



# Consequences of simulated microgravity on lipid metabolism in humans and effects of proteins supplementation tested as a counter measure

Floriane Rudwill

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*ÉCOLE DOCTORALE Vie et Santé*

IPHC – Département d'Ecologie Physiologie et Ethologie

**THÈSE** présentée par :

**Floriane RUDWILL**

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## Conséquences d'une simulation d'impesanteur de 21 jours chez l'homme sur le métabolisme des lipides et effets d'une supplémentation en protéines testée comme contremesure.

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« J'ai appris que l'essentiel dans la vie, ce n'est non pas d'être fort, mais de se  
sentir fort.  
Et le but c'est pas d'aller le plus loin mais c'est le chemin pour y arriver »

**Into the wild – Jon Krakauer**





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# Liste des communications et publications.

## *Communications :*

### Présentations orales:

- **What are the metabolic adaptations to a 21-d bed rest under thigh energy balance control?** Rudwill F., Bergouignan A., Chery I., Zahariev A., Gauquelin-Koch G., Simon C. Blanc S. *Waterloo, Canada, June 2014, ISGP meeting - Young researcher award -*
- **Unusual metabolic adaptations during a 21-days bed rest.** Rudwill F., Bergouignan A., Chery I., Zahariev A., Gauquelin-Koch G., Simon C. Blanc S. *Toyohashi, Japan, ISGP meeting, June 2013*

### Posters

- **Effects of different levels of physical inactivity on plasma visfatin in healthy normal-weight men.** Rudwill F., Blanc S., Gauquelin-Koch G., Chouker A., Heer M., Simon C., Bergouignan A., *New Orleans, LA. ASGSR meeting Nov 2012*
- **Dietary resveratrol prevents the metabolic, bone and muscle adaptations to simulated microgravity in the rat.** Rudwill F., Stevens L., Desplanches D., Picquet F., Momken I., Blanc S., Gauquelin-Koch G. *San José, CA. ASGSR & ISGP meeting Nov 2011.*

## *Publications :*

- **Effect of enforced physical inactivity induced by 60-d of bed rest on hepatic markers of NAFLD in healthy normal-weight women.** Rudwill F., Bergouignan A., Gastebois C., Gauquelin-Koch G., Lefai E., Blanc S., Simon C. *Liver international 2014 Nov. in press*
- **A combination of whey protein and potassium bicarbonate supplements during head-down-tilt bed rest: Presentation of a multidisciplinary randomized controlled trial (MEP study)** Buehlmeier J., Mulder E., Noppe A., Frings-Meuthen P., Angerer O., Rudwill F., Biolo G., Smith S. M., Blanc S., Heer M. *Acta Astronautica 2013, 95, 82-91.*
- **Effects of different levels of physical inactivity on plasma visfatin in healthy normal-weight men** Rudwill F., Blanc S., Gauquelin-Koch G., Chouker A., Heer M., Simon C., Bergouignan A., *Applied Physiology Nutrition and Metabolism 2013 June; 38(6):689-93. doi: 10.1139/apnm-2012-0434.*
- **Physical inactivity as the culprit of metabolic inflexibility: evidence from bed rest studies** Bergouignan A., Rudwill F., Simon C., Blanc S.. *Journal of Applied Physiology (1985). 2011 Oct; 111(4):1201-10. doi: 10.1152/jappphysiol.00698.2011. Review.*

- **Resveratrol prevents the wasting disorders of mechanical unloading by acting as a physical exercise mimetic in the rat** Momken I., Stevens L., Bergouignan A., Desplanches D., Rudwill F., Chery I., Zahariev A., Zahn S., Stein T P., Sebedio J. L., Pujos-Guillot E., Falempin M., Simon C., Coxam V., Adrianjafiniony T., Gauquelin-Koch G., Picquet F., Blanc S. *The FASEB Journal*. 2011 Oct; 25(10):3646-60. doi: 10.1096/fj.10-177295
- **Urinary C-peptide is not an accurate bioindicator of energy balance in humans** Bergouignan A., Habold C., Rudwill F., Gauquelin-Koch G., Normand S., Simon C., Blanc S. *Obesity* 2011, 20(3), 683-688. doi: 10.1038/oby.2011.116
- 

### *Publications en préparation :*

- **Consequences of 21-days of simulated microgravity on lipid metabolism in humans and effects of protein supplementation tested as a countermeasure** Rudwill F., Bergouignan A., Chery I., Zahariev A., Gauquelin-Koch G., Simon C., Blanc S. *in prep*
- **Enterocytes de novo lipogenesis after 21-days of severe physical inactivity.** *in prep*
- **Effects on different levels of physical activity on rhythms of dietary lipid oxidation.** *in prep*

# Abréviations

ACACB : Acetyl-CoA carboxylase 2  
ACADM : Acyl-coenzyme A deshydrogenase  
ACAA2 : Acetyl-CoA acyltransferase 2  
ACOX2 : Acyl-CoA oxidase 2  
ALT : Alanine aminotransferase  
ANGPTL3 : Angiopoietin-like protein 3  
aRED : advanced Resistive Exercise Device  
AST : Asparate aminotransferase  
AUC : Area under the curve  
BDC : Baseline data collection  
BM : body mass  
CEVIS : Cycle Ergometer with Vibration Stabilisation System  
CK18 : Cytokeratin-18  
CPT1 : Carnitine palmitoyltransferase 1  
CM : Chylomicron  
COX4l1 : Cytochrome c oxidase  
CRP : C-reactiv protein  
DGAT2 :Diacylglycerol O-acyltransferase 2  
EB : energy balance  
FAT/CD36 : Fatty acid translocase / cluster of differenciation 36  
FATP1 : Fatty acid transport protein 1  
FoxO3a : Forkhead box O3  
FFM : fat free mass  
FM : fat mass  
GAPDH : Glyceraldehyde-3 phosphate deshydrogenase  
GC/MS : Gas chromatography/mass spectrometry  
GLUT4 : Glucose transporter type 4  
GGT : Gamma-glutamyl transferase  
GSK3B : Glycogen synthase kinase 3 beta  
HDL : High density lipoprotein  
HDT : Head down tilt  
HOMA-IR : Homeostasis model assessment – insulin resistance  
iRED : interim Resistive Exercise Device  
IRS1 : Insulin receptor subunit 1  
IU : International unit  
LBM : Lean body mass  
LDL : Low density lipoprotein  
LPL : Lipoprotein lipase



MAP2K1 : Mitogen-activated protein kinase kinase 1  
MAPK1 :Mitogen-activated protein kinase 1  
MJ : Megajoules  
NAFLD : Non-alcoholic fatty liver disease  
NEFA : Non-esterified fatty acids  
OGTT : Oral glucose tolerance test  
PFKM : Phosphofructo kinase muscle  
PGC1 $\alpha$  : Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha  
PIK3R1: Phosphoinositide-3-kinase, regulatory subunit 1  
PIK3R2: Phosphoinositide-3-kinase, regulatory subunit 2  
PKB : Protein kinase B  
pPKB : Phosphorylated protein kinase B  
PRKCA: Protein kinase C, alpha  
qPCR : quantitative polymerase chain reaction  
R : Recovery  
RMR : Resting metabolic rate  
SAT : subcutaneous adipose tissue  
SEM : Standart error of the mean  
SPE : Solid phase extraction  
TG : Triglycerides  
TVIS : Treadmill with Vibration Isolation and Stabilisation System  
VLDL : Very low density lipoprotein

# Table des matières

Communications :	5
Présentations orales:	5
Posters	5
Publications :	5
Publications en préparation :	6
<b>Abréviations</b>	<b>7</b>
<b>Préambule</b>	<b>13</b>
1. <i>La conquête spatiale</i>	19
a. Les premières avancées dans la recherche spatiale	19
b. Les premières collaborations internationales	19
c. L'émergence de l'Asie dans l'exploration spatiale.	20
d. L'implication des compagnies privées dans l'exploration spatiale.	21
e. Le futur de l'exploration spatiale	21
2. <i>Les défis de la bioastronautique et les modèles d'étude.</i>	23
a. Adaptations à la microgravité	23
b. Les modèles d'étude au sol.	25
i. Simulation de la microgravité chez l'Homme	25
1. Alitement prolongé	25
2. Immersion sèche	27
ii. Simulation de la microgravité chez l'animal	29
1. Animaux suspendus	29
2. Cultures cellulaires	30
3. <i>La microgravité réelle et simulée : impact sur les fonctions physiologiques.</i>	31
a. Système neurophysiologique	31
b. Système immunitaire	31
c. Système cardiovasculaire	32
d. Système musculo-squelettique	33
e. Adaptation métabolique	37
4. <i>Programmes de contremesures : les solutions mises en place lors des études de microgravité réelle et simulée</i>	49
a. Gravité artificielle	49
b. Contremesures : exercices physiques	51
c. Contremesures nutritionnelles	54
i. Supplémentation en vitamines et antioxydants	55
ii. Supplémentation en protéines	57
5. <i>Objectifs et hypothèses</i>	62
a. Projet d'étude : objectifs	62
b. Hypothèses	63
<b>Etude 1 : Variation de la concentration plasmatique en visfatine après différentes interventions sur les niveaux d'activité physique chez l'homme.</b>	<b>67</b>
a. Design of the studies	67
b. Body composition	68
c. Physical activity energy expenditure	68
d. Fasting plasma visfatin, insulin and glucose concentrations	68
e. Statistical analysis	69
<b>Etude 2 : Impact de 60 jours d'inactivité physique sévère chez la femme sur l'évolution de marqueurs d'une stéato-hépatite.</b>	<b>71</b>
a. Study design	71
b. Body composition	71
c. Metabolic and hepatic markers measurements	71

d. Statistical analysis	72
-------------------------	----

**Etude 3: Conséquences d'une simulation d'impesanteur de 21 jours chez l'homme, sur le métabolisme des lipides et effets d'une supplémentation en protéines testée comme contre mesure.**

	73
a. Study design	73
b. Nutrition	76
c. Experimental protocols	77
i. Body composition and organ fat content	77
ii. Lipid trafficking and oxidation	77
iii. Glucose tolerance	78
d. Analyses and calculations	79
i. Organ fat content and regional fat depot	79
ii. Energy expenditure, total and dietary substrates oxidation	80
1. d <sub>31</sub> palmitate oxidation	80
2. 1- <sup>13</sup> C acetate oxidation	81
3. d <sub>7</sub> glucose oxidation during oral glucose tolerance test	82
4. Muscle and liver insulin resistance index	82
5. Energy expenditure and total substrates oxidation	82
iii. Dietary lipid trafficking	83
1. Separation of lipoproteins	83
2. Extractions of lipid fractions	83
3. Analyses and calculations of lipoproteins and NEFA enrichment in labeled and unlabeled palmitate.	84
iv. Molecular biology	84
1. Microfluidic real-time polymerase chain reaction (microfluidic RT-PCR)	84
2. Western Blot	85
v. Inflammation and oxidative stress	86
vi. Plasma hormones and metabolites	87
e. Statistics	87

**Etude 1: Variation de la concentration plasmatique en visfatine après différentes interventions sur les niveaux d'activité physique chez l'homme**

91

**Etude 2 : Impact de 60 jours d'inactivité physique sévère chez la femme sur l'évolution de marqueurs d'une stéato-hépatite.**

97

**Etude 3: Conséquences d'une simulation d'impesanteur de 21 jours chez l'homme, sur le métabolisme des lipides et effets d'une supplémentation en protéines testée comme contre mesure.**

105

**Summary**

105

a. Body mass and composition changes	108
i. Organ fat content and regional fat depot	111
b. Inflammation	112
c. Fasting metabolic variables during bed rest	114
d. Oral glucose tolerance	116
i. Muscle and liver insulin sensitivity index	116
ii. Exogenous d <sub>7</sub> glucose oxidation	116
e. Energy expenditure, total and dietary substrates oxidation	118
i. Energy expenditure and total substrates oxidation	118
ii. Plasma metabolic variables during test day	121
iii. Dietary d <sub>31</sub> palmitate oxidation	123
iv. 1- <sup>13</sup> C Acetate oxidation	125
v. Dietary lipid trafficking	126
1. d <sub>31</sub> palmitate enrichment and concentration in chylomicrons.	126
2. d <sub>31</sub> palmitate enrichment and concentration in VLDL.	129
3. d <sub>31</sub> palmitate enrichment and concentration in NEFA	131
f. Molecular biology	134

i. RT-PCR	134
ii. Western Blot	137
g. Correlations	138
<b>Perspectives</b>	<b>150</b>
<b>References</b>	<b>152</b>
<b>Annexe 1 : Resveratrol prevents the wasting disorders of mechanical unloading by acting as a physical exercise mimetic in the rat</b>	<b>169</b>
<b>Annexe 2 : A combination of whey protein and potassium bicarbonate supplements during head-down-tilt bed rest: Presentation of a multidisciplinary randomized controlled trial (MEP study).</b>	<b>182</b>



# Préambule

Les programmes scientifiques en recherche spatiale se sont développés autour de missions incluant des séjours de courtes durées. Dans le futur, ils tendront cependant à s'allonger avec la mise en place de projets plus ambitieux, en vue de l'exploration planétaire vers la Lune ou Mars. En biologie et physiologie, ces programmes se concentrent sur les adaptations qui affectent les performances et la santé des astronautes. Radiations, isolement et confinement, et adaptations biologiques liées à l'absence de gravité contribuent tous, à divers degrés, à la réduction des capacités des astronautes. Depuis le début des vols spatiaux, la perte osseuse et l'augmentation du risque de fractures, la diminution de la masse et de la force musculaire, le déconditionnement cardio-vasculaire ainsi qu'une réduction de la capacité à l'exercice, sont les adaptations qui ont attiré le plus l'attention. Au cours de la dernière décennie, des dérégulations du métabolisme ont été mises en évidence. Cette étude tardive du métabolisme est sans nul doute liée à sa complexité de mise œuvre au cours des vols spatiaux. De ce fait, l'atteinte métabolique a été majoritairement évaluée au sol au cours d'études de simulation de la microgravité, telles que l'alitement prolongé à tête déclive chez l'homme. Ces atteintes métaboliques affectent tous les systèmes physiologiques et ont été suggérées comme associées au développement d'un stress chronique. Parmi ces altérations, on retrouve notamment une inflammation de bas niveau, une diminution de la sensibilité à l'insuline, une diminution de l'oxydation des lipides au profit des glucides, une hypertriglycérémie ainsi qu'un stockage ectopique des lipides. Ces principaux troubles résulteraient de l'inactivité physique sévère, à laquelle sont confrontés les astronautes, et induite par la microgravité. Plus précisément, l'atteinte du métabolisme lipidique est caractérisée par une diminution de la clairance des lipides alimentaires, à l'origine d'une hypertriglycérémie et implique probablement le muscle et le foie. La réduction de la capacité oxydative des lipides est associée quant à elle, à leur stockage ectopique dans divers organes, tels que le muscle squelettique, cardiaque ou la moelle osseuse. De récentes études en pathologie humaine montrent que le stockage ectopique de lipides pourrait être responsable d'une résistance à l'insuline à travers de nombreux effecteurs cellulaires potentiels. Le stockage ectopique en tant que cause de la résistance à l'insuline observée au cours d'études au sol et en vol, apparaît donc comme une hypothèse qu'il convient de tester.

Au-delà de ces aspects fondamentaux, des programmes, appelés contremesures, visant à réduire et ou prévenir les conséquences négatives des vols spatiaux sur le corps humain, sont testés depuis le début des missions humaines, leur but étant de permettre à la fois le succès des missions et un retour sur Terre des astronautes dans les meilleures conditions. Dans la mesure où l'inactivité physique a depuis longtemps été considérée comme causale dans les adaptations à l'impesanteur, les contremesures à différents type d'activité physique, aérobique et résistive, font partie

intégrante des missions habitées. Ces dernières se sont avérées cependant peu efficaces. Plus récemment, un intérêt croissant pour les contremesures nutritionnelles s'est développé. En effet, des résultats prometteurs sur la supplémentation en protéines ou en acides aminés essentiels ont été obtenus chez l'homme et l'animal. Elle jouerait notamment un rôle favorable dans le maintien de la masse musculaire, sur le métabolisme lipidique ou encore la sensibilité à l'insuline. Ces résultats restent tout de même discutés du fait notamment de l'acidose, induite par l'augmentation de protéines ingérées. Celle-ci accélérerait notamment la déminéralisation osseuse, mais pourrait être évitée par un ajout de sels alcalins. D'un autre côté, il faut souligner que l'effet d'une supplémentation en protéines a jusqu'à présent été montré uniquement dans des expérimentations où le groupe contrôle ingérait 0.8 g/kg/jour de protéines, alors que des études récentes indiquent des apports minimaux de 1.2 g/kg/jour dans ces circonstances. L'impact de la nature des protéines données en supplémentation, notamment de leur richesse en acides aminés ramifiés, reste également l'objet de débats.

C'est dans ce contexte que s'inscrit ce travail de thèse. Nous nous sommes intéressés aux mécanismes responsables des dérégulations métaboliques induites par la simulation d'impesanceur. Nous avons tout d'abord rétrospectivement étudié les effets de différents niveaux d'inactivité physique sur l'évolution de marqueurs plasmatiques de stockage ectopique et d'inflammation de bas niveau. Ensuite, le travail principal de cette thèse a consisté à étudier le devenir métabolique des lipides alimentaires, en relation avec leur stockage dans différents organes, et l'insulino-résistance lors d'un alitement de 21 jours chez l'homme. L'ensemble de ces dérégulations a été étudié dans le cadre d'un apport protéique journalier de base de 1.2g/kg/jour. Au cours de cette étude, nous avons également testé les effets d'une supplémentation en protéines, amenant à un apport protéique de 1.8g/kg/jour, combinée à une alcalose induite par une supplémentation en bicarbonate de potassium, dans la prévention de ces dérégulations métaboliques. Cette contremesure est constituée plus précisément de protéines du petit lait, composées principalement d'acides aminés ramifiés, (leucine, isoleucine et valine) qui sont des acides aminés essentiels considérés comme de meilleurs stimulants de la synthèse protéique et de la rétention d'azote que les autres acides aminés.

Au cours de ce travail de thèse, l'introduction présentera les enjeux de l'exploration spatiale et décrira les dérégulations physiologiques dues au passage à la microgravité, en se focalisant sur les adaptations métaboliques et les contremesures actuelles, aussi bien en vol que sur des modèles au sol.

Après la présentation des objectifs et de l'approche expérimentale, les résultats seront organisés autour de trois sous-chapitres.

- 1) L'impact de différents niveaux d'inactivité physique induite, sur la concentration plasmatique en visfatine.

- 2) L'impact de 60 jours d'inactivité physique sévère sur l'évolution de marqueurs du développement d'une stéato-hépatite.
- 3) Les conséquences physiologiques d'une simulation d'impesanteur de 21 jours chez l'homme sur le métabolisme lipidique et d'une supplémentation en protéines, testée comme contremesure.

Enfin, ces résultats seront suivis par une discussion aboutissant aux perspectives des futures études de microgravité simulée.





# Introduction

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# 1. La conquête spatiale

## *a. Les premières avancées dans la recherche spatiale*

Dans l'histoire de la conquête spatiale, les années cinquante font figure de point de départ. En effet, à l'issue de la seconde Guerre Mondiale, l'utilisation des technologies allemandes en matière de missiles, ont permis à deux pays, les Etats-Unis et l'URSS, de se démarquer. Ces deux puissances se sont concentrées sur le développement de nouvelles technologies dans le but de permettre à l'homme d'explorer l'espace. Dans les années qui suivirent, les russes furent les premiers à placer un satellite artificiel en orbite, Spoutnik 1 en octobre 1957, suivi un mois plus tard de Spoutnik 2 qui fut le premier vol spatial habité par un être vivant, la chienne Laïka. Les américains suivirent peu de temps après avec le premier satellite américain Explorer-1. C'est en 1961, que Youri Gagarine, un astronaute russe, fut le premier homme à réaliser un vol orbital habité. A ce stade, l'URSS présentait un certain avantage face aux Etats-Unis dans la course à l'espace, jusqu'en 1969, date à laquelle, l'homme posa un pied sur la Lune en la personne de l'astronaute Neil Armstrong, lors de la mission Apollo 11. A la suite de ces premières missions, un bon nombre de questionnements ont été soulevés quant aux répercussions de la microgravité sur la santé des astronautes. En effet, bien que les séjours spatiaux aient été de courte durée, certains astronautes ont présenté différents problèmes liés aux changements d'environnement, responsables de modifications physiologiques et pathologiques (Armed Forces et al., 1961). A l'issue de ces premières années d'explorations, les deux pays durent réduire le nombre de missions spatiales du fait de leur coût. A partir de cette période, les deux puissances ont dès lors cherché à développer des moyens plus pérennes de rester dans l'espace par le développement de stations spatiales. Ces dernières permettent d'étudier les impacts réels de la microgravité sur la santé des astronautes tout en continuant les recherches sur l'utilisation de nouvelles technologies.

## *b. Les premières collaborations internationales*

Les deux nations leaders ont très vite compris l'enjeu capital d'une collaboration en vue de diminuer le coût des missions spatiales et surtout de maximiser leurs chances de réussite et de découverte. La première station spatiale fut réalisée par les Russes, Saliout en 1970, suivie par les Américains avec Skylab en 1973. Les programmes spatiaux à bord de ces deux stations incluaient plusieurs expérimentations de suivi des altérations physiologiques, physiques et psychologiques (Leach, 1981). La plus grande avancée dans les collaborations internationales fut réalisée avec le projet Mir, mis en route entre 1986 et 1996. La station actuellement en place est également le fruit d'une collaboration entre plusieurs pays européens, le Canada, le Japon, la Russie et le Brésil, lui donnant son nom actuel de Station Spatiale Internationale (ISS). De cette collaboration sont nés plusieurs projets à buts spécifiques aussi bien en biologie, qu'en physique ou en technologie. Certains de ces projets seront présentés

dans les paragraphes suivants. Ainsi, en septembre 2014, plus de 200 individus ont vécu à bord de la station, chacune des nations participatives contribuent au développement et à l'amélioration des conditions de vie des habitants à bord.

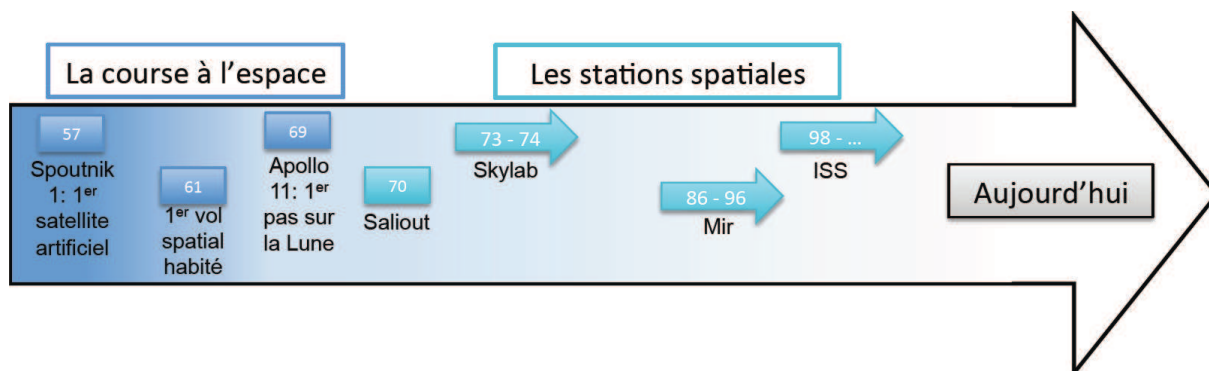


Figure 1. Chronologie de la conquête spatiale : les dates clés de la course à l'espace de 1957 à 1969 et des stations spatiales de 1970 à aujourd'hui.

### *c. L'émergence de l'Asie dans l'exploration spatiale.*

La collaboration entre les divers pays pionniers dans la recherche spatiale a permis de grandes découvertes. Cependant, aujourd'hui, ces pays ne sont plus les seuls à s'intéresser à ce sujet. En effet, de nombreux éléments restent encore à apprendre et à comprendre de l'exploration spatiale. Parmi les pays émergents, certains sont de plus en plus impliqués dans la recherche spatiale. En première ligne, se trouve la Chine. Cette puissance économique montante évolue petit à petit dans le milieu avec le lancement de son premier satellite en 1970 et le premier vol habité en 2003. Elle s'est ainsi retrouvée en 2013, le troisième pays à financer par ses propres fonds, un engin spatial, Yutu, qui s'est posé sur la Lune. La Chine est présente dans l'espace, mais également au sein des études de microgravité simulée chez l'homme avec un nombre rapidement croissant d'études au cours des dernières années.

L'Inde est le second pays émergent qui s'est démarqué cette dernière décennie. Grâce à de nombreuses collaborations, notamment avec l'agence spatiale française (CNES), les premiers projets ont vu le jour dans les années soixante pour aboutir au lancement de leur premier satellite (1975), puis de la mise en œuvre de leur premier lanceur (1993) et enfin de la première sonde spatiale vers la Lune (2008). A notre connaissance, aucune étude de microgravité simulée n'a été entreprise à ce jour par ce pays, limitant de ce fait l'accès à certaines données physiologiques importantes.

Aujourd'hui, malgré leurs progrès croissants, la Chine et l'Inde ne possèdent pas encore les outils suffisants pour atteindre le niveau d'expérience des nations qui ont été leaders dans le domaine de l'astronautique depuis des décennies. Cependant, force est de constater que la conquête de l'espace se déplace vers l'Asie et permet le développement de nouvelles collaborations entre les nations.

#### *d. L'implication des compagnies privées dans l'exploration spatiale.*

Aujourd'hui, les agences spatiales nationales ne sont plus les seules à s'intéresser à la conquête spatiale. En effet, plusieurs compagnies privées se sont lancées le défi de concurrencer les leaders dans le domaine. De plus, pour des raisons évidentes de coût, les agences spatiales nationales ont dû se diversifier et ont permis aux entreprises privées de s'imposer. Ainsi, en décembre 2010, la compagnie Space X fut la première à envoyer un engin spatial dans l'orbite basse de la Terre. En 2012, le cargo Dragon fut également le premier engin ravitaillant l'ISS à être entièrement financé par une société privée. Les missions futures de Space X se profilent également vers la planète Mars, avec le projet Red Dragon, une sonde du même type que Dragon. D'autres compagnies privées se partagent également le monopole du tourisme de l'espace telle que Virgin galactic, XCOR Aerospace ou encore Golden Spike Company. Ces compagnies proposent notamment d'emmener des touristes en vol suborbital dans une zone à plus de 100km d'altitude autour de la Terre.

Dans une optique plus utopique, l'un des derniers projets lancés par une compagnie privée est le projet Mars One, qui consiste en un aller simple vers la planète rouge, et l'établissement d'une colonie dès 2024.

Le tourisme de l'espace, bien qu'en plein essor, ne possède pas encore les capacités suffisantes pour réellement concurrencer les agences spatiales. En revanche, sur certains points, tels que le financement des missions, ces compagnies privées possèdent une avance non négligeable et pourraient accroître leur présence dans l'espace dans un futur proche.

#### *e. Le futur de l'exploration spatiale*

Actuellement, les missions à bord de l'ISS représentent la majorité des missions humaines dans l'espace au sein desquelles les astronautes réalisent différentes études mises en place par les scientifiques. Les projets réalisés ont pour but d'apporter des éléments de réponses aux questions posées aux cours de recherches sur Terre, mais également de préparer les futures missions spatiales, en prévenant les différents problèmes de santé auxquels sont sujets les astronautes.

Aujourd'hui, les agences spatiales nationales, mais également plusieurs compagnies privées sont impliquées dans l'exploration spatiale avec des projets de plus en plus ambitieux. Malgré la diminution des fonds alloués, la conquête spatiale reste une des priorités des nations impliquées avec d'importants enjeux géopolitiques. En effet, beaucoup de nations impliquées dans la conquête spatiale ont développé des projets, actuellement en place, ou sur les années, voire les décennies à venir avec l'exploration de Mars comme l'un des projets phares. À l'heure actuelle, seuls des robots ou sondes se sont approchés ou posés sur la planète rouge. Quelque peu essoufflée, en 2004 la ferveur pour la découverte de Mars a pris un regain avec

Curiosity, un robot développé par la NASA en collaboration avec des scientifiques de plusieurs pays. Il étudie et apporte encore aujourd'hui un bon nombre d'informations sur la composition de ses sols, ou encore l'atmosphère de Mars. Curiosity offre des photos des paysages martiens, permettant de comprendre un peu plus son histoire, sa formation et peut être la présence de vie antérieure. Plus récemment, la sonde Maven, développée par la NASA, a apporté ses premiers clichés sur la composition de l'atmosphère martienne. Une seconde sonde spatiale, MOM, cette fois-ci envoyée par l'Inde est quant à elle entrée dans l'atmosphère fin septembre 2014. Dans un futur proche, aux Etats-Unis, la NASA a planifié la capture d'un astéroïde, qui sera placé en orbite autour de la Lune. La décennie suivante sera également l'occasion de mettre en place des habitations sur Mars et de permettre à l'homme d'étudier au plus près la planète. En parallèle, une mission qui prendra place en 2016 et 2018 et qui réunit l'ESA et Roscosmos, ExoMars, devrait apporter des éléments de réponses quant à l'éventualité de la présence de vie par le passé sur Mars.

C'est dans ce contexte d'exploration spatiale que la durée des vols, actuellement de quelques mois, va s'allonger. Il devient donc urgent de prévenir au maximum l'impact de la perte ou de la diminution de la gravité exercée sur les astronautes et de développer des contremesures efficaces pour y parvenir. Les scientifiques en ont fait une priorité et se concentrent ainsi sur l'impact de la microgravité réelle ou simulée prolongée sur la physiologie humaine.

## 2. Les défis de la bioastronautique et les modèles d'étude.

### *a. Adaptations à la microgravité*

Depuis les premiers vols spatiaux, plusieurs conséquences physiologiques et pathologiques de ces vols ont été décrites chez les astronautes. Tout d'abord, sur terre, la composition de l'atmosphère permet une protection contre les radiations émises par le soleil. Dans l'espace, en revanche, cette barrière est absente et expose ainsi les hommes à une dose plus importante de radiations (Crucian et al., 2014). L'impact réel des radiations sur l'organisme est à considérer à court et à long terme (Petrov, 2002). En effet, d'après des études récentes, l'exposition prolongée aux radiations en condition de microgravité peut exposer les astronautes à des risques plus importants comme celui de contracter une maladie infectieuse. Ce risque serait multiplié par deux au cours des vols (Todd et al., 1999). Toutefois, les risques réels de l'exposition aux radiations restent difficilement évaluables, notamment ceux liés à une dose aiguë de radiations, telle que celle induite par des éruptions solaires par exemple (Durante, 2008). Sur Terre, il est bien connu qu'une exposition prolongée favorise le développement de maladies telles que des cancers (Einstein et al., 2007; Sodickson et al., 2009). Néanmoins, les données acquises au cours des missions spatiales ne permettent pas d'établir ce risque de façon précise. Afin de protéger au maximum les astronautes des effets indésirables des radiations, plusieurs stratégies ont été proposées : réduire la distance avec la source de radiation, réduire le temps d'exposition et/ou créer une protection efficace. La solution la plus simple à mettre en œuvre reste de développer une protection efficace contre les rayons cosmiques, en utilisant des matériaux présentant le meilleur compromis entre qualité de la protection, poids, disponibilité de la ressource et coût (Durante, 2008; Shavers et al., 2004). Dernièrement, des données sur les risques d'exposition aux radiations sur Mars ont été publiées grâce aux informations envoyées par la sonde Curiosity. A la lueur de ces données, il apparaît évident qu'avec les moyens de protection en place actuellement, l'exposition des astronautes aux radiations au cours de leurs futures missions martiennes dépasserait les seuils limites et entraînerait des risques importants pour la santé des astronautes (Hassler et al., 2014; Zeitlin et al., 2013).

L'exposition aux radiations ne représente pas la seule contrainte imposée par les vols en orbite. Le retentissement psychologique est un élément important à prendre en compte lors de l'évaluation des risques liés aux missions spatiales. Le confinement et l'isolation créés par les conditions de vie à bord de l'ISS peuvent, en effet, représenter un risque important pour la santé mentale des astronautes. Les collaborations entre les pays les amènent à travailler avec des sujets issus d'autres nations et, de ce fait, à s'adapter aux us et coutumes de chacun. Outre ces changements, ils sont également sujets à l'isolement, à une diminution de la vie



sociale, à la diminution des contacts avec leur famille et amis. Des études réalisées au cours des vols spatiaux, notamment dans Mir ou plus récemment à bord de l'ISS, rapportent des changements dans l'humeur ou un stress chez les astronautes ("Stress Challenges and Immunity in Space," 2011) tout comme les études de confinement et d'isolement réalisées sur Terre (Ishizaki et al., 2002; Seaton et al., 2009). Certaines différences sont toutefois à noter entre les deux modèles. Tout d'abord, les études réalisées sur Terre incluent des individus, certes triés sur le volet, mais avec des critères concernant le profil psychologique qui sont moins stricts que lors de la sélection des astronautes. Ces derniers sont souvent d'anciens militaires ou pilotes dotés d'une capacité d'adaptation importante. De plus, l'excitation créée par les missions spatiales pourrait jouer un rôle de prévention, en comparaison avec les missions réalisées sur Terre que cela soit dans la station d'étude Concordia en Antarctique ou dans les cliniques spatiales. L'ensemble de ces données souligne l'importance des préparations psychologiques des astronautes au stress, au changement d'environnement, à l'ennui et à l'isolement. Les méthodes de préparation, actuellement appliquées par l'ensemble des partenaires internationaux participent à la cohésion des équipes au sein de l'ISS. Enfin, la nutrition joue également une part importante dans l'équilibre psychologique des individus (Lane et al., 2002). En effet, une nutrition adaptée aux besoins énergétiques va permettre de maintenir et prévenir le niveau de fatigue, et de ce fait le stress des astronautes. L'humeur sera également impactée par la qualité de l'alimentation, sans oublier l'impact favorable de l'échange et du partage de moments conviviaux avec les autres habitants de la station spatiale durant les repas (Cena et al., 2003).

Outre l'impact psychologique, l'environnement microgravitaire va avoir un effet important sur les fonctions physiologiques de l'organisme. En effet, la perte de gravité va induire deux principaux changements: l'inactivité physique sévère induite par l'absence de force s'exerçant sur les structures de soutien du corps et une altération de la répartition des fluides corporels. Ces deux conséquences de l'environnement microgravitaire vont provoquer une adaptation du corps humain qui se traduit par un déconditionnement cardiovasculaire, une atrophie musculaire, une perte de la densité osseuse et des troubles métaboliques. Ces différentes adaptations physiologiques sont détaillées dans le chapitre suivant.

L'adaptation des astronautes au passage à l'environnement microgravitaire, aussi bien psychologique, physique que physiologique va conditionner le succès des missions spatiales. Il est ainsi nécessaire d'en connaître les mécanismes, afin de les prévenir au mieux. A cause des difficultés d'accès aux données en vol, du fait de la complexité de mise en place des protocoles ou du matériel nécessaire, il est vite apparu primordial de développer des modèles d'études plus accessibles. De ce fait, des modèles au sol sur l'homme et l'animal ont rapidement été développés dès les premiers voyages spatiaux.

## *b. Les modèles d'étude au sol.*

Les modèles de simulation de la microgravité sur Terre recréent les deux principales conséquences de l'absence de gravité sur l'organisme : l'inactivité physique et la redistribution des fluides corporels. Cette section présente les principaux modèles, humain et animal, de microgravité simulée.

### i. Simulation de la microgravité chez l'Homme

#### 1. Alitement prolongé

Chez l'Homme, les premières simulations de la microgravité ont été faites par immersion dans l'eau, afin de recréer cette sensation de « flottement » retrouvé dans l'espace. Il s'est très vite avéré que ce modèle présentait plus d'inconvénients que d'avantages, du fait de la difficulté d'étanchéité des appareils utilisés par exemple. Ce n'est que dans les années 60 qu'un parallèle entre les symptômes dont souffrent les astronautes et ceux retrouvés chez les sujets soumis à un alitement et une inactivité physique forcée, suite à des blessures ou une maladie par exemple, a été suggéré (Deitrick et al., 1948). Les premières études d'alitement ont cependant très vite démontré qu'une composante non négligeable des effets de l'impesanceur était manquante contrairement à l'immersion : le changement de répartition des fluides corporels. Ainsi c'est à partir d'une simple observation, que les Russes ont proposé chez l'homme, un modèle d'étude à tête déclive reproduisant la répartition thoraco-céphalique des fluides, caractéristiques des effets de la microgravité. Plusieurs degrés d'inclinaison ont été testés (-15°, -10°, -5°), jusqu'à arriver à une inclinaison de -6°, aboutissant au modèle d'étude chez l'homme encore utilisé à l'heure actuelle (Figure 2 et 3). A ce jour, ce modèle reste le plus utilisé et le plus simple à mettre en place. L'alitement prolongé, bien que ne reproduisant pas tous les effets de la microgravité, présente tout de même les principales caractéristiques d'un environnement dépourvu de gravité. Au niveau psychologique, le développement d'un stress tel qu'il est noté lors des vols spatiaux est également retrouvé au cours des études au sol (Ishizaki et al., 2002; Seaton et al., 2009). Les conditions environnementales, telles que la pression, la température ou le rythme circadien ne sont, en revanche pas reproduites à l'identique. Elles sont tout de même régulées et contrôlées au quotidien. Enfin, lors des premiers jours de vols, les astronautes sont très souvent sujet au « mal de l'espace ». Bien que les symptômes ne soient pas totalement équivalents à ceux retrouvés dans l'espace, les individus alités sont également sujets pour certains à des désordres tels que des maux de tête ou des troubles de l'orientation (Clement et al., 2004).

Enfin, plusieurs altérations physiologiques sont retrouvées : diminution du volume plasmatique, de la masse et de la force musculaire, de la densité osseuse ou encore de la sensibilité à l'insuline (Bergouignan et al., 2009; Pavy-Le Traon et al., 2007; Stein et

al., 2005). Ainsi, malgré ces quelques difficultés à recréer l'environnement exact dans lequel vivent les astronautes, l'alitement prolongé à tête déclive de 6° reste le modèle le plus adéquat chez l'homme permettant de simuler l'impact de la microgravité et de tester les contremesures nutritionnelles et/ou physiques sur des durées variables et dans des conditions expérimentales contrôlées.

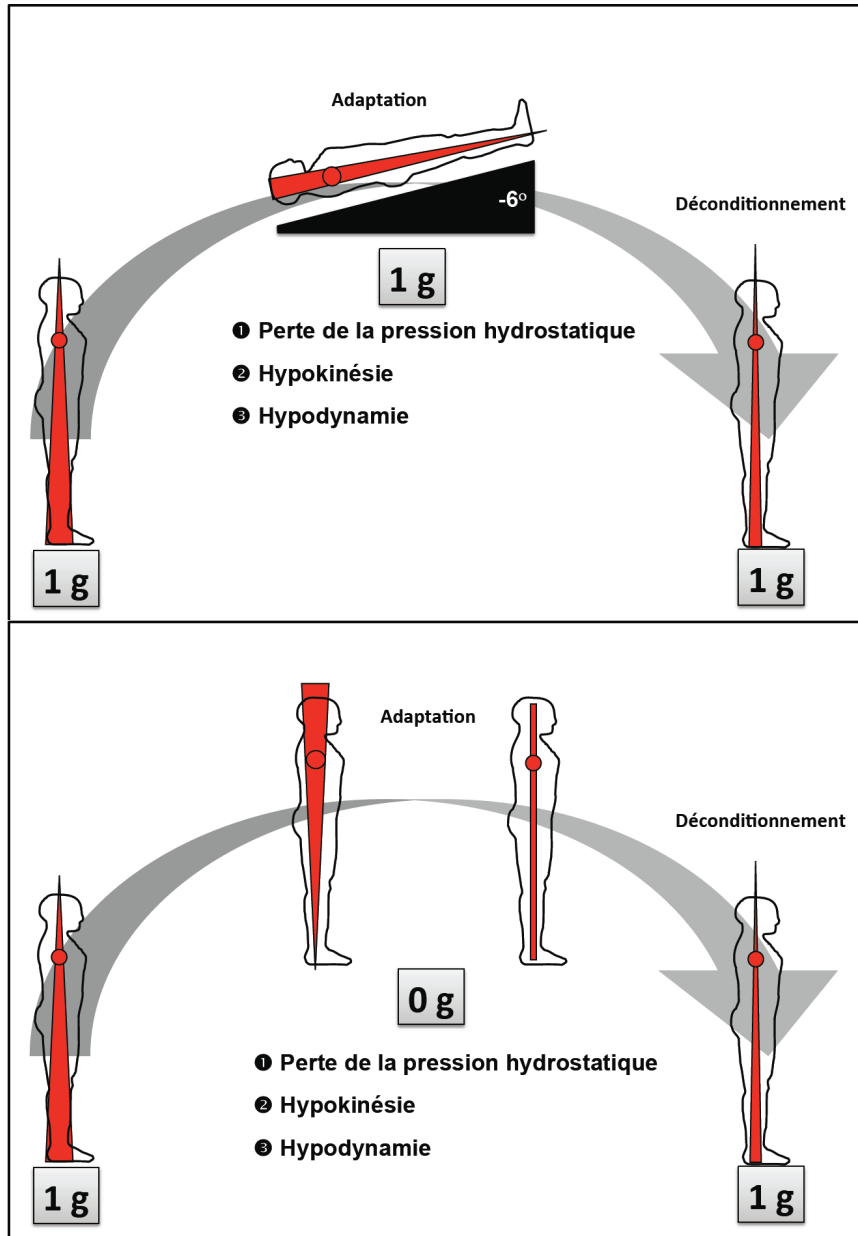


Figure 2:

**Figure du haut :** Distribution des fluides au cours d'un vol spatial.

**Figure du bas :** Distribution des fluides au cours d'une étude de simulation de la microgravité par alitement prolongé.

Sur Terre, en position debout, les fluides sont répartis préférentiellement dans les membres inférieurs. En absence de gravité, les fluides vont être redistribués vers les membres supérieurs. L'alitement prolongé à tête déclive de -6° va reproduire ces changements de distribution. Va s'en suivre une phase d'adaptation avec une perte de la pression hydrostatique, une hypokinésie ainsi qu'une hypodynamie. En conséquence, le retour à la gravité ou le passage à la position debout va provoquer un déconditionnement cardiovasculaire fréquemment accompagné d'un malaise vagal.



Figure 3. Alitement prolongé à tête déclive de  $-6^\circ$ . Crédit photo : Floriane Rudwill

## 2. Immersion sèche

Dans les années 70 un nouveau modèle d'immersion dite « sèche » a été proposé (Shulzhenko, 1975). Ce modèle consiste en l'utilisation d'un bassin rempli d'eau que l'on recouvre à l'aide d'un tissu. Le sujet s'installe sur le tissu, puis est recouvert d'une couverture contenant également de l'eau. Ainsi, les sujets sont soumis aux effets de l'immersion tout en restant au sec (Figures 4 et 5). En comparaison avec l'alitement, les adaptations physiologiques à l'immersion sèche se développent plus rapidement. De plus, contrairement à l'alitement, une accentuation des changements neuromusculaires est observée : diminution du tonus et de la tension musculaire ou encore augmentation de la raideur musculaire (Adams et al., 2003; Williams et al., 2009). Ces symptômes, parmi d'autres soulèvent un point important, nécessaire dans un modèle de simulation de microgravité : l'absence de force s'exerçant sur la structure corporelle. L'immersion sèche provoquerait ainsi une diminution plus importante de cette force et de ce fait un développement plus important des altérations métaboliques qui en découlent. Les autres systèmes physiologiques semblent être impactés de la même manière que lors d'une étude d'alitement prolongé (Navasiolava et al., 2011). D'un point de vue pratique, les études menées lors d'une immersion sèche restent tout de même plus difficiles à réaliser que lors d'un alitement classique en raison d'une moindre accessibilité et de la relative instabilité du sujet une fois dans le bassin mais aussi de la possibilité de n'avoir que deux bassins à la fois, ce qui augmente la durée de chaque étude.



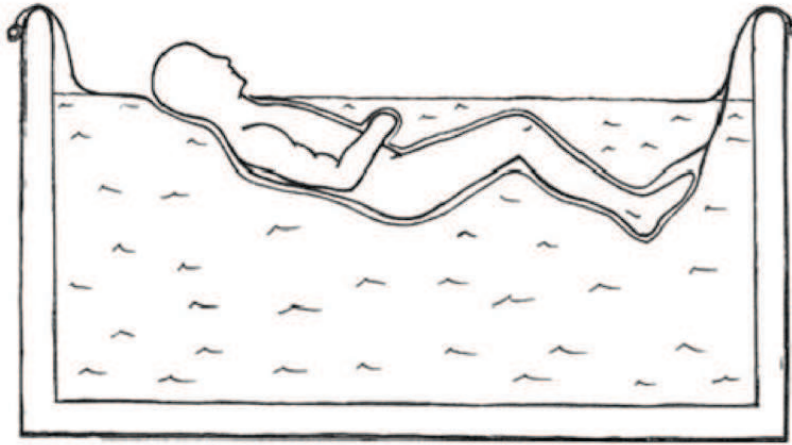


Figure 4 : **Immersion sèche** : Le sujet est immergé jusqu'au cou et séparé de l'eau par un tissu résistant à l'eau (Navasiolava et al., 2011).



Figure 5 : Photo d'un participant à une étude d'immersion sèche.  
Crédit photo : Loïc Treffel

## ii. Simulation de la microgravité chez l'animal

### 1. Animaux suspendus

Bien avant la conquête spatiale de l'homme, les animaux ont été les premiers à être soumis aux effets de l'impesanteur, avec la chienne Laïka en 1957. Par la suite, d'autres chiens, primates et rongeurs ont été envoyés dans l'espace afin de préparer les premiers vols habités par l'homme. De la même manière, l'emploi de modèles animaux au sol peut s'avérer utile, afin de compléter les données obtenues lors de simulation de l'impesanteur chez l'homme. Le modèle le plus couramment utilisé est le rat suspendu par les membres postérieurs avec un angle de 30°, qui permet de recréer l'inactivité physique en empêchant le rat de se mouvoir et en entraînant la redistribution des fluides dans les membres supérieurs (ou antérieurs ici), par une élévation des membres postérieurs (Chowdhury et al., 2013; Morey-Holton et al., 2002) (Figure 6). Le même modèle est également employé avec les souris (Arbogast et al., 2007; Morey-Holton et al., 2002). L'accès à certaines données physiologiques n'est possible que grâce à ces modèles animaux. Par exemple, certaines données mécanistiques sur des organes clés, tels que le foie ou le cerveau, peuvent être obtenus grâce aux modèles animaux. Ces modèles sont également très intéressants afin de tester différents types de contremesures nutritionnelles et/ou physiques, car moins coûteuses que lorsqu'elles sont réalisées sur l'homme. De plus, l'avantage non négligeable du modèle animal est de pouvoir tester les effets de la microgravité sur des animaux déficients pour certains gènes. En effet, des études ont montré des similitudes dans les altérations physiologiques retrouvées à la fois chez l'homme et l'animal : comme le changement de la répartition des fluides corporels ou encore une atrophie musculaire ainsi qu'une diminution de la force musculaire et osseuse (Machida et al., 2005; Momken et al., 2011; Stein et al., 2005). Des études comparatives entre des modèles animaux au sol et en vol ont démontré des modifications similaires concernant les altérations intestinales, immunitaires (Morey-Holton et al., 2002), la résistance à l'insuline (Hilder et al., 2005; Stuart et al., 1993) mais peu d'altérations ont été décrites au niveau du métabolisme hépatique (Morey-Holton et al., 2002), avec des variations uniquement pour la production hépatique de glucose et de triglycérides (Stein et al., 2005) ou au niveau de la quantité de globules rouges et des molécules plasmatiques (Morey-Holton et al., 2002).

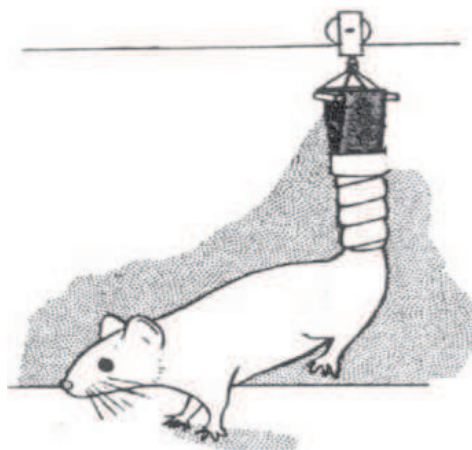


Figure 6 : modèle animal de rat suspendu  
(Barbosa et al., 2011)

## 2. Cultures cellulaires

Les derniers modèles utilisés classiquement sur terre sont les cultures cellulaires. Afin de simuler l'impesanteur, un système de centrifugeuse tridimensionnelle a été mis au point pour permettre une rotation des cellules de manière aléatoire dans les trois plans (Borst et al., 2008). En effet, la centrifugation de manière aléatoire est le meilleur compromis trouvé pour recréer les effets de la microgravité. Tout type de cellules peut être employé avec cette technique, des cellules musculaires (Blaber et al., 2004) aux cellules végétales (Martzivanou et al., 2006). Les cultures cellulaires peuvent représenter un avantage afin de tester *in vitro* différents paramètres, comme les contremesures nutritionnelles, des variations de pression ou température. Cependant il faut garder à l'esprit que ces études ne sont pas faites en conditions physiologiques réelles. Néanmoins, elles restent une bonne alternative, bien que contraignante en terme de survie cellulaire, au modèle animal classique.

En conclusion de cette section, le passage à un environnement microgravitaire entraîne une nécessité d'adaptation de l'organisme. La compréhension du développement de ces adaptations passe par l'utilisation de modèles adéquats de microgravité simulée chez l'homme et l'animal. Ces derniers permettent l'élaboration de solutions de prévention efficaces, notamment contre le développement des atteintes physiologiques.

### 3. La microgravité réelle et simulée : impact sur les fonctions physiologiques.

#### *a. Système neurophysiologique*

Le système neurophysiologique des astronautes est un des premiers impactés dès les premières heures dans l'espace. Un bon nombre d'astronautes souffrent du « mal de l'espace » qui se traduit par des nausées, maux de tête, sueurs, fatigue et perte d'appétit (Heer et al., 2006; Homick, 1979) et touche 80 à 90% des membres de l'équipage lors des premiers jours en impesanteur (Reschke et al., 1998). Il peut être apparenté au mal des transports dont souffrent certaines personnes sur terre. Les causes de ce mal être restent cependant encore mal définies. Fort heureusement ces effets se dissipent rapidement et n'ont qu'un impact mineur sur la santé des astronautes. L'impact neurophysiologique de la microgravité se manifeste également par des troubles de l'orientation et de l'équilibre (Layne et al., 2001) avec des sensations de sol mouvant ou d'illusions proprioceptives (Reschke et al., 1998), probablement dues au passage d'un environnement bidimensionnel à tridimensionnel. Un ajustement neurovestibulaire est donc nécessaire et peut prendre plusieurs jours pour se mettre en place (Lane et al., 1993). Les mécanismes sous-tendant ces symptômes ne sont cependant pas clairement élucidés. Des altérations visio-spatiales (Cheron et al., 2014) ou dans la perception haut/bas sont également décrites (De Saedeleer et al., 2013).

Sur terre, au cours d'une étude d'alitement prolongé de 60 jours chez la femme, une altération de la balance posturale statique et dynamique a été décrite, similaire aux problèmes rencontrés par les astronautes à leur retour sur terre (Viguiet et al., 2009; Young et al., 1993). Le système neurophysiologique interagit également avec le système musculaire par le biais de la commande neurologique des muscles. Sung et al. (2013) ont également démontré une diminution de l'impédance électrique du muscle, ainsi que de son état contractile (Koryak, 1995; Sung et al., 2013).

#### *b. Système immunitaire*

La microgravité, le changement d'environnement, les radiations et la privation de sommeil sont des facteurs de stress importants qui vont moduler les réponses immunitaires de l'organisme. La résultante de ces changements est une diminution des défenses immunitaires, propice au développement d'infections (Crucian et al. 2009, Sonnenfeld et al. 2002). Les dérégulations de la fonction immune vont varier en fonction de la durée des missions. Ainsi, sur les durées les plus longues, une diminution des cellules immunitaires est observée, touchant notamment les cellules T (Crucian et al., 2009; Sonnenfeld et al., 2002). De plus, sur des cultures cellulaires, on



observe une augmentation de cytokines proinflammatoires telles que le TNF- $\alpha$ , l'IL-1 ou encore l'interféron alpha (Chapes et al., 1992). L'atteinte du système immunitaire que cela soit à court ou à long terme peut provoquer des risques sévères pour la santé des astronautes.

Au sol, les études révèlent des résultats variables en fonction des modèles de simulation. En effet, certaines données ne sont pas totalement identiques à celles retrouvées en vol (Schmitt et al., 2000). Cependant l'alitement prolongé reste tout de même un bon modèle de simulation du stress développé au cours des vols (Chouker et al., 2001).

### *c. Système cardiovasculaire*

Sur terre, en raison de la force exercée par la gravité, les fluides corporels, tels que le sang ou l'eau, sont répartis à 70% en dessous du cœur. Dans l'espace, la distribution devient préférentiellement thoraco-céphalique. Physiquement, ce changement se traduit par un gonflement du visage, dû à l'apport sanguin plus important dans la partie supérieure du corps (Vernikos, 1996). D'un point de vue physiologique, l'hypervolémie thoracique est détectée par des barorécepteurs cardiaques situés au niveau du ventricule cardiaque, qui, en réponse, entraînent une diminution du volume sanguin en augmentant la diurèse (Perhonen et al., 2001) (Figure 2 - haut). L'une des conséquences de l'hypovolémie provoquée est une diminution de la taille et un remodelage du muscle cardiaque (Hargens et al., 2009). Cette adaptation du volume des fluides par une réduction du gradient hydrostatique se fait dès les premiers jours de vol et se maintient tout au long du séjour. Au retour sur terre, la gravité va rétablir la distribution initiale des fluides. Cependant, l'hypovolémie ainsi que l'atrophie cardiaque ne permettent plus un apport sanguin suffisant au cerveau pour assurer un fonctionnement optimal et provoquent le plus souvent une syncope. Ce phénomène est appelé hypotension orthostatique. Elle se manifeste chez l'homme lors d'un passage trop rapide de la position assise ou couchée à la position debout. Chez les astronautes, des études ont démontré que 15 à 64% d'entre eux font une syncope à leur retour sur terre (Yates et al., 1998). Au niveau vasculaire, plusieurs études au cours de vols spatiaux ont montré un remodelage qui contribuerait à cette intolérance orthostatique. En effet, la réponse vasculaire n'est pas la même au niveau des membres supérieurs et des membres inférieurs. Dans les membres inférieurs, le système vasculaire subit une hypotrophie, ainsi qu'une diminution de la vasoréactivité (Zhang et al., 2012), mais en revanche, on observe également une diminution de la contractilité vasculaire (Purdy et al., 1998). L'inverse est observé dans les vaisseaux des membres supérieurs et au niveau cérébral en réponse à l'adaptation à l'hypervolémie cérébrale (Zhang et al., 2012). Ces symptômes, lorsqu'ils sont couplés à une diminution de la capacité à l'exercice, un autre symptôme dû à la microgravité, provoquent ce que l'on appelle le déconditionnement cardiovasculaire.

Les études au sol induisent les mêmes altérations que celles subies par les astronautes : une intolérance orthostatique, une atrophie cardiaque (Yang et al.,

2011) en particulier du ventricule gauche (Hastings et al., 2012), une diminution du baroréflexe (Coupé et al., 2011) secondaire à la diminution de la pression sanguine ainsi qu'une atteinte endothéliale qui pourrait contribuer au développement des symptômes cités plus haut (Coupe et al., 2009). L'alitement prolongé induit également les mêmes atteintes cardiorespiratoires (Trappe et al., 2006). En revanche, le système de coagulation ne semble pas être impacté par la microgravité simulée (Haider et al., 2013). En conclusion, l'atteinte cardiovasculaire aussi bien sur terre que dans l'espace se caractérise par un remodelage cardiovasculaire dû principalement au changement de la distribution des fluides corporels. Sur terre, des études populationnelles ciblant notamment les personnes âgées, plus fréquemment sujettes à l'hypotension orthostatique, ont révélé que des carences en certaines vitamines, telles que la vitamine D (McCarroll et al., 2012; Soysal et al., 2014) ou encore B12 (Ganjehei et al., 2012) pourraient également contribuer au développement d'une intolérance orthostatique. Ces résultats indiquent ainsi que la nutrition pourrait également contribuer au déconditionnement cardiovasculaire.

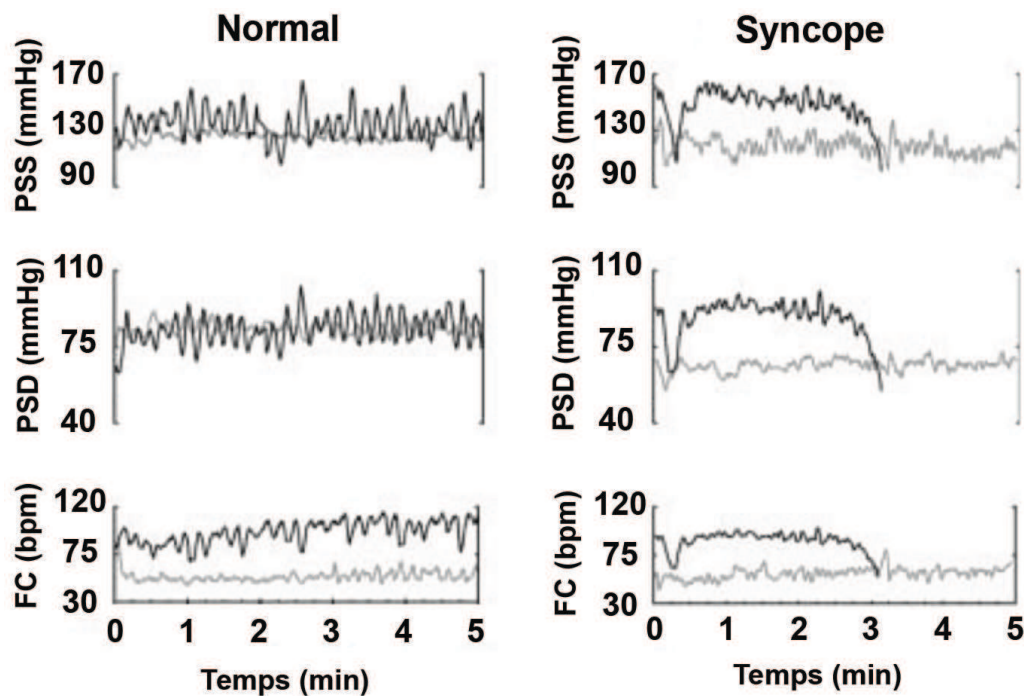


Figure 7 : Variations de la fréquence cardiaque (FC) et des pressions sanguines systolique (PSS) et diastolique (PSD) chez des astronautes sujet ou non à une syncope après un vol spatial (Sigaud-Roussel et al., 2001).

#### *d. Système musculo-squelettique*

Sur terre, la force gravitationnelle est responsable de la charge exercée sur la structure musculo-squelettique qui assure le soutien corporel et permet le mouvement et les déplacements. A l'opposé, dans l'espace, l'absence de gravité entraîne une forte diminution de cette force exercée sur les muscles et les os (LeBlanc et al., 2000b; LeBlanc et al., 2007; Stein, 2012; Vico et al., 2000). L'atteinte musculaire

se traduit, à plus ou moins long terme, par une atrophie (Figure 8), qui touche tous les muscles, et plus particulièrement ceux des membres inférieurs et les muscles posturaux (Adams et al., 2003; Narici et al., 2011). En effet, pour les membres inférieurs, l'atrophie musculaire peut atteindre 24% pour les muscles posturaux lors des vols de 197 jours comparés à un maximum de 12% pour un muscle non-postural (LeBlanc et al., 2000a). Une diminution de la synthèse protéique contribue à cette atrophie, résultant en une balance protéique négative au cours des vols spatiaux (Stein, 2012). Il a été montré que l'atrophie musculaire est due à une diminution en taille des fibres et non de leur nombre (Tischler et al., 1995). Parallèlement à cette perte de masse musculaire, on observe une diminution de la force musculaire (Figure 9). Après seulement quelques jours de microgravité, des cosmonautes russes avaient perdu 5% de leur force ; une diminution de 26% est observée pour des missions spatiales de plus de 80 jours (Narici et al., 2011). Ces atteintes musculaires s'accompagnent d'une augmentation de la fatigabilité musculaire partiellement expliquée par un changement phénotypique des fibres, (passage de fibres lentes de type 1 à rapides de type 2). En résumé, la microgravité est ainsi responsable d'une altération globale du système musculaire (Stein et al., 2005).

Outre les changements musculaires, des altérations du système squelettique et osseux ont également été décrites, directement liées à la diminution de la charge exercée par la gravité. L'un des principaux symptômes décrits est une augmentation de la fragilité osseuse, causée par une diminution de sa masse et de sa densité (Figure 10 et 11) (Clément, 2011; LeBlanc et al., 2000b; Smith et al., 1999; Vico et al., 2000; Vico et al., 2001). Comme pour l'atrophie musculaire, la perte osseuse est plus importante dans les membres inférieurs avec une perte moyenne de 0,34% par mois contre 0,04% pour le haut du corps suite à des vols allant de 4 mois à plus d'un an (LeBlanc et al., 2000b; LeBlanc et al., 2007). La perte la plus importante se situe néanmoins au niveau du trochanter avec 1,56% de perte osseuse par mois. Une précision a tout de même été apportée par une étude menée par Vico et al. (2000) qui relève toutefois une hétérogénéité de perte de densité au sein d'un même os probablement due à un remodelage local (Vico et al., 2000). Du fait de cette importante perte osseuse au cours des vols spatiaux, le risque de fracture et d'ostéoporose est également augmenté (Lang, 2006). Le système de régulation du calcium qui joue un rôle capital dans le maintien de la densité osseuse est également touché avec une augmentation de son excrétion dans les urines au cours des vols spatiaux (LeBlanc et al., 2007; Smith et al., 2012b; Smith et al., 1999). Impliqué dans l'homéostasie cellulaire, cette dérégulation du métabolisme calcique perturbe l'équilibre homéostatique de l'organisme (Vernikos, 1996). Les marqueurs du métabolisme osseux varient également au cours des vols spatiaux. Plusieurs marqueurs de la dégradation osseuse sont augmentés au cours d'étude d'alitement prolongé (Frings-Meuthen et al., 2011; LeBlanc et al., 2002). L'ostéocalcine est quant à elle plus accrue en période de récupération après vol, témoignant d'une augmentation de la synthèse osseuse (LeBlanc et al., 2007; Smith et al., 2005). D'un point de vue chronologique, l'impact de la microgravité sur le système musculo-

squelettique est graduel mais apparaît dès la première journée dans un environnement spatial (Williams et al., 2009). Lors des études d'alitement prolongé, les participants présentent les mêmes symptômes que les astronautes après les vols spatiaux avec, parallèlement à l'atrophie musculaire au niveau des membres inférieurs (Bamman et al., 1998; Paddon-Jones et al., 2006; Rittweger et al., 2005) et du muscle cardiaque (Perhonen et al., 2001), une déminéralisation osseuse (Lang et al., 2004; Smith et al., 2005) bien que pour certains auteurs cette dernière soit moins importante (Smith et al., 2012b).

Au vu de la littérature, il est important de noter une grande hétérogénéité dans l'atteinte musculaire entre les astronautes. Cette variabilité s'explique par des différences génétiques entre les individus, mais aussi des différences d'âge ou de condition physique avant le vol, les différentes contremesures appliquées pendant le vol ou encore les différences nutritionnelles entre les pays (Narici et al., 2011). Le point commun entre toutes les études réalisées est une grande variabilité inter-individuelle de la perte osseuse et musculaire (Sievanen, 2010; Vico et al., 2000). De la même manière, la récupération de la masse musculaire et osseuse est exponentielle après un vol spatial et variable selon les individus (Sibonga et al., 2007). Au retour sur terre, la récupération de la densité osseuse et musculaire peut prendre de quelques mois à plusieurs années suivant la durée des vols et varie en fonction des astronautes. Ainsi, la perte osseuse reste pour le moment un des points négatifs de tout vol spatial avec un pourcentage de récupération atteignant rarement la totalité de la perte (Sibonga et al., 2007), ce qui s'expliquerait par une involution graisseuse au sein même de l'os (Alexandre et al., 2011).

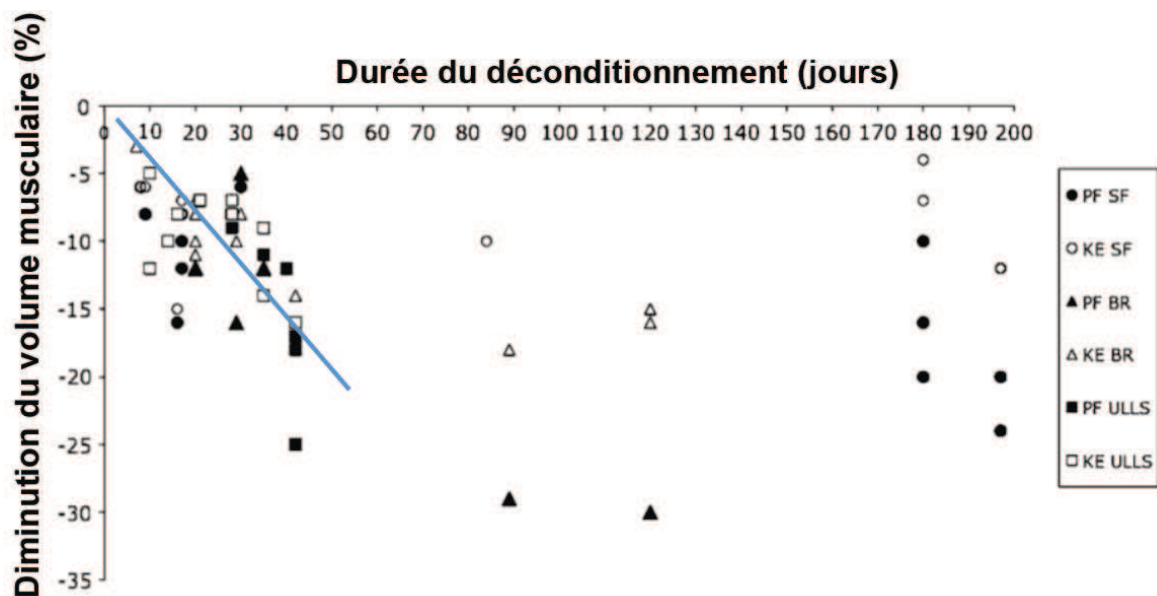


Figure 8 : Variation du volume musculaire (PF: plantar flexor et KE: muscle extenseurs du genou) en fonction de la durée de vol spatial (SF), d'alitement prolongé (BR) ou de suspension unilatérale des membres inférieurs (ULLS) (Narici et al., 2011).

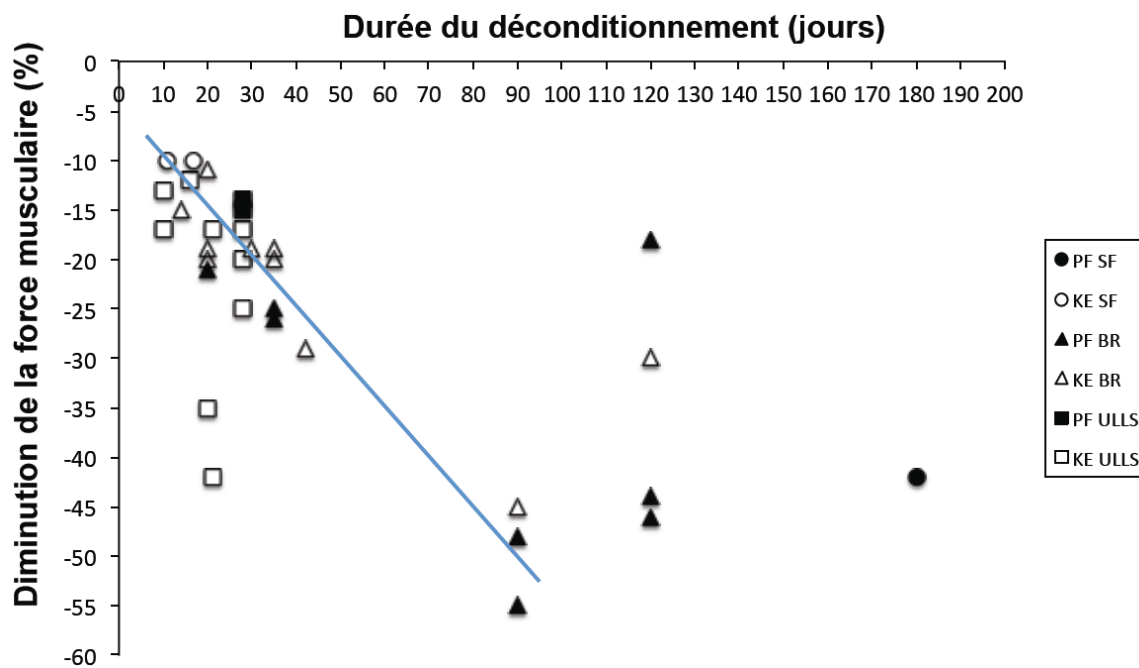
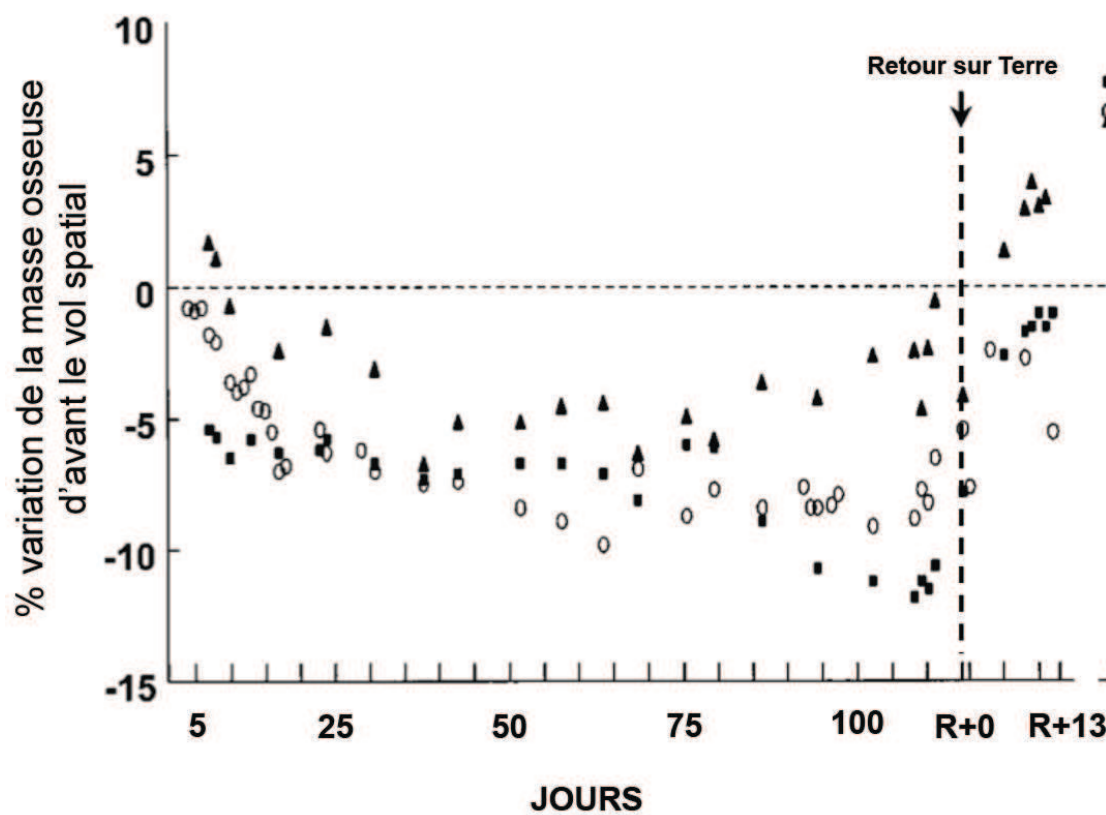


Figure 9 : Variation de la force musculaire (PF: plantar flexor et KE: muscle extenseurs du genou) en fonction de la durée de vol spatial (SF), d'alitement prolongé (BR) ou de suspension unilatérale des membres inférieurs (ULLS) (Narici et al., 2011).





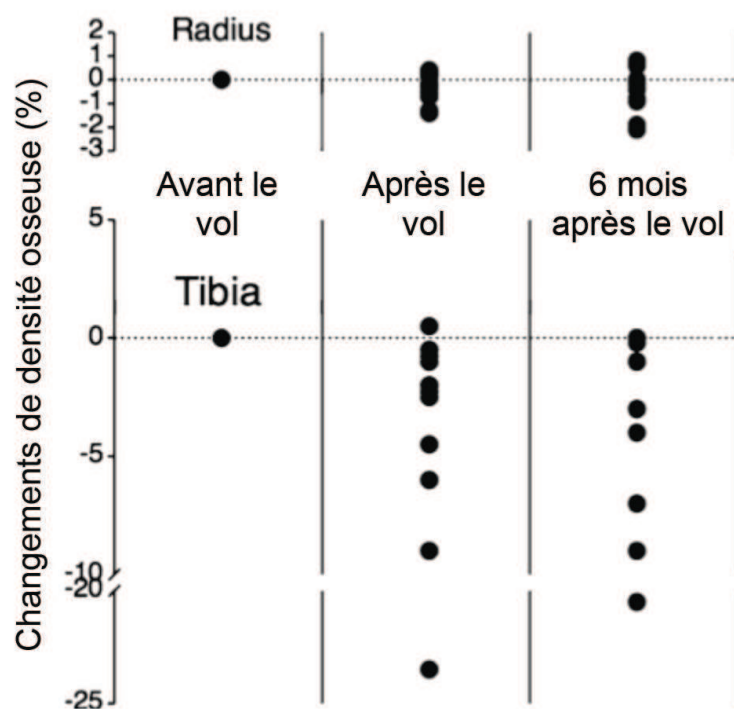


Figure 11 : Perte moyenne de densité osseuse (%) dans le radius et le tibia après 6 mois de vol spatial à bord de la station spatiale Mir et 6 mois après le vol en comparaison aux valeurs obtenues avant le vol (Clément, 2011).

### e. Adaptation métabolique

Dans les paragraphes précédents, une adaptation des systèmes physiologiques a été décrite. Elle est à mettre en parallèle avec une adaptation métabolique à la microgravité.

Au cours des vols spatiaux ou d'un alitement prolongé, certaines études ont montré une augmentation du taux de cortisol urinaire, traduisant le développement d'un stress (Blanc et al., 1998; Borchers et al., 2002; Chouker et al., 2001). Cette variation n'est cependant pas systématiquement décrite (Yi et al., 2014). L'augmentation du cortisol est parfois associée avec une chute des catécholamines aussi bien au cours d'études en vol que lors des études de simulation d'impesanteur au sol (Blanc et al., 1998; Stein et al., 1999). L'implication des catécholamines dans la régulation du système physiologique en fait des molécules clés du métabolisme.

En parallèle de ces résultats, certaines molécules inflammatoires, les cytokines, ont également été mesurées au cours d'un vol spatial ou d'une étude d'alitement. Il a été suggéré que ces molécules participeraient au développement d'une inflammation chronique de bas niveau. Cependant ces résultats restent encore discutés. Certaines cytokines proinflammatoires générales, telles que Tumor Necrosis Factor-alpha (TNF- $\alpha$ ) ou l'interleukin-1 (IL-1) et l'IL-8 ont été trouvées augmentées lors des situations d'impesanteur (Borchers et al., 2002; Chapes et al., 1992; Chouker et al., 2001; Crucian et al., 2014). De même certaines molécules inflammatoires plus spécifiques du muscle ou du tissu adipeux telles que la pentraxin 3 et de la protéine C-réactive, qui sont sécrétées au cours de la phase aigüe de l'inflammation, ont

également été augmentées après 14 jours d'alitement (Bosutti et al., 2008). Après 520 jours de simulation, les cytokines anti-inflammatoires telles que l'IL-4 et l'IL-10 sont quant à elles diminuées au cours de certaines études (Le Traon et al. 2007) ce qui concorde avec la diminution des défenses immunitaires au cours des missions spatiales (Sonnenfeld, 1999). *A contrario*, Muid et al. (2013) présente une diminution de l'expression des mRNA du TNF- $\alpha$ , une molécule proinflammatoire (Muid et al., 2013). De même Baqai et al. (2009) ont démontré une stimulation de la sécrétion des cytokines anti-inflammatoires IL-6 et IL-10 après un vol spatial (Baqai et al., 2009). En complément des molécules inflammatoires, le stress oxydant, dû à une surproduction de radicaux libres au sein de la mitochondrie, est également augmenté après un vol spatial (Baqai et al., 2009; Stein, 2002; Versari et al., 2013), de même qu'après plus de 60 jours de microgravité simulée (Zwart et al., 2009). A l'opposé, aucune variation n'a été observée après seulement 30 jours d'alitement (Morgan et al., 2012).

En résumé, la variabilité des résultats décrits dans la littérature peut s'expliquer par les nombreux paramètres qui diffèrent entre les études d'impesanceur en vol et au sol. Ces paramètres variables peuvent influencer la réponse inflammatoire à la microgravité. Ces données sont donc à interpréter avec précaution.

En parallèle du développement d'une inflammation de bas niveau, d'autres altérations métaboliques sont décrites : une résistance à l'insuline et une altération du métabolisme lipidique, caractérisées par une diminution de l'oxydation des lipides au profit des glucides, une hypertriglycémie ainsi qu'un stockage ectopique des lipides. La résistance à l'insuline ou encore l'accumulation de lipides dans les différents organes, muscle ou foie, entraînent le dysfonctionnement de ces organes et pourraient contribuer à la sécrétion de molécules inflammatoires.

Les adaptations métaboliques à la microgravité, notamment l'altération du trafic lipidique et la sensibilité à l'insuline, sont détaillées dans la revue « Physical inactivity as the culprit of metabolic inflexibility : evidence from bed rest studies » publiée en 2011 dans *Journal of Applied Physiology*, (Bergouignan et al., 2011).

## HIGHLIGHTED TOPIC | *Physiology and Pathophysiology of Physical Inactivity*

### Physical inactivity as the culprit of metabolic inflexibility: evidence from bed-rest studies

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**Bergouignan A, Rudwill F, Simon C, Blanc S.** Physical inactivity as the culprit of metabolic inflexibility: evidence from bed-rest studies. *J Appl Physiol* 111: 1201–1210, 2011. First published August 11, 2011; doi:10.1152/jappphysiol.00698.2011.—Although it is no longer debatable that sedentary behaviors are an actual cause of many metabolic diseases, the physiology of physical inactivity has been poorly investigated for this purpose. Along with microgravity, the physiological adaptations to spaceflights require metabolic adaptations to physical inactivity, and that is exceedingly well-simulated during the ground-based microgravity bed-rest analogs. Bed rest thus represents a unique model to investigate the mechanisms by which physical inactivity leads to the development of current societal chronic diseases. For decades, however, clinicians and physiologists working in space research have worked separately without taking full awareness of potential strong mutual questioning. This review summarizes the data collected over the last 60 years on metabolic adaptations to bed rest in healthy subjects. Our aim is to provide evidence that supports the hypothesis that physical inactivity per se is one of the primary causes in the development of metabolic inflexibility. This evidence will focus on four main tenants of metabolic inflexibility: 1) insulin resistance, 2) impaired lipid trafficking and hyperlipidemia, 3) a shift in substrate use toward glucose, and 4) a shift in muscle fiber type and ectopic fat storage. Altogether, this hypothesis places sedentary behaviors upstream on the list of factors involved in metabolic inflexibility, which is considered to be a primary impairment in several metabolic disorders such as obesity, insulin resistance, and type 2 diabetes mellitus.

fat oxidation; insulin resistance; ectopic fat storage; sedentary behaviors; lipolysis

OUR SOCIETIES have constructed during the 20th century an ecological niche in which sedentary behaviors and junk food became the new reference of living. Although this evolution is considered as an improvement in our living conditions, it created a mismatch between our evolutionary history and the environment for which humans adapted. Nowadays, exercise is typically presented as a therapy to several chronic diseases (16). This transition has even altered our appreciation of physical activities itself. Although 65% of the National Health and Nutrition Examination Survey American population self-reported to be as active as recommended by national guidelines, objectively measured, physical activity by accelerometry showed this number to be only 5% (70). This is a rather important observation when considering that physical inactivity has been classified as the second cause of death in the

United States (47) based on the 35% self-reported inactive people.

The hazards of physical inactivity have been described for a long time (43). A growing body of data suggests that reduced physical activity is causative in the development of modern chronic metabolic diseases, including obesity, insulin resistance, dyslipidemia, type 2 diabetes mellitus (T2D), hypertension, and others (16). Some data suggest that sedentary behavior is an independent predictor of metabolic risk, even when exercise is performed at a level that meets current recommendations (54). The causal relationships between physical inactivity and metabolic diseases are essentially based on epidemiological studies or on the indirect beneficial effects of exercise training. None of these studies provide, however, evidence to support a cause-and-effect relationship. Our poor knowledge on physical inactivity physiology can in part be attributed to the paucity of models to induce long-term physical inactivity in healthy subjects that account for the confounding effects of the positive energy balance induced by physical inactivity itself.

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Along with microgravity, the physiological adaptations to spaceflights require metabolic adaptations to physical inactivity that is exceedingly well simulated during the ground-based microgravity bed-rest analogs. In addition to space medicine-related questions, bed rest thus represents a unique model to investigate the mechanisms by which physical inactivity leads to the development of current societal chronic diseases. The degree of physical inactivity encountered during bed rest may be seen as too extreme compared with that seen in the general population, who spend more time sitting and with some level of movement rather than strictly lying down. However, it is important to note that the physical activity level (PAL) measured during bed-rest studies is close to that measured in sedentary individuals, i.e., a PAL of  $\sim 1.4$ – $1.5$  (5, 14), because of fidgeting movements in the bed. Bed rest remains a unique model to investigate the basic mechanisms of adaptation to short- or long-term physical inactivity in healthy subjects. For decades, clinicians and physiologists working in space research have worked separately without full awareness of potential strong mutual questioning.

The concept of metabolic inflexibility was first proposed ten years ago by Kelley et al. (33) from several metabolic disorders such as obesity, insulin-resistance, and T2D. These diseases are characterized by a metabolic deregulation that impairs the capacity to increase fat oxidation when fatty acid availability is increased and to switch from fat to glucose as the primary fuel source after a meal (19). Because these adaptations were not normalized after weight loss in some studies (11), several authors suggested that they might represent a primary impairment responsible for the development of the diseases. In this review, we provide evidence collected over the last 60 years from healthy, bed-rested subjects to support the hypothesis that physical inactivity per se is one of the primary causes involved in the development of metabolic inflexibility, even when changes in energy balance are not detectable. The data that support our hypothesis are divided into four main parts. The first section is dedicated to insulin resistance, the second, to the alterations in lipid trafficking, and the third, to the changes in the fuel mix being oxidized during bed rest. The fourth part provides evidence that insulin resistance and the decreased capacity to use fat as fuel during physical inactivity may trigger a generalized ectopic fat storage. We will provide some recent evidence to highlight the role of physical activity level in the regulation of metabolic flexibility. Last, we will combine all these data and describe the link between physical inactivity and metabolic inflexibility.

#### PHYSICAL INACTIVITY INDUCES INSULIN RESISTANCE

*Whole body evidence from bed-rest experiments in humans.* Numerous bed-rest studies of different durations showed a decrease in insulin sensitivity (1, 7, 8, 12, 22, 25, 38–40, 45, 46, 51, 57–59, 66, 67, 74). To our knowledge, Lutwack and Whedon (40) were the first in 1959 to have reported abnormal intravenous glucose tolerance tests after 1–3 wk of bed rest in healthy subjects. Other early reports in the 1970s also observed glucose intolerance and hyperinsulinemia following an oral glucose tolerance test (OGTT) after 10–14 days of bed rest (22, 38, 39, 51). Although the acute response of insulin to bed rest (1–2 days) has not been investigated, Yanagibori et al. (74) observed during a 20-day bed-rest period that an increase in

insulin and glucose responses during an OGTT appears as early as day 3 in 13 females and 10 males. In this same study, bed-rest studies also confirmed the development of insulin resistance in both sexes (7, 8, 12).

Studies were conducted to understand the mechanisms involved in the reduced whole body insulin sensitivity by specifically investigating the response to insulin in the main insulin-sensitive organs, i.e., skeletal muscle, adipose tissue, and liver. In a 7-day bed rest in six healthy men, Mikines et al. (45, 46) observed a decreased insulin action at muscle level on glucose uptake and glycogen storage. Even if the subjects were allowed to get up every other day to shower, Tabata et al. (68) also showed a 16% decrease in vastus lateralis glucose transporter 4 (GLUT-4) concentrations in nine healthy males after 20 days of bed rest. Altogether these results indicate the development of insulin resistance at the muscle level. No modification on hepatic glucose production was observed at both physiological and supraphysiological insulin concentrations in the males (45, 46, 67). However, by using stable isotopes to track endogenous and exogenous glucose metabolism, we observed that insulin resistance was only developed at the muscle level in men after 7 days of bed rest, as in the previous studies, but at both muscle and liver levels in women (12). This suggests a sex effect on liver insulin sensitivity during bed rest.

With regard to the adipose tissue insulin sensitivity, although the  $\beta$ -adrenergic receptors showed an increased sensitivity (4) during bed rest, plasma catecholamines are reduced (14), indicating a decreased activity of the sympathetic nervous system that does not allow an increase in lipolysis. On the basis of the fasting decrease in plasma free fatty acids (FFA) observed and increased fasting insulin in men and women after 7 days of bed rest, we can assume that physical inactivity does not reduce adipose tissue insulin sensitivity in both sexes (12). In support of that, recent bed-rest studies even associated whole body insulin resistance with a whole body reduction in lipolysis in male subjects (1, 2). Except in a very recent study (27) described later in this review, adipose tissue in the context of physical inactivity has been poorly investigated, and further studies are clearly needed.

Most of the above studies have a major limitation: they did not tightly control the diet of the subjects. An adequate nutrient supply to accurately derive the true effects of bed rest alone is of particular importance. A positive energy balance due to overfeeding will be a confounding factor that exaggerates the deleterious effects of physical inactivity (10). Thus the 67% increase in insulin response recently observed (25) may be due to the fact that subjects were provided with their habitual diet. Mikines et al. (45, 46) reduced energy intake by 15% only during the last 3 days of the bed rest. Furthermore, in the early study of Dolkas and Greenleaf (22), volunteers received a very high energy intake (3,073 kcal/day). Increased plasma leptin levels at the end of our first 7-day bed rest studies (13), with leptin being a bioindicator of energy intake (18, 56), indicate that our subjects were overfed. The diet macronutrient composition also influences the bed-rest outcomes. In a crossover design, 60-h bed rest in eight males (2 days of washout) with either a high-carbohydrate diet (70% of energy intake) or a high-saturated-fat diet (45% of energy intake as fat and 60% of saturated fatty acid), Settler et al. (65) showed that insulin

sensitivity decreased by 24% with the high-saturated-fat diet but did not change with the high-carbohydrate diet.

Because the lack of control on energy balance in early studies questioned the impact of physical inactivity per se on insulin sensitivity, we recently performed bed-rest studies, one study of 2 mo in women (8) and one of 3 mo in men (7) during which we tightly controlled the diet to clamp fat mass. Contrary to others (1, 2), we clamped fat mass rather than body mass, because due to bed-rest-induced muscle atrophy, a weight-clamping approach tends to increase fat mass (76), which reflects a positive energy balance. Tests investigating energy status of the volunteers confirmed that we succeeded (5). Interestingly, we showed that physical inactivity per se yet induces an increase in plasma insulin concentration without changes in plasma glucose concentration in fasting conditions and after a standard meal (Fig. 1). These results indicate that even in physiological conditions (i.e., not in supraphysiological insulinemic clamp) physical inactivity leads to the development of insulin resistance in both postabsorptive and postprandial situations (7, 8). A recent study (64) investigated the effect of 1 day of sitting,

a level of physical inactivity closer to that observed in the general population than strict bed rest, on insulin action. The authors showed that with and without energy surplus, reduced physical activity considerably alters insulin sensitivity. The fact that this effect still occurred when food intake matched energy expenditure emphasized the deleterious effects of physical inactivity alone.

All of these studies only studied lean healthy subjects without any genetic predisposition to obesity or T2D. Considering the increasing prevalence to these chronic diseases and the fact that the general population tends to become more and more sedentary (26), it appears relevant to investigate the interaction between physical inactivity and the predisposition to obesity and T2D. In this line, some authors investigated the effects of 9–10 days of bed rest in healthy lean males (control group); in males with a first-degree relationship of diabetes (FDR); and in males with a low birth weight (LBW), known to be at risk to develop T2D in adulthood (1, 2, 60). By investigating whole body and forearm insulin sensitivity using a hypersulinemic euglycemic clamp, Sonne et al. (60) reported that bed rest induces a marked reduction in whole body

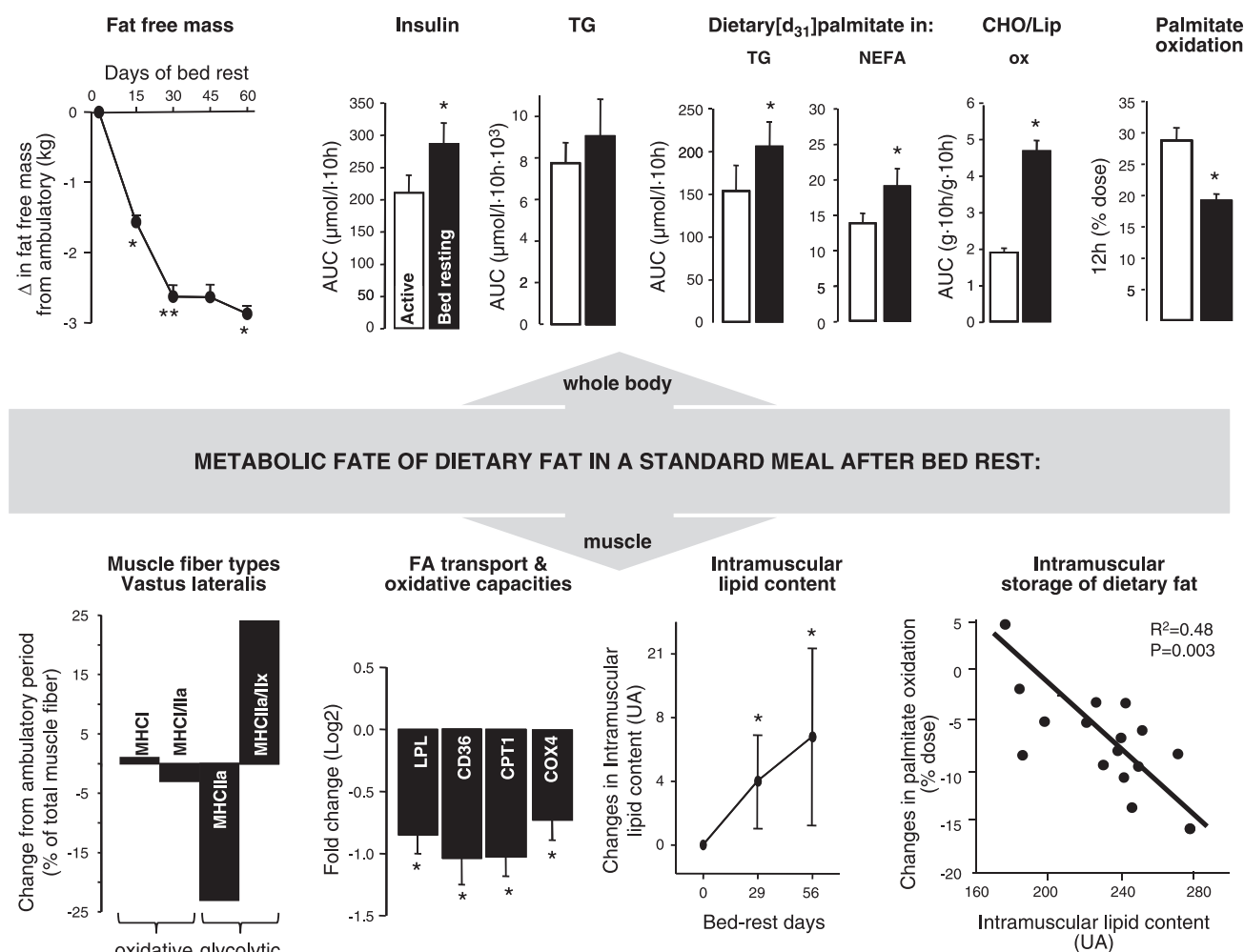


Fig. 1. Whole body and muscle metabolic alterations induced by 2 mo of bed rest in women postprandially following the ingestion of standard breakfast and lunch. TG, triglyceride; FA, fatty acid; NEFA, nonesterified FA; CHO, carbohydrate; AUC, area under the curve; \* $P < 0.05$  vs. active state. [Panel at bottom left reproduced from Trappe et al. (68a)]. [All the other panels modified from Bergouignan et al. (8). Copyright 2009 American Diabetes Association. Modified with permission from The American Diabetes Association.]

skeletal muscle and vascular insulin sensitivity in both control and LBW groups with more pronounced changes in the control group. Although insulin resistance was already present before to bed rest in the FDR group, these subjects still displayed a decrease in insulin sensitivity in response to bed rest. In these three groups, insulin resistance was fully accounted for by nonoxidative glucose metabolism, but hepatic insulin resistance only occurred in the groups with a predisposition for T2D (1, 2). It should however be noted that although not significant, the reduction in the hepatic glucose production was about the same magnitude in the control group (+35%) compared with the FDR group (+36%) (2).

In conclusion, these bed-rest studies provided indisputable evidence that physical inactivity per se induces a reduction in the whole body insulin sensitivity associated with the development of insulin resistance in muscle but not in adipose tissue. The liver remains an important area to investigate, especially given the recent data associating physical inactivity with characteristics of nonalcoholic fatty liver diseases.

*Mechanistic evidence from bed-rest experiments in humans and analogs in animals.* The classical insulin-signaling pathways can be summarized as follows. Skeletal muscle glucose transport is acutely regulated by insulin through the coordinated actions of several proteins. Briefly, insulin activation of tyrosine kinase in the  $\beta$ -subunit of the insulin receptor leads to tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1). Phosphorylated IRS-1 then interacts with the p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3K). Activated PI3K subsequently activates phosphoinositide-dependent kinases, which regulate the serine/threonine protein kinase (Akt, also known as protein kinase B). Although the specific role of Akt in muscle glucose remains controversial, it seems to conduce to the translocation of GLUT-4 to the plasma membrane to facilitate glucose transport via a diffusion process. In addition, the stress-activated p38 mitogen-activated protein kinase (p38-MAPK) may negatively interact with the upstream of the insulin-signaling cascade, particularly IRS-1, inducing insulin resistance (23).

Those pathways were investigated in animal models of physical inactivity. Using the hindlimb tail paradigm, O'Keffe et al. (49) showed that after 1 day of suspension, female rats have a decreased glucose tolerance and reduced stimulated glucose uptake. This acute development of insulin resistance was mainly attributable to changes in p38-MAPK phosphorylation, as it was noted by others (41), but not to changes in insulin receptor beta (IR-beta)/IRS1/PI3K/Akt signaling pathway. After 3 and 7 days of suspension, insulin resistance was however associated with increased insulin signaling pathway through IRS1 and PI3K and with decreased p38-MAPK (50), suggesting that PI3K-insulin-independent mechanisms may also play a role in the adaptations to suspension.

Another model has been developed to study the effects of physical inactivity in rats: the wheel lock model. After a certain period during which rats naturally run in their cage, the investigators lock their wheel. This model better simulates the decrease in habitual physical activity observed in the general population than the hindlimb unloading model that is closer to immobilization. By using this model (34–37), a rapid decrease in skeletal muscle insulin sensitivity has been associated with

an expansion of intra-abdominal fat storage. The authors especially observed a lower insulin-stimulated 2-deoxyglucose uptake into the epitrochlearis after 53 h of cessation of daily running (34). This impaired muscle glucose transport was associated with a reduction in insulin binding, IR-beta, submaximal insulin stimulated IR-beta tyrosine phosphorylation, and GLUT-4 level. Akt/protein kinase B Ser(473) phosphorylation was also reduced after 53 h, suggesting that both upstream and downstream insulin signaling was diminished following running cessation.

Alibegovic et al. (3) investigated the mechanisms involved in the development of insulin resistance by using the microarray technique in 20 healthy men. Bed rest altered the expression of >4,500 genes and downregulated 34 pathways, predominantly those of genes associated with mitochondrial function, including the peroxisome proliferator-activated receptor- $\gamma$  coactivator- $\alpha$  (PGC1 $\alpha$ ) that regulates oxidative phosphorylation (OXPHOS) in the mitochondria. Expression of several candidate genes involved in T2D was also impaired, such as the expression of PGC1 $\alpha$  and OXPHOS genes in general, suggesting a reduced oxidative capacity. Likely related to the depressed PGC1 $\alpha$  expression, a reduced expression of vascular endothelial growth factor A (VEGFA), which regulates the degree of capillarization and microvascular flow and thereby insulin action, was also observed. Hexokinase 2 (HK2), the protein that phosphorylates glucose after its entrance in the cell, and ras-related associated with T2D (RRAD) are considered the most prominent down- and up-regulated transcriptional alterations in skeletal muscle from diabetic patients that contribute to the altered insulin response and muscle glucose transport (29). The reduction in HK2 gene expression and the increase in RRAD level suggest that physical inactivity triggers modifications similar to those observed in T2D. The fact that 4 wk of intensive retraining did not completely normalize the expression of several metabolic genes underscores the importance of avoiding even short periods of physical inactivity.

These authors further observed an increase in genes involved in inflammation and endoplasmic reticulum stress in response to insulin stimulation during bed rest. Other studies have reported an upregulation of proinflammatory markers (10, 44) whereas some others did not (25). This is also true for oxidative stress (10, 42). Inflammation and oxidative stress are however processes known to negatively impact insulin sensitivity and should receive a greater attention in future bed-rest studies investigating the mechanisms underlying the development of insulin resistance. This appears even more important when considering that oxidative stress has been involved in muscle atrophy during disuse and that there is a tangled relationship between muscle fiber type, oxidative stress, and insulin sensitivity.

#### PHYSICAL INACTIVITY AFFECTS LIPID TRAFFICKING BETWEEN ADIPOSE TISSUE AND MUSCLE

*Evidence from bed-rest experiments in humans.* The whole body insulin resistance induced by bed rest is concomitant with the development of hypertriglyceridemia in both sexes (7, 8, 25, 73) (Fig. 1). From a mechanistic point of view, these higher levels of plasma triglycerides can be due to an increased adipose tissue lipolysis, a decreased plasma fat clearance, or both.

As we previously noted, whole body lipolysis is reduced during bed rest in subjects with or without a predisposition to T2D (i.e., LBW subjects) (1). The decrease in the whole body lipolysis in the subcutaneous femoral adipose tissue seems to be related to a decrease in hormone-sensitive lipase (HSL) activity in both groups (1). Hojberre et al. (27) compared glucose uptake and lipolysis from subcutaneous abdominal and femoral adipose tissue by microdialysis in healthy subjects and in offspring of patients with T2D. The aim was to investigate whether physical inactivity would unmask defects of adipose tissue metabolism in FDR subjects. Although 10 days of bed rest significantly decreased lipolysis and tended to increase glucose uptake in the subcutaneous femoral adipose tissue in both control and FDR subjects, subcutaneous adipose tissue glucose uptake only increased in control subjects. Thus physical inactivity is as deleterious in control as in FDR subjects and even tends to attenuate the differences in glucose uptake subcutaneous adipose tissue that was observed between the two groups before bed rest. In summary, these results suggest that bed-rest-induced hypertriglyceridemia is not due to a higher lipolysis but likely caused by an altered plasma fat clearance.

Few human studies investigated fat uptake by peripheral tissues in humans. We determined the plasma metabolic fate of dietary fat by using stable isotope labeling (8). We showed that 2 mo of bed rest in women increases the spillover of nonesterified fatty acid (NEFA) released after hydrolysis of lipoprotein-triglycerides (chylomicrons or VLDL) by lipoprotein lipase (LPL) (Fig. 1). This higher NEFA spillover suggests that NEFAs are less taken up by peripheral tissues. This result is in accordance with a diminished gene expression of skeletal muscle LPL and FAT/CD36 (Fig. 1). Muscle atrophy also likely contributes to the lower plasma fat clearance and indirectly to hyperlipemia. To our knowledge, no data on liver and adipose tissue fat uptake are available during bed rest. Such studies are required to determine the respective role of these three main peripheral tissues in the development of hyperlipemia, which is strongly related to the development of insulin resistance.

*Evidence from analogs in animals.* Studies in rats using the hindlimb tail paradigm (9) showed that from 11 h to 11 days of inactivity induces a diminution of 80% of the muscle LPL activity, supporting the fact that physical inactivity decreases muscle fat uptake. Using the wheel-locked model, Booth et al. (15) further investigated in a nicely conducted series of experiments the effects of physical inactivity on lipid metabolism at the adipose tissue level along with the alterations in insulin sensitivity previously described. After 53 h of wheel-running cessation following 21 days of running, the authors measured a 25–48% increase in epididymal and omental fat pad weights, respectively (35). This increase in fat storage was associated with increased palmitate incorporation into triacylglycerol in epididymal fat in relation to an increase in protein level and activity of mitochondrial glycerol-3-phosphate acyltransferase 1 (mtGPAT) (36), a key regulator of triacylglycerol synthesis. These changes were associated with increases in both plasma insulin and triglycerides (34). Interestingly, the enlargement in abdominal fat mass was observed in both ad libitum and pair feeding, suggesting that fat storage is the result of physical inactivity per se. The increase in epididymal, perirenal, and retroperitoneal fat masses was due to an increase in fat cell number, in the absence of changes in adipocyte size (37). In

other words, these data indicate that physical inactivity promotes fat storage through adipocyte hyperplasia rather than hypertrophy.

Taken altogether, these results collected in both humans during bed-rest experiments and in rats using analogs suggest that physical inactivity shunts the delivery of plasma lipids away from muscle to adipose tissue but also stimulates, at least in rats, lipogenesis. This altered trafficking is likely concomitant (as a cause or a consequence) with an altered fat partitioning between oxidation and storage in muscle.

#### PHYSICAL INACTIVITY DECREASES FAT OXIDATION

*Evidence from bed-rest experiments in humans.* In normal conditions, the switch in fuel oxidation depends on the type and amount of nutrient (glucose, fatty acids, and amino acids) available for oxidation at the cellular level and is regulated through the activation or inhibition of specific metabolic pathways. In all bed-rest studies, a shift in fuel metabolism is observed in favor of carbohydrate oxidation and in detriment of lipid oxidation (Fig. 1). Importantly, this shift is independent of energy balance (63). The increase in nonprotein respiratory quotient (NPRQ) varies between 4 and 14% according to the duration of the bed rest (7, 8, 12). For example, 3 mo of bed rest induces in healthy lean men (7) a decrease of 37% in lipid oxidation and an increase of 21% in carbohydrate oxidation in postabsorptive situations. Similarly, changes of –40% and of +6% in lipid and carbohydrate oxidation are, respectively, observed in postprandial conditions.

The reduction in lipid oxidation is in fact more complex. It is well known that lipid oxidation depends on the length and the degree of saturation of the fatty acids: the shorter the carbon chain and higher the number of unsaturations, the greater is the oxidation (21). Using a double-labeling fatty acid method, we recently showed that physical inactivity differently alters the metabolic fate of dietary saturated and monounsaturated fatty acids (7, 8). Although bed rest does not affect dietary monounsaturated fatty acid (oleate) oxidation, it significantly decreases dietary saturated fatty acid (palmitate) oxidation in both healthy males and females (Fig. 1). This reduction in oxidation was likely in favor of incorporation of dietary saturated fatty acids into intramuscular lipids (Fig. 1) (8). Although we do not have a clear explanation for these striking observations, we submitted a hypothesis in a recent review (6). Briefly, carnityl palmitoyl transferase I (CPT-I) regulates the entrance of fatty acids into mitochondria and thus fat oxidation. mtGPAT regulates the first step of glycerolipid biosynthesis and thereby triglyceride synthesis. Because both mtGPAT and CPT1 are located on the outer mitochondrial membrane, they can compete for acyl-CoAs and thus reciprocally regulate the partitioning of fatty acids between degradative and biosynthetic fates in liver and muscle. The coordinated regulation of CPT1 and mtGPAT is mediated by AMP-activated kinase (AMPK). AMPK is an energy sensor that is influenced by physical activity; it is stimulated by exercise but reduced by physical inactivity. An increase in AMPK reduces malonyl-CoA concentration, which attenuates the inhibition of CPT1 and thereby increases fat oxidation. Conversely, it inactivates mtGPAT, which reduces triglyceride synthesis. A



decrease in AMPK has the opposite effects. Because mtGPAT has a significantly higher affinity for saturated than monounsaturated fatty acids, we considered this pathway a good candidate to explain the differential effects of physical inactivity on dietary oleate and palmitate oxidation and storage. This hypothesis needs to be investigated and is yet to be confirmed.

*Mechanistic evidence of the shift in fuel use from bed rest in humans and analogs in animals.* It is important to note that changes in fuel use are independent of the bed-rest-induced muscle atrophy, i.e., of changes in metabolically active mass. Nevertheless, this shift may be linked to the shift in muscle fibers characterized by an increase in glycolytic muscle fibers and a decrease in oxidative muscle fibers (69) (Figs. 1 and 2). Glycolytic fibers have low rates of fat oxidation and lower insulin sensitivity compared with oxidative fibers, which have high mitochondrial density and oxidative enzyme activities. However, a study in rats showed that the shift in substrate use preceded the shift in muscle fiber type (24). Thus the mechanisms involved in these changes in substrates use are still poorly characterized. Some hypothesis can however be proposed on the changes in metabolic pathways.

Associated with the decreased muscle fat uptake, both human and rat studies showed that physical inactivity reduces gene expression of CPT-I (61) (Fig. 1), a protein responsible for the entrance of long-chain fatty acids into mitochondria. In addition of the altered fatty acid transport into myocyte and mitochondria, a reorganization of the metabolic pathways likely plays a role. Hindlimb suspension in rats for several weeks induces an increased glycolytic capacity in atrophied muscle (61, 72) via an increase in the gene expression of three key enzymes of glycolysis: hexokinase, phosphofructokinase, and kinase pyruvate (61). Conversely, a reduction in the capacity to oxidize lipids was indicated by a reduction in the gene expression of enzymes involved in the beta-oxidation (61).

Simultaneously to the changes in the glycolytic and oxidative pathways, a complementary hindlimb-tail rat study showed an increase in the gluconeogenic capacities of liver (62) that could explain the greater use of glucose by muscle. Another study using the wheel-locked model (53) also reported in the liver a lower mitochondrial oxidative capacity and a higher malonyl CoA concentration associated with increased levels of proteins involved in de novo lipogenesis, suggesting that similar alter-

ations are occurring in liver and muscle. Based on these results, we can assume that the increase in glucose availability increases malonyl CoA concentration, which, by inhibition of the CPT-I, decreases the entrance of fatty acids into mitochondria, and hence fat oxidation. This results in an accumulation of nonoxidized fatty acids that are, in turn, likely synthesized as intramuscular lipids. An increase in gluconeogenic capacity has however still not been observed in humans (12) but remains to be demonstrated.

The intramuscular lipids can be in the form of triacylglycerol, diacylglycerol, and/or ceramides. A growing body of data showed that they contribute to alterations in insulin sensitivity. Although a negative correlation between intramyocellular triacylglycerol and insulin sensitivity has been repeatedly reported (31, 32), it is now accepted that it is not muscle triacylglycerol per se that induces insulin resistance but rather lipotoxic intermediaries such as diacylglycerol and ceramides (17, 30, 48). Although the respective role of these two intermediary lipids in insulin responsiveness alteration is still in debate, they are thought to engage stress-activated serine kinases that interfere with insulin signal transduction (28, 75).

#### PHYSICAL INACTIVITY INDUCES ECTOPIC FAT STORAGE

As expected, the reduced fat oxidation is observed concomitantly to different ectopic fat storages during bed rest (8, 20). For example, the significant increase in intramuscular lipid (+2.7%) observed after 2 mo of bed rest in healthy women (8) was correlated with the reduction in dietary palmitate oxidation (Fig. 1). This suggests that dietary fat is diverted from oxidation in muscle toward incorporation into muscle lipids. Along this line, the study of Settler et al. (65) showed an increase in intramuscular lipids with both high-carbohydrate and high-saturated-fat diets, but the increase was more marked after the high-saturated-fat diet (+32 vs. +17%). Although Settler et al. (65) did not observe a relationship between intramuscular lipid and insulin resistance likely due to the short period of the bed rest (2 days), Cree et al. (20) reported that intramuscular lipid negatively correlated with the reduced glucose uptake after 28 days of bed rest. The effects of physical inactivity on the different lipid fraction content, i.e., triglycerides, diacylglycerol, and ceramides, are still unknown. Studies investigating the profile of the intramuscular lipids are clearly required to better understand the relationship between the reduced oxidative capacity, the muscle fat storage, and the development of insulin resistance under physical inactivity conditions.

The accumulation of fat during bed rest seems to be a general phenomenon. It has indeed also been observed in bone marrow and within muscle fibers (71). Ectopic fat storage is thus likely to occur in other tissues. Although no direct measurements of fat in liver have been done in humans for obvious ethical reasons, suspended rats exhibit a lipogenic profile (53, 62), a reduced mitochondrial oxidative capacity, higher malonyl CoA concentrations (53), and an increase in gluconeogenic pathways (62). Altogether these results suggest the development of a nonalcoholic fatty liver. These aspects are further reviewed by Rector et al. (52a) as part of this Highlighted Topic series in the

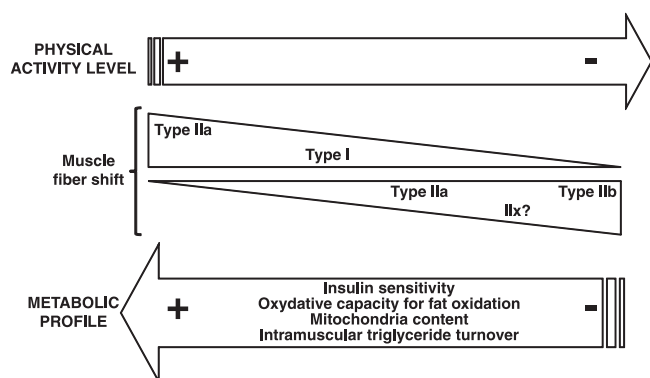


Fig. 2. Relationship between physical activity level and muscle fiber type profile and metabolic profile.

*Journal of Applied Physiology*. During three bed rests of 42, 90, and 60 days, in men and women, we have repeatedly observed that both aspartate aminotransferase (AST) and alanine aminotransferase (ALT) increased in a normal clinical range by almost 50% (unpublished data). Interestingly, epidemiological studies have shown that transaminases, and in particular elevated ALT, even in the normal range predict fatty liver and the new onset of T2D, independent of classical risk factors, metabolic syndrome, and C-reactive protein (55). It is likely that the increase in transaminases during bed rest reflects the development of a fatty liver. In support of that, some epidemiological studies in the general populations reported negative correlations between intrahepatic lipid content and habitual physical activity (52).

#### OVERALL, THE PHYSICAL ACTIVITY LEVEL PREDICTS METABOLIC FLEXIBILITY

The data collected during bed rest showed strong evidence for an effect of physical inactivity on fuel homeostasis. The largest body of data from the literature certainly suggests that physical training improves metabolic flexibility. A key question thus emerged: Will the body response to a continuum of changes in physical activity levels linearly translate into changes in metabolic flexibility? To answer this question we combined two studies from our laboratories that studied flexibility over a wide range of physical activity levels. Briefly, we measured metabolic flexibility in active women and highly trained men before and after an intervention that markedly reduced their physical activity level (bed rest in women and detraining in men). Plasma insulin concentration and NPRQ (indirect calorimetry) were measured hourly before and after the consumption of two standardized meals. For each individual we used the insulin and NPRQ data to calculate the mathematical variance of the daily NPRQ and insulin responses to the test meals. From this, we calculated the average and the standard error of these variances per group. We defined metabolic flexibility by a low variance in insulinemia associated with an elevated variance in NPRQ, i.e., a small shift in the fuel mix being oxidized for a high insulin secretion, and the converse. By using this approach that placed subjects in normal physiological conditions, rather than in supraphysiological conditions as during a hyperinsulinemic euglycemic clamp, we showed that the variances of insulinemia and NPRQ are inversely but linearly associated along a physical activity continuum (Fig. 3). This indicates that habitual physical activity predicts metabolic flexibility.

#### CONCLUSION : A SCENARIO TO EXPLAIN HOW PHYSICAL INACTIVITY INDUCES METABOLIC INFLEXIBILITY

The above review of the literature provides evidence that physical inactivity as investigated by bed rest induces, independently of measurable changes in energy balance, a reduced capacity to use fat as substrate, muscle atrophy, and a shift in muscle fiber type toward fast-twitch glycolytic type, a resistance to the effect of insulin and a hypertriglyceridemia along with an ectopic fat storage. Similar alterations in metabolic pathways are associated with metabolic disease state.

The following sequence of events can be hypothesized to explain the physical inactivity-induced metabolic alterations

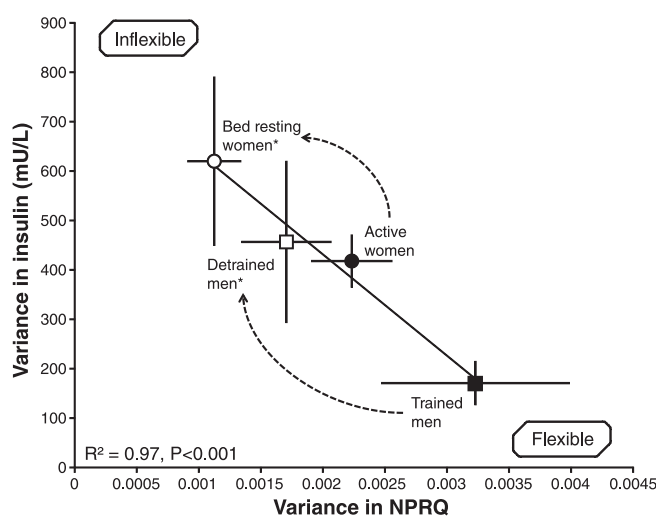


Fig. 3. Physical activity predicts metabolic flexibility. This figure represents the relationship between the daily individual intraindividual variances of plasma insulin concentrations and nonprotein respiratory quotient (NPRQ). These variances have been measured before and after 2 standardized meals over a period of 7–10 h before and after an intervention reducing physical inactivity in healthy men and women. Physically active women ( $n = 8$ ) were subjected to 2 mo of bed rest (cf Ref. 8), and trained men ( $n = 10$ ) were asked to stop for 1 mo both structured and spontaneous physical activity (unpublished data). For an equivalent food quotient, metabolically flexible subjects will greatly increase carbohydrate oxidation (i.e., high variation in NPRQ; right) after the consumption of a meal despite a low increase in plasma insulin concentration (i.e., low variation in insulin; bottom). A metabolically inflexible individual, i.e., a person who also displays an insulin resistance, will display a low increase in carbohydrate oxidation (i.e., low changes in NPRQ; left) despite an marked elevation in insulin secretion (i.e., high variation in insulin; top). We can observe that variances in NPRQ and insulin are linearly distributed along a continuum of physical activity level.

and thus metabolic inflexibility (Fig. 4). The physical inactivity induced by bed rest leads to insulin resistance in skeletal muscle, requiring a hyperinsulinemic response to properly dispose of glucose in daily postprandial conditions, whereas adipose tissue displays an appropriate response. At the same time, muscle fiber type shifts toward fast-twitch glycolytic fibers, and muscle increases glucose uptake and oxidation through insulin-independent pathways. This in turn inhibits fatty acid oxidation and ultimately uptake. During meal ingestion, hyperlipemia occurs due to a decreased plasma clearance of dietary fat. This increases the flux of dietary lipids to organs and results in ectopic fat storage with consequences on insulin sensitivity. The liver displays susceptibility to hyperinsulinemia and increased lipid synthesis and storage that overcomes rate of oxidations. Hepatic steatosis will likely ensue. With a reduced oxidative capacity, the liver will then contribute to an increased rate of atherogenic lipid products (VLDL) in which the contributions of FFA coming from the diet and neolipogenesis to the total VLDL-triglycerides will increase, feed-forwarding hyperlipemia and ectopic fat storage. Concomitantly, the steatotic liver will become insulin resistant and unable to suppress hepatic glucose production, which leads to increased gluconeogenesis and feed-forward worsening of hyperinsulinemia.

Although the hypothesis described above is plausible, the available data remain too scant to draw a more accurate kinetic

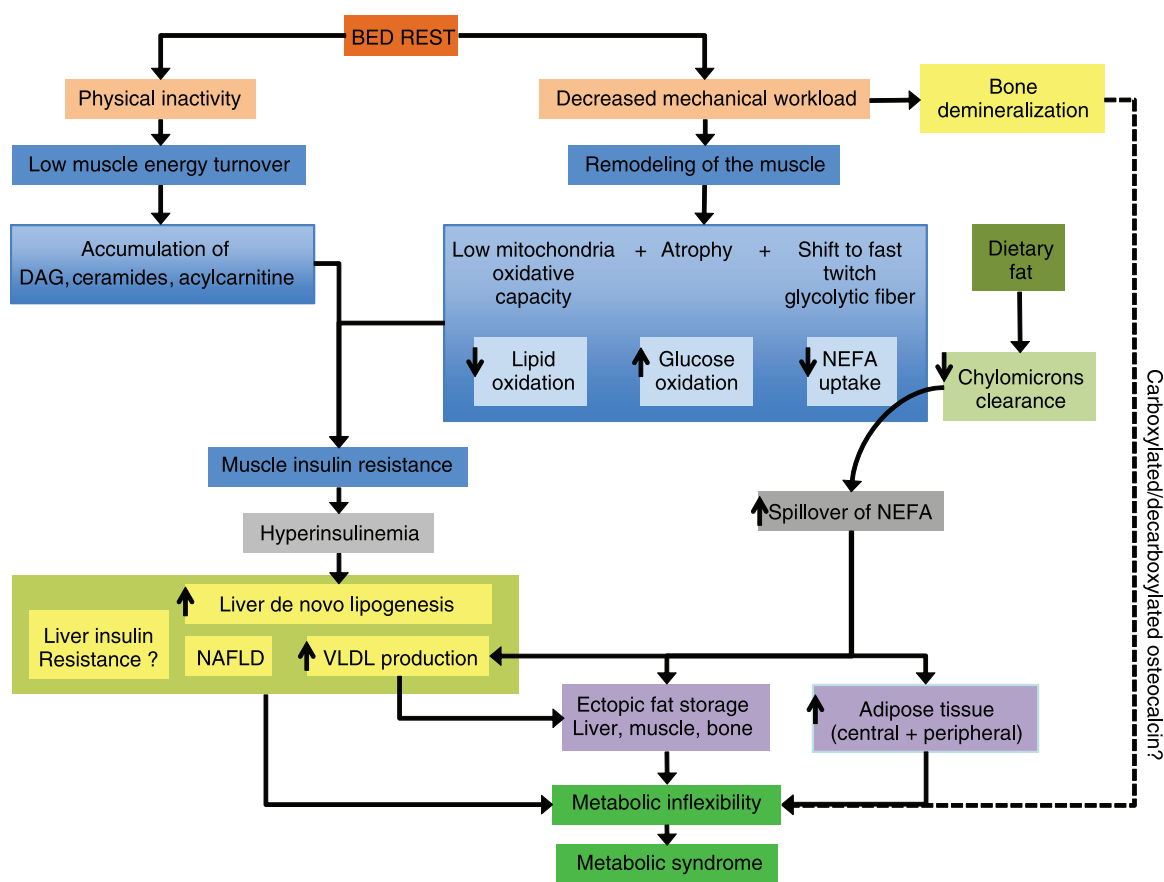


Fig. 4. Hypothetical metabolic alterations cascade induced by bed rest that can explain how physical inactivity induces metabolic inflexibility. VLDL, very-low-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; DAG, diacylglycerol.

of the adaptations to bed rest. More controlled and standardized studies are needed to better assess the time course of changes. The present review presents other limitations. Most of the results reviewed therein were obtained in studies with small sample sizes (usually between 4 and 8) and mainly in male subjects. Some of the conclusions may not apply to women. It can be also difficult to have a good estimate of the decrease in physical activity experienced by the subjects since measurements of physical activity and total energy expenditure were not systematically done, even before bed rest. This critical piece of information is necessary to know if the observed effects stem from a decrease in physical activity, or from detraining in very active people/athletes, and consequently the extent to which the conclusions of a study can apply to the general population. Furthermore, numerous bed-rest studies, especially the earlier ones, did not tightly control the subjects' diet, and the confounding effects of a potential positive energy balance should be kept in mind. In this present review, we finally did not mention one of the most important aspects of bed-rest studies: the countermeasures. The efficiency of pharmacological (e.g., pamidronate, resveratrol), nutritional (e.g., high-protein diet), and exercise (e.g., aerobic or resistive exercise training) countermeasures in alleviating the deleterious adaptations to space environment are tested in most bed rests. These results can provide valuable insight into the development of efficient strategies to counteract the effects of physical inactivity. Although these results are of great

importance in the battle against the general adoption of sedentary behaviors, we chose to only focus our review on the negative effects of bed rest.

Despite these limitations, we believe that the data accumulated over years from the bed-rest studies support the idea that physical inactivity by itself is a main factor contributing to the onset of numerous chronic diseases. This again raises the importance of investigating directly the physiology of physical inactivity in order to better understand the impact of the adoption of sedentary behaviors in the general population on general health. Although bed rest is a unique model to investigate mechanisms underlying defects induced by physical inactivity in healthy subjects, it is important to remember that bed rest induces a level of physical inactivity likely different (quantitatively and qualitatively) from that observed in the general population. Studies that investigate the exact decrease in physical activity encountered over the past century in the general population will thus be needed to confirm these results. Future studies also need to determine the physical activity level threshold below which metabolic inflexibility is developed in order to refine the physical activity recommendations in the general population.

#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).



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## 4. Programmes de contremesures : les solutions mises en place lors des études de microgravité réelle et simulée

Les chapitres précédents ont présenté les impacts de la microgravité sur les différents systèmes physiologiques. L'enjeu des recherches spatiales impose une maîtrise et une compréhension de l'adaptation de l'organisme à l'absence de gravité, afin de développer des solutions efficaces pour pallier à ces altérations physiologiques.

### *a. Gravité artificielle*

Dans l'environnement spatial, la première contremesure qui vient à l'esprit est de recréer la gravité absente dans l'espace. La gravité artificielle peut être créée en accélérant ou décélérant l'engin spatial par une accélération linéaire ou centripète d' $1g$ . A bord de l'ISS, il est actuellement impossible de réaliser une accélération linéaire d' $1g$  ; en revanche l'accélération centripète est, elle, réalisable par centrifugation. La centrifugation représente un défi tant au niveau spatial que financier. Au niveau de la durée des protocoles, il n'est pas forcément nécessaire pour les astronautes de subir la gravité artificielle durant 24h. Ainsi, une adaptation de la technique de gravité artificielle, aussi bien au niveau de la construction de la centrifugeuse au sein de la station qu'au niveau des protocoles de centrifugation, notamment leurs durées et force, est importante avant validation de l'utilisation d'une telle contremesure dans l'espace. Sur l'homme, quelques tests ont été réalisés durant la mission Gemini-11, Apollo ou Spacelab D-1. Ce n'est qu'en 1998, durant la mission Neurolab, que les astronautes ont pour la première fois été soumis à une gravité de 0,5 à  $1g$ . Lors de cette mission, les astronautes ont rapporté un impact positif de la centrifugation sur leur physiologie, comme par exemple une absence de mal des transports et chez certains astronautes une absence d'intolérance orthostatique après le vol. Ces résultats décrits au cours des missions spatiales sont très encourageants quant à l'aboutissement d'une contremesure efficace pas seulement sur un seul système physiologique mais sur l'organisme dans son ensemble (Clement et al., 2004). Seule l'exposition aux radiations ainsi que les contraintes environnementales, telles que l'isolation et le confinement ne sont pas, de façon évidente, influencée par la gravité simulée. Sur terre, au cours d'études d'alitement prolongé, la centrifugation a été utilisée avec deux objectifs principaux : tester l'impact du déconditionnement avant et après l'alitement en évaluant la tolérance à l'accélération des sujets, ou en tant que contremesure dans le but d'éviter ou de minimiser les effets du déconditionnement. Les premières études ont été

réalisées dans les années soixante et montraient déjà une prévention des malaises vagaux qui surviennent normalement au moment du lever, grâce à une série de centrifugation (Figure 15). Par la suite, d'autres études ont démontré des effets bénéfiques sur le système cardiovasculaire avec notamment prévention de l'intolérance orthostatique classiquement observée au cours des alitements prolongés (Iwase, 2005). Combinée avec de l'exercice aérobique, la centrifugation permettrait de prévenir l'augmentation de la fréquence cardiaque et de maintenir la fonction systolique cardiaque (Yang et al., 2011). La gravité artificielle n'a pas comme seule conséquence de prévenir l'intolérance orthostatique, mais est également connue pour son impact favorable sur la prévention de l'atrophie musculaire (Symons et al., 2009; Williams et al., 2009). En effet, 1h de gravité artificielle permettrait de maintenir la synthèse protéique dans le *vastus lateralis*. En revanche, la déminéralisation osseuse ne serait pas totalement évitée (Smith et al., 2009; Williams et al., 2009). Aussi bien au sol qu'en vol, cette approche intégrée du développement de contremesures est prometteuse, mais nécessite des études plus poussées, afin de déterminer la durée et la technique optimales.



Figure 15: Gravité artificielle recrée chez l'homme par une centrifugeuse. Crédit photo : ESA.

### *b. Contremesures : exercices physiques*

Dans un contexte d'inactivité physique sévère provoqué par l'absence de gravité, l'exercice physique est également l'une des contremesures testées pour pallier aux effets de la microgravité (Carter et al., 2013). L'objectif premier de ce type de contremesures est de cibler l'atteinte musculo-squelettique, tout en répondant aux besoins spécifiques de chaque astronaute. En effet, une grande variabilité entre les individus dans l'atteinte musculo-squelettique a été décrite au cours des vols, que cela concerne la diminution de la force, de la puissance et de l'endurance musculaire, mais aussi de la densité osseuse (Adams et al., 2003; Lang et al., 2004; LeBlanc et al., 2000b).

Il existe deux types d'exercices physiques utilisés au sein de l'ISS afin de maintenir les capacités physiques des astronautes : les exercices aérobiques et résistifs. Les exercices ont été développés pour faire travailler les membres inférieurs, mais également les membres supérieurs, qui avaient été négligés sur les précédentes missions. Ces deux types d'exercices physiques sont également testés au cours d'études d'alitement prolongé à tête déclive avec l'avantage est de pouvoir tester différents protocoles avec des durées ou des intensités différentes.

Basés sur des études réalisées sur terre, les protocoles d'entraînement aérobique en vol incluent plusieurs fois par semaine un travail sur un cyclo-ergomètre (Cycle Ergometer with Vibration Isolation and Stabilisation System : CEVIS) (Figure 16). Certains astronautes travaillent quant à eux sur un tapis roulant (Treadmill with Vibration Isolation and Stabilisation System : TVIS), également à disposition à bord de l'ISS. Ces protocoles visent à maintenir plus particulièrement les performances aérobiques des astronautes. Ils sont généralement réalisés à une intensité comprise entre 50 et 75% de la  $VO_{2max}$ , la consommation maximale d'oxygène des astronautes (Convertino, 2002). Des résultats variables ont été décrits après 84 jours de vols chez 3 astronautes, qui présentaient une augmentation de leur  $VO_{2max}$  (Convertino, 2002) après leur séjour. De manière intéressante, Trappe et al. (2009) a également montré un impact du type de machine utilisée sur la prévention de l'atrophie musculaire (Trappe et al., 2009). En effet, au cours de son étude, seuls les astronautes qui ont travaillé sur le TVIS ont présenté une atrophie musculaire moins importante, contrairement à ceux qui avaient réalisés les exercices sur CEVIS (-7% vs -17%). Au sol, les capacités d'endurance des participants, sont généralement mesurés sur un tapis roulant en position couchée. Après une étude d'alitement prolongé de 30 jours, les sujets qui ont eu un entraînement aérobique intensif à 60-90% de leur  $VO_{2max}$ , 5 jours/semaine, ont réussi à maintenir le  $VO_{2max}$  au niveau obtenu avant l'alitement (Greenleaf et al., 1982). Avec le même type d'exercice, Hastings et al. (2012) a montré de façon similaire une amélioration ou un maintien de la masse cardiaque, ainsi que de la capacité à l'exercice après 5 semaines d'alitement chez les femmes (Hastings et al., 2012). En comparant les résultats obtenus au sol avec ceux obtenus en vol, il est important de tenir compte de l'impact différencié de la position debout et couchée lors de la réalisation du même exercice aérobique. En effet, Quinn et al. (1995) ainsi

que Convertino et al. (1982) ont montré une fréquence cardiaque plus faible chez les sujets allongés que chez les sujets pratiquant un exercice physique sur l'ergomètre en position assise (Convertino et al., 1982; Quinn et al., 1995). De même, la capacité aérobique des sujets est diminuée suite à l'alitement après l'exercice physique effectué en position debout (Convertino et al., 1982). Ainsi, il est nécessaire que les tests soient réalisés de la même manière durant la période précédant et pendant l'alitement afin que les résultats obtenus correspondent au mieux à ceux qui pourront être potentiellement obtenus au cours des vols spatiaux.

En conclusion des effets de l'exercice aérobique aussi bien en vol qu'au sol, il ressort un effet positif de la course dans le maintien de la capacité aérobique des astronautes. Cependant, suivant l'intensité et/ou la fréquence de l'exercice appliqué, l'effet sur le métabolisme ne serait pas le même. Ainsi en modulant ces paramètres, les capacités aérobiques peuvent être maintenues même dans le cas de protocoles différents (Lee et al., 2010).

Dans le but de rendre l'entraînement physique le plus complet possible, les protocoles de contremesures en vol incluent également des exercices résistifs réalisés sur l'aRED (advanced Resistive Exercise Device). Le principal objectif de ce type d'appareil est de maintenir la force musculaire et osseuse. Ceux-ci sont réalisés sur une plateforme verticale permettant les flexions tout en poussant contre une plaque. La résistance de cette plateforme peut varier et permet d'entretenir la force physique de chaque sujet. De récentes études ont montré que l'exercice résistif impacterait positivement la perte osseuse en augmentant la formation de l'os (Shackelford, 2004). Les exercices résistifs sont très souvent combinés à d'autres contremesures, dont la plus courante est la vibration. Son efficacité a été prouvée sur l'atrophie des myofibres et dans le maintien de la force spécifique allouée à l'exercice (Bamman et al., 1998), contrairement à l'exercice résistif seul (Rittweger et al., 2005). De la même manière, l'exercice résistif vibratoire préviendrait la perte osseuse au niveau du tibia (Rittweger et al., 2010). Des résultats similaires ont été retrouvés après 60 jours d'alitement chez l'homme, dont la prévention de la perte osseuse a été meilleure dans le groupe sujet à un exercice résistif et vibratoire (Belavy et al., 2010). D'autres systèmes physiologiques sont également touchés par les contremesures physiques résistives. Ainsi au niveau cardiovasculaire, l'exercice résistif vibratoire a permis de maintenir la sensibilité du baroréflexe, mais n'a pas pu prévenir l'intolérance orthostatique (Coupé et al., 2011). Enfin, au cours d'une étude d'alitement prolongé, ce type d'exercice a permis de préserver l'atteinte cardiaque caractéristique des effets de la microgravité (van Duijnhoven et al., 2010).

Certaines études ont également analysé l'effet combiné d'un exercice aérobique et résistif. En effet, actuellement, à bord de l'ISS, les protocoles d'entraînement combinent les deux types d'exercices à des fréquences différentes, de 3 à 6 fois par semaine (Schneider et al., 2003; Shackelford, 2004). L'efficacité de la combinaison des deux types d'exercice reste cependant controversé. En effet, LeBlanc et al. (2000), ont démontré que la densité osseuse n'avait pas pu être maintenue à son taux normal durant la mission spatiale malgré la combinaison des deux types d'exercices



combinés (LeBlanc et al., 2000b). En revanche, après une étude d'alitement, la combinaison d'un exercice résistif et aérobique a eu un impact partiel sur l'altération du métabolisme lipidique. Ainsi, après 60 jours de microgravité simulée, la contremesure physique combinée a limité la diminution de l'oxydation des lipides alimentaires. Cependant il n'y a aucun effet sur le stockage ectopique des lipides dans le muscle (Bergouignan et al., 2009), contrairement à un effet positif d'un entraînement résistif seul sur le stockage ectopique des graisses dans la moelle osseuse (Trudel et al., 2012).

En conclusion, de réels effets positifs de l'exercice qu'il soit aérobique et/ou résistif et/vibratoire sont observés. Cependant, l'intensité et la fréquence des exercices restent encore à définir afin de trouver le meilleur compromis entre efficacité et faisabilité et de pouvoir l'appliquer à bord de la station spatiale internationale. Les futures recherches tendent à une diminuer le temps alloué aux exercices au profit d'une augmentation de l'intensité et en espérant une meilleure efficacité (Convertino, 2002). Ainsi, la combinaison de plusieurs types d'exercices à différentes intensités ou d'autres contremesures est une approche à ne pas négliger.



**aRED** : advanced Resistive Exercise Device



**CEVIS** : Cycle Ergometer with Vibration Stabilisation System



**TVIS** : Treadmill with Vibration Isolation and Stabilisation System

Figure 16 : Exercices physiques réalisés au sein de la Station Spatiale Internationale. Crédit photo : ESA

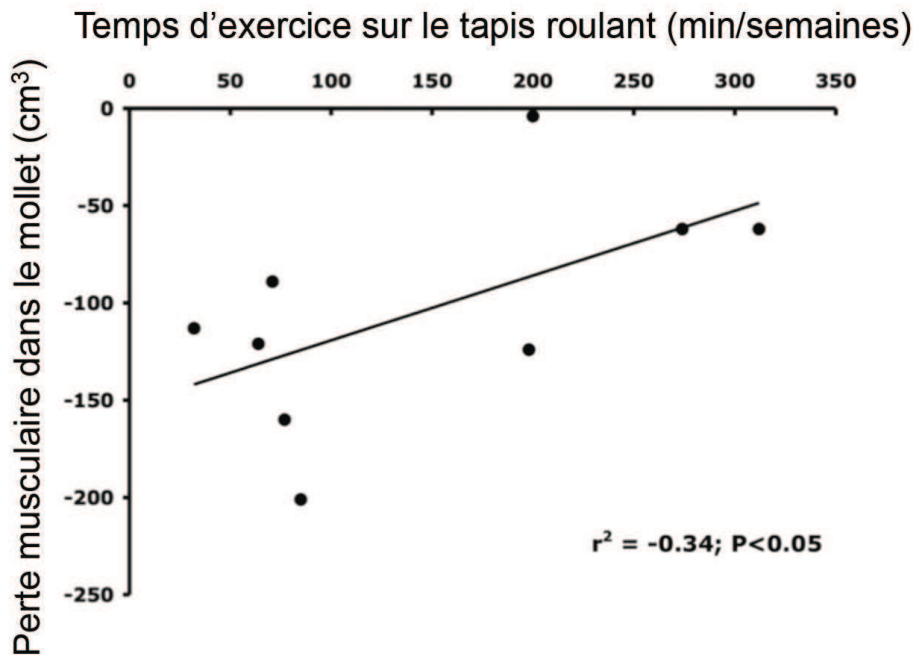


Figure 17 : Corrélation positive entre la perte musculaire du mollet et le temps d'exercice sur le tapis roulant. (Trappe et al., 2009).

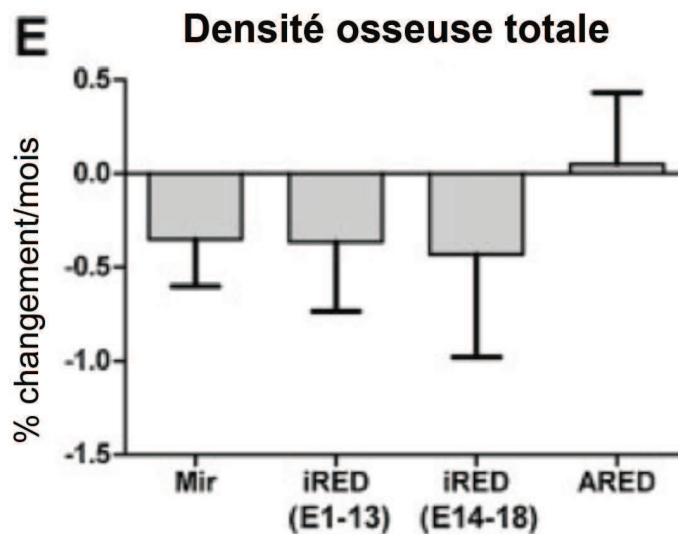


Figure 18 : Densité osseuse totale à bord de la station Mir, en fonction des différents types d'exercice (Smith et al., 2012a) : Mir (Sans exercice), iRED (interim Resistive Exercise Device) et aRED (advanced Resistive Exercise Device).

### c. Contremesures nutritionnelles

La nutrition qui correspond à l'assimilation des nutriments, les composants de base de nos aliments, joue un rôle primordial dans le métabolisme en apportant l'énergie nécessaire afin de maintenir l'équilibre physiologique et de ce fait, les performances des astronautes. A la suite de plusieurs études rapportant une déficience de l'organisme en certains nutriments pouvant être à l'origine du développement d'altérations physiologiques, diverses contremesures nutritionnelles ont été testées.

### i. Supplémentation en vitamines et antioxydants

Le contrôle de la nutrition au cours des vols spatiaux vise à prévenir le développement d'altérations physiologiques et notamment la déminéralisation osseuse ou l'atrophie musculaire. En effet, plusieurs molécules interviennent dans le processus de conservation de la densité osseuse, par le maintien de la concentration en calcium. La vitamine D est normalement synthétisée lors d'une exposition suffisante au soleil et permet d'augmenter l'absorption intestinale du calcium. Sa concentration au cours d'un vol spatial étant diminuée, elle est administrée au cours des vols spatiaux, combinée au calcium. Cependant, la supplémentation en vitamine D ne suffirait pas à prévenir l'ostéoporose (Holick, 1998).

Une autre vitamine, la vitamine K, est quant à elle responsable de la fixation du calcium sur les os et inhiberait la résorption osseuse (Heer, 2002). Sa supplémentation a été décrite comme augmentant la concentration plasmatique en marqueurs de la formation des os et réduisant ceux de la résorption osseuse. Elle stimulerait également la carboxylation de l'ostéocalcine, diminuant ainsi la fragilité osseuse (Caillot-Augusseau et al., 2000). Des études complémentaires restent cependant à réaliser afin de préciser les conséquences de la supplémentation en vitamine K chez les astronautes. En effet, Zwart et al. (2011) suggère l'inutilité d'une supplémentation en vitamine K, car aucun changement de concentration n'a été décrit chez 15 astronautes lors de leur mission à bord de l'ISS (Zwart et al., 2011).

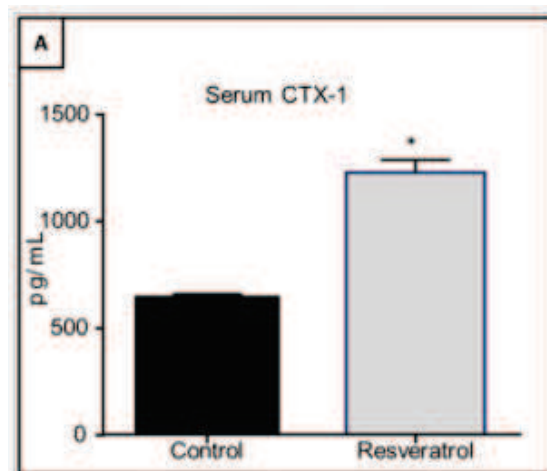
D'autres scientifiques ont également montré un possible effet préventif d'une supplémentation en micronutriments sur le développement de troubles métaboliques. C'est le cas de Savary-Auzeloux et al. (2013) qui ont montré qu'un cocktail antioxydant, extrait de thé vert, curcuma ou encore l'huile de noix, couplé à de la leucine stimulerait la synthèse protéique chez des rats suspendu. De ce fait, les rats supplémentés récupéreraient plus rapidement la masse musculaire perdue au cours de la suspension (Savary-Auzeloux et al., 2013). Toutes les études aussi bien au sol qu'en vol, ne démontrent pas d'effets ou des effets controversés des contremesures nutritionnelles, vitamines ou antioxydants, sur la déminéralisation osseuse (Smith et al., 2013a) ou l'atrophie musculaire, soulignant l'importance d'études complémentaires afin de trouver le dosage ou la composition adéquats.

Au-delà de l'impact sur la structure musculo-squelettique, la nutrition va avoir un rôle capital dans les changements hormonaux liés à la microgravité. Certaines hormones de stress (cortisol, épinephrine, norépinephrine etc.) sont sécrétées suite à une augmentation du stress. L'apport nutritionnel va probablement pouvoir jouer un rôle de temporisateur en permettant de maintenir le système électrolytique et notamment du sodium, dont la régulation implique les catécholamines (Cena et al., 2003). Dans le même contexte, le système immunitaire est également influencé par la nutrition. La composition nutritionnelle des repas, les suppléments en vitamines peuvent améliorer les défenses immunitaires diminuées dans l'espace. En effet, la diminution et l'altération de la synthèse des lymphocytes T a été décrite au cours d'un



vol, ou encore l'augmentation du risque de contracter une infection (Cena et al., 2003). Plusieurs micronutriments sont connus pour être impactés lors d'infections (Bhaskaram, 2002) et inversement : vitamines A, B12, C, riboflavine ou encore le zinc sont impliqués dans le développement de nombreuses maladies du fait de leur déficience. Par exemple, une déficience en zinc va entraîner une atrophie du thymus (Chandra, 1997), impliqué dans la formation des lymphocytes T. Enfin, au-delà de l'aspect physiologique de la nutrition, l'aspect social est également capital. Le déficit en certaines molécules dans l'alimentation peut à plus long terme altérer la mémoire, les fonctions cognitives des astronautes ou entraîner du stress ou de l'anxiété. L'ensemble de ces troubles peut altérer les relations sociales entre les astronautes et à plus long terme, le succès de la mission (Cena et al., 2003).

La plupart des contremesures nutritionnelles citées plus haut, supplémentation en micronutriments, vitamines ou antioxydants, sont testées dans un premier temps sur les animaux, facilitant l'accès à des données plus complètes et parfois inaccessibles chez l'homme. Ces études préalables permettent par la suite de mettre en place des protocoles chez l'homme. C'est dans ce contexte que nous avons testé une molécule anti-oxydante, plus précisément un polyphénol naturel, le resvératrol connu pour ses vertus activatrices du métabolisme lipidique mais également protéique (Aguirre et al., 2014; Chen et al., 2015; Feige et al., 2008). Au travers de la littérature, nous avons pu constater que le resvératrol pourrait avoir un effet bénéfique sur de nombreuses fonctions physiologiques, notamment sur la déminéralisation osseuse (Boissy et al., 2005; Durbin et al., 2014; Lee et al., 2014; Tou, 2014) (Figure 19). Nous avons montré entre autres que ce polyphénol pourrait prévenir la perte de la masse osseuse et musculaire, l'augmentation des risques de fractures et l'altération du métabolisme lipidique chez les rats suspendus durant 14 jours. Cet article est joint en annexe 1 de cette thèse.



**Figure 19** : Effets d'une supplémentation en resvératrol sur la concentration plasmatique en marqueur de la résorption osseuse (CTX-1). \* $p < 0.05$  (Lee et al., 2014)

## ii. Supplémentation en protéines

Sous l'effet de la microgravité réelle ou simulée, l'atteinte musculaire se traduit par une atrophie musculaire caractérisée par une augmentation de la dégradation protéique, visible par une augmentation de la concentration urinaire en azote, le produit du catabolisme protéique. Si la dégradation du muscle est plus importante que la synthèse, cela traduit une perte de masse musculaire. On parle de balance protéique ou azotée négative. Ces données ont notamment été mesurées au cours des missions Skylab et Life and Microgravity Sciences (LMS) (Lane, 1992; Stein, 2000). Sous l'effet de la microgravité, la balance protéique négative serait due à une diminution de la quantité d'acides aminés. Ils jouent un rôle particulièrement important dans la maintenance de la masse musculaire, surtout après des vols de longues durées (Cena et al., 2003). La majorité des études entreprises au sol montre également cette même diminution de la synthèse protéique (Biolo et al., 2004; Ferrando et al., 1996; Stuart et al., 1990). C'est à partir de ces résultats et de données populationnelles, qu'une supplémentation en protéines a été suggérée comme contremesure, afin de pallier à l'atrophie musculaire. En effet, sur terre, la supplémentation en protéines est une des solutions proposées aux personnes âgées dans le but de prévenir différentes altérations métaboliques qui surviennent avec l'âge, comme la sarcopénie également favorisée par le manque d'exercice physique (Freiberger et al., 2011).

La prévention de troubles métaboliques, comme la perte musculaire, par la supplémentation en protéines requiert cependant des études supplémentaires. En effet, toutes les études ne démontrent pas des résultats similaires (Stein et al., 2011). Plusieurs paramètres sont à prendre en compte pour expliquer ces résultats discordants, comme la quantité ou le type de protéines ingérées sous forme de supplément. D'un autre côté les caractéristiques des apports protéiques dans le groupe contrôle est également déterminant pour observer les effets de la supplémentation. A l'heure actuelle, l'apport journalier de base recommandé en protéines est de 0.8g/kg/jour alors que des études récentes suggèrent que les apports en protéines de base optimaux seraient plutôt entre 1.0 et 1.5g/kg/jour (Evans et al., 2013; Paddon-Jones et al., 2008; Stein et al., 2011; Stuart et al., 1990). De ce fait, comme le décrivent Stein et Blanc (2011), les effets bénéfiques d'une supplémentation en protéines au cours d'un alitement sont retrouvés uniquement dans les études dont le groupe/la condition contrôle est caractérisé par un apport protéique journalier dit faible d'en moyenne 0.9g/kg/jour. En revanche, lorsque le groupe/condition contrôle est caractérisée par un apport protéique plus élevé, d'environ 1.2g/kg/jour, les études ne montrent pas d'effets bénéfiques d'une supplémentation en protéines sur la perte musculaire (Stein et al., 2003; Trappe et al., 2007). L'hypothèse est que dans le second cas, l'apport protéique correspondrait aux besoins et n'aurait donc pas d'effets supplémentaires. Ainsi, la quantité de protéines ingérées serait donc importante dans le maintien de certaines fonctions

physiologiques, comme le présente Smith et al. (2012), avec une corrélation positive entre l'apport protéique journalier et la variation de densité osseuse du pelvis (Figure 20) (Smith et al., 2012a).

Il a également été démontré que la qualité des protéines ingérées peut influencer les effets de la contremesure sur le métabolisme. Il existe plusieurs types de supplémentation en protéines possibles. Ferrando et al. (2002) ont montré que la supplémentation en acides aminés (les briques de bases de la construction des protéines) permettent de prévenir la perte musculaire en stimulant la synthèse protéique et en maintenant la taille des fibres (Figure 21) (Ferrando et al., 2002). D'un autre côté, des tests ont été effectués après supplémentation en protéines de lait, contenant 80% de caséine et 20% de protéines du petit lait, qui ont montré un effet positif global de la supplémentation sur le développement de troubles métaboliques tels que l'hypertension, la dyslipidémie ou l'hyperglycémie chez l'enfant (McGregor et al., 2013). Il a été démontré que les protéines du lait limitent la conversion de l'angiotensine 1 en angiotensine 2, responsable de la vasoconstriction des vaisseaux sanguins et donc de la pression sanguine. De plus, ces protéines diminueraient la captation des lipides par le foie chez les rats atteints de stéatose hépatique. Enfin, la caséine stimulerait la sécrétion de l'insuline et aurait la capacité d'altérer le métabolisme glucidique en réduisant sa captation par les organes. De ce fait, les protéines de lait agiraient sur la dyslipidémie ou l'hyperglycémie. Enfin, chez des sujets sains, après un repas riche en protéines de lait, la réponse insulinémique est plus importante en comparaison avec d'autres protéines animales. De ce fait, la concentration plasmatique en glucose serait plus faible et les sujets auraient une sensation de satiété plus importante, limitant l'appétit, ainsi que la prise alimentaire (Pal et al., 2010). La supplémentation en protéines de lait d'origine animale a été comparée à la supplémentation en protéines d'origine végétale, comme les protéines de soja. A l'heure actuelle, peu d'études ont été réalisées sur le sujet humain et les résultats sont divergents (Bendtsen et al., 2013; Oliveira et al., 2012). Comme évoqué plus haut, les protéines de lait sont composées à 20% de protéines du petit lait qui ont la caractéristique d'être riches acides aminés branchés (BCAA pour branched-chain amino acids), composé d'environ 26% d'acides aminés essentiels (leucine, isoleucine et valine) (Ha et al., 2003). Plusieurs études montrent un effet stimulant d'une supplémentation en BCAA sur la synthèse protéique chez les personnes âgées, en créant une balance protéique positive (Ferrando et al., 2010). La même observation a été faite chez des adultes après un exercice résistif combiné à une supplémentation en BCAA (Rasmussen et al., 2000). Enfin, une augmentation de la rétention d'azote au cours d'études d'alitement prolongé est également décrite à la suite d'une supplémentation en BCAA (Stein et al., 1999). Cette contremesure nutritionnelle permettrait ainsi de prévenir l'atrophie musculaire (Figure 21) (Dillon et al., 2009; Ferrando et al., 2002; Paddon-Jones et al., 2004; Stuart et al., 1990). Les protéines du petit lait auraient également des effets bénéfiques sur d'autres systèmes que le système musculaire, comme le métabolisme lipidique. Il a été observé une diminution de l'hypertriglycémie (Bortolotti et al., 2011) ou encore de la

concentration plasmatique de différentes fractions d'acides gras : triacylglycérols, cholestérol total et LDL (Pal et al., 2010) chez des femmes obèses supplémentées. Les protéines du petit lait agiraient également favorablement sur la résistance à l'insuline et diminuerait la concentration plasmatique en insuline (Pal et al., 2010). L'une des hypothèses est que cet effet serait dû à l'effet de satiété produit par l'ingestion de protéines du petit lait. De ce fait, l'apport calorique en lipides étant moins important, on observe une diminution des différentes fractions d'acides gras plasmatiques.

A l'opposé, malgré ces effets encourageants, Weinheimer et al. (2011) n'a montré aucune amélioration de l'effet de l'exercice physique chez des sujets en surpoids après une supplémentation en protéines du petit lait (Weinheimer et al., 2012). Cette absence de résultats pourrait à nouveau être expliquée par un apport protéique journalier de base déjà plus élevé et probablement adéquat que les recommandations nutritionnelles de 0.8g/kg/jour. De même qu'aucun effet visible n'a été décrit sur les protéines inflammatoires telles que la CRP, IL-6 ou TNF- $\alpha$  chez des individus en surpoids (Pal et al., 2010). Dans les protéines du petit lait, l'un des acides aminés essentiels, la leucine, est considéré comme jouant un rôle clé dans la stimulation de l'anabolisme protéique et limitant ainsi la perte musculaire (Volpi et al., 2003). Le mécanisme sous-jacent précis n'a pas encore été élucidé. Les protéines du petit lait, qui font partie de ce que l'on appelle des protéines à « digestion rapide », augmenterait la vitesse de disponibilité de la leucine qu'elle contienne (Volpi et al., 2003).

L'ensemble de ces études a montré un impact de la quantité et de la qualité des protéines ingérées sur le métabolisme. La durée de supplémentation influencerait également l'efficacité de la contre-mesure. Ainsi Turner et al. (2014) n'ont montré un effet préventif des protéines de lait sur la sensibilité à l'insuline chez les personnes âgées qu'à partir de 12 mois de supplémentation. Le mécanisme sous-jacent reste cependant à déterminer (Turner et al., 2015). Une autre étude a montré que les protéines de lait agiraient sur la satiété et donc la prise alimentaire des personnes sur le court terme en comparaison avec la caséine seule qui agirait plus sur le long terme (Bendtsen et al., 2013) ce qui serait expliqué par le fait que les protéines du petit lait sont considérées comme des protéines à « digestion rapide » alors que la caséine est à « digestion lente ».

Pour finir, la supplémentation en protéines peut également induire des effets négatifs sur le métabolisme. En effet, l'apport trop important en protéines va entraîner une acidification du sang due à la diminution de molécules de bicarbonates ( $\text{HCO}_3^-$ ), caractéristique d'une acidose métabolique (Fettman, 2000). Cette acidification altère l'os en diminuant l'activité des ostéoblastes, mais également en inhibant l'activation de la vitamine D nécessaire pour le développement osseux. L'excès d'acides aminés entraîne également une exacerbation de la résorption osseuse, ainsi que de la perte calcique (Zwart et al., 2005). Cette dernière serait due à un excès de sodium et jouerait donc sur l'équilibre acido-basique de l'organisme (Frings-Meuthen et al., 2011; Heer et al., 2000). Afin de remédier à cette acidification, la combinaison de la supplémentation en protéines avec des sels alcalins, sous forme de potassium

bicarbonate ( $\text{KHCO}_3$ ) apparaît comme le meilleur compromis dans la littérature (Buehlmeier et al., 2012; Dawson-Hughes et al., 2009).

Au vu de l'impact positif de la supplémentation en protéines et particulièrement des protéines du petit lait, sur la prévention des altérations métaboliques liées aux effets de l'inactivité physique, nous avons choisi de tester cette contremesure nutritionnelle. L'apport protéique de base des participants à notre étude a été plus élevé que les recommandations nutritionnelles actuelles, soit 1.2g de protéines/kg/jour, et les participants à l'étude ont été supplémentés en protéines du petit lait combinée à du bicarbonate de potassium afin de prévenir l'acidification du sang.

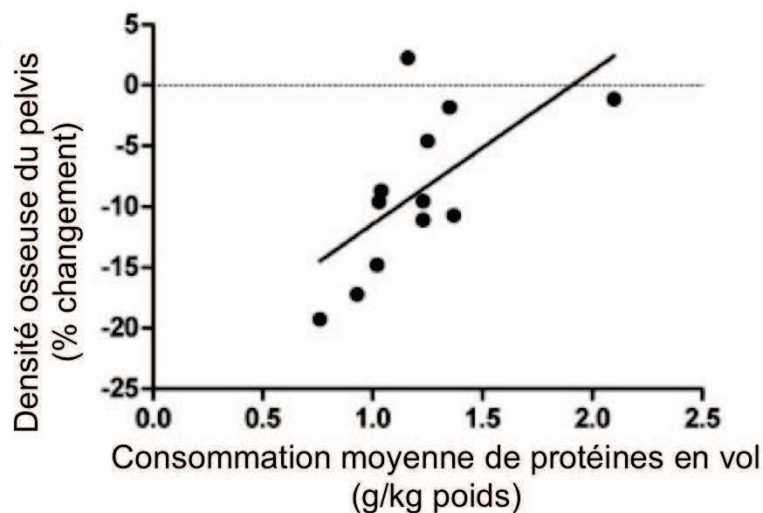


Figure 20: Corrélation entre la densité osseuse du pelvis et la consommation moyenne de protéines au cours d'un vol spatial de durée variable (4 à 6 mois) (Smith et al., 2012a).

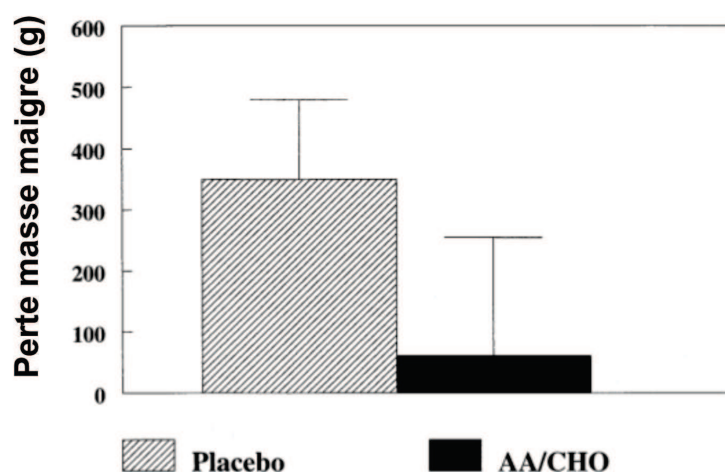


Figure 21: Perte de masse maigre après 28 jours d'alitement prolongé dans un groupe supplémenté en acides aminés et carbohydrates (AA/CHO) et sans supplémentation (placebo) (Ferrando et al., 2002)

En résumé de ce chapitre, les études entreprises en vol et au sol ont montré des résultats prometteurs des contremesures nutritionnelles dans la prévention des altérations métaboliques. Néanmoins, suivant les études, les résultats restent controversés et montrent la nécessité d'entreprendre des analyses complémentaires afin de préciser notamment la quantité, ainsi que la qualité des nutriments supplémentés, l'intérêt de cocktail de molécules ou de leur combinaison avec de l'exercice physique. Enfin, parmi les contremesures nutritionnelles, la supplémentation en protéines, et plus particulièrement en protéines du petit lait, présente des effets protecteurs spécifiques notamment sur la fonction musculo-squelettique, ou sur le métabolisme lipidique, l'inflammation ou la sensibilité à l'insuline.

.

## 5. Objectifs et hypothèses

### *a. Projet d'étude : objectifs*

Au travers de cette thèse, nous avons cherché à étudier l'impact différents niveaux d'activité physique sur le développement de dérégulations métaboliques, caractéristiques d'un environnement microgravitaire : une inflammation de bas niveau, une altération du métabolisme lipidique et de la sensibilité à l'insuline. En parallèle de ces travaux, nous avons voulu évaluer l'influence de contremesures nutritionnelles, la supplémentation en protéines du petit lait combinée à des sels alcalins sous forme de bicarbonate de potassium, sur ces altérations.

Ainsi, le premier chapitre de cette thèse se consacre à la variation de la concentration plasmatique en visfatine à la suite de différentes interventions réalisées sur le niveau d'activité physique des participants : 1 mois de désentraînement chez des sujets surentraînés, 10 jours de confinement et 3 mois d'inactivité physique sévère avec ou sans exercice résistif chez des sujets sains. La visfatine est une adipokine sécrétée préférentiellement par le tissu adipeux viscéral chez l'homme et connue pour son lien avec l'obésité ou le diabète. De ce fait, l'interaction entre les variations de concentration en visfatine et l'activité physique ou un index de résistance à l'insuline est également testée, indépendamment de toutes variations de la balance énergétique.

Le second chapitre se focalise sur l'impact de 60 jours d'inactivité physique sévère, sur l'évolution de marqueurs d'une stéato-hépatite, indépendamment de la variation de la balance énergétique. Pour cela quatre marqueurs hépatiques ont été choisi pour leur lien avec le développement d'une inflammation hépatique, ainsi que différents symptômes caractéristiques de maladies métaboliques: l'alanine et aspartate aminotransférase, la cytokératine 18 et l'angiopoietin-like 3. Ces données ont été complétées par le calcul de deux index de sévérité de l'inflammation hépatique. En parallèle, l'impact d'une contremesure physique, la combinaison d'un exercice aérobique et résistif, a également été testée.

Enfin, le dernier chapitre de cette thèse porte sur l'impact de 21 jours de simulation de la microgravité sur le métabolisme lipidique et sur la sensibilité à l'insuline. Le foie, le muscle et le tissu adipeux, impliqués dans l'altération métabolique liés à l'inactivité physique sévère, ont été suivis afin de déterminer leur rôle respectif dans l'oxydation et le stockage lipidique, ainsi que de la sensibilité à l'insuline. En parallèle de ces analyses, une contremesure nutritionnelle, la supplémentation en protéines et en bicarbonate a été testée dans la prévention de ces troubles métaboliques.



## *b. Hypothèses*

L'ensemble des travaux de cette thèse cherche à déterminer si les résultats suivent l'hypothèse d'un impact négatif d'une inactivité physique sévère sur le métabolisme lipidique, l'inflammation et la sensibilité à l'insuline.

- Plus spécifiquement, le premier chapitre se propose de tester l'hypothèse d'une augmentation de la visfatine, marqueur inflammatoire du tissu adipeux viscéral, après une diminution de l'activité physique chez les participants. Cette diminution serait plus faible ou inexistante chez les sujets surentraînés et dans le groupe ayant une activité physique plus importante.
- Le second chapitre testera l'hypothèse d'une augmentation des marqueurs hépatiques après un alitement prolongé de 60 jours chez la femme. Cette augmentation serait minimisée par la contremesure physique aérobic et résistive.
- Enfin, le dernier chapitre se propose de tester l'hypothèse d'un impact négatif de 21 jours d'alitement prolongé sur la sensibilité à l'insuline, avec le développement d'une résistance à l'insuline notamment dans le foie et dans le muscle. Le métabolisme lipidique serait également touché avec une diminution de l'oxydation des lipides totaux et alimentaires, le développement d'une hypertriglycémie, ainsi que d'un stockage ectopique des lipides dans le foie et le muscle. Enfin, nous testerons si la supplémentation en protéines et en bicarbonates préviendra totalement ou partiellement le développement de ces altérations métaboliques dans un contexte où l'apport protéique journalier de base suit les dernières recommandations nutritionnelles de 1.2g de protéines/kg de poids/jour.





# Matériels et méthodes

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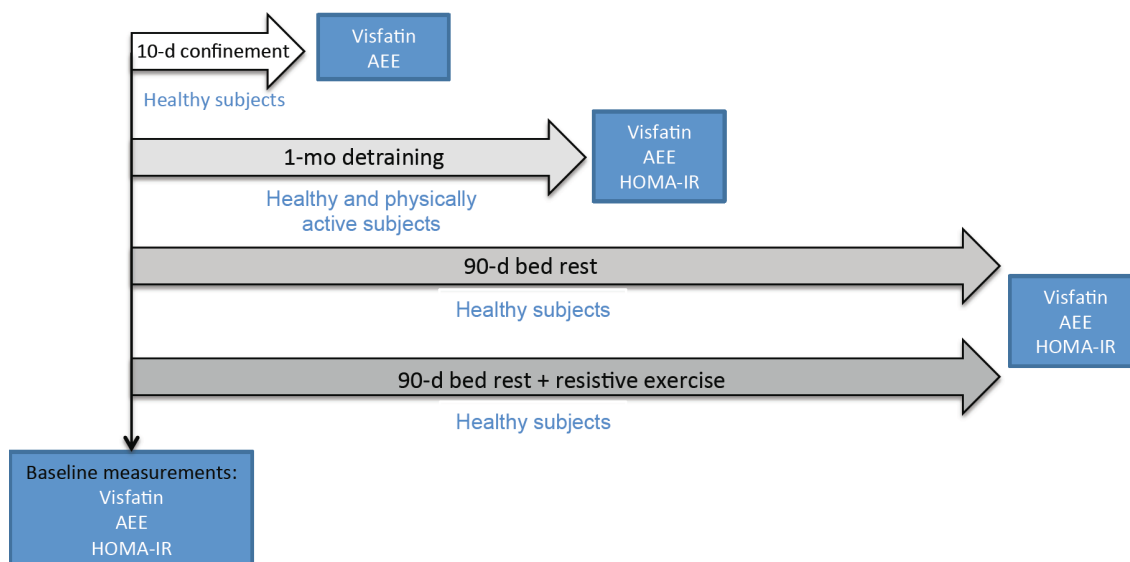
# Etude 1 : Variation de la concentration plasmatique en visfatine après différentes interventions sur les niveaux d'activité physique chez l'homme.

## *a. Design of the studies*

Healthy normal-weight volunteers (body mass index (BMI) = 20-25 kg/m<sup>2</sup>) participated in three studies. They were free of any chronic known diseases, and had no first-degree family history of obesity or type 2 diabetes, no alcohol or drug dependence and no medical treatment. Four different groups out of three different studies composed this retrospective study:

- The Strasbourg Lipid Oxidation study (LIPOX) was a detraining study on ten healthy trained and normal-weight men (Strasbourg, France, 2008), with a high physical activity level of 2.2 (defined as the ratio of the total energy expenditure over resting metabolic rate) (Bergouignan et al., 2013a). They were asked to stop structured exercise and reduce their spontaneous daily living activities during a month (1-mo detraining group) (Bergouignan et al., 2013b).
- The Thermo study was a confinement study conducted at the German Space Agency (DLR, Cologne, Germany, 2006). Eight healthy men were confined in a small metabolic ward for 10 days (10-d confinement group), with no access to any exercise facility.
- The third study was a 3-month bed rest (3-mo BR) study (Space Clinic, Toulouse, France, 2001), where fifteen healthy men were randomly divided into two groups: a control group (n=8; 3-mo BR group) who strictly remained in bed and an exercise group (n=7; 3-mo BR+EX group) who was subjected concomitantly to 3-mo of BR and a resistive exercise training program (Bergouignan et al., 2006).

Body composition, activity energy expenditure (AEE), fasting plasma visfatin were measured at baseline and at the end of each intervention on physical activity. Fasting plasma insulin and glucose were measured in the 1-mo detraining and 3-mo BR studies only (Figure 22).



**Figure 22:** Study design and protocol of the studies: Visfatin, Activity Energy Expenditure (AEE) and Homeostasis Model Assessment of Insulin resistance (HOMA-IR) were measured before and after 10-d of confinement, 90-d of bed rest with and without resistive exercise in healthy subjects and 1-mo detraining in healthy and physically active subjects.

### *b. Body composition*

In the 1-mo detraining and 10-d confinement studies, body composition was measured using the isotope dilution method (Blanc et al. 1998). In the 3-mo BR study, fat mass (FM) and fat-free mass (FFM) were measured by DXA (Bergouignan et al., 2006).

### *c. Physical activity energy expenditure*

AEE was calculated from the equation  $AEE = TEE - RMR - DIT$ , where TEE was total energy expenditure, RMR, resting metabolic rate and DIT, diet-induced thermogenesis. In the 1-mo detraining and 10-d confinement studies, RMR and TEE were respectively measured by indirect calorimetry and the doubly labeled water method over 10 days, as previously detailed (Blanc et al., 1998). DIT was assumed as 10% of TEE. In the 3-mo BR study, AEE was estimated from the same equation except that only RMR was measured. TEE was estimated by assuming a physical activity level (PAL) of 1.7 and 1.42 during control and bed rest periods respectively, and by using the following equation:  $PAL = TEE/RMR$  (Bergouignan et al., 2006).

### *d. Fasting plasma visfatin, insulin and glucose concentrations*

Visfatin was measured by Visfatin C-Terminal Enzyme Immunoassay kit (Phoenix Pharmaceuticals, Inc., EK-003-80, Burlingame, USA). Insulin was measured by radioimmunoassay (DSL, Inc. - 1600, Webster, USA) and glucose by enzymatic method (Biomérieux, n°61270, Craponne, France). Insulin resistance index was

estimated by calculating the homeostasis model of assessment – insulin resistance (HOMA-IR).

#### *e. Statistical analysis*

The effects of physical activity interventions on anthropometric and biological data and the associations between AEE, visfatin and HOMA-IR were analyzed by using mixed linear models taking into account the repeated individual data over time, with group and intervention as fixed effect and individuals as random effect. Heterogeneity of the intervention's effect across groups was tested. Least-square means and their SEM are presented. Post-hoc Tukey tests were used for pairwise comparisons. The models examining the associations between visfatin and HOMA-IR with AEE were adjusted for FFM and FM. Two-sided P-values are reported and significance was set to 0.05 and 0.10 for main and interaction effects respectively (SAS version 9.3, SAS Institute Inc., Cary, NC).





# Etude 2 : Impact de 60 jours d'inactivité physique sévère chez la femme sur l'évolution de marqueurs d'une stéato- hépatite.

## *a. Study design*

Sixteen healthy normal-weight women participated in a 60-d bed rest study (Bergouignan et al., 2009). Subjects were randomized into two groups (n=8, each): a control group subjected to strict bed rest and an exercise group that was subjected to supine resistive and aerobic exercise training protocol concomitantly to bed rest (Figure 23). Dietitians tightly controlled dietary intake during the whole period of the study to maintain subjects in energy balance. No alcohol consumption was allowed.

## *b. Body composition*

Body mass (BM), fat mass (FM) and fat free mass (FFM) were measured before and after 30 (BR30) and 60 days of bed rest (BR60) by DXA (Hologic, France).

## *c. Metabolic and hepatic markers measurements*

At baseline and BR30, dietary fat metabolism was fully investigated (Bergouignan et al., 2009). Fasting plasma insulin was measured by radioimmunoassay (DSL, Webster, USA) and glucose and triglycerides by enzymatic methods (Biomérieux, Lyon, France). Plasma leftovers were insufficient to measure fasting triglycerides and insulin at BR60.

From stored fasting plasma samples, we measured hepatic markers concentration at baseline, BR30 and BR60. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by enzymatic test using phosphate pyridoxal, as routinely done in hospital laboratories. Plasma angiopoietin-like 3 protein (ANGPTL3) and fragments of cytokeratin 18 (CK18) were, respectively, assessed by using Quantikine Human Angiopoietin-like 3 (R&D Systems Inc., Minneapolis, USA) and M30 Apoptosense (Peviva, Stockholm, Sweden) Elisa kits.

We considered two combined indexes of non-alcoholic fatty liver disease (NAFLD), the AAC index (an acronym for AST, ALT and CK18) calculated as the sum of reduced values (according to baseline values) of AST, ALT and CK18 and the CHA index (an acronym for CK18, HOMA-IR, AST Index) calculated as the product of homeostasis model assessment – insulin resistance (HOMA-IR), AST and CK18 (divided by 1000) (Polyzos et al. 2013). While we calculated the AAC index at both BR30 and BR60, the CHA index was obtained at BR30 only since fasting insulin was not measured at BR60.

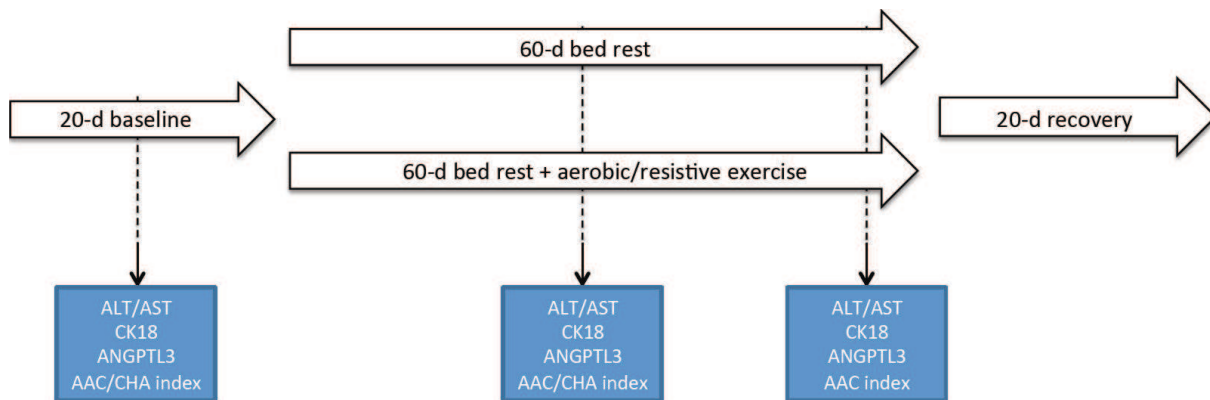


Figure 23: Study design and protocol of the bed rest: 20-d baseline followed by 60-d bed rest with or without aerobic and resistive exercise and 20-d of recovery period.

#### *d. Statistical analysis*

Data are presented as mean  $\pm$  SD. Analyses were performed with SAS software (SAS v.9.3., Institute Inc., Cary, North Carolina). Significance was set up to 0.05. Between-group comparisons at baseline were performed by using Student's t-tests. The evolution of anthropometric and biological variables was analyzed by using mixed linear models taking into account the repeated measures over time with individuals as random effects and baseline values of the dependent variable as fixed effect. Models considering biological variables were adjusted for FM. Pre-specified hypotheses were that bed rest will induce a significant increase in insulin, triglycerides and hepatic markers concentrations and that the exercise countermeasure will significantly reduce the effect of bed rest on the metabolic and hepatic markers leading to non-significant variations over time. One-tailed tests were used for these analyses. The associations between changes in hepatic markers concentrations and changes in anthropometric (FM) and metabolic (insulin, HOMA-IR, triglycerides) variables were tested by using Pearson correlations.

# Etude 3: Conséquences d'une simulation d'impesanceur de 21 jours chez l'homme, sur le métabolisme des lipides et effets d'une supplémentation en protéines testée comme contremesure.

## *a. Study design*

The impact of simulated weightlessness and protein supplementation on human physiology was evaluated during a 6° head down tilt (HDT) bed rest. The selection of the subjects began 6 months prior to the study, starting with approximately 200 subjects to select only 10 participants. All volunteers were healthy men, aged of  $31.0 \pm 2.1$  years old, non-smokers, with a normal body mass index ( $20\text{-}25 \text{ kg/m}^2$ ) and a maximum oxygen uptake of  $30\text{-}60 \text{ mL.kg}^{-1}.\text{min}^{-1}$ . No drug consumption, alcohol excess, vegetarianism were authorized. The volunteers subject to anemia, infectious diseases, migraine, diabetes, hyperlipidemia and hypocalcaemia or other metabolic diseases were excluded. The study protocol was approved by the ethics commission of the Ärztekammer Nordrhein (Düsseldorf, Germany) and the study was conducted in accordance with ethical principles stated in the Declaration of Helsinki (Acronym: MEP, ClinicalTrials.gov Identifier: NCT01655979).

The study took place at the German Aerospace center (DLR) in Cologne, Rheinland-Pfalz, Germany. It was organized over two sessions, 4 months apart, each starting by 7 days of baseline data collection (BDC), followed by 21 days of HDT bed rest and finally 5 days of recovery (R). During HDT period, the participants were continuously bed resting 24h a day, with seating and standing position strictly forbidden. During one session, while bed resting, half of the subjects were supplemented with a combination of whey protein ( $0.6\text{g/kg BW/d}$ ) and potassium carbonate ( $90 \text{ mmol KHCO}_3/\text{d}$ ), compared to the other half, which remained on a control diet. During the second session, they switched diet, so that each volunteer was his own control during the study (Figure 25).

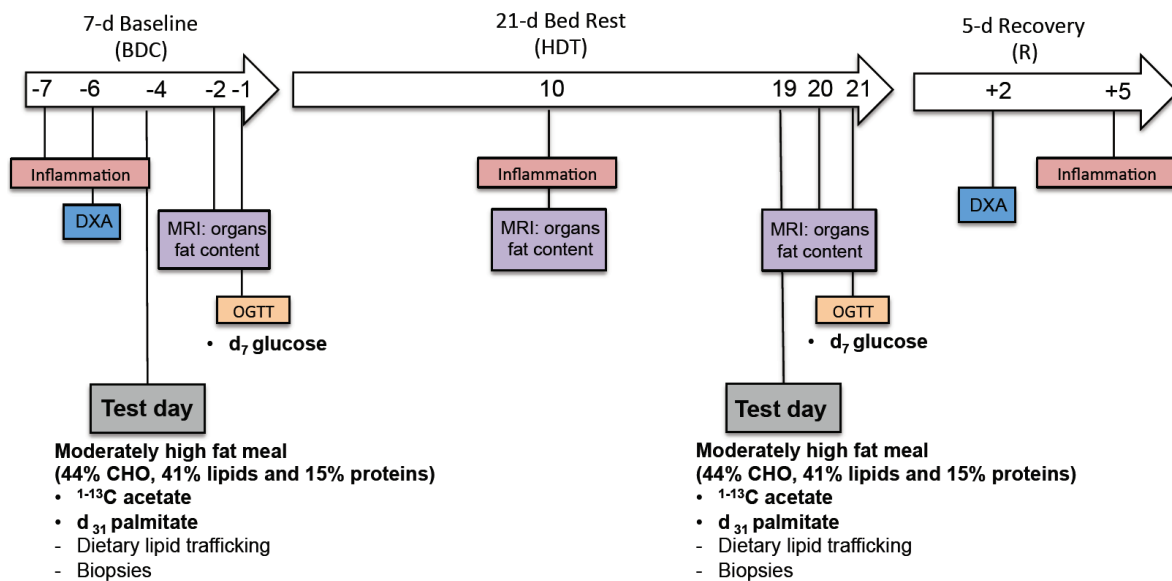


Figure 24: Overview of all experiments performed during one session of 21-d bed rest study.

The study is composed of 21-days of bed rest (HDT), preceded by 7 days of baseline (BDC) and followed by 5 days of recovery (R).

DXA: Dual X-Ray absorptiometry; MRI: Magnetic resonance imaging; OGTT: Oral glucose tolerance test

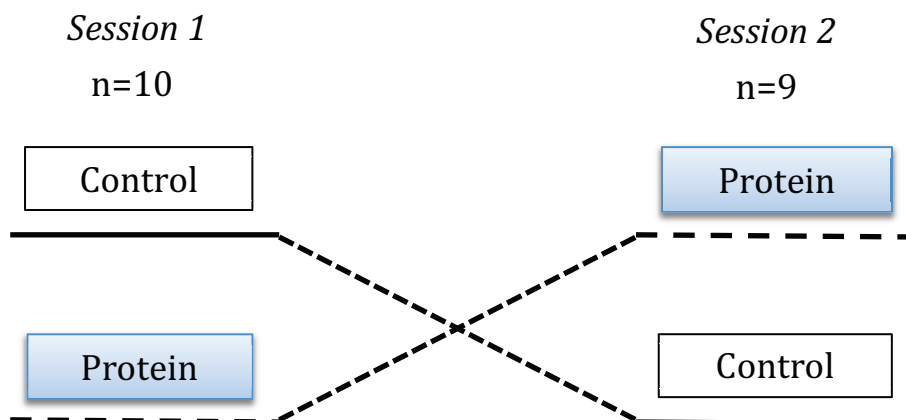


Figure 25: Crossover design of a 21-days head down tilt bed rest study with two conditions: control and supplemented in proteins

The organization of all experiments performed during the study is presented in Figure 24. Daily measures of body mass (BM) were made during the whole study. Body composition was made at baseline on BDC-6 and the second day of R period (R+2). We also measured liver, muscle and adipose tissue fat content three times during the study at BDC-2, HDT10 and HDT20 using magnetic resonance imagery (MRI).

To test our main hypotheses on the effect of bed rest on lipid metabolism as well as the effect of protein and sodium bicarbonate as countermeasures, we designed three complementary protocols to assess:

- Dietary fat metabolism: oxidation, trafficking and ectopic storage.
- Oral glucose tolerance.
- Inflammation and oxidative stress statuses

Dietary fat metabolism was dynamically investigated using dietary lipids labeled with stable isotopes administered in a standardized meal. Lipid trafficking and oxidation were evaluated twice during specific test days at BDC-4 and HDT19, using 1-<sup>13</sup>C acetate and d<sub>31</sub> palmitate.

Glucose tolerance was evaluated twice at BDC-1 and HDT21 using a typical oral glucose tolerance test in which d<sub>7</sub> glucose was added to assess exogenous glucose oxidation and improve our evaluation of insulin sensitivity.

Then, a longitudinal follow-up of different metabolic variables was conducted during the study using fasting blood samples.

Inflammation was investigated measuring hepatic markers of inflammation at BDC-7 and HDT10. Oxidative stress markers were also evaluated at BDC-4, HDT14 and HDT19 and so were measured lipoproteins. Finally, key metabolic markers were measured at BDC-4, BDC-2, HDT5, HDT10, HDT14, HDT19 and R+5.

### *b. Nutrition*

Nutrition was tightly controlled during the study. The first day, after 30 min of rest, RMR was measured by indirect calorimetry for 30min (Deltatrac II MBH 200 Metabolic Monitor, Datex Ohmeda, Madison, Wisconsin, USA.). RMR was then used to estimate energy requirement during the study based on the standardization document of ESA ("Standardization of bed rest study conditions" Version 1.5), 160% of the RMR during the baseline and recovery ambulatory period and 120% during the bed rest period to adjust to the decrease in physical activity. The nutrient daily intake followed the German recommendations of daily intake and was composed of 56% of carbohydrates ( $4.75 \pm 0.45$  g/kg/day), 30% of lipids ( $1.11 \pm 0.09$  g/kg/day) and 14% of proteins intake ( $1.21 \pm 0.01$  g/kg/day) during the ambulatory period. During HDT, in the control condition with no countermeasure, the carbohydrates were decreased to 51% ( $3.34 \pm 0.38$  g/kg/day), the lipids remained unchanged and the proteins increased to 19% ( $1.23 \pm 0.01$  g/kg/day). With protein supplementation, carbohydrates intake remained unchanged and an isocaloric replacement was done with an increase in protein intake to 28% ( $1.79 \pm 0.02$  g/kg/day) and decreasing of lipids intake to 25% ( $0.71 \pm 0.05$  g/kg/day).



Figure 26: Standard meal from subject F during baseline period.



### *c. Experimental protocols*

#### *i. Body composition and organ fat content*

BM was measured on fasting state, but after the first void and blood sampling (electronic scale, IS150IGG-S0CE, Sartorius, Göttingen, Germany). During the baseline and recovery periods, the measures were taken seated and in head down tilt position during the bed rest period.

Body composition, composed of FM and FFM was measured in a supine position, using a DXA scan (Hologic, Germany).

Liver, muscle and adipose tissue fat content was measured using an in-and-out of phase technique (1.5T, repetition time: 7.53, echo time: 2.38, image frequency: 64 Hz, Siemens Medical Systems, Erlangen, Germany) on each patient scanned in supine position.

#### *ii. Lipid trafficking and oxidation*

Test days started by providing baseline blood, breath and urine samples. It was followed by 30min of indirect calorimetry (Deltatrac II; General Electric) to measure RMR. The subjects then ingested a liquid meal (44% of carbohydrates, 41% of lipids and 15% of proteins) of approximately 4MJ, representing on average 52% of the RMR. It was composed of liquid meal (Renutril booster, Nestlé) mixed with colza and olive oil, 3g of 1-<sup>13</sup>C acetate (CLM-156-MG-0, Euriso-top, Saint-Aubin, France) and 2.2g d<sub>31</sub>-tripalmitate (660698-SPEC, Sigma-Aldrich, St-Louis, MO, USA). In addition homemade bread, butter and raspberry jam were provided. In the post-prandial period, hourly samples of blood, breath and urine were taken for 7 hours and indirect calorimetry performed every hour for 30 min. Plasma samples were obtained by centrifugation of total blood, 15min, 3000rpm at 4°C. Samples were stored at -80°C until analysis. Plasma samples were used to measure dietary palmitate oxidation and trafficking into NEFA and lipoproteins, insulin and glucose. Palmitate oxidation was further measured using spot urine samples collected for 3 days after the test. Breath tests were hourly collected to assess <sup>13</sup>C acetate oxidation to estimate tricarboxylic acid (TCA) cycle activity.

At the end of each test day, muscle and adipose tissue biopsies were taken between 9 to 11 hours after the moderately high fat meal and an extended snack. Muscle *vastus lateralis* and *soleus* biopsies were taken using a biopsy forceps inserted into the muscle after local anesthesia of the section. The biopsy is then cleaned with physiological serum and frozen into liquid nitrogen for further analyses. Adipose tissue biopsies were realized using a 50mL syringe. After anesthesia, the surgeon inserted the needle under the skin and made the vacuum by blocking the plunger. Slow movements under the skin allowed then the subcutaneous fat to get inside the syringe. The biopsy is then cleaned on a filter with physiological serum and frozen into liquid nitrogen. Samples were stored at -80°C until analysis.



Figure 27: Indirect calorimetry measurement performed on a participant



Figure 28: Standard breakfast eaten during a test day.

### iii. Glucose tolerance

Glucose tolerance was measured during an oral glucose tolerance test (OGTT) starting with baseline blood samples. The subjects then ingested 2.5g of d<sub>7</sub> glucose mixed in 300mL of glucose solution (0.25g/L) giving 75mg of glucose per subjects. Blood samples were taken every 30 min during 2 hours to measure plasma glucose and insulin concentrations and exogenous glucose oxidation. Exogenous labeled

glucose oxidation during an OGTT has been significantly correlated with insulin sensitivity measured by hyperinsulinaemic clamp (Bluck et al., 2013).

## *d. Analyses and calculations*

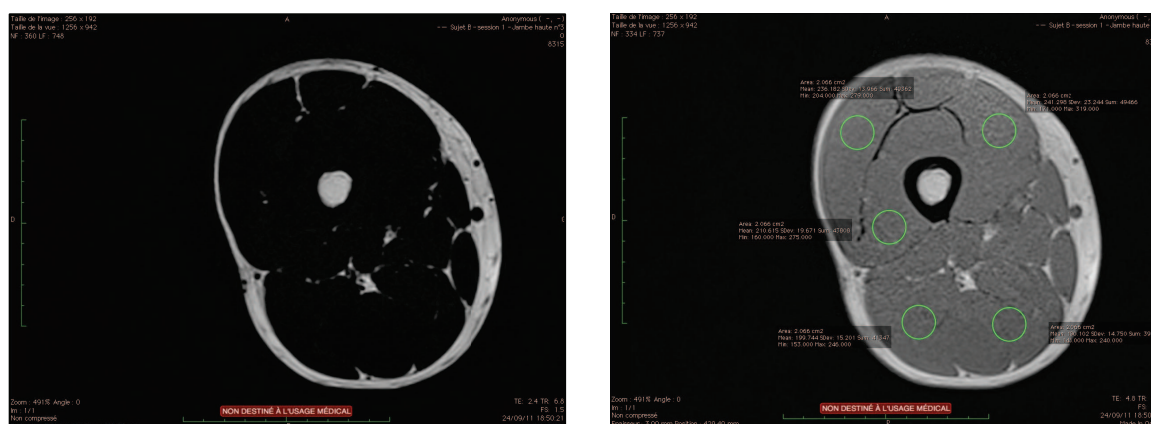
### *i. Organ fat content and regional fat depot*

An in-and-out of phase technique was used to evaluate organ fat content. In fact, water and fat protons have not the same resonance at 1.5T, thus we can differentiate them on an image. Two types of images are then measured with different spin echo pulse sequence. The first images are taking water and fat signals in-phase and the other out-phase. The combination of these measures gave us fat and water + fat images.

For liver fat content, the images were chosen avoiding artifacts and having the largest liver section. For thigh and calf fat content, the images were chosen at 1/3 of the femur and tibia lengths respectively, both starting from the knee.

In each sets of images, 5 round regions of interest (ROIs) from about 2cm<sup>2</sup>, were defined using Osirix software (Pixmeo Sarl, v5.5.2) and avoiding artifacts (Figure 29). The same ROIs were kept for every subject for the whole study. Using the number of pixels obtained out of the different ROI, the percentage of fat content in each organ was then calculated using this equation (Kim et al., 2008):

$$\text{Fat (\%)} = \frac{\text{number of pixels in fat image}}{\text{number of pixels in (water + fat) image}} * 100$$



**Figure 29:** In and out of phase technique left image: water and fat image of subject's B thigh. Five regions of interest have been selected; right image: fat image of subject's B thigh (OsiriX software).

We also assessed subcutaneous and visceral adipose tissue fat content. The segment chosen was taken between the 4<sup>th</sup> and 5<sup>th</sup> lumbar vertebrae to have the same reference for each subject during all measures. Thigh and calf subcutaneous fat contents were measured at the same level than intramuscular fat content. All

calculations were made by segmentation using Amira software (Visage imaging, Inc., Berlin).

## ii. Energy expenditure, total and dietary substrates oxidation

### 1. $d_{31}$ palmitate oxidation

Both plasma and urine samples were used to measure  $d_{31}$  palmitate oxidation. 0.5 ml of urine samples were decolorized with 20mg of black carbon and filtered to extract water using mini centrifuge filters with a cut-off of 10kDa and a membrane made of Polyethersulfone (PES) (Vivaspin 500, Sartorius, France). Plasma samples were only filtered.

The online determination of hydrogen isotope ratio in water was performed using a high-temperature conversion elemental analyser (HTC/EA) interfaced with a Delta V Plus, Isotope Ratio Mass Spectrometer (IRMS), through a ConFlo III (Thermo Scientific, Bremen, Germany for all instruments). The elemental analyser was equipped with the bottom feed connector and a glassy carbon tube, filled with glassy carbon chips heated at 1400°C. 0.1 µL of the sample was injected by a CTC PAL autosampler (CTC analytics, Zwingen, Switzerland). The water extracted from the sample was converted by pyrolysis to  $H_2$  and CO gases were separated with a GC column maintained at 110°C. Only  $H_2$  gas was measured during the injection.

In a given sequence, homemade water standards were included during the measure to calibrate the samples against the international scale (VSMOW2-SLAP2 scale).  $^2H/^1H$  ratios were calculated and expressed in delta notation ( $\delta$ ).

Cumulative recovery of  $^2H$  was calculated in total body water (TBW) as previously described using the raw data of isotope enrichment (Bergouignan et al. 2008). We estimated TBW from FFM measurement multiplied by a hydration factor of 0.73, based on previous data that we obtained (Blanc et al., 1998). Instantaneous and cumulative oxidation rate of palmitate were then calculated with these data (see equation below) and expressed as a kinetic or area under the curve (AUC).

We measured the instantaneous and cumulative recovery of  $^2H$  in urine and plasma samples and the results are expressed as a percentage of the dose per hour (Bergouignan et al., 2009).

$$^2H \text{ sample enrichment} = \text{delta enrichment} - \text{baseline delta enrichment}$$

$$\text{Constant} = \frac{R_{\text{std}} * MW}{\text{purity} * n}$$

Values for  $d_{31}$  palmitate:  $R_{\text{std}}$  ( $^2H/^1H$  of SMOW):  $1.558 \cdot 10^{-4}$ , MW (molecular weight): 287 g/mol, Purity ( $^2H$  isotope purity): 98%, n (number of labeled atoms per molecule): 31



$$^2H \text{ cumulative recovery } \left( \% \frac{\text{dose}}{\text{per}} \text{ hour} \right) = \frac{^2H \text{ instantaneous enrichment} * \text{constant} * \text{total body water}}{\text{dose}}$$

$$^2H \text{ instantaneous recovery } \left( \% \frac{\text{dose}}{\text{per}} \text{ hour} \right) = ^2H \text{ cumulative recovery}_t - ^2H \text{ cumulative recovery}_t - 1$$

## 2. $^{13}C$ acetate oxidation

$^{13}C/^{12}C$  isotope ratio from  $CO_2$  breath test enrichment was measured using a GasBench II (Thermo Fischer Scientific, Bremen, Germany) coupled with an IRMS. Every breath test samples contains water and nitrogen that are being removed using an on-line water trap made of Nafion and a gas chromatography system respectively, before analyze.

The isotope ratios values were corrected for linearity and for a time-dependent machine drift because of the long time analysis. To do so, the sequence started and ended with two enriched homemade gas standard and one quality control regularly interspersed between samples. Standards are calibrated against the international standard of Pee Dee Belemnite scale.  $^{13}C/^{12}C$  ratios were calculated and expressed in delta notation ( $\delta$ ).

We measured the instantaneous and cumulative recovery of  $^{13}C$  in expired  $CO_2$  per hour and the results are expressed as a percentage of the dose (Bergouignan et al., 2009).

$$^{13}C \text{ sample enrichment} = \text{delta enrichment} - \text{baseline delta enrichment}$$

$$\text{Constant} = \frac{R_{\text{std}} * MW}{\text{purity} * n}$$

Values for  $^{13}C$  acetate :  $R_{\text{std}}$  ( $^{13}C/^{12}C$  of the standard  $CO_2$ ): 0.011237, MW (molecular weight): 83.04 g/mol, Purity ( $^{13}C$  isotope purity): 99%, n (number of labeled atoms per molecule): 1

$$^{13}C \text{ instantaneous recovery } \left( \% \frac{\text{dose}}{\text{per}} \text{ hour} \right) = \frac{^{13}C \text{ instantaneous enrichment} * V_{CO_2} * \text{constant}}{\text{dose}}$$

$$^{13}C \text{ cumulative recovery } \left( \% \frac{\text{dose}}{\text{per}} \text{ hour} \right) = \text{instantaneous recovery}_t * (\Delta_{\text{time}} / 60) + \% \text{ cumulative recovery}_t - 1$$

We completed these variables by calculating the slope between 0 and 45 min of  $^{13}C$  instantaneous recovery curve that is linear during that window. We consider the latter to represent the liver activity of tricarboxylic acid cycle (TCA), due to the fast absorption of acetate and the liver first pass effect

### 3. $d_7$ glucose oxidation during oral glucose tolerance test

150 $\mu$ L of total blood was cryo-distilled to extract water and analyzed for deuterium enrichment by TC/EA IRMS. The protocol followed is the same as the one described in  $d_{31}$  palmitate oxidation paragraph.

Deuterium enrichment of water was then calculated using the followed equations:

$$^2H \text{ sample enrichment} = \text{delta enrichment} - \text{baseline delta enrichment}$$

$$\text{Constant} = \frac{R_{\text{std}} * MW}{\text{purity} * n}$$

Values for  $d_7$  glucose:  $R_{\text{std}}$  ( $^2H/^1H$  of SMOW):  $1.558.10^{-4}$ , MW (molecular weight): 193.15 g/mol, Purity ( $^2H$  isotope purity): 97%, n (number of labeled atoms per molecule): 7

$$^2H \text{ cumulative recovery} \left( \% \frac{\text{dose}}{\text{per}} \text{ hour} \right) = \frac{^2H \text{ enrichment}_t \text{ constant} * \text{total body water}}{\text{dose}}$$

$$\begin{aligned} ^2H \text{ instantaneous recovery} \left( \% \frac{\text{dose}}{\text{per}} \text{ hour} \right) \\ = ^2H \text{ cumulative recovery}_t - ^2H \text{ cumulative recovery}_{t-1} \end{aligned}$$

### 4. Muscle and liver insulin resistance index

According to Abdul-Ghani et al. (2007), we also calculated muscle and liver insulin resistance indexes using the data collected during the OGTT (Abdul-Ghani et al., 2007). Muscle insulin sensitivity was estimated with the slope of glucose concentration from the peak to nadir divided by the mean of insulin concentration. Liver insulin sensitivity was estimated with the product of the AUC of insulin and glucose between 0 and 30 min of test.

### 5. Energy expenditure and total substrates oxidation

Energy expenditure was calculated using  $V_{O_2}$  and  $V_{CO_2}$  measured by indirect calorimetry and  $N_2$  in urine by Kjeldahl method. Baseline data were used to calculate, energy expenditure and RMR, using Weir equation (Weir, 1949). RMR was then normalized by FFM of each subject.

$$\begin{aligned} \text{Energy expenditure (kcal/min)} &= (3.913 * V_{O_2}) + (1.093 * V_{CO_2}) - (3.341 * N_2) \\ \text{RMR (MJ/d)} &= \text{energy expenditure (kcal/min)} * (4.1816 * 60 * 24) / 1000 \end{aligned}$$

We also calculated Non-Protein Respiratory Quotient (NPRQ):

$$NPRQ = \frac{(V_{CO_2} - (\text{oxidized proteins} * 0.774))}{(V_{O_2} - (\text{oxidized proteins} * 0.966))}$$



Total substrates oxidation were also measured (Frayn, 1983) at fasting and fed state after a moderately high fat meal. All measures were extrapolated to 60min the duration of the measure.

$$\text{Carbohydrates oxidation (g/min)} = (4.55 * VCO_2) - (3.21 * VO_2) - (2.87 * N_2)$$

$$\text{Lipids oxidation (g/min)} = (1.67 * VCO_2) - (1.67 * VO_2) - (1.92 * N_2)$$

$$\text{Proteins oxidation (g/min)} = 6.25 * N_2$$

### iii. Dietary lipid trafficking

#### 1. Separation of lipoproteins

CM\_TG and VLDL\_TG were separated by ultracentrifugation (Beckman Coulter, Optima MAX-XP) at the end of the test day. One mL of density solution of 1.042 g/mL of NaBr was added to 2.5mL of plasma. The mix was put into thick wall tubes, coated with polyvinyl alcohol (PVA), where 2.5mL of 1.006g/mL density solution of NaCl was gently added on the top. The tubes were then centrifuged 21min at 35000rpm and 15°C and we obtained the top white layer containing chylomicron (CM). After equilibrating the tubes with 1.006g/mL density solution, we centrifuged again at 75000rpm during 9.8h to obtain the top white layer containing the VLDL fraction. Samples were stored at -80°C until analysis by mass spectrometry.

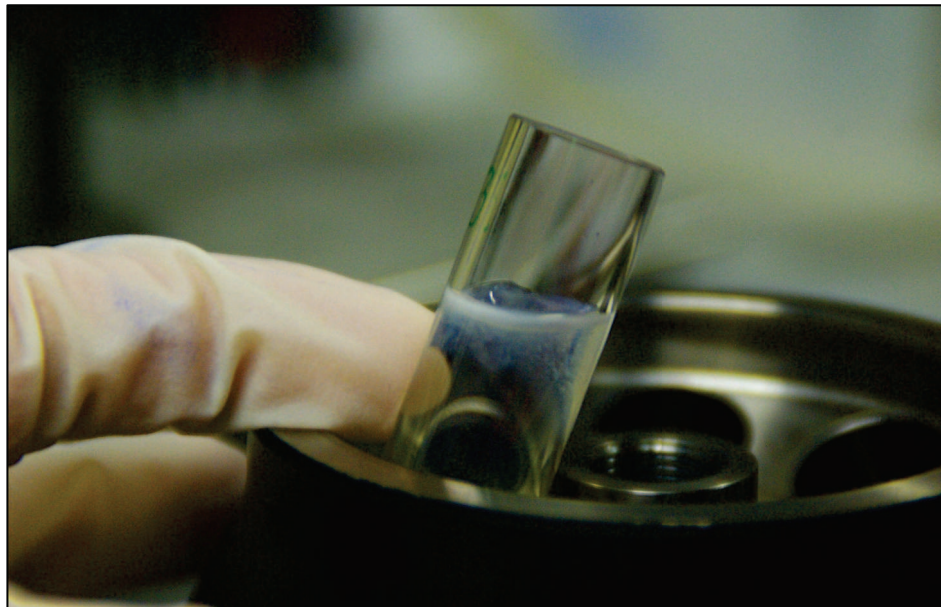


Figure 30: Top white layer of chylomicrons after ultracentrifugation of 2.5mL of plasma

#### 2. Extractions of lipid fractions

Total lipids were extracted from 200μL of CM\_TG or VLDL\_TG using the Folch technique after adding pentadecanoic (C15:0) acid used as an internal standard. TG

fraction was then obtained by thin layer chromatography. The last step of preparation was the methylation using methanol and sulfuric acid added to the fraction. The samples were incubated 2h at 50°C and a neutralizing solution was added to stop methylation. Then, hexane was added and after centrifugation, the top layer was taken and dried under nitrogen at 40°C and then retaken with 150µL hexane to be analyzed.

For NEFA, total lipids were extracted from 250µL of plasma placed into glass tubes. Internal standard (C17:0) was added with BHT solution and 1mL of KOH methanol (0.2M). After adding 1mL of NaCl (1M) and 10min centrifugation at 2500rpm at 4°C, total lipids were isolated from the bottom layer and dried under nitrogen gas. The second step consisted in the separation of different fractions of lipids on solid phase extraction (SPE) columns (aminopropylsilica cartridges, ref. 12102014, Varian): chloroform was used to elute triglycerides, chloroform/methanol (60/40) to elute PC and finally chloroform/methanol/glacial acid acetic (100/2/2) to collect the NEFA fraction. After collection, the NEFA were dried under nitrogen and retaken into toluene. The last step of preparation was the methylation of the NEFA fraction. Methanol and sulfuric acid was added to the fraction for methylation during 2h at 50°C. A neutralizing solution (KHCO<sub>3</sub> 0.25M, K<sub>2</sub>CO<sub>3</sub> 0.5M) was then added to the solution with 1mL of hexane and centrifuged during 10min at 2500rpm at 15°C. The top layer was taken and dried under nitrogen at 40°C and then retaken with 150µL hexane to be analyzed.

### 3. Analyzes and calculations of lipoproteins and NEFA enrichment in labeled and unlabeled palmitate.

The isotopic enrichment of labeled and unlabeled palmitate concentrations were calculated during the same measure on Gas Chromatography/Mass Spectrometry (GC/MS). The m/z ratio of 270 and 301 for palmitate was acquired during a dual acquisition. CM\_TG, VLDL\_TG and extracted NEFA were analyzed on an Agilent 5975 Inert XL and NEFA using a HP 6890 GC connected to HP 5973 MS (Agilent, Massy, France for all equipment).

Standard curves were made of different concentrations of tracer/trace i.e d<sub>31</sub> palmitate/unlabeled palmitate (moles). The concentrations were chosen according to results usually obtained in previous studies. Then, in each sample, the d<sub>31</sub> palmitate/palmitate ratio was calculated and the concentration of the d<sub>31</sub> palmitate was deduced from the standard curve.

### iv. Molecular biology

#### 1. Microfluidic real-time polymerase chain reaction (microfluidic RT-PCR)

Each biopsy sample of *soleus* muscle was powdered with steel balls into liquid nitrogen using a Mikro-Dismembrator (Sartorius Stedim Aubagne, France). Several

molecular analyzes were performed on muscle biopsies. DNA quantities of several genes involved in specific pathways were measured using microfluidic RT-PCR: Akt1, GLUT4, insulin receptor substrate 1 (IRS1), MAP2K1, MAPK1, PFKM, phosphoinositide-3-kinase regulatory subunit 1 and 2 (PIK3R1 and R2) and PRKCA involved in insulin resistance, ACOX2, FoxO3a, ACAA2 and ACACB, lipid transporters ACADL, ACADM and LPL in lipid oxidation, COX4I1, PGC1a, CPT1b and GSK3b in mitochondrial pathways. High throughput real-time quantitative PCR was performed using the Biomark microfluidic system from Fluidigm in which every sample-gene combination is quantified using a 96.96 Dynamic Array™ IFCs. Pre-amplification of the samples, chip loading and real time quantitative PCR were performed according to manufacturer's protocol. RT-qPCR results were analyzed using the Fluidigm real-time PCR analysis software v.4.1.2. Firstly, all primers targeting every gene on the study were pooled at a final concentration of 0.2  $\mu$ M each. This pool was used to pre-amplify 10 ng of each cDNA using Taqman PreAmp Master Mix from Applied Biosystems. Pre-amplified cDNAs and primer couples at 20  $\mu$ M each were then loaded and combined into 96.96 Dynamic Array™ IFC from Fluidigm and according to manufacturer's protocol. Firstly, 10ng of each cDNA were initially preamplified with Taqman PreAmp Master Mix (Applied Biosystems) and a pool containing all the primers targeting all the genes (200nM). In order to prepare samples for loading into the IFC, a mix was prepared consisting of 440 $\mu$ L 2X TaqMan Master Mix (Applied 430976), 44  $\mu$ L 20 $\times$  DNA Binding Dye Sample Loading Reagent (Fluidigm 100-3738), 44 $\mu$ L 20X Evagreen (Biotium 31000) plus 132 $\mu$ L TE, and 6 $\mu$ L of this mix was dispensed to each well of a 96-well assay plate. Two microliters of preamplified cDNA sample was added to each well and the plate was briefly vortexed and centrifuged. Following priming of the IFC in the IFC Controller HX, 5 $\mu$ L of the cDNA sample + reagent mix were dispensed to each Sample Inlet of the 96.96 IFC. For the assays, 5 $\mu$ L of each 10 $\times$  Assay (5 $\mu$ M each primer) were dispensed to each Detector Inlet of the 96.96 IFC. After loading the assays and samples into the IFC in the IFC Controller HX, the IFC was transferred to the BioMark and PCR was performed using the following thermal protocol: Thermal Mix of 50 °C, 2 min; 70 °C, 30min; 25°C, 10min, Hot Start at 50 °C, 2min; 95°C, 10 min, PCR Cycle of 35 cycles of (95 °C, 15 s; 60 °C, 60 s), and Melting analysis. Results were analyzed using the Fluidigm real-time PCR analysis software v.4.1.2.

## 2. Western Blot

The analyses of molecular biology were completed by western blot on powdered muscle samples comparing a few proteins expressions: CD36, CPT1, DGAT2, FATP1, GAPDH, Oxphos (complex of mitochondrial chain and ATP synthase), PKB and phosphorylated PKB. After the biopsies, a piece of frozen muscle was taken and crushed using a Mikro-dismembrator S (Sartorius, Göttingen, Germany) during 30 sec at 2500rpm using steel balls (steel balls 7mm Retsch, Thermo Fischer scientific, Waltham, MA, USA). Muscle samples were weighted and proteins were extracted

using SLB+ buffer (EDTA 5mM, Na<sub>3</sub>VO 41mM, NaF 20mM, DTT 1mM, protease inhibitors): 100µL of buffer for 10mg of muscle. After 30min, the samples were centrifuges 20 min at 13000 rpm at 4°C. The supernatant was collected and 5µL used to quantify the proteins by the Bradford method. The aliquot was added into Bio-Rad Protein Assay reactive (ref. 500-0006, Bio-Rad Laboratories) in a final volume of 1mL into each tank to measure the optic density at 595nm within an hour. The protein concentration was then calculated using the standard range made with known concentration of BSA. At the same time, migration gel was realized: separation gel with 10% acrylamide, Tris-HCl 1.5M, SDS 4%, H<sub>2</sub>O, Temed and APS 10% surmounted by a stacking gel of 15 wells with 30% acrylamide, Tris-HCl 0.5M, SDS 0.4%, H<sub>2</sub>O, Temed and APS. The equivalent of 30µg of proteins were inserted into each wells after denaturation at 75°C during 10 min in Laemmli buffer (Tris HCl 150mM, glycerol 36%, SDS 3%, beta-mercaptoethanol 12% and Bromophenol blue 0.03%). Migration was made during 1h at 60V in migration buffer (10% TG-SDS). The proteins were then transferred on a membrane during 2h at 800mA in a transfer buffer (10% TG and 20% methanol). At the end of the transfer, the membrane was incubated during 2 hours with BSA solution (4%), 10% TBS and 0.3% tween to block aspecific sites on the proteins. Following the night incubation at 4°C, with primary antibody, a session of washing was done. Finally, the membrane was incubated with the secondary antibody during 1h at room temperature and followed by a session of wash out before revelation using Lumina Forte Millipore (WBLUF0500) with ChemiDoc 01 Bio-Rad. After migration and transfer on a membrane, several antibodies were used to reveal proteins expression: anti-CD36 (sc9154, Santa Cruz, USA), anti-CPT1 (sc20670, Santa Cruz, USA), anti-DGAT2 (NB100-57851, Novus, USA), anti-FATP1 (sc25541, Santa Cruz, USA), anti-GAPDH (sc20357, Santa Cruz, USA), anti-Oxphos (ab110413, Abcam, UK), anti-PKB (9272, Cell signaling, USA) and anti-phospho PKB (9275, Cell signaling, USA). The signal was then quantified using ImageJ program (National Institute of Health, USA). Due to a lack of material, dropping out or total absence of biopsy the analyses couldn't be performed on all subjects.

#### v. Inflammation and oxidative stress

Specific hepatic inflammation markers, alanine transferase (ALT), aspartate transferase (AST), gamma glutamyl-transpeptidases (GGT), C-reactiv protein (CRP) and fibrinogen were measured in fasting plasma samples. These measurements were part of a larger evaluation of safety parameters performed by DLR.

Subject plasma antioxidant capacity was evaluated using KRL and RESEDA tests (Brevet M. Prost/Spiral). KRL test is measuring the global resistance of blood sample against free radical attacks. Blood sample is tested and the time until 50% of the red blood cells are lysed is measured. The result is representative of the global antioxidant power of the subject. With the same technique, RESEDA test is measuring antioxidant defenses after the action of three different hydrolases: glucosidase (R1),

sulfatase (R2), glucuronidase (R3) on anti-free-radicals groups. The results are expressed in percentage of variation against control blood without any enzyme action.

#### vi. Plasma hormones and metabolites

During the study, plasma glucose (GLUC-PAP, GL 364, Randox, Crumlin, UK) and insulin (Mercodia Insulin ELISA, 10-1113-01, Mercodia, Uppsala, Sweden) concentration were measured using respectively colorimetric and ELISA assays. Insulin concentration was calculated using the equation of a standard curve of known-concentration samples, specific of each assay. Intra-assay test were respectively 0.3% and 3.35%, and inter-assay test 1.14% and 3.0%. Insulin resistance index was calculated as HOMA-IR according to the followed equation:

$$HOMA - IR = \frac{(Glucose * Insulin)}{22.5}$$

With glucose in mmol/L

Total plasma TG was measured using colorimetric assays (TRIGS, TR212, Randox, Crumlin, UK). Intra-assay variation was < 1.5%.

These data were completed with fasting LDL, HDL and LDL/HDL ratio measured by a private company using plasmatic lipoprotein electrophoresis.

In parallel, non-esterified fatty acids (NEFA) were dosed by colorimetric assays (NEFA, FA115, Randox, Crumlin, UK). Intra-assay variation was 4.51%.

#### e. *Statistics*

One subject of the dietary supplementation group dropped out after the first session. Thus analyses were done on 10 subjects under control condition and 9 under protein supplementation. AUCs were calculated for the concentration or substrate oxidations time curves. We present the total AUC for the whole day of study (7-hours AUC), and both the 3 first (3-hours AUC) and the 4 last hours (4-hours AUC).

Data are presented as least-square means  $\pm$  SEM. Analyses were performed with SAS software (SAS v.9.3, Institute Inc., Cary, North Carolina). Significance was set up to 0.05 and 0.10 for interactions.

Baseline data from each bed rest sessions were first analyzed to identify the presence of a carry-over effect (i.e. an effect of from the treatment of the first session on the baseline values of the second session). For this we used mixed models taking into account the repeated measures over the time with treatment, period and sequence (i.e interaction between period and treatment) as fixed factors, and individuals as random effects. Significance was not reached for sequence or for period effects for any of the main variables. So, data of subjects of both sequences were pooled together according to treatment (control vs protein supplementation). The evolution of all variables during the two 21 days of bed rest were further analyzed using linear mixed

models taking into account the repeated measures over time, with time and condition and time\*condition as fixed effects, and subjects as random effects. Pre-specified hypotheses were that bed rest will induce a significant modification in variables of interest and that the protein countermeasure will significantly reduce the effect of bed rest on these variables leading to non-significant variations over the bed rest period. Two-tailed tests were used for these analyses.

Western blot analyzes were made with a mixed model testing bed rest effect in each condition separately.

Associations between variables were tested using Pearson correlations.



# Résultats

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# Etude 1: Variation de la concentration plasmatique en visfatine après différentes interventions sur les niveaux d'activité physique chez l'homme

Under simulated microgravity, the development of a low-grade inflammation has been described in previous studies. However, to our knowledge, the real impact of severe physical inactivity itself on the development of inflammation and more precisely organ inflammation has never been described. Using previous studies, we measured the variation of visfatin, an inflammatory molecule specifically secreted by visceral adipose tissue.

# Effects of different levels of physical inactivity on plasma visfatin in healthy normal-weight men

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**Abstract:** We tested whether physical inactivity (PI) is an independent predictor of plasma visfatin, a newly discovered adipokine likely involved in the relationship between obesity-associated low-grade inflammation and insulin resistance. PI was induced in healthy men (Body Mass Index =  $23.4 \pm 0.6 \text{ kg}\cdot\text{m}^{-2}$ ) by 10 days of confinement ( $n = 8$ ), 1 month of detraining ( $n = 10$ ), and 3 months of bed rest with ( $n = 7$ ) and without exercise ( $n = 8$ ). Visfatin was negatively associated with activity energy expenditure ( $p = 0.03$ ). No relationship was observed with insulin sensitivity. This suggested that PI itself increases visfatin concentrations.

**Key words:** physical activity level, PBEF (pre-B-cell colony-enhancing factor), Nampt (nicotinamide phosphoribosyltransferase), exercise, insulin, bed rest and confinement.

**Résumé :** La visfatine est une adipokine récemment découverte et vraisemblablement impliquée dans la relation entre l'inflammation de bas niveau associée à l'obésité et l'insulinorésistance. Nous avons testé l'hypothèse que l'inactivité physique est un prédicteur indépendant de la concentration plasmatique en visfatine. Pour ce faire, nous avons combiné différentes études dans lesquelles l'inactivité physique a été induite chez des hommes sains (Indice de Masse Corporelle =  $23,4 \pm 0,6 \text{ kg}\cdot\text{m}^{-2}$ ) par 10 jours de confinement ( $n = 8$ ), 1 mois de désentraînement ( $n = 10$ ) et 3 mois d'alitement avec ( $n = 7$ ) ou sans exercice physique ( $n = 8$ ). La visfatine est négativement associée avec la dépense énergétique liée à l'activité physique ( $p = 0,03$ ). Aucune relation n'a été observée avec la sensibilité à l'insuline. Ceci suggère que la concentration plasmatique en visfatine augmente avec l'inactivité physique. [Traduit par la Rédaction]

**Mots-clés :** niveau d'activité physique, PBEF, Nampt, exercice physique, insuline, alitement, confinement.

## Introduction

Visfatin, also known as Nampt (nicotinamide phosphoribosyltransferase), is preferentially produced by human visceral adipose tissue (Fukuhara et al. 2005) and acts as a cytokine with glucose-regulatory and pro-inflammatory properties (Kadoglou et al. 2010). Increased circulating visfatin levels have been observed in individuals with obesity, insulin resistance, type 2 diabetes, and atherosclerosis (Choi et al. 2007; Haider et al. 2006b; Chang et al. 2011). Although controversies exist regarding the mechanisms, regulation (Tanaka et al. 2007), and physiological effects of this protein, visfatin likely contributes to the pathological relationship between obesity-associated low-grade inflammation and the development of inflammation-related insulin resistance, type 2 diabetes, and atherosclerosis.

Physical inactivity is known, along with poor diet, to contribute to weight gain and obesity. The relationship between physical inactivity and low-grade inflammation may, however, be independent of obesity. Lower levels of physical activity have indeed been associated with a number of inflammatory markers (Hamer 2007), after adjustment for fat mass. In diabetic individuals, a cross-sectional study showed that after adjusting for sex, age, and BMI, physical activity is associated with plasma visfatin levels (Kadoglou et al. 2010). Most of the recent studies (Haider et al. 2006b; Jorge et al. 2011; Brema et al.

2008), but not all (Brema et al. 2008; Lee et al. 2010) have also reported that chronic aerobic exercise training decreases visfatin concentration. However, these studies have only been performed in overweight or obese, type 1 or type 2 diabetic individuals, which does not allow for determination of the impact of physical inactivity on plasma visfatin concentration independent of fat mass, glucose tolerance, insulin sensitivity, or inflammation. By taking advantage of 3 studies performed in our laboratories corresponding to 4 experimental groups, we investigated the effect of induced physical inactivity on plasma visfatin concentration. The advantage of our studies was that they induced various degrees of physical inactivity that were objectively quantified and induced either by (i) voluntary decrease in structured or spontaneous physical activity in free-living conditions, (ii) bed-resting with and without exercise, or (iii) confinement in a metabolic ward, in healthy and normal-weight subjects with contrasted physical activity levels at baseline. All of these studies were also performed under controlled fat mass, which allowed us to determine the direct effect of physical inactivity on visfatin, i.e., independent of confounding positive energy balance.

## Methods

### Design of the studies

Healthy normal-weight volunteers (Body Mass Index (BMI) =  $20\text{--}25 \text{ kg}\cdot\text{m}^{-2}$ ) were recruited for 3 studies, corresponding to

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4 experimental groups. In all of these studies, subjects were weight stable for at least 3 months prior to enrollment. They were free of any known chronic diseases, had no alcohol or drug dependence, and had no medical treatment. Subjects had no first-degree family history of obesity or type 2 diabetes.

The Strasbourg Lipid Oxidation (LIPOX) study was a detraining study in 10 healthy normal-weight men (Strasbourg, France, 2008), who performed 2–3 h of moderate to vigorous leisure physical activity per week in their daily life. Subjects had a high physical activity level of 2.2 on average and were asked to stop structured exercise and reduce their spontaneous daily living activities for 1 month (1-month detraining group). Although subjects were in free-living conditions, the investigators tightly controlled their compliance with the intervention and diet during the whole period of the study. Details of the study can be found elsewhere (Bergouignan et al. 2013).

The Thermo study was a confinement study conducted at the German Space Agency (DLR, Cologne, Germany, 2006). After a 5-day baseline period, 8 healthy men stayed in a metabolic ward for 10 days (10-day confinement group). Because the area was small and subjects did not have access to any exercise facility, body movement was limited, which reduced daily physical activity level.

The third study was a 3-month bed rest (3-month BR) study (Space Clinic, Toulouse, France, 2001). After a 20-day ambulatory baseline period, 15 healthy men were randomly divided into 2 groups: a control group ( $n = 8$ ; 3-month BR group) who strictly remained in bed, and an exercise group ( $n = 7$ ; 3-month BR + EX group) who was subjected concomitantly to 3 months of BR to resistive exercise training program in supine position on a fly-wheel ergometer (every 3 days, for 30 min at an intensity that represented an energy cost of about 640 kJ per session) (Bergouignan et al. 2006). This device allowed subjects to perform maximal concentric and eccentric actions in the supine squat and calf press.

Body composition, activity energy expenditure (AEE), and fasting plasma visfatin were measured at baseline and at the end of the intervention on physical activity in each study. Fasting plasma insulin and glucose were measured in the 1-month detraining and 3-month BR studies only.

The 1-month detraining, 10-day confinement, and 3-month BR studies were respectively approved by the Institutional Review Boards of Alsace I, Ethics commission of the Ärztekammer Nordrhein, and Midi-Pyrénées I. Each subject signed a written consent form.

### Body composition

In the 1-month detraining and 10-day confinement studies, body composition was measured using the isotope dilution method, as previously described (Blanc et al. 1998). In the 3-month BR study, fat mass and fat-free mass were measured by dual-energy X-ray absorptiometry (Bergouignan et al. 2006).

### Physical activity energy expenditure

AEE was calculated from the equation  $AEE = TEE - RMR - DIT$ , where TEE is total energy expenditure, RMR is resting metabolic rate, and DIT is diet-induced thermogenesis. In the 1-month detraining and 10-day confinement studies, RMR and TEE were measured by indirect calorimetry and with the doubly labeled water method, respectively, over 10 days, as previously detailed (Blanc et al. 1998). DIT was assumed 10% of TEE. In the 3-month BR study, AEE was estimated from the same equation except that only RMR was measured. TEE was estimated by assuming a physical activity level (PAL) of 1.7 and 1.42 during control and bed-rest periods, respectively, and by using the following equation:  $PAL = TEE/RMR$  (Bergouignan et al. 2006).

### Fasting plasma visfatin, insulin, and glucose concentrations

Visfatin was measured by Visfatin C-Terminal Enzyme Immunoassay kit (Phoenix Pharmaceuticals, Inc., EK-003-80, Burlingame, USA) with an intra-assay variation of <10% and an inter-assay variation of <15% capable of detecting concentration from 0.1 to 1000 ng·mL<sup>-1</sup>. Insulin was measured by radioimmunoassay (DSL-1600, DSL, Inc., Webster, Texas, USA) and glucose by enzymatic method (Biomérieux, No. 61270, Craponne, France). Not enough plasma was available from the 10-day confinement study to measure insulin and glucose. Insulin sensitivity was estimated by calculating the homeostasis model of assessment – insulin resistance (HOMA-IR).

### Statistical analysis

The effects of physical activity interventions on anthropometric and biological data and the associations between AEE, visfatin, and HOMA-IR were analyzed by using mixed linear models taking into account the repeated individual data over time, group, and intervention as fixed effect, and individuals as random effect. Heterogeneity of the intervention's effect across groups was tested. Least-square means and their standard errors of the mean (SE) are presented. Post-hoc Tukey tests were used for pairwise comparisons. The models examining the associations between visfatin and HOMA-IR with AEE were adjusted for fat-free mass and fat mass. Two-sided  $p$  values are reported and significance was set to 0.05 and 0.10 for main and interaction effects, respectively, (SAS version 9.3, SAS Institute Inc., Cary, North Carolina).

## Results

### Baseline subjects' characteristics

At baseline, age, body mass, and composition were not significantly different between the groups (Table 1). AEE was higher ( $p < 0.0001$ ) and visfatin tended to be lower ( $p < 0.09$ ) in the 1-month detraining subjects compared to both the 10-day confinement and 3-month BR subjects (Fig. 1, Table 1). Insulin, glucose, and HOMA-IR did not differ between the 1-month detraining and 3-month BR subjects.

### Effect of physical activity interventions on AEE and body composition

AEE was differently reduced according to the interventions on physical activity (interaction:  $p = 0.08$ ; Fig. 1, Table 1). AEE was more decreased by 10 days of confinement and by 3 months of BR with or without exercise (47.5%, 38.3%, and 46.5%, respectively) than by detraining (14.3%). Because of muscle disuse, 3 months of BR significantly decreased fat-free mass and consequently body mass (interaction:  $p < 0.05$  for both). However, that fat mass was unchanged indicates that energy balance was stable (Zahariev et al. 2005). None of the other physical activity interventions modified body mass and composition (Table 1).

### Effect of physical activity interventions on visfatin and insulin sensitivity

The physical activity interventions differently impacted visfatin concentrations (interaction:  $p = 0.1$ ). Whereas visfatin significantly increased by 54.9% and 49.7% in response to 10 days of confinement ( $p < 0.01$ ) and 3 months of BR ( $p = 0.02$ ), respectively, no change was noted in the 1-month detraining and 3-month BR + EX groups. No change was observed in glucose concentration. Physical activity interventions also differently affected insulin concentration and HOMA-IR (interaction:  $p < 0.10$  for both). Insulin and, consequently HOMA-IR, significantly increased in response to 3 months of BR even when exercise was performed ( $p < 0.001$  for both) but not in response to detraining.

**Table 1.** Subject characteristics.

Characteristic	1-month detraining (n = 10)		10-day confinement (n = 8)		3-month BR (n = 8)		3-month BR + EX (n = 7)	
	Baseline	After physical intervention	Baseline	After physical intervention	Baseline	After physical intervention	Baseline	After physical intervention
Age (years)	23.8±1.2		25.3±4.7		32.0±4.0		33.0±5.0	
BMI (kg·m <sup>-2</sup> )	22.2±0.6		24.2±0.5		23.5±0.6		23.5±0.5	
BM (kg)	71.7±2.8	71.4±3.1	79.4±2.0	79.2±2.2	70.3±2.2	67.7±1.9**	69.0±2.1	69.0±2.4
FM (kg)	10.5±1.1	11.4±1.4	16.3±1.7	16.1±1.9	13.0±1.5	12.6±1.1	10.5±1.2	11.0±1.3
FFM (kg)	61.2±2.4	60.0±2.6	63.2±1.3	63.2±1.2	57.4±1.6	55.0±1.0***	58.5±1.5	58.0±1.1
Visfatin (ng·mL <sup>-1</sup> )	10.4±1.4	11.3±3.4	14.4±1.2	22.3±2.8*	14.3±1.6	21.4±3.3*	19.1±4.6	17.9±2.1
Glucose (g·L <sup>-1</sup> )	0.84±0.01	0.86±0.02			0.88±0.03	0.84±0.01	0.82±0.03	0.88±0.02
Insulin (mIU·L <sup>-1</sup> )	3.63±0.29	5.24±0.73			3.71±0.55	8.79±1.05***	2.78±0.35	7.47±0.55***
HOMA-IR	0.74±0.05	1.11±0.14			0.80±0.13	1.83±0.23***	0.56±0.07	1.49±0.15***
AEE (MJ·day <sup>-1</sup> )	6.32±0.41	5.41±0.34***	4.40±0.35	2.31±0.33***	3.54±0.07	1.90±0.03***	3.65±0.09	2.25±0.06***

**Note:** Characteristics of the subjects from each experimental group represented as mean ± SE. BMI, Body Mass Index; BM, body mass; FM, fat mass; FFM, fat free-mass; HOMA-IR, homeostatic model assessment of insulin resistance; AEE, activity energy expenditure. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$  baseline vs after intervention. 3-mo BR, 3 months of bed rest; 3-mo BR + EX, 3 months of bed rest with concomitant resistive exercise.

### Relationships between AEE, visfatin, and insulin sensitivity

We examined the association between AEE and visfatin by taking all the subjects together. We similarly looked at the relationships between AEE and insulin, glucose, and HOMA-IR in the 1-month detraining and 3-month BR groups. After adjusting for body composition, AEE was negatively associated with visfatin ( $p = 0.03$ ). However, no significant associations were observed between visfatin and glucose, insulin, or HOMA-IR ( $p = 0.93$ ,  $p = 0.39$ , and  $p = 0.34$ , respectively). The inverse associations between AEE and insulin or HOMA-IR failed to reach significance ( $p = 0.34$  and  $p = 0.39$ , respectively).

### Discussion

Although physical inactivity may be a trigger of inflammation contributing to the development of inflammation-related diseases independent of overweight or obesity, the role of physical activity per se in the regulation of circulating visfatin concentration is still scarce. By compiling 3 of our studies corresponding to 4 experimental groups, we observed a negative association between AEE and circulating visfatin levels in healthy and normal-weight men. We also showed that a severe reduction in physical activity, independent of detectable changes in fat and energy balance, increases plasma visfatin concentration. These results along with the decrease in visfatin observed in response to chronic aerobic exercise training (Haider et al. 2006b; Jorge et al. 2011; Brema et al. 2008) show that plasma visfatin concentration is modified by changes in physical activity. The underlying mechanisms are however unknown.

Although visfatin was originally thought to have insulin mimetic effects by binding and activating the insulin receptor (Fukuhara et al. 2005), subsequent studies have been unable to repeat those results, and thus have been retracted (Fukuhara et al. 2005). We reported a decrease in insulin sensitivity in association with an increase in plasma visfatin in response to physical inactivity, but like others (Oki et al. 2007), we did not observe a significant relationship between visfatin and insulin sensitivity levels or changes over time. Visfatin has been suggested to instead be regulated by glycaemia and respond to nutrient availability (Haider et al. 2006a). However, we did not observe relationships between visfatin and glucose concentrations or energy intake (data not shown), but with energy expended during physical activity. Visfatin under its extracellular form has recently been suggested to be, like the intracellular form Nampt, one of the rate-limiting enzymes of the biosynthesis of NAD<sup>+</sup> from nicotinamide (Freyssenet 2007). Through its activation of SIRT1 but also of AMPK, the NAD/NADH ratio is known to be part of the energy-sensing pathway at least at the intracellular level (Freyssenet 2007). One may speculate that through its action on

NAD regulation, plasma visfatin (or Nampt) contributes to the regulation of energy metabolism, and thus responds to changes in AEE. Further studies are required to understand the mechanisms underlying the regulation of plasma visfatin in response to changes in physical activity.

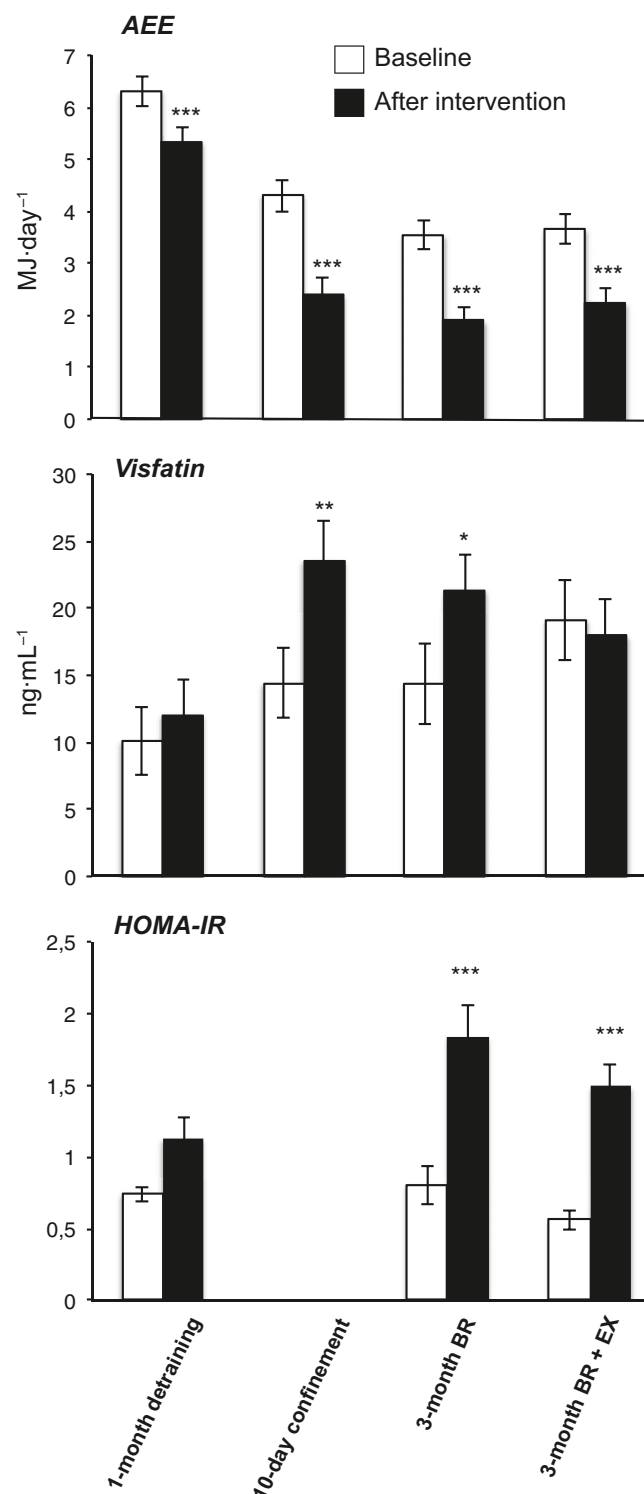
An alternative explanation to the increased visfatin concentration in response to physical inactivity is a modification in fat distribution. It has been shown that only 2 weeks of decrease in daily steps (from 10 501 to 1344 steps·day<sup>-1</sup>) induce a significant 7% increase in visceral adipose tissue with no change in total fat mass (Olsen et al. 2008). A similar scenario is conceivable in our studies and may explain the increase in plasma visfatin induced by physical inactivity in absence of a detectable increase in fat mass. The persistence of a relatively high AEE in the 1-month detrained group and the exercise performed during the 3 months of BR may have, however, prevented accumulation of visceral adipose tissue, which would explain the absence of increase in visfatin in these groups. However, it is important to note that Curat et al. (2006) have suggested that visfatin is produced not by visceral adipose tissue itself but by macrophages that have infiltrated the adipose tissue as a result of inflammation. Of interest, visfatin has been correlated with inflammatory markers such as C-reactive protein and interleukin 6 (Oki et al. 2007). Given that physical inactivity is known to increase inflammation (Højbjerg et al. 2011), it seems possible that the elevation in circulating visfatin may be the result of macrophage infiltration and (or) increased inflammation. Visfatin would thus be the result of an increased inflammation rather than the cause of an inflammatory state. Further studies are required to better delineate the cause-and-effect relationship between physical inactivity and inflammation and the role that visfatin plays in it.

Some limitations exist. The relationship between visfatin and insulin sensitivity have been weakened by the absence of glucose and insulin measures in the 10-day confinement study. The relationships were, however, far from significant, even with a sample size of 25 subjects, and we don't believe that increasing the sample size would have changed the results. Measures of visceral and subcutaneous adipose tissues, macrophages, and inflammatory markers are lacking to better understand the underlying mechanisms. Finally, our results cannot necessarily be applied to other populations than healthy and normal-weight men.

Visfatin remains a controversial molecule, whose role and regulation still need to be defined. Our results propose a regulation by physical activity, independent of measurable changes in energy balance. Physical activity impacts plasma visfatin concentration in fasting state, with an increase in circulating levels under physical inactivity conditions. Further studies are required to bet-



**Fig. 1.** Activity energy expenditure (AEE), fasting plasma visfatin concentration, and fasting homeostasis model of assessment – insulin resistance (HOMA-IR) at baseline and after the following interventions on physical activity: 1 month of detraining ( $n = 10$ ), 10 days of confinement ( $n = 8$ ), 3 months of bed rest (3-month BR;  $n = 8$ ), and 3 months of bed rest with concomitant resistive exercise (3-month BR + EX;  $n = 7$ ). Interaction effect between time and interventions: AEE ( $p = 0.08$ ), visfatin ( $p < 0.10$ ), and HOMA-IR ( $p < 0.1$ ). \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$  baseline vs. after intervention.



ter understand the mechanisms underlying the link between physical inactivity and inflammation-related diseases.

#### Conflict of interest statement

The authors have no conflict of interest and nothing to disclose.

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## *Etude 2 : Impact de 60 jours d'inactivité physique sévère chez la femme sur l'évolution de marqueurs d'une stéato- hépatite.*

After the study of visfatin, a protein specific of adipose tissue inflammation, we investigated the development of liver inflammation under severe physical inactivity, using the measure of specific hepatic markers of non-alcoholic fatty liver disease (NAFLD). Beside classical aminotransferases, alanine and aspartate aminotransferase, we also used hepatic markers involved in more severe stages of NAFLD, cytokeratin 18 (CK18) and angiopoietin-like protein 3 (ANGPTL3).

## ORIGINAL ARTICLE

# Effect of enforced physical inactivity induced by 60-day of bed rest on hepatic markers of NAFLD in healthy normal-weight women

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## Keywords

angiopoietin-like 3 – cytokeratin 18 – exercise – physical inactivity – steatohepatitis – transaminases

## Abbreviations

AAC, alanine transferase, aspartate transferase and cytokeratin 18 index; ALT, alanine transferase; ANGPTL3, angiopoietin-like 3; AST, aspartate transferase; BM, body mass; BR30, 30-days of bed rest; BR60, 60-days of bed rest; CHA, cytokeratin 18, homoeostasis model assessment – insulin resistance and Aspartate transferase index; CK18, cytokeratin 18; FFM, fat free mass; FM, fat mass; HOMA-IR, homoeostasis model assessment – insulin resistance; LPL, lipoprotein lipase; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

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Non-alcoholic fatty liver disease (NAFLD) is the hepatic component of the metabolic syndrome and a progressive and chronic liver disease that goes from simple hepatic steatosis to non-alcoholic steatohepa-

## Abstract

**Background & Aims:** Physical inactivity leads to a cluster of metabolic disorders that have been associated with non-alcoholic fatty liver diseases (NAFLDs). To test whether physical inactivity increases hepatic biomarkers of NAFLDs. **Methods:** Sixteen normal-weight healthy women (body mass index =  $21.2 \pm 0.5$  kg/m<sup>2</sup>) were studied under controlled energy balance conditions during a previous 60-day bed rest with ( $n = 8$ ) or without ( $n = 8$ ) a combined aerobic/resistive exercise protocol. We retrospectively used stored samples to measure plasma hepatic markers, i.e. steatosis-related alanine and aspartate transaminases, cytokeratin 18 and angiopoietin-like 3, at baseline, after 30 and 60 days of bed rest. Fasting insulin and triglycerides were measured at baseline and after 30 days of bed rest. Two indexes were calculated, one combining alanine, aspartate transferase and cytokeratin 18 and another cytokeratin 18, homoeostasis model assessment – insulin resistance and aspartate transferase. **Results:** Sixty days of bed rest increased all hepatic markers ( $P < 0.05$  for all) and the two indexes ( $P < 0.01$  for both). Exercise significantly reduced the elevation in aspartate transaminase, cytokeratin 18 and both indexes ( $P < 0.02$  for all) but not the increase in alanine transferase and angiopoietin-like 3. Changes between baseline and 30 days of bed rest in triglycerides were positively associated with changes in aspartate transferase ( $R^2 = 0.28$ ,  $P = 0.04$ ) suggesting a role of hypertriglyceridaemia in the alteration of liver metabolism under inactive conditions. **Conclusion:** Physical inactivity increases, independent of fat mass, hepatic markers of steatosis and steatohepatitis. Regular exercise can limit these physical inactivity-induced metabolic alterations. Future studies need to elucidate the underlying mechanisms.

titis (NASH). Although the pathophysiology of NAFLD requires further elucidation, according to the 'two-hit' hypothesis (1), the development of hepatic steatosis associated with insulin resistance and alteration of lipid oxidation (2) is followed by a phase of inflammation and oxidative stress leading to activation of stellate cells, increased collagen deposition

\*F. Rudwill and A. Bergouignan contributed equally to this work as first authors.

**Key points box:**

- Steatohepatitis
- Transaminases, cytokeratin 18 (CK18) and angiotensin-like 3 (ANGPTL3)
- Physical inactivity
- Exercise

and fibrosis (3). Worldwide prevalence is estimated between 2.8% and 46% in the adult population and up to 90% in overweight and obese individuals (4, 5). Common explanation for the increased prevalence of NAFLD is the increased rate of obesity. The risk of developing NAFLD, however, is not limited to overweight individuals. A better understanding of its determinants is needed.

Beside obesity, low levels of exercise are thought to represent a major risk factor of developing metabolic syndrome (6). Recent epidemiological data even showed that time spent in sedentary activities has a harmful effect independent of the time spent exercising on insulin sensitivity and a number of cardio-metabolic biomarkers (7). The effects of physical activity and sedentary behaviours may extend to NAFLD. Cross-sectional population studies observed a negative association between time spent in leisure activity and NAFLD prevalence (8) and in old adults between intrahepatic fat and physical activity level (9, 10). A 3-month low-to-moderate intensity exercise training has also been shown to improve, independent of weight loss, liver enzymes and insulin sensitivity in patients with NAFLD (11). Longitudinal investigations to directly examine the impact of physical inactivity on liver metabolism are, however, missing.

By using plasma samples collected during a previous 60-day bed-rest study in healthy female adults under stable energy balance conditions, we retrospectively assessed whether enforced physical inactivity affects markers of hepatic steatosis and NAFLD. Bed-rest studies are validated models of simulated weightlessness during which countermeasures are tested to prevent and/or decrease the adaptations to the physical deconditioning occurring during space flights. In this study, we tested, concomitantly to bed rest, the impact of a combined aerobic and resistive exercise protocol as countermeasure. We specifically examined changes in plasma concentrations of four hepatic markers known to be associated with steatosis, steatohepatitis or NAFLD-related metabolic features, i.e. the two liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), fragments of cytokeratin 18 (CK18), a marker of hepatic apoptosis considered a good predictor of steatohepatitis (12, 13), and angiotensin-like 3 (ANGPTL3), a liver derived plasma protein that modulates plasma trigly-

cerides clearance (14) and is increased in more severe stage of NASH (15).

**Methods****Study design**

Details of the study can be found elsewhere (16, 17). Briefly, 16 healthy normal-weight women participated in a 60-day bed-rest study. This study was divided into three periods: a 20-day baseline period, a 60-day bed rest, and a 20-day recovery period. Subjects were randomized into two groups ( $n = 8$ , each): a control group subjected to strict bed rest and an exercise group that was subjected to supine resistive and aerobic exercise training protocol concomitantly to bed rest. Nineteen resistive training sessions of 35-min were performed every 3-day at maximal effort on a flywheel ergometer. Aerobic training was performed three to four times per week (29 sessions of  $50 \pm 2$  min on average), with intensities varying from 40 to 80% pre-bed rest maximal oxygen uptake. Dietary intake was tightly controlled by dietitians during the whole period of the study to maintain subjects in energy balance. No alcohol consumption was allowed. Every subject signed a written informed consent form. The protocol was approved by the Midi-Pyrénées I Institutional Review Board.

**Body composition**

Body mass (BM), fat mass (FM) and fat free mass (FFM) were measured before and after 30 (BR30) and 60 days of bed rest (BR60) by dual-energy X-ray absorptiometry (DXA, Hologic, France).

**Metabolic and hepatic markers measurements**

At baseline and BR30, dietary fat metabolism was fully investigated (17). Fasting plasma insulin was measured by radioimmunoassay (DSL, Webster, TX, USA) and glucose and triglycerides by enzymatic methods (Biomérieux, Lyon, France).

From stored fasting plasma samples, we measured hepatic markers concentration at baseline, BR30 and BR60. Plasma ALT and AST were measured by enzymatic test using phosphate pyridoxal, as routinely done in hospital laboratories. Plasma ANGPTL3 and fragments of CK18 were, respectively, assessed by using Quantikine Human Angiotensin-like 3 (R&D Systems Inc., Minneapolis, MN, USA) and M30 Apoptosense (Peviva, Stockholm, Sweden) Elisa kits. Plasma leftovers were insufficient to measure fasting triglycerides and insulin at BR60.

We considered two combined indexes of NAFLD, the AAC index (an acronym for AST, ALT and CK18) calculated as the sum of reduced values (according to base-

line values) of AST, ALT and CK18 and the CHA index [an acronym for CK18, Homoeostasis Model Assessment – Insulin Resistance (HOMA-IR), AST Index] calculated as the product of HOMA-IR, AST and CK18 (divided by 1000) (18). While we calculated the AAC index at both BR30 and BR60, the CHA index was obtained at BR30 only since fasting insulin was not measured at BR60.

### Statistical analysis

Data are presented as mean  $\pm$  SD. Analyses were performed with SAS software (SAS v.9.3., Institute Inc., Cary, NC, USA). Significance was set up to 0.05. Between-group comparisons at baseline were performed by using Student's *t*-tests. The evolution of anthropometric and biological variables was analysed by using mixed linear models taking into account the repeated measures over time with individuals as random effects and baseline values of the dependent variable as fixed effect. Models considering biological variables were adjusted for FM. Prespecified hypotheses were that bed rest will induce a significant increase in insulin, triglycerides and hepatic markers concentrations and that the exercise countermeasure will significantly reduce the effect of bed rest on the metabolic and hepatic markers leading to non-significant variations over time. One-tailed tests were used

for these analyses. The associations between changes in hepatic markers concentrations and changes in anthropometric (FM) and metabolic (insulin, HOMA-IR, triglycerides) variables were tested by using Pearson correlations.

## Results

### Body composition and metabolic variables

No between-groups differences were noted at baseline (Table 1). After 60 days of bed rest, BM had decreased in both groups by 6% on average ( $P < 0.001$ ). In the control group, BM reduction was mainly related to a loss of FFM ( $-7\%$ ;  $P < 0.001$ ). In the exercise group, weight loss was because of reduction in both FFM ( $-3\%$ ;  $P = 0.001$ ) and FM ( $-14\%$ ;  $P = 0.001$ ). Compared to the control group, exercise nevertheless limited the loss of muscle ( $P < 0.001$ ).

Bed rest significantly increased fasting insulin and HOMA-IR at BR30 in the control group ( $+36\%$  and  $+49\%$  respectively;  $P < 0.01$  for both). These increases were significantly lower in the exercise group compared to the control group ( $+17\%$  and  $+18\%$ ;  $P = 0.02$  and  $P < 0.01$  respectively) and no more significant. Fasting triglycerides, however, increased by 35% on average from baseline to BR30 in both the control and exercise groups ( $P = 0.03$  for both).

**Table 1.** Anthropometry, metabolic characteristics and hepatic markers according to groups and time

Characteristics	Control ( $n = 8$ )			Exercise ( $n = 8$ )		
	Baseline	BR30	BR60	Baseline	BR30	BR60
<b>Body composition</b>						
Age (years)	34.0 $\pm$ 1.4					
BMI (kg/m <sup>2</sup> )	21.0 $\pm$ 0.5	20.0 $\pm$ 0.5	19.7 $\pm$ 0.4	21.4 $\pm$ 0.6	20.6 $\pm$ 0.5	20.2 $\pm$ 0.5
BM (kg)	55.6 $\pm$ 3.9	52.9 $\pm$ 4.1 <sup>c</sup>	52.3 $\pm$ 3.8 <sup>c</sup>	58.1 $\pm$ 6.4	55.8 $\pm$ 6.0 <sup>c,d</sup>	54.9 $\pm$ 6.1 <sup>c</sup>
FM (kg)	14.7 $\pm$ 3.8	14.7 $\pm$ 3.8	14.3 $\pm$ 3.5	14.3 $\pm$ 3.1	13.1 $\pm$ 3.5 <sup>c,f</sup>	12.4 $\pm$ 3.6 <sup>c,f</sup>
FFM (kg)	40.9 $\pm$ 3.1	38.3 $\pm$ 3.2 <sup>c</sup>	38.0 $\pm$ 3.0 <sup>c</sup>	43.8 $\pm$ 5.7	42.7 $\pm$ 5.5 <sup>c,f</sup>	42.5 $\pm$ 5.7 <sup>c,f</sup>
<b>Metabolic parameters</b>						
Insulin (mIU/l)	4.7 $\pm$ 1.4	6.2 $\pm$ 2.1 <sup>b</sup>	–	4.3 $\pm$ 1.3	4.7 $\pm$ 0.9 <sup>d</sup>	–
HOMA-IR	0.97 $\pm$ 0.30	1.41 $\pm$ 0.51 <sup>b</sup>	–	0.86 $\pm$ 0.26	0.97 $\pm$ 0.18 <sup>e</sup>	–
Triglycerides (g/L)	0.68 $\pm$ 0.21	0.94 $\pm$ 0.65 <sup>a</sup>	–	0.65 $\pm$ 0.12	0.88 $\pm$ 0.20 <sup>a</sup>	–
<b>Hepatic biomarkers</b>						
AST (IU/L)	18.8 $\pm$ 4.7	24.0 $\pm$ 6.1 <sup>b</sup>	24.3 $\pm$ 5.8 <sup>b</sup>	15.9 $\pm$ 2.8	19.0 $\pm$ 4.0	17.8 $\pm$ 3.2 <sup>d</sup>
ALT (IU/L)	13.5 $\pm$ 5.3	25.3 $\pm$ 11.5 <sup>c</sup>	22.8 $\pm$ 9.4 <sup>b</sup>	13.4 $\pm$ 4.6	18.6 $\pm$ 7.8 <sup>d</sup>	18.3 $\pm$ 6.7
CK18 (U/L)	122.3 $\pm$ 44.5	161.6 $\pm$ 71.6	194.2 $\pm$ 95.4 <sup>b</sup>	126.1 $\pm$ 35.7	117.1 $\pm$ 7.6 <sup>d</sup>	139.5 $\pm$ 18.7 <sup>d</sup>
ANGPTL3 (ng/ml)	70.2 $\pm$ 11.6	76.2 $\pm$ 10.1	85.8 $\pm$ 24.8 <sup>a</sup>	68.6 $\pm$ 13.1	75.9 $\pm$ 18.4	75.2 $\pm$ 13.6
CHA	2.2 $\pm$ 1.3	5.3 $\pm$ 2.6 <sup>b</sup>	–	1.7 $\pm$ 0.7	2.1 $\pm$ 0.4 <sup>d</sup>	–
AAC	10.6 $\pm$ 2.5	15.4 $\pm$ 3.9 <sup>c</sup>	15.7 $\pm$ 3.3 <sup>c</sup>	10.0 $\pm$ 1.7	11.6 $\pm$ 2.4 <sup>e</sup>	11.8 $\pm$ 2.1 <sup>e</sup>

AAC (Alanine transaminase, Aspartate transaminase, Cytokeratin 18 index); ALT, alanine transaminase; ANGPTL3, angiotensin-like protein 3; AST, aspartate transaminase; BM, body mass; BMI, body mass index; BR30, after 30 days of bed rest; BR60, after 60 days of bed rest; CHA (Cytokeratin 18, Homoeostasis Model Assessment – Insulin Resistance, Aspartate transaminase index); CK18, cytokeratin 18; FFM, fat free mass; FM, fat mass; HOMA-IR, homoeostasis model assessment – insulin resistance.

Data are presented as mean  $\pm$  SD. Analyses were done using linear mixed models taking into account repeated measures, with adjustment for baseline values and for FM for hepatic biomarkers.

<sup>a</sup> $P \leq 0.05$ , <sup>b</sup> $P \leq 0.01$  and <sup>c</sup> $P \leq 0.001$  vs baseline; <sup>d</sup> $P \leq 0.05$ , <sup>e</sup> $P \leq 0.01$  and <sup>f</sup> $P \leq 0.001$  vs control group variations.



### Hepatic markers of NAFLD

At baseline, no significant difference in hepatic markers was noted between the two groups (Table 1). In the control group, 30 days of bed rest induced a significant increase in the transaminases (AST: +28%,  $P < 0.01$  and ALT: +87%,  $P < 0.001$ ) that was maintained at BR60 (AST: +32% and ALT: +75%;  $P < 0.01$  for both). While no significant modifications were noted at BR30, both CK18 (+76%;  $P < 0.01$ ) and ANGPTL3 (+29%;  $P = 0.02$ ) were significantly higher after 2 months of bed rest than at baseline. In the exercise group the increases at BR60 in AST (+13%) and CK18 (+22%), but not in ALT (+37%) and ANGPTL3 (+10%), were significantly reduced compared to the control group ( $P = 0.02$  for both). These increases did not reach significance for any of the hepatic markers.

Hepatic index AAC increased by 51% ( $P < 0.001$ ) after 30 days of bed rest; this elevation was maintained **3** for the next 30 days (+52%,  $P < 0.001$ ) (Fig. 1). Hepatic index CHA was 162% higher at BR30 than at baseline ( $P < 0.001$ ). In the exercise group, no significant changes were noted and the increase in both AAC (+17% and +19% at BR30 and BR60 respectively) and CHA (+48% at BR30) were significantly lower than those measured in the control group ( $P < 0.01$  for all) and not significant.

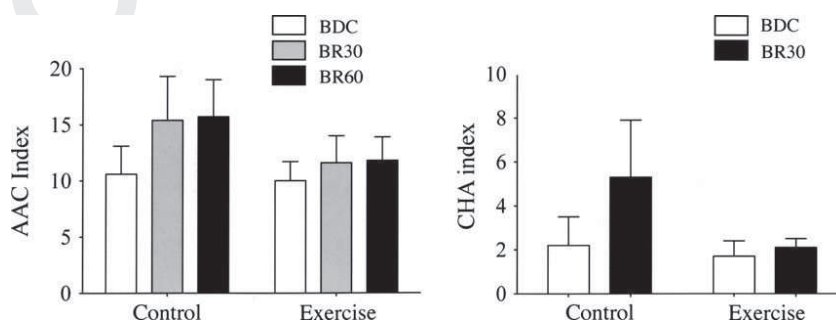
### Correlation analyses

Baseline-to-BR30 changes in AST were positively associated with concomitant changes in ALT both in the control ( $R^2 = 0.81$ ,  $P < 0.01$ ) and exercise group ( $R^2 = 0.64$ ,  $P = 0.02$ ). In the control group only, baseline-to-BR30 changes in fasting triglycerides were also positively associated with concomitant changes in AAC ( $R^2 = 0.66$ ,  $P = 0.02$ ), CHA ( $R^2 = 0.74$ ,  $P < 0.01$ ) and AST ( $R^2 = 0.44$ ,  $P = 0.07$ ). Changes in FM and insulin did not significantly correlate with any of the hepatic markers.

### Discussion

Although it is admitted that physical inactivity plays a key role in the development of metabolic diseases, its impact on liver metabolism and in the development of NAFLD are still poorly characterized. We retrospectively used samples from a previous 60-day bed-rest study, taking thus advantage of a unique opportunity to follow the evolution of hepatic markers of several stages of NAFLD when transitioning from physically active status to severe physical inactivity. We furthermore investigated the protective effect of combined aerobic and resistive exercise on hepatic markers changes. In this study, we showed that, under controlled energy balance conditions, enforced physical inactivity increases hepatic markers that are associated with hepatic steatosis, steatohepatitis and even more severe stages of NAFLD. This supports the observation from cross-sectional studies of higher prevalence of NAFLD in sedentary normal-weight men than in their physically active counterparts (19). It is also consistent with rodents studies showing that transition from an active to sedentary state induces an increased risk of liver steatosis along with an accumulation of lipogenesis intermediates (20), a reduction in hepatic fat oxidation (20) and hypertriglyceridaemia, features associated with NAFLD (21).

Of note, after 2 months of bed rest, transaminases that were positively associated were still in a normal range of concentrations. This, however, does not diminish the impact of physical inactivity on liver metabolism. Similar elevated but normal concentrations are indeed observed in a large number of patients with NAFLD (22) and are associated with greater risks to develop metabolic disorders and diabetes (23). Furthermore, if elevated transaminases are widely used to detect the presence of NAFLD (24), they represent a poor predictor of NASH when used alone. That is why we combined them with measures of more specific steatohepatitis markers. Caspase-cleaved fragments of CK18, the major intermediate filament protein in the liver, are released in the blood stream following the



**Fig. 1.** Combined hepatic indices according to group and time. \*Data are presented as mean  $\pm$  SD. Analyses were done using mixed models taking into account repeated measures, with adjustment for baseline value and for FM. AAC (Alanine transaminase, Aspartate transaminase, Cytokeratin 18 index); BR30, after 30 days of bed rest; CHA (Cytokeratin 18, Homoeostasis Model Assessment – Insulin Resistance, Aspartate transaminase index). \*\*\* $P \leq 0.001$  vs baseline; \*\* $P \leq 0.01$  vs concomitant control value.

apoptotic cell death of injured hepatocytes. Because liver apoptotic activity is associated with steatohepatitis, but not with isolated hepatic steatosis, levels of CK18 fragments can differentiate the two states of NAFLD (12). CK18 is recognized as the most promising single non-invasive biomarker of NASH by the guidelines for the diagnosis and management of NAFLD (25). We showed that enforced physical inactivity increases CK18, and this elevation even reached the 200 cut-off values proposed for the detection of NASH (26) in half of our control subjects. This, in addition to the augmentation of AAC index in the control subjects, suggests an onset of the development of NASH under physical inactivity conditions. However, the fact that the increase in ANGPTL3 was only moderate indicates that our volunteers did probably not reach more severe stages of NAFLD. The development of NAFLD could not be verified using invasive liver biopsies or non-invasive imaging of liver fat content, because the study was retrospective. Of note it is important to indicate that ectopic fat storage in those very same subjects were observed in both the bone marrow (27) and the skeletal muscle (17). This likely reflects a general response to bed rest and the present results on liver biomarkers do support the development of a fatty liver.

Because bed rest induced hypertriglyceridaemia and ANGPTL3 is a hepatic actor of lipid metabolism, an association between changes in plasma triglycerides and ANGPTL3 was expected. The reduced clearance of triglycerides may, however, be rather because of defects in fat uptake at muscle level rather than to an inhibition of hepatic lipoprotein lipase (LPL) by ANGPTL3 leading to a reduced fat uptake by liver. Previously reported results from this same bed rest study showed that physical inactivity decreases muscle gene expression of the fatty acid transporters into muscle and mitochondria, i.e. FAT/CD36 and CPT1, respectively, suggesting a reduced uptake of fatty acid by the muscle. This alteration was associated with a reduced muscle oxidative capacity and whole-body fat oxidation. Altogether these results support the hypothesis that hypertriglyceridaemia is the result of an altered clearance at muscle level. However, in the absence of direct measurements of muscle and liver LPL activity, it is difficult to conclude on the exact cause of hypertriglyceridaemia in our subjects. We, however, reported a positive correlation between AST and triglycerides, as it has been previously observed in obese patients (28), suggesting a role of ectopic fat storage in the alteration of liver metabolism under inactive conditions. It is finally important to note that not only the combined index including the hepatic markers increased during the bed-rest period, but also CHA that also considered insulin sensitivity in its calculation. Furthermore, changes in both NAFLD indexes, CHA and AAC, were positively associated with increased plasma triglycerides. Altogether

these results suggest relationships between insulin sensitivity, ectopic fat storage and liver metabolism under inactive conditions. As we recently reviewed it (29), enforced physical inactivity leads to the development of insulin resistance, hypertriglyceridaemia along with a reduced muscle fat uptake and oxidation. To understand the underlying time course of these different metabolic alterations induced by physical inactivity and thus their cause-to-effect relationships will help gaining a better understanding on the mechanisms underlying the onset of hepatic diseases as well as on the role of sedentary behaviours in the risk of developing NAFLD and metabolic syndrome. **5**

We further showed that training programme combining resistive and aerobic exercise prevents, at least in part, the increase in hepatic markers induced by physical inactivity. Previous studies showed that moderate-intensity aerobic exercise improves ALT concentration in patients with NAFLD (30) and decreases liver fat content (31) and inflammation (32). Both aerobic and resistive exercises, performed independently, improve intrahepatic fat (33, 34) and insulin sensitivity (33) even in absence of weight loss in obese adolescents. Consistent with our results, aerobic exercise training has also been shown to decrease CK18 in obese patients (35) and ANGPTL3 in patients with coronary artery disease (36). In our study, the increase in the hepatic markers was, however, limited but not fully prevented in the exercise group. These results are consistent with previous observations we had made on these same participants. In an in-depth investigation of the effect of physical inactivity on the intermediary metabolism (17), we had demonstrated that the combined aerobic/resistive exercise training prevented the development of insulin resistance induced by bed rest, but not the development of hypertriglyceridaemia, the reduced exogenous and total fatty acid oxidation, the decrease in fatty acid uptake by the muscle, the reduced oxidative capacity, and the accumulation of fat in skeletal muscle. Population studies have recently suggested that the deleterious effect of sedentary behaviours on metabolic health is independent of the practice of moderate-intensity exercise. This exercise-induced partial prevention of the negative effects induced by bed rest may be explained by the absence of light-intensity physical activity (e.g. walking, taking stairs or any body movement of daily life) in our bed-rested exerciser subjects. To better understand the independent role of sedentary behaviours and physical exercise countermeasure in the development of metabolic and hepatic abnormalities, further studies will be needed.

Some limitations have to be acknowledged. As previously evoked hepatic fat content measurements and liver biopsy are missing. Hepatic markers, and in particular CK18 (13, 37, 38), however, are considered good markers to evaluate the degree of hepatic impairments (39), especially when not limited to transaminases but



used as combined indexes like we did. The low number of subjects participating to this study can be seen as a limiting factor. However, this is a typical sample size for bed rests (17, 27, 40) and the design of any bed rest study undergoes thorough *a priori* power calculation for sample sizes on the principal outcomes. Finally, these results have been obtained in healthy lean female adult and will need to be extended to other populations (male, children, adolescents, elderly, overweight, etc.).

Hepatic markers of steatosis and steatohepatitis are impacted by severe physical inactivity, induced by 60 days of bed rest in healthy normal-weight women. Despite the absence of hepatic diseases, transaminases and markers of more severe stages like CK18 and ANGPTL3 increased independent of FM variation. Two NAFLD combined indexes (AAC and CHA), with one including insulin resistance index also worsened during this study. Our results are in accordance with the suggested role of physical inactivity in the development of metabolic diseases and more particularly NAFLD. In the second part of our study, we proposed a potential partial protective effect of aerobic and resistive exercise, by maintaining or limiting the increase in hepatic markers under severe physical inactivity. Complementary studies on healthy persons are still required to focus on an effective prevention against the development of NAFLD. Interesting studies suggest a combination of nutritional and physical countermeasures.

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# Etude 3: Conséquences d'une simulation d'impesanteur de 21 jours chez l'homme, sur le métabolisme des lipides et effets d'une supplémentation en protéines testée comme contremesure.

## Summary

The two precedent studies presented the impact of severe physical inactivity on inflammation markers specific from visceral adipose tissue and liver, and the effect of physical countermeasure. Simulated microgravity is also inducing other metabolic disorders. During this study, we tested whether bed rest can affect several metabolic variables implicated in insulin sensitivity, lipid oxidation and trafficking, inflammation and oxidative stress. Secondly, we tested whether whey protein supplementation combined with bicarbonate salts could prevent it.

First of all, in control condition, BM had a tendency to decrease and no changes were noted in FFM. However, legs LBM, which is a better indicator of muscle atrophy than FFM, was severely decreased in both conditions. Otherwise, no significant changes in FM could be detected in both groups even if several changes in fat storage was detected during bed rest. In fact, in control condition, liver fat content decreased after bed rest compared to an absence of changes in SAT. In protein condition, the opposite was observed. Furthermore, bed rest affected muscle in control and protein condition by increasing calf, but not thigh fat content.

At fasting time, bed rest did not particularly affect baseline metabolites concentrations involved in insulin sensitivity, like insulin, glucose and HOMA-IR or lipid metabolism; like TGs, NEFA or HDL/LDL. Unusual results were found in the development of metabolic alterations under simulated microgravity. First of all, no sign of inflammation or increase in oxidative stress have been described. Insulin sensitivity was evaluated using an OGTT. No changes were observed in exogenous glucose oxidation, neither in insulin plasma concentration. However, glucose concentration increased the last hour of the test, but only in protein condition. Finally

liver and muscle insulin sensitivity only tended to decrease after bed rest. Lipid metabolism was evaluated during a test day, after a standardized meal. Numerous metabolic variables were measured. Firstly, RMR was decreased after 19 days of bed rest in control condition, but fasting NPRQ and DIT were not affected by bed rest at all. Fasting macronutrients oxidation did not change after bed rest either. Primarily, on fed state, total lipid oxidation remained stable regardless to the condition, however it still increased during the first 3 hours of the test, but stabilized afterwards. Secondly, total carbohydrates oxidation showed an important decrease in both conditions, only due to a significant decrease during the first hours after the meal. Total protein oxidation improved, but only in protein condition due to an effect of protein supplementation on bed rest. The same observation was made during the first and the last hours of the test. Hypertriglyceridemia, usually found in previous bed rest studies, was not described in ours. To precise these results, we then followed dietary lipids oxidation measured in  $d_{31}$  palmitate oxidation. It was not impacted by 19 days of bed rest, however, the 4 hours cumulative recovery of  $d_{31}$  palmitate showed a significant increase after bed rest in both conditions, but remained stable 7 and 23 hours after the dose, whatever the condition. Total  $1-^{13}C$  acetate oxidation showed an increase in control and protein condition. Moreover, the slope of  $1-^{13}C$  acetate oxidation, representing tricarboxylic acid cycle (TCA) activity, was only increased in protein condition. To complete these data on lipid metabolism, dietary lipid trafficking was followed and evaluated by measuring  $d_{31}$  palmitate enrichment in different lipid fractions: CM, VLDL and NEFA. The analyze showed no specific impact of bed rest in CM isotopic enrichment or total unlabeled palmitate concentration leading to a stable enrichment of  $d_{31}$  palmitate. Nevertheless, during the first hours of the test, unlabeled palmitate concentration still increased after bed rest in control condition, but was prevented by protein supplementation. Plasma TG included in CM was also stable. VLDL or NEFA dietary lipid enrichment was not changed after bed rest whatever the condition. Still, NEFA enrichment in  $d_{31}$  palmitate was increased during the first hours of the test under both conditions and thus the variation was not prevented by protein supplementation. Both plasma concentration of NEFA and VLDL\_TG were stable. Finally, we followed metabolic variables implicated in insulin sensitivity after a standardized meal. Glucose concentration was impacted by protein supplementation leading to an increase in protein condition, in total, during the first and last hours of test. Total insulin concentration did not show any sign of changes nevertheless an important increase in insulin concentration was described during the last hours of the test. Similarly, HOMA-IR also increased during the last hours only.

Finally, a more specific study of metabolic pathway involved in lipid trafficking and insulin resistance was also assessed using molecular biology performed on muscle biopsies of *vastus lateralis* and *soleus*. In microfluidic RT-PCR analyzes on *soleus* muscle, the results showed very few genes impacted by bed rest. Regardless to the condition, two enzymes involved in lipid transport, ACADM and ACOX2, were decreased after bed rest. So were COX4l and PGC1 $\alpha$ , both involved in mitochondrial

pathway, and ACAA2, the last enzyme of beta-oxidation pathway. Regarding insulin resistance, only IRS1 was decreased, but in control condition only. Western blot analyzes showed no impact of bed rest in *vastus lateralis* in control condition. In protein condition, only CPT1 and Oxphos-3 protein expression increased after bed rest. At the opposite, in *soleus*, no effect of bed rest was found in protein condition. In control condition, only PKB expression increased after 19 days of bed rest.

To conclude, BM and legs LBM were decreased after bed rest. A stable FM was described, however liver fat content was decreased at the opposite of calf fat content. Unusual results were found in metabolic alterations usually found after bed rest. In fact, no sign of inflammation was found in our study. Furthermore, bed rest did not particularly affect whole body insulin sensitivity, only with a slight increase in glucose concentration in the protein group after OGTT. Liver and muscle insulin sensitivity were also unchanged. During test day, we described an increase in glucose concentration, but only in the protein condition due to a decrease in glucose oxidation. Insulin and HOMA-IR also increased the last hours of the test. The study of lipid metabolism during test day showed interesting results. In fact, lipid oxidation increased, due to augmentation in dietary lipid oxidation. However, no changes in lipid trafficking were observed. Finally, in all these results, protein supplementation only increased protein oxidation and glucose concentration.

Baseline data of the 21-days bed rest study are presented in annex 2 at the end of the manuscript. The paper is titled: "A combination of whey protein and potassium bicarbonate supplements during head-down-tilt bed rest: Presentation of a multidisciplinary randomized controlled trial (MEP study)" and was published in *Acta Astronautica* in 2013.



As indicated in the methodology chapter, each subject was tested for bed rest effect under two conditions: “control” (n=10), without nutritional countermeasure, and “protein” (n=9), with a supplementation of proteins during bed rest period. The order of bed rest sessions was random. As no baseline differences between groups and no carry-over effect (i.e. effect from the treatment of the first session on the baseline values of the second session) were observed; data were pooled according to the nutritional condition (“control” or “protein”).

Baseline values are referred here as BDC (for baseline data collection) whatever the day of measure during the BDC period. In the same way, as specified in the methodology chapter, the impact of bed rest and of the protein countermeasure (referred here as HDT for head down tilt bed rest) were tested at different times of HDT. Of note, unfortunately, the effect of bed rest on body composition was only assessed during the recovery period (referred here as R), which is characterized by an important fluid redistribution leading to an underestimation of fat-free mass loss.

As detailed in the methodology chapter, based on general mixed models, for each variable we first tested the impact of the bed rest (HDT vs BDC in control condition). If the bed rest effect was significant we further tested whether 1) protein supplementation at least partly prevented the variations observed (comparison of HDT-BDC variations according the condition), 2) there was still a significant impact of bed rest in subjects supplemented with protein BDC vs HDT in protein condition).

### *a. Body mass and composition changes*

Daily BM measures during the whole study are presented in Figure 31 in each nutritional condition (“control” and “protein”).

Measures of BM showed a significant decrease of 2.9% after 21 days of bed rest in control condition ( $74.9 \pm 1.8\text{kg}$  at HDT21 vs  $77.1 \pm 1.9\text{kg}$  at BDC-1;  $p<0.001$ ). Protein supplementation did not prevent this decrease during HDT (interaction time\*condition:  $p=0.78$ ;  $74.6 \pm 1.5\text{kg}$  at HDT21 in protein condition) with a decrease of 3.1% compared to BDC-1 ( $77.0 \pm 1.5\text{kg}$  at HDT21;  $p<0.001$ ).

As body composition were measured only at BDC-6 and R+2, we also tested bed rest effect on BM at these times to be consistent with FM and FFM analyzes. BM and body composition data are summarized in Table 1.

Between BDC-6 and R+2, BM was still, but less, decreased by 1.1% in control condition, where bed rest effect nearly reached significance ( $p=0.11$  vs BDC-6). Protein supplementation did not prevent decrease of BM (interaction time\*condition:  $p=0.78$ ), which was of the order as in the control condition (R+2 vs BDC-6, -1.2%;  $p=0.02$ ). The decrease in FM was not significant and did not explain BM variation because of an absence of bed rest effect in FM at R+2 (-3.0%,  $p=0.50$  vs BDC-6 in both conditions). By contrast we observed an approximately 1.0% decrease in FFM in both conditions (interaction time\*condition:  $p=0.66$ ) although the decrease reached significance in the protein condition only: difference of 0.9% in the protein condition ( $p=0.04$ ) and of 1.0% in the control condition ( $p=0.24$ ). Nevertheless, LBM in legs is more representative of muscle atrophy than whole body FFM. And as illustrated in

Table 1, the variation of FFM was mainly explained by a decrease in lean body mass (LBM) of lower limbs. In legs, a significant and similar decrease was observed in both the control condition (R+2 vs BDC-6, -3.3%,  $p<0.01$ ) and the protein condition (R+2 vs BDC-6, -2.5%;  $p<0.001$ ; interaction condition\* time:  $p=0.75$ ). On the opposite, arms LBM was not affected by the bed rest in control condition (R+2 vs BDC-6, +0.21%,  $p=0.81$ ).

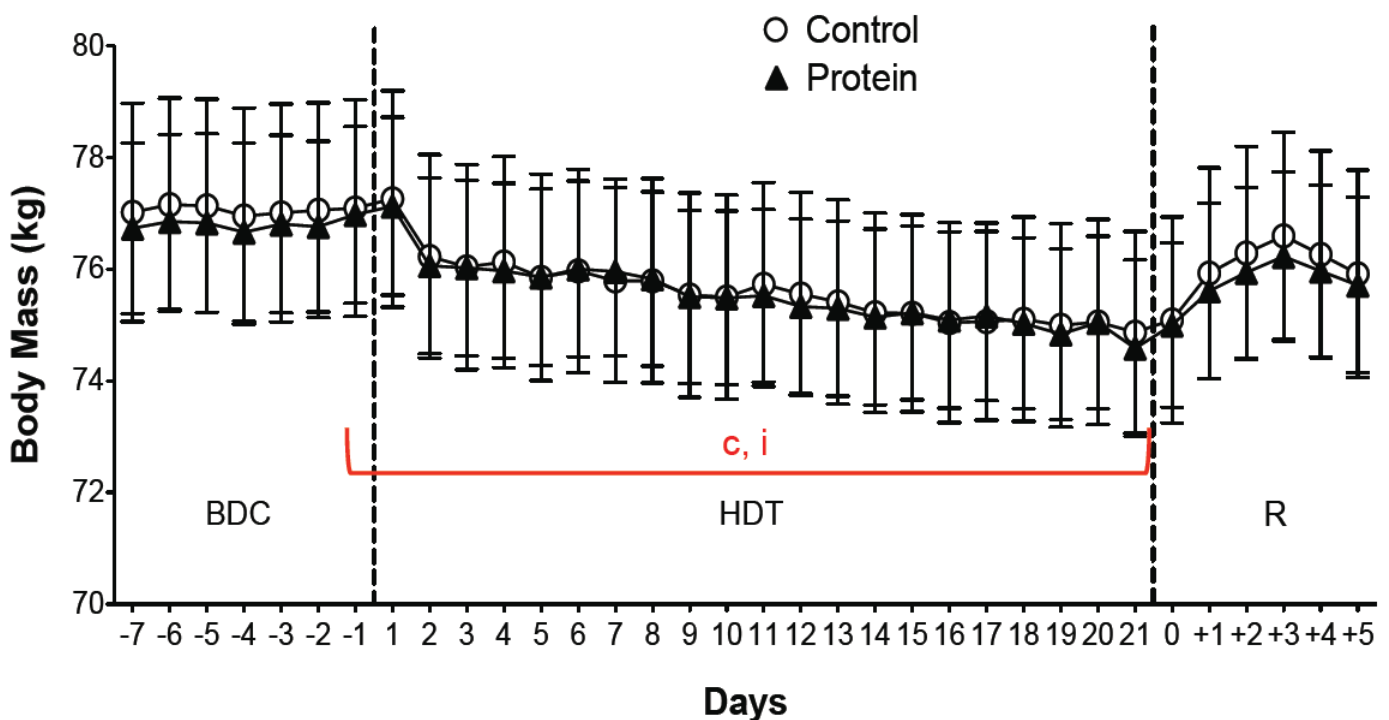


Figure 31: Body mass variation.

The graph is presenting body mass variations of the subjects under control (white) and protein (black) conditions, during BDC (7 days), HDT (21 days) and R (5 days) periods.

Data are means  $\pm$  SEM. Analyses were performed using linear mixed model and specified hypotheses

<sup>a</sup> $p<0.05$ , <sup>b</sup> $p<0.01$ , <sup>c</sup> $p<0.001$  vs baseline in control condition

<sup>d</sup> $p<0.05$ , <sup>e</sup> $p<0.01$ , <sup>f</sup> $p<0.001$  vs variation in control condition

<sup>g</sup> $p<0.05$ , <sup>h</sup> $p<0.01$ , <sup>i</sup> $p<0.001$  vs baseline in protein condition



<u>Characteristics</u>	Control (n=10)		Protein (n=9)	
	Baseline	2 <sup>nd</sup> day of recovery	Baseline	2 <sup>nd</sup> day of recovery
Age (years)	31.0 ± 2.1		31.6 ± 2.0	
Height (meters)	1.8 ± 0.02		1.8 ± 0.02	
VO <sub>2</sub> max	4.0 ± 0.2		4.1 ± 0.2	
Number of steps	3268.7 ± 269.6		3312.3 ± 301.3	
Body mass index (kg/m <sup>2</sup> )	23.8 ± 0.5	23.6 ± 0.5	23.7 ± 0.5	23.4 ± 0.5
<u>Body mass and composition</u>				
Body mass (kg)	77.2 ± 1.9	76.3 ± 1.9	76.9 ± 1.5	75.9 ± 1.5 <sup>g</sup>
Fat mass (kg)	18.6 ± 1.3	18.0 ± 0.1	17.3 ± 1.1	17.6 ± 1.1
% Fat mass	23.8 ± 1.1	23.5 ± 1.0	22.5 ± 1.0	23.1 ± 1.0
Fat free mass (kg)	59.0 ± 1.3	58.4 ± 1.1	59.0 ± 0.9	58.5 ± 0.7 <sup>g</sup>
% Fat free mass	76.0 ± 1.0	76.6 ± 1.0	77.3 ± 1.0	77.2 ± 1.0
Arm lean body mass (kg)	6.6 ± 0.2	6.8 ± 0.2	6.6 ± 0.2	6.7 ± 0.1
Leg lean body mass (kg)	19.5 ± 0.6	19.3 ± 0.4 <sup>b</sup>	18.9 ± 0.5	18.8 ± 0.3 <sup>i</sup>

Table 1: Characteristics, body mass and composition.

The table is presenting the effect of bed rest under each condition, control (n=10) or protein (n=9), at baseline and the 2<sup>nd</sup> day of recovery period.

Data are means ± SEM. Analyzes were performed using linear mixed model and specified hypotheses.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition

### i. Organ fat content and regional fat depot

The variations in organ fat contents, as measured by MRI, are presented in Table 2. For readability, and although analyses have been performed using all data over tie, only data at baseline and HDT20 are presented.

Contrary to expected results, in control condition, bed rest resulted in a 15.8% decrease in liver fat content (HDT20 vs BDC-2:  $p < 0.01$ ). Although there was no time\*condition interaction ( $p = 0.72$ ), the decrease in liver fat content tended to be lower in the protein condition and did not reach significance (HDT20 vs BDC-2; -5.4%,  $p = 0.24$ ).

Concomitantly and inversely a non-significant decrease of 6.5% in subcutaneous adipose tissue (SAT) was observed in the control condition ( $p = 0.15$  vs BDC-2). The decrease in SAT was significantly higher in the protein condition (-14.8%,  $p < 0.01$  vs BDC-2). No significant impact of bed rest was found for visceral adipose tissue.

Muscle fat content was measured by MRI in calf and thigh. An effect of bed rest was observed in calf intra-muscular fat content with a similar increase of about 7.5% in both condition (interaction time\*condition:  $p = 0.65$ ), while no significant variation in calf subcutaneous fat was noted in either condition. By contrast high intra-muscular and subcutaneous fat content remained stable between baseline and HDT20 whatever the condition.

<u>Organ fat content</u>	Control (n=10)		Protein (n=9)	
	Baseline	20 days of bed rest	Baseline	20 days of bed rest
Liver (% fat)	3.2 ± 0.2	2.8 ± 0.2 <sup>b</sup>	3.0 ± 0.1	2.8 ± 0.2
Subcutaneous adipose tissue (pixels)	8573.7 ± 980.3	8049.1 ± 841.7	8501.0 ± 993.9	7404.2 ± 944.7 <sup>h</sup>
Visceral adipose tissue (pixels)	3878.0 ± 548.6	3538.4 ± 543.2	3389.8 ± 407.5	3473.5 ± 341.7
Thigh (% fat)	5.7 ± 0.2	5.7 ± 0.2	5.7 ± 0.2	5.7 ± 0.3
Thigh subcutaneous fat (pixels)	3877.6 ± 477.7	3949.1 ± 463.4	3776.0 ± 420.3	3719.5 ± 439.0
Calf (% fat)	4.5 ± 0.1	4.8 ± 0.2	4.6 ± 0.1	5.0 ± 0.3 <sup>g</sup>
Calf subcutaneous fat (pixels)	1483.3 ± 167.0	1433.7 ± 189.6	1471.4 ± 178.5	1383.0 ± 160.5

Table 2: Organ fat content and regional fat content

The table is presenting liver, thigh and calf intra-muscular and subcutaneous fat content, and subcutaneous and visceral adipose tissue in subjects in control (n=10) and protein (n=9) condition at baseline and after 20-days of bed rest.

Data are means ± SEM. Analyses were performed using linear mixed model and specified hypotheses.

<sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , <sup>c</sup> $p < 0.001$  vs baseline in control condition

<sup>d</sup> $p < 0.05$ , <sup>e</sup> $p < 0.01$ , <sup>f</sup> $p < 0.001$  vs variation in control condition

<sup>g</sup> $p < 0.05$ , <sup>h</sup> $p < 0.01$ , <sup>i</sup> $p < 0.001$  vs baseline in protein condition

### *b. Inflammation*

Plasma aminotransferases, CRP and fibrinogen concentration are presented in Table 3.

Hepatic markers were not significantly affected by 10 days of bed rest (+11.8%,  $p=0.43$  vs BDC-7; -9.9%,  $p=0.10$  vs BDC-7; -2.9%,  $p=0.76$  vs BDC-7) for plasma aminotransferase ALT, AST and GGT respectively. The same conclusion was found for CRP (+2.0%,  $p=0.80$  vs BDC-7) and fibrinogen (+0.5%,  $p=0.95$  vs BDC-7).

Global blood resistance and plasma antioxidant power of plasma is presented in Figure 32.

Antioxidant power of plasma in subject in control condition did not significantly change after bed rest (-5.6%;  $5.5\pm0.2\text{mmol}$  at BDC-4 vs  $4.7\pm0.2\text{mmol}$  at HDT19;  $p=0.26$ ) (Figure 32). Neither did the antioxidant defenses after glucosidase (-8.8%;  $10.4\pm1.1\%$  at BDC-4 vs  $9.5\pm0.5\%$  at HDT19;  $p=0.56$ ) and glucuronidase action (-9.8%;  $11.5\pm1.0\%$  at BDC-4 vs  $10.4\pm0.7\%$  at HDT19;  $p=0.41$ ) on free radicals in subjects in control condition. Similarly, after sulfatase action on free radicals, antioxidant defenses did not vary after 19 days of bed rest in control condition (+106%;  $0.05\pm0.93\%$  in BDC-4 vs  $-1.0\pm1.0\%$  in HDT19;  $p=0.43$ ).

<u>Inflammation markers</u>	<b>Control (n=10)</b>		<b>Protein (n=9)</b>	
	<i>Baseline</i>	<i>10 days of bed rest</i>	<i>Baseline</i>	<i>10 days of bed rest</i>
ALT (IU/L)	14.1 ± 1.3	15.8 ± 1.1	16.2 ± 1.7	20.4 ± 2.3
AST (IU/L)	19.2 ± 1.9	17.3 ± 1.3	19.5 ± 1.4	18.3 ± 1.3
GGT (IU/L)	18.8 ± 2.9	18.3 ± 2.9	16.8 ± 2.1	17.5 ± 2.1
CRP (mg/dL)	0.053 ± 0.014	0.054 ± 0.013	0.049 ± 0.010	0.045 ± 0.010
Fibrinogen (mg/dL)	268.7 ± 25.6	270.1 ± 20.1	214.5 ± 13.8	244.5 ± 14.2

**Table 3:** Plasma inflammation markers.

Plasma ALT, AST, GGT, CRP and fibrinogen concentration in subjects in control (n=10) and protein (n=9) condition at baseline and 10 days of bed rest.

Data are means ± SEM. Analyses were performed using linear mixed model and specified hypotheses.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition

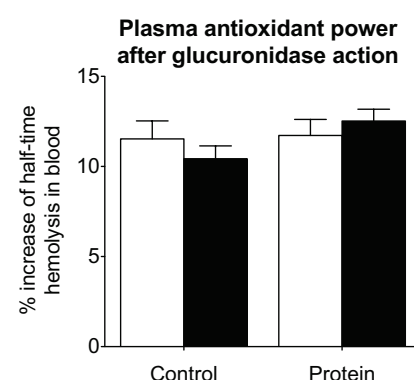
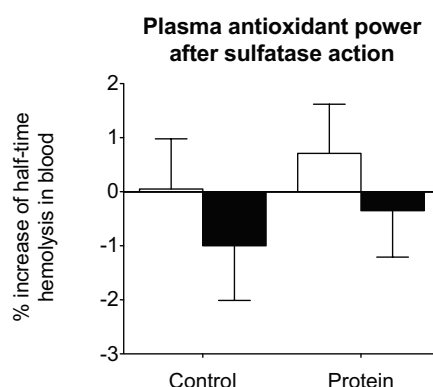
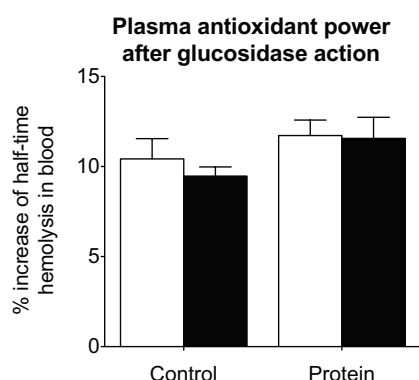
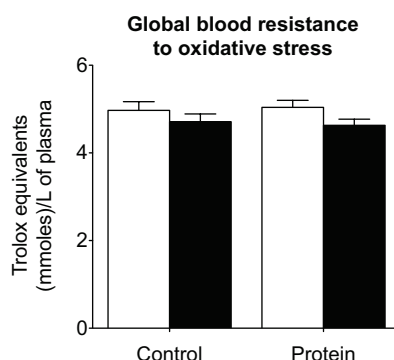


Figure 32: Oxidative stress: KRL and RESEDA tests

Global blood resistance and plasma antioxidant power after glucosidase, sulfatase and glucuronidase action in subjects in control (n=10) and protein (n=9) condition at baseline (white) and after 19 days of bed rest (black).

Data are least square means  $\pm$  SEM. Analyses were performed using linear mixed model and specified hypotheses.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition

*c. Fasting metabolic variables during bed rest*

Fasting hormones and metabolites plasma concentrations and their evolutions are presented in Table 4 and 5.

At fasting, bed rest did not impact glucose (+0.2%; p=0.94 vs BDC-4) and insulin concentration (+1.3%; p=0.91 vs BDC-4) or insulin resistance estimated by HOMA-IR (+2.7%, p=0.83 vs BDC-4) (Table 4).

Fasting plasma TG, NEFA and TG in CM and VLDL concentrations were not impacted by bed rest (+5.8%, p=0.29 vs BDC-4; +14.3%, p=0.28 vs BDC-4; -7.1%, p=0.67 vs BDC-4, -5.9%, p=0.87 vs BDC-4 resp.) (Table 4)

Plasma LDL, HDL concentrations and ratio LDL/HDL showed no impact of bed rest in control condition (-7.4%, p=0.86 vs BDC-4; -10%, p=0.33 vs BDC-4; 9.2%, p=0.58 vs BDC-4 resp.) (Table 5).

	Control (n=10)		Protein (n=9)	
	Baseline	19 days of bed rest	Baseline	19 days of bed rest
<b><u>Glucose tolerance</u></b>				
Fasting glucose (g/L)	0.84 ± 0.02	0.84 ± 0.02	0.86 ± 0.03	0.87 ± 0.03
Fasting insulin (IU/L)	6.01 ± 0.73	6.09 ± 0.43	4.98 ± 0.45	5.38 ± 0.58
Fasting HOMA-IR	0.012 ± 0.001	0.013 ± 0.001	0.011 ± 0.001	0.012 ± 0.001
<b><u>Lipid trafficking</u></b>				
Fasting TG (mmol/L)	0.77 ± 0.08	0.81 ± 0.05	0.81 ± 0.07	0.77 ± 0.08
Fasting NEFA (mmol/L)	0.33 ± 0.04	0.37 ± 0.05	0.33 ± 0.04	0.37 ± 0.04
Fasting TG in CM (mmol/L)	0.036 ± 0.006	0.033 ± 0.005	0.034 ± 0.005	0.028 ± 0.006
Fasting TG in VLDL (mmol/L)	0.39 ± 0.10	0.36 ± 0.08	0.32 ± 0.09	0.31 ± 0.09

**Table 4: Fasting concentration of glucose tolerance and lipid trafficking variables.**

Glucose tolerance variables: fasting glucose, insulin and HOMA-IR concentration are presented in control (n=10) and protein condition (n=9) at baseline and after 19 days of bed rest. We also presented lipid trafficking variables: fasting triglycerides (TG), non-esterified fatty acids (NEFA), triglycerides in chylomicron (TG in CM) and triglycerides in VLDL (TG in VLDL).

Data are means ± SEM. Analyzes were performed using linear mixed model and specified hypotheses.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition

	Control (n=10)		Protein (n=9)	
	Baseline	19 days of bed rest	Baseline	19 days of bed rest
LDL (%)	0.534 ± 0.036	0.540 ± 0.017	0.547 ± 0.037	0.554 ± 0.013
HDL (%)	0.030 ± 0.007	0.026 ± 0.004	0.024 ± 0.003	0.028 ± 0.003
LDL/HDL (%)	0.145 ± 0.015	0.151 ± 0.016	0.149 ± 0.012	0.164 ± 0.008

**Table 5:**

LDL, HDL concentration and LDL/HDL ratio in control (n=10) and protein (n=9) condition at baseline (white) and after 19 days of bed rest (black).

Data are means ± SEM. Analyzes were performed using linear mixed model and specified hypotheses.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition



#### *d. Oral glucose tolerance*

Glucose and insulin concentrations after glucose administration are presented in Figure 33 (top).

Bed rest did not impact significantly impact glucose and insulin concentrations in the control condition, as indicated by the non-significant 2.0% increase of glucose AUC ( $16.9 \pm 0.6$  g/L/hr at BDC-1 vs  $17.3 \pm 0.6$  g/L.hr at HDT21;  $p=0.50$ ) (Figures 32). As indicated by the first and second hour glucose AUC, the kinetics of post-prandial glucose concentration was not affected by the bed rest in the control condition. In the protein condition the bed rest induced a nearly significant 5.6 % increase in total glucose AUC and a significant increase of the second hour glucose AUC (+9.4%;  $p=0.02$ ; time\*condition interaction,  $p=0.16$ ).

The insulin response to OGTT was not impact impacted by the bed rest whatever the condition (control or protein).

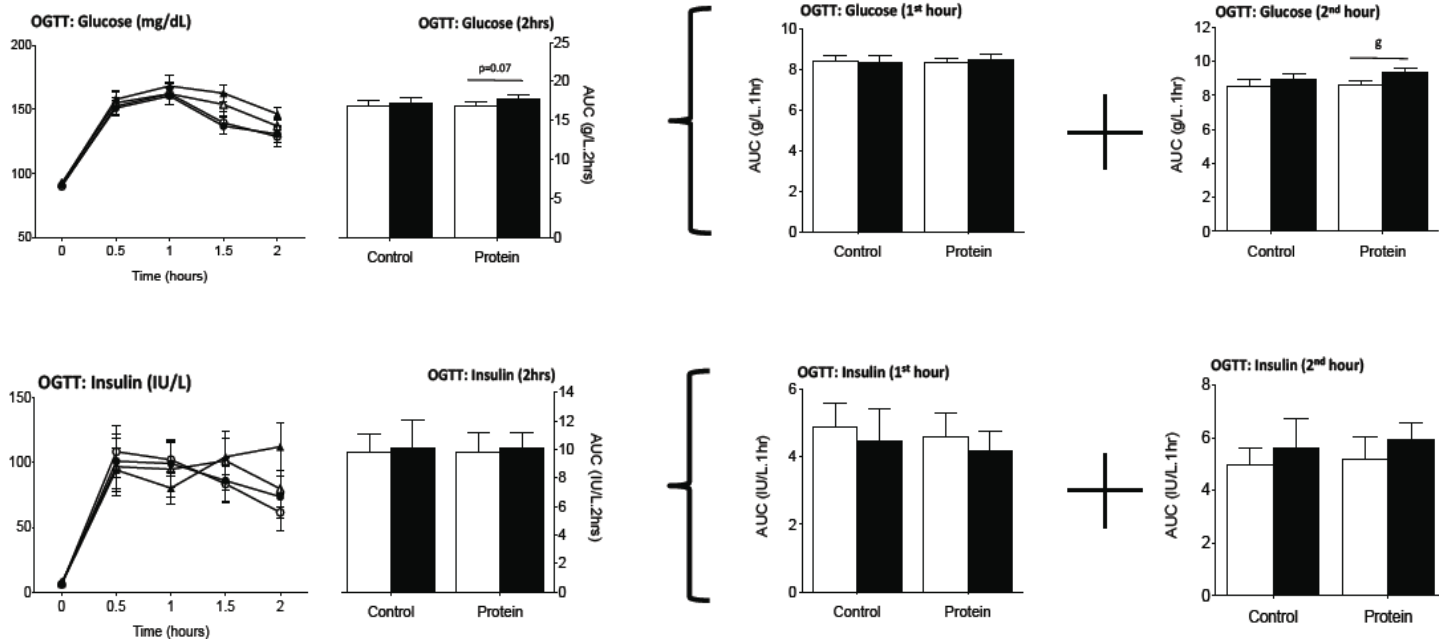
#### *i. Muscle and liver insulin sensitivity index*

Based on these data from OGTT, we calculated organ insulin sensitivity index (Figure 32, middle). Three subjects had an increase of glucose concentration at the end of OGTT. We removed them from the calculation of insulin sensitivity. The results showed that bed rest tended to decrease the insulin sensitivity of both muscle and liver but these variations were not significant.

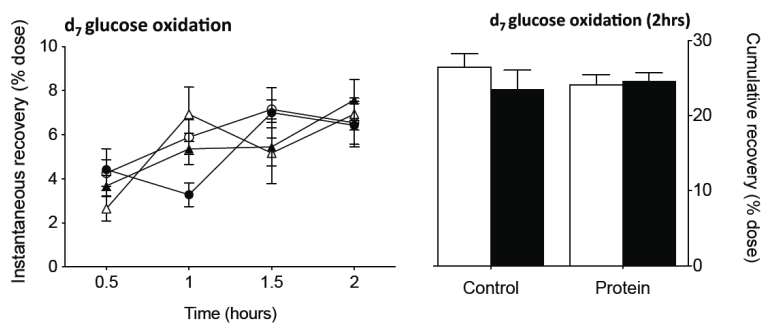
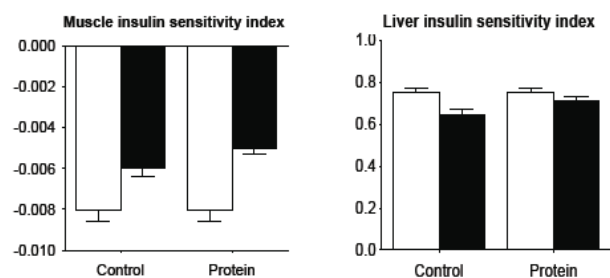
#### *ii. Exogenous $d_7$ glucose oxidation*

Instantaneous and cumulative recoveries of  $d_7$  glucose oxidation are presented in Figure 33 (bottom).

Maximum instantaneous  $d_7$  glucose oxidation did not change after 21 days of bed rest in control condition (-1.6%,  $6.5 \pm 1.0$  %dose at BDC-1 vs  $6.4 \pm 1.0$  %dose at HDT21;  $p=0.91$ ) (Figure 32). Similarly, no impact of bed rest was found on the 7hrs cumulative recovery of  $d_7$  glucose oxidation (-11%,  $26.5 \pm 1.9$  %dose at BDC-1 vs  $23.5 \pm 2.6$  %dose at HDT21;  $p=0.11$ ) and not effect of protein supplementation was noted.



## Organ insulin sensitivity



**Figure 33: Oral glucose tolerance test and organ insulin sensitivity.**

Kinetics of plasma glucose and insulin concentrations are shown and the 2-hours cumulative concentration after glucose administration at t0 are presented in the right bars. The third and fourth columns are respectively presenting the first hour and the last hour of glucose and insulin concentration.

Muscle and liver insulin sensitivity are presented as an index calculated based on Abdul-Ghani et al. 2007 equation.

Kinetic of d<sub>7</sub> glucose oxidation is presented in control (n=10, white) and protein condition (n=9, black) at baseline (rond) and after 21-days of bed rest (triangle) and cumulative recovery is shown with the right bars (white: baseline, black: bed rest).

Data are least-square means ± SEM. Analyzes were performed using linear mixed model and specified hypotheses.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition

## *e. Energy expenditure, total and dietary substrates oxidation*

### *i. Energy expenditure and total substrates oxidation*

Energy expenditure and NPRQ data are presented in Table 6.

A 7.8% decrease in RMR normalized on FFM was described in control condition (p<0.01 vs BDC-4) and was not significantly prevented by protein supplementation (interaction: time\*condition: p=0.79)(Table 6). No changes were noted in fasting NPRQ after bed rest in control condition (-1.2%, p=0.49 vs BDC-4). When normalized on breakfast energy, dietary induced thermogenesis (DIT) was not modified during bed rest in either condition.

	Control (n=10)		Protein (n=9)	
	Baseline	19 days of bed rest	Baseline	19 days of bed rest
<b><i>Energy expenditure and NPRQ</i></b>				
RMR adj FFM (MJ/kg)	0.126 ± 0.006	0.116 ± 0.002 <sup>c</sup>	0.118 ± 0.003	0.115 ± 0.003
Fasting NPRQ	0.84 ± 0.02	0.83 ± 0.01	0.82 ± 0.04	0.81 ± 0.01
Post prandial energy expenditure (kcal/7hrs)	0.616 ± 0.206	0.584 ± 0.016 <sup>c</sup>	0.606 ± 0.013	0.579 ± 0.016 <sup>g</sup>
DIT (%)	8.39 ± 0.92	9.65 ± 0.68	8.93 ± 1.02	8.81 ± 0.74

**Table 6: Energy expenditure and NPRQ**

Resting metabolic rate (RMR), Fat free mass (FFM), NPRQ (Non-protein respiratory quotient), DIT (Dietary induced thermogenesis) are presented in this table in control (n=10) and protein condition (n=9) at baseline and after 19 days of bed rest.

Data are means ± SEM. Analyzes were performed using linear mixed model and specified hypotheses.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition

Fasting and postprandial oxidation of macronutrients are presented in Table 7 and Figure 34 respectively. In the control condition, bed rest did not induce any significant effect on fasting macronutrient oxidation although the decrease in fasting protein oxidation was nearly significant (-3.0%,  $p=0.79$  vs BDC-4; -24.6%,  $p=0.12$  vs BDC-4; +0.7%,  $p=0.94$  vs BDC-4, respectively for glucose, protein and lipid oxidation in the control condition and -9.9%,  $p=0.45$  vs BDC-4; -9.6%,  $p=0.57$  vs BDC-4; +6.4%,  $p=0.45$ ).

Post-prandial oxidations of macronutrients were differently impacted by 19 days of bed rest and protein supplementation. Total carbohydrates oxidation decreased by 18.9% in control and by 23.6% in protein condition ( $p<0.001$  vs BDC-4 for both) with a nearly significant interaction between time and condition (interaction time\*condition:  $p=0.12$ ). This decrease was mostly occurring during the 3 first hours after the meal (-27.9% in control condition and -32.0% in protein condition;  $p<0.001$  vs BDC-4 for both, interaction time\*condition:  $p=0.13$ ). No changes were observed the last 4 hours after the meal (+1.4% in the control condition,  $p=0.87$  vs BDC-4; -5.2% in the protein condition,  $p=0.55$  vs BDC-4; interaction time\*condition:  $p=0.39$ ). Total lipids oxidation did not change after 19 days of bed rest (+3.6% in the control condition,  $p=0.58$  vs BDC-4; +1.3% in the protein condition,  $p=0.84$ ; interaction time\*condition,  $p=0.83$ ). However, post meal, lipid oxidation during the 3 first hours was significantly higher after bed rest than during BDC both in the control condition (+18.3%;  $p=0.02$ ) and in the protein condition (+13.5%,  $p=0.06$ ; time \*condition interaction:  $p=0.98$ ). This early increase in postprandial oxidation was associated with a non-significant decrease during the 4 subsequent hours in both conditions (-10.7% vs BDC in control condition,  $p=0.15$  and -11.4% in protein condition,  $p=0.12$ ; time\*condition interaction,  $p=0.74$ ).

Finally, post-prandial protein oxidation was not impacted by bed rest in control condition (+7.4%,  $p=0.19$  vs BDC-4), while, as expected it increase with protein supplementation (+26.6%,  $p<0.001$  vs BDC-4; interaction time\*condition:  $p<0.01$ ). Similar variations were found when the first and last hours of the post-meal period were considered with no significant effect of the bed rest in the control condition and a significant increase of protein oxidation with protein supplementation (interaction time\*condition:  $p<0.01$ ).

		Control (n=10)		Protein (n=9)	
		Baseline	19 days of bed rest	Baseline	19 days of bed rest
<b>Oxidized carbohydrates</b>	Fasting (mg/kg BM/min)	1.51 ± 0.16	1.46 ± 0.21	1.28 ± 0.13	1.15 ± 0.09
	Post prandial AUC (g/7hrs)	3981.9 ± 179.3	3229.5 ± 251.5 <sup>c</sup>	3802.0 ± 144.4	2904.8 ± 149.2 <sup>i</sup>
	Post prandial AUC (g/3hrs)	2754.7 ± 142.1	1985.0 ± 167.3 <sup>c</sup>	2603.6 ± 122.3	1768.3 ± 75.2 <sup>i</sup>
	Post prandial AUC (g/4hrs)	1227.3 ± 68.2	1244.5 ± 116.4	1198.4 ± 81.0	1136.4 ± 79.8
<b>Oxidized lipids</b>	Fasting (mg/kg BM/min)	0.74 ± 0.09	0.75 ± 0.07	0.80 ± 0.08	0.85 ± 0.07
	Post prandial AUC (g/7hrs)	1462.9 ± 68.3	1516.0 ± 64.6	1475.4 ± 68.3	1493.8 ± 82.7
	Post prandial AUC (g/3hrs)	723.1 ± 27.0	855.5 ± 40.4 <sup>a</sup>	749.3 ± 48.3	850.6 ± 49.5
	Post prandial AUC (g/4hrs)	739.8 ± 46.7	660.5 ± 35.9	726.0 ± 29.8	643.2 ± 35.8
<b>Oxidized proteins</b>	Fasting (mg/kg BM/min)	0.78 ± 0.10	0.59 ± 0.08	0.67 ± 0.05	0.61 ± 0.10
	Post prandial AUC (g/7hrs)	1524.6 ± 50.5	1636.7 ± 72.1	1514.7 ± 88.7	1917.5 ± 89.7 <sup>f,i</sup>
	Post prandial AUC (g/3hrs)	870.8 ± 30.6	917.6 ± 40.3	858.6 ± 49.5	1070.2 ± 48.3 <sup>f,i</sup>
	Post prandial AUC (g/4hrs)	653.8 ± 21.5	719.1 ± 32.5	656.1 ± 39.4	847.4 ± 42.3 <sup>f,i</sup>

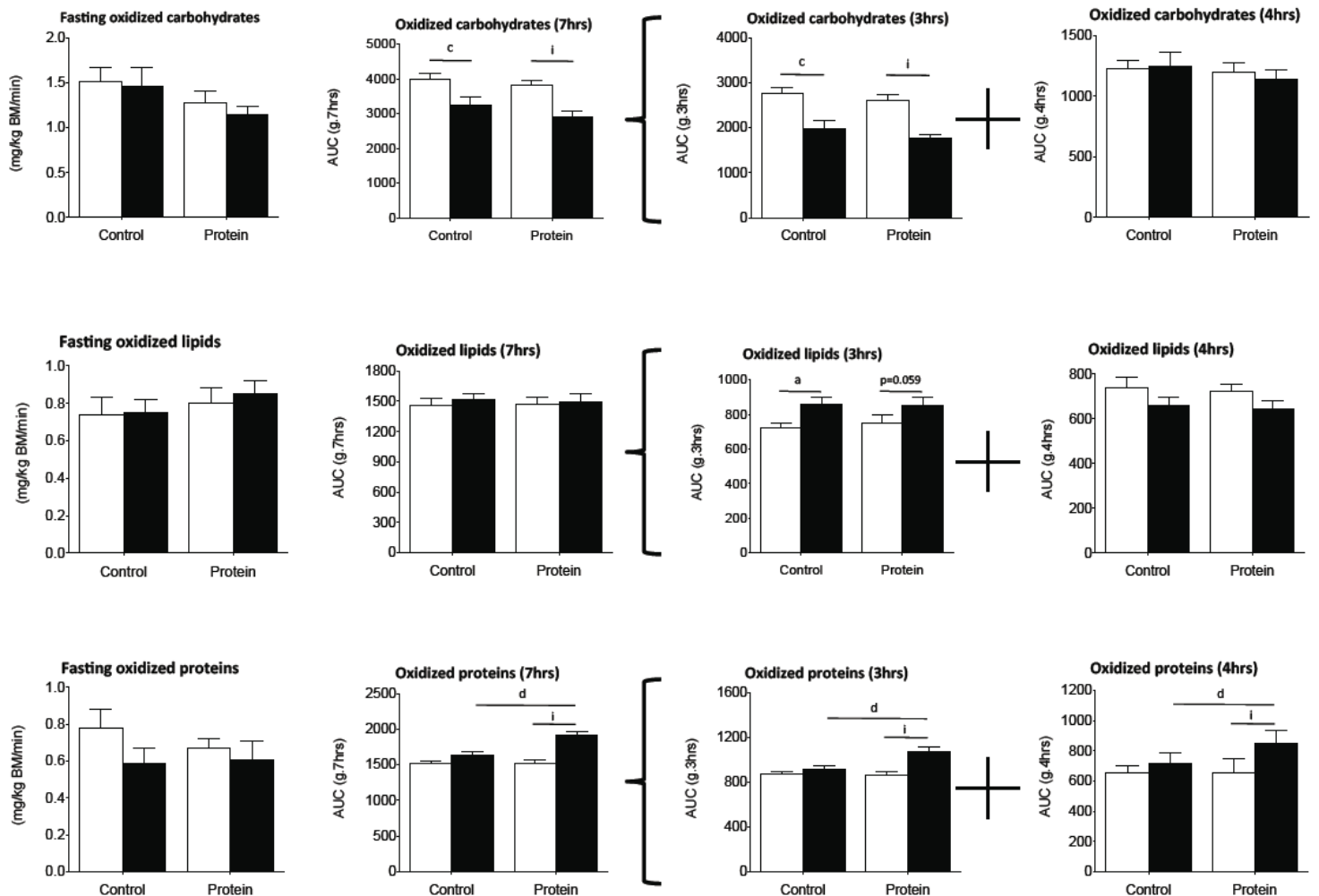


Table 7 & Figure 34: Total Fasting and post-prandial carbohydrates, lipids and proteins oxidations.

The figure is presenting total fasting and post-prandial oxidized carbohydrates, lipids and proteins at baseline (white) and after 19 days of bed rest (black) in subjects under control (n=10) and protein (n=9) condition.

The first column of histograms are fasting oxidized macronutrients. The second column presents the area under the curve (AUC) of 7 hours oxidized carbohydrates, lipids and proteins. The third and fourth columns are respectively presenting the 3 first and 4 last hours of oxidized macronutrients.

Data are least-square means  $\pm$  SEM. Analyses were performed using linear mixed model and specified hypotheses.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition

ii. Plasma metabolic variables during test day

An overall effect of bed rest was observed in plasma glucose concentration (Figure 35). After 19 days of bed rest, a 4.3% increase in total glucose nearly reached significance ( $396.7 \pm 9.0$  g/L.7hrs at BDC-4 vs  $413.7 \pm 12.3$  g/L.7hrs at HDT19; p=0.12). Protein supplementation reinforced this variation (interaction time\*condition: p<0.01) leading to a 13.4% increase in protein condition ( $395.0 \pm 11.9$  g/L.7hrs at BDC-4 vs  $447.7 \pm 15.7$  g/L.7hrs at HDT19; p<0.001). Almost the same observation can be made during the first hours following the meal. In fact, no changes were shown in control condition (+0.01%,  $182.1 \pm 5.8$  g/L.3hrs at BDC-4 vs  $182.1 \pm 8.2$  g/L.3hrs at HDT19, p=1) and also no effect of protein supplementation (interaction time\*condition: p=0.14). However, there still was a significant increase of 9.3% ( $176.0 \pm 6.2$  g/L.3hrs at BDC-4 vs  $192.3 \pm 8.3$  g/L.3hrs at HDT19; p=0.03) in protein condition. Finally, the major variation is occurring the last hours of the test. A significant increase under physical inactivity (+7.9%,  $214.7 \pm 5.3$  g/L.4hrs at BDC-4 vs  $231.7 \pm 5.8$  g/L.4hrs at HDT19; p=0.02) was exacerbated by protein supplementation (interaction time\*condition: p<0.01) leading to a more important increase of 16.6% in protein condition ( $219.0 \pm 7.4$  g/L.4hrs at BDC-4 vs  $255.4 \pm 9.7$  g/L.4hrs at HDT19; p<0.001).

Conversely, bed rest did not significantly impact total insulin concentration (+5.0%,  $10.6 \pm 1.9$  IU/L.7hrs at BDC-4 vs  $11.1 \pm 1.1$  IU/L.7hrs at HDT19; p=0.72) (Figure 35). The same conclusion was found the first hours after the dose in subjects under bed rest (-8.8%,  $7.7 \pm 1.4$  IU/L.3hrs at BDC-4 vs  $7.1 \pm 1.0$  IU/L.3hrs at HDT19; p=0.60). Nevertheless, insulin concentration was significantly increased the last four hours of the test, with a 42.1% increase after bed rest in control condition ( $2.9 \pm 0.8$  IU/L.4hrs at BDC-4 vs  $4.1 \pm 0.3$  IU/L.4hrs at HDT19; p<0.01) and 100.1% when supplemented with proteins ( $2.0 \pm 0.4$  IU/L.4hrs at BDC-4 vs  $3.9 \pm 0.3$  IU/L.4hrs at HDT19; p<0.001). Protein supplementation did not prevent this variation under strict bed rest (interaction: p=0.73).

Finally, in control condition, 19 days of bed rest did not significantly impact HOMA-IR (+2.9%,  $28.4 \pm 6.0$  at BDC-4 vs  $29.3 \pm 3.6$  at HDT19;  $p=0.87$ ). The same conclusion was made the 3 first hours following the meal in control condition (-12.9%;  $23.3 \pm 5.3$  at BDC-4 vs  $20.3 \pm 3.3$  at HDT19;  $p=0.49$ ). Interestingly, in control condition, a 74.7% significant increase of HOMA-IR was observed after 19 days of bed rest ( $5.1 \pm 1.0$  at BDC-4 vs  $9.0 \pm 0.9$  at HDT19;  $p<0.01$ ). Protein supplementation nearly significantly influenced this variation (interaction time\*condition:  $p=0.11$ ), leading to a more important increase of 130.0% in protein condition ( $4.9 \pm 1.2$  at BDC-4 vs  $11.2 \pm 1.3$  at HDT19;  $p<0.001$ ).

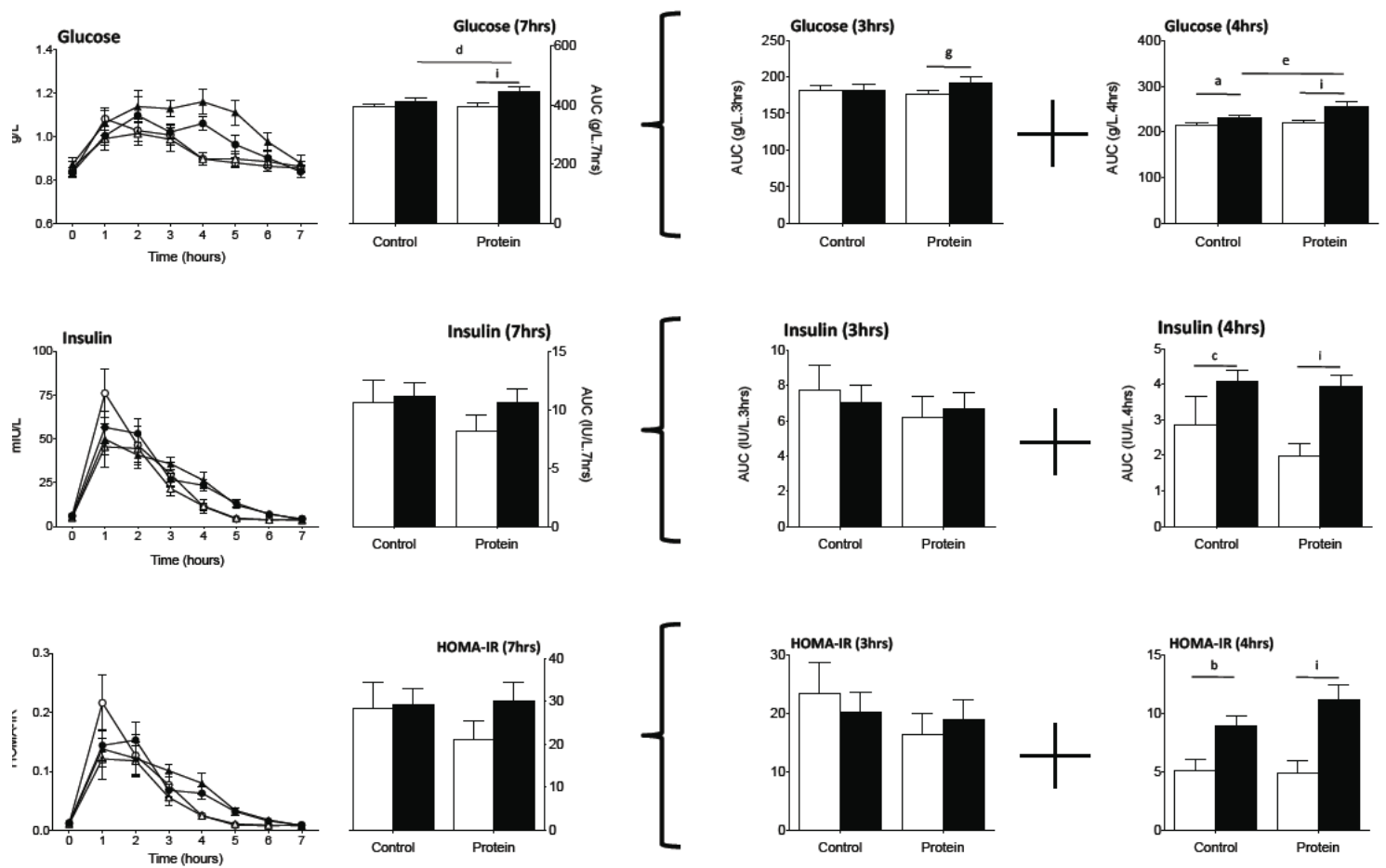


Figure 35: Glucose, insulin and HOMA-IR concentrations after a standardized meal.

Kinetics of glucose, insulin and Homeostasis Model of Assessment – Insulin Resistance (HOMA-IR) are presented after a standardized meal administered at t0 in subjects under control (n=10, white) and protein condition (n=9, black) at baseline (rond) and after 19-days of bed rest (triangle). The right graphs are area under the curve (AUC) of the concentrations of each variable over 7 hours post meal at baseline (white) and after 19 days of bed rest (black). The two last graphs are presenting the AUCs over the first 3 hours and the last four hours of the test.

Data are least square means  $\pm$  SEM.

<sup>a</sup> $p<0.05$ , <sup>b</sup> $p<0.01$ , <sup>c</sup> $p<0.001$  vs baseline in control condition

<sup>d</sup> $p<0.05$ , <sup>e</sup> $p<0.01$ , <sup>f</sup> $p<0.001$  vs variation in control condition

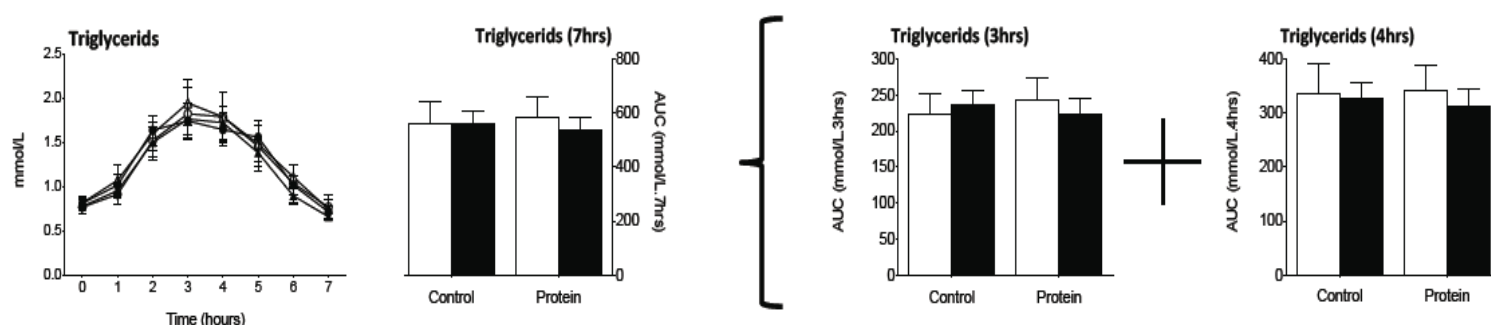
<sup>g</sup> $p<0.05$ , <sup>h</sup> $p<0.01$ , <sup>i</sup> $p<0.001$  vs baseline in protein condition



### iii. Dietary d<sub>31</sub> palmitate oxidation

TG plasma concentrations are presented in Figure 36.

Total TG concentrations remained unchanged after 19 days of bed rest in control condition (+0.8%,  $p=0.91$  vs BDC-4). In control condition, the same conclusions can be made for the concentration over the first and the last hours after the meal with no impact of bed rest in TG (+5.5%,  $p=0.41$  vs BDC-4 and -2.3%,  $p=0.79$  vs BDC-4 resp.).



**Figure 36:** TG kinetics concentrations after a standardized meal

Kinetic of triglycerids (TG) is presented after a standardized meal administered at t0 in subjects under control (n=10, white) and protein condition (n=9, black) at baseline (rond) and after 19-days of bed rest (triangle). The right graphs are area under the curve (AUC) of the concentrations of each variable over 7 hours post meal at baseline (white) and after 19 days of bed rest (black). The two last graphs are presenting the AUCs over the first 3 hours and the last four hours of the test.

Data are least square means  $\pm$  SEM.

<sup>a</sup> $p<0.05$ , <sup>b</sup> $p<0.01$ , <sup>c</sup> $p<0.001$  vs baseline in control condition

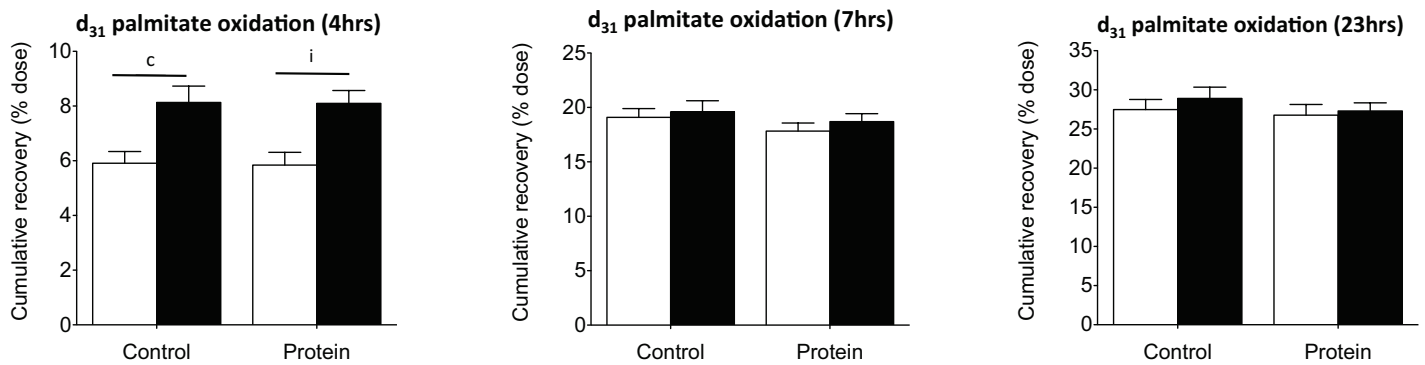
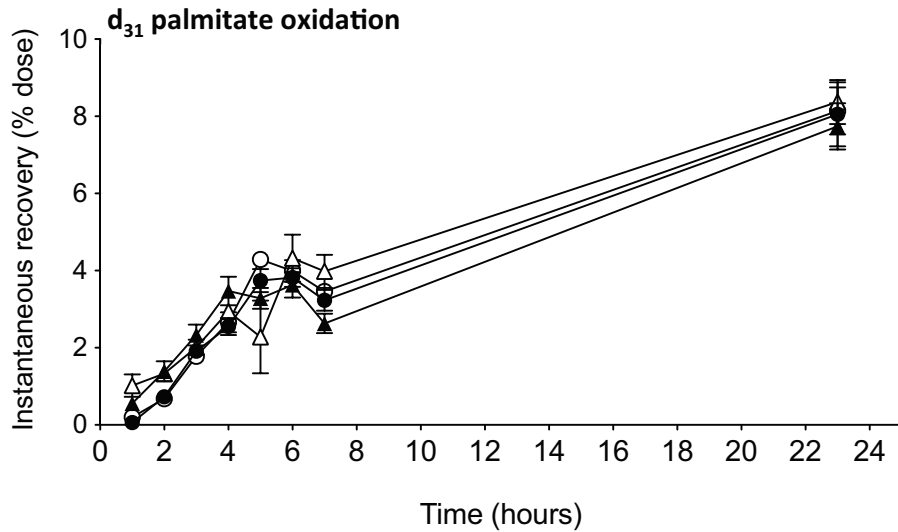
<sup>d</sup> $p<0.05$ , <sup>e</sup> $p<0.01$ , <sup>f</sup> $p<0.001$  vs variation in control condition

<sup>g</sup> $p<0.05$ , <sup>h</sup> $p<0.01$ , <sup>i</sup> $p<0.001$  vs baseline in protein condition

Instantaneous and cumulative recoveries per hour of d<sub>31</sub> palmitate oxidized are presented in Figure 37.

Bed rest did not impact d<sub>31</sub> palmitate oxidation. In fact, maximum instantaneous recovery of d<sub>31</sub> palmitate oxidation did not vary in control condition (+15.2%,  $3.5 \pm 0.2$  %dose/hour at BDC-4 vs  $4.0 \pm 0.4$  %dose/hour at HDT19;  $p=0.55$ ).

When looking closely to the results, cumulative recovery of d<sub>31</sub> palmitate during the first 4 hours after the dose was significantly increased by 37.6% in control condition ( $5.9 \pm 0.4$  %dose/hour at BDC-4 vs  $8.1 \pm 0.6$  %dose/hour at HDT19;  $p<0.01$ ). Protein supplementation did not prevent this increase (interaction time\*condition:  $p=0.97$ ) that was still observed after bed rest in protein condition (+38.6%;  $5.8 \pm 0.5$  %dose/hour at BDC-4 vs  $8.1 \pm 0.5$  %dose/hour at HDT19;  $p<0.001$ ). There was no significant effect of bed rest anymore for data measured 7 hours (+2.7% in control condition;  $19.1 \pm 0.8$  %dose/hour at BDC-4 vs  $19.6 \pm 1.0$  %dose/hour at HDT19,  $p=0.68$ ) and 23 hours after palmitate dose administration (+5.2% in control condition;  $27.5 \pm 1.4$  %dose/hour at BDC-4 vs  $28.9 \pm 1.4$  %dose/hour at HDT19;  $p=0.46$ ).



**Figure 37:** Hourly instantaneous percent recovery of dietary  $d_{31}$  palmitate are presented in subjects under control ( $n=10$ , White) and protein ( $n=9$ , noir) condition at baseline (rond) and after 19 days of bed rest (triangle). Time 0 corresponds to the dose ingestion. Cumulative recoveries of  $d_{31}$  palmitate are presented 4 hours, 7 hours and 23 hours post dose at baseline (white) and after 19-days of bed rest (black).

Data are least square means  $\pm$  SEM. Analyzes were performed using linear mixed model and specified hypotheses.

<sup>a</sup> $p<0.05$ , <sup>b</sup> $p<0.01$ , <sup>c</sup> $p<0.001$  vs baseline in control condition

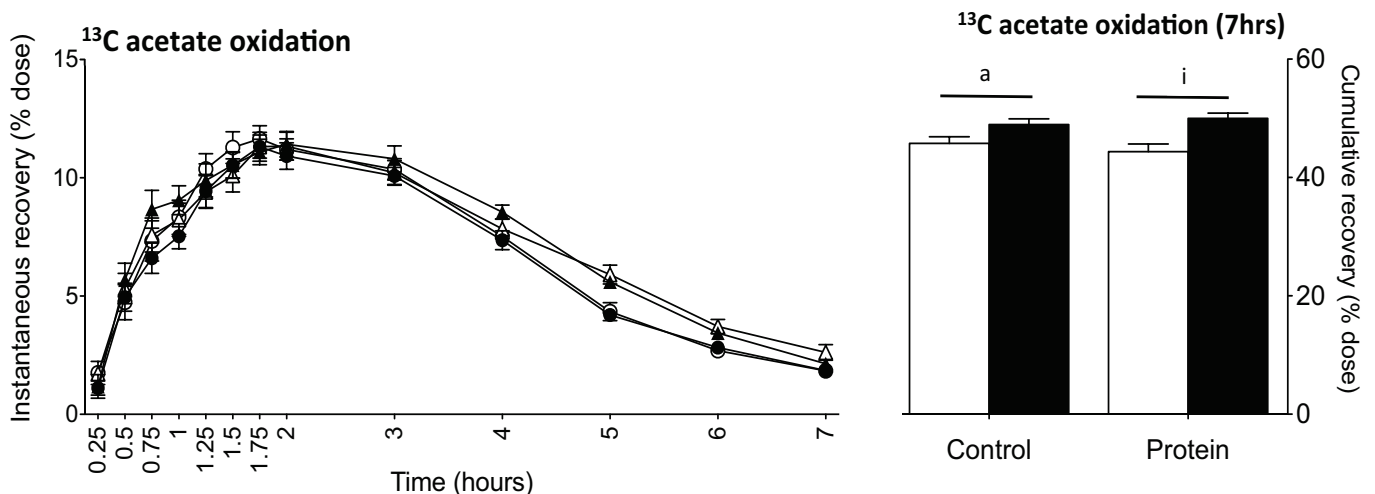
<sup>d</sup> $p<0.05$ , <sup>e</sup> $p<0.01$ , <sup>f</sup> $p<0.001$  vs variation in control condition

<sup>g</sup> $p<0.05$ , <sup>h</sup> $p<0.01$ , <sup>i</sup> $p<0.001$  vs baseline in protein condition

#### iv. $1\text{-}^{13}\text{C}$ Acetate oxidation

Instantaneous and cumulative recoveries of  $1\text{-}^{13}\text{C}$  acetate oxidized are presented in Figure 38.

Maximum instantaneous  $1\text{-}^{13}\text{C}$  acetate oxidation was not changed after 19-days of bed rest in the control condition ( $-1.7\%$ ,  $11.7 \pm 0.6$  %dose/hour at BDC-4 vs  $11.5 \pm 0.5$  %dose/hour at HDT19;  $p=0.91$ ). However, the 7-hours recovery of  $^{13}\text{C}$  acetate showed a significant increase of  $7.4\%$  during bed rest both in the control condition ( $42.9 \pm 1.1$  %dose/hour at BDC-4 vs  $46.0 \pm 1.0$  %dose/hour at HDT19;  $p=0.03$ ) and with protein supplementation (interaction: time\*condition:  $p=0.47$ ;  $+12.8\%$ ,  $41.8 \pm 1.3$  %dose/hour at BDC-4 vs  $47.1 \pm 0.9$  %dose/hour at HDT19,  $p<0.001$ ). However the increase in the slope of  $^{13}\text{C}$  oxidation during the first 45 min of measures induced by the bed rest was significantly higher in protein condition  $+35.9\%$ ,  $0.18 \pm 0.02$  at BDC-4 vs  $0.25 \pm 0.03$  at HDT19;  $p=0.04$ ) as compared to control condition ( $+6.9\%$ ;  $0.18 \pm 0.02$  at BDC-4 vs  $0.20 \pm 0.02$  at HDT19;  $p=0.66$ ; interaction time\*condition:  $p=0.07$ ).



**Figure 38:** Kinetic of instantaneous  $^{13}\text{C}$  acetate recovery in subjects under control ( $n=10$ , White) and protein ( $n=9$ , black) condition at baseline (round) and after 19 days of bed rest (triangle). Time 0 corresponds to the dose ingestion. 7-hours cumulative recovery of the dose is presented at baseline (white) and after 19 days of bed rest (black).

Data are least square means  $\pm$  SEM. Analyzes were performed using linear mixed model and specified hypotheses.

<sup>a</sup> $p<0.05$ , <sup>b</sup> $p<0.01$ , <sup>c</sup> $p<0.001$  vs baseline in control condition

<sup>d</sup> $p<0.05$ , <sup>e</sup> $p<0.01$ , <sup>f</sup> $p<0.001$  vs variation in control condition

<sup>g</sup> $p<0.05$ , <sup>h</sup> $p<0.01$ , <sup>i</sup> $p<0.001$  vs baseline in protein condition

## v. Dietary lipid trafficking

### 1. $d_{31}$ palmitate enrichment and concentration in chylomicrons.

TG in CM kinetics is presented in Figure 39 and  $d_{31}$  palmitate enrichment and concentration in CM\_TG are presented in Figure 40.

In control condition, no changes were found in TG concentration in CM (+14.5%,  $p=0.39$  vs BDC-4) (Figure 40). In control condition, the same conclusions can be made for the concentration over the first and the last hours after the meal with no impact of bed rest in TG in CM (+28.7%,  $p=0.06$  vs BDC-4 and +6.2%,  $p=0.76$  vs BDC-4 resp.)

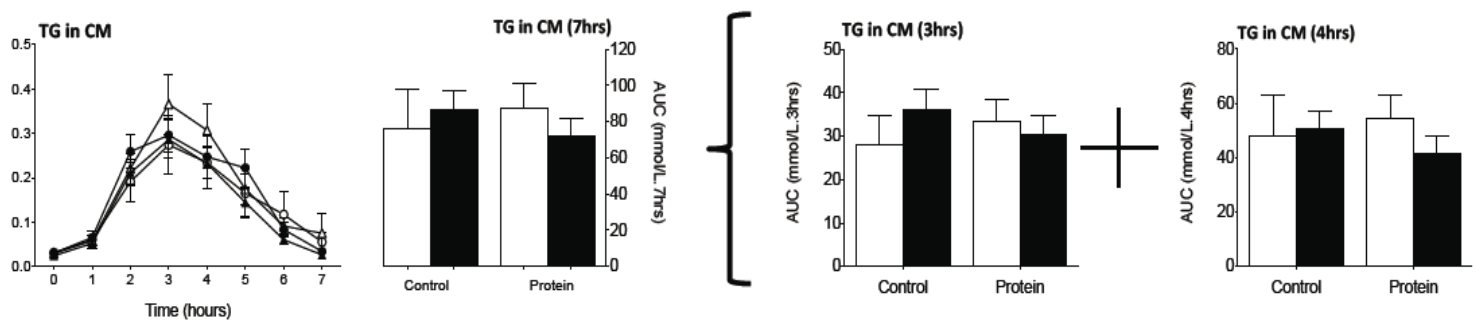


Figure 39: TG in CM kinetics concentrations after a standardized meal

Kinetic of triglycerids in CM is presented after a standardized meal administered at t0 in subjects under control (n=10, white) and protein condition (n=9, black) at baseline (rond) and after 19-days of bed rest (triangle). The right graphs are area under the curve (AUC) of the concentrations of each variable over 7 hours post meal at baseline (white) and after 19 days of bed rest (black). The two last graphs are presenting the AUCs over the first 3 hours and the last four hours of the test.

Data are least square means  $\pm$  SEM.

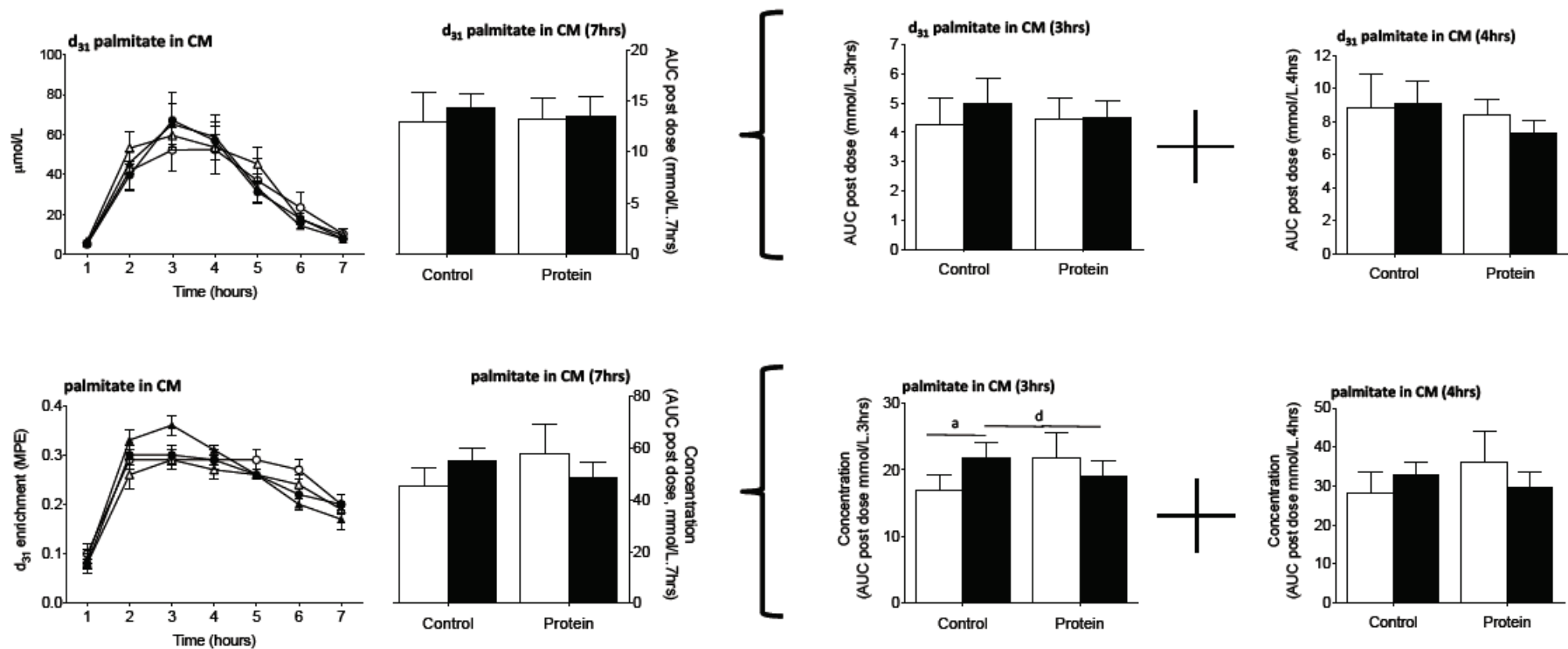
<sup>a</sup> $p<0.05$ , <sup>b</sup> $p<0.01$ , <sup>c</sup> $p<0.001$  vs baseline in control condition

<sup>d</sup> $p<0.05$ , <sup>e</sup> $p<0.01$ , <sup>f</sup> $p<0.001$  vs variation in control condition

<sup>g</sup> $p<0.05$ , <sup>h</sup> $p<0.01$ , <sup>i</sup> $p<0.001$  vs baseline in protein condition

Total isotopic enrichment of CM\_TG in control condition (-4.1%;  $94.6 \pm 1.9$  at BDC-4 vs  $90.7 \pm 3.1$  at HDT19;  $p=0.41$ ) and for total palmitate concentration in CM\_TG did not vary significantly after bed rest (21.2%;  $45.3 \pm 7.2$  mmol/L.7hrs at BDC-4 vs  $54.9 \pm 5.2$  mmol/L.7hrs at HDT19;  $p=0.14$ ). However, during the first hours of the test, palmitate concentration was significantly increased during bed rest in control condition (29.3%;  $16.9 \pm 2.4$  mmol/L.3hrs at BDC-4 vs  $21.8 \pm 2.3$  mmol/L.3hrs at HDT19;  $p=0.02$ ) and protein supplementation did significantly prevent it in the protein condition (interaction time\*condition:  $p=0.05$ ; -12.7% vs  $19.0 \pm 2.4$  mmol/L.3hrs at HDT 19 in protein condition) with a disappearance of bed rest effect in protein condition (vs  $21.8 \pm 3.9$  mmol/L.3hrs at BDC-4;  $p=0.14$ ). No significant variation of palmitate concentration was observed in control condition during the last 4 hours of the test (+16.4%;  $28.4 \pm 5.2$  mmol/L.4hours at BDC-4 vs  $33.0 \pm 3.3$  mmol/L.4hrs at HDT19;  $p=0.47$ )

With no surprise, the rate of  $d_{31}$  palmitate appearance, isotope enrichment and palmitate concentration in CM\_TG is presented in Figure 40. In control condition, bed rest did not have any impact on 7-hours cumulative  $d_{31}$  palmitate appearance in CM\_TG (+10.5%;  $12.9 \pm 2.9$  mmol.L/7hrs at BDC-4 vs  $14.3 \pm 1.4$  mmol.L/7hrs at HDT19;  $p=0.50$ ), but also during the 3 first (+16.9%;  $4.3 \pm 0.9$  mmol.L/3hrs at BDC-4 vs  $5.0 \pm 0.7$  mmol.L/3hrs at HDT19;  $p=0.19$ ) or the last 4 hours of the test (3.1%;  $8.8 \pm 2.1$  mmol.L/4hrs at BDC-4 vs  $9.1 \pm 1.0$  mmol.L/4hrs at HDT19;  $p=0.84$ ).



**Figure 40:**

Kinetics of d<sub>31</sub> palmitate rate of appearance and isotope enrichment in chylomicrons (CM) are presented in subjects under control (n=10, white) and protein condition (n=9, black) at baseline (round) and after 19-days of bed rest (triangle). Total cumulative rate of appearance of d<sub>31</sub> palmitate and unlabeled palmitate concentration in CM are presented as area under the curve (AUC) 7 hours post dose and separated in the 3 first hours and the 4 last one (baseline (white) and after 19-days of bed rest (black)).

Data are least-square means  $\pm$  SEM. Analyses were performed using linear mixed model and specified hypotheses.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

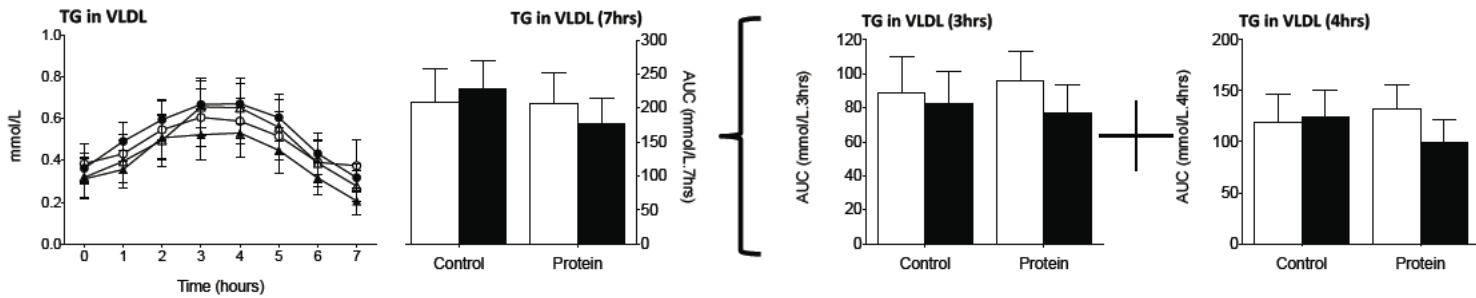
<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition

## 2. $d_{31}$ palmitate enrichment and concentration in VLDL.

TG in VLDL kinetics is presented in Figure 41 and  $d_{31}$  palmitate enrichment and concentration in VLDL\_TG are presented in Figure 42.

In control condition, no changes were found in TG concentration in VLDL\_TG (+9.9%,  $p=0.73$  vs BDC-4). In control condition, the same conclusions can be made for the concentration over the first and the last hours after the meal with no impact of bed rest in TG in VLDL (+8.7%,  $p=0.77$  vs BDC-4 and 10.9%,  $p=0.70$  vs BDC-4).



**Figure 41:** TG in VLDL kinetics concentrations after a standardized meal

Kinetic of triglycerids in VLDL is presented after a standardized meal administered at t0 in subjects under control ( $n=10$ , white) and protein condition ( $n=9$ , black) at baseline (rond) and after 19-days of bed rest (triangle). The right graphs are area under the curve (AUC) of the concentrations of each variable over 7 hours post meal at baseline (white) and after 19 days of bed rest (black). The two last graphs are presenting the AUCs over the first 3 hours and the last four hours of the test.

Data are least square means  $\pm$  SEM.

<sup>a</sup> $p<0.05$ , <sup>b</sup> $p<0.01$ , <sup>c</sup> $p<0.001$  vs baseline in control condition

<sup>d</sup> $p<0.05$ , <sup>e</sup> $p<0.01$ , <sup>f</sup> $p<0.001$  vs variation in control condition

<sup>g</sup> $p<0.05$ , <sup>h</sup> $p<0.01$ , <sup>i</sup> $p<0.001$  vs baseline in protein condition

The rate of  $d_{31}$  palmitate appearance, isotope enrichment and palmitate concentration in CM is presented in Figure 42.

Isotopic enrichment in VLDL\_TG did not vary after bed rest (-4.5%;  $28.5 \pm 1.4$  at BDC-4 vs  $27.2 \pm 1.0$  at HDT19;  $p=0.53$ ) and so did unlabeled palmitate concentration in total (+20.4%;  $151.3 \pm 18.4$  mmol/L.7hrs at BDC-4 vs  $182.2 \pm 17.0$  mmol/L.7hrs at HDT19;  $p=0.053$ ), during the first (+19.5%;  $67.7 \pm 7.8$  mmol/L.3hrs at BDC-4 vs  $80.9 \pm 7.2$  mmol/L.3hrs;  $p=0.79$ ) and last hours of the test (+21.2%;  $83.6 \pm 10.7$  mmol/L.4hrs at BDC-4 vs  $101.4 \pm 9.9$  mmol/L.4hrs at HDT19;  $p=0.21$ ). Protein supplementation had no significant effect on these parameters ( $p>0.30$  for all).

After 19 days of bed rest, the rate of  $d_{31}$  palmitate appearance in VLDL\_TG remained unchanged (+11.0%;  $22.5 \pm 5.0$  mmol/L.7hrs at BDC-4 vs  $27.3 \pm 4.0$  mmol/L.7hrs at HDT19;  $p=0.42$ ). Similarly, no changes were noted during the first (-4.0%;  $2.6 \pm 0.4$  mmol/L.3hrs at BDC-4 vs  $2.5 \pm 0.3$  mmol/L.3hrs at HDT19;  $p=0.79$ ) and last hours of test (+18.4%;  $7.9 \pm 1.1$  mmol/L.4hrs at BDC-4 vs  $9.3 \pm 1.0$  mmol/L.4hrs at HDT19;  $p=0.21$ ).



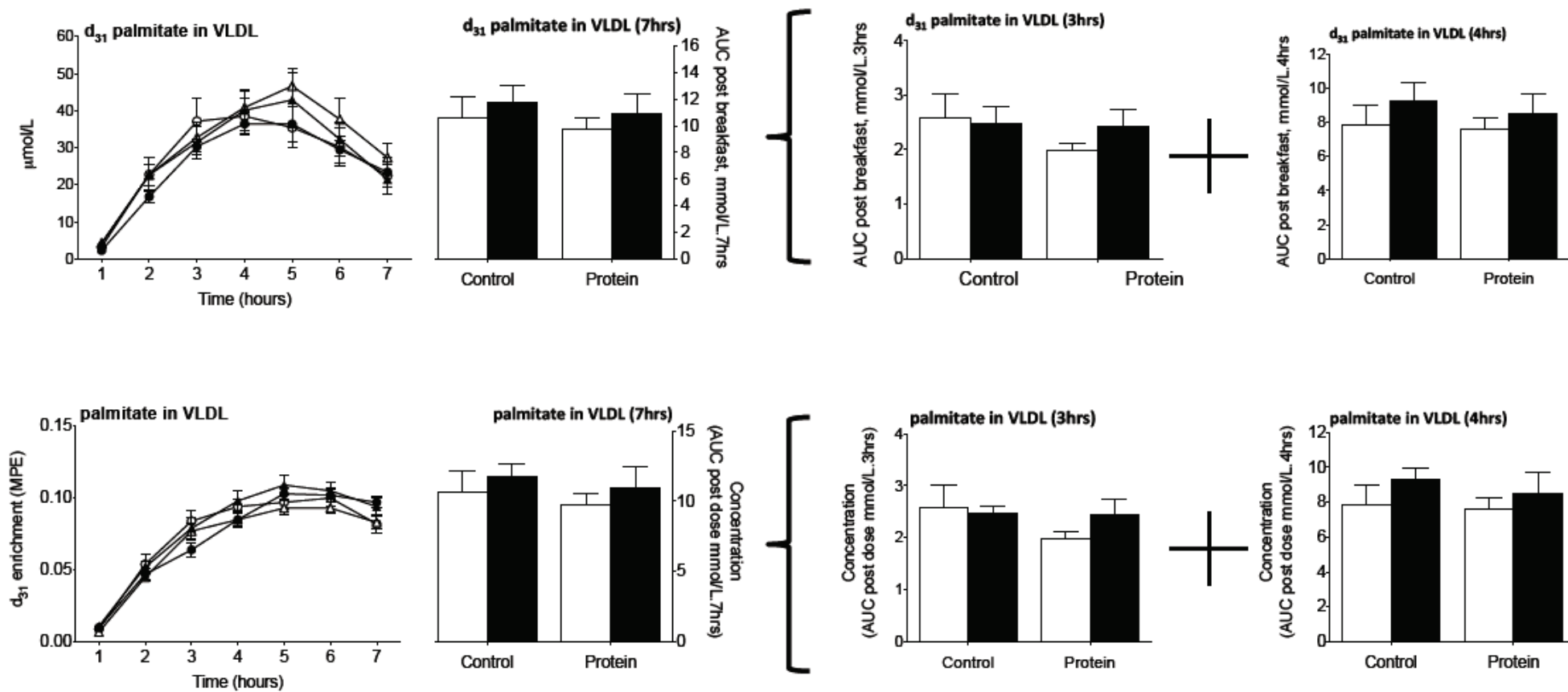


Figure 42:

Kinetics of  $d_{31}$  palmitate rate of appearance and isotope enrichment in VLDL are presented in subjects under control ( $n=10$ , white) and protein condition ( $n=9$ , black) at baseline (round) and after 19-days of bed rest (triangle). Total cumulative rate of appearance of  $d_{31}$  palmitate and unlabeled palmitate concentration in VLDL are presented as area under the curve (AUC) 7 hours post dose and separated in the 3 first hours and the 4 last one (baseline (white) and after 19-days of bed rest (black)).

Data are least-square means  $\pm$  SEM. Analyses were performed using linear mixed model and specified hypotheses.

<sup>a</sup> $p<0.05$ , <sup>b</sup> $p<0.01$ , <sup>c</sup> $p<0.001$  vs baseline in control condition

<sup>d</sup> $p<0.05$ , <sup>e</sup> $p<0.01$ , <sup>f</sup> $p<0.001$  vs variation in control condition

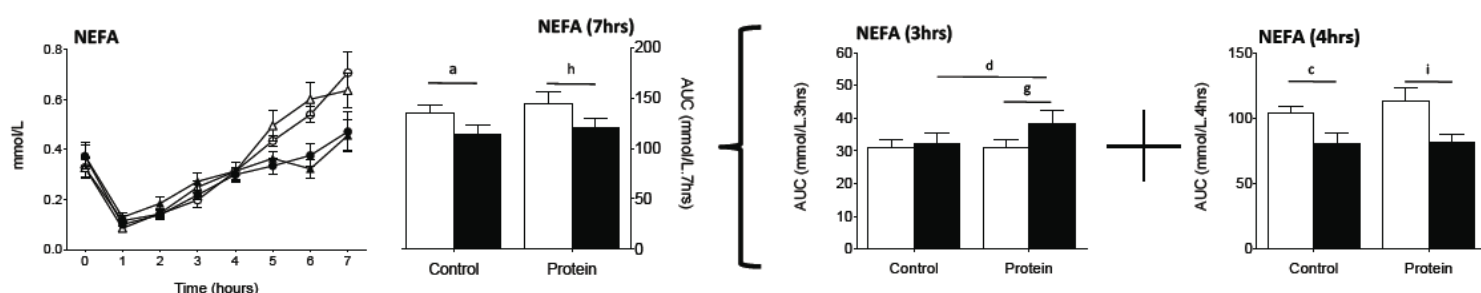
<sup>g</sup> $p<0.05$ , <sup>h</sup> $p<0.01$ , <sup>i</sup> $p<0.001$  vs baseline in protein condition

### 3. $d_{31}$ palmitate enrichment and concentration in NEFA

NEFA kinetics after the meal is presented in Figure 43.

Total NEFA concentrations were significantly decreased in control condition (-16.3%,  $p=0.02$  vs BDC-4) (Figure 43). Protein supplementation did not prevent this change (interaction time\*interaction:  $p=0.49$ ) leading to 17.0% decrease in subjects with protein supplementation ( $p<0.01$  vs BDC-4).

During the first hours following the meal, no variation of NEFA concentration was observed after bed rest in control condition (+3.1%,  $p=0.73$  vs BDC-4). However, protein supplementation did not maintain this concentration (interaction time\*condition:  $p=0.03$ ) by inducing a significant 22.8% increase when combined to bed rest in protein condition ( $p<0.01$  vs BDC-4). At the opposite, during the last hours of the test, NEFA concentration decreased by 22.1% in control condition ( $p<0.01$  vs BDC-4) and protein countermeasure did not prevent this change (interaction time\*condition:  $p=0.99$ ). Indeed, it led to a 27.9% decrease ( $p<0.001$  vs BDC-4) in protein condition.



**Figure 43:** NEFA kinetics concentrations after a standardized meal

Kinetic of triglycerids NEFA is presented after a standardized meal administered at  $t_0$  in subjects under control ( $n=10$ , white) and protein condition ( $n=9$ , black) at baseline (rond) and after 19-days of bed rest (triangle). The right graphs are area under the curve (AUC) of the concentrations of each variable over 7 hours post meal at baseline (white) and after 19 days of bed rest (black). The two last graphs are presenting the AUCs over the first 3 hours and the last four hours of the test.

Data are least square means  $\pm$  SEM.

<sup>a</sup> $p<0.05$ , <sup>b</sup> $p<0.01$ , <sup>c</sup> $p<0.001$  vs baseline in control condition

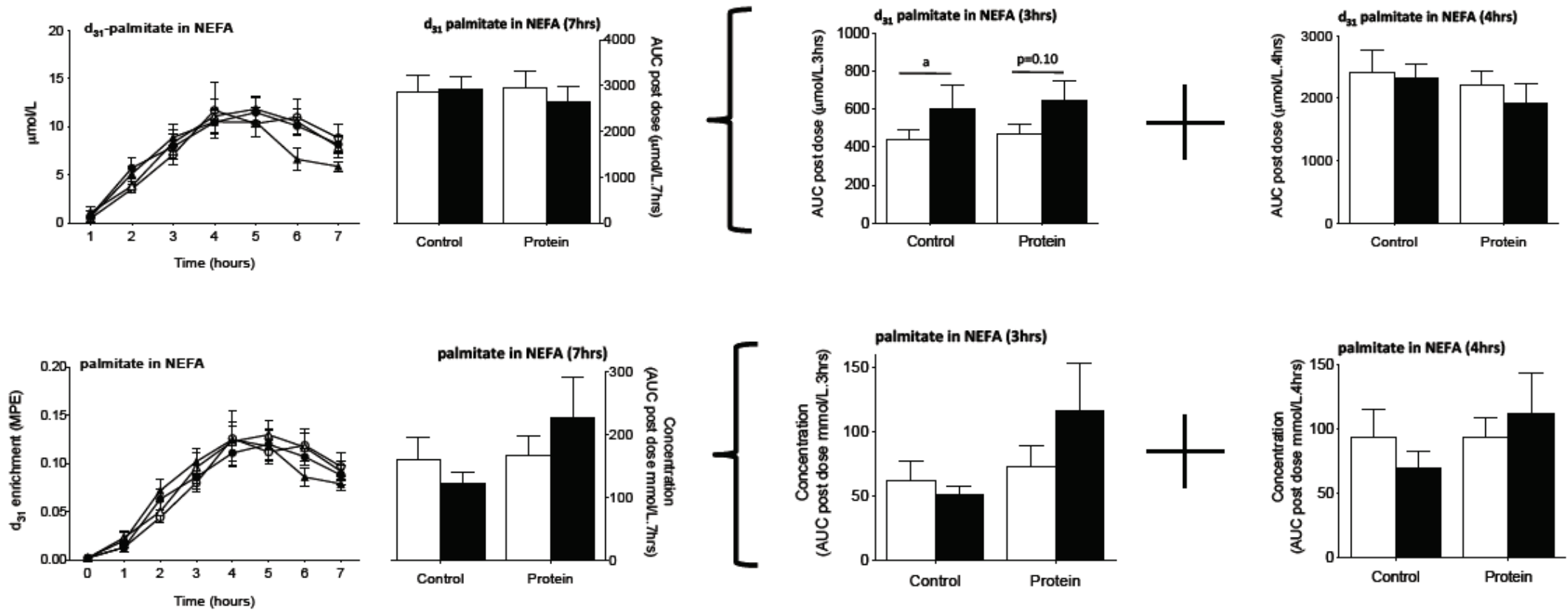
<sup>d</sup> $p<0.05$ , <sup>e</sup> $p<0.01$ , <sup>f</sup> $p<0.001$  vs variation in control condition

<sup>g</sup> $p<0.05$ , <sup>h</sup> $p<0.01$ , <sup>i</sup> $p<0.001$  vs baseline in protein condition

The rate of  $d_{31}$  palmitate appearance, isotope enrichment and palmitate concentrations in NEFA is presented in Figure 44.

After bed rest, total, the first hours and the last hours unlabeled palmitate concentration in NEFA did not vary in control condition. The results were not modified by protein supplementation. Moreover, isotope enrichment in NEFA did not significantly increase during bed rest in the control condition (+2.7%;  $3201.5 \pm 335.8$  at BDC-4 vs  $3288.0 \pm 222.2$  at HDT19;  $p=0.86$ ). Thus, the 7-hours cumulative rate of  $d_{31}$  palmitate appearance in NEFA was not significantly changed during the bed rest

(+2.9%;  $2853.6 \pm 359.3$   $\mu\text{mol/L}$ .7hrs at BDC-4 vs  $2935.6 \pm 269.8$   $\mu\text{mol/L}$ .7hrs;  $p=0.86$ ). However, when looking closely, during the first 3 hours post dose, the appearance of  $d_{31}$  palmitate increased significantly in control condition (+54.3%;  $440.4 \pm 52.5$   $\mu\text{mol/L}$ .3hrs at BDC-4 vs  $679.4 \pm 108.9$   $\mu\text{mol/L}$ .3hrs at HDT19;  $p=0.04$ ). The protein supplementation did not prevent this increase (time\*condition interaction:  $p=0.75$ ; +38.5%;  $466.7 \pm 59.3$   $\mu\text{mol/L}$ .3hrs at BDC-4 vs  $646.4 \pm 104.5$   $\mu\text{mol/L}$ .3hrs at HDT19;  $p=0.10$ ). There were no significant changes in the rate of  $d_{31}$  palmitate appearance during the last hours of the test.



**Figure 44:**

Kinetics of  $d_{31}$  palmitate rate of appearance and isotope enrichment in non-esterified fatty acids (NEFA) are presented in subjects under control (n=10, white) and protein condition (n=9, black) at baseline (rond) and after 19-days of bed rest (triangle). Total cumulative rate of appearance of  $d_{31}$  palmitate and unlabeled palmitate concentration in NEFA are presented as area under the curve (AUC) 7 hours post dose and separated in the 3 first hours and the 4 last one (baseline (white) and after 19-days of bed rest (black)).

Data are least-square means  $\pm$  SEM. Analyzes were performed using linear mixed model and specified hypotheses.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition

## *f. Molecular biology*

Two principal pathways were studied using molecular biology: insulin resistance and lipid trafficking pathway.

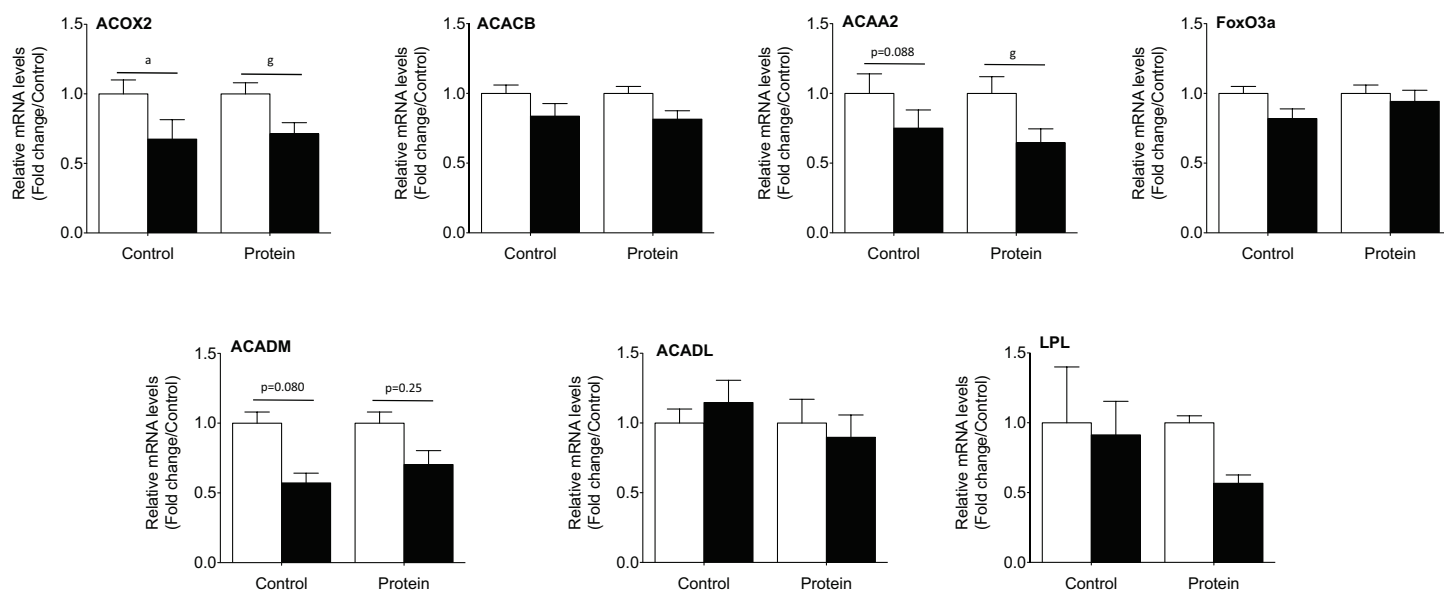
### i. RT-PCR

The variations of each genes expression are represented in Figure 45, 46 and 47. Each gene expression measured at BDC-4 has a fixed value of 1.0 and HDT19 gene expression is expressed relative to BDC value.

The study of lipid trafficking showed a decrease in a few gene expressions using microfluidic RT-PCR. In fact, two enzymes expression, ACADM and ACOX2, respectively involved in medium and long chain fatty acid degradation were impacted by bed rest. After 19 days of bed rest in control condition, ACADM nearly reached significance by decreasing by 30.5% (0.7 at HDT19,  $p=0.08$  vs BDC-4). Protein supplementation did not influence this variation (interaction time\*condition:  $p=0.49$ ). In parallel, ACOX2 expression significantly decreased in both conditions: by 32.5% (0.64 at HDT19,  $p=0.014$  vs BDC-4) in control condition and by 28.7% (0.71 at HDT19,  $p=0.02$  vs BDC-4) in protein condition (Figure 45). In the same way, a trend of decrease of ACAA2 expression, the last enzyme of beta-oxidation, is observed both in control condition (+24.9%; 0.75 at HDT19;  $p=0.09$  vs BDC-4) and in protein condition (+26.7%; 0.73 at HDT19;  $p=0.04$  vs BDC-4)(Figure 45).

Similarly the last enzyme of mitochondrial respiratory chain, COX4l1 was also decreased both in control and protein condition (-29.5%; 0.71 at HDT19;  $p=0.05$  vs BDC-4 and -28.7%; 0.71 at HDT19;  $p=0.03$  vs BDC-4 resp.; interaction time\*condition:  $p=0.95$ )(Figure 46). Mitochondrial biogenesis is usually estimated by the measure of PGC1 $\alpha$  expression. In our study, a trend of decrease in its expression was observed both in the control (+28.9%; 0.71 at HDT19;  $p=0.06$  vs BDC-4) and in the protein condition (+25.6%; 0.74 at HDT19;  $p=0.06$  vs BDC-4; interaction time\*condition:  $p=0.81$ )(Figure 46). No particular impact of bed rest was observed in insulin resistance gene pathway, albeit a slight nearly significant decrease for IRS1 in the control condition (-32.5%; 0.68 at HDT19;  $p=0.075$  vs BDC-4)(Figure 47). Changes in protein condition were no significant (-13.6%; 0.86 at HDT19;  $p=0.39$  vs BDC-4).

Other genes involved in lipid trafficking pathway were not affected after 19 days of bed rest. This absence of variation concerned ACADL, CPT1b, GSK3B, ACACB, FOxO3a and LPL gene expression ( $p>0.08$  for all). In addition, insulin resistance pathways were poorly impacted by bed rest, with no impact on Akt1, PIK3R1, PIK3R2, MAP2K1, MAPK1, PRKCA, GLUT4 and PFKM expression ( $p>0.20$  for all).



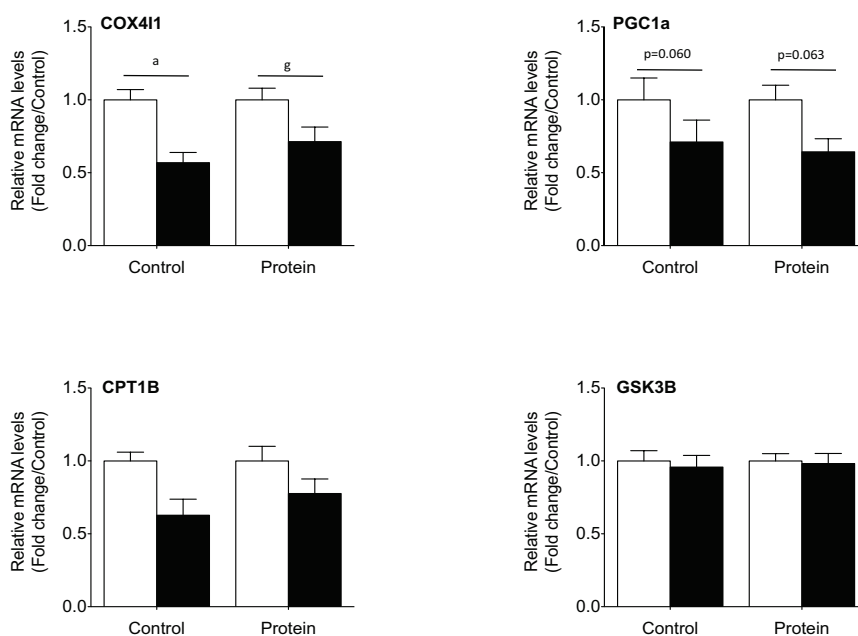
**Figure 45:** mRNA expression of genes involved in lipid oxidation and transport pathway: ACOX2, ACACB, ACAA2, FoxO3a, ACADM, ACADL and LPL.

Data are means  $\pm$  SEM.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition



**Figure 46:** mRNA expression of mitochondrial genes: COX4I1, PGC1a, CPT1B and GSK3B.

Data are means  $\pm$  SEM.

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 vs baseline in control condition

<sup>d</sup>p<0.05, <sup>e</sup>p<0.01, <sup>f</sup>p<0.001 vs variation in control condition

<sup>g</sup>p<0.05, <sup>h</sup>p<0.01, <sup>i</sup>p<0.001 vs baseline in protein condition

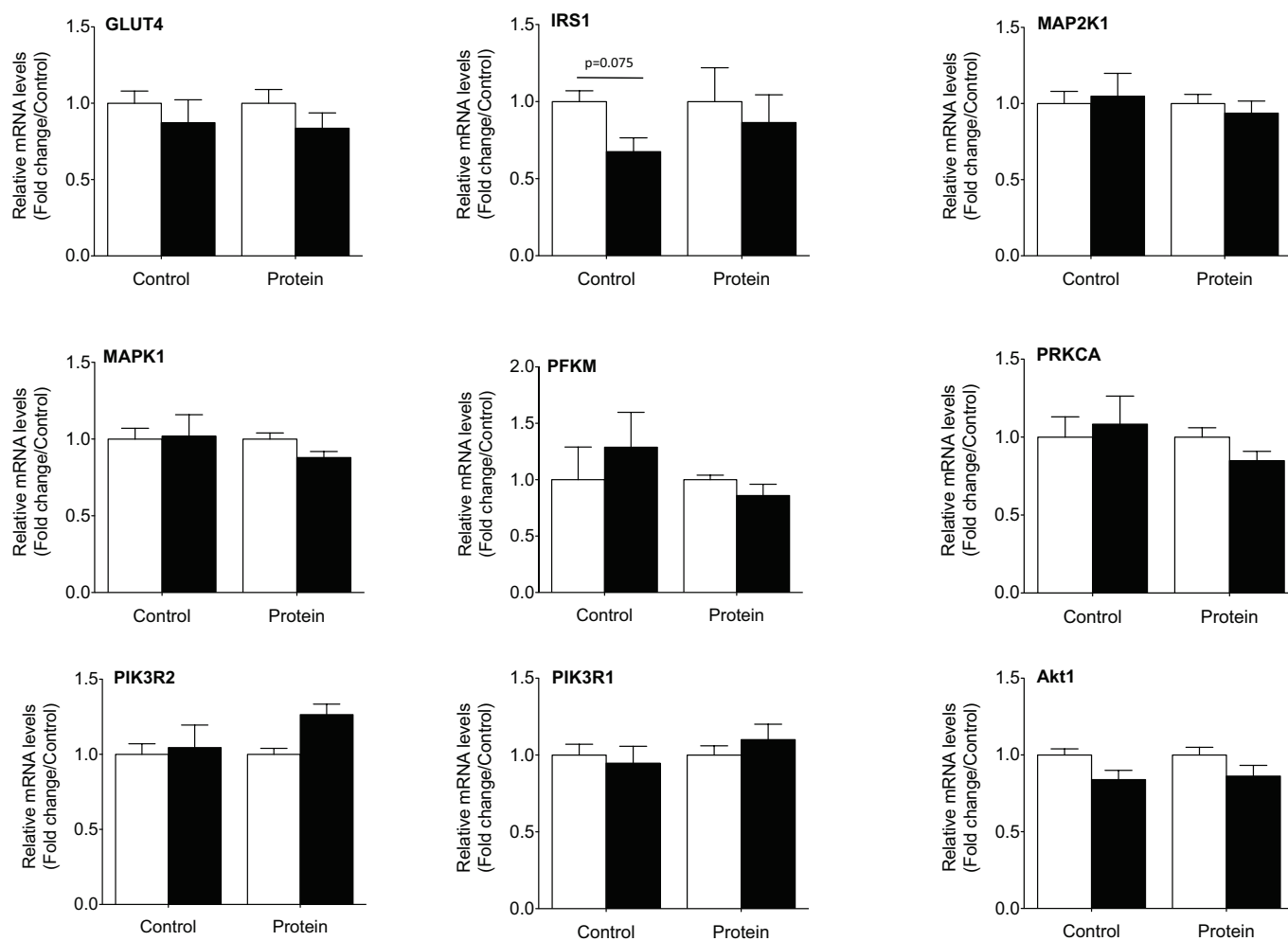


Figure 47: mRNA expression of genes involved in insulin resistance pathway: GLUT4, IRS1, MAP2K1, MAPK1, PFKM, PRKCA, PIK3R2, PIK3R1 and Akt1.

Data are means  $\pm$  SEM.

<sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , <sup>c</sup> $p < 0.001$  vs baseline in control condition

<sup>d</sup> $p < 0.05$ , <sup>e</sup> $p < 0.01$ , <sup>f</sup> $p < 0.001$  vs variation in control condition

<sup>g</sup> $p < 0.05$ , <sup>h</sup> $p < 0.01$ , <sup>i</sup> $p < 0.001$  vs baseline in protein condition



## ii. Western Blot

Each analyzes were performed in each muscle, *vastus lateralis* and *soleus*, in each condition, control and protein, separately. Thus, due to a lack of biopsies for each subject in each condition, we were not able to test the effect of protein supplementation on bed rest effect.

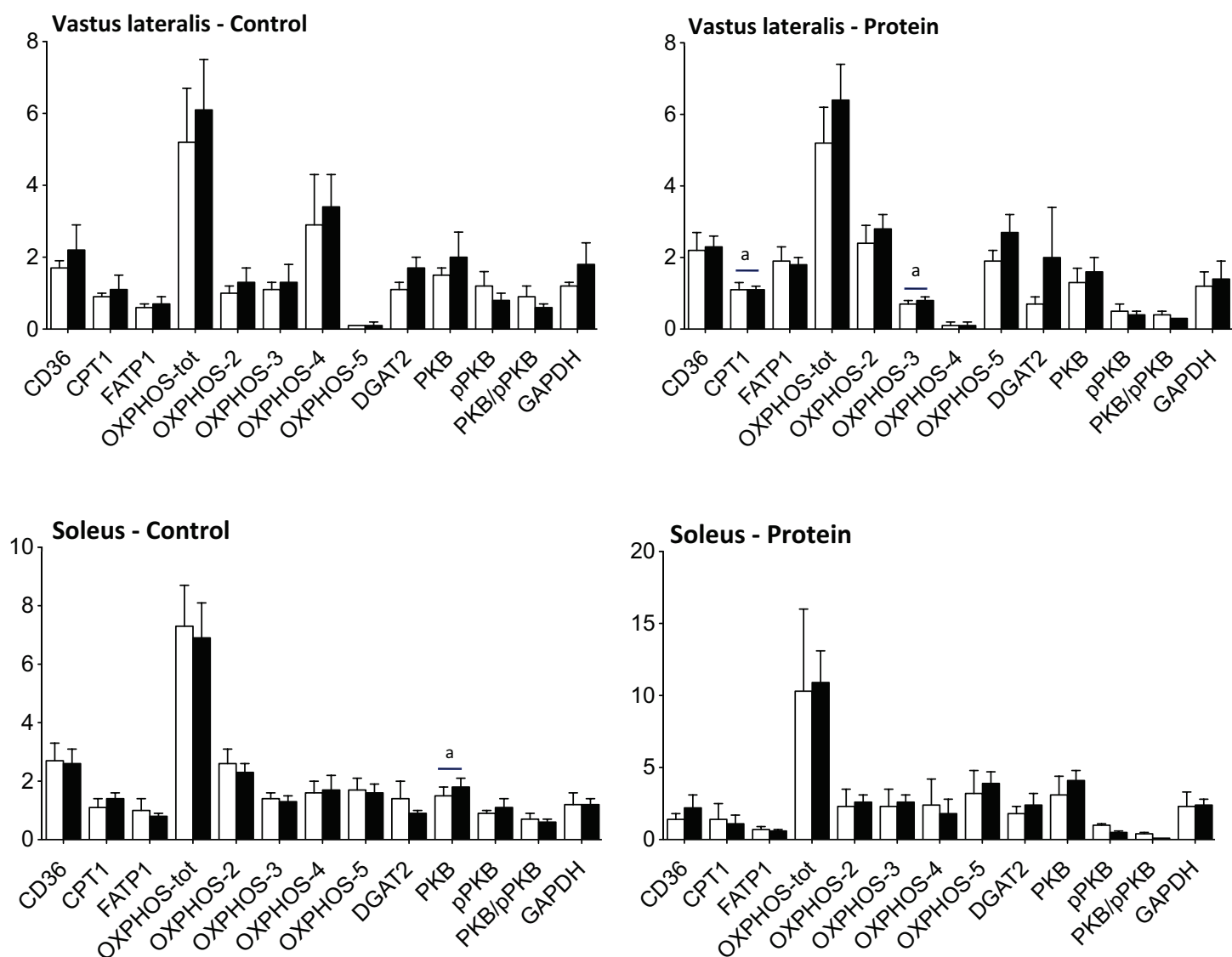
An example of western blot analyze, PKB expression in soleus muscle is presented in Figure 48A. The variation of each protein expression in *vastus lateralis* and *soleus* are presented in Figure 48B.

Bed rest did not have any impact on protein concentration in western blot analysis performed on *vastus lateralis* of subjects in control condition ( $p > 0.10$  for all proteins). In protein condition, only CPT1 and OXPHOS-3 increased significantly ( $+2.1\%$ ,  $1.1 \pm 0.2$  at BDC-4 vs  $1.1 \pm 0.1$  at HDT19;  $p = 0.03$  and  $+17.4\%$ ,  $0.7 \pm 0.1$  at BDC-4 vs  $0.8 \pm 0.1$  at HDT19;  $p = 0.03$ ). However, we cannot see any changes in CD36, CPT1, FATP1, all OXPHOS proteins, DGAT2, PKB and GAPDH protein concentrations ( $p > 0.25$  for all) (Figure 41B).

After 19 days of bed rest, protein concentration remained unchanged in *soleus* in control condition, except a significant increase in PKB ( $+23.4\%$ ,  $1.5 \pm 0.3$  at BDC-4 at  $1.8 \pm 0.3$  at HDT19;  $p = 0.02$ ). In the same way, in protein condition, no particular variations were found in protein expression in *soleus* ( $p > 0.45$  for all).



Figure 48A: Western Blot analyze of PKB expression in soleus muscle in control condition, at baseline (BDC) and after bed rest (HDT). Bed rest significantly impacted PKB expression ( $p = 0.02$ ) .



**Figure 48B:** Protein concentration in *vastus lateralis* and *soleus* under control and protein conditions at baseline (white) and after 19 days of bed rest (black).

Data are least square means  $\pm$  SEM.

<sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , <sup>c</sup> $p < 0.001$  vs baseline in control condition

<sup>g</sup> $p < 0.05$ , <sup>h</sup> $p < 0.01$ , <sup>i</sup> $p < 0.001$  vs baseline in protein condition

### *g. Correlations*

In the control condition, delta correlations between HDT and baseline were performed. A positive correlation was found between insulin sensitivity index in the liver and fasting lipid oxidation ( $R^2=0.551$ ,  $p=0.04$ ), but also with  $d_{31}$  palmitate oxidation ( $R^2=0.615$ ,  $p=0.04$ ).

# Discussion et perspectives

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Our study tested the impact of bed rest on lipid metabolism and whey protein supplementation as countermeasure against the effects of severe physical inactivity induced by simulated microgravity. In previous bed rest studies both in men and women with duration ranging from 7 to 90 days (Alibegovic et al., 2009; Belavy et al., 2010; Bergouignan et al., 2006; Bergouignan et al., 2009; Biensø et al., 2012; Biolo et al., 2008; Blanc et al., 1998; Cree et al., 2010; Heer et al., 2014; Inniss et al., 2009), BM decreased significantly mostly due to a decrease in FFM. In the present study, in control condition, BM decreased and muscle atrophy occurred as highlighted by the drop in legs LBM.

Organ fat storage was also impacted by bed rest. Liver fat content was decreased after bed rest, which is unexpected, as several studies have shown a causal effect of physical inactivity in the development of NAFLD. Indeed, cross-sectional studies have showed a negative influence of physical inactivity on NAFLD patients (Rector et al., 2011), but also, in animals, which presented an increased fatty liver when physical activity is decreased (Linden et al., 2013; Rector et al., 2008). Moreover, human studies usually show a decrease in fatty liver or prevalence of NAFLD when physical activity, resistive or aerobic exercise is increased (Harrison et al., 2007; Krasnoff et al., 2008; Zelber-Sagi, 2011). To our knowledge, liver fat content was never studied during bed rest, however during this thesis we investigated the impact of physical inactivity on hepatic markers, specific of liver inflammation. We demonstrated a significant increase of hepatic markers under severe physical inactivity that was prevented by an increased physical activity (Rudwill et al., 2014). In our results, the decrease in liver fat content could be explained by an energy deficit leading to an increased hepatic oxidation. In fact, total and dietary lipid oxidations were increased after bed rest during the first hours right after the dose and so was acetate oxidation and TCA cycle activity in protein condition. Also, this augmentation of lipid oxidation could rather be due to an increased hepatic oxidation than muscle oxidation, as muscle fat content increased in our study and some genes involved in lipid oxidation were decreased in the muscle. In fact, an impaired lipid metabolism pathway in the muscle could lead to an accumulation of lipids byproducts in the muscle (Cooney et al., 2013). At the opposite of the liver, muscle fat variation in our study is actually supporting previous studies on the effect of physical inactivity on muscle showing that the lack of activity is inducing several changes in muscle metabolism. Indeed, 60 days of bed rest increased muscle fat content in women and showed a positive correlation between intramuscular lipids and bed rest duration. This changes in muscle fat was also negatively correlated with percentage of palmitate oxidation. A decrease in genes involved in lipid oxidation and transport in muscle like LPL, or FAT/CD36, CPT1 and COX4, because of severe physical inactivity was responsible for this increase in muscle fat content (Bergouignan et al., 2011; Bergouignan et al., 2009). Also a few more studies from 28 and 53 days of bed rest showed an increased intramuscular fat content (Belavý et al., 2014; Brooks et al., 2008). Along with this data, Stettler et al. (2005) described an increased intramyocellular fat content when 60h bed rest is combined either with high fat or high carbohydrates diet in healthy

men (Stettler et al., 2005). The accumulation of muscle fat content can also be explained by a shift in fiber type from slow to fast. In fact, fast type of fibers has a decreased lipid oxidation rate compared to slow type of fiber (Grichko et al., 2000; Helge et al., 1999). Nevertheless, in our study, Blottner et al. (2014) showed that the change in fiber was quite questionable (Blottner et al., 2014). Depending on the session, the impact of bed rest was not the same on muscle and the proportion of fast and slow fibers vary importantly in *soleus* and *vastus lateralis*.

According to our results, the variation of lipids storage in different organs traduced a shuffle of lipids during bed rest. However, no hypertriglyceridemia was described after bed rest. This is in contradiction with previous results obtained in women after 60 days of bed rest (Bergouignan et al., 2009), where a hypertriglyceridemia, and an increase in dietary NEFAs were described. Several causes could explain these results, like greater lipids absorption from enterocytes and/or a decreased clearance of TGs. Actually, it appeared that a greater spillover of dietary NEFAs was observed after 60 days of bed rest, probably due to a decreased muscle uptake induced by a drop in muscle LPL expression. In our study, we observed no particular changes in TG concentration, both in CM and more specifically in dietary CM, suggesting no changes in lipids absorption after the meal. This observation is at the opposite of the decrease in dietary CM found in active subjects severely detrained for 1 month (Bergouignan et al., 2013a). A drop in NEFAs concentration was still observed after bed rest and especially the last hours of the test. Based the literature (Bergouignan et al., 2009; Ramos-Roman et al., 2012), we could suggest either an increased NEFA uptake by peripheral tissue and/or a limitation of adipose tissue NEFAs release. In our case, an important increase of insulin concentration was observed the last hours of the test, and insulin is known to limit NEFAs release by adipose tissue and increase muscle NEFA uptake. As a matter-of-fact, Ramos-Roman et al. (2012) have suggested an increased peripheral tissue uptake, by the liver or muscle for example, and a limitation of adipose tissue lipolysis, along with augmented insulin secretion (Ramos-Roman et al., 2012). Furthermore, LPL expression in muscle did not change, which do not suggest any variation in of NEFA uptake by muscle. Bed rest did not impact dietary NEFAs except an increase described during the first hours right after the meal. NEFAs are also the principal origin of VLDL synthesis through the liver. Even though NEFAs concentration decreased after bed rest, VLDL concentration did not vary. As dietary NEFAs was not changed after bed rest, we did not observe an impact on dietary VLDL concentration. The same observation was also made in active subjects detrained for 1 month (Bergouignan et al., 2013a). To explain this absence of variation, as liver fat content decreased, it could be due to a higher liver activity, and by that a higher VLDL production that maintained the VLDL concentration in plasma. Indeed, *de novo* lipogenesis is the second most important source of VLDL after NEFA during fed state (Barrows et al., 2006). Finally, with no surprise, severe physical inactivity did not particularly impact HDL and LDL concentration either. To resume, the variations in lipid trafficking in our study revealed the implication of muscle and liver at different levels in lipid metabolism.

During severe physical inactivity, the development of an insulin resistance has been described in different bed rest studies from 60h to 60 days in both men and women (Bergouignan et al., 2009; Stettler et al., 2005) (Alibegovic et al., 2009; Biensø et al., 2012; Cree et al., 2010; Mazzucco et al., 2010). However in our study, neither the evolution of glucose and insulin throughout the time course of the study, nor the results from the OGGT did allow highlighting changes in glucose tolerance. In our study, as no changes were found in  $d_7$  glucose oxidation after 21-days of bed rest, we conclude that whole-body insulin sensitivity is unlikely modified during the bed rest. Nevertheless, as glucose oxidation was decreased importantly, we still suspect changes in glucose homeostasis that need to be highlighted. A comparable 21-days bed rest study, also organized at the DLR facility in Germany, showed a decrease of the whole-body insulin sensitivity of their subjects, but not hepatic insulin sensitivity (Heer et al., 2014). The absence of impaired insulin sensitivity in our study is however probably not due to the bed rest duration. In fact, a shorter bed rest of 60h already showed a decrease in insulin sensitivity (Stettler et al., 2005). The same observation was found after 9 days of severe physical inactivity in subjects who had at least one parent with type 2 diabetes and their control (Alibegovic et al., 2009). These subjects also became insulin resistant probably due to a decrease of activity and GLUT4 expression in muscle leading to a decreased storage of glucose as glycogen. On the contrary, in our study, expression of genes involved in insulin resistance pathway or protein concentration was not decreased, except a slight decrease in IRS1. Muscle did not show a sign of insulin resistance as presented in bed rest studies from 7 days to 60 days in both men and women (Bergouignan et al., 2009; Biensø et al., 2012; Mazzucco et al., 2010). The presented studies also showed the settlement of insulin resistance and decreased insulin sensitivity in peripheral organs, like in the muscle (Bergouignan et al., 2009; Cree et al., 2010) or the liver (Alibegovic et al., 2009). No variation of fasting glucose concentration were described in our study, so by deduction, insulin sensitivity of the liver was not impacted. We corroborated this hypothesis with the calculation of liver insulin sensitivity using OGTT glucose and insulin concentration based on the equations from Abdul-Ghani et al. 2007. Indeed, severe physical inactivity did not decrease liver insulin sensitivity as expected and neither did muscle insulin sensitivity (Abdul-Ghani et al., 2007). Finally, we completed the investigation on lipid metabolism and insulin sensitivity with inflammation measures. After bed rest, no particular changes in oxidative stress or hepatic plasma markers were described. These results are in total opposition with the studies presented in the first and second chapter. In fact, we showed a specific impact of severe physical inactivity on visfatin and several hepatic markers, including liver aminotransferase (Rudwill et al., 2014). Several studies have also presented the development of oxidative stress after simulated microgravity (Margaritis et al., 2009; Powers et al., 2012; Roth, 2007). In the same way, in bed rest, inflammation markers have been showed to increase even after short duration. Indeed, Hojbjerg et al. (2011) showed an increase of C-reactiv protein, IL-6, MCP-1, IL-10 and TNF $\alpha$  in insulin resistant patients after only 10 days of bed rest (Hojbjerg et al., 2011). In the



same way, after 14 days of bed rest, C-reactiv protein and pentraxin 3 have increased (Bosutti et al., 2008). Also Drummond et al. (2013) showed an increase of IL6 and TLR4 expression in muscle after only 7 days of bed rest in elderly patients (Drummond et al., 2013). To conclude, as presented in these studies and the introduction part, the development of metabolic alterations in a context of simulated microgravity will enhance the development of low-grade inflammation. Thus we were expecting changes in inflammation, but as our subject did not clearly present metabolic alterations, the absence of changes in inflammation markers follows our actual results.

The second part of our study consisted in the impact of whey protein supplementation on bed rest effect. Only a few impacts of the nutritional countermeasure were described. Indeed, protein supplementation had an impact on bed rest effect on protein oxidation, by increasing it. And secondly, in protein condition, we also showed an increase in glucose concentration after bed rest. A recent study, on a 14-d bed rest showed a significant increase in protein oxidation after severe physical inactivity in subjects supplemented with whey proteins (Stein et al., 2003). This increase was even greater than casein supplementation, explained by the rapid digestion of essential amino acids contained in whey proteins. This follows our results, as a significant increase in protein oxidation was already found the first hours right after the meal ingestion in the supplemented participants. The impact of whey protein supplementation on glucose oxidation has not been particularly studied. Nevertheless, as whey protein intake has been shown to increase insulin secretion; it should decrease glucose concentration (Turner et al., 2015). However, the opposite was clearly observed in our study, due to a decreased glucose oxidation, that could not maintain glucose concentration. One explanation for these changes is involving the isocaloric replacement of fat by protein intake during bed rest in protein condition. Protein supplementation being eucaloric, it became needed for energy. In fact, Tappy et al. (1992) showed that amino acids infusion induced an increase in glucose concentration, due to a decreased glucose oxidation (Tappy et al., 1994). They concluded that the principal way of amino acids degradation was through neoglucogenesis.

The absence of whey protein supplementation effect could also be due to the isocaloric replacement of fat by proteins. Indeed, in the literature compared to hypercaloric diets, isocaloric diets have been shown to prevent the development of metabolic impairments. For example, Smith et al. (2013) showed that even after 1 day of overfeeding, men are already presenting an increased insulin resistance and glucose rate of appearance compared to an isocaloric feeding (Smith et al., 2013b). However, no changes in VLDL-TG, NEFA or beta-hydroxybutyrate concentration were found. An increase in diet intake also increased TNF-related apoptosis ligand, compared to eucaloric diet and hypocaloric diet (Biolo et al., 2012). They also showed no changes in FM and leptin concentration in participants with eucaloric diet.

Furthermore, Weigle et al. (2005) still showed an increased insulin secretion and thus satiety with an isocaloric replacement of fat by proteins (from 15% to 30%) (Weigle et al., 2005). Not all studies are showing differences between isocaloric and hypercaloric diet depending if fat or carbohydrates are increased. Indeed, Adochio et al. (2009) showed an increase of insulin, free-fatty acids and TG concentration only in subjects fed with high carbohydrates diet (60%), compared to a high fat diet (50%) and isocaloric diet (Adochio et al., 2009). In the same way, Bortolotti et al. (2009) presented interesting results when subjects are fed with a high fat-high carbohydrates diet (26% fat, 40% carbohydrates and 24% proteins) (Bortolotti et al., 2011). In fact, compared to isocaloric diet, a decrease in NEFA, lipid and protein oxidation were described. However, intrahepatocyte lipids still increased. Based on these data, as whey protein supplementation did not change calorie intake in our study, we are in line with the prevention effect of isocaloric calorie replacement on metabolic impairment.

In the introduction section, we showed the interesting effect of whey protein supplementation on several different metabolic disorders: insulin resistance development (Pal et al., 2010), ectopic storage of lipids and particularly in the liver (Bortolotti et al., 2011), hypertriglyceridemia (Bortolotti et al., 2011) and muscle atrophy (Volpi et al., 2003). However, the questionable effect of this nutritional countermeasure on muscle atrophy raises the subject of combining or not whey protein supplementation and exercise. In fact, in our study, muscle atrophy, evaluated with legs LBM, was not prevented by the nutritional countermeasure. In the same way, muscle fat content, and also genes expression decreases were not prevented either. The lack of muscle activity could be one reason for explaining these results.

In our study, we described a decrease of glucose oxidation in favor of lipid oxidation that was observed in both conditions, regardless to the whey protein supplementation. This suggests that an outside parameter could have influenced the absence of bed rest effect in most of the metabolic variables and induced new ones in the subjects in both conditions. As specified in the methodology part, the actual recommendations of protein intake are of 0.8g/kg/day. Nevertheless, in the literature, it has been suggested that the quantity is not sufficient to fill the protein requirements, especially in the case of real or simulated microgravity (Humayun et al., 2007). In fact, according to a recent paper written by Stein et Blanc (2011), not all studies testing an increased protein diet, showed a significant impact on metabolic variables (Stein et al., 2011). Out of three studies, it came out that when subjects have a baseline protein intake of 1.2g/kg/day, protein supplementation has no supplemental benefit, because they already reached their protein requirements. Our results follow this hypothesis, with a protein intake of 1.2g/kg/day at baseline period and in the control condition during bed rest period, and up to 1.8g/kg/day in the protein condition, with a supplementation in whey proteins. In fact, beside the effect of protein supplementation in total proteins oxidation and glucose concentration, no other changes were described. A point must still be made upon the baseline protein intake of 1.2g/kg/day. In fact, an improvement of liver fat content has been made in

our subjects under severe physical inactivity, even though we know that sedentary lifestyle can aggravate the development of liver steatosis (Rector et al., 2011). Nutrition could be the key to the prevention of metabolic impairments. Earlier in the discussion, we suggested an increased hepatic fat oxidation. In fact, it has been described that a higher protein intake can provoke an increase in lipid oxidation and a decrease in glucose oxidation (de Wit, 1998; Stepien et al., 2010) as observed in our results. Based on de Wit et al. (2012), the assimilation by the liver of protein supplemented requires an increase of hepatic lipid oxidation, to provide more energy (de Wit, 1998). The augmented oxidation of lipids in the liver will lead to a decrease in liver fat content. Moreover, the impaired glucose oxidation could also be due to an accumulation of byproducts of lipids that suppress key steps of this pathway (Newgard, 2012). In fact, this sudden increase in lipids flux will lead to a higher production of catabolic intermediates in the liver and the muscle that can disturb glucose and insulin action. In final, it could lead to a decrease in insulin sensitivity. Our results follow that hypothesis with the increase of lipid oxidation, during the first hours after the meal, leading to a decrease in glucose oxidation. As a result, we saw an increase of both glucose and insulin concentrations the last hours of test. To conclude on this part, the isocaloric replacement of fat by protein supplementation probably prevented the effect of bed rest. Furthermore, we also follow the hypothesis proposed by Stein et Blanc (2011) that suggested an absence of bed rest effect when baseline protein requirement is reached (Stein et al., 2011). We still observed a possible settlement of impaired insulin sensitivity, with an increased hepatic lipid oxidation leading to a decreased glucose oxidation and an accumulation of lipid intermediates in the liver.

To explain these unexpected results, a point attracted our attention and could explain the absence of bed rest effect. As presented in the paper in Buehlmeier et al. 2014, physical activity level of the subjects was evaluated using steps count (Buehlmeier et al., 2014). In average, in free-living condition, the subjects walked approximately 10000 steps per day. During baseline period, due to the confinement, the subjects dropped to 3200 steps per day in average. Based on Tudor-Locke et al. 2004, the subjects can be classified as sedentary during baseline period compared to active during free-living period (Tudor-Locke et al., 2004). This important drop in physical activity between the two stages of the study could be seen as a preconditioning to the bed rest following baseline period. Moreover, the decrease between free-living and baseline period is even more important than the one between baseline and bed rest period, suggesting that the adaptation to baseline period would have more impact than the adaptation to bed rest. Moreover as presented in Table 6, our subjects had a %FM more important than previous studies. In fact, in average they had 24% FM compared to 17.5% in average in other studies. Along with the preconditioning to physical inactivity and the less important fitness of the subjects, their response to bed rest could have been minimized or even avoided. To corroborate this hypothesis, RMR measurements during the whole baseline period show a decline for the whole 7-

days pre bed rest period. Effectively, the first measure made at BDC-7, when the subjects arrived at the facility, showed a RMR of 7.06MJ/d compared to 6.56MJ/d at BDC-4.

To explain these unexpected results in our bed rest study, we compared the characteristics of our study with previous European bed rest. In that context, a difference needs to be underlined: the variation of energy balance. Over a bed rest study, energy balance variation can be calculated based on FM and FFM loss. Assuming that the energy equivalent of fat mass and fat free mass were respectively 38.9MJ/kg and 23.5MJ/kg (Tremblay et al., 1992), we were able to calculate the energy balance in MJ for the whole study. Then, we compared it to previous bed rest studies. To normalize the comparison all energy balance were adjusted over 30 days (Table 6). All studies are presenting a negative energy balance after bed rest, but our study is presenting one of the most important deficits when standardized to 30 days (-50.59MJ), but with an almost equivalent loss in FM and FFM (-0.80kg vs -0.83kg). As the energy equivalent of FM is higher than FFM, the contribution of FM in energy balance variation is more important than FFM (-61.5% vs -38.5%). At the opposite, in other studies, FFM was the principal contributor to energy balance decrease, except for one study but the energy balance remained almost unchanged (Biolo et al., 2007). To complete these observations in energy balance, we calculated the food quotient (FQ) for each subjects, based on Westerterp (1993) work, and compared it to respiratory quotient (RQ) (Westerterp, 1993). In fact, he assumed that when FQ is higher than RQ, it traduced an increase of fat oxidation into energy, whereas when RQ is higher than FQ, proteins or carbohydrates are transformed in body fat. We presented the values in Figure 49. As we can see, in both baseline periods in control and protein conditions, FQ was higher than RQ, suggesting that our subjects were burning fat, probably due to a decreased fat intake in comparison with the free-living period (30% vs 34% respectively). This also shows that our subjects were not adapted to their food consumption during BDC period. During HDT period, FQ was quite equivalent to RQ, but was still a little less important than RQ. According to Westerterp, this suggest that we nearly reached the perfect balance, where neither glycogen or protein storage were used to produce energy. This FQ/RQ comparison during HDT period shows that during bed rest the subjects were perfectly adapted to food intake. Out of this ascertainment, we might suggest that a thigh control of energy balance with the control of energy intake could prevent the effect of severe physical inactivity.

As a conclusion, even though the results obtained during this bed rest study were not expected, they raise a lot of interesting and positive questions on underlying mechanisms involved in metabolic disorders, but also on the study design of bed rest studies.

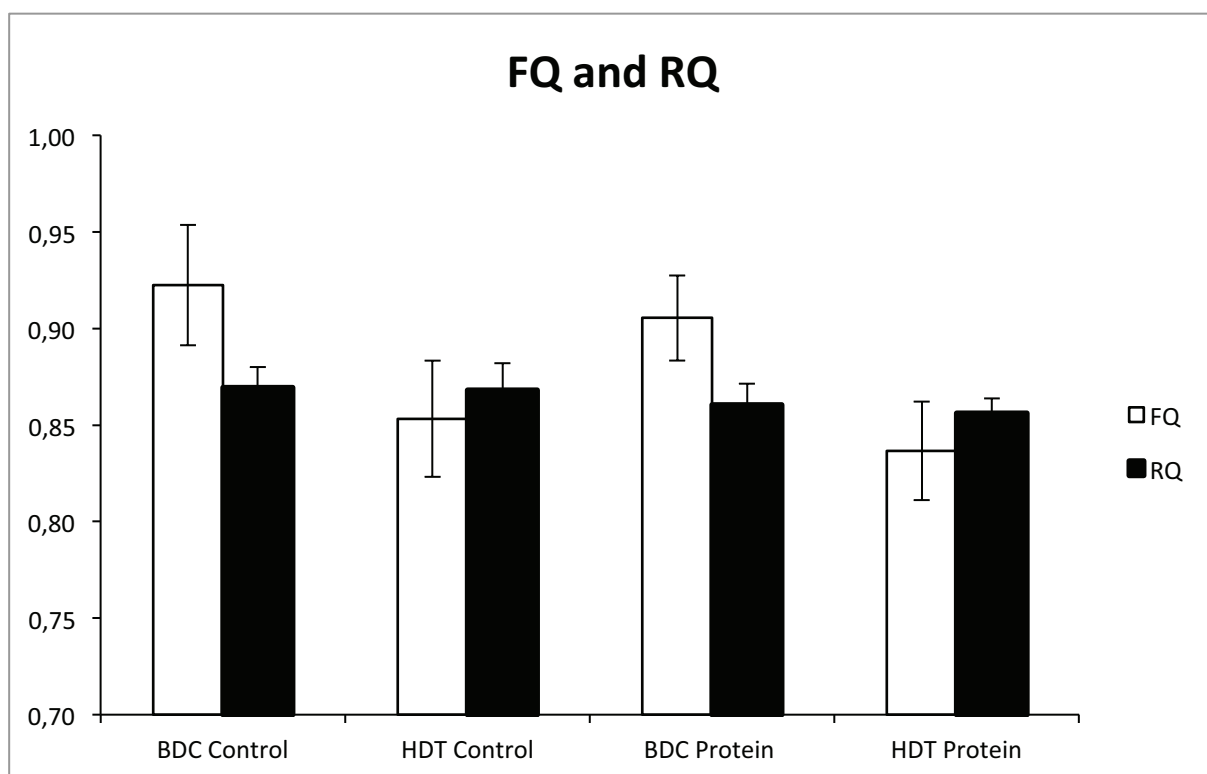


Figure 49: Food quotient (FQ, white) and Respiratory quotient (RQ, black) in baseline (BDC) and bed rest (HDT) period.

<b>Studies</b>	<b>Duration</b>	<b>%FM (Baseline)</b>	<b>EB/day (Control group)</b>	<b>FM/30d</b>	<b>FFM/30d</b>	<b>EB/30d</b>	<b>FM Contribution/30d</b>	<b>%FM contribution</b>
<b>Present study</b>	<b>21-d</b>	<b>24.0%</b>	<b>Negative (-1.69 MJ/d)</b>	<b>-0.80kg</b>	<b>-0.83kg</b>	<b>-50.59MJ</b>	<b>-31.12MJ</b>	<b>-61.5%</b>
Bergouignan et al. (2006)	90-d	18.6%	Negative (-0.2 MJ/d)	-0.13kg	-0.80kg	-23.99MJ	-5.19MJ	-21.6%
Blanc et al. (1998)	42-d	17.8%	Negative (-1.7 MJ/d)	-0.14kg	-1.93kg	-50.88MJ	-5.56MJ	-10.9%
Biolo et al. (2008)	35-d	16.1%	Negative (-0.15 MJ/d)	1.8kg	-3.17kg	-4.51MJ	70.02MJ	+48.4%
Heer et al. (2014)	21-d	/	Negative (-0.73 MJ/d)	+1.86kg	-4.00kg	-21.76MJ	72.24MJ	+43.4%
Biolo et al. (2007)	14-d	16.1%	Negative (-0.11 MJ/d)	-0.21kg	+0.21kg	-3.30MJ	-3.89MJ	-63.6%
Blanc et al. (2000)	6-d	17.9%	Negative (-1.61MJ/d)	+4.5kg	-9.5kg	-48.2MJ	175.05MJ	+43.9%

Table 6: Energy balance variations in 6 different bed rest studies in men compared to the present study. We presented % of fat mass (FM) in the subjects at baseline, energy balance (EB)/day in the control group, fat mass (FM), fat free mass (FFM) and energy balance (EB) over 30 days and fat mass (FM) contribution over 30 days (MJ) and finally the percentage of fat mass (FM) contribution.

## Perspectives

The unexpected results described after 21 days of bed rest in our study, allowed us to propose several perspectives for future studies in simulated microgravity.

First of all, some complementary analyzes would permit us to better understand our results. Firstly, hepatic lipid oxidation is increased after bed rest in our study with a baseline protein intake of 1.2g/kg/day. To confirm this hypothesis, we are going to measure beta-hydroxybutyrate concentration. In fact, lipid oxidation through the liver is producing two different products: carbon dioxide and beta-hydroxybutyrate. As liver fat content was decreased in our study, we suggested an increase in hepatic oxidation. We will be able to measure the proportion of dietary lipids that has been oxidized through the liver, by measuring deuterium enrichment in beta-hydroxybutyrate fractions. It will give us the proportion of lipids oxidized that came from endogenous lipids stored in the liver or from dietary origin.

Secondly, to complete the study of lipid trafficking, we are currently developing a technique to measure the proportion of dietary lipids captured by muscle. Lipid oxidation pathway is involving several steps in the degradation process. It can lead to an accumulation of byproducts during the oxidation, due to a defect in important enzymes expression and/or to an increased lipids uptake. Thus, ectopic fat storage in the muscle will happen. Using this technique we will measure the dietary lipids proportion in different lipid intermediates: diacylglycerols, NEFA or ceramides. These analyzes will give us the opportunity to precise the question of lipid metabolism in the muscle and if bed rest under the described conditions is responsible for an accumulation of lipids oxidation byproducts in the muscle.

Finally, metabolomics in urine will give us an overview from the whole metabolism. Indeed, metabolites are the intermediates and product of the metabolism, and by comparing analyzes before and after bed rest, will probably help us better understand our results.

As we showed in our study, whey protein supplementation had no particular impact on metabolic variables variation. However, the question of a prevention of bed rest effect with protein supplementation still remains. In fact, the only variations observed and induced by severe physical inactivity were not prevented by whey protein supplementation. Actually, protein supplementation even induced changes in metabolic variables, like protein oxidation or glucose concentration and oxidation. Nevertheless, in a context of simulated microgravity, it looks like baseline protein intake up to 1.2g/kg/day participated to the prevention of metabolic disorders development, like an impaired lipid metabolism, inflammation or insulin resistance. However, a hypothesis on the impairment of insulin sensitivity as a consequence of higher protein intake was proposed. In that way, future studies should also focus on the prevention of insulin sensitivity impairment. Furthermore, in our study even though the participants had an increase in lipid oxidation and in glucose concentration, we did not really observe impaired insulin sensitivity. It could be due



to the medium duration of the study. Thus it might be interesting to test the same countermeasure and baseline protein intake on insulin sensitivity in a longer bed rest study. Other suggestions could be made out of our results. As whey protein supplementation and alkaline salts had no particular impact, other nutritional countermeasure should also be tested, with the same baseline protein intake.

Finally, regarding future bed rest studies, a point deserves to be considered: maintaining physical activity level of the participant during baseline period. Indeed, it could prevent the possible preconditioning to physical inactivity before bed rest. One solution could be to give pedometers to the subjects and a number of steps per day to reach during the baseline period. The value will be based on free-living steps number. More parameters could also be measured during free-living period to compare them to baseline period. For example, along with steps count, we might use accelerometers to better evaluate physical activity during free-living period. Also, RMR measures would also be informative before and during the study. By that we will be able to affirm the presence or absence of preconditioning during baseline period.

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*Annexe 1 : Resveratrol prevents the wasting disorders of mechanical unloading by acting as a physical exercise mimetic in the rat*

## Resveratrol prevents the wasting disorders of mechanical unloading by acting as a physical exercise mimetic in the rat

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**ABSTRACT** Long-term spaceflight induces hypokinesia and hypodynamia, which, along microgravity *per se*, result in a number of significant physiological alterations, such as muscle atrophy, force reduction, insulin resistance, substrate use shift from fats to carbohydrates, and bone loss. Each of these adaptations could turn to serious health deterioration during the long-term spaceflight needed for planetary exploration. We hypothesized that resveratrol (RES), a natural polyphenol, could be used as a nutritional countermeasure to prevent muscle metabolic and bone adaptations to 15 d of rat hindlimb unloading. RES treatment maintained a net protein balance, soleus muscle mass, and soleus muscle maximal force contraction. RES also fully maintained soleus mitochondrial capacity to oxidize palmitoyl-carnitine and reversed the decrease of the glutathione *vs.* glutathione disulfide ratio, a biomarker of oxidative stress. At the molecular level, the protein content of Sirt-1 and COXIV in soleus muscle was also preserved. RES further protected whole-body insulin sensitivity and lipid trafficking and oxidation, and this was likely associated with the maintained expression of FAT/CD36, CPT-1, and peroxisome proliferator-activated receptor- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) in muscle. Finally, chronic RES supplementation maintained the bone mineral density and strength of the femur. For the first time, we report a simple countermeasure that prevents the deleterious adaptations of the major physiological functions affected by mechanical unloading. RES could thus be envisaged as a nutritional countermeasure for spaceflight but remains to be tested in humans.—Momken, I., Stevens, L., Bergouignan, A., Desplanches, D., Rudwill, F., Chery, I., Zahariev, A., Zahn, S., Stein, T. P., Sebedio, J. L., Pujos-Guillot, E.,

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**Key Words:** sedentary behavior • polyphenol • spaceflight • weightlessness

THE SCIENTIFIC RESULTS ACCUMULATED from the 40-yr human spaceflight experience, including both short- and medium-duration missions, have demonstrated that spaceflight has a dramatic effect on almost all physiological systems. Tension on the weight-bearing components of the musculoskeletal system is greatly reduced, as is the work required for movement. The body responds to this muscle disuse by a remodeling of the musculoskeletal system that has been found on all flight missions with humans and with all ground-based models, *i.e.*, bed rest in humans and hindlimb unloading in rats (1, 2). The major sites of the losses are the muscles and bones with antigravity functions located in the trunk and legs. Bone mineral density (BMD) is lost, and fracture risk increases (1, 3). The loss of muscle mass is responsible, at least in part, for the decrease in

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muscle strength observed after spaceflight and is accompanied by a partial and progressive shift from slow-twitch oxidative to fast-twitch glycolytic phenotypes and decreased resistance to fatigue (4–6). Another consequence of disuse consists of metabolic stress, including the development of resistance to the effects of insulin along with moderate hypercortisolemia (4). Microgravity exposure during spaceflight thus affects crew health and performance and interferes not only with the overall success of the mission but also with a healthy return to Earth.

The human spaceflight program has now entered the next phase of space exploration, toward the moon and Mars, and there are clearly inherent medical challenges with such a goal, among which countermeasure development is a clear priority. In fact, since the beginning of human spaceflight, numerous countermeasures have been tested to mitigate the maladaptive changes associated with prolonged spaceflight. These have included drugs, nutrition, physical exercise on various workout devices, and others (7–10). So far, however, none of them has proven to be fully effective.

In recent years, the biological effects of resveratrol (RES; 3,4',5-trihydroxystilbene), a natural polyphenol, have been the topic of numerous studies due to its action as a hormetic factor. Integrative studies showed that RES strongly improves metabolism. Indeed, through activation of the sirtuins, a family of nuclear deacetylase enzymes, AMPK, and thus peroxisome proliferator-activated receptor (PPAR)- $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), RES was shown to enhance insulin sensitivity, stimulate mitochondrial biogenesis, and regulate antioxidant status (11–15). Interestingly, during an obesigenic high-fat-diet treatment in mice, RES was also shown to increase the expression of genes involved in protein synthesis and muscle contraction, which improved strength, endurance, and maximal oxygen consumption, thus preventing weight gain. During that study, resistance to the high-fat diet seemed also to include a transition of glycolytic fibers to a more oxidative type in different skeletal muscles. More recently, RES was shown to preserve BMD during hypokinesia and aging in rats and mice (16) through enhanced osteoblast differentiation and neovascularization (17, 18). Taken together, all those combined results show that RES protects most of the physiological systems impaired during microgravity exposure.

The aim of our study was thus to test RES as a preventive countermeasure against mechanical unloading using the rat hindlimb unloading model that has been validated for decades as a model of hypokinesia and hypodynamia (19). We hypothesized that RES would maintain whole-body physiological homeostasis mainly by protecting the structural, mechanical, and metabolic functions of the soleus muscle, a slow-twitch oxidative skeletal muscle; protecting insulin sensitivity; and averting bone loss during unloading. Because the responses to microgravity share strong analogies with what is observed in aging, sedentary behaviors, and periods of bed rest in ill patients and pregnant women,

the results of the present studies have major Earth-related benefits.

## MATERIALS AND METHODS

### Chemicals

RES was purchased from Sequoia Research Products (Pangbourne, UK).  $\text{H}_2^{18}\text{O}$ ,  $^2\text{H}_2\text{O}$ ,  $\text{d}_{31}$ -palmitate, and  $^{15}\text{N}$ -glycine were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). EGTA/Ca-EGTA buffer, propranolol, free  $\text{Mg}^{2+}$ , taurine, phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail, diaminobenzidine, MY32, sodium pyruvate, atractyloside potassium, fatty acid-free BSA, phosphocreatine disodium salt glyceraldehyde-3-phosphate, malic acid, pyruvate, glutamate, and imidazole were from Sigma-Aldrich (St. Louis, MO, USA); BRL 37344 was from Life Technologies (Carlsbad, CA, USA); and MgATP was from Amersham Biosciences (Piscataway, NJ, USA). Dithiothreitol (DTT) and adenosine-5' diphosphate disodium salt were purchased from Fluka (Buchs, Switzerland). NCL-myosin heavy chain (MHC) was obtained from Novocastra (Newcastle-upon-Tyne, UK), and SC-71 was from DSMZ (Braunschweig, Germany).

### Animals

Male Wistar rats weighing 240–250 g were purchased from Harlan (Horst, The Netherlands). After 7 d of acclimatization (25°C with 12-h light-dark cycle), rats were randomly divided into 4 groups: control (C), hypokinetic and hypodynamic hindlimb suspended (HH), controls treated with RES (CR), and hindlimb suspended treated with RES (HHR). Control rats were housed in standard laboratory cages, whereas HH rats were housed in special cages allowing tail suspension. Both cages presented the same surface. All rats were fed standard rodent laboratory pellets (type A03; Safe, Augey, France) and water *ad libitum*. The 14 d of hindlimb unloading was performed as described previously (20). The model has been used for decades to induce hypodynamia (decreased mechanical loading) and hypokinesia (decreased motor activity). Briefly, the tail is secured and connected to an overhead swivel, placed on the top of the cage, which permits a free 360° rotation of the rat. The rat is then progressively unloaded at a 30° head-down angle. The investigation was carried out under authorization 67-223 from the Direction Départementale des Services Vétérinaires du Bas-Rhin. Experiments consisted of several sessions of suspension realized over 2 yr to avoid interference between protocols and collect enough biological materials for the numerous planned analyses. The exact number of rats involved in each substudy is indicated in Results. MHC profile, muscle mass and strength, mitochondrial respiration, RT-quantitative (q)PCR, and Western blot assays were conducted in a first series of suspensions. A second series was used for insulin sensitivity tests. A third series was dedicated to protein turnover, metabolomics, bone structure parameters, and plasma markers. A fourth series was committed to energy expenditure and body composition measurements and thermoregulation by telemetry and, upon death, oxidative stress data. A last series was used to assess dietary lipid trafficking and oxidation along with heart rate by telemetry. The first series was realized in the laboratory of the Université de Lille (L.S.) and the three others at the Hubert Curien Institute (Université de Strasbourg; S.B.). All series were conducted in strictly similar conditions by the same scientist (I.M.).



## RES supplementation

RES was first dissolved in rapeseed oil and then gently emulsified in a liquid meal (Resource Protein; Nestle, Noisiel, France) in warm water (40°C) for 3 h. Daily RES supplementation lasted 6 wk in the treated groups (4 wk before unloading and during the 2 wk of unloading). At 30 min before administration, the mixture was warmed and mixed. Rats were fed RES in a dose equivalent to 400 mg/kg through a 16-gauge stainless steel curved feeding needle. This dose was reported to be well tolerated by animals (12), and recent data failed to show a toxic effect of RES in rats even at doses reaching 700 mg/kg/d (21).

## Total energy expenditure (TEE) and body composition

TEE was determined by the doubly labeled water (DLW) method, as described by Blanc *et al.* (22). On d 11 of suspension and after a basal urine collection, 1.40 mg/kg of total body water (TBW) of DLW diluted in 3% NaCl to physiological osmolarity was injected intraperitoneally. The doses were calculated to measure an *in vivo* enrichment of ~250 and 1200 ‰ for  $^{18}\text{O}$  and  $^2\text{H}$ , respectively [ $\delta\text{‰} = R_{\text{sample}}/(R_{\text{standard}} - 1) \times 1000$ , where  $R$  is the ratio of heavy to light isotope]. Then fresh urine samples were collected daily after 1 h of equilibration for 4 d. Urine samples were stored in cryogenic tubes at -20°C until analyses. The urine samples were filtered and analyzed by isotope ratio mass spectrometer (Delta V; Thermo, Bremen, Germany) interfaced with a pyrolysis system (TC/EA; Thermo), as described previously (23).

TBW was measured from the dilution space of  $^{18}\text{O}$  after correction for exchange by factor 1.007 (24). TEE was calculated using the 1-pool model equation as previously validated in rats (22). Fat-free mass (FFM) was calculated from TBW by assuming a hydration coefficient of 73.2%. Fat mass (FM) was calculated by the difference of FFM from body mass.

## Thermoregulation

A telemetric transmitter (TA10TA-F20, 3.2 g; Data Sciences, St. Paul, MN, USA) was implanted into the abdominal cavity under general anesthesia (valium: 10 mg, 2 mg/100 g i.m.; anesthesia: furan 2% in  $\text{O}_2$ ) as routinely done in the laboratory. Animals were included in the experimental protocol after 2 wk of recovery. A receiver board (RPC-1; Data Sciences) positioned close to the cages collected the radio frequency signals. Body temperature was recorded continuously for 1 min every 5 min during the whole experiment. Data were analyzed by Dataquest LabPro software (Data Sciences). Brown adipose tissue responsiveness was tested through the increase in body temperature during the 180 min following an intraperitoneal injection of the  $\beta_3$ -adrenergic ( $\beta_3$ -ADR) receptor agonist (2  $\mu\text{g/g}$ ; 37344; Life Technologies).

## Oral glucose tolerance test and inflammatory markers

On d 10 of suspension, unfed rats underwent an insulin tolerance test (0.75 U/kg i.p.), and plasma glucose concentrations were measured by Dextrostix-Eyetone system (Accu-Chek Performa; Roche Diagnostics, Meylan, France) every 30 min for 2 h from a blood drop collected from the tip of the tail with light anesthesia (2% xylocaine s.c.). After 5 d, an oral glucose load test (2 g/kg) was performed under the exact same conditions.

## Whole-body dietary fat oxidation and dietary fatty acid spillover

On d 15 of suspension, unfed rats received by oral gavage 40 mg/kg body mass [ $\text{d}_{31}$ ]-palmitate (Cambridge Isotope Laboratories) mixed in a liquid meal (Renutryl; Nestle). Animals were killed at 6 h after the dose. The oxidation of palmitate was measured as the recovery percentage of deuterium in TBW sampled in spot urine (23). Deuterium analyses were conducted as described above for the DLW-derived measurement of TEE. Plasma was sampled. After total plasma lipids were extracted using the Folch method, nonesterified fatty acid (NEFA) and triglycerides were separated by solid-phase extraction, and concentrations of the [ $\text{d}_{31}$ ]-palmitate in the plasma triglycerides and NEFA fractions were assessed by GC-MS, as described previously in detail (25). The spillover of dietary palmitate was calculated as the ratio of labeled palmitate appearing in the blood in form of free fatty acids over the labeled palmitate appearing in the blood in form of triglycerides and expressed as percentage.

## Nitrogen balance and whole-body protein turnover

Rats were individually housed in metabolic cages for 48 h and were given *ad libitum* food and water on d 13 of suspension. During the last 24 h, food intake was measured, and cumulated urine and feces were collected over ice. Total nitrogen in urine, feces, and food was measured by chemoluminescence (Antek 7000; Alytech, Juvisy Sur Orge, France) and the Kjeldhal method (26). Nitrogen balance was calculated as difference between nitrogen intake and the excretion in urine and feces over 24 h. Protein turnover was determined by the [ $^{15}\text{N}$ ]-glycine end-product technique over 24 h. Rats were orally administered a 7 mg/kg body mass [ $^{15}\text{N}$ ]-glycine solution, and  $^{15}\text{N}$ -urea and  $^{15}\text{N}$ -ammonia enrichments were assayed in 24-h urine pools by isotope ratio mass spectrometry, as described previously (27). Nitrogen flux ( $Q$ ) was calculated as  $Q = d/e$ , where  $d$  is the given dose and  $e$  is the cumulated excretion of  $^{15}\text{N}$  end products in urine. Catabolism ( $C$ ) and synthesis ( $S$ ) rates were calculated by the equation  $Q = S + E = C + I$ , where  $E$  is excretion of nitrogen in urines and feces, and  $I$  is the dietary nitrogen intake (28).

## In situ isometric contractile properties of the soleus muscle

On the morning of suspension day 15, the rats were deeply anesthetized with intraperitoneal injections of sodium pentobarbital (60 mg/kg), prolonged if necessary by supplementary doses (30 mg/kg). The dissection protocol was previously described (29, 30). Briefly, all the muscles of the right hindlimb were denervated, except the soleus muscle. Then, the limb was fixed in isometric conditions and was immersed in a bath of paraffin oil thermostatically controlled (37°C). The limb was stabilized by using a combination of bars and pins, and the soleus muscle was maintained in a horizontal position. Afterward, the soleus muscle was isolated from surrounding tissues, and its distal tendon was connected to a force transducer (Grass FT 10; Grass Instruments, West Warwick, RI, USA). The muscle length was adjusted to produce a maximal isometric tension. Stimulating and reference electrodes (Teflon-coated platinum) were maintained by micromanipulators. Contractions were induced by stimulation of the soleus nerve (0.2-ms pulses) through monopolar platinum electrodes at twice the minimum voltage required to obtain the maximal twitch response. The reference electrode was inserted into adjacent denervated muscle mass. The following parameters were recorded: a single maximal twitch, from which the maximal twitch tension, the time to peak, and

the half-relaxation time were measured; and the tension/frequency relationship (for stimulation frequencies ranging from 16 to 100 Hz), which allowed the determination of maximum tetanic tension  $P_0$  obtained for a 100-Hz stimulation frequency and of index  $P_{20}/P_0$  (ratio of the tetanic tension at 20 Hz to  $P_0$ ). The soleus muscles were then removed and weighed.

### Characterization of muscle fiber types

After the suspension protocol and at the midbelly, the soleus muscles were cut into serial 10- $\mu$ m-thick cross-sections in a cryostat microtome at  $-20^\circ\text{C}$ . The protocol relative to immunohistochemically treated sections has been described in detail previously (30). The following antibodies were used: NCL-MHC, which recognizes MHC I; SC 71 (DSMZ) against MHC IIA; and MY32 against MHC IIA, IIB, and IIX. These antibodies were diluted at 1:400, 1:400, and 1:2000, respectively. The binding of the primary antibodies was localized by an immunoperoxidase reaction utilizing the Novostain Universal Quick Kit (Novocastra). Serial cross-sections were incubated in prediluted normal blocking serum for  $\sim 10$  min. The excess serum was blotted, and sections were incubated with primary antibodies diluted in PBS for 2 h at room temperature. Serial cross-sections were washed for 5 min in PBS solution. Then, they were incubated for 30 min in prediluted biotinylated universal secondary antibody. At the end of the 30 min, sections were washed with PBS for 5 min and incubated in ready-to-use streptavidin/peroxidase complex reagent for 30 min. To label the serial cross-sections, the peroxidase substrate solution (DAB; Sigma-Aldrich) was added after the sections had been washed for 5 min with PBS. Finally, after dehydration with alcohol and toluene, the slides were mounted in Eukitt resin. Positive fibers were characterized by a brown color, while negative fibers remained unlabeled. The cross-sectional area (CSA) of the fibers was also measured on immunohistochemical sections by use of an image analyzer (Mac Biophotonics ImageJ; <http://www.macbiophotonics.ca/imagej/>). CSA measurements were made in  $\sim 300$  fibers for type I and in all type IIA, IIB, and IID/x fibers.

### Measurement of mitochondria oxidative capacity of soleus muscle

Respiratory parameters of the total mitochondrial population were studied *in situ* in saponin permeabilized fibers using method of Veksler and Ventura-Clapier (31) after 2 wk of HH. Fiber bundles (100–250  $\mu$ m in diameter) were excised from skeletal muscles after hindlimb suspension. The bundles were incubated with intense shaking for 30 min in solution S containing 50  $\mu$ g/ml saponin to selectively destroy the integrity of the sarcolemma. Once the fibers were permeabilized by saponin, they were transferred into solution R twice for 10 min to wash out adenine nucleotides and phosphocreatine. All procedures were carried out at  $4^\circ\text{C}$ .

Both S and R solutions contained 10 mM EGTA/Ca-EGTA buffer (free  $\text{Ca}^{2+}$  concentration, 100 nM), 3 mM free  $\text{Mg}^{2+}$ , 20 mM taurine, 0.5 mM DTT, and 20 mM imidazole. Ionic strength was adjusted to 0.16 M by addition of potassium methanesulfonate. Solution S (pH 7.1) also contained 5 mM MgATP and 15 mM phosphocreatine. Just before the experiment was started, 2 mg/ml of fatty acid-free BSA solution was added to solution R (pH 7.1). Respiratory rates were determined by a Clark electrode (Hansatech; Hardwick Industrial, Royaume, UK) in an oxygraphic cell containing 15–20 fiber bundles in 3 ml solution R at  $24^\circ\text{C}$  with continuous stirring. The solubility of oxygen was taken to be 230 mmol  $\text{O}_2/\text{L}$ .

Depending on skeletal muscle type, 2 mM ADP, 4 mM glyceraldehyde-3-phosphate, 2 mM malic acid, 1 mM pyruvate, 5 mM glutamate, and 150  $\mu$ M palmitoylcarnitine were added during the experiment to solution R. Atractyloside was added to stop the respiration. After measurements, the bundles were removed and dried. Respiration rates were expressed as nanomoles of  $\text{O}_2$  per minute per gram of dry weight and are presented as percentage of the C group.

### RT-qPCR and Western blotting

We measured the gene expression of SIRT-1, COXIV (mitochondria marker), citrate synthase (CS; marker of TCA cycle activity), CPT-1 (long-chain fatty acid transporter into the mitochondria), and FAT/CD36 (fatty acid transporter into the cell) after the 2 wk of mechanical unloading on a piece of the same muscle used for mitochondrial respiration.

Soleus muscles kept at  $-80^\circ\text{C}$  were crushed into powder by using a Microdismembrator (Sartorius, Goettingen, Germany). Total RNA was extracted using Trizol (Invitrogen, Cergy Pontoise, France). The total RNA (1  $\mu$ g) was reverse transcribed to cDNA using Multiscribe reverse transcriptase (high-capacity reverse transcriptase kit with RNase inhibitor; Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using SYBR Green technology (Applied Biosystems) on Mastercycler ep realplex (Eppendorf, Le Pecq, France). Five-fold serial dilutions from total RNA were analyzed for each target gene and allowed us to construct linear standard curves from which the concentration of the test sample was calculated. Forward and reverse primers (Supplemental Table S1) were each designed in a different exon of the target sequence, eliminating the possibility of amplifying genomic DNA.

A basic local alignment search tool (BLAST) search performed for each set of primers revealed that sequence homology was obtained only for the target gene. Hypoxanthin phosphoribosyl-transferase (HPRT) was chosen as a housekeeping gene for normalization, as its expression did not differ between the groups. Results were thus normalized to HPRT transcription to compensate for variation in input RNA amounts and efficiency of reverse transcription.

The same soleus muscles used for RT-qPCR analysis were used for Western blotting. Soleus muscle powder was further homogenized by sonication in the presence of 200  $\mu$ l Laemmli buffer and centrifuged at 14,000 rpm for 30 min at  $4^\circ\text{C}$ . The supernatant was removed for protein quantification, and then PMSF and protease inhibitor cocktail were added. A total of 30  $\mu$ g of protein was suspended in 1%  $\beta$ -mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol, with total volume completed to 30  $\mu$ l with water. Protein samples were boiled, and 15  $\mu$ g was loaded on 10% polyacrylamide gel and after migration transferred to nitrocellulose membranes (Novalab, Malakoff, France). Signals for COXIV (mouse monoclonal; Abcam, Cambridge, MA, USA) at 17 kDa, PGC-1 $\alpha$  (rabbit polyclonal; Abcam) at 110 kDa, and Sirt-1 (sir-2 rabbit polyclonal; Millipore, Bedford, MA, USA) at  $\sim 92$  kDa were detected.

### Glutathione levels and antioxidant enzyme activities

After 2 wk of suspension, the reduced glutathione (GSH) and oxidized glutathione (GSSG) states induced by the HH protocol were measured in soleus according to the method of Anderson (32) by monitoring the reduction by GSH of 5,5'-dithio-bis-(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoate at 412 nm ( $25^\circ\text{C}$ ). Proteins were precipitated with ice-cold 5% metaphosphoric acid and centrifuged at 5000 g for 5 min. For GSH measurements, samples were derivatized with 2-vinyl-

pyridin. The assay was initiated by the addition of 10  $\mu$ l of 50 U/ml glutathione reductase. GSH was used as a standard and was assayed in parallel under the same condition as the tissue samples.

A portion of soleus muscle was homogenized with a potter Elvehjem at 4°C in buffer containing  $\text{KH}_2\text{PO}_4$  (100 mM), DTT (1 mM), and EDTA (2 mM), pH 7.4. After centrifugation (3000 g/min, for 5 min), the supernatant was used for enzymatic assays. Superoxide dismutase (SOD) activity was assayed by monitoring the rate of acetylated cytochrome *c* reduction by superoxide radicals generated by the xanthine-xanthine oxidase system. One activity unit of SOD was defined as the quantity of SOD required to cause a 50% inhibition of the rate of acetylated cytochrome *c* reduction. The total activity of glutathione peroxidase (GPX) was assayed with cumene hydroperoxide as a substrate according to Pierce and Tappel (33). All enzyme activities were expressed in units per gram of protein.

### Bone function

After a 2-wk suspension protocol, the rats were killed in unfed conditions, the femurs were dissected, and blood was collected. Femoral BMD was assessed by dual-energy X-ray absorptiometry using a Hologic QDR-4500 A X-ray bone densitometer (Hologic, Massy, France). For mechanical testing, femoral length and mean diaphyseal diameter were measured with a precision caliper (Mitutoyo, Telford, UK). The femoral failure load was determined using a 3-point bending test, with a universal testing machine (Instron 4501; Instron, Canton, MA, USA). The two lower supports were separated by 20 mm, and an upper crosshead roller was applied in front of the middle of the bone at a speed of 0.5 mm/min until fracture.

Plasma osteocalcin was measured with the RAT-MID assay (Immunodiagnostic Systems, Paris, France) as a marker of bone synthesis (intraassay variation of 5%). Similarly, urine dextroxypridinoline (DPD) was measured as a marker of bone degradation by  $^{125}\text{I}$  radioimmunoassay (Immunodiagnostic Systems).

### Cardiovascular function

A telemetric transmitter (TA11PA-C40; Data Sciences) was implanted into the abdominal cavity under the same surgical conditions as above described 3 wk prior HH to allow full recovery. Electrodes were subcutaneously tunneled to the heart and pelvis positions to record heart rate. Heart rate was recorded continuously for 1 min every 5 min during the whole experiment.

### Fasting plasma markers

On d 15 of suspension, the rats were killed, and blood was collected to assess insulin (measured by the rat insulin ELISA kit; Mercodia, Uppsala, Sweden), glucose and inflammation markers. ELISA immunoassay kits (Arcus Biologicals Euromedex, Souffelweysheim, France) specific for rat were used to measure specific pro- and anti-inflammatory markers: ghrelin (intraassay variation coefficient of 9%), TNF- $\alpha$  (intraassay variation coefficient of 10%), IL-1 $\beta$  (intraassay variation coefficient of 11%), and IL-6 (intraassay variation coefficient of 10%). The D-ROM and Oxy-adsorbent kits (Diacron International, Grosseto, Italy) were used to evaluate the oxidant ( $\text{H}_2\text{O}_2$ ; intraassay variation coefficient of 2%) and antioxidant (HCLO; intraassay variation coefficient of 9%) status. Plasma corticosterone was measured after death using

the AssayMax Corticosterone ELISA kit for rats (AssayPro, Paris, France).

### Determination of urinary RES metabolites by mass spectrometry

A metabolomic approach was used to determine urinary RES metabolites. First, an untargeted method consisting of the simultaneous analysis of a high number of metabolites allowed the determination of all metabolic perturbations induced by the HH protocol.

A sample of 24 h urine (500  $\mu$ l) was defrosted at room temperature, centrifuged at 7000 g for 5 min at 4°C, and then diluted 4-fold with distilled water. Analyses were performed on a Waters Acquity UPLC system (Waters Corp., Manchester, UK) coupled to a Waters QToF-Micro mass spectrometer, equipped with an electrospray source. To avoid possible differences between sample batches, a Latin square was carried out to obtain a randomized list of samples for analysis. Separation was carried out at 30°C using a 2.1-  $\times$  100-mm Acquity UPLC BEH Shield RP18 column (Waters), with a particle size of 1.7  $\mu$ m at a flow rate of 0.4 ml/min. Samples were eluted from the column using the following gradient: 0–2 min, 100% A; 2–7 min, 0 to 10% B; 7–22 min, 95% B. Solvent A was water, and solvent B was acetonitrile; both solvents contained 0.1% formic acid. Injection volume was set to 8  $\mu$ l. The mass spectrometer source temperature was set to 120°C with a cone gas flow of 50 L/h, a desolvation temperature of 300°C, and a nebulization gas flow of 400 L/h. The capillary voltage was set at 3000 V and the cone voltage to 30 V. The mass spectrometric data were collected in continuum full-scan mode with a mass-to-charge ratio (*m/z*) of 100–1000 from 0 to 22 min in positive and negative modes. All analyses were acquired by using the lockspray with a frequency of 5 s to ensure accuracy. Leucine enkephalin was used as the lock mass at a concentration of 0.5 ng/L (in MeOH:water, 50:50 vol/vol, with 0.1% formic acid).

The raw data were transformed to centroid mode and mass corrected before being analyzed with MarkerLynx Applications Manager v4.1 (Waters). The data were peak detected and noise reduced for both the liquid chromatography and mass spectrometry components. Each peak in the resulting 3-dimensional data set was represented by retention time *m/z* and its ion intensity in each sample.

The matrix obtained was filtered to eliminate the background ions present in blank samples and then exported for statistical analysis. The 3077 selected ions were analyzed by 2-way ANOVA using multivariate ANOVA R function. ANOVA was performed on log-transformed data to assess the effects of RES, hanging, and interaction on each ion. To deal with multiple testing issues, the Benjamini-Hochberg procedure was applied to adjust the *P* values.

Ions with adjusted values of *P* < 0.10 for at least 1 of the 3 effects were kept for supervised multivariate discriminant analysis. Partial least-square discriminant analysis (PLSDA) was performed on the log-transformed centered data. PLSDA produced a linear discriminant cross validated model that highlighted a discriminant combination of ions. The quality of the model is given by the  $Q_2$  parameter. A  $Q_2$  value close to 1 indicates a good predictive model.

Metabolites contributing to the discrimination of d 0 and 14 samples were identified on the basis of their exact masses, which were compared with those registered in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/>).

### Statistical analyses

Data were analyzed by 2-way ANOVA with group and treatment as main factors. When significance was noted, a *post hoc*



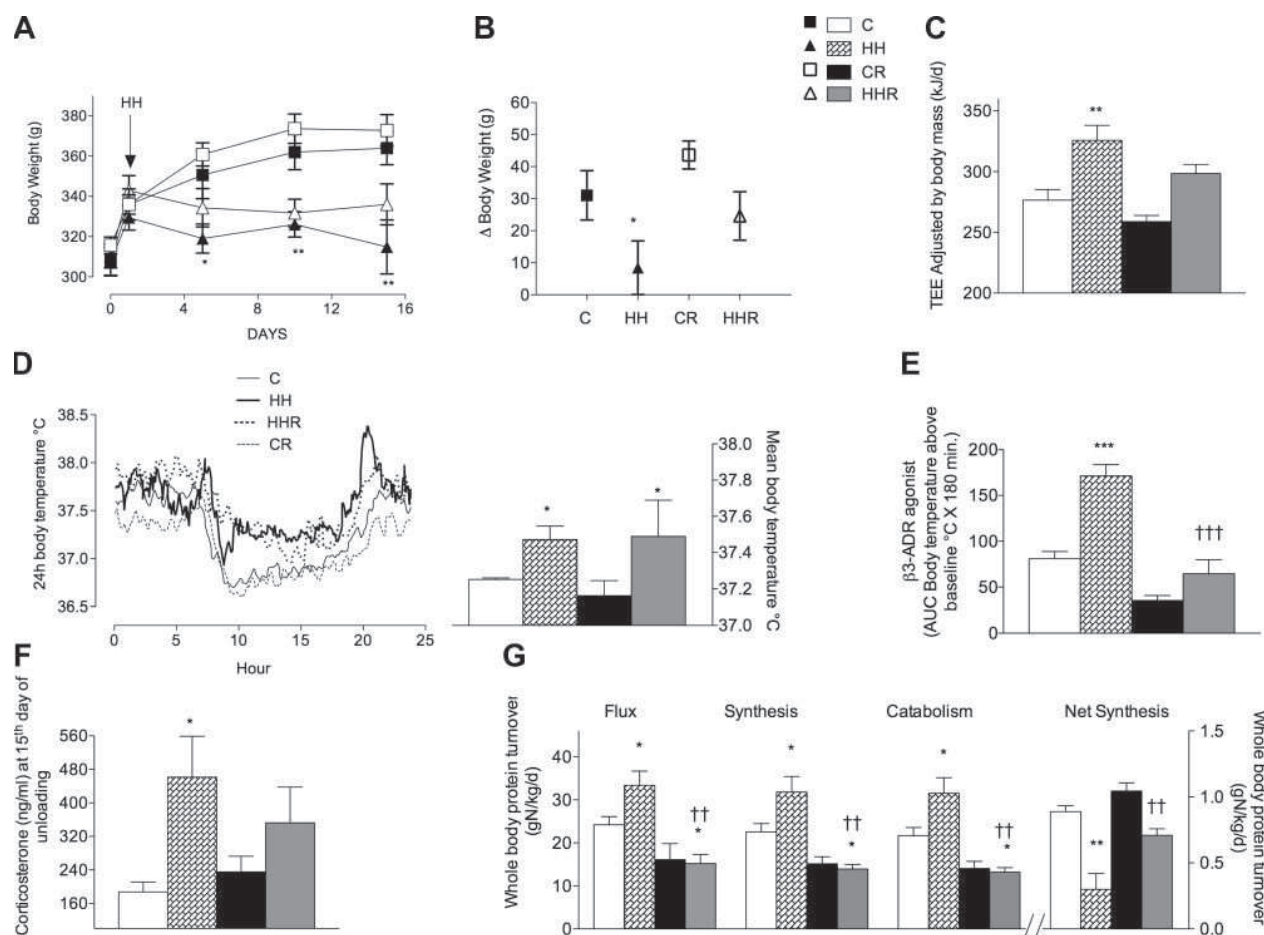
analysis with the PLSD Fisher's test was performed. To improve readability, only *post hoc* tests are mentioned in the text and in the figures. All data are presented as means  $\pm$  SE, and statistical significance was set at  $P < 0.05$ . All statistics were performed using JMP 5.1 (SAS Institute, Cary, NC, USA).

## RESULTS

### Effects of RES on main physiological variables

To investigate the effect of RES on known hypokinesia and hypodynamia responses during hindlimb unloading, we first characterized general physiological parameters. After a week of unloading, body mass gain reached a plateau (Fig. 1A). RES treatment tended to increase body mass in the supplemented groups. At the end of the experiment, body masses of the C and HHR groups were not statistically different (Fig. 1A, B).

Although HH rats showed a trend for a lower FM, body composition was not affected by unloading or by RES treatment (data not shown). The difference in body mass during unloading was likely explained by a 25% increase in TEE that did not reach significance in HHR rats compared with C rats (Fig. 1C). To investigate whether those energy adaptations were due to thermoregulatory defects, as previously shown in suspended rats (4, 20), body temperature was measured daily. Average 24-h body temperature was modestly but significantly higher during unloading, independent of RES treatment (Fig. 1D). In HH animals, the thermogenic capacity of brown adipose tissue was increased after injection of a  $\beta$ -3 agonist that mimics cold exposure (Fig. 1E). In support of that result, the observation that resting heart rate was increased in HH rats by 8% ( $P < 0.05$ , data not shown) compared with C rats demonstrated an increased sympathetic nervous system tonus during unloading that supported the notion that



**Figure 1.** A) Effects of RES on main physiological variables. Body mass gain of all groups during 15 d of suspension. B) Delta of body mass evolution from the beginning of the experiment in the same groups. C) TEE adjusted by body mass. D) Nycthemeral evolution of body temperature and 24-h average body temperature during unloading period. E) Nonshivering thermogenesis capacity through body temperature increases above baseline after intraperitoneal injection (2  $\mu$ g/g) of the  $\beta$ 3 agonist (BRL 37344; Life Technologies), expressed as area under the curve (AUC) during the 3 h postinjection. F) Fasting plasma corticosterone concentration, measured after death following 15 d of the protocol. G) Whole-body protein turnover, protein synthesis, catabolism, and net synthesis rates, measured by the end-product technique, following an oral ingestion of  $^{15}$ N glycine (7 mg/kg) over 24 h. Sample size:  $n = 8$ –10 animals/group, except for corticosterone ( $n = 5$ /group) and thermoregulation tests ( $n = 6$ /group). \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  vs. C; † $P \leq 0.05$ , †† $P \leq 0.01$ , ††† $P \leq 0.001$  vs. HH; *post hoc* ANOVA using Fisher test.

nonshivering thermogenesis was chronically stimulated.

Corticosterone concentration increased significantly in HH animals. No increase in corticosterone plasma levels was noted in the HHR group (Fig. 1*F*). Because cortisol is also known to affect protein turnover and influence muscle function during spaceflight in humans (34), we assessed whole-body protein fluxes. The unloaded rats showed the highest nitrogen flux but the lowest, although positive, net protein synthesis rates (synthesis minus catabolism). Conversely, RES treatment decreased nitrogen flux and maintained net protein synthesis to the level observed in the control rats (Fig. 1*G*).

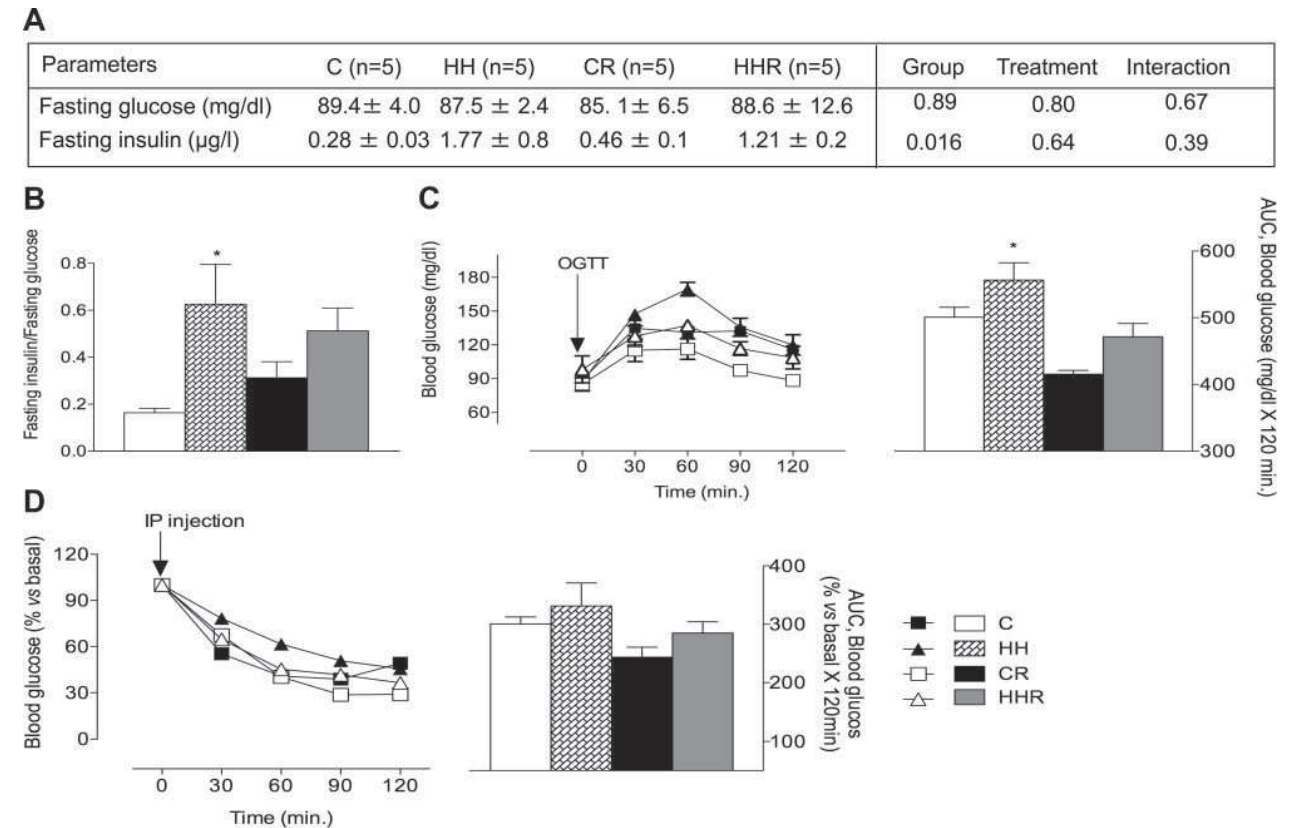
**RES prevents hypokinesia/hypodynamia-induced insulin resistance**

Numerous studies showed that muscle disuse during bed rest in humans and HH in rats leads to insulin resistance (35–37). To test how RES modulates insulin resistance in HHR rats, we measured fasting blood glucose and insulin and observed a strong hyperinsulinemia in the unloaded animals, independent of RES treatment (Fig. 2*A*). This translated to higher fasting insulin to glucose ratio in all suspended groups (Fig.

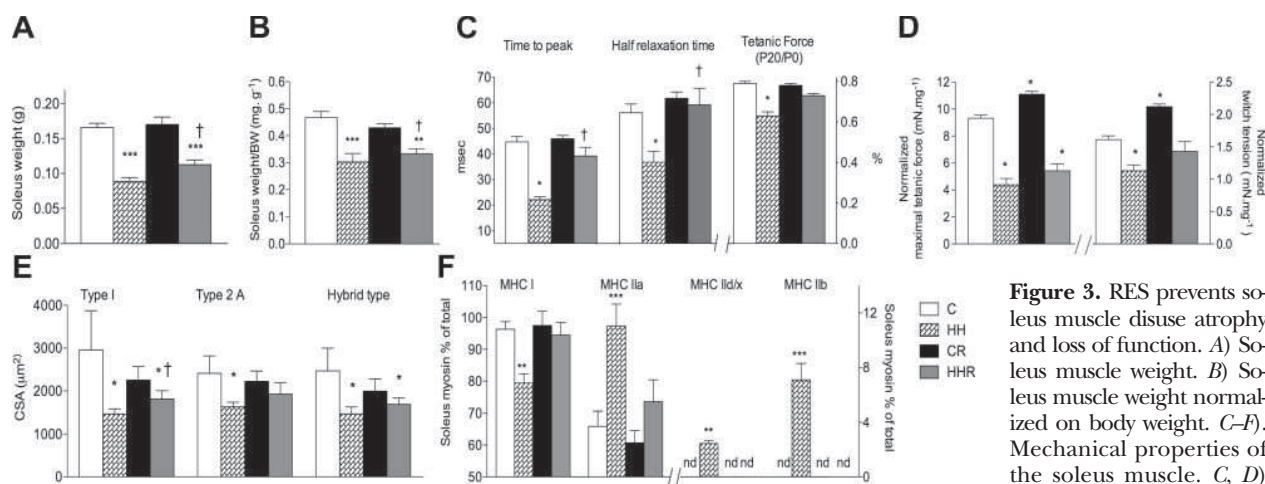
2*B*). The significant treatment-by-group interaction ( $P>0.05$ ) suggested, however, that RES treatment mitigated the insulin resistance induced by HH during fasting. Those observations were confirmed during the dynamic responses to both an oral glucose tolerance test and an intraperitoneal insulin challenge (Fig. 2*C, D*).

**RES prevents soleus muscle disuse atrophy and loss of function**

Skeletal muscle plasticity plays a key role in performance and maintenance of whole-body homeostasis. Slow-twitch skeletal muscle force diminishes and contractile protein profile changes with severe physical inactivity in humans; this is also well described in unloaded rats (for review, see Fitts, ref. 2). RES treatment significantly mitigated the soleus disuse atrophy by 26 and 10% without and with normalization, respectively (Fig. 3*A, B*). The mechanical properties of the soleus muscle observed *in situ* showed that the rate of force development was reduced by 30% and the interval from peak tension to half peak tension was reduced by ~40% after unloading (Fig. 3*C*). In the same direction, the maximal tetanic force  $P_0$  (normalized on muscle mass) decreased by 53% and twitch tension by



**Figure 2.** RES prevents hypokinesia/hypodynamia-induced insulin resistance. *A, B*) Fasting glucose and insulin levels (*A*) and fasting insulin to glucose ratio (*B*) after 14 d of mechanical unloading in rats. *C, D*) Kinetics of blood glucose and the corresponding AUC after a 120-min oral glucose tolerance test (OGTT) at 2 g/kg (*C*) and kinetics of blood glucose and the corresponding area under the curve after a 120-min intraperitoneal insulin tolerance test at 0.75 IU/kg (*D*). Sample size:  $n = 6$ /group. \* $P \leq 0.05$  vs. C; *post hoc* ANOVA using the Fisher test.



**Figure 3.** RES prevents soleus muscle disuse atrophy and loss of function. **A)** Soleus muscle weight. **B)** Soleus muscle weight normalized on body weight. **C–F).** Mechanical properties of the soleus muscle. **C, D)** Time to peak and half

relaxation time tetanic force at 20 Hz/ total tetanic force ( $P_{20}/P_0$ ) after 15 d of mechanical unloading (**C**) and corresponding maximal tetanic force and twitch tension (**D**). **E)** Soleus CSA. **F)** MHC profiles of the soleus. Sample size:  $n = 9–11$  for soleus weight;  $n = 5–7$  for other data.  $*P \leq 0.05$ ,  $**P \leq 0.01$ ,  $***P \leq 0.001$  vs. C;  $^{\dagger}P \leq 0.05$  vs. HH;  $\dagger$  post hoc analysis using Fisher PLSD test.

30% (Fig. 3D). RES treatment did not preserve  $P_0$ ; however, it did maintain tetanic force at 20 Hz ( $P_{20}/P_0$ ; Fig. 3C) and twitch tension (Fig. 3C). To confirm the beneficial effect of RES on contractile property of slow-twitch muscle, we further detected the fiber type composition and myosin profiles of the soleus. The CSA results were in line with the classical muscle response to disuse atrophy; all areas were decreased after unloading (Fig. 3E). Only type 2A fiber with RES treatment was maintained at the CSA level observed in control animals. However, the RES treatment clearly alleviated the loss in type I fiber observed in the HH animals.

The protective effect of RES on soleus atrophy was confirmed on the MHC pattern. Whereas slow MHC I expression decreased by 18% in HH animals in favor of an increase in fast MHC IIa and an apparition of mixed MHC IIx and MHC IIb, no changes were noted after RES treatment (Fig. 3F). Overall, the RES treatment preserved muscle contractile properties during a well-designed protocol to induce slow-twitch muscle disuse atrophy in the rat.

### RES maintains soleus oxidative capacity and whole-body fat utilization

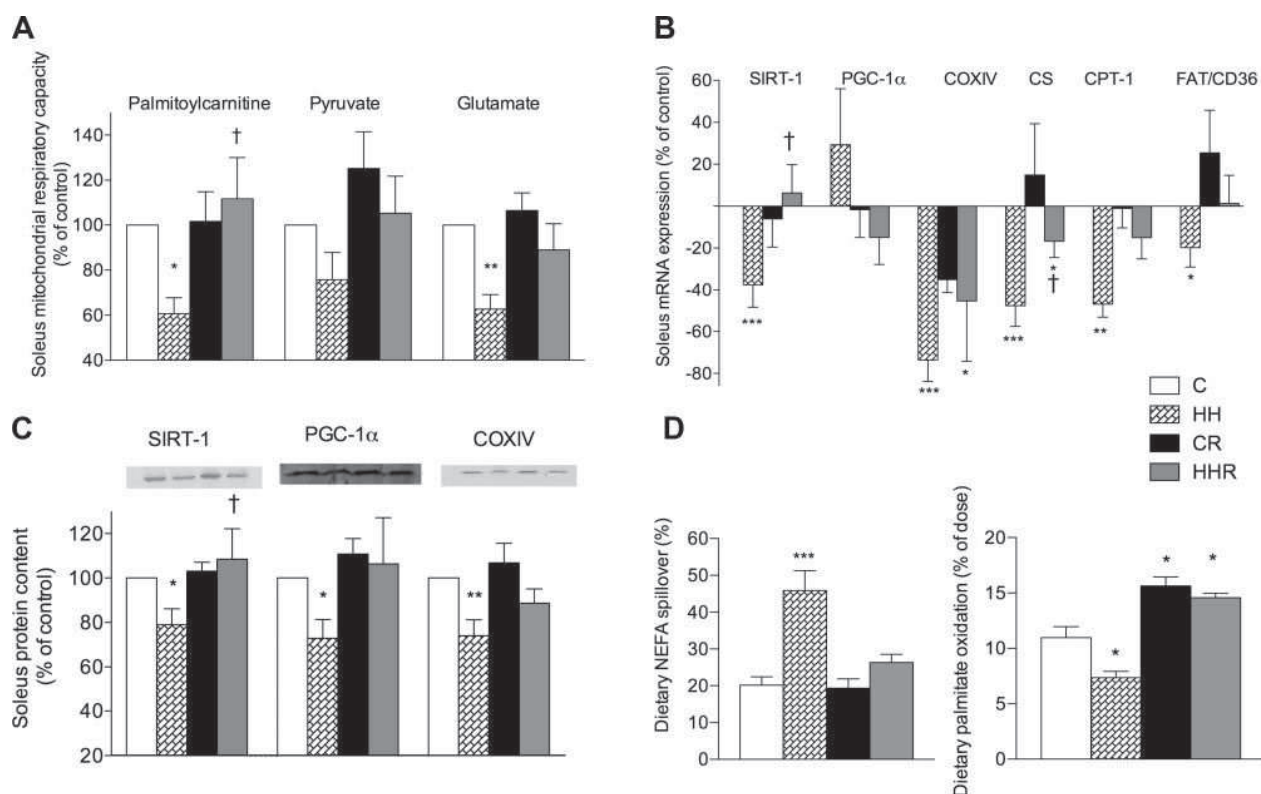
Evidence suggests that physical inactivity, either in humans during outpatient studies or longitudinal bed rest, or in rats using hindlimb unloading or artificial selection for low aerobic capacity, decreases the ability of the oxidative skeletal muscle to oxidize fatty acids for energy (5, 36). This was associated with the development of insulin resistance and increased muscle fatigability. Interestingly, some studies in rats suggested, but only suggested, that those metabolic defects were observed prior to any loss in muscle function (38, 39). Mechanistically, this was associated with a reduction in mitochondrial density or decrease in expression or

activity of enzymes participating in transport of fatty acids and in  $\beta$ -oxidation (2). In the present study, unloading caused mitochondrial respiratory capacity of soleus muscle to drop by 40% in the presence of palmitoyl carnitine, by 24% in the presence of pyruvate, and by 38% in the presence of glutamate (Fig. 4A). RES fully maintained soleus oxidative capacity in the presence of all these substrates. Recently, Lagouge *et al.* (12) proposed that RES, by activating SIRT-1, increases the number of mitochondria. To investigate the underlying mechanisms explaining the protective effects of RES during unloading, we measured the mRNA expression of several enzymes and cofactors involved in energy metabolism.

The mRNA expression of SIRT-1, COXIV, CS, CPT-1, and FAT/CD36 decreased in HH rats. RES supplementation mitigated the reduction in the mRNA expression of COXIV, CS, and CPT-1 and fully prevented the reduction in mRNA expression of SIRT-1 and FAT/CD36 (Fig. 4B). The results at the mRNA levels were confirmed at the protein levels for SIRT1, PGC-1 $\alpha$ , and COXIV (Fig. 4C).

Muscles are the main location of fatty acid oxidation. To investigate how the metabolic adaptations we observed at the muscle level extended to the whole body, we measured an index of plasma clearance and oxidation of exogenous palmitate labeled with deuterium. The palmitate spillover increased during unloading, suggesting an important decrease in the plasma clearance of fatty acids (Fig. 4D). Those results were supported by the decreased soleus expression of FAT/CD36. RES treatment fully prevented this reduction in fatty acid clearance from plasma (Fig. 4D). Overall, the whole-body oxidation rate of palmitate was decreased in the HH rats, but, again, RES was effective in preventing this decrease in oxidative capacity (Fig. 4E).





**Figure 4.** RES maintains soleus oxidative capacity and whole-body fat utilization. *A*) Soleus mitochondrial respiratory capacity in the presence of palmitoylcarnitine, pyruvate, and glutamate. *B*) mRNA expression of SIRT-1, PGC-1 $\alpha$ , COXIV, CS, CPT-1, and FAT-CD36 in soleus. *C*) Protein levels in soleus muscle of SIRT-1, PGC-1 $\alpha$ , and COXIV. *D*) Index of plasma dietary fatty acid clearance, measured as ratio of exogenous d<sub>31</sub>-palmitate in NEFA fraction over d<sub>31</sub>-palmitate in the triglyceride fraction, expressed as percentage at 6 h postingestion. *E*) Whole-body d<sub>31</sub>-palmitate oxidation, measured as percentage recovery of the dose given (40 mg/kg). Sample size:  $n = 11$ – $14$ , except for Western analyses ( $n = 5$ /group). For mitochondrial oxidative activity, protein levels, and qPCR expression, C group is taken as 100%. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  vs. C; † $P \leq 0.05$  vs. HH; *post hoc* analysis using Fisher PLSD test.

#### RES counteracted the soleus disuse-induced oxidative stress

Several lines of evidence link muscle-derived oxidants and inflammatory cytokines to muscle-unloading-induced atrophy *via* regulation of proteolysis (40, 41). As RES is known to present both antioxidant and anti-inflammatory properties, we evaluated whether the beneficial effects of RES observed so far were in part explained by a prevention of muscle oxidative stress and inflammation. As shown in **Fig. 5A**, no change occurred in basal plasma oxidant and antioxidant status in various treatments, whether hindlimb unloading or RES supplementation. Regarding the fasting plasma inflammatory markers, we also failed to observe any difference between groups and treatment, although a trend was noted for a positive effect of RES on IL6 plasma concentrations (Fig. 5D).

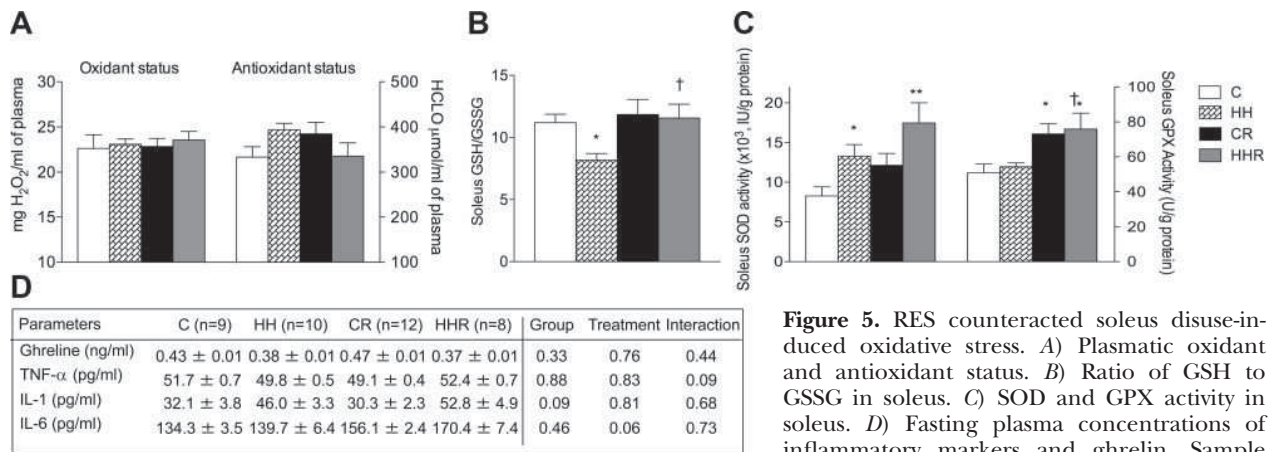
However, in the soleus muscle, unloading resulted in a decrease of GSH/GSSG ratio (Fig. 5B), an effect that was prevented by RES treatment. Simultaneously, the antioxidant activity of SOD increased in both unloaded groups and to a larger extent during RES treatment (Fig. 5C). This last result confirms the general action of RES as an antioxidant. In support of that result, GPX

activity increased only in the RES-treated groups (Fig. 5C).

#### RES prevented bone demineralization and loss of strength due to unloading

Bone demineralization is a classic observation during spaceflight and represents a major risk for long-term missions, especially because recovery is a long-term process on return to Earth (42). The mechanical unloading due to the physical inactivity induced by microgravity is a major drive for bone loss. In support of that observation, the risk for bone fracture is increased in sedentary population on Earth (43). In the aged population, bone fracture is an important overall risk for death (43). Studies conducted in humans during physical inactivity induced by bed rest have demonstrated how mechanical loading is critical to the maintenance of bone formation, structure, and strength (43). It has been suggested that RES might prevent osteoporosis through enhancing proliferation and osteoblastic differentiation (17). As expected, the BMD and the femoral load to break the femur were decreased by 13 and 32%, respectively, after 2 wk of





**Figure 5.** RES counteracted soleus disuse-induced oxidative stress. **A)** Plasmatic oxidant and antioxidant status. **B)** Ratio of GSH to GSSG in soleus. **C)** SOD and GPX activity in soleus. **D)** Fasting plasma concentrations of inflammatory markers and ghrelin. Sample size:  $n = 6/\text{group}$ , except as indicated.  $*P \leq 0.05$ ,  $**P \leq 0.01$  vs. C;  $^{\dagger}P \leq 0.05$  vs. HH; *post hoc* analysis using Fisher PLSD test.

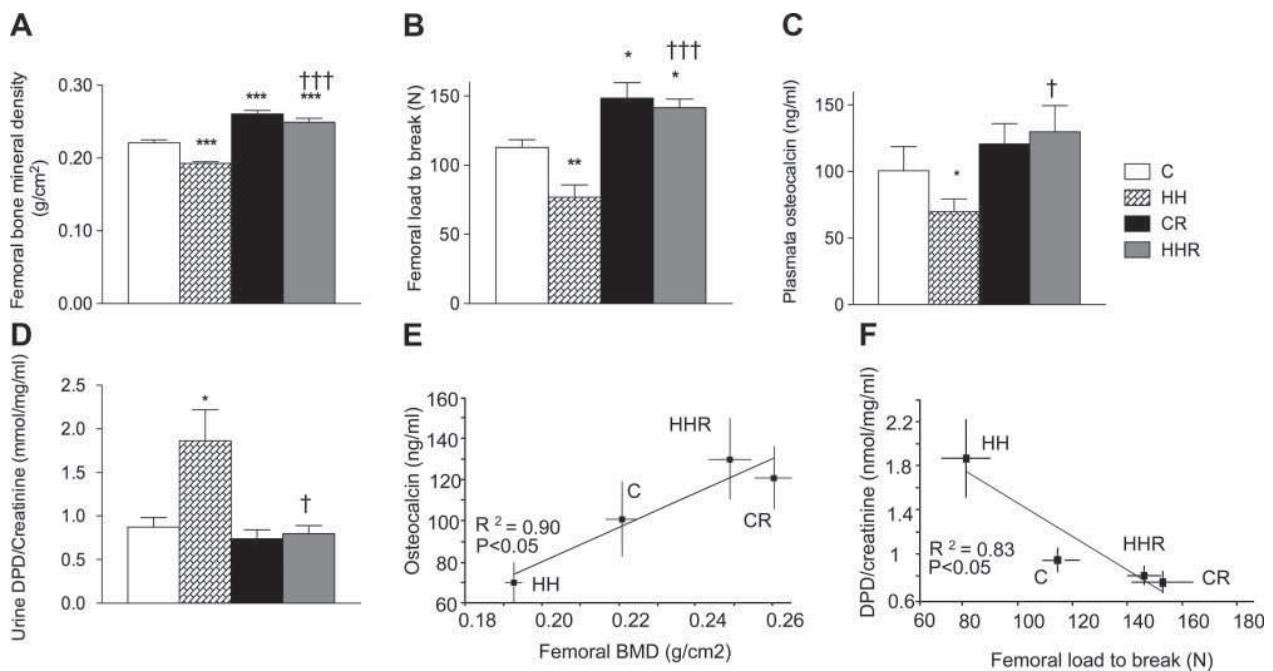
unloading (**Fig. 6A, B**). RES not only prevented these changes but actually increased both BMD and resistance of the femur (**Fig. 6A, B**).

Those functional changes were reflected in the plasma markers of bone synthesis and degradation. Osteocalcin (secreted by osteoblasts) was decreased during unloading, and levels in RES-treated animals were maintained at the control levels (**Fig. 6C**). The bone degradation marker DPD measured in 24-h urine showed a mirrored response, with a 2-fold increase during unloading; this increase was fully alleviated with RES supplementation (**Fig. 6D**). The homogeneity of these results is further confirmed by the strong relationship observed between the plasma markers of bone

synthesis and degradation and the bone BMD and strength (**Fig. 6E, F**). These results overall indicate that RES is able to prevent bone resorption and to maintain bone strength and could be considered an efficient dietary strategy to decrease fracture risk in astronauts and sedentary populations.

#### Orally ingested RES showed good bioavailability in our model

Although the vast effects of RES observed in the present study suggest that the rats correctly assimilated RES, we conducted a metabolomic study in the 24-h urine to



**Figure 6.** RES prevented bone demineralization and loss of strength due to unloading. **A)** Femoral BMD. **B)** Femoral breaking load. **C)** Plasma osteocalcin concentration. **D)** Urine DPD. **E)** Correlation analyses between osteocalcin and BMD. **F)** DPD with femoral breaking load. Sample size:  $n = 6-7$ , except for osteocalcin and DPD assay ( $n=9-11$ ).  $*P \leq 0.05$ ,  $**P \leq 0.01$ ,  $***P \leq 0.001$  vs. C;  $^{\dagger}P \leq 0.05$ ,  $^{\dagger\dagger\dagger}P \leq 0.001$  vs. HH; *post hoc* analysis using Fisher PLSD test.

look for metabolites of RES. The data showed that most of the RES metabolites were found in the urine, demonstrating the bioavailability of the molecule given *per os* (Supplemental Table S2). The PLSDA results (Supplemental Fig. S1) further confirmed the overall metabolic effects of dietary supplementation by RES that differed among groups.

## DISCUSSION

The loss of body functions in response to microgravity exposure has been a medical and physiological concern since the early Gemini, Soyuz, and Skylab missions. Early research on countermeasures was initiated to maintain health and fitness for long-duration flights. To be effective, a countermeasure must be easy to implement during spaceflight and present positive effects that go beyond the protection of a single function. Although such an integrated physiological approach has long been recognized as a required scientific line of attack, researchers have focused on some organ systems and symptoms in a piecemeal fashion rather than simultaneously targeting all of the physiological systems affected by gravity.

The present results provide evidence that dietary RES at 400 mg/kg/d is able to prevent the metabolic, muscle, and bone deconditioning caused by mechanical unloading. To our knowledge, this is the first study to report such broad counteractive effects that extend beyond a single function.

### Whole-body homeostasis

We observed that energy expenditure is higher during mechanical unloading, in line with the finding of Lew *et al.* (44) on suspended mice. Such expenditure likely explains the weaker body weight gain in HH rats observed in our study and others (44, 45) during unloading. The higher TEE could be related to an impaired thermoregulation capacity, as previously suggested, and/or stress (ref. 46; for review, see ref. 47). In support of that result, the response to a  $\beta$ 3-adrenergic agonist mimicking cold exposure was increased during unloading. Similarly, Yamashita *et al.* (48) showed that chronic hindlimb suspension in rats leads to an increase in both thermogenic capacity and activity in brown adipose tissue. The higher corticosterone level also likely contributed to some extent to the higher TEE (49). Hypercortisolemia was observed in some but not all bed-rest studies in humans but is certainly a common feature in rats during spaceflight of varying duration (50–52). As chronic and acute treatments by corticosterone in rats resulted in muscle wasting and reduction of growth rate and insulin sensitivity (53, 54), it is, however, an important parameter to consider in the adaptation to the space environment. The positive effect of RES on cortisol secretion warrants further analyses. However, such results are supported by Supornsilchai *et al.* (49), showing that RES exerts a suppressive

effect on corticosterone production in isolated rat adrenocortical cells.

Cortisol is also strongly involved in protein turnover regulation. In rats, unloading was shown to result in both an increase in muscle protein degradation and a decrease in muscle protein synthesis, leading to muscle atrophy (55). Treatment with RES normalized protein turnover and inhibited the protein degradation. In the literature, there have been debates over the proteogenic effects of RES through SIRT activation. Rathbone *et al.* (56) proposed that SIRT-1 stimulation enhances muscle precursor cells to proliferate, while Wyke *et al.* (57) proposed that RES inhibits the nuclear transcription factor nuclear factor- $\kappa$ B, which is an inductor of proteasome expression and proteolysis-inducing factor. Further studies on the different pathways regulated by RES during unloading are now clearly needed.

It is well known that muscle disuse due to tail suspension and human bed rest contributes to the development of insulin resistance (58, 59). It was hypothesized that insulin resistance affects protein metabolism in skeletal muscle and may contribute to the protein catabolism (58, 60). Stein and Wade (4) proposed that the alteration of muscle metabolism in HH is due to an increased availability of gluconeogenic precursors (amino acids) as a result of muscle disuse, independently of myosin profile. The results of the present study certainly confirmed that HH induced insulin resistance, as assessed from the fasting insulin to glucose ratio and the oral glucose tolerance test. The fact that only trends for insulin resistance were observed when using the insulin tolerance test might be explained by the higher IL6 levels. Indeed, a number of studies suggested that IL-6 contributes to blood glucose clearance (61, 62). Certainly RES was efficient in preventing the development of insulin resistance due to HH. RES is known to improve glucose tolerance, enhance oxygen consumption, and increase insulin sensitivity, notably through its effect on liver and skeletal muscle (11, 12). The muscle factors involved in the improvement in insulin sensitivity after HH are discussed in the following sections.

There is a tangled relationship between insulin sensitivity and muscle lipid metabolism, and insulin-resistant humans present a lower capacity for fat oxidation (63). Consistent with that picture, dietary palmitate clearance and oxidation were reduced during HH. These alterations are likely related to the lower mRNA expression of CPT-1 and FAT/CD36 but also lipoprotein lipases that were not measured in this study. RES treatment was efficient in fully preserving most of these changes. The positive effect of RES on all the key cofactors, transporters, and mitochondrial enzymes, and the preservation of type I fiber type, all involved in the partitioning of fat toward oxidation, likely contributed to the maintenance of insulin sensitivity. The studies conducted in rats and mice during high-fat trials confirmed those positive effects of RES (12, 13), although they showed more details of the molecular mechanisms, whereas our approach demonstrated the

physiological outcomes lacking from the molecular studies.

### Muscle function

In our study, the soleus muscle was atrophied after 15 d of suspension, and muscle strength was lost, as observed in many other studies on HH rats (64, 65). Numerous studies during human space missions and bed-rest studies showed that exercise or nutrition (amino acid supplementation) countermeasures do not fully protect muscle transition and atrophy (8, 66–68). Those studies show either an effect on mass or on strength parameters but rarely on both. RES supplementation alleviates losses in both soleus mass and strength as the contractile profile (time to peak, half relaxation time, and tetanic force  $P_{20}/P_0$ ) were maintained in HHR rats. Previous results from the literature support those effects. Feige *et al.* (13) showed that high-fat-diet mice treated with SRT1720 (analog to RES) improved their endurance capacity on a treadmill but also locomotor functions and muscle strength. Another study, Murase *et al.* (69), proposed that combination of RES and exercise would increase the endurance capacity to exhaustion. The maintenance of muscle strength could be explained by the ability of RES to preserve skeletal muscle fiber, probably through the maintenance of myosin phenotypes, but also muscle metabolic profile. HHR rats had the same myosin phenotypes as C rats and had a significantly higher proportion of type I and IIA fibers compared with HH rats. In support of that result, Lagouge *et al.* (12) showed that high-fat-diet mice treated with RES increased the expression of genes involved in skeletal muscle contraction, and the researchers suggested that RES influences the transition of glycolytic fiber type to a more oxidative type. This effect seems to involve the action of RES on PGC-1 $\alpha$  expression through SIRT-1. Indeed enhanced expression of PGC-1 $\alpha$  was shown to contribute to the maintenance of oxidative capacity characteristic of slow-twitch fiber type (70) and the prevention of skeletal muscle disuse atrophy in a denervated mouse model (71).

Further, RES maintained both skeletal muscle oxidative capacities during suspension. This is likely related to a better preservation of mitochondrial capacity and quantity. In support of that conclusion, we observed a lower decrease in gene expression of COXIV and CS and in the protein amounts of COXIV, SIRT1, and PGC-1 $\alpha$  in HHR rats compared with the HH group. The HH treatment reduced the mRNA expression of SIRT-1. SIRT-1 is proposed to enhance the activity of the genes participating in mitochondrial biogenesis, notably through PGC-1 $\alpha$  (12, 14, 72). We detected a reduction of mRNA of COXIV and CS but not PGC-1 $\alpha$  in HH rats, while this decrease was less important in HHR rats. In addition, the lower SIRT-1, PGC-1 $\alpha$  (contrary to mRNA), and COXIV protein levels strengthen the hypothesis of a lower mitochondrial respiratory capacity in HH but not in HHR rats. Enhanced mitochondria

biogenesis contributes to an increased uptake and utilization of substrates and respiration rates (73). Whether RES promotes PGC-1 $\alpha$  expression through SIRT-1 is still controversial. In a study by Barger *et al.* (74) on the retarding effects of RES on aging, PGC-1 $\alpha$  expression remained unchanged in mouse skeletal muscle. In our study, the expression of PGC-1 $\alpha$  was also not modified. This suggests that RES may prevent a drop in expression rather than induce overexpression. Taken together with a maintained expression of CPT1 in HHR rats, the results of the present study suggest that RES seems to prevent the deleterious consequences of HH mainly by preventing/boosting the deterioration/function of the mitochondria.

Whether cellular redox imbalance is a key trigger or a byproduct of unloading-induced muscle atrophy is still debated (74, 75). Pharmacological agents (vitamin E, trolox, allopurinol, Bowman-Birk inhibitor) have been proposed to limit muscle oxidative damage (76, 77). However, muscle benefits were not always related to their antioxidant activities. For example, long-term supplementation of the lipid-soluble antioxidant vitamin E partly prevented soleus muscle atrophy induced by 14 d of hindlimb unloading, but the protective effect of vitamin E was due to the modulation of muscle proteolysis-related genes rather than to its antioxidant function (78). Given the role of RES as a scavenger of hydroxyl, superoxide, and metal-induced radicals (79), the present data show that RES supplementation restored the decreased GSH/GSSG ratio, a biomarker of oxidative stress caused by unloading. Furthermore, a complex cytoprotective system including muscle enzymes scavenging ROS is recruited during hindlimb unloading (65, 69). SOD but also GPX activities increased in HH and even more in HHR rats, which is in line with recent results observed by Jackson *et al.* (80) in unloaded gastrocnemius muscles treated with RES (12.5 mg/kg/d).

### Bone function

Bone mass and density decrease especially in weight-bearing bones in long space missions. Bone structure normally will respond to application of mechanical force; thus, unloading enhances weakening of the bone. Accelerated bone loss is one of the major consequences of long-term spaceflight and is mainly exposed through the loss of the BMD (81). So far, the osteogenic stimulus from exercise countermeasures has been insufficient to maintain bone mass, probably due to insufficient dose of exercise (82). In addition, no convincing results were obtained from pharmaceutical agents such as vitamin K<sub>2</sub>, testosterone, or biophosphonates (82). Only one study (83) on HH showed that vitamin E supplements have a modest protective effect. In the past few years, RES was proposed as a protector against bone loss, notably due to estrogen-like effects (84). RES is also known to increase the osteogenic responses of osteoblasts (17, 74). Our findings extend those results by showing that HHR rats were able to




maintain BMD in tibia and femur but also the breaking load.

### Dosing issue for translational studies in humans

The outcomes of this study permit classification of RES as an efficient countermeasure against the overall metabolic and functional consequences of mechanical unloading and leave us with the obvious question: that is, whether we can apply an equivalent dose of RES to humans during spaceflight. Using the average body surface area of humans and the constant of Michaelis-Menten for RES in rats and humans, we calculated that the dose of 400 mg/kg in rats is equivalent to a dose of 65 mg/kg in humans (85). For an adult of 70 kg, such a dose would result in a daily intake of 4.5 g. Recently, la Porte *et al.* (86) provided healthy humans with a dose of 2 g of RES 2×/d and did not report any adverse effects. Similarly, Boocock *et al.* (87) did not report serious adverse effects after a single dose of 5 g in healthy humans. However, the limited number of subjects studied in these two studies does not permit conclusions on the toxicity of RES. Furthermore, the effects of chronic administration of RES in humans still need to be investigated.

### CONCLUSIONS

In contrast to most investigations published to date on the effectiveness of countermeasures against unloading, our results support RES as a valid countermeasure against most of the metabolic and functional adaptations to mechanical unloading, likely by acting as an exercise mimetic. Further investigations are necessary to validate the beneficial effects of RES in bedridden humans. Those results further present obvious interest for sedentary populations and bedridden patients. 

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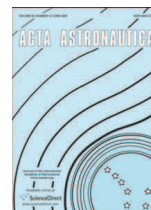
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*Annexe 2 : A combination of whey protein  
and potassium bicarbonate supplements  
during head-down-tilt bed rest: Presentation  
of a multidisciplinary randomized controlled  
trial (MEP study).*



## Invited Paper

# A combination of whey protein and potassium bicarbonate supplements during head-down-tilt bed rest: Presentation of a multidisciplinary randomized controlled trial (MEP study) <sup>☆</sup>



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## ABSTRACT

Inactivity, as it appears during space flight and in bed rest, induces reduction of lean body and bone mass, glucose intolerance, and weakening of the cardiovascular system. Increased protein intake, whey protein in particular, has been proposed to counteract some of these effects, but has also been associated with negative effects on bone, likely caused by a correspondingly high ratio of acid to alkali precursors in the diet.

The main hypothesis of the presented cross-over study (MEP study) was that supplementing high protein intake (1.2 g/kg body weight/d plus 0.6 g/kg body weight/d whey protein) with alkaline salts (90 mmol potassium bicarbonate/d) will maintain lean body mass during bed rest without increasing bone resorption. A 21-day head-down-tilt bed rest study was performed to examine several physiological systems in a multidisciplinary approach. Ten healthy men (age:  $31 \pm 6$  years; body weight:  $76.5 \pm 5.6$  kg) were randomly assigned to the dietary countermeasure or isocaloric control first, one test subject randomized to the dietary countermeasure first dropped out after the first campaign.

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<sup>☆</sup> This reference paper documents and discusses standardized study conditions, as well as implementation and acceptance of the nutritional intervention.

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## 1. Introduction

Gravity virtually affects all physiological systems of the human body. Major impairments observed in weightlessness are cardiovascular decrements as well as bone and muscle loss [1,2]. To meet the daunting challenge of keeping space travelers healthy on long-term missions to the ISS and potential exploration missions beyond low Earth orbit, space agencies often resort to using ground-based analogs of space flight to simulate the physiological adaptations in microgravity and to test potential countermeasures, such as

artificial gravity, exercise, dietary supplements, and pharmaceuticals. The head-down-tilted bed rest model (HDTBR) is regarded as a gold standard for these types of studies [3]. An ideal countermeasure to microgravity-associated adaptations would allow the simultaneous preservation of muscle mass and function and of locomotion, cardiovascular fitness, and bone mass, along with the prevention of metabolic consequences due to changes in body composition and the endocrine milieu. This multisystem approach underlines the importance of multidisciplinary countermeasure research. To optimize cost-effectiveness, scientific research is performed with a relatively small number of subjects, which requires minimizing any confounding factors and thus highly standardizing study conditions.

So far HDTBR-tested exercise regimes alone have failed to fully counteract changes in the musculoskeletal system [4–7]. Supplemented nutritional intake has been proposed to enhance the anabolic effects of exercise, and protein and amino acid intake have been a key focus of these proposals. The reason for studying protein and amino acids is that supplementation of essential amino acids has been shown to acutely stimulate muscle protein synthesis in bed rest, and supplementation of branched-chain amino acids (BCAAs) led to improved nitrogen retention during bed rest [8] as well as recovery from the same [9]. Whey protein in particular contains high amounts of BCAAs and offers comparatively fast digestion and absorption kinetics [10]. Hence, whey protein promotes postprandial protein synthesis during bed rest [11] as well as insulin sensitivity in ambulatory but obese subjects [12]. Protein and glucose metabolism affecting properties of whey protein have been reviewed recently [13]. On the other hand – if it is not compensated by adequate intake of alkali precursors – high intake of animal protein contributes to systemic acidity through metabolism of sulfur-containing amino acids [14,15] and thus may increase bone demineralization to buffer the induced acid load [16–18]. Indeed, evidence from cohort studies shows that bone loss at the femoral neck, as well as forearm and hip fractures, are associated with high intake of animal protein [19,20]. Intervention studies show differences in calcium excretion related to the level of protein intake [21,22]; an increase in dietary protein of 1 g has been related to additional renal calcium losses of 1.6 mg [23]. Bed rest studies showed that the ratio of animal protein to potassium intake, used as an index of the ratio of acid to alkali precursor consumption, also correlates with markers of bone resorption [24]. Therefore, combination of a whey protein supplement with an alkalizing agent, such as potassium bicarbonate, seems reasonable to prevent the described detrimental effect of protein supplementation on bone turnover.

The study presented here, sponsored by the European and German Space Agency, consisted of 19 experiments examining the influences of whey protein supplementation combined with alkaline salt administration as a potential nutritional countermeasure in multiple physiological systems. In this paper we present the implementation of the nutritional countermeasure, describe the standardized study conditions, the acceptance of and compliance with the intervention, and discuss aspects of cross-over designed bed rest studies in light of our experience.

## 2. Methods

### 2.1. Study design

The present study (Medium-term bed rest whey protein, acronym: MEP, ClinicalTrials.gov Identifier: NCT01655979) tested the effects of combined supplementation of 0.6 g whey protein (WP)/kg body weight (BW) and 90 mmol potassium bicarbonate (KHCO<sub>3</sub>) on several physiological systems during 21 days of head-down-tilt bed rest (HDTBR) in 10 healthy male test subjects (Table 1). The study was conducted in a classical cross-over design (Fig. 1), and the test subjects were randomized 1:1 to the sequences Control first and WP+KHCO<sub>3</sub> first. In each campaign (MEP-1 and MEP-2) the subjects were confined to the metabolic ward of the German Aerospace Center (DLR) for 7 days of environmental and dietary adaptation, 21 days of HDTBR, and 6 days of stationary recovery. Subjects were discharged from the metabolic ward after the 6 days of reambulation, but returned 14 and 28 days after completion of HDTBR for follow-up measurements. The first campaign was conducted in September and October 2011; the second campaign was conducted in February and March 2012.

### 2.2. Ethics and funding

The study protocol was approved by the ethics commission of the Aerztekkammer Nordrhein (Duesseldorf, Germany) and the study was conducted in accordance with ethical principles stated in the Declaration of Helsinki. Test subjects were exposed to X-radiation (peripheral quantitative computed tomography, dual-energy X-ray absorptiometry measurements) in the screening process as well as during the study. The exposure was approved by the Federal Authority of Radiation Protection (Bundesamt für Strahlenschutz, Germany). Written informed consent to the study conditions was obtained from all test subject candidates.

The study was funded by the European Space Agency and the German Aerospace Center. All contributing scientific parties organized funding for performance of their experiments independently.

**Table 1**  
Test subject characteristics at study entrance.

	Per protocol n=9	Intention to treat n=10
<b>Age</b> (Years)	31 ± 6	32 ± 6
<b>Height</b> (cm)	180.6 ± 5.8	180.6 ± 5.8
<b>Weight</b> (kg)	76.5 ± 5.6	76.1 ± 5.5
<b>25-OH-D</b> (µg/L)	28.42 ± 3.76	28.44 ± 3.54
<b>VO2max</b> (l/min)	4.03 ± 0.58	4.06 ± 0.61
<b>Activity levels</b>		
<b>MET score</b>	113 ± 42	110 ± 40
<b>kcal</b>	1250 ± 535	1214 ± 517
<b>Step count</b>	10119 ± 3202	9807 ± 3176
<b>PAT*</b>	111 ± 52	108 ± 51
<b>Summed activity*</b>	10067 ± 6767	9675 ± 6681

Data are presented as mean ± SD. MET=metabolic rate for specific activity/ resting metabolic rate; PAT=physical activity time.

\* n=7.

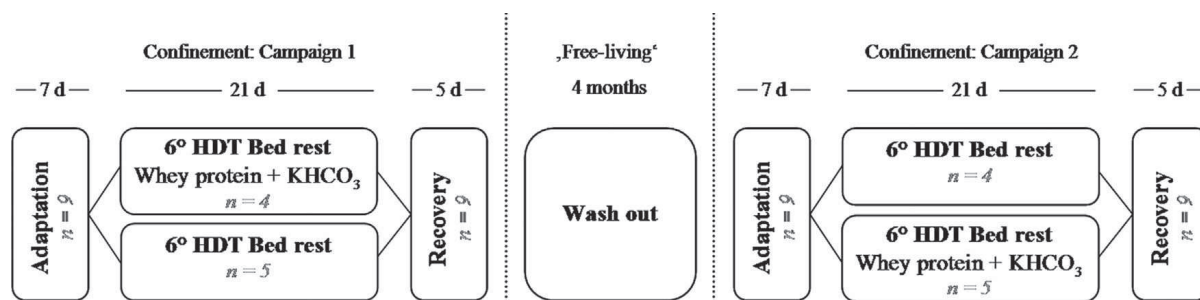


Fig. 1. Study design. HDT= head-down-tilt.

### 2.3. Study outcomes

An interdisciplinary set of 19 experiments was established with the recommendations of a board of nutrition experts. The primary outcome measurement for the study was change in body composition; secondary outcomes were manifold and included changes in body weight; acid base balance; fat-, glucose-, and energy metabolism; nitrogen balance; oxidative stress; glucocorticoid activity; the hematopoietic system; bone metabolism; bone mineral density and content; fat accumulation in bone marrow; muscle metabolism; muscle free water and fat content; muscle fatigue and volume; isometric torque; standing balance; locomotion; cartilage metabolism and thickness; Achilles tendon structure; plasma volume; maximum volume of oxygen uptake; orthostatic tolerance; sympathetic activity and plasma galanin and adrenomedullin during orthostatic stress; visual orientation; intracranial pressure; and headache.

Most of the related measurements were conducted in the facilities of the DLR, but some required transportation to local hospitals. All experiment settings were implemented under standardized conditions, and the proceedings were identical in both study campaigns.

### 2.4. Subject recruitment and screening

Volunteers were recruited by mailings and Internet advertisements. The selection process started 6 months before the study onset and included 5 stages: a general prescreening by telephone ( $n=195$ ); a detailed information session followed by a questionnaire-based psychological prescreening ( $n=67$ ); a medical screening including an intensive medical history, a physical examination, an eye examination, an orthostatic tolerance test, a resting electrocardiogram (ECG), a spiroergometry test (including stress ECG), blood and urine biochemistry, analysis of vitamin status, nicotine and drug screening ( $n=38$ ), a psychological interview ( $n=22$ ), and a dual-energy X-ray absorptiometry scan ( $n=18$ ).

The target population was healthy, nonsmoking men of average weight (body mass index 20–25 kg/m<sup>2</sup>), aged between 20 and 45 with a maximum relative oxygen uptake of 30–60 ml kg<sup>-1</sup> min<sup>-1</sup>. The following exclusion criteria were defined: drug consumption, alcohol excess, vegetarianism, claustrophobia, porphyria, blood dyscrasia, Achilles tendon injury, cruciate ligament fracture, any fracture within 1 year before study onset, femur and lumbar spine bone mineral density 1.5 SD  $\leq$  T-score, severe

orthostatic intolerance, kinetosis, hiatal hernia, hyperlipidemia, renal insufficiency, anemia, infectious diseases, intraocular hypertension, as well as any history of psychiatric diseases, migraine, muscle/cartilage/joint diseases, herniated disc, chronic back pain, diabetes, rheumatism, renal stones, hyper/hypothyroidism, hyper/hypouricemia, hyper/hypocalcemia, hyperhomocysteinemia. Any nutrient deficiency was also defined as an exclusion criterion. As iron or folic acid insufficiency (Hb < 13.5 g/dL, transferrin saturation < 15%, or ferritin < 11 mg/dL, folic acid < 5.4 ng/ml) appeared very often at the initial screening (15 out of 22), candidates were advised to supplement iron or folic acid, respectively. Levels were checked again when subjects entered the lab, and subjects were allowed to start the study only if their iron or folic acid levels were sufficient. Because subjects would be exposed to 21 days of immobilization, negative results of a thrombophilia screening panel (ATIII, Protein C and S, F-V-Leiden, Prothrombinmut, Lupus-PTT) were mandatory for final inclusion in the study. A total of 14 participants successfully completed all screening stages.

### 2.5. Test subjects

Ten healthy male test subjects were enrolled to participate in the study. One test subject randomized to WP+KHCO<sub>3</sub> first discontinued the study for medical reasons.

### 2.6. Evaluation of baseline characteristics

#### 2.6.1. Habitual nutrient intake

Habitual nutrition was assessed by 7-day food diaries before the start of the first study campaign. All entries were analyzed using the computer-based calculation software PRODI 4.2 (Nutri-Science GmbH, Hausach, Germany) (Table 2).

#### 2.6.2. Habitual activity levels

To evaluate whether habitual physical activity levels of the test subjects were comparable before both campaigns, physical activity was assessed by self-reported questionnaires, step counters, and accelerometers. Each volunteer filled out the "Freiburger questionnaire of physical activity" [25] roughly 1 month before the start of each campaign. The validated original questionnaire contains 12 questions concerning the type, frequency, and duration of various activities in daily life, leisure time, and sport over the last week or month. Items 9–12 (questions about

**Table 2**  
Habitual daily nutrient intake.

	Mean daily intake	Intersubject variability (SD)	Intrasubject variability (SD)
Energy (kcal/d)	2573	1014	626
Protein (g/d)	114	69	33
Protein (g/kgBW/d)	1.5	0.8	0.4
Protein (E%)	17	4	3
Fat (g/d)	97	42	38
Fat (E%)	34	6	7
Carbohydrates (g/d)	294	100	76
Carbohydrates (E%)	50	8	7
Fiber (g/d)	33	19	18
Fluid (ml/d)	2379	1758	567
Calcium (mg/d)	1592	666	495
Potassium (mg/d)	3135	2524	1068
Sodium (mg/d)	6	5	6
Magnesium (mg/d)	442	162	130
Phosphorus (mg/d)	1718	1274	581

Data are presented as mean  $\pm$  SD ( $n=9$ ). BW=body weight.

relaxation times and sleep duration) were omitted in the present study, as they were not directly related to physical activity [26]. The different activities filled in by the subjects were subsequently coded into metabolic equivalents (MET) according to Ainsworth et al. [27] and reference values provided by the U.S. National Cancer Institute [28]. A MET is defined as the ratio of the associated metabolic rate for a specific activity divided by the resting metabolic rate, which is by definition 1 MET. Accordingly, every activity-specific MET value was multiplied by the individual weekly amount of time spent on the activity. To assess the additional energetic cost of the physical activity, resting MET scores were subtracted from the total MET score, and a mean daily MET score was constructed.

To additionally quantify daily steps and intensity of activity, digital step counters (Yamax Digiwalker SW200) and 3-axis digital accelerometers (X-16 A, Gulf Coast Data Concepts, USA) were used for 7-day phases roughly 1 month before the start of each campaign. Each subject received individual marked devices, which continuously recorded from 5:30 am to 11:00 pm (accelerometry: 20 Hz). The accelerometer was taped horizontally to a stretch belt and centered anteriorly on the sagittal plane. Each subject received a logbook and was instructed to record specific activities or events that could be taken into account during the analysis of the recordings. The data were resampled to 8 Hz with the Java-based software program “XLR8R” provided by Gulf Coast Data Concepts. To assess physical activity, all data were then converted from voltage to acceleration by dividing each data point by 1024. The resultant acceleration ( $G_R$ ) for each time point was calculated as  $G_R = \sqrt{G_x^2 + G_y^2 + G_z^2}$ . Subsequently 1 G (representing zero activity) was subtracted from the resultant accelerations. Data points with delta G-levels that ranged from  $-1$  to  $0$  G, and with delta G-levels that exceeded  $0$  G, were classified as physical activity. As we wanted to have an estimate for the duration and “intensity” of physical activity, in terms of G-load, the negative delta G-levels were converted to positive values. To limit the possibility of erroneously accrediting all delta accelerations, only G-levels exceeding  $0.05$  G were classified as

physical activity. Physical activity time (PAT, [min]) was calculated from the number of data points representing physical activity ( $N$ )/8/60. Total daily activity was calculated as the sum (area under the curve) of the G-level histogram. Results of step counts were entered into logbooks at the end of each day. Mean daily values were subsequently constructed for comparisons.

### 2.6.3. Standardization of vitamin D status

Circulating 25-hydroxyvitamin D (25-OH-D) levels of  $20$ – $30$   $\mu\text{g/L}$  are regarded as adequate to prevent the risk of osteomalacia [29,30]. In the northern European population, 25-OH-D levels not only are often insufficient, but also vary widely [31]. This heterogeneity in vitamin D status before a study starts can confound the precise analysis of the effects of nutritional intervention during bed rest on various physiological systems, such as bone metabolism. To ensure adequate and thus more homogeneous baseline levels of 25-OH-D in all test subjects, we recommended daily vitamin D3 supplementation  $8$  and  $4$  weeks before the start of the first and the second campaign, respectively. Total doses were calculated on an individual basis according to Van Groningen et al. [32], taking body weights and initial 25-OH-D3 levels into account: total loading dose (IU) =  $40 \times (75 - 25\text{-OH-D3 (nmol/L)}) \times \text{body weight (kg)}$ . Daily doses were calculated and were  $1500 \pm 800$  IU before the first campaign and with respect to the shortened timeframe ( $8$  weeks vs.  $4$  weeks) were  $3100 \pm 1100$  IU before the second campaign. Serum 25-OH-D2/3 and  $1,25\text{-(OH)}_2\text{-D2/3}$  have been analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS, Waters, Eschborn, Germany).

### 2.7. Metabolic ward conditions

The subjects were encouraged to keep a constant day and night cycle during the entire study, and they were verifiably awake for  $16$ – $17$  h and advised to sleep for  $7$ – $8$  h during the night. Ward lights were turned off from  $11$  pm to  $6$  am. Temperature and humidity inside the metabolic ward were controlled during the study ( $21.5 \pm 1$  °C,  $40 \pm 6.3\%$ ).

#### 2.7.1. Activity levels during stationary adaptation

During the adaptation and recovery periods, the subjects were free to walk around inside the metabolic ward. All daily activities such as reading, eating, and watching television were performed in a seated position with both feet on the ground. Activity levels were assessed by digital step counters and accelerometry (cf. 2.6.2 Habitual Activity Levels) from  $6:30$  am– $11:00$  pm.

#### 2.7.2. 6° Head-down-tilt bed rest

During the intervention periods, subjects were constrained to HDTBR  $24$  h/day. All activities of daily living such as eating, body weight measurements, and hygienic procedures took place in bed. For any measurements performed outside the metabolic ward or that area of the DLR, transportation was arranged in a head-down-tilt position. The subjects were allowed to change their position horizontally, as long as at least one shoulder remained



in contact with the mattress. Muscular activity of the lower extremities was prohibited. For tension release a passive physical therapy was included regularly at 3- to 4-day intervals.

Adherence to the study rules was controlled by study nurses. Additionally, compliance with the requirements of bed rest was verified by 24 h/day video monitoring, with random real-time control.

### 2.7.3. Medical care

Blood pressure, heart rate, and body temperature were measured daily, and clinical safety parameters were measured at the start of confinement, after 10 days of HDTBR, and on the last day of confinement. All safety parameters were assessed by an independent medical doctor who monitored the subjects' health status during daily ward rounds and was responsible for documentation of adverse events according to good clinical practice. Medications prescribed during the study were mild antiemetics (metoclopramide, Dorithricin; two subjects in MEP-1) and analgesics (paracetamol, ibuprofen; 3 subjects in MEP-1 and one subject in MEP-2). To benefit the well-being of the test subjects, psychological support was provided around the midpoint of the bed rest period, when stress tends to peak.

## 2.8. Sample collection

### 2.8.1. Blood and urine sampling

Fasting (9 h) blood samples were collected shortly after awakening, with the subject in the supine or head-down-tilt position, according to study phase. Samples were taken from an antecubital vein through a short catheter into provided tubes that met the requirements of the respective experiments (Monovetten<sup>®</sup>, Sarstedt, Germany; Vacutainer<sup>®</sup>, BD, USA). The pre-analytic processing of the samples was realized in accordance with the requirements of the respective experiments. In each campaign a total of 630 ml blood was collected from each test subject.

Urine was collected void by void, stored at 4 °C, and pooled in the laboratory for 24-h collection periods.

### 2.8.2. Urinary pH

The pH of 24-h urine pools was measured on given study days with a pH-sensitive electrode (inoLab pH720; WTM, Weilheim, Germany).

### 2.8.3. Body weight measurements

Body weight was measured each morning in the fasted state (electronic scale, IS150IGG-S0CE, Sartorius, Göttingen, Germany) in the head-down-tilt or seated position after the first void of the day, and after any blood draws. Subjects were asked to exhale and hold the breath during the measurement.

## 2.9. Diet

Diet composition followed the requirements given by a standardization document of ESA ("Standardization of bed rest study conditions" Version 1.5) to compare results from all human ground-based and flight studies.

During both stationary study campaigns the test subjects received an individually tailored and strictly controlled diet with relatively constant nutrient intake from day to day, to avoid any impact of nutrient supply on study outcomes. On the first day in the ward, resting metabolic rate (RMR) was computed by indirect calorimetry (Delta-trac II MBH 200 Metabolic Monitor, Datex Ohmeda, Madison, Wisconsin, USA). To account for the low physical activity level during the adaptation and recovery periods, the number of calories provided was based on 160% of RMR (RMR, plus 0.5 RMR for activity level, and 0.1 RMR for diet-induced thermogenesis), whereas during the bed rest period this was reduced to 120% of RMR (0.1 RMR for activity, and 0.1 RMR for diet-induced thermogenesis) [33]. Methylxanthine derivatives (e.g., caffeine), alcohol, and flavor enhancers were prohibited. Intake of all nutrients at least matched the Recommended Dietary Allowances (RDAs) [34] and was strictly controlled by tight tolerance limits for each nutrient to reduce variance. The average nutrient intake is documented in Table 3. As the ward is underground, subjects were excluded from sunlight during the study campaigns and were supplemented with 1000 IU vitamin D3/day during both campaigns. Compliance was supervised by study nurses. As the contribution of dietary vitamin D to 25-OH-D levels is regarded as negligible for people consuming Western diets [35], dietary vitamin D intake was not controlled.

Individual menus for each test subject were determined with the nutritional calculation software PRODI (Nutri-Science GmbH, Germany). To avoid discrepancies in nutrition facts of processed foods, these were either made in house (bread, sauces) according to the method of weighed intake [36] to account for water losses, or provided by a local manufacturer (meat, fish) (Apetito AG, Rheine, Germany) with accurate nutrition facts, or chemically analyzed (ham, cheese). All meals were prepared in the metabolic kitchen according to the method of weighed intake [36] with an accuracy of 0.01 for NaCl and 0.5 g for all other foods. Subjects received six meals per day at 8 am, 10:30 am, 1 pm, 4 pm, 7 pm, and 9:30 pm; the majority of energy intake was given with the main meals (8 am, 1 pm, 7 pm). If experiment conditions required a fasted state, energy and nutrient intake were transferred to the remaining meals. To compensate for energy and water loss following physically demanding experiments additional fluid and energy intake was administered in the form of water and diluted-apple juice. Energy loss was calculated based on oxygen uptake; water loss was metered from body weight changes.

## 2.10. Intervention

The intervention of the presented study was a combined supplementation protocol of 0.6 g whey protein/kg BW/d and 90 mmol of KHCO<sub>3</sub>/d. The whey protein isocalorically replaced fat and carbohydrates in a ratio of 1:1. To distribute the load over the day, the daily doses were divided into 6 subdoses (6 × 0.1 g WP/kg BW/d + 15 mmol KHCO<sub>3</sub>) and given with each meal. If experiment conditions required a fasted state, the doses were allocated to the remaining meals. All subjects strictly adhered to the



**Table 3**

Daily nutrient intakes throughout the study.

	Adaptation (Mean Campaign 1/2)	6° HDT bed rest control	6° HDT bed rest whey protein + KHCO <sub>3</sub>	Recovery (mean campaign 1/2)
Energy (kcal/d)	2698 ± 209	2029 ± 178	2028 ± 124	2693 ± 205
Protein (g/d)	94 ± 6	94 ± 7	136 ± 8	93 ± 6
Protein (g/kgBW/d)	1.21 ± 0.01	1.23 ± 0.01	1.79 ± 0.02	1.22 ± 0.01
Protein (% TEE/d)	14 ± 1	19 ± 2	28 ± 2	14 ± 1
Fat (g/d)	86 ± 7	65 ± 6	54 ± 4	86 ± 7
Fat (% TEE/d)	30 ± 0	29 ± 0	25 ± 0	30 ± 0
Carbohydrates (g/d)	369 ± 35	255 ± 29	236 ± 22	369 ± 34
Carbohydrates (% TEE/d)	56 ± 1	51 ± 2	48 ± 2	56 ± 1
Fiber (g/d)	34 ± 1	34 ± 2	36 ± 2	33 ± 1
Fluid (ml/d)	3856 ± 254	3853 ± 292	3860 ± 227	3856 ± 254
Fluid (ml/kgBW/d)	49.9 ± 0.4	50.8 ± 0.5	50.1 ± 0.6	50.6 ± 0.6
Calcium (mg/d)	1042 ± 23	1070 ± 32	1232 ± 24	1079 ± 34
Chloride (mg/d)	6240 ± 400	6241 ± 449	6094 ± 281	6271 ± 415
Potassium (mg/d)	3554 ± 66	3526 ± 149	3785 ± 104	3557 ± 125
Sodium (mg/d)	3834 ± 268	3895 ± 316	3861 ± 237	3826 ± 291
Sodium (mmol/kgBW/d)	2.16 ± 0.02	2.23 ± 0.03	2.21 ± 0.03	2.18 ± 0.04
Magnesium (mg/d)	463 ± 13	432 ± 26	487 ± 31	451 ± 17
Phosphorus (mg/d)	1609 ± 24	1623 ± 72	1709 ± 64	1644 ± 50
Iron (mg/d)	17.6 ± 0.8	15.3 ± 1.3	15.8 ± 0.6	17.2 ± 1.0
Fluoride (μg/d)	3252 ± 147	3192 ± 283	3290 ± 164	3304 ± 177
Iodide (μg/d)	209 ± 10	208 ± 19	211 ± 11	212 ± 14
Zinc (mg/d)	12.4 ± 0.3	12.0 ± 0.5	11.0 ± 0.4	12.2 ± 0.5
Copper (μg/d)	2054 ± 66	1873 ± 137	1957 ± 83	1967 ± 64
Biotin (μg/d)	64.4 ± 2.0	65.2 ± 3.6	68.1 ± 3.8	64.8 ± 4.0
Folic acid (μg/d)	1012 ± 37	928 ± 79	1056 ± 42	1049 ± 38
Niacinequivalent (μg/d)	37114 ± 1876	36678 ± 3167	39667 ± 2705	38593 ± 2223
Pantothenicacid (mg/d)	6.2 ± 0.1	6.5 ± 0.4	7.2 ± 0.4	6.3 ± 0.3
Retinolequivalent (μg/d)	1718 ± 151	2267 ± 248	2263 ± 236	2222 ± 202
Vit B1 (mg/d)	2.0 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	2.0 ± 1.0
Vit B12 (μg/d)	3.9 ± 0.4	4.3 ± 0.4	4.1 ± 0.2	4.7 ± 0.5
Vit B2 (mg/d)	1.9 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	1.9 ± 0.1
Vit B6 (mg/d)	2.4 ± 0.1	2.2 ± 0.2	2.3 ± 0.1	2.4 ± 0.1
Vit C (mg/d)	195 ± 9	191 ± 16	221 ± 8	204 ± 20
Vit E (mg/d)	18.1 ± 1.6	16.1 ± 3.1	18.0 ± 2.8	18.9 ± 2.4
Vit K (μg/d)	179 ± 10	196 ± 12	196 ± 12	180 ± 11
PRAL (mEq/d)	15 ± 3	13 ± 1	26 ± 2	17 ± 2

Data are presented as mean ± SD, *n*=9. Energy intake matched total energy expenditure (TEE). HDT=head-down-tilt, BW=body weight, PRAL=potential renal acid load of the diet.

supplementation protocol and the given doses. Compliance with the respective diet was supervised by study nurses.

Nutrient information about the selected whey protein isolate (Diaprotein<sup>®</sup>, Dr. Steudle, Linden, Germany), determined by an independent lab (Eurofins Analytis, Cologne, Germany), is presented in Table 4. Content description is based on true analysis except that energy and carbohydrate content refer to approximate analysis. To neutralize the bitter taste of the protein, the powder was solved in yoghurt, cream, or water and enriched with mixed fruit or herbs. The potassium bicarbonate was provided by a local manufacturer (Krueger GmbH, Bergisch-Gladbach, Germany) in effervescent form. The tablets were dissolved in 100 ml of tap water immediately before the meal was served (mineral content of tap water [mg/100 g]: Ca<sup>2+</sup>: 7, Mg<sup>2+</sup>:1, Cl<sup>-</sup>:3, Na<sup>+</sup>:2, K<sup>+</sup>:0, P: 0).

With respect to the very high protein intake of the WP+KHCO<sub>3</sub> group and an increasing satiety with protein compared to carbohydrates or fat [37,38], tolerance of the supplement was evaluated by use of subjective equilateral rating scales. After dinner the subjects filled out a visual analog scale to assess their satiety and feelings of nausea. Provided in its native matrix (i.e., dairy products), the

**Table 4**

Whey protein isolate.

Intake (g/d)	46.3 ± 2.8
Energy (kcal)	384.4
Protein (g)	85.4
Fat (g)	0.2
Carbohydrates (g)	7.9
Fluid (ml)	3.8
Calcium (mg)	540
Potassium (mg)	450
Sodium (mg)	190
Magnesium (mg)	95
Chloride (mg)	< 10
Phosphorus (mg)	220
PRAL (mEq)	22.1

Mean intake ± SD (*n*=9) and nutrient content (in 100 g). PRAL=Potential renal acid load of the diet.

whey protein isolate did not cause any reported feelings of nausea, and despite the high protein content of the diet, satiety did not reach the upper limit of the rating scale: on a subjective equilateral rating scale from 1 to 10, the mean

feeling of nausea was 0.4 with an intra- and intersubject variability of 0.6. Mean satiety was documented as 4.8 on a subjective equilateral rating scale from 1 to 6, with an intrasubject variability of 0.4 and an intersubject variability of 1.3. Judging by results from previous studies [39–41], we did not expect any adverse reactions to the  $\text{KHCO}_3$  supplementation. For safety reasons serum potassium levels were checked 10 days after the intervention started, but no hyperkalemia was observed.

### 2.11. Dietary acid load

Potential renal acid load of the diet (PRAL) was calculated as  $0.268\text{Cl}^- + 0.0366\text{PO}_4^{3-} + (0.488 \times 10^{-3}) \times \text{Protein} - 0.0413\text{Na}^+ + 0.0205\text{K}^+ + 0.063\text{Mg}^{2+} + 0.0155\text{Ca}^{2+}$  according to Remer [14]. The model was modified with respect to different absorption rates of calcium during bed rest [42]. The control group received a basic diet with a protein content based on habits of astronauts and the Western terrestrial population [43–45], but still higher than the typical recommendations (1.2 g protein/kg body weight/day) [34]. The PRAL was moderately acidifying ( $13 \pm 1$  mEq/d), according to findings from common omnivorous diets [46]. The mean PRAL of the diet during  $\text{WP} + \text{KHCO}_3$  was  $26 \pm 2$  mEq/d (Table 4), of which the whey protein supplement generated a net acid load of 24.5 mEq/100 g ( $75 \text{ kg} \triangleq 45 \text{ g WP/d} \triangleq 11 \text{ mEq PRAL/d}$ ). On the contrary, with an average absorption of 80%, the PRAL of 90 mmol  $\text{KHCO}_3/\text{d}$  amounts to  $-72$  mEq/d. Accordingly the overall PRAL of the diet during  $\text{WP} + \text{KHCO}_3$  was  $-46$  mEq/d. The net difference in overall dietary acid load between Control and  $\text{WP} + \text{KHCO}_3$  was  $-59$  mEq/d.

### 2.12. Statistical analysis

Statistics were calculated with the software STATISTICA (StatSoft, Germany). All results are presented as means  $\pm$  SD of 9 test subjects who finished the protocol unless otherwise noted. Consistency of baseline vitamin D status, body weight, and physical activity (based on questionnaires) between campaigns (MEP-1 vs. MEP-2) and treatment groups (Control vs.  $\text{WP} + \text{KHCO}_3$ ) was evaluated by Student's paired *t*-test for dependent samples. For evaluation of all other data a general linear model was used. To compare habitual and stationary step count and accelerometry data between study campaigns and treatment groups, categorical predictors “study campaign” (MEP-1 vs. MEP-2) and “treatment groups” (Control vs.  $\text{WP} + \text{KHCO}_3$ ) were used. For comparison of body weight during the two study campaigns, “treatment” (Control vs.  $\text{WP} + \text{KHCO}_3$ ) and “study phase” (Adaptation vs. HDTBR vs. Recovery) were the categorical predictors analyzed. When differences in “study phase” occurred, a post hoc test (Tukey) was performed to differentiate between the three study phases. To analyze the extent of alkalization during  $\text{WP} + \text{KHCO}_3$ , “treatment” (Control vs.  $\text{WP} + \text{KHCO}_3$ ) was analyzed as a categorical predictor for urinary pH. Differences between means were considered statistically significant when  $p < 0.05$ .

## 3. Results

### 3.1. Baseline characteristics

Baseline characteristics are presented in Table 1 for the 10 test subjects who started the study, and the 9 test subjects who finished the protocol.

#### 3.1.1. Habitual nutrient intake

Habitual nutrient intake was very heterogeneous (Table 2, intersubject variability and intrasubject variability), and no statistically significant differences were detected. In general mean fat and protein intake seemed to be greater under free-living conditions than in the ward (cf. 2.8 Diet, Table 3), whereas the relative proportion of carbohydrate was less.

#### 3.1.2. Habitual activity levels

The mean daily MET score before the first study campaign was significantly higher than the score before the second ( $113 \pm 42$  vs.  $83 \pm 32$ ,  $p=0.026$ ). Expressed in daily kilocalories expended on physical activity, this accounts for  $1250 \pm 534$  kcal and  $914 \pm 345$  kcal, respectively ( $p=0.034$ ). The step counts were also significantly higher before the first study campaign ( $10119 \pm 3202$  vs.  $7252 \pm 3212$ ,  $p < 0.001$ ). Accelerometry data were analyzed for  $n=7$  due to hardware failure. No differences in physical activity time were observed before the campaigns ( $111 \pm 53$  vs.  $85 \pm 60$  min ( $p=0.204$ ), although the summed activity before the second campaign tended to be less than the activity before the first ( $7824 \pm 4593$  G vs.  $10067 \pm 6768$  G,  $p=0.073$ ). However, no differences in baseline levels between the treatment groups were observed in any measure of activity levels (MET score: Control,  $98 \pm 47$ ;  $\text{WP} + \text{KHCO}_3$ ,  $98 \pm 32$ ;  $p=0.957$ ; kcal: Control,  $1090 \pm 580$ ;  $\text{WP} + \text{KHCO}_3$ ,  $1075 \pm 362$ ;  $p=0.937$ ; step count: Control,  $8967 \pm 3496$ ;  $\text{WP} + \text{KHCO}_3$ ,  $8334 \pm 2717$ ;  $p=0.678$ ; PAT: Control,  $95 \pm 38$  min;  $\text{WP} + \text{KHCO}_3$ ,  $109 \pm 69$  min;  $p=0.278$ ; summed activity: Control,  $8197 \pm 4155$  G;  $\text{WP} + \text{KHCO}_3$ ,  $9478 \pm 6937$  G;  $p=0.307$ ).

#### 3.1.3. Vitamin D status

Individualized vitamin D supplementation before the first campaign increased pre-study 25-OH-D3 levels from  $20.7 \pm 8.2$  to  $28.4 \pm 3.8$   $\mu\text{g/L}$  within 8 weeks ( $p=0.033$ ). Simultaneously levels of 1,25-(OH) $_2$ -D3 tended to be higher ( $34.7 \pm 13.9$  vs.  $50.0 \pm 10.0$  ng/L;  $p=0.062$ ) ( $n=7$ , due to missing samples). Contribution of D2 metabolites was negligible. When the same approach was used within a shortened timeframe before the start of the second campaign, baseline 25-OH-D3 levels were significantly higher than at the start of the first campaign ( $33.3 \pm 2.2$  vs.  $28.4 \pm 3.8$   $\mu\text{g/L}$ ,  $p=0.006$ ). However, this difference is considered of minor relevance. As targeted at the start of both study campaigns, vitamin D levels were in the range of 20–30  $\mu\text{g/L}$ , to avoid any confounding influence of vitamin D insufficiency. There was no difference in 25-OH-D3 baseline levels between the treatment groups (Control,  $29.2 \pm 1.2$   $\mu\text{g/L}$ ;  $\text{WP} + \text{KHCO}_3$ ,  $32.5 \pm 2.9$   $\mu\text{g/L}$ ;  $p=0.106$ ).

### 3.1.4. Body weight

No differences in baseline body weight were apparent between the two study campaigns (MEP-1,  $76.5 \pm 5.6$  kg; MEP-2,  $77.7 \pm 4.8$  kg;  $p=0.106$ ) or between the treatment groups (Control,  $77.0 \pm 5.9$  kg; WP+KHCO<sub>3</sub>,  $77.2 \pm 4.6$  kg;  $p=0.809$ ).

## 3.2. Activity levels during stationary adaptation

During both campaigns and in both treatment groups step counts during stationary adaptation were comparable, being  $3343 \pm 897$  in the Control group and  $3244 \pm 914$  in the WP+KHCO<sub>3</sub> group (MEP-1:  $3285 \pm 805$ , MEP-2:  $3312 \pm 601$ ,  $p=0.960$ ). The amount of 3000–3500 steps/day generally classifies the subjects as sedentary [47]. Likewise, neither the mean daily physical activity time ( $58 \pm 28$  vs.  $55 \pm 9$  min,  $p=0.764$ ), nor the mean daily summed activity  $3606 \pm 1304$  vs.  $3241 \pm 1864$  G ( $p=0.301$ ) differed between campaigns or treatment groups (PAT: Control,  $51 \pm 24$  min; WP+KHCO<sub>3</sub>,  $62 \pm 63$  min;  $p=0.649$ ; summed activity: Control,  $3237 \pm 1139$  G; WP+KHCO<sub>3</sub>,  $3602 \pm 2020$  G;  $p=0.290$ ). However, when pooled data sets of free-living (cf. 2.5.2 Habitual Activity Levels) and stationary conditions were compared, the mean step count, PAT, and summed daily activity were significantly less during stationary conditions (all  $p < 0.001$ ). The relative losses in step count, PAT, and summed daily activity were  $-58 \pm 8\%$ ,  $-43 \pm 18\%$ , and  $-59 \pm 10\%$ , respectively.

## 3.3. Basic results of the intervention

### 3.3.1. Body weight

No overall difference in body weight dependent on the treatment was observed (mean Control,  $75.6 \pm 5.2$  kg; mean WP+KHCO<sub>3</sub>,  $76.0 \pm 4.5$  kg;  $p=0.410$ ). A significant decrease in body weight of 1.4 kg was observed in both groups with HDTBR (overall mean adaptation phase,  $77.2 \pm 5.0$  kg; overall mean HDTBR,  $75.8 \pm 4.9$  kg;  $p=0.019$ ). However, we did not see increased fluid excretion during HDTBR relative to the adaptation period (overall mean adaptation phase,  $2870 \pm 395$  ml; overall mean HDTBR,  $2886 \pm 355$  ml;  $p=0.935$ ).

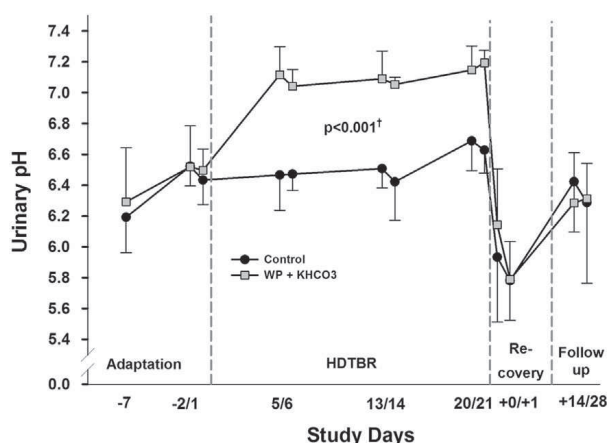


Fig. 2. Urinary pH throughout the study. Data are presented as mean  $\pm$  SD ( $n=9$ ). <sup>†</sup>“Treatment effect”.

### 3.3.2. Urinary pH

With an average PRAL of 13 mEq/d in the control subjects, the urinary pH was  $6.53 \pm 0.20$ , while – as expected – during WP+KHCO<sub>3</sub> supplementation (mean PRAL:  $-46$  mEq), urinary pH was significantly higher ( $7.11 \pm 0.14$ ,  $p < 0.001$ ) (Fig. 2). Accordingly the PRAL reduction of  $-59$  mEq/d led to an increase in urinary pH of 0.58 units.

## 4. Discussion

In this reference paper we describe the standardized and strictly controlled study conditions of a multidisciplinary bed rest study examining the effects of whey protein and potassium bicarbonate supplementation and portrait the quite homogenous group of 9 healthy young volunteers who finished the protocol.

The nutritional intervention was chosen to constitute an accentuated contrast to the basic diet, to detect changes within a short timeframe of 3 weeks: whereas the control diet was moderately acidifying with a rather high protein intake, matching Western habits [45], the nutritional intervention was composed of an additional protein load but nonetheless an alkalizing diet. More alkaline urine confirmed an alkali over acid production under this supplement regime. However, though protein intake increased to a very high level ( $1.8$  g/kgBW/d), administration of the whey protein isolate in the form of naturally flavored dairy products did not cause any obvious adverse reactions. On the other hand, providing the whey protein in dairy products may have changed dietary patterns and led to a higher consumption of animal fat and protein. Additionally the isocaloric reduction of fat and carbohydrate intake may influence metabolism itself, independently from protein supplementation.

Individual responses to the nutritional intervention and to immobilization itself may have been caused by differences in activity levels before the study campaigns began. We observed an average of 25% higher activity levels during the summer (campaign 1) than in mid-winter (campaign 2), which likely resulted from a seasonal influence. However, as the intervention was applied in a cross-over design and we did not find systematic differences in baseline levels before the treatments (Control vs. WP+KHCO<sub>3</sub>), we assume that an overall confounding of the effects expected by the intervention was minimized by application of the cross-over design. Elaborate training sessions between campaigns of bed rest studies having a cross-over design might lessen the risk of seasonal influences on baseline activity and at the same time reduce the possibility of carrying over disuse-induced deconditioning to the subsequent campaigns. Although in this study a washout period of 4 months was set to reduce the possibility of carrying over influences of the nutritional countermeasure or of bed rest, such influences may not have been fully eliminated.

Comparable training sessions also seem reasonable during stationary adaptation, as our results imply. Pooled data sets show a physical preconditioning during stationary adaptation in both campaigns and in both treatment groups. This may dilute potential bed rest effects and accordingly influence the magnitude of the total response to the supplementation of whey protein and KHCO<sub>3</sub>. For

future campaigns an appropriate imitation of habitual activity patterns, to result in more activity and avoid preconditioning to inactivity during a stationary adaptation period, seems reasonable. This imitation should be based on habitual activity patterns, classified by means of the described combination of questionnaires, step counts, and accelerometry. An active population sample ( $> 10,000$  steps/d), homogenous in activity patterns (i.e., proportion of resistive vs. endurance exercise) would complete this approach.

## 5. Conclusion

In conclusion, 3 weeks of daily supplementation of  $6 \times 0.1$  g whey protein isolate/kg BW on top of a 1.2-g protein/kg BW diet seemed to be a tolerable approach during 3 weeks of HDTBR. Simultaneous alkalization was achieved by daily administration of  $6 \times 15$  mmol  $\text{KHCO}_3$ . Differing activity levels may have affected specific outcome measures. The results of the presented study may provide evidence of the influence of a diet high in protein, but nonetheless alkaline, on several physiological systems affected by  $6^\circ$  head-down-tilt bed rest. Altogether these results can constitute a further step in multidisciplinary countermeasure research, in particular for immobilized people such as those in bed rest or space flight.

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## Conséquences d'une simulation d'impesanceur de 21 jours chez l'homme sur le métabolisme des lipides et effets d'une supplémentation en protéines testée comme contremesure.

### Résumé

Au cours d'une simulation de la microgravité de 21 jours, le développement de plusieurs altérations métaboliques ont été étudiées : une inflammation de bas-niveau et une altération de la sensibilité à l'insuline et du métabolisme lipidique. Connues pour leurs impacts positifs sur le métabolisme, une supplémentation en protéines du petit lait, combinée à des sels alcalins, a également été testée. Contrairement aux études précédentes, aucune inflammation, ni altération du métabolisme lipidique n'a été clairement décrite dans notre étude. En revanche une diminution de l'oxydation des glucides en faveur des lipides est observée, suggérant le début d'une insensibilité à l'insuline. Nos données suggèrent qu'il serait possible de prévenir les dérégulations métaboliques associées à l'inactivité physique sévère par un remplacement isocalorique des lipides par les protéines, avec un apport protéique de base de 1,2g/kg/jour et un contrôle strict de la balance énergétique par l'apport calorique.

Mots clés : microgravité, inactivité physique, métabolisme, nutrition, lipides

### Résumé en anglais

During 21 days of simulated microgravity, the development of several metabolic alterations has been studied: a low-grade inflammation and an alteration of insulin sensitivity and lipid metabolism. Known for their positive effect on metabolism, whey proteins supplementation combined with alkaline salts have also been tested. At the opposite of previous studies, no inflammation, nor lipid metabolism alterations have clearly been described. Nevertheless, a decrease in carbohydrates oxidation in favor of lipids is observed, suggesting the development of insulin insensitivity. Our data suggest that it may be possible to prevent the metabolic disorders associated with severe physical inactivity by an isocaloric replacement of lipids by proteins in the diet, along with a protein intake of 1.2g/kg/day and tight control of energy balance by adjusting energy intake.

Key words: microgravity, physical inactivity, metabolism, nutrition, lipids