadeh, 2004).

More subjective assessment methods can also be implemented, particularly for mental workload study and workplace ergonomic risk factors. Examples of such methods,

applicable to specific case studies concerning pilots or astronauts, are: Cooper-Harper Scale (Cooper and Harper, 1969), the Bedford Scale (Roscoe and Ellis, 1990), the Subjective Assessment Technique (SWAT) (Reid and Nygren, 1988) and the NASA Task Load Index (NASA-TLX) (Hart and Staveland, 1988), the Workload Profile (WP) (Tsang and Velazquez, 1996). According to one study (Rubio et al., 2004) NASA-TLX has the highest sensitivity, as well as strongest operator acceptance (Hill et al., 1992) compared to SWAT, WP. Also, as a result of the same study comparing these three tools, NASA-TLX's validity assessment gave a positive correlation coefficient between them. Furthermore, NASA-TLX shows a higher correlation with human performance than SWAT and WP. Rubio et al. (2004) suggested that the SWAT and NASA-TLX is credible for composite natural world tasks. Rubio et al. (2004) concluded that if the goal is to predict human performance when performing a specific task, NASA-TLX is suggested because of its high correlation with this parameter.

The combination of Task Intensity - ET estimation and NASA-TLX formed the basis of the approach presented in this study. We assumed that HG would increase participant productivity, by reducing physical fatigue, and also reduce the mental workload of participants compared to Earth's gravity. Such a model can be used to assess physiological limitations of a given workplace environment in HG as well as 1G. For HG in particular, maximum admissible weight and forces for various percentages of a population can be designed based on this data, reducing the number of cumulative trauma and disorders related to specific motions. Although there is critically little data collected about the time of real experiments, considering the simulation of motion under HG, digital human modeling (DHM) is now widely used for testing various manual scenarios without real participants.

2. MATERIALS AND METHODS

2.1. Participants

A total of 32 volunteers participated in the study (18 males, 14 females). All participants were right-hand dominant and reported that they did not currently have health issues. Two sets of experiments were conducted in a 1G and then underwater. Underwater conditions were used because Archimedes force counteracts the action of gravity.

During the experiments in the water tank (Swissub, Vaud, Switzerland) all participants were seated with their heads above the water level. The capacity of the parallelepiped water tank, adapted for experiments, is 8.3 tons. The two sides of the water tank are made of glass and the participant can be observed from the outside. The temperature in the water was constant at 29 degrees. Ballasts were selected for the different body parts to provide the necessary level of buoyancy, equivalent to gravity on the Moon ($G = 1.626$ m/s ^{2}) and Mars ($G = 3.72076$ m/s ^{2}).

2.2. Statistical Methods

Data distribution was analyzed using Excel and statistical analysis package Stata 17 (StataCorp, California, US). We used empirical and a subjective model for physical fatigue of upper extremity fatigue and mental workload assessment with three levels of gravity, six task intensities, and four types of tasks [outstretched arm (S1), arm bent at the elbow (S2), dynamic (D) and repetitive (R)], with gender as an independent variable, and Endurance time (min), mental workload, hand and back-chest-leg muscle contraction as dependent variables. Statistical significance was defined at $\alpha = 0.05$. The power model were was used to determine the Endurance Time—Task Intensity curves for all types of tasks. In accordance with these models R-squared values and power model coefficients were investigated. Then an assessment of the normality of distribution, *p*-values calculation, one tailed paired samples *t*-test were conducted. To investigate the level of significance of the dependence between ET (min) and WWL,% and muscle contraction from the gravity level (1G, ⅓ G and ⅙ G), as well as the character of this dependence we conducted statistical analysis by means of a multivariate regression with correlation coefficients, standard errors and *p*-values assessment.

Continuous variables are presented as means and standard deviations. Effect sizes were calculated using G*Power software version (3.1.9.7). Thresholds were defined as 0.2 (small effect), 0.5 (moderate effect), and 0.8 (large effect) (Cohen, 1992) between four experimental groups. With 100 participants split in four different groups, a *post-hoc* power analysis suggests a power of 0.88 for average sized group effects. The power analysis is presented in **Supplementary Section 1.1**. Descriptive statistics of anthropometric data of the participants are presented below in **Table 1**.

Participants' body part masses and lengths were measured in accordance with statistical data (Plagenhoef et al., 1983), see **Supplementary Figure S2** and **Supplementary Table S1**. The center of mass (COM) of the body segments were estimated according to the statistical data of the same author, for example, for males for the forearm, shoulder and the whole body, COM is equal 43, 43.6, and 63%, respectively and for females 43.4, 45.8, and 56.9 %, respectively.

2.3. Experimental Set Up

Direct and subjective methods were combined because they provided a larger range of parameters to understand a phenomenon. Direct methods involved the use of a timer, dynamometers (hand digital dynamometer (Camry scale store, North America) to measure hand muscle contraction, back-leg-chest (BLC) (Baseline, New York, USA) to measure leg-back muscle contraction and based on Bioelectrical impedance analysis (BIA) scale (Nokia Health, Body +, China) to determine body composition (percentage of body fat, water percentage, muscle and bone mass) of participants. In the subjective method, NASA-TLX was implemented.

First, a numerical model for the ballast calculation was developed, based on a simplified model of the human body. To assess the whole torso volume the photogrammetrie method was used due to the non-invasiveness for the participants in the experiment and the speed of measurements. However, in some cases, the reconstruction of the 3D volume of the hands required a lot of refinement and improvement of the mesh, and for this it was decided to measure this volume by the method of water displacement. To estimate the whole torso volume,

TABLE 1 | Descriptive statistics for the main characteristics of the participant.

Study variable	Total ($N = 32$)		Male ($N = 18$)	Female ($N = 14$)	p-value
	Mean (SD)	Min/Max	Mean (SD)	Mean (SD)	
Age (year)	33.59 (8.16)	25/55	34 (9.62)	33.07 (6.11)	0.742
Height (m)	1.75 (0.11)	1.54/1.95	1.83 (0.07)	1.66 (0.06)	<0.001
Body mass (kg)	71.22 (17.01)	43.8/114.10	82.92 (13.02)	56.19 (5.95)	<0.001
BMI (kg/m ^{2})	22.91 (3.78)	16.09/37.43	24.83 (3.77)	20.43 (1.93)	<0.001
Muscle mass (kg)	53.64 (12.51)	35.10/75.80	63.3 (7.38)	41.22 (2.60)	<0.001
Body fat (%)	19.67 (6.29)	8.50/36.40	17.77 (6.46)	22.11 (5.32)	0.046
Body fat (kg)	14.18 (6.75)	5.36/41.53	15.38 (8.23)	12.65 (3.93)	0.227
Body water (%)	54.74 (6.13)	30.30/66.00	56.89 (4.52)	51.98 (6.94)	0.032
Body water (kg)	38.99 (9.97)	17.85/55.28	46.74(4.94)	29.02 (3.86)	<0.001
Bone mass (kg)	2.87 (0.65)	2.00/4.00	3.37 (0.38)	2.23 (0.12)	<0.001
Upper arm (m)	0.34 (0.04)	0.25/0.40	0.35 (0.33)	0.32 (0.03)	0.007
Forearm (m)	0.28 (0.03)	0.20/0.33	0.30 (0.02)	0.25 (0.02)	<0.001
V torso (dm ^{3})	37.00 (11.00)	28.00/61.00	44.71 (6.93)	27.00 (4.42)	<0.001
V upper arm (dm ^{3})	2.00 (0.80)	0.8/3.8	2.70 (0.67)	1.48 (0.38)	<0.001
V forearm (dm ^{3})	1.00 (0.30)	0.4/2.00	1.37 (0.20)	0.72 (0.16)	<0.001

BMI, body mass index.

the photogrammetrie method of the Agisoft Metashape 1.7.2 software (Agisoft LLC, St.Petersburg, Russia) was applied (Jebur et al., 2018). The bodies of all participants were photographed using a high-resolution camera (12 MP), focal length (4.25 mm), f/1.8, and 1,000 photographs were taken for each participant. All models were calculated with high or medium dense cloud quality and DSM 114 = 10 cm/Pixel resolution (see **Figure 1B**) First, a mesh of participants body created by photogrammetry was imported to Blender, called a modified mesh. Then a target mesh was added for each body segment, and a boolean modifier[1] with difference option was applied to calculate the volume of a specific body segment (Freixas et al., 2006). The volumes of the participants were scaled and adjusted according to the height measured using a stadiometer (NutriActivia, Minnesota, USA). In some cases proportional editing[2] (Guevarra, 2020) was required to modify irregular mesh. The Boolean method with a combination of proportional editing function allowed us to achieve a result that provides a difference in the estimate of the 3D model equal to 5 cm ^{3} for the whole body volume, compared to the water displacement method.

The volumes of the hand, forearm and shoulder were assessed as follow. First, hand up to wrist was immersed and a mark was made with the water level 1, then the hand was immersed up to the elbow and a new mark was made with the water level 2, and then the hand was immersed in the cylinder with water along the shoulder and a new mark wit ha level 3 was made. Then the difference between the water levels was calculated. The volume of the shoulder is equal to the difference in water displacement between the level 3 and 2 marked when the upper arm and elbow are immersed. The volume of the forearm was calculated based on the difference in water level 2 and 1 noted when the elbow and wrist were immersed. We neglected the volume of the wrist, because this volume is about less than 1% of the total body volume. This can be included in the overall error estimation.

Figure 1A shows experimental setup with a participant-adjusted chair. Electronics and sensors were not used in this study. All participants were strapped at hips and legs. The participant's legs were attached to the footrest in accordance with their anthropometry, taking into account the length of the legs from knee to foot in particular.

For experiments in the water tank, ballasts are added on different parts of the body of the participants. A weight vest (Thorn+Fit Schweiz, Bale, Switzerland) was used for the torso, and adjustable weights (Strength shop.ch, Switzerland) for the forearm and upper arm. The participants' upper limb and trunk ballast weights were calculated as follows:

$$m_{b.p} \cdot g_m = (m_{\mathrm{b.p}} + m_{\mathrm{b}}) \cdot g_{\mathrm{e}} - (V_{\mathrm{b.p}} + \frac{m_{\mathrm{b}}}{\rho_b}) \cdot g_e \cdot \rho_{H2O} \quad (1)$$

where $m_{\mathrm{b.p}}$ is the mass of the body part,
$V_{\mathrm{b.p}}$ - the volume of the body part,
m_b - the mass of the ballast,
g_m - the acceleration of gravity on the surface of the Moon,
g_e - the acceleration of gravity on the surface of the Earth,
ρ_b - the density of ballast weights,
ρ_{H2O} - the density of water.

Sand ($\rho_b = 2816.9$ kg/ m^3) and lead ($\rho_b = 11340$ kg/ m^3) and polystyrene ($\rho_b = 30$ kg/ m^3) were used for ballast. For example

[1]**Boolean modifier** allows to perform logical operations with complex 3D meshes. These operations include intersect, difference and union options. Difference option allows to subtract the target mesh from the modified mesh so that anything outside the target mesh is retained.

[2]**Proportional editing** is a way of modifying selected model elements (such as mesh elements) with a scaling effect. This method is convenient for smooth deformation of the mesh surface.

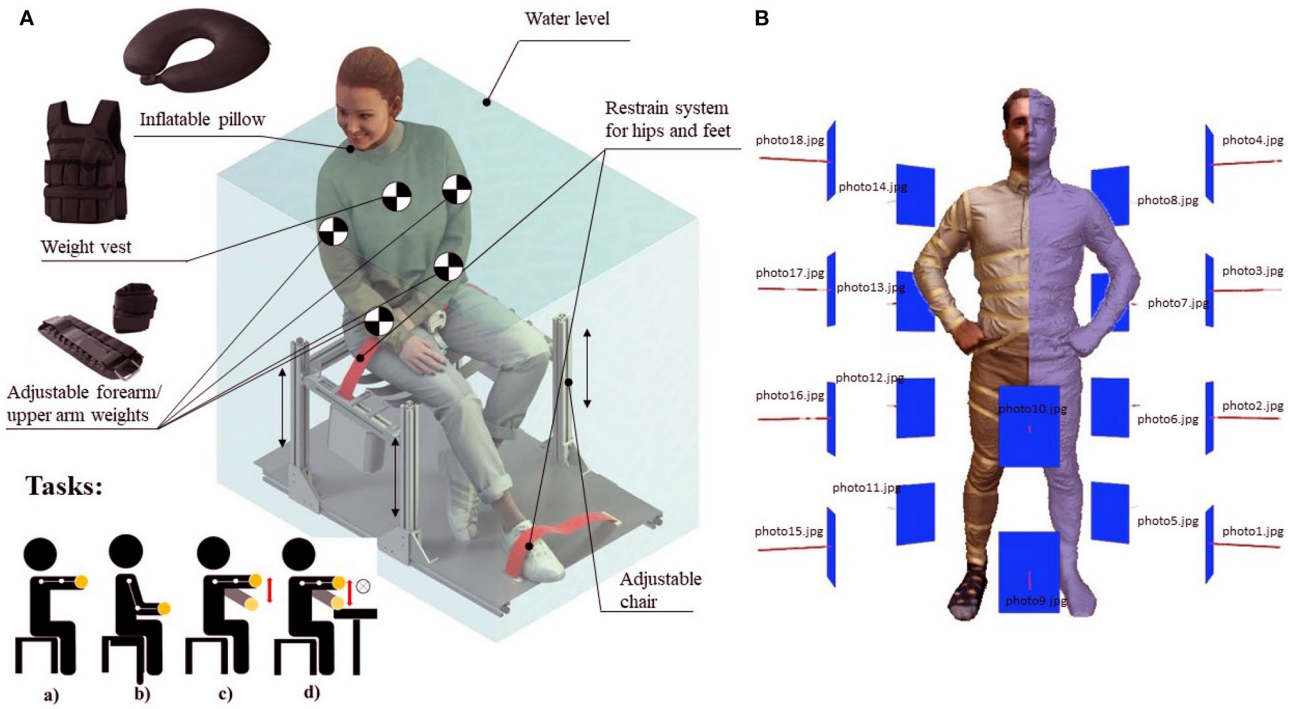

FIGURE 1 | (A) Technical aspects of experimental setup. Tasks: a) Holding weight with an outstretched arm, Task (S1); b) Holding weights in an arm bent at the elbow, Task (S2); c) Slow 1 (lifting)-2 (lowering)-1-2 hand motion with weight (3 sec range of motion), Task (D); d) Repetitive motion with load 1(lifting)-2 (horizontal transfer)-3 (lowering)-4 (pause without load)-1-2-3-4 with constant repetition, Task (R). Image from: open source model https:/humano3D.com. **(B)** Participants 3D scan example with a visual explanation of the principle of photogrammetry (Agisoft Metashape).

for participant with trunk volume of 28.41 dm^3, and mass of the trunk of 29.26 kg, ballast mass (lead) is 5.7 kg.

2.4. Tasks Description and Experimental Measurements

Four different one handed tasks were performed by the participants: holding weights with an outstretched arm (S1), holding weights in an arm bent at the elbow (S2), slow dynamic motion (D), and repetitive motion (R). Due to the fact that not 32 all participants were available for the same tasks, then 22 participants were invited for the task (S1), 27 participants conducted task (S2), 25 participants conducted task (D) and 26 participants conducted task (R) under 1G and ⅙ G. And only 6 participants completed similar tasks under ⅓ G condition. Six different task intensities were investigated 0.5, 1, 3, 5, 7, and 9 kg taking into account the corresponding level of gravity. The different intensities were different for each participant, especially the lowest and highest intensities. Lower intensities were tested less often because it was more time consuming and the participants were not always motivated to do monotonous work, and time was essential to conduct experiments with other intensities. Higher intensities were also tested less often since not all participants had the appropriate physical capabilities. The choice of maximum load was dependent on the physical capabilities of the participant and the gender (HSE, 2020). All task types, gravity and task intensity levels were randomized. All participants were asked to answer a questionnaire adapted for everyone to assess their readiness for physical activity (Warburton et al., 2019). It can be found in the **Supplementary Figure S1**. This questionnaire is an internationally renowned tool for participants screening. This questionnaire contained 7 questions related to general health and two options choices: “Yes” or “No.” Only those participants who answered "No" to all questions were cleared for physical activity and our experiments.

All participants in the experiment received detailed oral instructions to perform the tasks. More complex tasks, such as those involving repetition, were demonstrated in a video and each participant could consult video instructions throughout the exercise. All participants participated in warm-up exercises to be ready for physical activity. They performed a 3 kg dumbbell lift in their right hand for 5 min, 15 times with a break of 1 min. The tasks were first conducted in 1 G and then repeated in the water tank.

The right hand strength of each participant was measured with a hand digital dynamometer three times before and three times after the task. Then, the mean values were evaluated. The same approach was applied with a calibrated back-leg-chest (BLC) dynamometer. Due to the difficulty of the simulation of the tasks underwater, back measurements were not taken. These dynamometers provides kilograms (kg) and pounds (lb)

estimates based on a certain amount of applied force. All measurements were taken in a seated position and took into account the position of the participant's limb (outstretched arm or arm bent at the elbow). For measurements with a BLC dynamometer, the length of the chain was adjusted to the participant's sitting height by asking the participant to sit on a chair and put their legs on the base of the BLC dynamometer, bent at 90 degrees. All tasks were performed until volitional failure. Between all tasks, participants were able to take micro-pauses (very short intermittent breaks) equal at least 1/5 of working time (Australia, 2011). In practice, rest breaks are largely the result of a worker's personal feedback of sufficient free time to allow workers to complete the activity with relative comfort (Brown, 1994). Therefore, if the participant needed more time to recover, then additional time was provided according to their individual needs. This time was not recorded by authors.

In an underwater environment, the speed of motions can play a crucial role and significant loads can occur from the water during fast movements. A rough estimate of this can be obtained by presenting the forearm of the participant, moving rectilinearly and evenly in the water, as an equivalent cylinder, and calculating the force of hydrodynamic resistance (F_{res}). In this case, the diameter of the equivalent cylinder is:

$$d = 2 \cdot \sqrt{\frac{V_{b.p}}{\pi \cdot l_{b.p}}} \tag{2}$$

where $l_{b.p}$ is the length of the body part (or equivalent cylinder), Then the resistance force:

$$F_{res} = C_x \cdot S \cdot \frac{\rho_{H2O} \cdot V^2}{2} \tag{3}$$

where $S = l_{b.p} \cdot d$ - flow obstruction area,

$C_x = 0.5$ - drag coefficient (Savitsky, 1972),

V - the speed of the object in a fluid.

The limiting speed of the forearm was calculated from the condition that water resistance force did not exceed 10% of the weight of the forearm: $F_{fa} = m_{\mathrm{b.p}} \cdot g_e$. In this case speed of the forearm should be less than 47 cm $\cdot$ s^{-}1. The range and speed of the participant's dynamic motions were designed in accordance with the found value and were approximately 10 cm $\cdot$ s^{-}1 for a 3-s motion cycle. This means that we had a margin equal to 3.

2.5. NASA-TLX

The NASA-TLX scale was originally developed for the aviation industry. Then this scale was applied to power plants, remote control systems, and space applications. Different human factors were assessed with this approach: team collaboration (6%), fatigue (2%), tensity (3%), experience (4%), and disability (1%) (Hart, 2006).

In our study, the NASA-TLX (Hart and Staveland, 1988) scale was applied to assess the mental workload of the participants. This assessment was carried out immediately after the execution of each task under 1G and simulated ⅙ G, as well as ⅓ G. The benefit of this approach is that it reveals a specific demand of each participant.

NASA-TLX is based on independent subjective demands related to: Mental (MD), Physical (PD), and Temporal Demands (TD), Performance (P), Effort (EF) and Frustration (FR). Before completing the task, the participants were asked to read the detailed description of these subjective demands, see **Supplementary Figure S3**. NASA - TLX consisted of two parts. The first part is based on individual weighing of subjective demands through 15 pairwise weighing. Participants were asked to select the most appropriate subjective demand for the workload from each pair. The 15 pairs for pairwise comparison included: MD or PD, MD or EF, PD or FR, PD or P, PD or TD, FR or MD, FR or EF, P or MD, P or TD, EF or PD, EF or P, TD or FR, TD or MD, TD or EF, P or FR. The total number of selected specific subjective demands is called task load index or $Weight_{demand}$. The calculation of which will be discussed below. The second part is based on 100 point range $Rating_{demand}$. For this evaluation the following questions for all subjective demands were asked (Hart, 2006):

- MD - How mentally demanding was the task?
- PD - How physically demanding was the task?
- TD - How hurried or rushed was the pace of the task?
- P - How successful were you in accomplishing what you were asked to do?
- EF - How hard did you have to work to accomplish your level of performance?
- FR - How insecure discouraged, irritated, stressed, and annoyed were you?

Then each subjective demand of participants was weighted, resulting in a composite mental workload, see Equation 4. The calculation of overall mental workload is as follows:

$$WWL = \frac{\sum(Weight_{demand} \cdot Rating_{demand})}{15} \tag{4}$$

where $Weight_{demand}$ - task load index based on pairwise comparison of subjective demand (total 15 pairs) $Rating_{demand}$ - a total score of 0 corresponds to a very low subjective demand, a total score of 100 corresponds to a very low subjective demand.

3. RESULTS

3.1. Fatigue Curves for 1G, ⅙ G

The following dataset were combined in an Excel: sample size, sex (male, female), mean age, ET (min), task intensity in kg and in newtons with respect to 1G, ⅙ G and ⅓ G, muscle voluntary contraction (hand and leg-spine) measured before and after each task, mental workload data. The main descriptive statistics of the participants with p-value calculation are presented in **Table 1**. The males were significantly taller, heavier than the females ($p < 0.001$).

First, we built curves for different gravity conditions (1G and ⅙ G). Each participant's ET (min) is normalized for the participants' ratio of muscle mass (kg) and body mass (kg).

Task Intensity is expressed in Newtons for 6 different loads (0.5, 1, 3, 5, 7, and 9 kg) taking into account the corresponding level of gravity. We defined two trends in the data

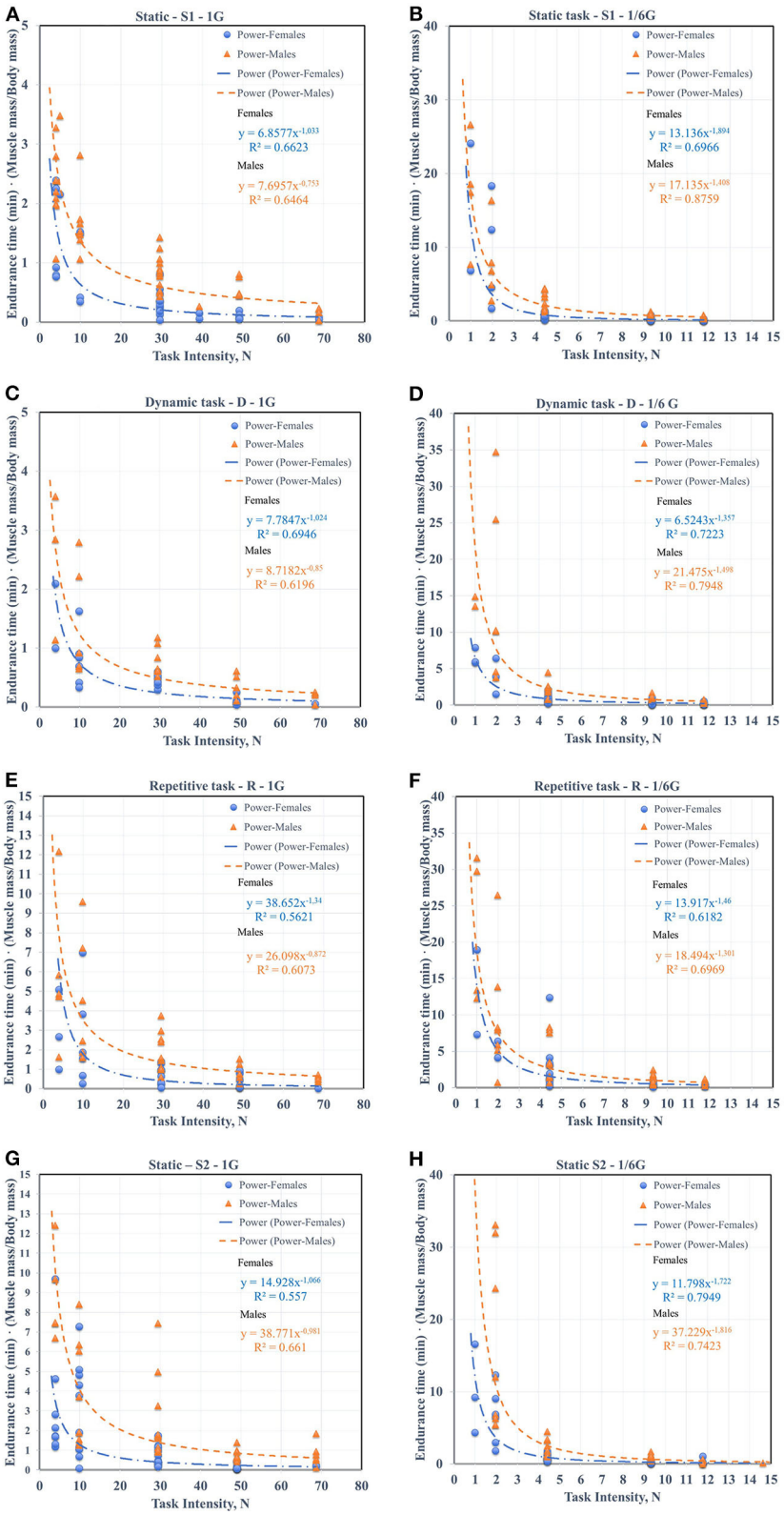

FIGURE 2 | The ET power models (N = 520 studies, 6 task intensities, **(A)** static task (S1)-1G, **(B)** static task (S1) ⅙ G, **(C)** dynamic task (SD)-1G, **(D)** dynamic task (D)-⅙ G, **(E)** repetitive task (R)-1G, **(F)** repetitive task (R)-⅙ G, **(G)** static task (S2)-1G, and **(H)** static task (S2)-⅙ G.

set corresponding to females and males. The power model has superior fit over exponential fit all data for each task and specific environment due to slightly greater R^2, as seen in **Figures 2A–H**. Thus, we used power models for 1G and ⅙ G comparisons. Then the constants b_o, b_1, and R^2 values of power trendline equation for all models are provided in **Table 2**.

According to the results, Coefficients b_o vary greatly, while coefficients b_1 have quite similar values for 1G and ⅙ G for males and females. The average values of the coefficient $b_1 = 0.86$ for males and $b_1 = 1.11$ for females for 1G, while the average values of $b_1 = 1.50$ for males and $b_1 = 1.61$ for females for ⅙ G.

All models for all tasks have the average value $R^2 = 0.63$ for males and average value $R^2 = 0.62$ for females for 1G and average $R^2 = 0.77$ for males, average value $R^2 = 0.70$ for females conducting the tasks under ⅙ G. The lower values of R^2 for 1G are most likely due to the fact that a smaller number of participants could work with loads of 5 and 7 kg. For example 80% of females couldn't work with 5–7 kg load under 1G.

Consistent with all curves, ET (min) increased for simulated lunar gravity in comparison with 1G. We found the average ET (min) for 1, 3, and 5 kg for all types of tasks to identify the growth rate. For a static task (S1) with a load of 1 kg, the ET of male increased 4.62 times and 6.53 times for female for ⅙ G compared to 1G. For the same task with a load of 3 kg, the ET of male increased 3.11 times and 1.91 for female for ⅙ G compared to 1G. And for load of 5 kg, the ET of male increased 1.64 times and 2.14 for female for ⅙ G compared to 1G. As can be seen from the given example, with increasing load, the ratio between ET (min) under ⅙ G and 1G decreases.

3.1.1. Hand and BLC Muscle Contraction

In **Table 3** you can see the average values for all task intensities from 0.4 to 9 kg for hand (H) and BLC strength measured for each participant before and after each task in an appropriate environment, 1G, ⅓ G and ⅙ G. The percentage change in values after each task is calculated for hand and BLC muscle contraction to compare the results.

Estimated averages of muscle contraction force indicate a greater reduction in physical strength under ⅙ G than 1G. This is consistent with the fact that the participants were able to work longer and their ET (see **Table 3**) is higher in a simulated ⅙ G environment due to lower loads and the weight of the participants themselves. Overall, all participants showed a greater decrease in hand strength after doing all tasks under 1G and

TABLE 2 | Task Intensity-Endurance time power model.

Power model	b_o	b_1	R^2	mn ET (min) load (1 kg)	mn ET (min) load (3 kg)	mn ET (min) load (5 kg)
			22 participants			
S1-1G	7.70/6.86	–0.75/–1.03	0.65 / 0.66	1.67/0.95	0.85/0.34	0.39/0.14
S1-⅙ G	17.13/13.14	–1.40/–1.89	0.87/0.70	7.73/6.21	2.65/0.65	0.64/0.30
			25 participants			
D-1G	8.72/7.79	–0.85/–1.02	0.62/0.69	1.30/0.80	0.79/0.35	0.28/0.08
D-⅙ G	21.47/6.52	–1.50/–1.36	0.79/0.72	14.93/9.34	2.16/0.82	0.77/0.34
			26 participants			
R-1G	26.10/38.65	–0.87/–1.34	0.61/0.56	6.39/2.54	1.93/0.72	0.75/0.21
R-⅙ G	18.49/13.92	–1.30/–1.46	0.70/0.62	9.80/5.31	4.43/2.84	1.06/0.49
			27 participants			
S2-1G	38.77/14.93	–0.98/–1.07	0.66/0.56	2.43/0.65	0.80/0.69	0.80/0.16
S2-⅙ G	37.22/11.80	–1.81/–1.72	0.74/0.79	15.72/6.63	2.38/0.94	0.82/0.29
			6 participants	(M/F)		
S1-1G	1.56	–1.19	0.77	1.43	0.56	0.22
S1-⅓ G	2.71	–1.32	0.72	2.62	0.77	0.32
S1-⅙ G	7.89	–1.55	0.84	10.34	1.29	0.60
D-1G	1.57	–1.03	0.56	1.70	0.62	0.32
D-⅓ G	3.38	–1.34	0.80	3.70	0.92	0.37
D-⅙ G	5.49	–1.27	0.77	6.57	1.60	0.68
R-1G	5.25	–1.85	0.57	3.15	0.89	0.33
R-⅓ G	6.64	–1.37	0.60	6.24	1.39	1.08
R-⅙ G	10.20	–1.38	0.71	9.69	2.06	1.00
S2-1G	2.51	–0.94	0.52	4.26	1.20	0.51
S2-⅓ G	6.12	–1.46	0.82	6.36	1.28	0.55
S2-⅙ G	7.66	–1.47	0.85	9.55	1.37	0.69

Power ($ET = b_0 \cdot (TaskIntensity)^{b_1}$) model with coefficients b_0 and b_1. The results are presented first for males and then for females in the first four groups and mixed males and females for 6 participants. Task types: holding weights with an outstretched arm (S1), holding weights in an arm bent at the elbow (S2), slow dynamic motion (D), and repetitive motion (R). Task intensity is presented for 1, 3, and 5 kg (⅙ G).

TABLE 3 | Before and after variation hand (H) and back-leg-chest (BLC) strength values, including % of variation.

G-level Task	H (Before) Mean (SD)	H (After) Mean (SD)	Δ (%) H	BLC (Before) Mean (SD)	BLC (After) Mean (SD)	Δ(%) BLC
			22 participants			
1G-S1	48.84 /24.95	48.95/24.66		7.81 /2.83	7.89 /2.43	
	(2.92)/(1.82)	(2.05)/(2.09)	0.21/–1.21	(0.83)/(0.16)	(0.82)/(0.22)	0.99/–16.12
⅙ G-S1	47.30/26.28	45.86/24.82				
	(1.12)/(3.53)	(1.46)/(2.78)	–4.95/–4.91	NA	NA	NA
			25 participants			
1G-D	46.02/22.15	45.49/20.84		8.03/2.55	8.01/2.18	
	(7.57)/(1.09)	(5.73)/(1.50)	–1.17/–6.29	(1.34)/(0.25)	(1.36)/(0.41)	–0,19/–16.41
⅙ G-D	46.37/24.46	44.96/24.91				
	(2.61)/(5.10)	(1.13)/(5.10)	1.83/–3.14	NA	NA	NA
			26 participants			
1G-R	48.47/23.79	47.41/22.65		8.57/2.88	8.13/2.65	
	(4.18)/(3.41)	(3.65)/(2,33)	–2.23/–5.04	(0.86)/(0.51)	(0.88)/(0.59)	–5.47/–8.87
⅙ G-R	44.00/22.96	43.75/22.15				
	(5.44)/(3.02)	(5.22)/(2.85)	–0.58/–3.64	NA	NA	NA
			27 participants			
1G-S2	44.56/20.52	42.49/19.48		13.46/5.15	12.73/4.75	
	(3.52)/(1.21)	(2.39)/(0.71)	–4.87/–5.37	(1.22)/(1.49)	(1.61)/(1.36)	–5.73/–8.49
⅙ G-S2	43.27/22.03	41.70/20.67				
	(2.84)/(2.05)	(1.48)/(2.24)	–3.77/–6.61	NA	NA	NA
			6 participants			
1G-S1	53.05/26.05	49.12/25.94	–8.00/–0.41	NA	NA	NA
⅓ G-S1	51.02/23.09	49.02/21.88	–4.09/–5.55	NA	NA	NA
1G-D	47.85/26.57	47.81/26.55	–0.08/–0.08	NA	NA	NA
⅓ G-D	51.04/25.07	49.55/24.51	–3.02/–2.29	NA	NA	NA
1G-R	51.22/27.42	46.93/24.50	–9.13/–11.91	NA	NA	NA
⅓ G-R	48.76/25.57	46.65/24.89	–4.53/–2.76	NA	NA	NA
1G-S2	46.76/24.76	42.63/22.29	–9.68/–11.10	NA	NA	NA
⅓ G-S2	48.75/23.42	47.15/22.89	–3.39/–2.33	NA	NA	NA

The results are presented first for males and then for females for all cases. Task types: holding weights with an outstretched arm (S1), holding weights in an arm bent at the elbow (S2), slow dynamic motion (D), and repetitive motion (R). Analyzed task intensities: 0.5, 1, 3, 5, 7, and 9 kg, respectively. All values are presented as mean and standard deviation (SD). NA-the values that could not be measured for logistic reasons of the experiment.

⅙ G. The same pattern is seen for the BLC measurements. There is very rarely an increase in muscle contraction after the tasks. And this may be due to non-compliance by participants with the instructions for using anemometers or the individual characteristics of the participants.

3.2. NASA-Task Load Index for 1G, ⅙ G

To investigate the mental workload of participants in 1G and ⅙ G, overall mental workload (WWL) in %, as well as average values of MD, PD, TD, P, EF, FR are calculated with NASA-TLX, see **Table 4**. In this table we gave one example of a study of the effect of 3 kg load on participants' mental workload. The results of the subjective questionnaire taking into account the responses collected in the ⅓ G simulation with 6 participants are included in the same table.

In our study, we found that all average values of WWL,% are lower for all type of tasks for males and females for simulated ⅙ G compared to to the data obtained under 1G. We found 12% decrease in average value of WWL,% for static task (S1), 33% for dynamic task (D), 15% for repetitive task (R) and 23% for static task (S2) for males under ⅙ G vs. 1G. The average WWL,% values decreased for static task (S1) by 8%, for dynamic task (D) the values remained the same, for the repetitive task (R) the values increased by 4 % and for static task (S2) increased by 12% for females in ⅙ G compared to 1G. It is important also to analyse the impact the different demands of the overall workload. In accordance with all presented data, physical demand (PD) and effort (EF) have the highest values for males and females. It was also observed that WWL,% is systematically higher for females performing tasks under ⅙ G than for males, **Table 2**.

3.3. Comparison 1G, ⅙ G, ⅓ G

Due to the experimental conditions and the need to use large ballast weights, which may be invasive for the participants, a small number of participants were invited (3 males and 3 females). We had only a small amount of data to carry out a comparative analysis of the effect of gravity on participant's

TABLE 4 | Summary of the calculated NASA-TLX parameters for the tasks with 3 kg load.

Task G-level	MD mean	PD mean	TD mean	P mean	EF mean	FR mean	WWL(%)
			22 participants				
S1-1G (M)	40.72	275.54	71.09	118.63	232.91	56.45	53.02
S1-1G (F)	49.77	264.69	102.92	101.08	252.61	49.08	54.67
S1-⅙ G (M)	42.70	198.10	94.50	163.90	181.30	10.4	46.6
S1-⅙ G (F)	75.00	226.67	145.78	120.67	179.44	8.11	50.37
			25 participants				
D-1G (M)	47.00	279.60	30.10	148.50	286.00	87.10	58.55
D-1G (F)	35.20	303.80	64.90	105.70	204.60	14.30	48.57
D-⅙ G (M)	73.00	187.90	82.00	108.90	134.60	0.40	39.12
D-⅙ G (F)	12.25	231.25	107.75	143.75	214.50	21.50	48.73
			26 participants				
R-1G (M)	115.00	190.22	132.11	130.89	197.89	9.89	51.73
R-1G (F)	48.09	285.09	75.45	92.73	194.45	44.64	49.36
R-⅙ G (M)	92.50	167.00	106.50	126.00	158.50	6.00	43.77
R-⅙ G (F)	54.30	215.80	142.10	135.80	184.20	39.40	51.44
			27 participants				
S2-1G (M)	58.08	247.46	88.69	119.46	165.54	48.38	48.51
S2-1G (F)	58.92	318.58	125.58	118.42	272.75	12.92	60.48
S2-⅙ G (M)	57.89	184.89	66.67	91.33	181.44	13.56	39.72
S2-⅙ G (F)	59.64	269.64	127.00	151.00	211.36	11.64	55.35
			6 participants				
S1-⅙ G (M,F)	40.92	129.92	121.25	72.50	115.42	26.00	33.73
S1-⅓ G (M,F)	31.36	150.90	89.09	147.27	142.27	40.90	40.12
S1-1G (M,F)	21.91	278.73	54.64	111.73	149.45	16.91	42.22
D-⅓ G (M,F)	61.25	114.17	53.75	90.42	143.33	38.42	33.42
R-⅓ G (M,F)	44.09	118.18	75.45	105.45	146.82	51.82	36.12
S2-⅓ G (M,F)	49.50	97.00	68.50	74.50	130.00	40.80	30.69

The results are presented first for males and then for females in the first four groups and mixed males and females for 6 participants. Task types: holding weights with an outstretched arm (S1), holding weights in an arm bent at the elbow (S2), slow dynamic motion (D), and repetitive motion (R). Analyzed Task Intensities: 0.5, 1, 3, 5, and 7 kg. All values in table are means. Subjective demands were multiplied by task load index, thus some parameters are higher than 100 range due to weighting parameters.

fatigue in ⅓ G doing static, dynamic and repetitive tasks. **Figure 3A** shows an example of the fatigue curves for 1G, ⅙ G and ⅓ G, where the fatigue curve for simulated lunar gravity is located above the curve for Martian gravity and Earth gravity. We built only one power trend curve due to this limited data, and we took into account the intensity of the task from 1 to 7 kg. The values of b_o, b_1, R^2 mean muscle mass ET ratio of muscle mass (kg) and body mass (kg) of the participant for 1 kg load, 3 kg and combined 5 and 7 kg loads for all types of tasks are presented in the **Table 2**.

We found the ratio between the mean ET (min) values for 1, 3, and 5–7 kg loads, normalized to the participants' ratio of muscle mass (kg) and body mass (kg) for ⅙ G and 1G, and then for ⅓ G and ⅙ G. For static tasks (S1), the ratio between ET (min) for ⅙ G and 1G is 4.10, and the ratio between ET(min) for ⅓ G and 1G is 1.60. For dynamic tasks (D) the ratio between ET(min) for ⅙ G and 1G is 2.87, and the ratio between ET (min) for ⅓ G and 1G is 1.25. For repetitive tasks (R), the ratio between ET(min) for ⅙ G and 1G is 2.82, and the ratio between ET (min) for ⅓ G and 1G is 2.30.

For a more complete understanding of the phenomenon, we also conducted a NASA-TLX survey after the participant completed the task. **Figure 3B** shows the Box-and-whisker plots with outliers for Earth, simulated lunar and martian gravity. For static tasks we see the normal distribution for ⅙ G, ⅓ G and 1G. According to the respective median values for each environment and each box plot we can say that there is little difference between the three groups of data, but there is nonetheless a tendency for the workload to increase with increasing gravity. We found 10.54% increase of average values of WWL,% for static task for males and females under 1G vs. ⅙ G. And we found 4.15 % increase of average values of WWL,% for static task for males and females under 1G vs. ⅓ G.

The results of dependence between ET (min) and WWL,% and muscle contraction from the gravity level (1G, ⅓ G and ⅙ G), as well as the character of this dependence are presented in **Table 5**. It shows the ET (min) and WWL% predictors for Static (S1) and dynamic (D) tasks under 1G, ⅓ G and ⅙ G for males and females.

These results indicate that HG increases a participant's productivity by reducing overall physical fatigue expressed in

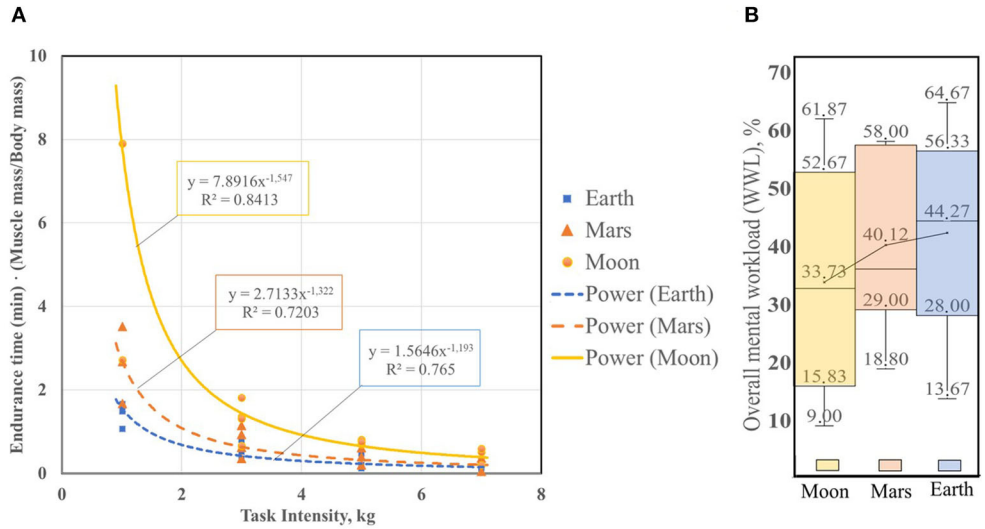

FIGURE 3 | (A) Endurance time-gravity level dependence for static tasks for Earth, simulated Moon and Mars gravity levels. Task Intensity in kg. **(B)** Overall mental workload (WWL,%) [example for static task (S1)], for males and females for loads (1, 3, 5, and 7 kg)-gravity level dependence.

TABLE 5 | The ET (min) and WWL% predictors of different types of tasks under 1G, ⅓ G, ⅙ G.

Task G-level	ET (min)	WWL%	(H) before (kg)	(H) after (kg)
		n = 131		
S1 (M/F)	−0.25 (0.08)**	0.03 (0.02)*	−0.22 (0.12)*	0.24 (0.13)**
		n = 102		
D (M/F)	−0.33 (0.11)**	0.04 (0.02)**	−0.09 (0.11)	0.08 (0.12)
		n = 115		
R (M/F)	−0.08 (0.06)	0.00 (0.02)	−0.14 (0.14)	0.01 (0.15)
		n = 237		
S2 (M/F)	−0.09 (0.05)**	0.00 (0.01)**	0.03 (0.09)	−0.01 (0.09)

*All results are mixed for Males (M) and Females (F). Task types: holding weights with an outstretched arm (S1), holding weights in an arm bent at the elbow (S2), slow dynamic motion (D), and repetitive motion (R). **$p < 0.05$, *$p < 0.10$. Robust standard errors in parentheses.*

ET (min) compared to Earth's gravity. This was confirmed by a defined significant positive (p=0.002) relationship between Endurance time and gravity level (⅙ G, Moon, ⅓ G, Mars, 1G) with negative coefficient for male and female participants for a static task. This means that increasing gravity will reduce ET (min).

There is a significantly positive relation ($p < 0.05$) between ET (min) and gravity levels (1G, ⅓, ⅙) with a negative coefficient of correlation equal (−0.25, −0.33, and −0.09) for all tasks except repetitive one. At the same time there is a moderate relation ($p < 0.1$) for static (S1) task and significantly positive relation ($p < 0.05$) for static (S2) and dynamic (D) tasks between WWL,% and gravity levels (1G, ⅓, ⅙) with a positive coefficients of correlation. We found a significant relation ($p < 0.05$) between hand muscle contraction force and gravity level only for static task (S1).

4. DISCUSSION

This is the first study to provide insight into the effect of HG (⅙ G and ⅓ G) on data such as ET (min) of the upper extremities of participants and their mental workload. In addition, this study contributes to a clearer understanding of the relationship between physical fatigue of the upper limbs and mental workload when participants perform tasks under HG.

The results indicate that HG increases a participant's productivity by reducing overall physical fatigue expressed in ET (min) compared to Earth's gravity. This was confirmed by a defined significant positive ($p = 0.002$) relationship between Endurance time and gravity level (⅙ G, Moon, ⅓ G, Mars, 1G) with negative coefficient for male and female participants for a static task. This means that increasing gravity will reduce ET (min).

Our results also show a general decrease in overall mental workload, WWL,% under the same conditions. This is due to a decrease in PD and EF demands multiplied by task load index under ⅙ G in comparison with 1G and lower frustration values for males and females. It was also observed that WWL,% is systematically higher for females performing tasks under ⅙ G than for males, because most subjective demands are also higher for females, with the exception of P, EF and FR for the tasks (S1) and MD for the tasks (D) and (R). We assume that this is due to the female's higher sensitivity to the loads and weaker physical strength.

This study shows a moderate relation ($p < 0.1$) between overall mental workload and gravity level with a positive coefficient for male and female participants for the static task (S1), see **Table 5**. Lower p-values for WWL,% may be related to each participant's individual understanding and interpretation of the survey. Thus, increasing gravity will increase the mental workload. Other variables, such as hand muscle contraction

after task, also had significant relation ($p < 0.05$) and positive correlation (0.024) with gravity level. The same trend was observed for dynamic and repetitive tasks.

These results could be applied to practical application for astronauts training for missions to the Mons and Mars, because by defining the level of physical fatigue in specific environments we can develop guidelines with respect to defined capacities of males and females. And these correlations may suggest that NASA-TLX assessment can be an appropriate tool for preliminary studies of mental workload, independently or in combination with other tools.

With all our participants we found that power function better matched the data of ET (min) - Task Intensity, but without specification of upper limb joints. This is similar to such studies as Rohmert (1960), Monod and Scherrer (1965), Huijgens (1981), Sato et al. (1984), Rohmert et al. (1986), and Sjøgaard (1986). The exponential model was used by the following authors Manenica (1986), Matthijsse et al. (1987), and Rose et al. (2000). The power model was chosen because of better fit of curve to the data and higher R^2. We also subsequently found that the data for males and females should be divided into separate data sets because it results in a better curves fit and higher R^2. This can be related to the different physical capacities of the male and female participants as well as their anthropometry.

The main finding of the study with 24 participants conducting Static task (S1) was related to three variables: ET (min), mental workload and contraction force. ET increased by an average of 3.54 times for females and 3.14 for males under ⅙ G, in comparison with 1G. Another interesting finding is related to the ratio value between the average ET (min) values for loads of 1, 3, and 5–7 kg, normalized to the ratio of muscle mass (kg) and body mass (kg) of participants, measured under ⅙ G and 1G. This is systematically higher for females than for males, see **Table 2**. For example, for male for the task (S1) with an intensity of 1 kg this ratio is 4.62 and for females is 6.54. This may confirm that females in general are more fatigue resistant and have a faster recovery during prolonged intense activity. Conversely our results suggest that males have a higher maximal power output.

We assume that it is also due to the higher sensitivity of females to loads, especially under 1G, due to skeletal muscle function. For example, while most males are able to perform the task with a load of 5 kg, whereas few females can complete it, and accordingly there is a large gap in ET results. If under 1G and underwater measurements of ET (min) for males are on average two times higher than for females, there will be a greater difference for females. Similar trends and relations were found for the dynamic (D), repetitive (R) and static (S2) tasks.

With the same 24 participants we found that under ⅙ G, mental workload reduced by an average 1.15 times for males and 1.08 for females in comparison with 1G for the static tasks (S1). We found that PD and EF demands have the highest impact on the overall mental workload which is consistent with Brown (1994). According to Xu et al. (2018), the "control of movement is a kind of mental activity that can cause mental fatigue," because the participant must make more effort to complete the task after increasing physical fatigue. Even if according to Rubio et al. (2004) NASA-TLX highly correlates with performance and according to our results it increases with reduction of the gravity level, the physical demand and effort significantly reduced with reduction of the gravity level.

Regarding assessment of muscle contractions of the hand before and after the tasks, a higher change in % for ⅙ G than for 1G was observed. This is because, in general, all participants worked longer at ⅙ G and became weaker in terms of upper limb strength. It should be noted, however that back-leg-chest measurements were not collected due to logistic reason.

With six participants we found that under ⅓ G, ET increased by a factor of 1.60 and ET increased by a factor of 4.10 and mental workload decreased by a factor of 1.26 for males and females in comparison with 1G. The same trend was found for the dynamic (D), repetitive (R) and static (S2) tasks. Although there is a pattern of increasing ET (min) with decreasing gravity, these results do not fully converge with those obtained in the experimental group with more participants, because of size effect. To increase reliability of the data, an experiment with a larger number of participants with a simulation of reduced gravity and the same tasks is recommended. Nevertheless, it is critical to have the third environment ⅓ G with an additional gravity level for statistical predictions for different effect studies.

The state of upper extremities under HG has in general been studied very little in comparison with lower extremities.The most of the studies focused on running, hopping and jumping under HG (Lacquaniti et al., 2017; Richter et al., 2017, 2021; Weber et al., 2019). G-level effect was most significant on peak planar force, gait cycle duration, pace and mechanical work in the HG conditions reduced compared to 1G. Additionally, reduced gravity below 0.4 G is insufficient to support musculoskeletal and cardiopulmonary systems for a long period of time (Richter et al., 2017). Another study Lauer et al. (2018) showed that water reduces the mechanical load on the shoulder by up to 75%. This is also seen in our findings: since all the movements were performed in water, even though each participant was given additional ballasts to make the body heavier, the total body weight still remained 6 or 3 times (⅙ G, ⅓ G, respectively) lighter than under 1G.

While previous research has focused on lower extremities under HG, our results demonstrate that the study of upper extremities is also important and will play a crucial role for short term and long-term missions and regular work on the Moon. These results should be taken into account when considering how to design manual tasks, including astronaut training tasks for specific environments under HG. The motions with extension and ulnar deviation should be investigated during future experiments.

In parallel and in combination with posture studies, it can be also scaled to working space ergonomics under HG in general. With this data we will be able to design and optimize workplace and manual operations. Such design will require less effort due to physical fatigue optimization. Also, this has potential for reducing repetitive strain injury and muskuloskeletal disorders which are commune in many workplace situations and contribute to absenteeism and additional costs for workforce.

The developed model could be used to assess physically limiting situations in industry in 1G and HG to propose alternative solutions. Furthermore, we recommend applying it to digital human modeling, which requires experimental data for modeling and further predictions. Consequently, this will lead to the development of new guidelines and standards for workplace design under HG.

5. CONCLUSIONS

This paper proposes an empirical and a subjective model for physical fatigue of upper extremity fatigue and mental workload assessment with three levels of gravity, six task intensities, and four types of tasks. These new models show excellent agreement between experimental data and subjective data.

With ET (min) assessment, we found that participant performance reduced with increased gravity level for all types of tasks. With mental workload assessment, we found that the workload in ⅙ G is lower than in 1G for the same tasks. In our additional test with comparison of impact of 1G, ⅓ G, ⅙ G on six participants' physical strength we found constituency and a certain linearity, expressed by increasing the physical fatigue and workload with gravity level increasing. According to the small sample we can see that for all tested tasks the level of physical fatigue and mental stress for the simulated gravity of Mars is between levels estimated during the experiments under 1G and ⅙ G. It can be certainly be generalized.

Our results could be integrated into digital human simulations, helping to carry out longer-period simulations, for example, over years. If we can measure productivity in such an environment, we can improve the workplace design and develop a new hypothesis.

We recommend using an empirical fatigue model with a subjective assessment tool. It provides a better understanding of the phenomena and suffices to predict the fatigue curves for a particular task. Application of subjective mental workload assessment can be critical for workplace equipped with human-machine systems designed to ensure higher levels of comfort, performance, and safety.

6. LIMITATIONS

Although some valuable findings were obtained, there are still several limitations to this study. The main limitation arises from variations in the age, anthropometry and conducting asymmetric tasks of the participants. In order to determine these effect, younger and older, weaker, stronger populations, should be analyzed in future studies. As well as symmetric tasks should be investigated. In addition, we recommend validating ⅓ G fatigue curve with a larger number of participants. Finally, we recommend validating all defined empirical and subjective models with parabolic flights adapted to specific environments. The study of the fatigue effects on the posture of the participants may be a new attempt to explore the participant's fatigue states.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://figshare.com/s/03b02a630edfde797a12.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee-EPFL [HREC 507 024-2021/09.03.2021 amendment to initial protocol HREC 001-2020/20.12.2019)]. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TV organized the experiments, invited participants, collected relevant data during experiments, performed the statistical analysis, processed data, and drafted manuscript. CN an ESA astronaut, provided space-related expertise for the preparation of the experiments, helped coordinate the experiments, and draft the manuscript. VG participated in the design and coordination of the study, helped to draft the manuscript, and provided the founding for this study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.832214/full#supplementary-material

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Between-Subject and Within-Subject Variaton of Muscle Atrophy and Bone Loss in Response to Experimental Bed Rest

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To improve quantification of individual responses to bed rest interventions, we analyzed peripheral quantitative computer tomography (pQCT) datasets of the lower leg of 76 participants, who took part in eight different bed rest studies. A newly developed statistical approach differentiated measurement uncertainty U_{Meas} from between-subject-variation (BSV) and within-subject variation (WSV). The results showed that U_{Meas} decreased 59.3–80% over the two decades of bed rest studies ($p < 0.01$), and that it was higher for muscles than for bones. The reduction of U_{Meas} could be explained by improved measurement procedures as well as a higher standardization. The vast majority (89.6%) of the individual responses pc_i exceeded the 95% confidence interval defined by U_{Meas}, indicating significant and substantial BSV, which was greater for bones than for muscles, especially at the epiphyseal measurement sites. Non-significant to small positive inter-site correlations between bone sites, but very large positive inter-site correlation between muscle sites suggests that substantial WSV exists in the tibia bone, but much less so in the calf musculature. Furthermore, endocortical circumference, an indicator of the individual's bone geometry could partly explain WSV and BSV. These results demonstrate the existence of substantial BSV bone, and that it is partly driven by WSV, and likely also by physical activity and dietary habits prior to bed rest. In addition, genetic and epigenetic variation could potentially explain BSV, but not WSV. As to the latter, differences of bone characteristics and the bone resorption process could offer an explanation for its existence. The study has also demonstrated the importance of duplicate baseline measurements. Finally, we provide here a rationale for worst case scenarios with partly effective countermeasures in long-term space missions.

Keywords: between-subject variation, within-subject variation, measurement uncertainty, bed rest, muscle atrophy, bone loss

INTRODUCTION

Microgravity exposure is associated with profound adaptations of the human body, including muscle atrophy and bone loss, both of are usually interpreted as a result of unloading and the lack of mechanical forces (Vico et al., 2000; LeBlanc et al., 2007; Fitts et al., 2010; Rittweger et al., 2018). Especially, the lower limb is affected by these adaptations resulting in muscle wasting of 6 and 24% of the baseline volume after 8 days and 6 month of microgravity exposure, respectively (LeBlanc et al., 1997; Narici and de Boer, 2011). Bone losses amount to an average loss rate of 1–1.5% bone mineral content (BMC) per month (Pavy-Le Traon et al., 2007), which, however, is subject to substantial between-subject variation (BSV). Furthermore, besides BSV there is a within-subject variation (WSV) shown by differences in bone loss at different body sites after bed rest (Rittweger et al., 2005), the latter being a suitable analog of microgravity exposure on earth. The −6° head down tilt (HDT) bed rest induces an unloading of muscles and bones of the lower extremities and the spine evoking adaptations that are comparable to those from space-related adaptations. Bed rest studies unanimously show loss of muscle volume, of muscle strength and of BMC (Hargens and Vico, 2016). The loss of the muscle volume is exponential and in a comparable manner followed by losses in muscle power and muscle strength (Narici and de Boer, 2011). After 7–14 days of unloading, there are moderate effects, larger effects occur within 35 days of bed rest (Winnard et al., 2019). Furthermore, there is a correlation between reduction in muscle cross-sectional area (CSA) and strength loss. Because of that, CSA is a good approximation for muscle strength (Maughan et al., 1983; Rittweger et al., 2000; Fukunaga et al., 2001). Mitigating these profound adaptations and maintaining a sufficient physiological status is the aim of suitable countermeasures (Winnard et al., 2019). Yet, different training effort using such countermeasures can lead to variations in the musculoskeletal response (Timmons, 2011; Rittweger et al., 2018), however, in contrast, comparable training effort can also lead to BSV in muscle loss (English et al., 2015) or bone loss (Sibonga et al., 2015). The variation may be explained by the fact that there are responders and non-responders toward a training intervention (Mann et al., 2014; Hecksteden et al., 2015; Ahtiainen et al., 2016) resulting in a BSV (McPhee et al., 2010; Ross et al., 2019).

Until now, there are few approaches for quantifying BSV and WSV in response to bed rest (Narici and de Boer, 2011; Debevec et al., 2018; Scott et al., 2021). Thus, previous approaches have compared the standard deviation (SD) of changes between intervention and control group (Atkinson and Batterham, 2015; Hopkins, 2015). But so far, most researchers do not perform such analysis getting information about individual differences (Atkinson and Batterham, 2015). Moreover, systematic attempt to separate BSV and WSV from measurement-related uncertainty are rare except Swinton et al. (2018). Following Scott et al. (2021), bed rest is a good possibility to quantify "true" and "false" individual differences, because due to the conditions of such a study, several influencing parameters like nutrition or daily activities are controlled. Atkinson et al. (2019) explained that there is an additional factor, which cannot be controlled. There is a random WSV due to the period of time between the baseline data collection (BDC) and the follow up measurements, mostly influenced by non-standardized behavior of the participants. Understanding BSV and WSV is of general interest. Thus, large BSV in the presence of small WSV would favor genetic and epigenetic mechanisms, whilst large BSV in combination with large WSV would require more complicated explanation models. Moreover, detailed information about BSV and WSV is highly relevant in practical terms when the health and well-being of single individuals is at stake. Therefore, the aim of the present work was to explore variation in musculoskeletal responses to bed rest, and to separate BSV, WSV and measurement-related uncertainty.

MATERIALS AND METHODS

Selected Studies

We selected data sets of eight bed rest studies (AGBRESA, BBR, LTBR, MEP, NUC, Planhab, RSL, Valdoltra), which took place in France, Slovenia, and Germany, respectively, from 2001 till 2019 to explore the variation in musculoskeletal responses after bed rest. The studies varied in bed rest duration as well as bed rest condition (horizontal bed rest or head down tilt bed rest). The full study names, the year, the location, and the characteristics, which were important for this manuscript, are presented in **Table 1**. Additionally, **Table 1** includes references, which explain the exact study details.

Three out of the eight studies (LTBR, RSL, and Valdoltra) included two baseline measurements of the peripheral quantitative computed tomography (explained later on). This enabled a calculation of the measurement uncertainty, because the time between the two measurements was short and no intervention took place. As it is very consistent in literature, the bone losses, but not muscle wasting, continue for another 10–20 days following re-ambulation (Rittweger et al., 2005, 2010; Hargens and Vico, 2016), muscle losses were quantified at the last day of bedrest and bone losses at 14 days of follow-up after re-ambulation. Due to comparable durations of bed rest, BBR was presented as AGBRESA and RSL with 60 days of bed rest in the results section.

Participants

In total, we analyzed datasets of 76 participants, who took part in the described eight different bed rest studies. For the purpose of this manuscript only the data of the control groups, which experienced no intervention excluding bed rest, were analyzed. Only for calculation of the measurement uncertainty of LTBR, RSL and Valdoltra we used the data sets of control and intervention group getting more precise values. Additionally, from 11 subjects of the LTBR study (control and intervention group), both legs were measured and were also used for analysis of the measurement uncertainty.

TABLE 1 | Overview about the included studies with information about the year, the location, the number of subjects, the duration of bed rest, bed rest position, and measurement sites.

k	Study	Full study name	Year	Location	Number of subjects (n_k)	Duration of bed rest	Position	Muscle sites	Bone sites	References
1	AGBRESA	Artificial Gravity Bed Rest with ESA	2019	DLR, Cologne (GER)	8	60	HDT	TIBIA_38, TIBIA_66	TIBIA_04, TIBIA_38, TIBIA_66, TIBIA_98	Frett et al., 2020
2	BBR	Berlin Bed Rest	2003/2004	Benjamin Franklin Campus, Berlin (GER)	10	56	HDT	TIBIA_66	TIBIA_04, TIBIA_38, TIBIA_66	Rittweger et al., 2006
3	LTBR	Long-Term Bed Rest	2001/2002	MEDES, Toulouse (FR)	9	90	HDT	TIBIA_66	TIBIA_04, TIBIA_66	Rittweger et al., 2005
4	MEP	Medium-term Bed Rest Whey Protein Study	2011/2012	DLR, Cologne (GER)	8	21	HDT	-	TIBIA_04, TIBIA_38, TIBIA_66	Blottner et al., 2014
5	NUC	Nutritional Countermeasure	2010	DLR, Cologne (GER)	7	21	HDT	-	TIBIA_04, TIBIA_38, TIBIA_66	Heer et al., 2014
6	Planhab	Planetary Habitat Simulation Study	2012/2013	Olympic Sports Center, Planica (SLO)	13	21	HBR	TIBIA_66	TIBIA_04, TIBIA_38, TIBIA_66, TIBIA_98	Debevec et al., 2014
7	RSL	Reactive Jumps in a Sledge Jump system as a countermeasure during long-term bed rest	2015/2016	DLR, Cologne (GER)	11	60	HDT	TIBIA_38, TIBIA_66	TIBIA_04, TIBIA_38, TIBIA_66, TIBIA_98	Kramer et al., 2017
8	Valdoltra	Valdoltra	2007	Orthopedic Hospital, Valdoltra (SLO)	10	35	HBR	-	TIBIA_04, TIBIA_38, TIBIA_98	Borina et al., 2010

HDT, 6° head down tilt bed rest; HBR, horizontal bed rest.
The position with 6° head down tilt (HDT) and horizontal bed rest (HBR), the observed measurement sites for the muscles and the bones.

Peripheral Quantitative Computed Tomography Measurements

The tibia is composed of (1) distal and proximal epiphysis, which are both rich in trabecular bone and devoid of thick compact bone, (2) distal and proximal metaphysis, which are composed both of compact and trabecular bone, and (3) the diaphysis (shaft), which is mainly composed of thick compact bone and almost devoid of trabecular bone (Capozza et al., 2010), whereas at the proximal epiphysis the cortical shell consisting of compact bone is often not thicker than trabeculi. At diaphyseal sites, pQCT also allows segmentation of muscle from skin and bone tissues, thereby yielding the anatomical muscle cross-sectional area (CSA), albeit without distinction of individual muscle groups (**Figure 1**). All selected studies included a pQCT measurement with either a XCT2000 or a XCT3000 device (Stratec Medizintechnik, Pforzheim, Germany) (**Table 2**). The measurement process for pQCT has been described in detail elsewhere (Rittweger et al., 2010). In brief, the XCT devices use single-beam rotation-translation CT technology, and distal and proximal tibial sites are identified from tibio-talar and femoro-tibial scout views, respectively, whilst sites at the diaphysis are either found manually or *via* a scout view (**Figure 1**). Over the two decades that the studies have been performed, the procedures have been slightly modified to maximize repeatability of the measurements. Moreover, whilst earlier studies had solely focused on the distal tibia, the proximal tibia has also been studied since 2007, initially using femoro-tibial scout views in the frontal plane, and with sagittal scout views since 2016 (**Table 2**). As bed rest induces minimal to no changes in the metaphysis and in the forearms, the present manuscript focusses on epiphyseal and diaphyseal bone sites including two muscles sites at the diaphysis, thereby allowing WSV assessment in addition to BSV.

Image Analysis and Data Processing

All image analysis was performed with the integrated XCT software Stratec XCT Console in its version 6.20 in accordance with the manufacturer's instructions. The operator marked the regions of interest (ROI) in each picture (tibia, fibula, muscle including tibia and fibula at 38 and 66% of tibia length) and defined the segmentation threshold for the outer bone or muscle contour. The threshold differed between measurement sites, studies, and could even differ between subjects, respectively. The threshold was chosen yielding a satisfactory contour of the ROI and was kept for each subject throughout the study. To differentiate between compact and trabecular bone tissue at the epiphyseal measurement sites, we run a second analysis with a segmentation threshold at 650 mg/cmł, which enabled quantifying the compact bone at these sites (Rittweger et al., 2010). After analyzing all pQCT scans, an analysis was performed by the XCT software that encompassed all CT numbers included in the present analysis. The resulting data base was further

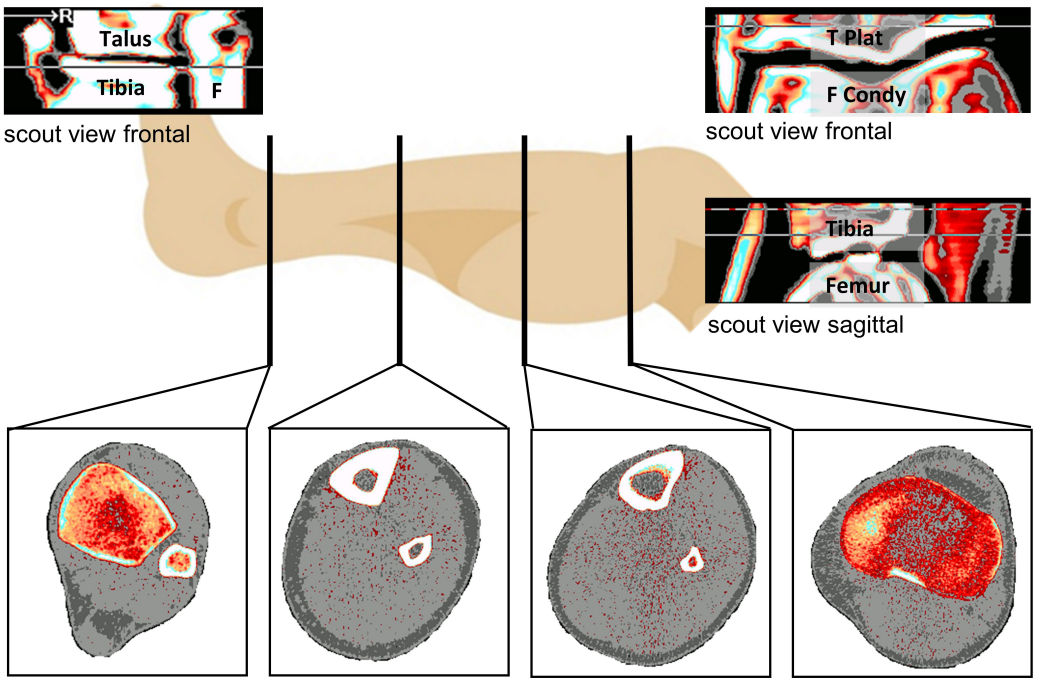

FIGURE 1 | Overview about the analyzed measurement sites (at the bottom) from left to right with TIBIA_04 (Epiphysis at the ankle), TIBIA_38 and TIBIA_66 at the diaphysis and TIBIA_98 (Epiphysis at the knee joint). 04, 38, 66, and 98 represent the relative position regarding the tibia length from ankle joint to knee joint. Top left is the distal frontal scout view (Tibia, Talus, F: Fibula), top right the frontal proximal scout view (F Condy: Femur Condyles, T Plat: Tibia Plateau), below that the sagittal proximal scout view.

processed with custom-made R-scripts (Version 3.6.0[1]), using the RStudio environment (Version 1.2.1335, Boston, MA, United States). Thus, BMC was identified as the product of the XCT-variables "TOT_DEN" and "TOT_A" divided by 1,000. The endocortical circumference (ENDO) was identified from "ENDO_C". Muscle CSA was calculated as the differences between "TOT_A" of the ROI of the muscle and "TOT_A" of tibia and fibula.

Statistical Computation

We assumed that measurement uncertainty (U_{Meas}) was variable across measurement sites and studies, but invariant within a given study and measurement site. Moreover, we assumed that U_{Meas} and the individual response to the bed rest intervention (U_{IR}) depict normal distribution. It follows then from the principle of linear superposition that the observed response's uncertainty U_{Obs} can be conceptualized as the sum of these two parameters.

$$U_{Obs} = U_{Meas} + U_{IR} \tag{1}$$

We defined the observed individual loss of bone or muscle of subject i (i = 1, . . ., n_k with n_k as the total number of subjects of study k, $k = 1, \ldots, 8$) after bed rest intervention as the percent change pc_i:

$$pc_{ik} = \frac{x_{IR,\,ik} - x_{BDC,\,ik}}{x_{BDC,\,ik}} \times 100 \tag{2}$$

[1]www.r-project.org

pc_{ik} is defined as the difference either BMC or CSA between the individual result after the intervention ($x_{IR,ik}$) and the result of the first baseline measurement ($x_{BDC,ik}$) divided by the baseline result ($x_{BDC,ik}$). This result is multiplied by 100 getting the percent change pc_{ik}. The mean among all subjects of a study is denoted by pc_k and is calculated for each measurement site of each of the eight included studies.

Empirically, the observed uncertainty $\hat{U}_{Obs,k}$ (k indicating the study) could be assessed as the variance of the individual percent change pc_{ik} of each participant of each study's control group, n_k is indicating the number of participants in the control group of study k, and $\overline{pc_k}$ the mean percent change of these participants:

$$\hat{U}_{Obs,\,k} = \frac{1}{n_k - 1} \sum_{i=1}^{n_k} (pc_{ik} - \overline{pc_k})^2 \tag{3}$$

$\hat{U}_{Meas}$ denote measurement uncertainties, which are usually assessed during baseline through repetition of measurements. Typically, we use two baseline measurements ($n_i = 2$) to compute the variance within each participant. The mean of these variances of all participants of one study and measurement site represents $\overline{U}_{Meas,k}$. As we compared individual's response toward the measurement uncertainty, the calculation of the variance was more practical resulting in absolute values compared to the coefficient of variation. For calculation of $\overline{U}_{Meas,k}$ we used the baseline measurements of all subjects of study k, irrespective of the group (control or intervention group) to get more precise

TABLE 2 | Overview about the way of the determination of the measurement site of TIBIA_98 and TIBIA_66.

k	Study	pQCT device	Scout view orientation at TIBIA_98	TIBIA_66 detection
1	AGBRESA	XCT3000	Sagittal	Automatic
2	BBR	XCT2000	Frontal	Manual
3	LTBR	XCT2000	Frontal	Manual
4	MEP	XCT3000	Frontal	Manual
5	NUC	XCT3000	Frontal	Manual
6	Planhab	XCT3000	Frontal	Automatic
7	RSL	XCT3000	Sagittal	Automatic
8	Valdoltra	XCT2000	Frontal	Automatic

values. n_i represents the number of baseline measurements, $x_{BDC,mk}$ the value of each baseline measurement (BMC or CSA), and $\overline{x_{BDC,k}}$ the mean of all baseline measurements of the specific participant.

$$\hat{U}_{Meas,ik} = \left(\frac{1}{n_i - 1} \sum_{m=1}^{n_i} (x_{BDC,mk} - \overline{x_{BDC,k}})^2\right) \times \left(\frac{100}{\overline{x_{BDC,k}}}\right)^2 \quad (4a)$$

$$\overline{U}_{Meas,\,k} = \frac{\sum_{i=1}^{n_k} \hat{U}_{Meas,\,ik}}{n_k} \quad (4b)$$

Solving Equation 1 for $\hat{U}_{IR}$ and replacing the parameters yields

$$\hat{U}_{IR,k} = \hat{U}_{Obs,k} - \overline{U}_{Meas,k} \quad (5)$$

$$\hat{U}_{IR,k} = \frac{1}{n_k - 1} \sum_{i=1}^{n_k} (pc_{ik} - \overline{pc_k})^2 - \frac{\sum_{i=1}^{n_k} \hat{U}_{Meas,\,ik}}{n_k}$$

This variable has been obtained for each study and measurement site. As only LTBR, RSL and Valdoltra included two baseline measurements, it was impossible to calculate $\overline{U}_{Meas,\,k}$ for all study and measurement sites. So, for studies with only one baseline measurement, we used the result of the study with the most similar measurement condition (**Table 2**). If this was not possible due to missing data (e.g., Valdoltra provided no data about MUSCLE_66, which is needed for Planhab MUSCLE_66 calculations), we used the higher $\overline{U}_{Meas,\,k}$ of the other studies (in the case of the example, we used $\overline{U}_{Meas,\,k}$ of LTBR).

Additionally, to compare results of studies with different durations of bed rest, we introduced the adjusted between subject deviation (ABD) for each study *k*. ABD_k is defined as the square root of the uncertainty of individual response divided by the mean of the percent change of each study and measurement site. This result is divided by each study's duration in weeks. By using the mean percent change and the study duration, we normalized the uncertainty to bed rest duration and bed rest conditions as well as other factors like age or sex of the participants:

$$ABD_k = \frac{\sqrt{\hat{U}_{IR,k}}}{\overline{pc}_k} \div weeks \quad (6)$$

Besides these analyses of variances, we calculated the absolute bone loss (BL) as difference of BMC of the first baseline measurement and the post bed rest value:

$$BL_{ik} = BMC_{BDC,ik} - BMC_{IR,ik} \quad (7)$$

Between-Subject Variation and Within-Subject Variation

The main focus of this manuscript is on the between-subject variation (BSV) and the within-subject variation (WSV) separated from the measurement uncertainty. For computation of the BSV, we calculated the mean percent change of each measurement site and study. Furthermore, we defined a 95%-confidence interval by using the term $1.96 \cdot U_{Meas}$ (measurement uncertainty of the specific measurement site and study or the most similar condition as described earlier). Thus, the interval was defined as the range between mean percent change added up and subtracted by the term based on the measurement uncertainty. If the individual percent change of a participant exceeded this interval, there was a BSV.

We defined WSV in this manuscript that there was no correlation between loss of muscle volume and/or BMC within one subject along the measurement sites. For this purpose, we used the Pearson's correlation coefficient between the different measurement sites for each study. As there are differences regarding the bone tissue content between the bone sites, we additionally analyzed the loss of compact and trabecular bone loss and its relationships toward the other measurement sites.

Statistics

All computations and statistics were performed with RStudio (Version 1.2.1335, Boston, MA, United States) based on the R-environment (Version 3.6.0,see text footnote 1). Firstly, a mean value of the baseline BMC of the studies with two baseline measurements was calculated followed by calculation of the difference from the mean to the first baseline data collection (BDC1) and was checked for normal distribution. To assess changes of BMC and CSA after the bed rest intervention to BDC1, a paired sample *t*-test was performed separately for each measurement site and study. An ANOVA was used to evaluate differences in U_{Meas} (studies with two baseline measurements: LTBR, RSL, Valdoltra). The mean of U_{Obs} and U_{IR} for each measurement site was compared by an ANOVA. In case of a significant result for the *F*-test of the ANOVA, a *post-hoc* Tukey Honest Significant Differences test was performed to further analyze differences between the studies and measurement. BSV is shown by exceeding the 95%-confidence interval, which was calculated based on U_{Meas} (as described before). The correlations of the percent changes for muscles and bones were analyzed by the Pearson correlation coefficient and scored as defined by Hinkle et al. (2009). Based on these results of the Pearson's correlation coefficient we could do statements about WSV. The relationship between ENDO and BL was analyzed by a linear regression analysis for each study and all studies combined. This approach was performed based on the results of Rittweger et al. (2009) and adapted that we did not use the compact bone loss, but the total BMC loss. Finally, we fitted a linear mixed model

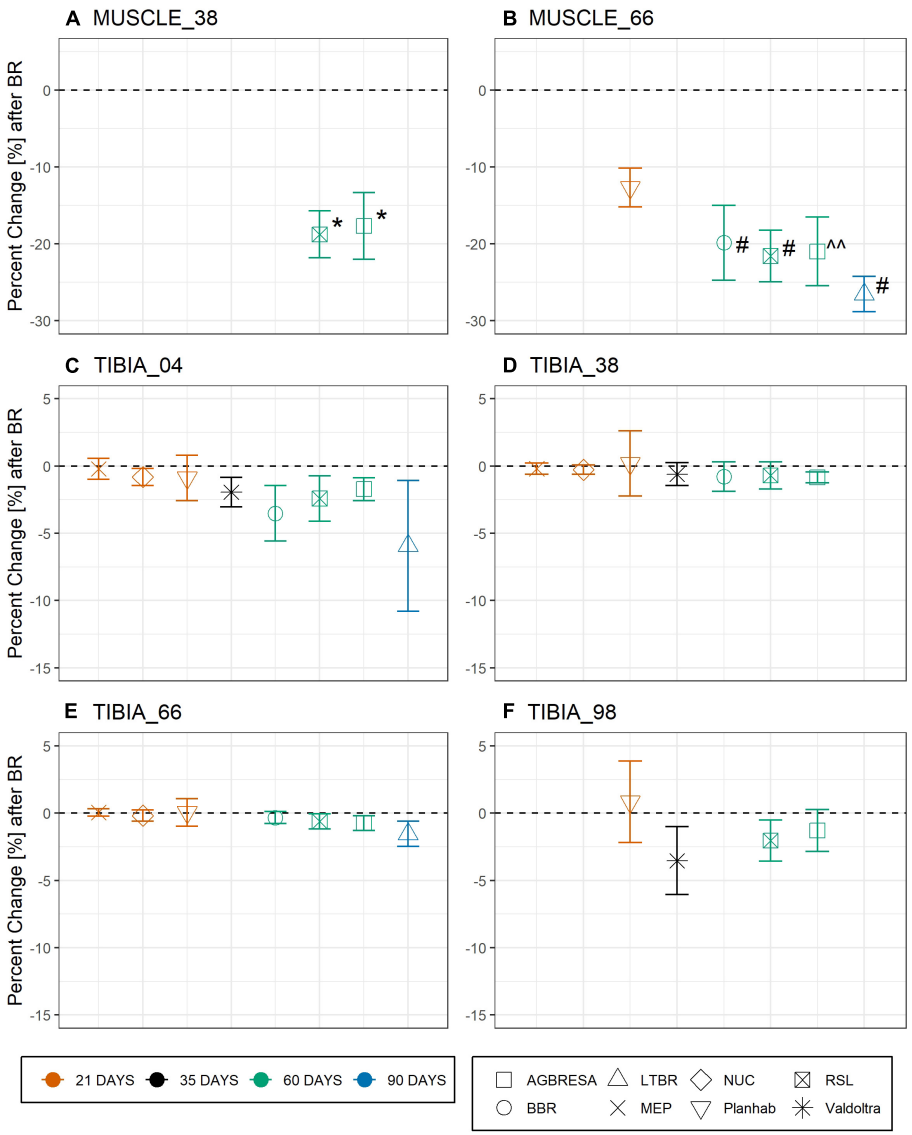

FIGURE 2 | Percent change pc_k as response toward bed rest intervention on the basis of muscle cross section area (CSA) for **(A)** MUSCLE_38 (RSL: $p = 0.0018$; AGBRESA $p = 0.0033$) and **(B)** MUSCLE_66 (BBR: $p = 6.58\text{-}04$; RSL: $p = 0.0002$; AGBRESA: $p < 0.05$; LTBR: $p = 3.35\text{–}07$) and bone mineral content (BMC) for **(C)** TIBIA_04, **(D)** TIBIA_38, **(E)** TIBIA_66, and **(F)** TIBIA_98, respectively. Numbers indicate the relative measurement position regarding the entire tibia length from distal to proximal. The average response is shown, error bars represent standard deviation. The color indicates the bed rest duration and the shape represents the study. ˜ denotes significant changes with $p < 0.05$; * denotes significant changes with $p < 0.01$; # denotes significant changes with $p < 0.001$.

(LMM) for analyzing the outcome variables BMC and CSA with fixed effects of measurement site, study days, amount of bed rest and ENDO (only for BMC), respectively, and random intercepts for study and subjects within a study allowing different variances for each measurement site (in case of heteroscedasticity of the within-group errors).

RESULTS

Differences between values of baseline data collection 1 (BDC1) and the within-subject means of the two baseline measurements were near-normally distributed without any substantial deviation, thereby justifying the approach outlined in the equations above. The difference between BDC1 and post bed rest was significant for MUSCLE_38 (AGBRESA: $p = 0.003$ and RSL: $p = 0.002$) and MUSCLE_66 for AGBRESA ($p = 0.01$), BBR ($p < 0.001$), LTBR ($p < 0.001$) and RSL ($p < 0.001$), but not for the 21-day Planhab study ($p = 0.07$) (**Supplementary Table 1**). **Figure 2** shows the study's percent change at each measurement site.

For computation of the measurement uncertainty U_{Meas}, we used the results of the studies with two baseline measurements, meaning LTBR, RSL, and Valdoltra, respectively. ANOVA

TABLE 3 | Measurement Uncertainty U_{Meas} [%^{2}] of studies with two baseline measurements per body site.

	LTBR	Valdoltra	RSL	Mean	*p*-value
MUSCLE_38	-	-	0.73	0.73 ± 1.02	-
MUSCLE_66	2.95	-	1.20	2.26 ± 3.50	0.053
TIBIA_04	0.38	0.08	0.09	0.24 ± 0.56	0.10
TIBIA_38	-	0.02	0.10	0.09 ± 0.16	0.16
TIBIA_66	0.15	-	0.03	0.10 ± 0.16	0.005
TIBIA_98	-	1.92	0.60	1.00 ± 2.22	0.12

Mean and standard deviation (SD) are shown. Mean and SD were computed by including all values of all studies, which provided data for the specific measurement site. P-value represents the results of ANOVA for analyzing significant differences for U_{Meas}.

indicated a significant difference of U_{Meas} between RSL and LTBR at TIBIA_66 ($p = 0.005$) (**Table 3**). As can be seen from **Figure 3**, the vast majority (89.9%) of the observed individual percent change pc_i exceeds the confidence intervals, indicating significant and substantial BSV. By subtracting the calculated U_{Meas} from U_{Obs}, U_{IR} was calculated (**Table 4**).

U_{Obs} and U_{IR} were significantly greater for MUSCLE_66 compared to TIBIA_38 ($p = 0.009$ and $p = 0.04$) and TIBIA_66 ($p = 0.005$ and $p = 0.02$), as well as for MUSCLE_38 compared to TIBIA_66 ($p = 0.03$) (**Supplementary Table 2**). Generally, it could be seen a trend that both U_{Obs} and U_{IR} were greater for the muscle measurement sites than for the bone measurement sites, except for LTBR, where U_{Obs} and U_{IR} were greater for TIBIA_04 than for MUSCLE_66.

As shown in **Figure 4**, the adjusted between-subject deviation ABD_k for the Planhab study depicts three outliers, namely for TIBIA_04 and TIBIA_66. In the absence of any bone loss for TIBIA_66 in the MEP study, ABD_4 could not be computed. **Figure 5** shows the results of the inter-site correlation analyses. A very high positive correlation of pc_i between MUSCLE_38 and MUSCLE_66 was observed ($r = 0.90$, $p < 0.001$). For BMC, there was no correlation seen between TIBIA_38 and TIBIA_98 ($r = 0.29$, $p = 0.07$), whereas the correlation ranged from 0.34 (TIBIA_04 and TIBIA_38; $p = 0.006$; low positive correlation) to 0.52 (TIBIA_38 and TIBIA_66; $p < 0.001$; moderate positive correlation) between the remaining bone site pairs. When differentiating compact and trabecular bone tissue at the epiphyseal bone sites, there were significant correlations between TIBIA_04_Comp and TIBIA_38 ($r = 0.51$; $p < 0.001$), and TIBIA_66 ($r = 0.39$; $p = 0.002$), respectively, but no correlation to TIBIA_98_Comp ($r = 0.29$; $p = 0.19$). Additionally, TIBIA_98_Comp showed no correlation to either TIBIA_38 ($r = 0.12$, $p = 0.54$) or TIBIA_66 ($r = 0.10$, $p = 0.62$). The loss of trabecular bone within the epiphyseal sites showed no correlation ($r = -0.12$, $p = 0.36$). With regards to muscle-bone inter-relationships, there was a moderate positive correlation between MUSCLE_38 and TIBIA_98 ($r = 0.68$, $p = 0.001$), MUSCLE_66 and TIBIA_66 ($r = 0.56$, $p < 0.001$) and MUSCLE_66 and TIBIA_98 ($r = 0.56$, $p < 0.001$), and a low positive correlation between MUSCLE_66 and TIBIA_04 ($r = 0.47$, $p < 0.001$) and MUSCLE_66 and TIBIA_38 ($r = 0.34$, $p = 0.03$; **Table 5**).

Figure 6 shows the relationship between ENDO and BL for all studies that provided BMC data for three or more bone sites. Linear regression analysis showed significant associations between ENDO and BL across the different studies (**Supplementary Table 3**) except for BBR ($p = 0.93$) and MEP ($p = 0.07$). However, these associations disappeared completely, when percent bone losses were plotted against the ratio of endocortical circumference to BMC, a maker of surface-to-volume ratio (**Figure 7** and **Supplementary Table 4**).

Analyses of BMC (mg/mm) of all bone sites with LMM showed that the variability among subjects (SD 47.1 mg/mm) was greater than the variability among studies (SD 4.9 mg/mm), and also greater than residuals (SD 37.7 mg/mm). The residual variability in BMC differed with measurement site: it was about 2.6 times greater for TIBIA_98 than for TIBIA_04, but lower for TIBIA_38 (by the factor 0.5) and TIBIA_66 (by the factor 0.07). For muscle (CSA in mm^2), the variability among studies (SD 3.0 mm^2) and subjects (8.9 mm^2) were similar. Measurement site and study day were significantly associated with BMC and there was no association of ENDO or bed rest duration and BMC. Similar results were observed for CSA (**Supplementary Tables 5**, **6**).

DISCUSSION

This paper has separated within-subject variation, between-subject variation and measurement uncertainty in a set of control groups from previously bed rest studies, applying a statistical framework for quantifying these aspects. As previously published, bone loss and muscle wasting were generally observed across all studies, underlining the view of Hargens and Vico (2016). The main result of the present paper is that measurement uncertainty of the pQCT was small, and that it cannot explain the large variation of the adaptations after bed rest. In addition, results also demonstrate prominent within-subject variation for bone losses, but not for muscle wasting. Moreover, although some differences were found between studies, the general outcome was relatively homogeneous across studies.

Measurement Uncertainty

Over the almost two decades during which these studies were performed, we generally note a trend for an improvement in measurement precision, e.g., the difference from 2001/2002 during LTBR till 2015/2016 during RSL. The phenomenon is explained by the steady methodological improvements, especially in the scout view procedures. For example, during LTBR TIBIA_66 positioning was performed manually without scout views, due to the short z-range of the specific XCT2000 device used in that study, which did not allow using distal scout views. Moreover, a proximal scout view was not available at that time. A proximal scout view had become available in the Valdoltra study, albeit in the frontal plane only (**Table 2** and **Figure 1**). These frontal plane knee scout views are often difficult to interpret and definition of a landmark on either of the two knee condyles or on the tibia plateau is inherently difficult. Therefore, introduction of sagittal scout

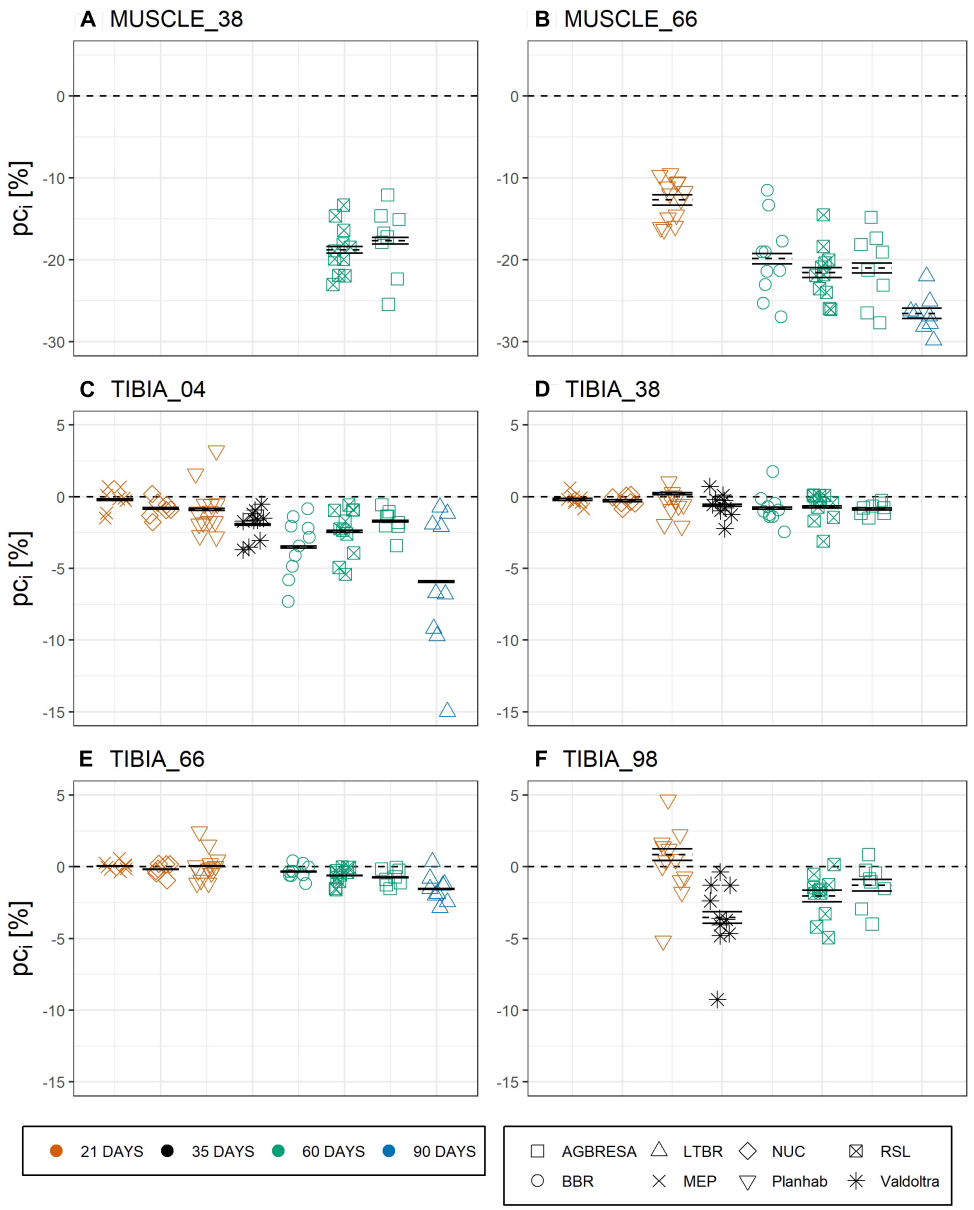

FIGURE 3 | Chart of the individual percent change (pc_i) by measurement sites with **(A)** CSA at MUSCLE_38, **(B)** CSA at MUSCLE_66, **(C)** BMC at TIBIA_04, **(D)** BMC at TIBIA_38, **(E)** BMC at TIBIA_66, and **(F)** BMC at TIBIA_98, where the numbers indicate the relative measurement position regarding the entire tibia length from distal to proximal. The color indicates the bed rest duration and the shape represents the study. Each chart is separated by the studies, who performed measurements at the measurement site. Mean of the pc as dashed line, upper and lower limit of the 95%-confidence interval based on measurement uncertainty U_{Meas} as solid lines. The vast majority of pc_i exceeds the confidence interval indicating significant and substantial between-subject variation.

viewing in the RSL study constituted further improvements as such sagittal scouts are much easier to interpret than frontal scout view.

In general, the measurement uncertainty was smaller for bone than for muscle sites. The observation may be explained by the fact that segmentation of muscle cross sections is somewhat more difficult than segmentation of bones. Moreover, orthostasis affects muscle volume. Although all leg muscle measurements were obtained after at least 60 min in the supine position, it is possible that these supine periods prior to pQCT sessions varied across studies, as well as prescription of fluid intake. As fluid redistribution is unlikely to affect BMC, inhomogeneity of fluid redistribution could have contributed to relative large measurement uncertainty for muscle sites. Possibly, different pQCT devices (**Table 2**) (LTBR: XCT2000 with short z-range; after LTBR XCT-devices with long z-range) are associated with different measurement error, but these are small (0.37% for XCT3000).

TABLE 4 | Overview about Observed Uncertainty U_{Obs} [%^{2}] and Uncertainty of individual response U_{IR} [%^{2}] by studies and body sites.

	AGBRESA		BBR		LTBR		MEP		NUC		Planhab		RSL		Valdoltra	
	U_{Obs}	U_{IR}	U_{Obs}	U_{IR}	U_{Obs}	U_{IR}	U_{Obs}	U_{IR}	U_{Obs}	U_{IR}	U_{Obs}	U_{IR}	U_{Obs}	U_{IR}	U_{Obs}	U_{IR}
MUSCLE_38	18.85	18.12	-	-	-	-	-	-	-	-	-	-	9.35	8.62	-	-
MUSCLE_66	19.96	18.77	23.56	20.63	5.37	2.42	-	-	-	-	6.36	3.41	11.30	10.10	-	-
TIBIA_04	0.73	0.63	4.27	3.89	23.62	23.25	0.61	0.24	0.40	0.02	2.81	2.74	2.81	2.72	1.22	1.15
TIBIA_38	0.17	0.06	1.20	1.10	-	-	0.18	0.08	0.12	0.02	5.88	5.86	1.01	0.91	0.73	0.72
TIBIA_66	0.31	0.28	0.21	0.06	0.89	0.75	0.08	-0.07*	0.18	0.04	1.04	0.89	0.32	0.29	-	-
TIBIA_98	2.39	1.79	-	-	-	-	-	-	-	-	9.20	7.28	2.32	1.72	6.37	4.44

**Negative value, because there was no bone loss during MEP at TIBIA_66 and U_{Meas} was subtracted.*

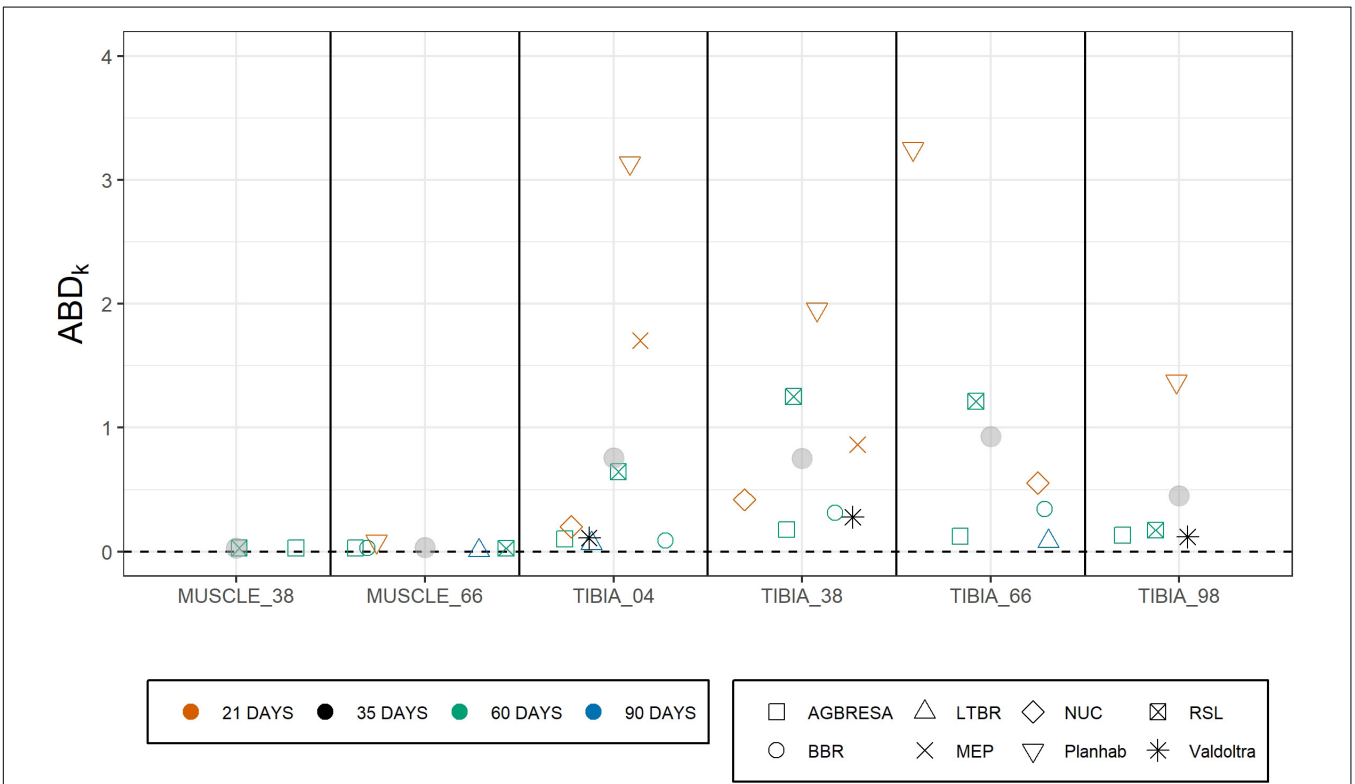

FIGURE 4 | Adjusted between subject deviation (ABD_k) of all studies separated by measurement sites. ABD_k represented the square root of uncertainty of individual response U_{IR} divided by pc_k enabling the comparison of several studies with different bed rest durations. The color indicates the bed rest duration and the shape represents the study. The data of MEP for TIBIA_66 are missing due to negative values of the uncertainty of individual response U_{IR}. Gray points represent the mean value of the specific measurement site.

Finally, operator experience and skills also have a strong bearing on measurement precision when using pQCT. Therefore, it is recommendable to assess the measurement uncertainty in all bed rest studies, e.g., by performing two baseline measurements. The approach serves to differentiate measurement uncertainty from biological variation between subjects (Swinton et al., 2018) and also provides better estimates of baseline values, thus, increasing the statistical power of the experiment.

Between-Subject Variation

In general, the responses toward bed rest were homogenous across studies (**Figure 2**). Turning to between-subject variation, **Figure 3** and **Table 4** clearly demonstrate that it exists, both for bone loss as well as for muscle wasting, and that between-subject variation was greater for muscle than for bone measures. In **Figure 3**, measurement uncertainty values were remarkably small for TIBIA_04, TIBIA_38 and TIBIA_66, and substantially larger for TIBIA_98 and the muscle sites. Regardless of the confidence interval width, the vast majority (89.9%) of the individual changes exceeded the interval. Notably, some individual participants showed positive values. The finding implies gains in CSA or BMC in the face of bed rest immobilization. However, such paradoxical gains were observed in the Planhab study only. The Planhab study involved only 21-day of bed rest, and average

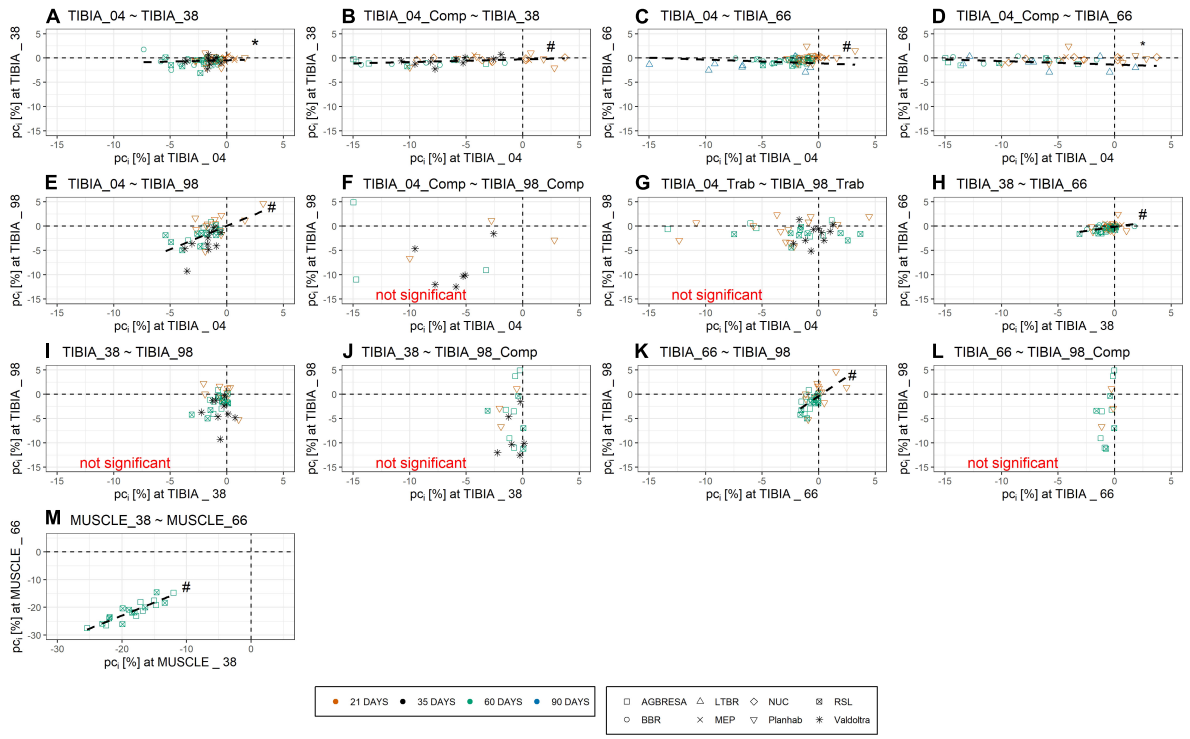

FIGURE 5 | Pearson Correlation of pc_i of **(A)** TIBIA_04 and TIBIA_38, **(B)** TIBIA_04_Comp and TIBIA_38, **(C)** TIBIA_04 and TIBIA_66, **(D)** TIBIA_04_Comp and TIBIA_66, **(E)** TIBIA_04 and TIBIA_98, **(F)** TIBIA_04_Comp and TIBIA_98_Comp, **(G)** TIBIA_04_Trab and TIBIA_98_Trab, **(H)** TIBIA_38 and TIBIA_66, **(I)** TIBIA_38 and TIBIA_98, **(J)** TIBIA_38 and TIBIA_98_Comp, **(K)** TIBIA_66 and TIBIA_98, **(L)** TIBIA_66 and TIBIA_98_Comp, and **(M)** MUSCLE_38 and MUSCLE_66. Numbers indicate the relative measurement position regarding the entire tibia length from distal to proximal. Comp and Trab indicate compact and trabecular loss, respectively. The color indicates the bed rest duration and the shape represents the study. Several outliers are not shown in the figure due to graphical reasons, but are included in the linear regression analysis. Within the muscle sites, there was a strong positive correlation. The inter-site correlation of the bone sites ranged from no correlation to low positive correlation and moderate positive correlation. Strong positive correlation indicates no within-subject variation for the muscles, no correlation or low correlation indicates within-subject variation. Dashed line represents significant correlation. * denotes significant correlation with $p < 0.01$; # denotes significant correlation with $p < 0.001$.

losses were therefore smaller than in studies with longer bed rest phases. In addition, there was only one baseline measurement in the Planhab study, which led to a less reliable baseline estimate and consequently also to a compromised reliability of the percent change. We therefore speculate that gains in bone mass and muscle CSA measurements may have been produced by a combination of small true changes in the study groups and limited reliability of individual percent changes. However, given the substantial between-subject variation observed in this study, by Scott et al. (2021) and the repeated observation of responders and non-responders to training interventions (McPhee et al., 2010; Mann et al., 2014; Hecksteden et al., 2015; Ahtiainen et al., 2016; Ross et al., 2019), blunted or even paradoxical responses to bed rest cannot be ruled out. We suggest that future bed rest studies should make further attempts at improving and unifying standard operating procedures for pQCT. In particular, two separate baseline measurements should be included whenever possible.

Figure 4 shows that the adjusted between-subject deviation (i.e., uncertainty of individual response relative to the averaged change per week for each study group) was greater for bone than for muscle. The observation is also confirmed by statistical analysis with linear mixed model, which has shown smaller adjusted between-subject deviation for muscle than for bone sites. Due to the fact that this manuscript referred to the data sets of the control groups only, it was impossible to use the already established approach of Hopkins (2015) and Atkinson and Batterham (2015), who compared the standard deviation of control and intervention group.

Within-Subject Variation

In addition to between-subject variation, the present study has also explored variation within subjects. A previous publication from the LTBR study reported significant correlation of bone losses at Tibia 66% with the other bone sites, but no such correlation among these other sites (Rittweger et al., 2005). In the present analysis of a much larger data base, these findings are replicated in that TIBIA_66 losses were correlated with losses at all other bone sites, even with the loss of compact bone tissue at TIBIA_04, but not at TIBIA_98. Yet, the present work did find correlations among the other bone sites, except between TIBIA_38 and TIBIA_98 (**Figure 5** and **Table 5**). These significant correlations are consistent with individual traits in

TABLE 5 | Correlation (p-value and r) of percent change pc_k for CSA and BMC.

Measurement site	MUSCLE_38	MUSCLE_66	TIBIA_04_total	TIBIA_04_comp	TIBIA_04_trab	TIBIA_38	TIBIA_66	TIBIA_98_total	TIBIA_98_comp	TIBIA_98_trab
MUSCLE_38	-	$p < 0.001$ $r = 0.90$	$p = 0.30$ $r = 0.25$	-	-	$p = 0.09$ $r = 0.40$	$p = 0.49$ $r = 0.17$	$p = 0.001$ $r = 0.68$	-	-
MUSCLE_66	$p < 0.001$ $r = 0.90$	-	$p < 0.01$ $r = 0.47$	-	-	$p = 0.03$ $r = 0.34$	$p < 0.001$ $r = 0.56$	$p < 0.001$ $r = 0.56$	-	-
TIBIA_04_total	$p = 0.30$ $r = 0.25$	$p < 0.01$ $r = 0.47$	-	-	-	$p = 0.006$ $r = 0.34$	$p < 0.001$ $r = 0.47$	$p < 0.001$ $r = 0.51$	-	-
TIBIA_04_comp	-	-	-	-	-	$p < 0.001$ $r = 0.51$	$p = 0.002$ $r = 0.39$	-	$p = 0.19$ $r = 0.29$	-
TIBIA_04_trab	-	-	-	-	-	-	-	-	-	$p = 0.36$ $r = -0.18$
TIBIA_38	$p = 0.09$ $r = 0.40$	$p = 0.03$ $r = 0.34$	$p = 0.006$ $r = 0.34$	$p < 0.001$ $r = 0.51$	-	-	$p < 0.001$ $r = 0.52$	$p = 0.07$ $r = 0.29$	$p = 0.54$ $r = 0.12$	-
TIBIA_66	$p = 0.49$ $r = 0.17$	$p < 0.001$ $r = 0.56$	$p < 0.001$ $r = 0.47$	$p = 0.002$ $r = 0.39$	-	$p < 0.001$ $r = 0.52$	-	$p < 0.001$ $r = 0.51$	$p = 0.62$ $r = 0.10$	-
TIBIA_98_total	$p = 0.001$ $r = 0.68$	$p < 0.001$ $r = 0.56$	$p < 0.001$ $r = 0.51$	-	-	$p = 0.07$ $r = 0.29$	$p < 0.001$ $r = 0.51$	-	-	-
TIBIA_98_comp	-	-	-	$p = 0.19$ $r = 0.29$	-	$p = 0.54$ $r = 0.12$	$p = 0.62$ $r = 0.10$	-	-	-
TIBIA_98_trab	-	-	-	-	$p = 0.36$ $r = -0.18$	-	-	-	-	-

Included were all datasets which provide data of both measurement sites. The epiphyseal measurement sites TIBIA_04 and TIBIA_98 were shown as total BMC and divided into compact (comp) and trabecular (trab) BMC. Significant correlations are marked in gray.

the bed rest responses of bone and muscle. However, all inter-bone site correlations (r between 0.34 and 0.52) were substantially weaker than the inter-muscle site correlation ($r = 0.90$). Looking at figures in **Table 5**, one might recognize a pattern of stronger correlations among diaphyseal sites (TIBIA_38 and TIBIA_66, $r = 0.52$), and of weaker correlations between epiphyseal and diaphyseal sites (r-values ranging between 0.29 and 0.51). Additionally, differentiating into compact and trabecular bone tissue at the epiphyseal bone sites showed that there were significant correlations between TIBIA_04_Cort and the diaphyseal sites, but no correlation to TIBIA_98_Cort, which could be explained by the fact that the compact bone tissue is often not thicker than the trabeculi at TIBIA_98. The comparison of the trabecular bone loss of the epiphyseal sites did not show any correlation, too. Overall, there seems to be little variability in the bed rest response within an individual's calf musculature, while the tibia's response exhibits substantial within-subject variation, even after dividing the epiphyseal measurement sites into compact and trabecular bone tissue.

Finally, it was observed that there were significant correlations between bone losses and muscle wasting, which are large for TIBIA_98 vs. either muscle sites (**Table 5**), but only moderate at best for the diaphyseal bone sites and for TIBIA_04. Bones adapt their structure to their mechanical environment (Frost, 1987; Rubin and Lanyon, 1987), and the greatest forces that bones are exposed to originate from regional muscle contractions (Rittweger, 2007). Consequently, bone strength measures typically depict large correlations with measures of muscle strength (Schiessl et al., 1998; Rittweger et al., 2000). Accordingly, previous studies had hypothesized to find correlations between individual muscle wasting and bone losses. However, such correlations never substantiated (Rittweger et al., 2005, 2010), at least at the diaphyseal and distal epiphyseal tibia sites and it had been proposed that bed rest is permissive, rather than inductive of bone loss (Rittweger et al., 2005). Large and highly significant correlations for the proximal epiphysis, which were observed in the present paper, are therefore unexpected. The question in how far the distal and proximal epiphysis differ deserves further study beyond the differences presented in this manuscript regarding adaptation of compact and trabecular bone tissue. However, the present study has much greater sample size than the aforementioned papers. Finding of a joint muscle-bone response also resonates with a recent report that habitual physical activity predicts space-flight induced bone losses (Gabel et al., 2021).

Origins of Variability

Muscles and bones fulfill mechanical roles for our organism and bed rest is foremost a model for the withdrawal of mechanical challenges. This withdrawal encompasses not only any habitual locomotor activities, but also participation in exercise and sports and it is also associated with metabolic derailments such as insulin resistance (Biensø et al., 2012). Given that there likely was substantial between-subject variation in exercise participation prior to participating in the bed rest studies, one could hypothesize that reductions in mechanical challenges varied

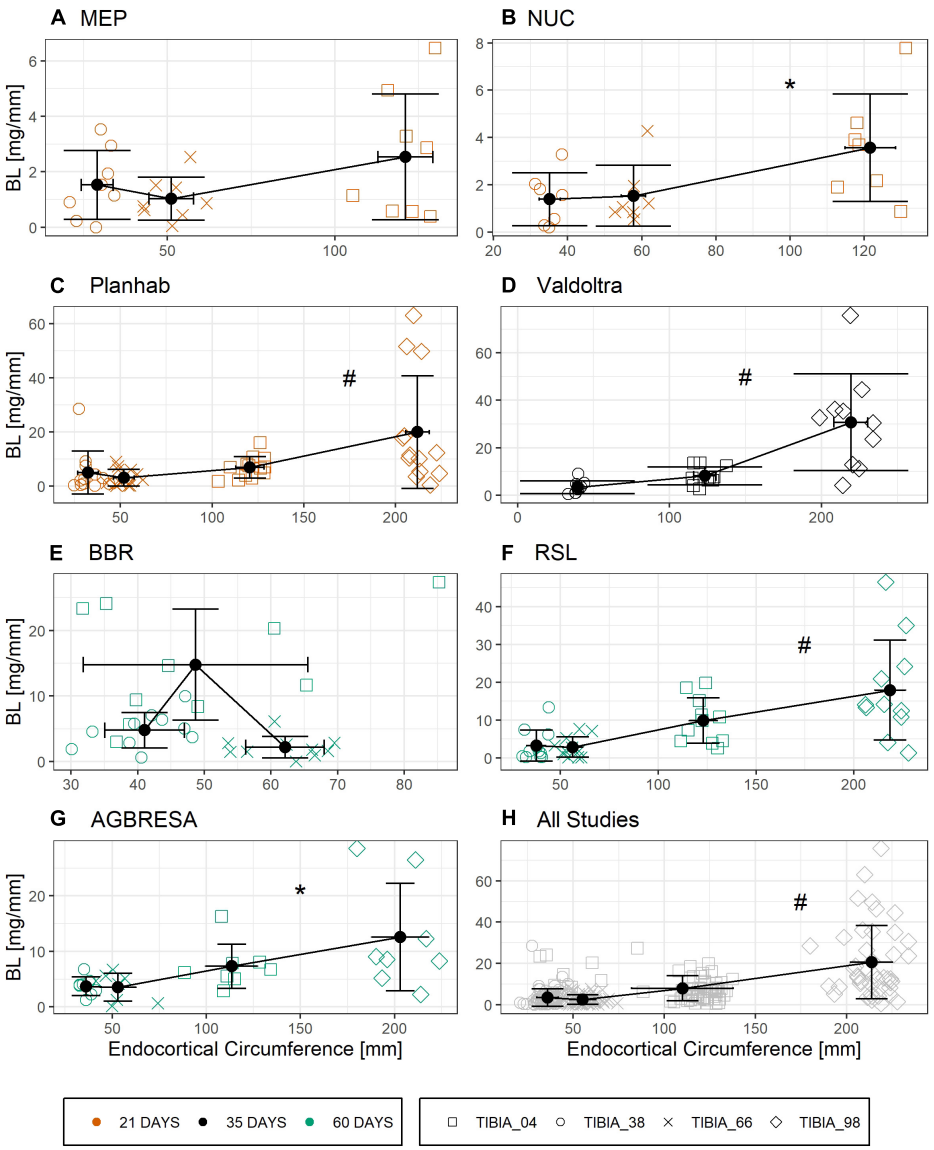

FIGURE 6 | Linear relationship of Bone Loss BL in [mg/mm] and endocortical circumference ENDO [mm] divided by study with **(A)** MEP, **(B)** NUC, **(C)** Planhab, **(D)** Valdoltra, **(E)** BBR, **(F)** RSL, **(G)** AGBRESA, and (H) all studies. The color indicates the bed rest duration and the shape represents the measurement site. Numbers in the measurement site names indicate the relative measurement position regarding the entire tibia length from distal to proximal. Presented are studies with at least three out of four bone sites. Black points are the mean value of each measurement site with error bars of BL and ENDO. Linear regression analysis showed significant associations across the different studies except for BBR and MEP. * denotes significant relationship with $p < 0.01$; # denotes significant relationship with $p < 0.001$.

between subjects, and that this variable reduction constitutes one origin of variability in bed rest response. Bed rest as well as spaceflight data support the idea (Rittweger et al., 2005; Gabel et al., 2021). Similarly, pre-study dietary habits may have varied between subjects, while the diet that is typically provided in ESA or NASA-funded bed rest studies is highly standardized. In many cases that diet will considerably deviate from the habitual intake patterns with expected effects on metabolism and adaptive processes. In addition, the well-established view of individual responsiveness to exercise interventions (McPhee et al., 2010; Mann et al., 2014; Hecksteden et al., 2015; Ahtiainen et al., 2016; Ross et al., 2019) also needs to be considered. This variable responsiveness may result from genetic and epigenetic pre-dispositions, which has been demonstrated, e.g., for the ACE ii/dd polymorphism (Montgomery et al., 1999; Valdivieso et al., 2017). Quite as much as in responses to increased mechanical and metabolic challenges, as in exercise training, genetic predispositions could also modulate the response to bed rest.

Finally, sizable variation in bone losses was observed between different tibia sites and bone tissues, but not between muscle sites. As a conclusion, changes in lifestyle like exercising and nutrition

as well as genetic predisposition may largely explain variation in muscle wasting. But obviously, there may be additional factors causing the different inter-site bone losses. Possibly, the differences between muscle and bone variation could be explained by their participation in metabolic processes. For bone, the metabolic involvement is primarily in calcium and phosphate. Of the 1,000 g calcium in the human body, only 1 g is located outside bone. Moreover, rapid calcium transients cause electrophysiological disruptions that are potentially lethal. Accordingly, serum calcium levels have to be kept within bands that are extremely narrow when considering that the bone reservoir is 1,000 times larger than the extra-osseous pool. Moreover, bone surfaces are covered by bone lining cells, which constitute a relatively small amount of biomass in relation to the huge bone mass, and whose function it is to separate the ionic milieu in bone from the other fluid spaces (Rubinacci et al., 2002; Marenzana et al., 2005). High calcium-phosphate levels in the body fluids are known to foster extra-osseous calcifications, and, sub-clinical renal calculus formation can occur in spaceflight and bed rest alike, highlighting possible limitations in phospho-calcic excretion capability (Watanabe et al., 2004). Naturally, between-subject variation can be pertinent to that capability as well. This all results in the adaptive responses in bone being relatively slow, potentially with a variable degree of individual traits. Hence, extra-osseous factors involved in the handling end excretion of calcium could constitute another important source of between-subject variation in spaceflight- and immobilized bone losses.

Another important peculiarity of bone is the cellular mechanism by which it is degraded. When active, multi-nucleated osteoclasts resorb bone in a specific space, contrasting with skeletal muscle, where protein degradation likely occurs more uniformly in all cells. Moreover, different bone turnover rates strongly differ between tissue compartments as shown for the iliac crest by Balena et al. (1992), which is not direct transferable to the tibia, but could be a clue for osseous within-subject variation. These compartment-specific differences in the bone's remodeling activity are likely the origin of the correlation between endocortical perimeter and BMC losses in **Figure 6**, thus confirming a previous finding. However, when using the ratio of endocortical perimeter to BMC as a marker of surface-to-volume ratio, all correlations with percent bone losses disappeared, indicating that bone "geometry" indicators do not predict individual losses (**Figure 7**). In addition, the linear mixed model showed that there was no association between endocortical circumference and BMC (**Supplementary Table 5**).

Preventing Worst Case Scenarios

Bed rest studies have so far focused on decrements in bone mass, muscle size etc., that were averaged within groups, and that were compared between control and countermeasure groups. This approach is straightforward to treat population means, but it could be problematic for single individuals. Provided the average bone loss in a long-term space mission at a given site is 1% per month and the between-subject SD of that loss is also 1% per month, the expected average loss is 12% during a 12-month mission. In addition, Gaussian distribution would predict that the largest bone loss in a 6-person crew will amount to $12\% + 12\% \cdot 0.967 = 23.6\%$, with 0.967 being the upper tail quantile for 1/6 of the normal distribution. Therefore, when the aim is to safeguard the strongest responder to microgravity exposure of the crew members, a better understanding of between-subject variation becomes as important as averaged effectiveness of countermeasures.

Consequences of Variability

These above findings have important implications for the design and interpretation of bed rest studies. With regards to sample size estimation, if the scientists are interested in mean effects, one way to enhance study power is to increase the number of participants. Alternatively, one might try to diminish the influence of individual variations in study endpoints by better controlling for habitual physical activity and dietary habits. These covariates are highly controlled during, but not prior to the bed rest studies. However, subject recruitment for bed rest studies is already quite a challenge and expanding the list of inclusion and exclusion criteria would certainly hamper the feasibility of such studies. Moreover, even if it was possible to fully homogenize the response to the bed rest between subjects, the same homogenization would probably not be feasible for space missions. Therefore, it might be best to monitor, rather than to control putative pre-bed rest covariates in the future. At least, the habitual daily diet and physical activity need to be assessed by a detailed questionnaire. Definitely, these aspects should be transferred to future Astronaut recruitment, increased by analysis of the genetic predisposition and hormone analysis to make full use of the current available possibilities of individual response predictions. Especially the manned missions to Moon and Mars, which lasts clearly longer than the current missions and will have a greater demand on physical health, make an intense analysis of the predisposition regarding greater individual variations in bone loss and muscle wasting imperative.

Limitations

The assessment of measurement uncertainty was limited as only three out of eight studies provided two baseline measurements and the evaluated measurement sites differed among studies. To overcome this, the authors summarized results using the most similar condition. Enhanced estimation of measurement uncertainty could be achieved by increasing the baseline measurements of the same subjects on different days without any intervention. The analyses focused only on individual data of the control groups, thus the results of this paper are just valid for participants without any additional intervention besides bed rest. A further investigation of the intervention groups is needed. The intervention groups were excluded, because the aim of the paper was to do a first exploration of the relationship of measurement uncertainty, between-subject variation and within-subject variation, thus, additional intervention next to bed rest would complicate this approach. It needs to be mentioned that some of the included data sets are from cross-over design studies (Planhab, MEP, NUC), where participants underwent additional intervention, but there were well-dosed wash-out phases between interventions. The present evidence

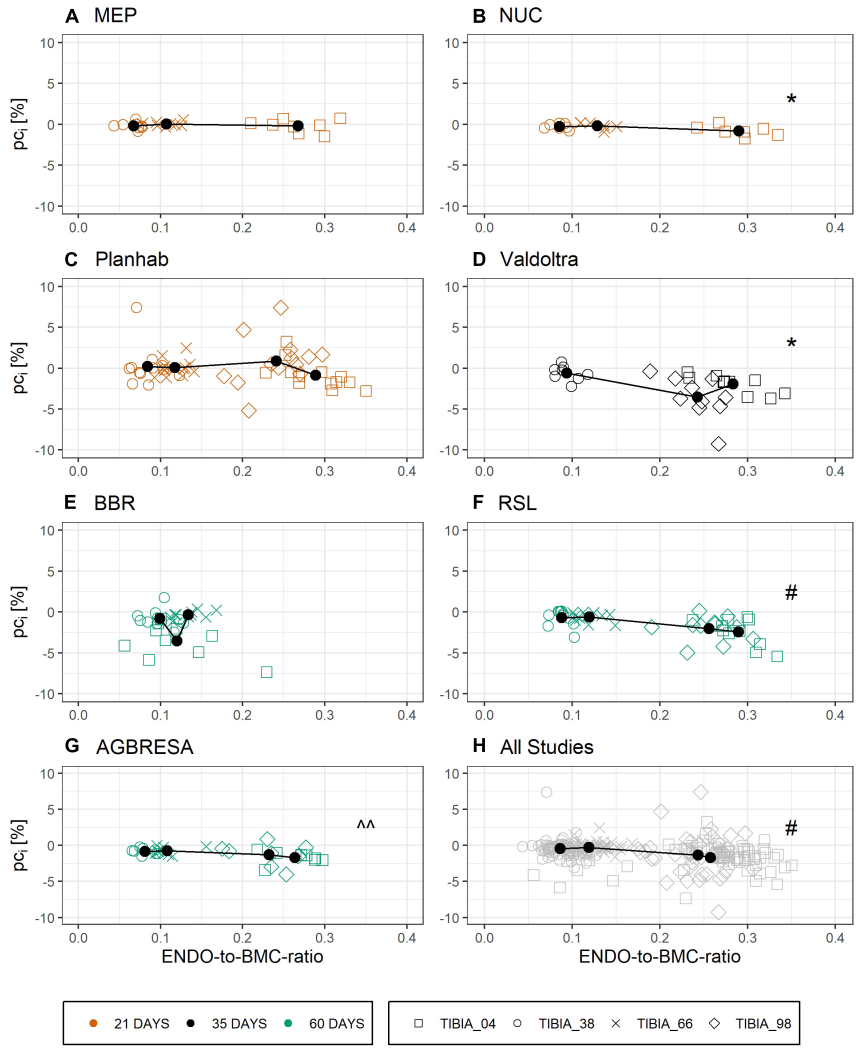

FIGURE 7 | Linear relationship of ENDO-to-BMC-ratio at baseline and the individual pc_i by study with **(A)** MEP, **(B)** NUC, **(C)** Planhab, **(D)** Valdoltra, **(E)** BBR, **(F)** RSL, **(G)** AGBRESA, and **(H)** all studies. The color indicates the bed rest duration and the shape represents the measurement site. Numbers in the measurement site names indicate the relative measurement position regarding the entire tibia length from distal to proximal. Presented are studies with at least three out of four bone sites. Black points are the mean value of each measurement site. Linear regression analysis showed significant associations across the different studies except for BBR, MEP, and Planhab. ^ denotes significant relationship with $p < 0.05$; * denotes significant relationship with $p < 0.01$; # denotes significant relationship with $p < 0.001$.

base comprises only two lower leg muscle sites and four tibia bone sites. However, obtaining data from more diverging bone and muscle sites would demand substantially greater subject time budgets, and also probably different technology such as full-size computed tomography or magnetic resonance imaging. By contrast, the strength of our data base is that it is used one single technology. Therefore, we trust that the principles of within-subject and between-subject variation in bone and muscle responses to bed rest, which have been firstly laid out here, may also apply to other anatomical sites. Yet, past studies have already shown that there is no bone loss in the upper extremity after bed rest (Hargens and Vico, 2016). Lastly, there may be additional factors affecting individual response that have not been captured.

CONCLUSION

Variation in muscle and bone responses to bed rest primarily results from between-subject and within-subject variation rather than measurement uncertainty. Nevertheless, measurement uncertainty should be considered in each data analysis, regardless of variation. It was observed that between-subject variation and within-subject variation were both lower for muscle than for bone sites. Training status, diet, and genetic predisposition may have contributed to the variation. The substantial variation in bone and muscle responses to deconditioning, be it in bed rest or during space missions, provides an impetus for a more individualized approach to countermeasure prescription.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the local ethics committees. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JB and JR created the idea of the manuscript. JB, M-TS, UM, and JR worked on the statistical approach. JR and UM provided the data of the measurements. JB analyzed the data and wrote the manuscript. JB, JR, M-TS, UM, and JJ edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.743876/full#supplementary-material

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Increased Variation in Body Weight and Food Intake Is Related to Increased Dietary Fat but Not Increased Carbohydrate or Protein in Mice

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A variety of inbred mouse strains have been used for research in metabolic disorders. Despite being inbred, they display large inter-individual variability for many traits like food intake and body weight. However, the relationship between dietary macronutrients and inter-individual variation in body weight and food intake of different mouse strains is still unclear. We investigated the association between macronutrient content of the diet and variations in food intake, body composition, and glucose tolerance by exposing five different mouse strains (C57BL/6, BALB/c, C3H, DBA/2, and FVB) to 24 different diets with variable protein, fat, and carbohydrate contents. We found only increasing dietary fat, but not protein or carbohydrate had a significant association (positive) with variation in both food intake and body weight. The highest variation in both body weight and food intake occurred with 50% dietary fat. However, there were no significant relationships between the variation in fat and lean mass with dietary protein, fat, or carbohydrate levels. In addition, none of the dietary macronutrients had significant impacts on the variation in glucose tolerance ability in C57BL/6 mice. In conclusion, the variations in food intake and body weight changes increased with the elevation of dietary fat levels.

Keywords: protein, fat, carbohydrate, mice, strain, variation

INTRODUCTION

Obesity is a major worldwide health issue. Obesity increases the risk of many chronic diseases, including type 2 diabetes, cardiovascular diseases, hypertension, and cancer (1). There is a continuous debate on how food macronutrient composition relates to body weight control (2, 3). It is still uncertain whether high-fat, high-glycemic-index carbohydrates, including sugar, low protein, or all the three macronutrients, are the cause of the elevated energy intake and obesity in humans (4–6). However, in mice, we have established that only an increased dietary fat content

was associated with an elevated energy intake and adiposity by exposing 5 different mouse strains (C57BL/6, BALB/c, C3H, DBA/2, and FVB) to 29 different diets with varying protein, fat, and carbohydrate contents (7).

Despite being inbred, mice fed with high-fat diets display large individual variations in weight gain (8–10). Several studies have indicated that different mouse strains also differ in their physiological phenotype when treated with a high-fat diet (11–14). There is debate, however, about whether specific mouse strains should be classed as obesity-prone or obesity-resistant. For example, FVB and DBA/2 mouse strains have been described as both obesity-prone and obesity-resistant by different laboratories (13, 15). The C57BL/6 mouse strain has been suggested to be the best strain for studying metabolic diseases, such as obesity and type 2 diabetes (16, 17). It is consistently described as "obesity prone"; however, it can be defined as either "diabetic prone" or "diabetic resistant" depending on which sub-strain was used (18). This strain also shows a considerable non-genetic-related variation in body weight gain when fed with a high-fat diet (19, 20). Several studies have investigated the potential mechanism related to variation in weight gain when fed with high-fat diets. Diet-induced obese mice had increased hypothalamic orexigenic and decreased anorexigenic neuropeptide gene expressions compared to diet-resistant mice when fed with a high-fat diet in the C57BL/6 mouse strain (21, 22). Furthermore, it has been recently shown that the inter-individual variability for high-fat intake in C57BL/6 mice was linked to dopamine neuron activity (23). These non-genetic variations in later-life responses to a high-fat diet seem to stem from the early-life environment of the individual mice, in particular the litter size they were raised in and hence their early-life nutritional status (15, 24).

Increased adiposity is linked to the higher risk of the development of type 2 diabetes (25). However, elevated adiposity is not inevitably linked to metabolic dysfunction (26, 27). For example, there is a population of people who have obesity but are metabolically healthy (27). In addition, it has been indicated in mice that 50% of mice became obese and diabetic, 10% lean and diabetic, 10% lean and non-diabetic, and 30% showed intermediate phenotype after being fed with a high-fat diet for 9 months (28). In our own studies, however, glucose tolerance was strongly linked to changes in body mass and fatness, but there was considerable residual variation at any given level of adiposity that was not related to the diet (29). Such variation in insulin resistance and glucose production in C57BL/6 and AKR mouse strains has been related to the differential expression of GLUT4 protein in adipose tissue (30).

All the work, thus far, on non-genetic variation in body mass and food intake has concerned the responses of mice to high-fat diets (29). It is interesting to know the extent to which the variation observed in response to high-fat diets is also observed in response to the intake of other macronutrients. Do mice, e.g., show an elevated variation in food intake and body weight when fed with low protein, or diets with high levels of high-glycemic-index carbohydrates. We have previously studied the responses of mice to a matrix of 24 different diets with varying protein, fat, and carbohydrate contents (6). This study included 5 different strains: C57BL/6 (24 diets), BALB/c, C3H, FVB, and DBA/2 (12 diets) exposed to the various diets from age 16 weeks onward for 10 weeks. We previously investigated the impact of these diets on mean body weight, food intake, hypothalamic gene expression (7), glucose tolerance ability (26, 31), and senescent cell populations in the liver (32). In the present study, we analyzed the associations between dietary macronutrient levels and individual variations of several metabolic phenotypes in different strains.

TABLE 1 | Summary of experiments performed.

Experiments	Design
Experiment 1: manipulation of dietary protein levels under fixed fat contents	a. Two series of 6 diets with varying protein level (5, 10, 15, 20, 25, and 30%) b. Series 1 had 60% fat and series 2 had 20% fat content c. C57BL/6 mice exposed to all 12 diets d. BALB/c, C3H, DBA/2, FVB mouse strains exposed to 6 diets with high fat (series 1)
Experiment 2: manipulation of dietary fat levels under fixed protein contents	a. Two series of 6 diets with varying fat level (10% to 80% and 8.3% to 66.6%) b. Series 3 had 10% protein and series 4 had 25% protein content c. C57BL/6 mice exposed to all 12 diets d. BALB/c, C3H, DBA/2, and FVB mouse strains exposed to 6 diets with 10% protein (series 3)

EXPERIMENTAL PROCEDURES

Mice and Experimental Diet

Data in the current article pertain to mice involved in a large dietary manipulation experiment, some aspects of which have already been published. These previous publications have included patterns of body weight, adiposity, hypothalamic gene expression (7, 31), and glucose homeostasis (29). All procedures in this study were reviewed and approved by the Institutional Review Board, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. C57BL/6N, DBA/2, BALB/c, FVB, and C3H mouse strains were used. C57BL/6N mouse strain was fed with 4 different diet series (series 1, 2, 3, and 4), and DBA/2, BALB/c, FVB, and C3H mouse strains were treated with 2 diet series (series 1 and 3) (**Table 1**). In the first two diet series (series 1: D14071601–D14071606, series 2: D14071607–D14071612), we fixed the level of fat 60 or 20% by energy and varied the protein content from 5 to 30% (5, 10, 15, 20, 25, and 30%, respectively) by energy. In the second two series of diets (series 3: D14071613–D14071618 and series 4: D14071619–D14071624), we fixed the level of protein at 10% (series 3) (10, 30, 40, 50, 70, and 80%, respectively) or 25% (series 4) (8.3, 25, 33.3, 41.7, 58.3, and 66.6%, respectively) by energy and varied the fat content from 8.3 to 80% by energy. For full details of the diets, refer to **Supplementary Tables S4–S8**. During the whole experimental period, mice were singly housed under controlled 22–24°C temperature and 12:12 light-dark cycle conditions. Mice were killed by rising concentrations of CO_2 for the collection of tissues and serum, which were quickly snap-frozen in liquid

nitrogen and then stored in an −80°C freezer until analysis. More information about procedures and experimental designs can be found in **Table 1** and in our previous articles [**Table 1**, (7, 31)].

Statistical Analysis

We used the coefficient of variation (CV) to express the variation in average body weight, food intake, and glucose tolerance ability of mice exposed to different diets with varying protein, fat, and carbohydrate contents. CV was calculated by dividing SD by the mean values of the dataset of body weight and food intake of each diet group. To reduce the skew, we used log transformed data. Multiple regression analysis with logged dietary protein, fat, and carbohydrate as predictors logged CV of body weight, food intake, fasting glucose level, and AUC as responses was used to analyze the relationships between predictor and responses. We also analyzed variation data using generalized linear modeling (GLM) with CV as the dependent variable, strain as a factor, and dietary levels of fat, protein, and carbohydrate contents as the covariates.

RESULTS

Variations in Body Weight and Food Intake Were Significantly Related to the Dietary Fat Content

In our previous study (6), we found no significant correlation between energy intake or body composition when the dietary protein content varied between 5 and 30%. However, an increased dietary fat content (8.3–80% fat) was associated with an elevated energy intake and adiposity up to around 50–60% fat; thereafter, there was a slight decrease. These effects were replicated at different levels in all the five mouse strains. To investigate the relationships between dietary macronutrient levels and variation in physiological traits, we used multiple regression analysis including data across all the diets with the percent dietary protein, fat, and carbohydrate contents as predictors and the CV of body weight and food intake during the last week (10th week) of dietary exposure as the dependent variables. Each strain by the diet combination generated a unique data point for the analysis. We found the CV of body weight and food intake were both significantly related to dietary fat levels ($p = 0.036$, R^2 (unadj) $= 0.07$, R^2 (adj) $= 0.07$, $\beta = 0.251$) and ($p = 0.024$, R^2 (unadj) $= 0.08$, R^2 (adj) $= 0.038$, $\beta = 0.415$, respectively) (**Figures 1B,E**), whereas there were no significant relationships between CV of body weight or food intake and dietary protein or carbohydrate content ($p > 0.05$) (**Figures 1A,C,D,F**). We also analyzed variation data using GLM with CV as the dependent variable, strain as a factor, and dietary levels of fat, protein, and carbohydrate contents as the covariates. In this analysis, we also found that CV of body weight and food intake was significantly affected by the dietary fat content ($p = 0.008$ and 0.019, respectively) but not the protein or carbohydrate content ($p > 0.05$). Furthermore, there was a significant effect of different strains on the CV of body weight and food intake ($p = 0.014$ and 0.025, respectively).

The highest average variation (CV) of body weight and food intake across all the five strains was observed at the 50% dietary fat level (CV = 10.6 and 13.3%, respectively) (**Figures 2A,B**). When we compared the variation in body weight and food intake of each strain when exposed to 50% fat, the DBA/2 and C57BL/6 strains had the highest variation (CV = 9.8 and 14.1%, respectively) (**Figures 2C,D**). However, the highest variation in different strains across different fat content diets appeared at different fat levels. The C57BL/6, BALB/c, and FVB strains had the highest variation at 50% fat (12.5, 10.9, and 12.7%, respectively), whereas for C3H mice, variation at 80% dietary fat was highest (10.1%) and for DBA/2 mice variation was highest at 40% dietary fat (14.9%) (**Figures 2E–I**).

To explore whether the variation in food intake is the cause of the variation in body weight, we also used regression analysis between these two variables. We found no significant relationship between variations in body weight and food intake ($p > 0.05$). We also used multiple regression analysis with the percent dietary protein, fat, and carbohydrate contents and variation in food intake as predictors and variation in body weight as the dependent variables, we also did not find any significant relationship between variation in food intake and body weight in this analysis ($p > 0.05$).

Variations in Fat Mass and Lean Mass Were Not Significantly Correlated With the Dietary Macronutrient Content

Body composition analysis of our previous large diet manipulation studies indicated that body fat mass and lean mass changes were the same as the changes in body weight. That is, the increasing dietary fat content up to 60% fat caused increased fat and lean contents; however, further increase in the fat content led to a slight decrease in the fat mass. To investigate whether the significant dietary fat effect on the variation in body weight was the result of the variation changes in the body fat mass or lean mass, we also used multiple regression between variation (CV) in body fat mass or body lean mass and dietary protein, fat, or carbohydrate content. We found there were no significant associations between any of the dietary macronutrients and variation in body fat mass or lean mass ($p > 0.05$) (**Figures 3A–F**).

Variations in Body Weight and Food Intake Across Different Diet Treatment Periods

The mice were treated with diets with varying content of protein, fat, and carbohydrate for 10 weeks (C57BL/6 mouse strain was treated for 12 weeks). The changing patterns of mean body weight and food intake with the alteration of dietary protein, fat, and carbohydrate contents were the same whether we used the average data over the entire 10 weeks or the average data of final weeks in all 5 strains. Therefore, we also used multiple regression to explore dietary impacts on variation in average body weight and food intake during weeks 1 and 4 of the dietary exposures. We found only the variation in body weight of 10 weeks was significantly correlated with dietary fat content ($p = 0.0326$, R^2 (unadj) $= 0.07$, R^2 (adj) $= 0.07$, $\beta = 0.251$) (**Figure 1B**). The variations in body weight after 1 and 4 weeks of dietary exposure had no significant associations with either dietary fat content or protein and carbohydrate contents ($p > 0.05$) (**Figures 4A–F**).

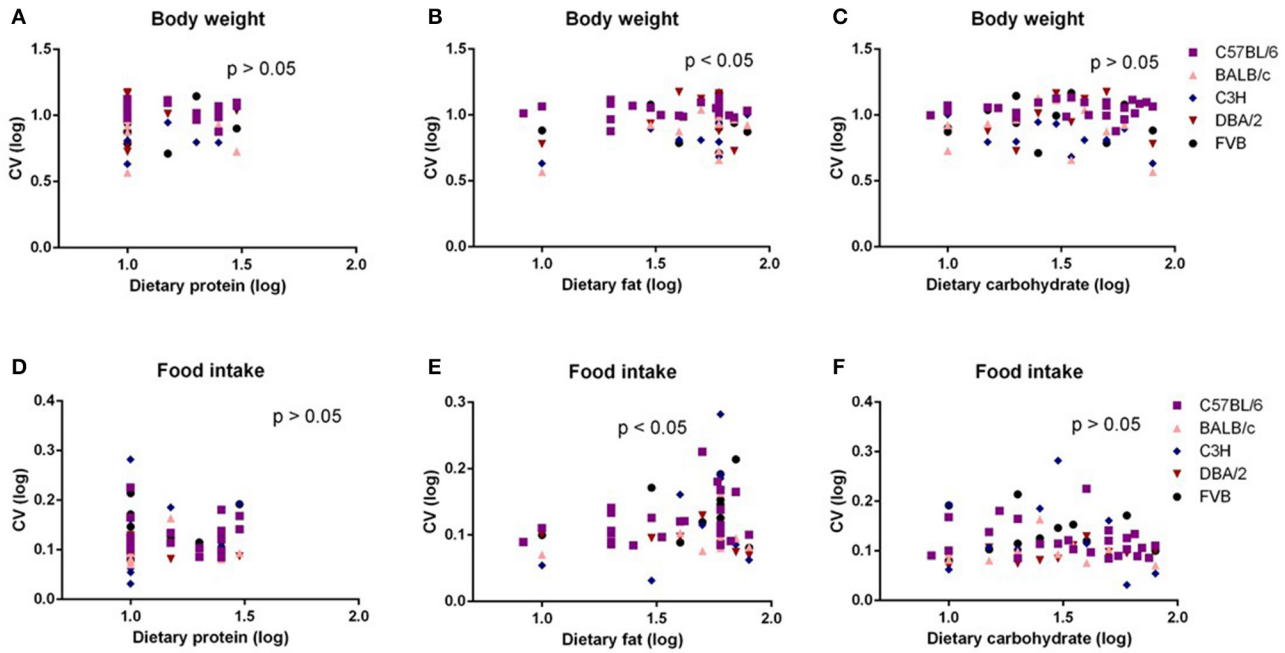

FIGURE 1 | The diagram showing the relationship between dietary macronutrient content and the coefficient of variation (CV) of body weight and food intake. The correlations between logged dietary fat, protein, and carbohydrate contents and **(A–C)** logged CV of body weight and **(D–F)** food intake of the last week (10th week) at different diet treatment periods, respectively (n = 7–21).

However, the variation in food intake during the first week of diet exposure was significantly related to the dietary fat content ($p = 0.014$, R^2 (unadj) = 0.16, R^2 (adj) = 0.12, $\beta = 0.358$) (**Figure 5B**) but not protein and carbohydrate contents ($p > 0.05$) (**Figures 5A,C**). In week 4, there were significant associations between the variation in food intake and both dietary fat and carbohydrate contents of the diets ($p < 0.001$, R^2 (unadj) = 0.18, R^2 (adj) = 0.14, $\beta = 0.516$ for fat and $p = 0.007$, R^2 (unadj) = 0.16, R^2 (adj) = 0.12, $\beta = 0.376$ for carbohydrate, respectively) (**Figures 5D–F**).

Variation in Glucose Tolerance Ability Was Not Related to the Dietary Macronutrients

In our previous study, we fed C57BL/6 mice with 29 different diets with variable macronutrients for 12 weeks, and an intraperitoneal glucose tolerance test (IPGTT) was used after 10 weeks. We found that the area under the glucose curve (AUC) was strongly associated with body fat mass, but once that effect was taken into account, AUC was not associated with different dietary macronutrients (29). In the present study, we also used correlations between the dietary protein, fat, and carbohydrate contents, and the CV in fasting glucose levels and AUC. There were no significant associations between CV of fasting glucose level or AUC and dietary protein, fat, or carbohydrate level ($p > 0.05$) (**Figures 6A–F**). Analysis of covariance (ANCOVA) with fat mass as a covariate (to remove the fat mass effect) also showed that CV of AUC was not significantly affected by dietary protein, fat, or carbohydrate content.

DISCUSSION

The C57BL/6 mice have been previously shown to display high variation in various metabolic traits when fed with high-fat diets (generally comprising 45–60% fat) (19, 20, 28, 33, 34). Consistent with these previous studies, we also found a high variation in body weight and food intake in C57BL/6 mice when fed with different macronutrient content diets, and that the highest variation in these traits occurred at the 50% dietary fat level. It has been indicated in a previous study that variations in body weight gain under a high-fat diet in male C57BL/6 mice were related to baseline fat mass, fat-free mass, and physical activity (19). A further study confirmed that the baseline fat mass and the change in energy intake on exposure to the new diet were predictors of body weight gain when fed with a high-fat diet in both male and female C57BL/6 mice (34). We have traced this baseline variation in body fatness and changes in energy intake to differentiate the nutritional environment experienced by young mice during lactation (15). Maternal milk production is constrained by the capacity to dissipate body heat (35); hence, as litter size increases, the pups must share a limited resource, and this means mice from larger litters wean with smaller body sizes and fatness, traits which persist into later life (15). Feeding the mother a high-fat diet can also affect milk production (36) and pup size/fatness at weaning.

As demonstrated here, the extent of variation among the individuals in both food intake and body weight was only related to differences in the dietary fat content. It is important to note

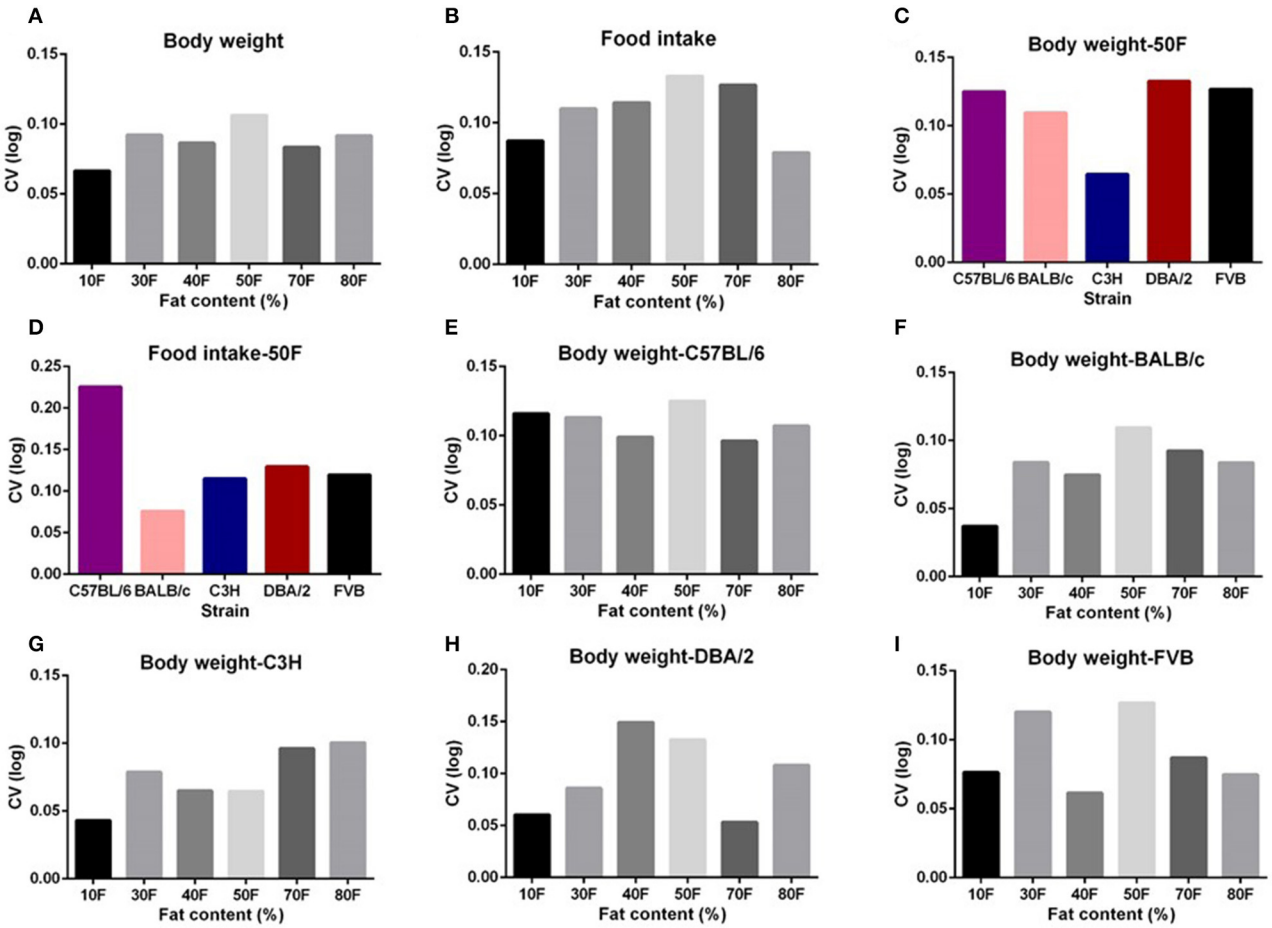

FIGURE 2 | The coefficient of variation (CV) of body weight and food intake of different strains and different diet treatment groups. The CV of body weight and food intake of **(A,B)** graded levels of fat content diet treatment group, **(C,D)** different strains ($n = 7–21$). **(E–I)** Variations in body weight gain (CV) of five different strains (C57BL/6, BALB/c, C3H, DBA/2, and FVB) when fed with 10, 30, 40, 50, 70, and 80% content fat diets, respectively ($n = 7–21$).

that because we expressed the variation as a CV, this was not simply a reflection of the changing mean levels of body weight under different exposures, which were also highest under high-fat feeding. Mice not only became on average heavier when fed with high-fat diets but also became more variable in their body weights. In contrast when exposed to, e.g., low-protein or high-carbohydrate diets, they did not become more variable in their responses. This suggests that the early-life programming in lactation seems only to prime the individuals to respond differently to dietary fat, and not to the other macronutrients. The reasons for this difference are unclear at present. It is also unclear if the early-life priming by other macronutrients (e.g., low protein or high sugar) would generate similar later-life differences in variation between individuals in response to the same macronutrients.

A variety of mouse strains have been used to study metabolic disorders. Early studies investigated the strain differences in metabolic phenotypes like weight gain and insulin resistance (10, 37–39). For example, they found that C57BL/6, DBA/2, FVB, BALB/c, and 129X1 mice are all susceptible to diet-induced weight gain when fed with a high-fat diet, but BALB/c mice displayed unchanged glucose tolerance and insulin action compared to the other strains that showed impaired glucose tolerance after fed with a high-fat diet (39). However, there is controversy about whether those mouse strains were obesity-prone or obesity-resistant. For example, FVB and DBA/2 mouse strains have been described as both obesity-prone and obesity-resistant (13, 15), whereas few studies investigated the variations in metabolic traits of different strains and their relationship to the dietary macronutrient content. It was interesting that the variations in fat mass and lean mass were not significantly associated with the dietary fat content. Therefore, the variations in body weight gain with the increase of dietary fat content cannot be explained by the variations in fat mass or lean mass alone. There were also no significant relationships between variations in body weight and food intake, which means the higher variation in food intake is not the direct factor caused by the higher variation in body weight. Therefore, the variations in both energy intake and expenditure may cause higher variations in body weight.

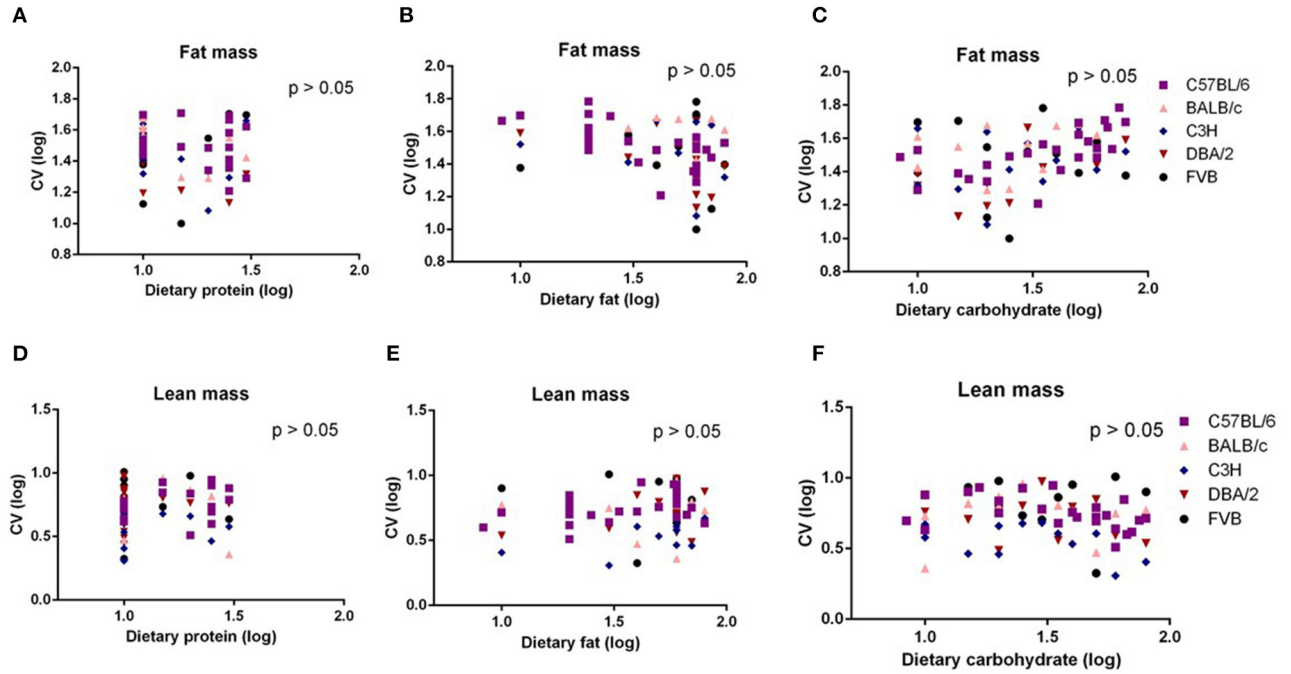

FIGURE 3 | The diagram showing the relationship between dietary macronutrient content and the coefficient of variation (CV) of fat mass and lean mass. The correlations between logged dietary fat, protein, and carbohydrate contents and **(A–C)** logged CV of fat mass and **(D–F)** lean mass of the last week (10th week) at different diet treatment periods, respectively ($n = 7–21$).

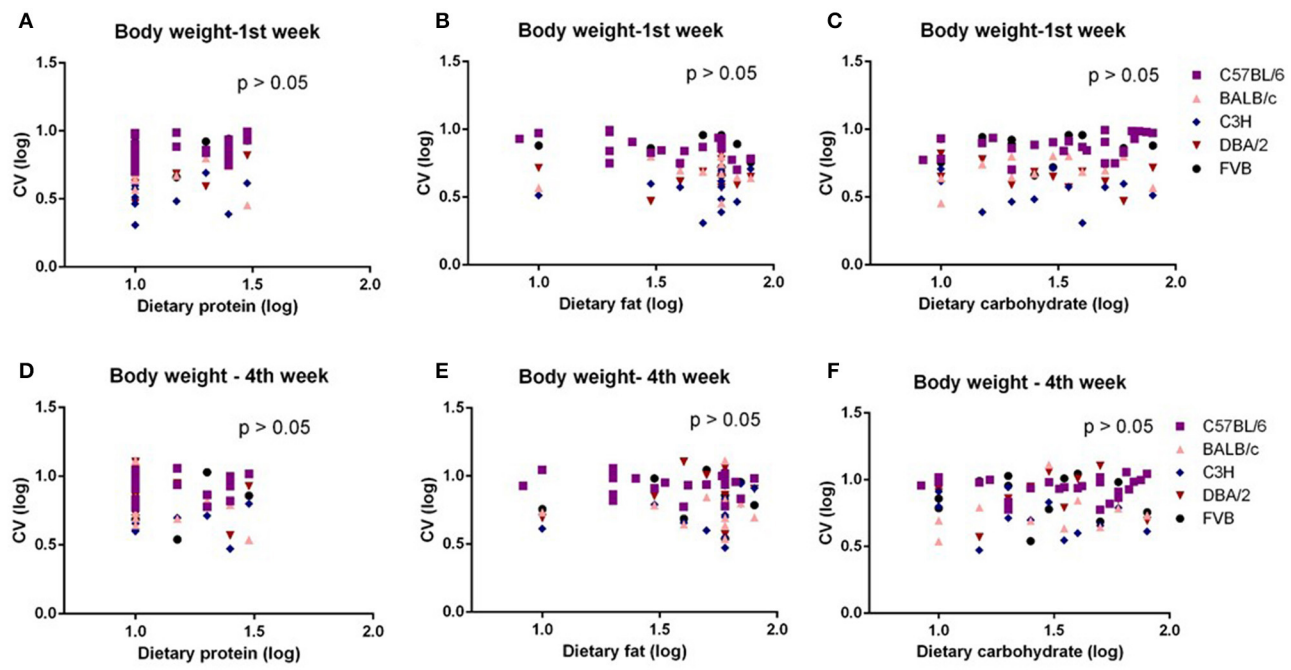

FIGURE 4 | The diagram showing the relationship between dietary macronutrient content and coefficient of variation (CV) of body weight of the 1st week and 4th week after exposure to different diets. The correlations between logged dietary fat, protein, and carbohydrate contents and **(A–C)** logged CV of body weight of the 1st week and **(D–F)** 4th week at different diet treatment periods, respectively ($n = 7–21$).

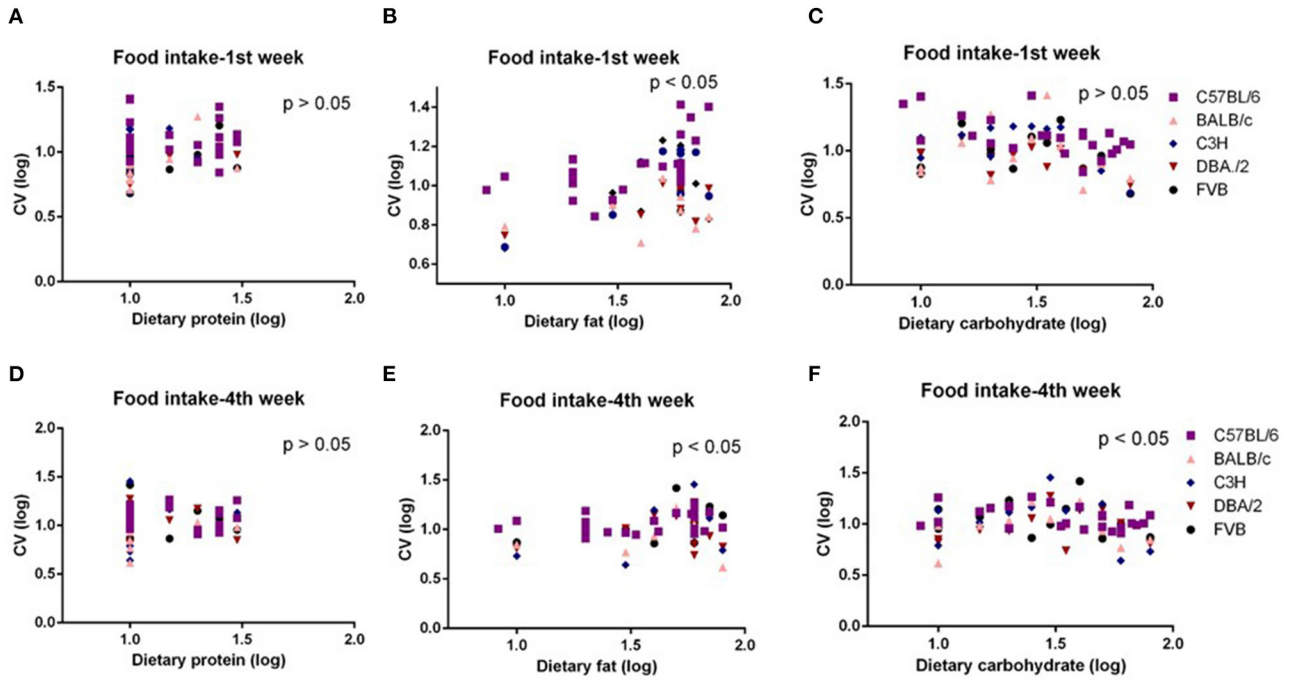

FIGURE 5 | The diagram showing the relationship between dietary macronutrient content and coefficient of variation (CV) of food intake of the 1st week and 4th week after exposure to different diets. The correlations between logged dietary fat, protein, and carbohydrate contents and **(A–C)** logged CV of food intake of the 1st week and **(D–F)** 4th week at different diet treatment periods, respectively (n = 7–21).

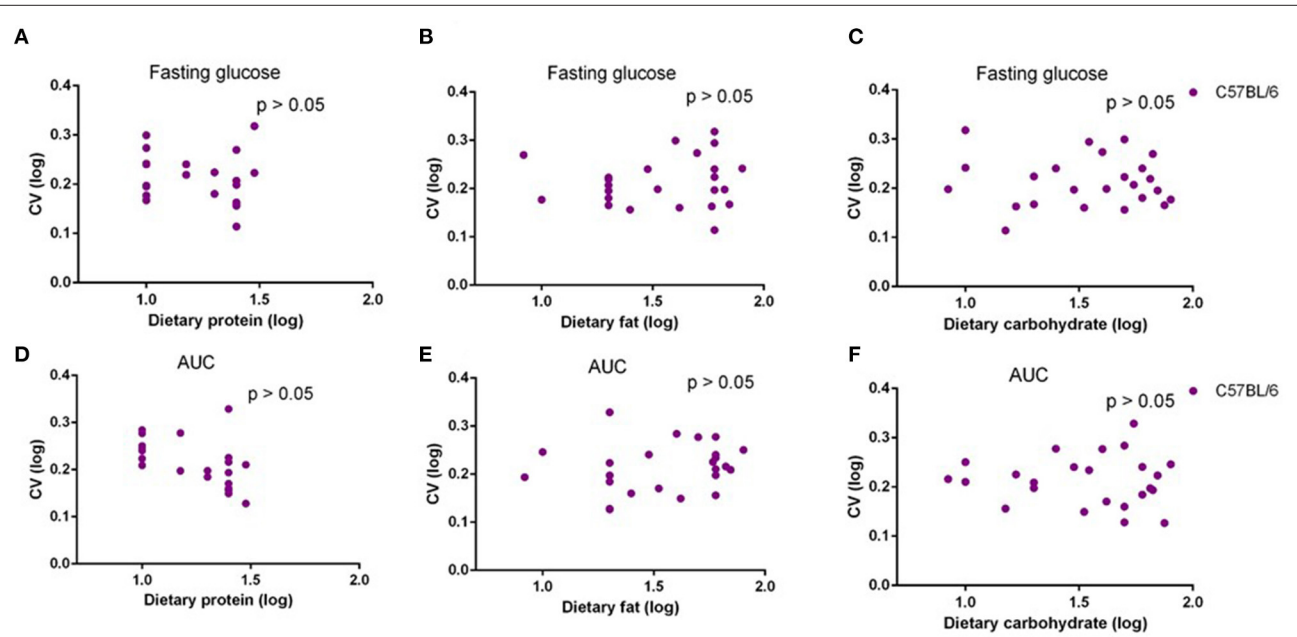

FIGURE 6 | The diagram showing the relationship between dietary macronutrient content and coefficient of variation (CV) of fasting glucose and area under the curve (AUC) of C57BL/6 mice after exposure to different diets. The correlations between logged dietary fat, protein, and carbohydrate contents and **(A–C)** logged CV of fasting glucose and **(D–F)** AUC of last week at different diet treatment periods, respectively (n = 10–11).

We found previously that only an increased dietary fat content was associated with an elevated energy intake and adiposity, and this was related to the increased gene expression in 5-HT receptors, and the dopamine and opioid signaling pathways in the hypothalamus in C57BL/6 mice (7). Because variations in body weight and food intake were both increased with an elevation of dietary fat content, the potential mechanism creating this variation may be linked to differences in these hypothalamic pathway changes (opioid and dopamine) in these conditions. Supporting this, C57BL/6 mice displayed a significant food intake variance when exposed to a high-fat diet for 4 consecutive days, and mice displaying higher-fat intake showed an increased c-Fos expression in dopamine neurons in the ventral tegmental area (VTA) compared to lower-fat intake group (23). However, there is no mechanism study to prove causality in this association. Further studies are required to elucidate the potential mechanism in the hypothalamic signaling pathway that may underlie the variation in weight gain under high-fat diet conditions.

Increased adiposity is linked to the development of type 2 diabetes (25). In the present study, we found there were no significant associations between the variations in fasting glucose level and AUC of glucose tolerance test and any of the dietary macronutrient contents. However, several previous studies reported that elevated adiposity is not linked to metabolic dysfunction (26, 27). For example, the percent of the mice that became obese and diabetic after feeding C57BL/6 mice with a high-fat (72% fat) carbohydrate-free diet for 9 months (28) included 47% of mice that became obese and diabetic, 10% lean and diabetic, 10% lean and non-diabetic, and 30% showed intermediate phenotype (28). We analyzed the percent of the mice that were diabetic or not using the previously established criteria (27). We found that under both 50% fat (higher variation occurred) and 70% fat (the same fat level as the previous study), only 10% of mice became diabetic, 50% of mice remained nondiabetic, and 40% of mice displayed an intermediate phenotype. The difference between studies was similar because the diet exposure in our study was only 10 weeks compared to 9 months earlier, indicating that progression from obesity to diabetes is time-dependent. Furthermore, it has been shown that the variations in glucose tolerance and insulin resistance are also strain-dependent as they indicated that C57BL/6 mice are more insulin-sensitive than AKR mice (30).

In summary, we demonstrated that the variations in body weight and food intake were significantly increased in relation to the elevation of dietary fat level in all the five mouse strains but not in relation to changes in the level of dietary carbohydrates and protein. Since we previously traced this individual variability to individual differences in early-life nutrition, it is unclear why such early-life experience leads to such high variability only in response to high levels of dietary fat exposure. More study to understand the basis of the non-genetic variability in responses to diets is needed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Review Board, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences.

AUTHOR CONTRIBUTIONS

YW contributed to the data collection, analyzed the data and co-wrote the manuscript. JS directed the project, conceived and designed the experiments, contributed to the analysis, and co-wrote the manuscript. DY, BL, LL, GW, LW, ML, JL, SH, CN, XZ, and YX contributed to the data collection. All the authors approved the final version prior to submission for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.835536/full#supplementary-material

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