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Original
High-protein diet with excess leucine prevents inactivity-induced insulin resistance in women / Mangogna, A., Di Girolamo, F.G., Fiotti, N., Vinci, P., Landolfo, M., Mearelli, F., Biolo, G.. - In: CLINICAL NUTRITION. - ISSN 0261-5614. - 42:12(2023), pp. 2578-2587. [10.1016/j.clnu.2023.10.028]

Availability:
This version is available at: 11368/3065359 since: 2023-12-01T13:34:30Z
Publisher:

Published
DOI:10.1016/j.clnu.2023.10.028
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08 October 2026

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Archivio della ricerca – postprint

High-protein diet with excess leucine prevents inactivity-induced insulin resistance in women

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SUMMARY

Background and aims: Muscle inactivity leads to muscle atrophy and insulin resistance. The branched-chain amino acid (BCAA) leucine interacts with the insulin signaling pathway to modulate glucose metabolism. We have tested the ability of a high-protein BCAA-enriched diet to prevent insulin resistance during long-term bed rest (BR).

Methods: Stable isotopes were infused to determine glucose and protein kinetics in the postabsorptive state and during a hyperinsulinemic-euglycemic clamp in combination with amino acid infusion (Clamp + AA) before and at the end of 60 days of BR in two groups of healthy, young women receiving eucaloric diets containing 1 g of protein/kg per day (n = 8) or 1.45 g of protein/kg per day enriched with 0.15 g/kg per day of BCAAs (leucine/valine/isoleucine = 2/1/1) (n = 8). Body composition was determined by Dual X-ray Absorptiometry.

Results: BR decreased lean body mass by 7.6 ± 0.3 % and 7.2 ± 0.8 % in the groups receiving conventional or high protein-BCAA diets, respectively. Fat mass was unchanged in both groups.

At the end of BR, percent changes of insulin-mediated glucose uptake significantly ($p = 0.01$) decreased in the conventional diet group from 155 ± 23 % to 84 ± 10 % while did not change significantly in the high protein-BCAA diet group from 126 ± 20 % to 141 ± 27 % (BR effect, $p = 0.32$; BR/diet interaction, $p = 0.01$; Repeated Measures ANCOVA). In contrast, there were no BR/diet interactions on proteolysis and protein synthesis Clamp + AA changes in the conventional diet and the high protein-BCAA diet groups.

Conclusion: A high protein-BCAA enriched diet prevented inactivity-induced insulin resistance in healthy women.

1. Introduction

Insulin resistance is one of the critical factors for the cause-effect relationship between the physical inactivity-related sedentary lifestyle [1] and the metabolic syndrome; the latest includes abnormalities of lipid pattern, arterial hypertension, increased glycemia, and endothelial dysfunction [2,3]. Experimental bed rest (BR) in healthy volunteers is an excellent model to explore the detrimental effects of muscle disuse on physiological functions [4,5]. Furthermore, *in vivo* experiments show that protein/amino acid intake modulates insulin metabolic action and β-cell function [6,7]. Although the close interactions between the metabolic pathways of carbohydrates and amino acids have been known for years, the impact of high dietary protein on glycemic control is still unclear [8,9]. Elevation of plasma amino acids after administration of an amino acid mixture acutely induces insulin resistance in skeletal muscle by inhibiting glucose transport and glucose phosphorylation in humans [10,11]. By contrast, a high protein, low-carbohydrate diet is responsible for postprandial glucose and insulin reduction, blood glucose stabilization, and improvement of dyslipidemia for individuals with type 2 diabetes or obesity [12–14].

Among all amino acids, leucine, valine, and isoleucine, also known as branched-chain amino acids (BCAAs), show unique

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effects not only as stimulators of muscle protein synthesis via activation of the mammalian target of rapamycin (mTOR) signaling pathway but also as enhancers of insulin-mediated glucose uptake [15]. In particular, leucine [16] plays multiple roles in numerous metabolic processes, including translation regulation of protein synthesis in skeletal muscle [17,18] and modulation of the insulin/phosphoinositide 3-kinase (PI3K) signal cascade [19]. Moreover, leucine is a primary nitrogen donor for alanine and glutamine production in skeletal muscle [20] and a modulator of the pancreatic β-cell concerning insulin release [21]. Through these mechanisms, leucine and the other BCAAs may directly influence insulin secretion, muscle protein synthesis, and insulin action on glucose homeostasis [15,22,23].

However, recent research has shown plasma BCAAs are elevated in obesity and type 2 diabetes mellitus, conditions characterized by insulin resistance [24–26]. In addition, several studies have demonstrated that dietary supplementation with BCAAs could be a contributing cause of impaired glucose metabolism [27–29]. Two possible mechanisms for this condition of insulin resistance are a persistent activation of the mTOR signaling pathway, uncouples the insulin receptor from insulin receptor substrate 1 (IRS) [30], and an accumulation of toxic BCAA metabolites (caused by abnormal BCAA metabolism) triggering mitochondrial dysfunction which is associated with insulin resistance [31]. In contrast, other studies reported a positive association between dietary BCAA intake and improved glucose metabolism in a large population-based cohort and obese, prediabetic subjects [32,33]. Therefore, these contradictory reports suggest an inconsistent relationship between BCAAs and glucose metabolism or its disorders, such as type 2 diabetes mellitus and obesity.

The present study aimed to assess the effects of a high-protein diet enriched with BCAA on insulin resistance and whole-body protein kinetics over two months of experimental BR in healthy young female volunteers. BR is a suitable model of experimental insulin resistance in healthy volunteers. We have assessed whole-body glucose and protein kinetics by stable isotopes before and at the end of the BR periods in the post-absorptive state and during euglycemic hyperinsulinemic clamp with amino acid infusion.

2. Material & methods

2.1. Subjects

Sixteen healthy, young female volunteers (age: 32.1 ± 4 years, body mass index: 21 ± 2 kg/m^{-2}) from the European Union were recruited for a 2-month head-down tilt position (−6°) BR to test the effects of simulated microgravity. This study was part of the 'Women International Space Simulation for Exploration" (WISE-2005), a comprehensive, international collaborative study [34–36]. It was performed in Toulouse at MEDES, the French Institute for Space Medicine and Physiology. The entire experimental protocol was approved by the *Ethical Comite Consultatif de Protection des Personnes dans la Recherche Biomedicale, Midi-Pyrenees* (France, 2005) and agreed with the declaration of Helsinki (2002). All volunteers signed an informed consent to be enrolled in the study. Routine medical and laboratory analyses, as well as a complete medical history, were collected to exclude chronic diseases. None of the volunteers was on medication. All of them were physically active before enrollment.

The study consisted of 20 days of baseline data collection in controlled ambulatory conditions, 60 days of BR, and a recovery period. During the adaptation period, volunteers were able to adapt to a standardized diet while a defined level of physical activity was monitored daily. Participants were randomly divided into two groups receiving conventional (CD; n = 8) or high-protein and BCAA (HPBD; n = 8) diets.

Being a strict BR study, volunteers maintained the 6° head-down tilt position for all activities y (including the use of rest-room facilities) throughout the BR period.

2.2. Dietary control

Six meals were served daily: three main meals (breakfast, lunch, and dinner) and three snacks. Served food was precisely weighed, and participants were asked to consume the whole meal. Energy requirements were calculated during the study periods (adaptation, BR, and recovery) for each volunteer according to the FAO/WHO equations [37–39]. While, for a tailored energy intake, the resting energy expenditure of each volunteer was determined by indirect calorimetry before BR. Body composition was measured by dual-energy X-ray absorptiometry (DXA) (Hologic QDR-2000, Waltham, MA, USA) at the end of the adaptation period and the beginning of the recovery period. Enhanced whole-body scans were analyzed to determine lean body mass (LBM). The daily energy content of diets was modified/reduced to maintain the energy balance during the BR period. Indeed, participants had a precisely prepared diet containing 1.4 times their basal metabolic rate during the ambulatory period, while the value was reduced to 1.1 times during the BR period. An additional 10 % of the total kilocalories were considered to account for dietary-induced thermogenesis [40]. To maintain energy balance, caloric intake was weekly corrected in each subject according to changes in body weight and fat mass. Due to the study design, protein intake varied between participants: eight volunteers received a tailored CD that included a protein content of 1 g/kg/d, while a HPBD containing 1.45 g/kg/d of protein and a fixed supplementation of BCAA (3.6 g/d of free leucine, 1.8 g/d of free isoleucine, and 1.8 g/d of free valine, Friliver; Bracco, Milano, Italy) was consumed by the other eight volunteers.

Based on the protein intake content, dietary fat was calculated to provide ~30 % of the total energy requirement, while the remaining energy supply was carbohydrates. Dietary energy intake was adjusted every two weeks.

For all volunteers, the maximum consumption of liquid was 60 ml/kg/d; sodium was between 1.2 and 1.6 mmol/kg/d; potassium was between 0.9 and 1.1 mmol/kg/d; calcium was 1 g/d; and phosphorus was between 1.2 and 1.6 mmol/kg/d. Folate intake was strictly monitored during all study phases at 400 μg/d, Methylxanthine (including caffeine) or alcohol were forbidden.

2.3. Metabolic test

During the last days of both the ambulatory and the BR periods, early in the morning, after a 12 h overnight fast, a metabolic study was performed in the two groups of participants, i.e., CD and HPBD. The metabolic study consisted of an infusion procedure of stable labeled isotopes. A polyethylene catheter was inserted into an antecubital vein for infusion of tracers, while a second polyethylene catheter was inserted in a wrist vein of the opposite hand for blood sample collections. This arm was heated to +50 °C to obtain arterialized venous blood. First, a blood sample was taken to determine basal enrichment of D-[6,6-^{2}H2]glucose, L-[ring-^{2}H5]phenylalanine, L-[ring-^{2}H4]tyrosine, L-[3,3-^{2}H2]tyrosine in arterialized plasma. Then, at minute 0, primed continuous infusions of D-[6,6-^{2}H2]glucose (infusion rate: 0.11 μmol/kg LBM priming dose 11 μmol/kg LBM), L-[ring-^{2}H5]phenylalanine (infusion rate 4.8 μmol/kg LBM/h, priming dose 4.8 μmol/kg LBM), L-[3,3-^{2}H2] tyrosine (infusion rate 1.2 μmol/kg LBM/h, priming dose 1.2 μmol/kg LBM) were started and maintained for 480 min. In addition, at minute 0, a single bolus of L-[ring-^{2}H4]tyrosine (0.45 μmol/kg LBM)

was administered. At minute 300 of infusion, a hyperinsulinemic-euglycemic clamp and a parallel administration of a primed continuous intravenous infusion of an amino acid solution (Vintene, Baxter, Deerfield, IL, USA) were started and continued for 180 min (i.e., until the eighth hour of tracer infusion). During the hyperinsulinemic-euglycemic clamp, regular insulin was continuously infused at a rate of 1/mU kg^{-1}/min^{-1}, while glycemia was kept constant at approximately at baseline value, constantly adjusting (10 μl blood draw every 5 min) the infusion rate of a 20 % dextrose solution. Hepatic glucose production was assessed by D-[6,6-^{2}H2]glucose as a tracer. The priming dose of the amino acid mixture (0.13 g/kg LBM^{-1}) infusion was calibrated to rapidly attain steady-state plasma phenylalanine concentrations [41,42]. Thereafter, the amino acid mixture infusion rate was maintained at 0.13 g/kg LBM/h. The amino acid concentrations reported by the manufacturer (expressed as mg/dl) were reported elsewhere [43]. The infusion rates of the unlabeled phenylalanine were 1.46 ± 0.04 and 1.49 ± 0.03 μmol/min^{-1}/kg LBM^{-1} during the ambulatory and BR phases, respectively. During the infusion protocol, blood samples were taken at 280, 290, 300 and, 460, 470, 480 min after infusion beginning for enrichment assessment in steady state. Moreover, at baseline and the end of the BR period, plasma insulin, leptin, ghrelin, C-reactive protein (CRP), glucose, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, free-fatty acids (FFA), creatinine, alanine aminotransferase (ALT), as well as aspartate aminotransferase (AST) concentrations were determined.

2.4. Analytical procedures

Analytical procedures were previously described [43–45]. Hs-CRP and plasma leptin concentrations were measured by using an enzyme-linked immunosorbent assay (Diagnostics Biochem, London, Canada, and human Leptin Quantikine kit, DLP00; R&D Systems, Minneapolis, MN, respectively). Plasma insulin and total plasma ghrelin concentrations were assessed by using a radioimmunoassay (Adaltis insulin kit; Adaltis Inc, Montreal, Canada, and Total Human Ghrelin, GHRT-89HK; Linco, St Charles, MO, respectively). Plasma glucose and lipid pattern (i.e., total cholesterol, HDL, and triglyceride) were measured by using commercially available kits (Olympus System Reagents; Olympus Diagnostica GmbH, Hamburg, Germany) and an autoanalyzer (Olympus AU400 System; Olympus, Tokyo, Japan). LDL cholesterol was calculated by using the Friedewald equation [46]. Plasma FFA concentrations were measured by enzymatic colorimetric assay (Free Fatty Acid Quantification Kit, ab65341; Abcam, Cambridge, United Kingdom). The plasma triglycerides-to-HDL cholesterol ratio was calculated as a surrogate marker for insulin resistance [47]. An enzymatic colorimetric assay assessed creatinine plasma concentrations (QuantiChrom Creatinine assay kit; BioAssay Systems, Hayward, CA, USA). Colorimetric methods allowed the assessment of ALT (ALT Alat Ls; MTI diagnostics, Idstein, Germany) and AST (AST Asat, MTI diagnostics, Idstein, Germany) plasma concentrations. As previously described, plasma amino acid concentrations (C_{AAs}) were determined by the internal standard technique [48]. Briefly, known amounts of stable isotopic tracer of each individual amino acid were added to a known plasma volume. Amino acid enrichments were determined by gas chromatography–mass spectrometry (HP 5890; Agilent Technologies, Santa Clara, CA) and expressed as tracer-to-tracee ratios. C_{AAs} were calculated as follows: $C_{AA} = C_{IS}/(E_{IS}/V_{PL})$, where C_{IS} is the concentration of the internal standard added to the plasma samples, E_{IS} is the isotopic enrichment of the internal standard, and V_{PL} is the volume of the analyzed plasma.

2.5. Glucose and phenylalanine kinetics

Whole body glucose kinetics had been determined using D-[6,6-^{2}H2]glucose according to the isotope dilution approach in steady state conditions of glucose concentrations and enrichments, i.e., during the last 30 min of the basal period and of the clamp study with amino acid infusion [42,49]. The total glucose rate of appearance (Ra) into plasma is calculated by the following standard equation: Glucose Ra = D2glucoseIR/ED2glucose, where D^2glucoseIR is the infusion rate of D-[6,6-^{2}H2]glucose and ED2glucose is the plasma enrichment, expressed as tracer-to-tracee ratio, of D-[6,6-^{2}H2]glucose. In the postabsorptive state, glucose Ra equals endogenous glucose production by the liver and other tissues. During the clamp study with amino acid infusion, glucose Ra equals the rate of endogenous glucose production plus the rate of glucose infusion. Thus, endogenous glucose production is calculated as the difference between glucose Ra and glucose infusion during the clamp to maintain euglycemia. In steady state conditions, both in the postabsorptive state and during the clamp, glucose Ra equals glucose rate of disappearance (Rd), reflecting glucose uptake in the whole body. Glucose clearance is calculated as glucose uptake divided by plasma glucose concentrations [45].

Phenylalanine is an essential amino acid. Tracing plasma phenylalanine turnover in the fasting state and during amino acid infusion allows a reliable estimation of whole-body protein turnover [43,44]. Total phenylalanine Ra into plasma is calculated by a standard equation according to the isotope dilution approach in steady state, as follows: Total Ra = IRpheD5/EpheD5, where IRpheD5 is the infusion rate of L-[ring-^{2}H5]phenylalanine and EpheD5 is the enrichment of L-[ring-^{2}H5]phenylalanine. Enrichments are expressed as tracer-to-tracee ratios. In the postabsorptive state, Ra from proteolysis equals total phenylalanine Ra. During amino acid infusion, Ra from proteolysis equals total phenylalanine Ra into plasma minus exogenous phenylalanine infusion rate. In steady state conditions, total Ra into plasma equals total Rd in both the postabsorptive state and during amino acid infusion. Total Rd equals the sum of Rd to protein synthesis and hydroxylation. Hydroxylation can be calculated as follows: Hydroxylation = [IRtyrD2/(EtyrD2/EtyrD4)]/EpheD5, where IRtyrD2 is the infusion rate of L-[3,3-^{2}H2]tyrosine, EtyrD2 is the enrichment of L-[3,3-^{2}H2]tyrosine, EtyrD4 is the enrichment of L-[ring-^{2}H4]tyrosine and EpheD5 is the enrichment of L-[ring-^{2}H5] phenylalanine. Rd to protein synthesis is calculated as total Rd minus hydroxylation.

2.6. Statistical analysis

All data were expressed as mean ± standard error (SE). We have investigated the effects of 60 days of BR in two groups of women receiving a CD (n = 8) or a HPBD (n = 8). Results of lean and fat mass, hormones, substrates, and biomarker concentrations, as well as of glucose and phenylalanine kinetics in the basal postabsorptive state (Basal) and, when appropriate, during hyperinsulinemic-euglycemic clamp with amino acid infusion (Clamp + AA) as well as change from Basal were analyzed with repeated-measures ANOVA with activity (ambulatory and BR) as within-subject factor and diet (CD and HPBD) as between-subject factor. *Post hoc* analysis was performed, when appropriate (i.e., significant activity × diet interaction), by paired *t*-test with Bonferroni's adjustment to assess the effects of BR in either group. The effects of Clamp + AA versus Basal were evaluated by paired *t*-test with Bonferroni's adjustments. All comparisons were considered significant at the conventional $p < 0.05$. Statistical analysis was conducted with SPSS statistical software (version 12; SPSS Inc, Chicago, IL).

3. Results

LBM during the two-month BR period significantly decreased by 7.6 ± 0.3 % and 7.2 ± 0.8 % in the groups receiving conventional or high-protein intakes, respectively. There was no BR/diet interaction on lean mass at the end of the 60-day BR period. There was no BR effect or BR/diet interaction on fat mass during the 60-day BR period. Results of lean and fat mass have been previously presented [36].

Table 1 shows the plasma concentrations of selected hormones, mediators, metabolites, and liver enzymes. In the basal post-absorptive state, there is an activity effect on leptin, triglycerides, HDL cholesterol, and ALT concentrations. Euglycemic hyperinsulinemic clamp with amino acid infusion significantly decreased ghrelin concentrations and free fatty acids. The triglycerides-to-HDL-cholesterol ratio, an index of insulin resistance [47], significantly increased following BR; nonetheless, the changes were significantly more significant ($p < 0.05$) in the CD group (+54 ± 17 %) as compared to the HPBD group (+17 ± 8 %).

Tracer isotopic enrichment reached a steady state in the basal postabsorptive state and during hyperinsulinemic-euglycemic clamp with amino acid infusion in ambulatory conditions and during long-term BR at CD or HPBD (Table 2). Table 3 shows the effects of BR at CD or HPBD on glucose metabolism in the basal postabsorptive state and during the euglycemic hyperinsulinemic clamp with amino acid infusion. In the basal postabsorptive state, glucose concentration, glucose appearance, and disposal rates were not significantly different in the ambulatory period and at the end of the two-month BR period, both in high and standard protein intake groups. In contrast, BR significantly affected on basal insulin concentration and glucose clearance, suggesting insulin resistance following inactivity. During the clamp study, plasma glucose concentrations were maintained at a basal level by glucose infusion at a variable rate. At the end of the clamp studies, at steady-state, plasma insulin concentrations were not significantly different in all conditions. BR significantly decreased glucose infusion rates in both the CD and HPBD groups; nonetheless, BR induced decrease of glucose infusion was greater in the CD group than that in the HPBD (−31 ± 6 % and −8±4 %; $p < 0.006$). The rates of endogenous glucose production were suppressed during the euglycemic hyperinsulinemic clamp studies with amino acid infusion in all conditions. In the CD group, insulin-mediated glucose uptake and clearance rates significantly decreased by −21 ± 6 % and −22 ± 6 % ($p < 0.05$). In contrast, these rates did not significantly change in the HPBD group (Fig. 1).

Table 4 shows whole-body protein synthesis and degradation results determined by phenylalanine kinetics. In the fasting state, whole-body protein synthesis and degradation rates were not significantly different in the ambulatory period and at the end of the two-month BR period, both in high and standard protein intake groups. Nonetheless, there was a significant BR effect on the rate of phenylalanine hydroxylation, which reflects the difference between protein degradation and synthesis in the postabsorptive state. There were no BR/diet interactions on protein kinetics and phenylalanine hydroxylation in the postabsorptive state. Following the euglycemic hyperinsulinemic clamp combined with amino acid infusion, the rates of proteolysis significantly decreased in all conditions by about 17 ± 2 %. In contrast, the rate of protein synthesis significantly increased in all conditions by about 100 ± 3 %. There were no BR/diet interactions on changes in proteolysis and protein synthesis following the euglycemic hyperinsulinemic clamp combined with amino acid infusion in the CD and the HPBD groups.

Table 5 shows significant changes mediated by amino acid mixture infusion and hyperinsulinemic-euglycemic clamp both before and after BR in plasma concentrations of leucine, phenylalanine, tyrosine, alanine, glycine, methionine, cysteine, glutamine, and glutamate.

Table 6 shows the effect of a conventional meal during BR in the two dietary regimens on selected plasma C_{AAs}. Two hours after meal, leucine concentrations are increased only in the high protein group, similarly to methionine and glutamine. Phenylalanine, tyrosine, and alanine are significantly increased in postprandial conditions both in the standard and high protein intake groups. Leucine, tyrosine, and methionine concentrations in the postprandial condition are significantly higher in the high protein group when compared to the standard protein group. Glycine cysteine and glutamate are not affected in the postabsorptive state in both groups.

Table 1
Hormones, substrates, and biomarker concentrations in the basal postabsorptive state and during hyperinsulinemic-euglycemic clamp with amino acid infusion in ambulatory conditions and during long-term bed rest at conventional or high protein-BCAA diets.

		CONVENTIONAL DIET		HIGH PROTEIN-BCAA DIET		EFFECT (p value)	
		Ambulatory	Bed rest	Ambulatory	Bed rest	Activity	Activity × diet
Ghrelin (μg/mL)	Basal	1.04 ± 0.11	1.04 ± 0.12	1.14 ± 0.14	1.04 ± 0.11	0.16	0.19
	Clamp + AA	0.72 ± 0.06[a]	0.72 ± 0.05[a]	0.85 ± 0.09[a]	0.78 ± 0.06[a]	0.25	0.16
Leptin (ng/mL)	Basal	4.7 ± 0.8	4.8 ± 0.7	5.8 ± 1.3	7.6 ± 1.5	0.017	0.073
	Clamp + AA	5.0 ± 0.9	5.0 ± 0.6	5.8 ± 1.3	7.7 ± 1.4	0.051	0.14
CRP (μg/mL)	Basal	0.92 ± 0.45	0.30 ± 0.18	0.29 ± 0.07	0.28 ± 0.09	0.22	0.25
	Clamp + AA	1.02 ± 0.53	0.25 ± 0.15	0.31 ± 0.07	0.29 ± 0.09	0.19	0.21
FFA (mmol/L)	Basal	0.58 ± 0.06	0.51 ± 0.06	0.60 ± 0.06	0.54 ± 0.04	0.14	0.80
	Clamp + AA	0.05 ± 0.01[a]	0.05 ± 0.01[a]	0.04 ± 0.01[a]	0.03 ± 0.005[a]	0.20	0.13
Triglycerides (mg/mL)	Basal	97 ± 14	120 ± 16[§]	100 ± 5	104 ± 11	0.042	0.071
HDL-cholesterol (mg/mL)	Basal	47 ± 4	39 ± 3	53 ± 2	47 ± 4	0.009	0.63
LDL-cholesterol (mg/mL)	Basal	90 ± 7	90 ± 7	100 ± 8	105 ± 8	0.23	0.24
Triglycerides/HDL-cholesterol	Basal	2.3 ± 0.6	3.3 ± 0.7	2.0 ± 0.1	2.3 ± 0.2	<0.001	0.05
ALT (U/L)	Basal	13 ± 1	18 ± 2	23 ± 4	36 ± 8	0.011	0.26
AST (U/L)	Basal	16 ± 1	18 ± 1	25 ± 3	27 ± 4	0.063	0.86

All data were expressed as means ± SE. All data were analyzed by repeated-measures ANOVA with activity (ambulatory or bed rest days) and diet (conventional or high protein-BCAA diet) as the two factors.

[a], $p < 0.05$, Clamp + AA *versus* Basal (paired *t*-test with Bonferroni's adjustment). Clamp, hyperinsulinemic-euglycemic glucose clamp; AA, amino acids; BCAA, branched-chain amino acids; CRP, C-reactive protein; FFA, free fatty acid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ALT, alanine transaminase; AST, aspartic transaminase; SE, standard error.

Table 2
Steady-state isotopic tracer enrichments in the basal postabsorptive state and during hyperinsulinemic-euglycemic clamp with amino acid infusion in ambulatory conditions and during long-term bed rest at conventional or high protein-BCAA diets.

		CONVENTIONAL DIET		HIGH PROTEIN-BCAA DIET	
		Ambulatory	Bed rest	Ambulatory	Bed rest
D-[6,6-$^2H_2$]glucose	Basal	0.0078 ± 0.0007	0.0080 ± 0.0005	0.0083 ± 0.0005	0.0083 ± 0.0005
	Clamp + AA	0.0032 ± 0.0003	0.0044 ± 0.0003	0.0039 ± 0.0005	0.0039 ± 0.0006
		6.95 ± 0.18	7.56 ± 0.19	6.90 ± 0.09	7.41 ± 0.08
L-[ring-$^2H_5$]phenylalanine	Basal	0.0865 ± 0.0022	0.0928 ± 0.0024	0.0872 ± 0.0030	0.0921 ± 0.0027
	Clamp + AA	0.0425 ± 0.0009	0.0444 ± 0.0011	0.0440 ± 0.0014	0.0462 ± 0.0014
L-[3,3-$^2H_2$]tyrosine	Basal	0.0401 ± 0.0007	0.0423 ± 0.0016	0.0418 ± 0.0014	0.0406 ± 0.0020
	Clamp + AA	0.0411 ± 0.0011	0.0402 ± 0.0016	0.0418 ± 0.0010	0.0390 ± 0.0017
L-[ring-$^2H_4$]tyrosine	Basal	0.0224 ± 0.0010	0.0242 ± 0.0007	0.0218 ± 0.0018	0.0245 ± 0.0012
	Clamp + AA	0.0245 ± 0.0006	0.0251 ± 0.0008	0.0230 ± 0.0013	0.0239 ± 0.0014

All data were expressed as means ± SE. Clamp, hyperinsulinemic-euglycemic glucose clamp; AA, amino acids; BCAA, branched-chain amino acids; SE, standard error.

Table 3
Whole body glucose kinetics, insulin, and glucose concentrations in the basal postabsorptive state and during hyperinsulinemic-euglycemic clamp with amino acid infusion in ambulatory conditions and during long-term bed rest at conventional or high protein-BCAA diets.

		CONVENTIONAL DIET		HIGH PROTEIN-BCAA DIET		EFFECT (p value)	
		Ambulatory	Bed rest	Ambulatory	Bed rest	Activity	Activity x diet
Insulin μU/L	Basal	6.0 ± 0.5	6.8 ± 0.7	4.0 ± 0.4	5.8 ± 0.5	0.005	0.25
	Clamp + AA	121 ± 13[b]	127 ± 14[b]	123 ± 10[b]	125 ± 13[b]	0.72	0.88
	Change	115 ± 13	120 ± 14	119 ± 10	119 ± 13	0.81	0.84
Glucose concentration mmol/L	Basal	5.2 ± 0.1	5.2 ± 0.1	5.4 ± 0.2	5.0 ± 0.2	0.051	0.07
	Clamp + AA	4.9 ± 0.1	4.9 ± 0.1	4.9 ± 0.2	4.9 ± 0.1	1.00	0.56
	Change	−0.3 ± 0.1	−0.3 ± 0.2	−0.5 ± 0.2	−0.1 ± 0.2	0.12	0.26
Glucose infusion [μmol/(kg LBM·min)]	Clamp + AA	43 ± 3	29±2[a]	37 ± 4	34 ± 3	<0.001	0.003
Endogenous glucose production [μmol/(kg LBM·min)]	Basal	19 ± 1	20 ± 1	18 ± 1	19 ± 1	0.09	0.73
	Clamp + AA	5 ± 6	8±3[b]	4±2[b]	14 ± 6	0.06	0.22
	Change	4 ± 5	3 ± 2	4 ± 2	−5±5	0.09	0.21
Glucose uptake [μmol/(kg LBM·min)]	Basal	19 ± 1	20 ± 1	18 ± 1	19 ± 1	0.09	0.73
	Clamp + AA	49±4[b]	37±2[a,b]	41±5[b]	48±8[b]	0.37	0.006
	Change	29 ± 4	17±2[a]	23 ± 4	29 ± 7	0.19	0.005
Glucose clearance [μmol/(kg LBM·min)]	Basal	3.7 ± 0.2	3.9 ± 0.2	3.3 ± 0.2	3.8 ± 0.2	0.009	0.21
	Clamp + AA	10.0 ± 0.9[b]	7.4 ± 0.3[a,b]	8.3 ± 1.0[b]	9.7 ± 1.5[b]	0.33	0.006
	Change	6.3 ± 0.9	3.6 ± 0.3[a]	5.0 ± 0.9	5.8 ± 1.4	0.12	0.008

All data were expressed as means ± SE. All data were analyzed by repeated-measures ANOVA with activity (ambulatory or bed rest) and diet (conventional or high protein-BCAA diet) as the two factors.
[a] , $p < 0.05$, Bed rest *versus* Ambulatory, *post hoc* analysis was performed, when appropriate, by using paired *t*-test with Bonferroni's adjustment.
[b] , $p < 0.05$, Clamp + AA *versus* Basal (paired *t*-test with Bonferroni's adjustment). Clamp, hyperinsulinemic-euglycemic glucose clamp; AA, amino acids; BCAA, branched-chain amino acids; LBM, lean body mass; SE, SE, standard error.

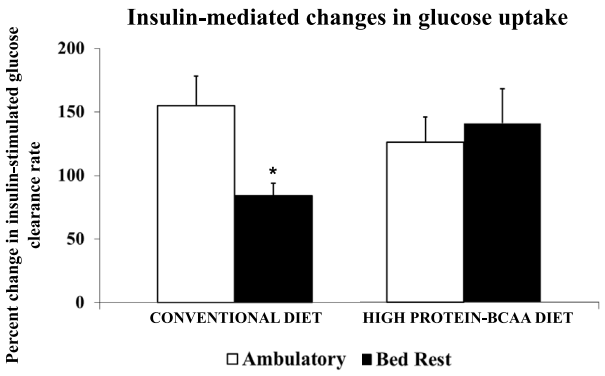

Fig. 1. Insulin-mediated changes in glucose uptake during hyperinsulinemic-euglycemic clamp with amino acid infusion in ambulatory conditions and during long-term bed rest at conventional or high protein-BCAA diets. Data were analyzed by repeated-measures ANCOVA with activity (ambulatory or bed rest) and diet (conventional or high protein-BCAA diet) as the two factors using ambulatory values as covariates. Effects: activity, $p = 0.32$; activity x diet, $p = 0.01$. *, $p < 0.01$, bed rest *versus* ambulatory, *post hoc* analysis was performed, when appropriate, by using paired *t*-test with Bonferroni's adjustment. BCAA, branched-chain amino acids.

4. Discussion

Long-term BR is an experimental model to investigate the consequences of physical inactivity in healthy volunteers, devoid of complicating factors linked to pathological conditions [2,50]. In our recent two-month study involving young women, a CD during experimental BR resulted in a substantial 7 % loss of LBM, intensified protein catabolism, and development of insulin resistance, evidenced by a diminished capacity of insulin to facilitate glucose utilization. Conversely, a high-protein diet supplemented with BCAAs, which raised leucine intake from 5 to 11 g/day, completely prevented the onset of insulin resistance due to inactivity. However, it did not mitigate the loss of LBM or protein catabolism throughout the 60-day BR period [36].

The study was designed to uphold energy balance during the 60-day BR duration in both participant groups. Energy balance was assessed by monitoring changes in fat mass as determined by DXA scans every 15 days. The initially predicted energy requirements were adjusted if individual alterations in fat mass occurred. Consequently, fat mass exhibited minimal fluctuations during the experimental period in both groups, whether on a conventional or high-protein, BCAA-enriched diet. This underscores that the beneficial effects of the high protein/amino acid diet on insulin

Table 4
Whole body phenylalanine kinetics in the basal postabsorptive state and during hyperinsulinemic-euglycemic clamp with amino acid infusion in ambulatory conditions and during long-term bed rest at conventional or high protein-BCAA diets.

		CONVENTIONAL DIET		HIGH PROTEIN-BCAA DIET		EFFECT (p value)	
		Ambulatory	Bed rest	Ambulatory	Bed rest	Activity	Activity x diet
Ra from proteolysis [μmol/(kg LBM·h)]	Basal	81 ± 3	82 ± 3	80 ± 4	81 ± 3	0.59	0.98
	Clamp + AA	71 ± 5	69±5[a]	66±6[a]	62±6[a]	0.16	0.58
	(delta from basal)	0 ± 4	2 ± 4	4 ± 4	9 ± 3	0.08	0.54
Rd to protein synthesis [μmol/(kg LBM·h)]	Basal	70 ± 3	70 ± 2	70 ± 4	69 ± 3	0.86	0.69
	Clamp + AA	140±6[a]	144±5[a]	137±6[a]	137±6[a]	0.43	0.54
	(delta from basal)	70 ± 5	74 ± 4	67 ± 4	68 ± 4	0.17	0.49
Hydroxylation [μmol/(kg LBM·h)]	Basal	11 ± 1	12 ± 1	10 ± 0.3	12 ± 1	0.003	0.18
	Clamp + AA	25±2[a]	27±2[a]	22±1[a]	25±1[a]	<0.001	0.45
	(change)	13 ± 1	15 ± 1	11 ± 0.5	12 ± 1	0.004	0.73

All data were expressed as means ± SE. All data were analyzed by repeated-measures ANOVA with activity (ambulatory or bed rest days) and diet (conventional or high protein-BCAA diet) as the two factors.
[a], $p < 0.05$, Clamp + AA *versus* Basal (paired *t*-test with Bonferroni's adjustment). Exogenous phenylalanine infusion rates during hyperinsulinemic-euglycemic clamp with amino acid infusion were as follows: conventional diet in ambulatory and bed rest conditions: 94 ± 2 and 102 ± 3 μmol/(kg LBM·h), respectively; high protein-BCAA diet in ambulatory and bed rest conditions: 92 ± 1 and 100 ± 1 μmol/(kg LBM·h), respectively. Ra, rate of appearance; Rd, rate of disappearance. Clamp, hyperinsulinemic-euglycemic glucose clamp; AA, amino acids; BCAA, branched-chain amino acids; LBM, lean body mass; SE, SE, standard error.

Table 5
Plasma amino acid concentrations in the basal postabsorptive state and during hyperinsulinemic-euglycemic clamp with amino acid infusion in ambulatory conditions and during long-term bed rest at conventional or high protein-BCAA diets.

		CONVENTIONAL DIET		HIGH PROTEIN-BCAA DIET		EFFECT (p value)	
		Ambulatory	Bed rest	Ambulatory	Bed rest	Activity	Activity x diet
Leucine	Basal	102 ± 3	110 ± 3	98 ± 4	106 ± 4	0.007	0.86
	Clamp + AA	246 ± 15[a]	291 ± 11[a]	228 ± 12[a]	232 ± 11[a]	0.002	0.009
Phenylalanine	Basal	54 ± 0.4	55 ± 1	54 ± 2	56 ± 1	0.078	0.69
	Clamp + AA	162±7[a]	163±6[a]	162±3[a]	156±5[a]	0.49	0.34
Tyrosine	Basal	47 ± 2	46 ± 3	46 ± 2	49 ± 2	0.37	0.19
	Clamp + AA	40±3[a]	45 ± 3	38±2[a]	44±3[a]	0.002	0.98
Alanine	Basal	383 ± 29	406 ± 49	369 ± 26	306 ± 34	0.14	0.005
	Clamp + AA	370 ± 57	379 ± 62	350 ± 48	365 ± 47	0.27	0.77
Glycine	Basal	312 ± 23	336 ± 27	292 ± 25	303 ± 30	0.14	0.58
	Clamp + AA	497 ± 37[a]	482 ± 47[a]	449 ± 37[a]	445 ± 47[a]	0.68	0.80
Glutamine	Basal	545 ± 25	527 ± 31	528 ± 20	521 ± 22	0.22	0.59
	Clamp + AA	497 ± 26[a]	479 ± 34	481 ± 20	486 ± 21[a]	0.59	0.33
Glutamate	Basal	78 ± 7	75 ± 5	70 ± 4	72 ± 3	0.86	0.35
	Clamp + AA	94 ± 7	83 ± 7	78 ± 4	76 ± 4	0.016	0.094

Units are μmol/L. All data were expressed as means ± SE. All data were analyzed by repeated-measures ANOVA with activity (ambulatory or bed rest days) and diet (conventional or high protein-BCAA diet) as the two factors.
[a], $p < 0.05$, Clamp + AA *versus* Basal (paired *t*-test). Clamp, hyperinsulinemic-euglycemic glucose clamp; AA, amino acids; BCAA, branched-chain amino acids; SE, standard error.

Table 6
Plasma concentrations of selected amino acids in the postabsorptive state and 2 h after a randomly chosen lunch at conventional or high protein-BCAA diets.

	CONVENTIONAL DIET		HIGH PROTEIN-BCAA DIET	
	Postabsorptive state	Postprandial state	Postabsorptive State	Postprandial state
Leucine	121 ± 6	118 ± 8	119 ± 3	243 ± 26[a,b]
Phenylalanine	57 ± 2	64±4[a]	60 ± 1	72±3[a,b]
Tyrosine	56 ± 3	64±5[a]	60 ± 1	84±6[a,b]
Alanine	412 ± 26	550 ± 33[a]	361 ± 22	614 ± 33[a]
Glycine	376 ± 38	407 ± 41	343 ± 35	346 ± 45
Methionine	47 ± 3	47 ± 4	46 ± 1	67±7[a,b]
Cysteine	217 ± 6	217 ± 8	220 ± 6	220 ± 5
Glutamine	572 ± 17	630 ± 38	541 ± 15	643 ± 26[a]
Glutamate	52 ± 2	54 ± 2	48 ± 5	56 ± 3

All data were expressed as means ± SE.
[a], $p < 0.05$, Postprandial *versus* postabsorptive state (paired *t*-test with Bonferroni's adjustment).
[b], $p < 0.05$, High protein-BCAA *versus* Conventional diet (unpaired *t*-test with Bonferroni's adjustment). BCAA, branched-chain amino acids; SE, standard error.

sensitivity were independent of changes in body fat and energy balance.

Insulin sensitivity was evaluated via the euglycemic hyperinsulinemic clamp technique. This approach aimed to simulate insulin action in a controlled, steady-state setting, mirroring postprandial conditions. Notably, the plasma leucine levels achieved during the clamp with amino acid infusion (Table 5) resembled those observed 2 h after consuming BCAA-enriched meals (Table 6).

Simultaneous infusions of glucose and phenylalanine stable isotopes allowed the examination of insulin's impact on glucose and protein kinetics.

Long-term BR reduced insulin's ability to facilitate glucose uptake and clearance in the group adhering to a CD. In contrast, the high-protein, BCAA-supplemented diet completely prevented these alterations. However, insulin effects on endogenous glucose production, mainly in the liver, were not significantly modified by BR or protein and BCAA supplementation, probably due to fat accumulation in the liver during physical inactivity [51].

In agreement with the findings on glucose metabolism, alterations in plasma lipids, specifically the triglyceride-HDL cholesterol ratio, induced by BR were effectively prevented by the BCAA-enriched, high-protein diet. Conversely, the capacity of insulin and amino acid infusions to suppress whole-body proteolysis, stimulate protein synthesis, and enhance phenylalanine hydroxylation remained unaffected by the high protein-BCAA diet.

We have recently shown that a high protein-BCAA diet was associated with early preservation of LBM within the initial 15 days of BR, which decreased over the medium and long term [36]. Consistently, whole-body protein kinetics displayed non-significant changes after the full 60 days of BR. Skeletal muscle atrophy is an outcome of extended muscle disuse [52,53], accompanied by numerous metabolic alterations, including a reduced capacity for skeletal muscle to oxidize fatty acids and an increased reliance on carbohydrates as a fuel source. In a previous inactivity study by Bilet et al., an accumulation of fat in the muscle also occurs, which may be a plausible explanation for the development of insulin resistance [54]. This state is associated with increased insulin resistance in skeletal muscle, the liver, and adipocytes [55,56]. Insulin signaling impairment significantly contributes to the insulin-resistant state observed in type 2 diabetes, obesity, and metabolic syndrome [57]. Notably, insulin resistance, along with certain metabolic features of the metabolic syndrome, appears within the initial 5–7 days of BR [58–61]. Furthermore, BR-induced insulin resistance primarily affects skeletal muscle, with limited alterations in insulin action in other organs, including the liver and adipocytes [58].

The molecular basis of insulin resistance in most patients primarily resides at the post-receptor level of insulin signaling [62]. Insulin initiates the activation of the insulin receptor tyrosine kinase, which subsequently phosphorylates and recruits various substrate adaptors, particularly the IRS family of proteins, including IRS-6 [62]. The mTOR pathway is closely interconnected with the insulin signaling cascade. While insulin activates mTOR pathway by Akt/PKB, mTORC1 hampers insulin/IGF signaling by inhibiting IRS [62]. Notably, mTOR and S6K activation occur downstream from insulin signaling and inhibit it by increasing serine phosphorylation while reducing IRS tyrosine phosphorylation [62]. Evidence demonstrates that amino acids significantly enhance phosphorylation and activate numerous proteins linked to mRNA translation (e.g., S6K1 and 4E-BP1) through the mTOR pathway [63,64]. Simultaneously, amino acids are well-established for their ability to enhance insulin's stimulation of protein synthesis through the mTOR pathway [65]. As explained earlier, this biochemical cascade is not solely involved in protein synthesis but also plays a crucial role in various biological effects of insulin, such as regulating glucose uptake [66]. Among the amino acids, BCCAs, particularly leucine, are known for their potent activation of the mTOR pathway, especially in adipocytes and skeletal muscle. mTOR can be activated through two independent pathways: the insulin-stimulated PI3K-PKB/Akt pathway and an Akt/PKB-independent pathway triggered by amino acids [67]. Consequently, mTOR is a mediator that orchestrates the interaction between amino acids and insulin signaling [65]. Furthermore, this effect can be potentiated by combining amino acid intake with physical exercise [68,69].

Elevated circulating BCAAs are positively correlated with conditions like obesity and insulin resistance. Moreover, elevated plasma levels of these amino acids can serve as predictive markers for future insulin resistance and are frequently observed in individuals with insulin-resistant obesity and type 2 diabetes [15,24–26,70–72]. These observations raise debates about whether increased BCAAs are causative factors or merely outcomes of insulin resistance [73]. Furthermore, the hypothesis that hyperactivation of mTOR pathways works as a plausible mechanism linking insulin resistance with elevated BCAAs is challenged by findings that contradict the results obtained in our study. Instead, the most likely explanation for elevated BCAAs in obesity and type 2 diabetes is impaired BCAA catabolic pathways [62,73,74] leading to BCAA buildup in the circulation [15,72]. BCAAs are enzymatically transaminated by branched-chain aminotransferase in organs such as muscle, kidney, heart, and adipose tissue, generating branched-chain α-keto acids (BCKAs). Subsequently, BCKAs are irreversibly decarboxylated by branched-chain α-keto acid dehydrogenase (BCKDH), a pivotal step in BCAA catabolism, predominantly occurring in the liver [75,76]. Decreased BCKDH activity is the primary cause of elevated BCAA levels and BCKAs, contributing to their heightened presence in type 2 diabetes and obesity. Indeed, diminished BCKDH protein expression has been observed in skeletal muscle, liver, and subcutaneous adipose tissue under conditions of insulin resistance, particularly in genetically obese rodents, diabetic rats, and diet-induced obese mice [15,77–79]. Moreover, there is evidence to suggest that BCAAs or BCKAs can trigger hyperactivation of the mTOR pathway [80,81], mitochondrial dysfunction [82], insulin resistance and/or impaired glucose metabolism [80,83–88], which are the critical factors involved in the pathogenesis of diabetes. These findings imply that excessive BCAA intake in obesity and type 2 diabetes, conditions marked by reduced BCKDH activity, leads to the overactivation of the mTOR cascade and subsequently increases the inhibition of IRS signaling. However, the precise role of this regulatory loop in developing and maintaining insulin resistance remains to be entirely determined.

Research by Guo et al. has demonstrated that chronic leucine supplementation can reduce HbA1c levels and improve glucose and insulin regulation in mouse models of obesity and diabetes [89]. Subsequent studies have supported the notion that leucine when included as a long-term dietary supplement, could enhance insulin sensitivity, and promote insulin-induced glucose transport in adipocytes of obese mice subjected to a high-fat diet, which closely mimics the most common form of insulin resistance in humans [90–97]. However, these effects were not as pronounced in adipocytes from control rats. Leucine, and potentially isoleucine, have been proposed to restore the insulin pathway, particularly at the Akt/PKB level, partially rescuing glucose transport. Notably, when rat adipocytes were exposed to wortmannin, a PI 3-kinase inhibitor that significantly reduces insulin-stimulated glucose transport, adding amino acids, particularly leucine, counteracted the inhibitor's effect. Moreover, leucine has been found to facilitate glucose uptake and enhance insulin sensitivity in skeletal muscle cells, especially in situations where the insulin-signaling pathway is impaired, particularly at the PI 3-kinase level [90–97].

These findings highlight the complex and inconsistent relationship between BCAAs and glucose metabolism in conditions like type 2 diabetes and obesity. The BCKDH enzyme activity potentially holds the key to unraveling this contradiction. Individuals with obesity or insulin resistance have reduced BCKDH activity, which increases circulating BCAAs [15,72,98]. Furthermore, insulin resistance in skeletal muscle may stem from impaired BCAA oxidation [99]. Consequently, additional BCAA supplementation could perpetuate chronic mTOR activation, mediating insulin resistance in both muscle and adipose cells. Conversely, in high-fat diet-

induced obese rats, liver oxidative deamination activity for BCKAs could intensify to compensate for reduced adipose tissue activity, thereby maintaining whole-body BCAA homeostasis [100,101].

Our results should be interpreted in the specific context of the BR model. We hypothesize that BCAAs, particularly leucine, may benefit BR-induced insulin resistance, where the insulin signaling pathway is compromised, especially at the IRS and PI 3-kinase [61]. In this context, when combined with insulin, leucine can activate Akt/PKB independently of PI 3-kinase, suggesting the potential significance of this pathway, which remains latent under normal conditions but becomes crucial when the classical PI 3-kinase cascade is impaired. Consequently, this pathway may offer an alternative route, bypassing the traditional PI 3-kinase pathway and restoring insulin-mediated glucose transport, albeit partially [91,97]. An alternative reasoning is that with the supplementation of leucine, the oxidation of BCAAs was stimulated, resulting in higher anapleurosis of the tricarboxylic acid cycle and, consequently, a high glycolysis rate [102,103].

The study's limitations are that the whole BR experimental protocol did not include an indirect calorimetry measurement, any signaling data, the phosphorylation status of critical insulin-signaling intermediates, and the small number of participants.

Our research demonstrates that a high-protein/amino acid diet, notably enriched in BCAAs such as leucine, effectively mitigates and prevents insulin resistance induced by physical inactivity in healthy women. These positive effects are not contingent on changes in body fat. This suggests that the insulin resistance encountered in the context of BR differs from that observed in obesity and type 2 diabetes, indicating the existence of additional factors that mask the protective influence of BCAA supplementation. Nonetheless, the varying results observed in long-term leucine supplementation studies may be attributed to disparities in study design. Consequently, further investigation is warranted to ascertain the optimal leucine dosage, supplementation format, and timing to achieve optimal health outcomes.

Funding statement

This work, Women International Space Simulation for Exploration (WISE), was supported by the European Space Agency and the Italian Space Agency (ASI). This study has been performed by MEDES, Institute for Space Physiology and Medicine in Toulouse, France.

Author contribution

Conceptualization: AM, and GB; Data curation: FDG; Formal analysis: GB, FDG, and NF; Funding acquisition: GB; Methodology: FDG, GB, and NF; FM, ML, and PV contributed to data interpretation; Original manuscript draft: AM, and GB; Review and editing: all. AM and GB had primary responsibility for the final content; all authors have read and approved the final manuscript.

Conflict of interest

The authors have declared that no conflict of interest exists.

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