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**BIOMARKERS TO DEFINE OPTIMAL
PROTEIN REQUIREMENT**

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PROTEIN REQUIREMENT**

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ABSTRACT

BACKGROUND. Dietary proteins are the source of the amino acids required by the body for tissue growth and maintenance. The Population Reference Intake (PRI) for proteins, as defined by the European Food Safety Authority (EFSA) for healthy adults, including the elderly, is 0.83 g/kg body weight/day. This amount is defined on the net balance of body protein (or “nitrogen balance”, given by the difference between dietary nitrogen intake and losses) equivalent to 0.66 g/kg/day plus a safety factor for interpersonal variability and differences in proteins quality of mixed diets. The PRI, however, is the minimum daily amount of protein needed to maintain the nitrogen balance and avoid a progressive loss of lean body mass in healthy people with moderate physical activity. Therefore nitrogen balance may not be adequate to define protein requirement in adults and especially in ageing characterized by loss of muscle mass and function (sarcopenia). Furthermore until recently the prevalent idea was that a protein intake above PRI had no further benefits and on the contrary could impair health. These believes are now under discussion, diets with higher protein intake have been shown beneficial in the prevention and treatment of conditions such as sarcopenia, COPD and type 2 diabetes mellitus. There is a need of more precise methods to define protein requirement.

AIM. The aim of the present thesis is to investigate in human healthy volunteers new biomarkers adequate to define optimal protein intake. Recent studies have determined protein needs by measuring whole-body protein metabolism using stable labeled isotope-amino acids.

METHODS. Our research group has applied two different metabolic methods based on the most widely used tracer, i.e. D5-Phe stable isotope, in two experimental bed rest campaigns (FP7 PLANHAB and INTERREG PANGaA) in healthy volunteers. BR is a suitable model to investigate physiologic adaptation to inactivity.

MAIN RESULTLS. FP7 PLANHAB. We applied the stable isotope infusion technique, to assess the effect of physical inactivity and/or hypoxic condition on whole body protein turnover as previously described in Biolo et al 2008. Chronic hypoxia has been associated with an overall reduction in protein synthesis and in total plasma and skeletal muscle protein content. During the PLANHAB study we investigated, through a crossover randomization, the net effects of 10 days normobaric hypoxia (4000 mt.), associated with either ambulatory conditions or BR, in 11 young (age 24±4 yr), healthy and normal weight male subjects maintained on eucaloric diets. *Main results.* Hypoxia

in ambulatory conditions significantly decreased whole body protein turnover by reducing both protein synthesis ($-8\pm 2\%$) and protein degradation ($-8\pm 3\%$). Hypoxia during bed rest did not caused significant changes in protein metabolism.

INTERREG PANGaA. The skeletal muscle loss in aging is caused mainly by the “anabolic resistance” i.e. the inadequate increase in the rate of protein synthesis in response to nutritional-metabolic stimuli, including exercise, protein and amino acid intake as well as insulin and insulin-like growth factor stimulation. As a consequence, the net protein balance becomes negative leading to sarcopenia. The effects of ageing on the anabolic resistance induced by inactivity are poorly investigated. During the PANGeA study we had the opportunity to perform the second documented experimental BR in in healthy elderly volunteers and the first comparing aged with young subjects. To evaluate the anabolic resistance associated with ageing and inactivity, we enrolled 7 young ($23\pm 1\text{yr}$) and 8 elderly ($59\pm 1\text{yr}$) normal weight individuals, in a 14-d experimental BR protocol. We replaced our previous infusion method with a new, simpler, safer and quicker technique, by which tracers are given orally instead of parenterally, the all procedure is completed in two hours, instead of 6, and only two blood draws versus 7 are sufficient. **Main results.** At baseline parameters of anabolic sensitivity were comparable between young and elderly individuals. The anabolic resistance significantly increased after BR in both groups (bed-rest effect $p<0.01$), with a statistically significant bed-rest \times group interaction ($p=0.01$). Anabolic resistance increased significantly in elderly ($18.5\%\pm 7.3\%$) more than in young ($5.2\%\pm 9.4\%$) subjects.

DISCUSSION. In the PLANHAB study, hypoxia in ambulatory conditions reduced by the same level both protein synthesis and catabolism, as measured by isotope infusions, suggesting an adaptive mechanism: the lower energy production and availability induced by hypoxia associated with ambulatory condition. These modifications could not have been revealed by the use of nitrogen balance method, showing the relevance of more sophisticated analysis. The direct evaluation of the muscle protein metabolism through an infusion of stable-labeled isotope tracer, considered the golden standard methodology, gave us, in the PLANHAB study, reliable results in the early protein metabolism changes during hypoxia and/or BR. This method however has the limit of being complex, onerous and invasive, therefore being unsuitable for clinical evaluation. In the PANGeA study we could confirm the presence of a reduced sensitivity to anabolic stimuli in the elderly population compared to the young men. The elderly subjects are therefore, more at risk to develop changes of protein metabolism induced

by inactivity. The simpler, timesaving and less invasive method we have developed for the PANGeA study, on the other hand, could be applied to a wider ranges of experimental conditions and clinical settings.

1. INTRODUCTION

1.1. Protein composition and metabolism

Proteins are the major nitrogen components of the protoplasm of animal tissue, representing 50% of the dry weight of animal cells. They are formed by a sequence of amino acids (AA), the basic structural units, attached by covalent chemical bonds (i.e. peptide bond). AA are characterized by the presence of both a carboxyl group (R-COOH) and an amino group (R-NH₃), nitrogen being equivalent to about 16% of protein weight (Shils ME et al. 2006; Caballero B et al. 2012). There are 20 different AAs commonly classified in *essentials*, EAA (or indispensable), which, since their carbon skeleton cannot be synthesized by the animal body, need to be introduced by diet, and *non-essentials*, NEAA (or dispensable), made in the body from carbon and nitrogen precursors. In humans the EAA include: histidine, valine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and possibly arginine, while the NEAAs are reported on figure 1. Cysteine and tyrosine are synthesized in the body from the EAAs methionine and phenylalanine. AAs are further divided in: *dibasic* (arginine lysine and histidine), *diacidic* (aspartic, glutamic acid), *neutral-aliphatic* (glycine, alanine, serine, threonine, cysteine, cystine, methionine and the three branched-chain AAs -BCAA- valine, leucine, isoleucine) and *neutral-aromatic* (phenylalanine, tyrosine and tryptophan). In the human body proteins have multiple roles. From structural to regulatory (e.g. hormonal activities) to functional (e.g. enzymes, muscle contraction, transport, osmolality regulation, etc.) (Shils ME et al. 2006; Caballero B et al. 2012).

Proteins, consumed with the diet are enzymatically hydrolyzed in the digestive system and then reach the peripheral circulation as free amino acids. These AAs mix with those coming from the tissues protein catabolism forming the circulating free AA pool. Amino acids follow one of the follow three major metabolic ways: a) incorporation into tissue proteins (protein synthesis); b) catabolism by oxidation and nitrogen excretion; c) synthesis of other nitrogen compounds, including purine bases, creatine and epinephrine. The absorbed AAs through the *vena porta* reach the liver, where, with the exclusion of the BCAAs, are catabolized at a rate influenced by the body requirements; therefore when EAA intake is elevated, their catabolism is also increased. Through this mechanism the liver regulates the amount of EAA obtained from the diet, which will be available to the rest of the body. A small portion of the absorbed EAAs is used for the

synthesis of liver proteins and of visceral plasma proteins, secreted by the liver, including albumin, transferrin, pre-albumin, etc. Finally, about one quarter of the diet EAAs reach the general circulation. The BCAAs from the liver are transported to other tissues, being metabolized mainly by muscle and kidney. On the other hand, NEAA plasma concentration is not modified by the passage through the liver. The metabolic pathways of NEAAs are showed in figure 1 (Shils ME et al. 2006; Caballero B et al. 2012).

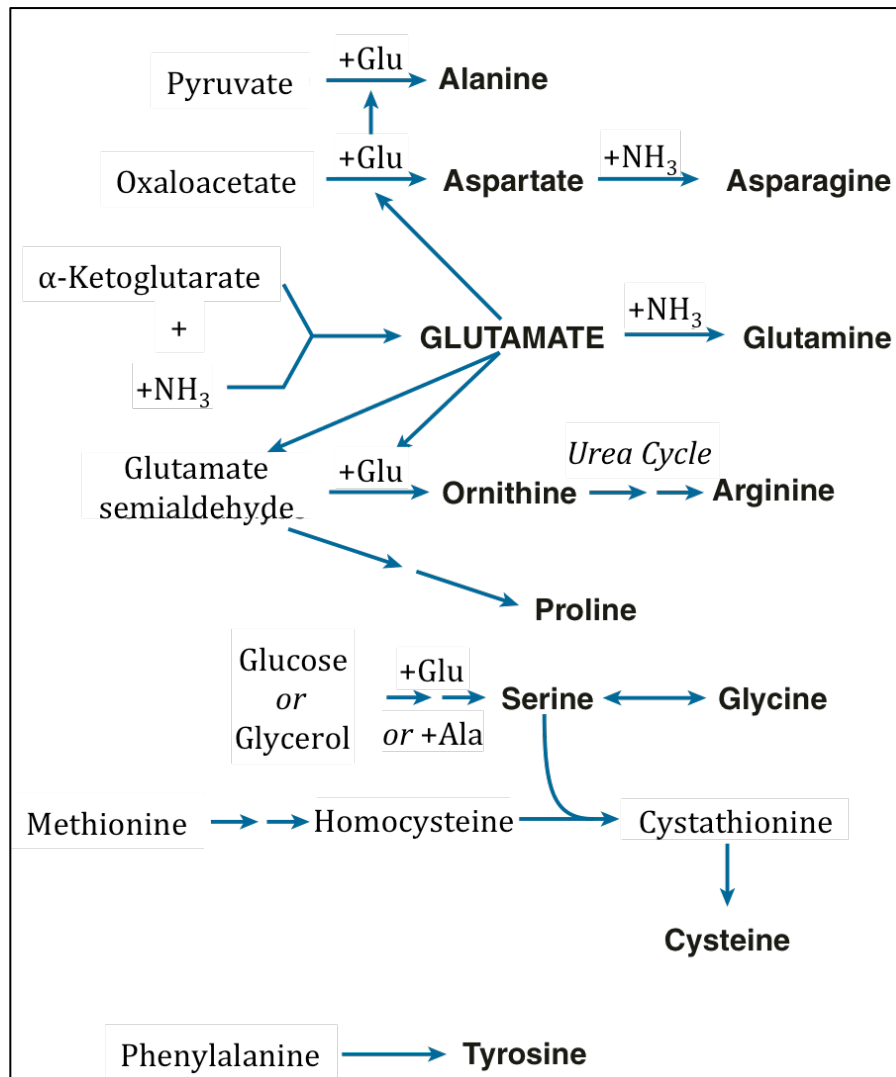


Figure 1. Metabolic pathways of the dispensable amino acids.

From Shils ME et al. 2006.

Skeletal muscle is the major body site of protein metabolism, being the largest tissue in the body (Shils ME et al. 2006; Caballero B et al. 2012). Muscle protein synthesis (MPS) is influenced by AA availability after the intake of a meal containing proteins. The post-prandial anabolic response is followed in the post-absorptive period, by a catabolic phase (i.e. muscle protein breakdown, MPB) (Figure 2), the magnitude of these metabolic cycles being influenced by the quantity and quality of protein intake and the meal nutrient composition and pattern. During fasting the muscles release AAs, mainly as alanine and glutamine (with a daily loss equivalent to about 50g of protein in an 70kg individual) (Shils ME et al. 2006; Caballero B et al. 2012). Alanine is derived by transamination between pyruvate from glucose, and the amino-groups of AAs originating from MPB. Alanine carries nitrogen to the liver where gluconeogenesis takes place. Glucose is formed from the carbon skeleton of alanine, while the amino-group is either converted to urea or transaminated. Glutamine, formed in skeletal muscle by transamination with glutamate, is transported to the intestine, where 50% gets transaminated to alanine, thereafter carried to the liver for gluconeogenesis and urea synthesis (Shils ME et al. 2006; Caballero B et al. 2012). Part of the glucose produced by gluconeogenesis in the liver returns to the muscles, the all process being called “glucose-alanine cycle” (Figure 2B).

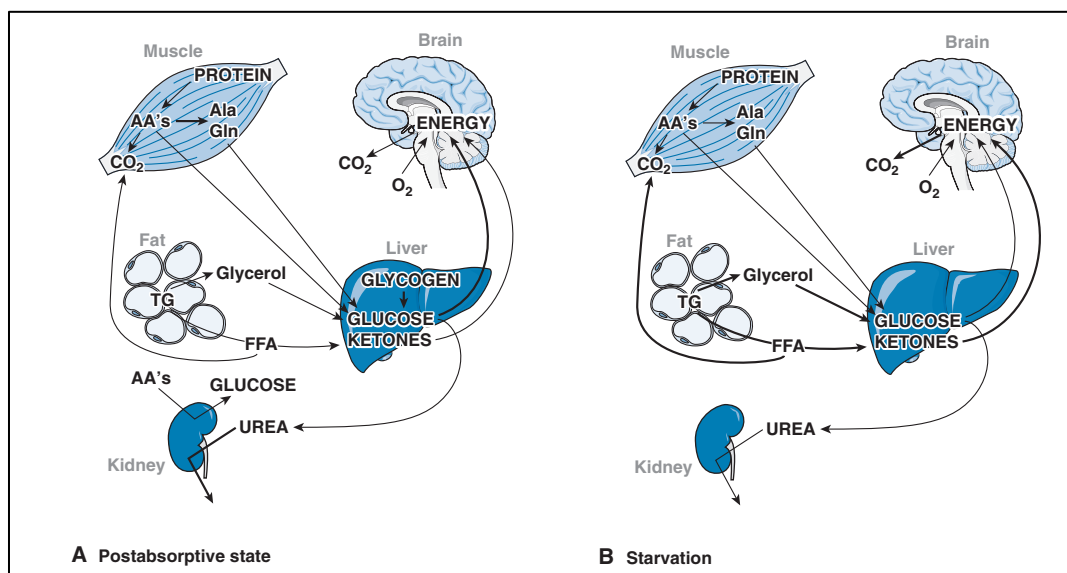


Figure 2. Substrate interorgan flow in the post-absorptive state (A) and in starvation (B).

From Shils ME et al. 2006.

The plasma levels of amino acids are also affected by dietary carbohydrate through a mechanism involving insulin secretion. After digestion of dietary carbohydrates and monosaccharides absorption, the raising plasma glucose concentration stimulates insulin secretion, favoring an increased insulin-mediated transport of most plasma amino acids into the muscle cells (Caballero B et al. 2012). This effect is maximal for BCAAs, whose plasma levels can fall as much as 40% after glucose intake (Caballero B et al. 2012).

When amino acids are degraded for energy rather than entering in anabolic pathways, the ultimate products are CO₂ and water, produced through the pathways of intermediary metabolism involving the tricarboxylic acid cycle, and urea, whose synthesis allows the removal of toxic metabolites, such as ammonia (Shils ME et al. 2006; Caballero B et al. 2012).

The whole body protein turnover is the result of protein synthesis and breakdown processes (Antonione R et al. 2008).

1.1.1. Protein synthesis

At the molecular level, regulation of protein synthesis depends both on transcriptional and translational mechanisms. The concentration of ribosomes in tissues determines the capacity for protein synthesis, therefore controlling the protein turnover rate. The concentration of ribosome, inside the cells is influenced by nutrient intake (protein and energy) and hormones (i.e. insulin, thyroid, growth hormone and glucocorticoids). The regulation of the translational processes is exerted mainly through initiation, with reversible phosphorylations known to control at least four separate steps of the initiation cycle. This allows very rapid changes in protein synthesis. Peptide hormones (insulin and insulin-growth factor 1, IGF-1), glucocorticoids and amino acids have all been implicated in such regulation.

Studies performed in experimental cell models have revealed the importance of Akt factors (also called protein kinase B) in the regulation of protein synthesis in skeletal muscle. Akt factors are serine/threonine-specific protein kinases playing a critical role in muscle hypertrophy (Bodine SC et al., 2001). Activation of signaling cascades involving IGF-1 and phosphatidylinositol 3-kinase (PI3K) induces Akt phosphorylation and activation (Bodine SC et al., 2001). Akt, in turn, activates the eukaryotic translation initiation factor 2B (eIF2B) by stimulation of glycogen synthase kinase-3 β (GSK-3 β) (Rhoads RE 1999). In parallel, Akt can activate initiation of protein translation

stimulating p70S6 kinase (p70S6K) by mammalian target of rapamycin (mTOR) protein-kinase (Terada N et al., 1994). Interestingly, a period of resistance training exercise induces muscle hypertrophy associated with increases in phosphorylated Akt, GSK-3b and mTOR (Leger B et al., 2009).

Insulin is a potent anabolic stimulus for MPS (Fujita, S et al. 2006). Insulin deficiency leads to a protein catabolic state with loss of muscle mass, reversible only by insulin therapy (Abu-Lebdeh HS & Nair KS 1996). Nonetheless, the mechanisms by which insulin enhances muscle protein anabolism are still debated (Biolo G et al 1995; Nygren J & Nair KS 2003; Wolf RF et al 1992; Denne SC et al 1991; Heslin MJ et al 1992; Moller-Loswick AC et al 199). Some studies reported that this effect was due to an increase in protein synthesis with no major changes, or some reduction, in proteolysis (Biolo G et al 1995; Nygren J & Nair KS 2003; Wolf RF et al 1992); other studies found a significant reduction in protein degradation with no significant changes in protein synthesis (Denne SC et al 1991; Heslin MJ et al 1992; Moller-Loswick AC et al 1994).

A stimulatory effect of insulin on protein synthesis has been demonstrated in various tissues, including skeletal muscle (Garlick PJ & Grant I 1988; O'Connor PM et al 2003). Furthermore, recent human experiments have shown that insulin can acutely stimulate muscle protein synthesis by increasing the initiation of mRNA translation (Guillet C et al 2004; Kimball SR et al 1997). Insulin can also reduce protein breakdown by stabilizing lysosomes and reducing the activity of the ubiquitin-proteasome pathway (Lee SW et al 2004).

Protein intake and amino acid availability are key regulators of muscle protein synthesis. Acute AA administration up regulates muscle protein synthesis and such effect is enhanced if the intake is associated with resistance or aerobic exercise (Biolo G et al., 1997). In contrast to exercise, the anabolic efficiency of amino acid administration is decreased during inactivity. The stimulatory effect of an amino acid load on whole body protein synthesis in healthy volunteers at the end of a period of experimental bed rest was reduced (-20%), when compared to individuals with normal physical activity (Biolo G et al., 2004). Furthermore during bed rest, the rate of protein turnover, in the fasting state, was decreased both at skeletal muscle and whole body levels (Biolo G et al., 2004). Other studies showed that dietary protein restriction (i.e., 0.6g protein/kg/d) led to 23% suppression of whole body protein turnover, in the fasting state, when compared to adequate levels (i.e., 1.0g protein/kg/d) of protein intake (Stuart CA et al., 1990). Other Authors, maintained leg mass and ameliorated the

muscle strength losses following 4 weeks bed rest by a daily supplementation of about 50g of essential amino acids (Paddon-Jones D et al., 2004).

1.1.2. Protein breakdown

Several proteolytic systems can contribute to the degradation of muscle proteins, among these, the most relevant are the following:

- The lysosomal-autophagic system which is present in all cells and involves acid proteinases (i.e. cathepsins), active within a vacuolar structure capable of engulfing and degrading complete organelles, ribosomes, as well as intracellular proteins and proteins entering the cells via endocytosis.
- The ubiquitin-proteasome ATP-dependent system, widely distributed among tissues, is characterized by relative broad protein specificity for the hydrolysis of proteins and peptides. It involves two components: a) the recognition system responsible for targeting the protein substrates toward proteolysis and b) the multifunctional proteasome, causing the proteolysis. In the proteasome system, degradation is carried out by the 26S subunit (Grune T et al., 2003; Grune T & Davies KJ 2003), which is composed by the 20S and by the regulatory 19S complex, the latter playing an important role in adenosine triphosphate (ATP)-dependent degradation (DeMartino GN & Ordway GA 1998). Protein substrates are marked for degradation in the 26S proteasome pathway by covalent addition of ubiquitin. This requires the ubiquitin-activating enzyme (E1), specific ubiquitin-conjugating enzymes (E2), and ubiquitin protein ligase enzymes (E3). The ubiquitin E3 ligases, atrogin1 and muscle ring finger-1 (MuRF-1), are involved in skeletal muscle atrophy (Bodine SC et al. 2001; Gomes MD et al., 2001). MuRF-1 was shown to be directly and indirectly upregulated by Forkhead family of transcription factors (FoxO) (Stitt TN et al., 2004) and by the NF- κ B transcription factor (Sandri M et al., 2004). Ubiquitinated proteins are recognized and bound by the 19S regulators of the 26S proteasome, that removes the polyubiquitin chain and unfolds the substrate protein for final degradation into the 20S core proteasome (Grune T et al., 2003).
- The calcium-activated calpain and calpastin pathway is responsible for the initiation of proteolysis. In vitro and animal studies, showed that Ca^{2+} -activated proteases (Calpain) and the proteasome system play important roles in muscle protein breakdown during muscle atrophy (Furuno K & Goldberg AL 1986; Ikemoto M et al., 2001; Purintrapiban J et al., 2003). Moreover, caspase-3 may also contribute to selected forms of muscle atrophy (Du J et al., 2004). Actomyosin complexes represent 50–70% of muscle proteins (Tidball JG & Spencer MJ 2002). The proteasome system can degrade only monomeric contractile proteins (i.e. actin and myosin) (Goll DE et al.,

2003), which, therefore, must be released from actomyosin complexes to be degraded by the proteasome (Goll DE et al., 2003). Both calpain and caspase-3 can play a key role in producing actomyosin disassociation (Du J et al., 2004; Goll DE et al., 2003; Tidball JG & Spencer MJ 2002). Calpain activity is increased by an elevation in cytosolic calcium concentrations (Goll DE et al., 2003). Caspases are cascade-activated proteases triggered by several signaling pathways (Primeau AJ et al., 2002): whose activation can result in protein breakdown and apoptosis.

1.2. Protein requirement in adults

Daily protein requirement is influenced by multiple factors the most relevant being the obligatory nitrogen losses which were measured in fasting subjects, with stable body weight, with modest levels of physical activity (Shils ME et al. 2006; Caballero B et al. 2012; Guarnieri G et al 1998). On the average this value is equal to 4,4 g/day, about 27 g of proteins considering a conversion factor of 6,25 (1g N = 6.25g protein), being nitrogen about 16 % of proteins by weight. This minimum protein requirement (0.34 g/kg/day) were raised to 0,8 g/day to adjust for the individual variability and for factors such as the differences in biological values and of the net protein utilization of proteins from different food sources. The biological value (BV) of proteins is an index of the body capacity to utilize them in anabolic processes. It is equal to the ratio between the retained nitrogen, RN and the absorbed nitrogen AN.

$$VB = RN/AN.$$

Where

$$AN = [\text{Nitrogen intake} - (\text{nitrogen excreted in the feces after the intake of proteins} - \text{nitrogen excreted on an aprotic diet})]$$

and

$$RN = AN - (\text{urinary nitrogen after the intake of proteins} - \text{urinary nitrogen excreted on an aprotic diet}).$$

Proteins from animal sources have higher biological values than those from vegetable food. The net protein utilization (NPU) defines, besides biological value also protein digestive efficiency and it is equal to the ratio between the retained and the dietary nitrogen (nitrogen intake).

$$NUP = R/I.$$

The correction factor for the definition of protein requirements considers an NPU of a mixed diet, with proteins from different sources. The NPU of various proteins present in the same food may also differ, as in the case of milk proteins, with whey and casein being considered respectively a fast protein and a slow protein, in reaching the sites of body protein synthesis (Shils ME et al. 2006; Caballero B et al. 2012; Guarnieri G et al 1998).

1.2.1. Recommended dietary allowances for protein in adults

The latest recommended dietary allowances (RDAs) in the USA, Europe and Italy have maintained the indication for a daily protein intake of 0,8 g/kg body weight, in all healthy adults, males and females, excluding pregnancy or lactation, independently from age (World Health Organization (WHO) 2007; European Food Safety Authority (EFSA) 2012). The adaptation of the body to a low or zero nitrogen intake is shown in figure 3: urinary N excretion drops dramatically in response to the protein-deficient diet over the first 3 days and thereafter stabilize a new lower level of N excretion by day 8.

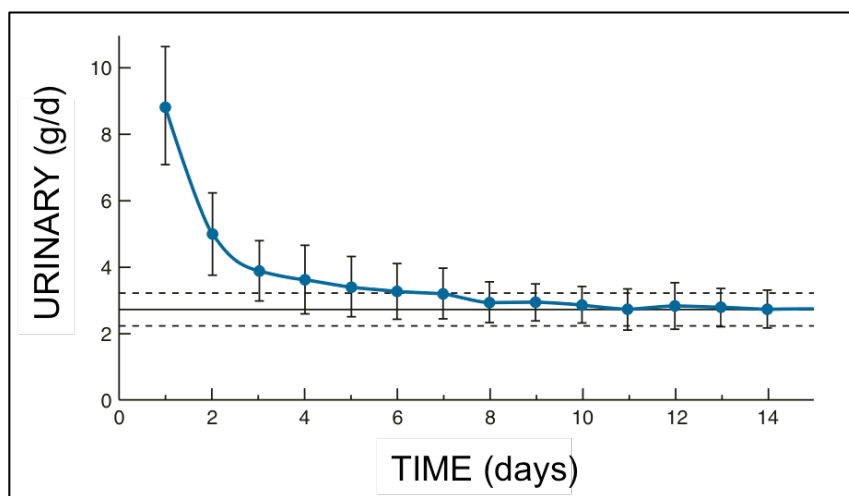


Figure 3. Physiological adaptation to a low or absent nitrogen intake

From Caballero B et al. 2012

Table 1 also illustrates how urea production is related to N intake and how the body adapts oxidation of amino acids to their supply.

Table 1. Physiological nitrogen-related urea production in the body

N SPECIES	HIGH-PROTEIN DIET (g N/d)	LOW-PROTEIN DIET	FASTING (DAY 2)
Urea	14.7 (87%)	2.2 (61%)	6.6 (75%)
Ammonia	0.5 (3%)	0.4 (11%)	1.0 (12%)
Uric acid	0.2 (1%)	0.1 (3%)	0.2 (2%)
Creatinine	0.6 (4%)	0.6 (17%)	0.4 (5%)
Undetermined	0.8 (5%)	0.3 (8%)	0.5 (6%)
Total	16.8 (100%)	3.6 (100%)	8.7 (100%)

From Caballero B et al. 2012

In other words, with a high intake, excess amino acids are oxidized and urea production is high, but with an insufficient supply of dietary amino acids, amino acids are conserved and urea production is greatly decreased (Shils ME et al. 2006; Caballero B et al. 2012; Guarnieri G et al 1998). A reduced protein intake increases the efficiency of nitrogen retention and may therefore not be indicative of an improved tissue protein anabolism. The validity of nitrogen balance in the definition of protein requirement have been therefore questioned. Furthermore, any change in protein intake requires time to reflect metabolic changes. Methods of assessment may also not be precise, collection of urine may not be complete over the 24 hours and it is difficult to estimate unmeasurable nitrogen losses through non-urinary, non-fecal routes (Shils ME et al. 2006; Caballero B et al. 2012; Guarnieri G et al 1998), shown in Table 2.

Table 2. Physiological nitrogen-related urea production in the body

	DAILY NITROGEN LOSS	
	AS NITROGEN (mg N/kg/day)	AS PROTEIN EQUIVALENT (g PROTEIN/kg/day)
Urine	38	0.23
Feces	12	0.08
Cutaneous	3	0.02
Other	2	0.01
Total	54	0.34
Upper limit (2 standard deviations)	70	0.44

From Caballero B et al. 2012

Recent studies have shown that nitrogen requirements are increased in the elderly or during acute and chronic illnesses, as being confirmed through higher-level methodologies.

1.2.2. *Tracer methods to define amino acid kinetics.*

Tracers are compounds, chemically identical to the endogenous metabolites, but with one or more atoms substituted by its nonradioactive stable isotope, thus allowing a “labeling” useful to follow the metabolic fate of the compound (the tracee) being evaluated (Wolfe RR, 2002). This substitution makes the tracers distinguishable and measurable from the normal metabolites (Wolfe RR, 2002). Because isotopes differ only in the number of the constituent neutrons they can be identify by mass spectrometry (GCMS). The molar ratio of the amount of tracer isotope divided by the amount of unlabeled material is called “tracer-to-tracee ratio” (TTR) or enrichment (Wolfe RR, 1992). Isotopic methods are frequently used to study protein and amino acid metabolism. Amino acids are constantly exchanged between the intracellular and the extracellular pools, through the action of specific transporter. Amino acids from intracellular protein catabolism enter the intracellular pool and can then follow three paths: protein synthesis, catabolism inside the cell or passage into the plasma. Amino acids have a Rate of Appearance (Ra) in the intracellular pool, since they can either derive from plasma and/or protein catabolism, and a Rate of Disappearance (Rd) due to their incorporation in a protein or degradation. Generally it is assumed that during the study period every amino acid entering in the protein pool will be, either metabolized or incorporated in to a protein, with an irreversible loss (Wolfe RR, 1992).

Furthermore it is also assumed that when the enrichment of the tracer in the pool is at the plateau (i.e. isotopic equilibrium) the Ra of the compound (endogenous production + exogenous infusion) is equal to its Rd:

$$Ra=Rd$$

If the tracer is administered through a constant infusion, the kinetics is described by the single-pool model. When the isotopic equilibrium is reached, the Ra of the tracee can be calculated as the ratio between the amount of the infused tracer and the isotopic enrichment at its plateau. A priming dose (bolus), at the start of the infusion, allows to reduce the time required to reach the isotopic equilibrium (Wolfe RR, 1992).

Isotopic methods are applied through the following techniques:

1. Precursor incorporation
2. Tracer dilution

The *precursor incorporation* technique requires a constant infusion of a tracer, usually after priming bolus, to measure, when reaching the isotopic equilibrium, anabolism from its incorporation rate into a protein (Wolfe RR, 1992). The *tracer dilution* technique is applied to measure the Ra and the Rd of a metabolite in a given pool. Since the tissues do not separate the tracer from the trace, the Rd does not modify the tracer substrate ratio, therefore any isotopic enrichment change is the consequences of the dilution effects of the unlabelled substrate (Wolfe RR, 1992).

The most frequently used stable-labelled isotopes for the investigation of protein metabolism are: ^{15}N -Glycine, ^{13}C -Leucine, ^{15}N -Leucine, L-[ring- $^2\text{H}_5$]-Phenylalanine.

L-[ring- $^2\text{H}_5$]-Phenylalanine (D5-Phe) is very useful in the study of protein metabolism. This essential amino acid is not peripherally catabolized, therefore, in the fasting state, its irreversible disappearance from the pool can be due only to protein synthesis or irreversible hydroxylation to tyrosine. On the other side, the appearance of the unlabelled phenylalanine can only derive from protein catabolism. Thus the administered D5-Phe produces an amount of $^2\text{H}_4$ -Tyrosine (D4-Tyr) indicative of net protein catabolism. If together with the labelled phenylalanine a labelled tyrosine is administered (e.g. $^2\text{H}_2$ -Tyr), it is possible to determinate the rate of phenylalanine hydroxylation (Figure 4) (Matthews DE 2007).

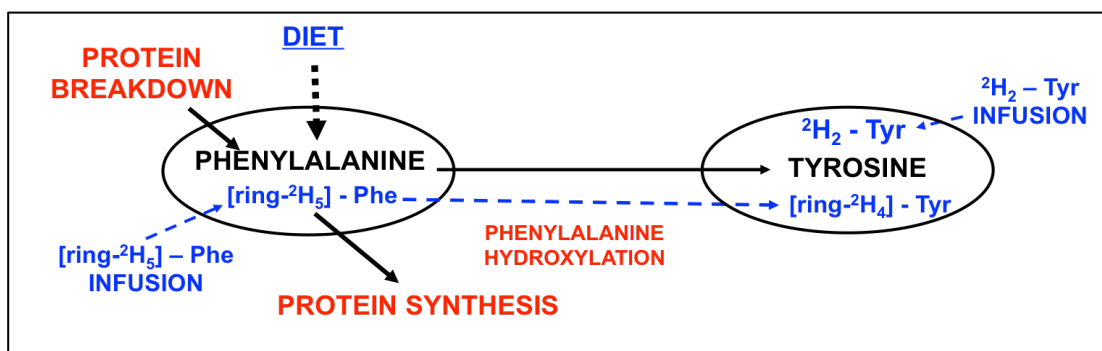


Figure 4. Phenylalanine and tyrosine kinetics in human

These methods, when requiring intravenous infusions of an extemporarily-made solutions, administered through two catheters, imply the preparation of apyretic and aseptic infusate, a process which needs a dedicated area and controlled conditions, not always readily available. Furthermore, the infusion protocols are usually time-consuming needing up to 6 to 7 hours. All these factors can reduce the compliance of the examined subjects and increase costs and efforts.

There are also other methods limitation that should be taken in to account. The model shown in the figure 4 assumes that all proteins have a slow turn-over and therefore will

not modify the amino acids entering and living the pool, from catabolism and synthesis respectively. However some proteins have a very short half-life (e.g. enzymes) (Wolfe RR, 1992).

1.3. Factors influencing protein requirements in different conditions.

As argued above, the guidelines for dietary protein need traditionally consider a similar requirement for all adults, regardless of age or sex (0.8g/kg/day of protein) (WHO 2007; EFSA 2012). However, new evidences (Bauer J et al. 2014) have shown that a higher dietary protein intake is effective to support good health, promote recovery from illness and preserve functionality in elderly subjects (aged over 65 years) (Walrand S et al 2011; Gaffney-Stomberg E et al 2009; Kurpad AV& Vaz M 2000; Morse MH et al 2001; Chernoff R. 2004; Morley JE et al 2010). An increased consumption of proteins with diet can overcome the decline of the anabolic response to dietary protein, and offset inflammatory and catabolic states associated with chronic and acute disease. These conditions occur often in elderly individuals (Walrand S et al 2011), whose generally consume less protein than young adults (Bauer J et al. 2014). An insufficient protein provision can also lead to loss of muscle mass and strength and, as a consequence, older people are at higher risk for conditions such as sarcopenia and osteoporosis than are younger individuals (CederholmTE, et al 2011; Cruz-Jentoft AJ et al 2010; De Souza et al 2010).

1.3.1. Sarcopenia

Sarcopenia from the Greek σάρξ, "flesh" and πενία, "paucity" defines a condition characterized by reduced muscle mass, associated with loss of strength or performance. The European Working Group on Sarcopenia in Older People (EWGSOP) has recently defined sarcopenia as a "syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength, with a risk of adverse outcomes such as physical disability, poor quality of life and death." (Cruz-Jentoft AJ et al. 2010). Moreover, The EWGSOP group classified sarcopenia as primary (or age related, associated with the physiological changes induced by the aging process), and secondary (caused by other factors such as inactivity, illnesses and nutritional problems, including malabsorption or other gastrointestinal disorders or medications, causing appetite loss). Recently, however, some authors disagreed with this etiological classification since in most cases, muscle loss is induced by multiple factors, difficult to identify separately (Biolo G et al. 2014). This is particularly true in the ageing population, characterized by a higher prevalence of chronic and acute diseases.

Causes and consequences

Sarcopenia and the associated body composition changes are caused by multiple factors including the so called “anorexia of aging”, i.e. reduced food intake, seen with advancing age, hormonal changes, such as reduced synthesis of growth and sex hormones, resistance to leptin, insulin and thyroid hormones, and neurodegenerative processes. Other contributing factors include heritability, intake of proteins (type and quantity) and energy, vitamin D status, physical inactivity, increased adiposity and chronic and acute diseases (Bauer J et al. 2013) (Figure 5).

Aging is characterized by changes in protein metabolism: protein turnover is reduced with lower protein synthesis and increased catabolism, leading to a net decline in protein synthesis. These changes are sustained by a higher splanchnic extraction of amino acids, that impairs their availability, a blunted response to the anabolic stimulus (anabolic resistance) of protein feeding and a reduced anticatabolic effect of insulin.

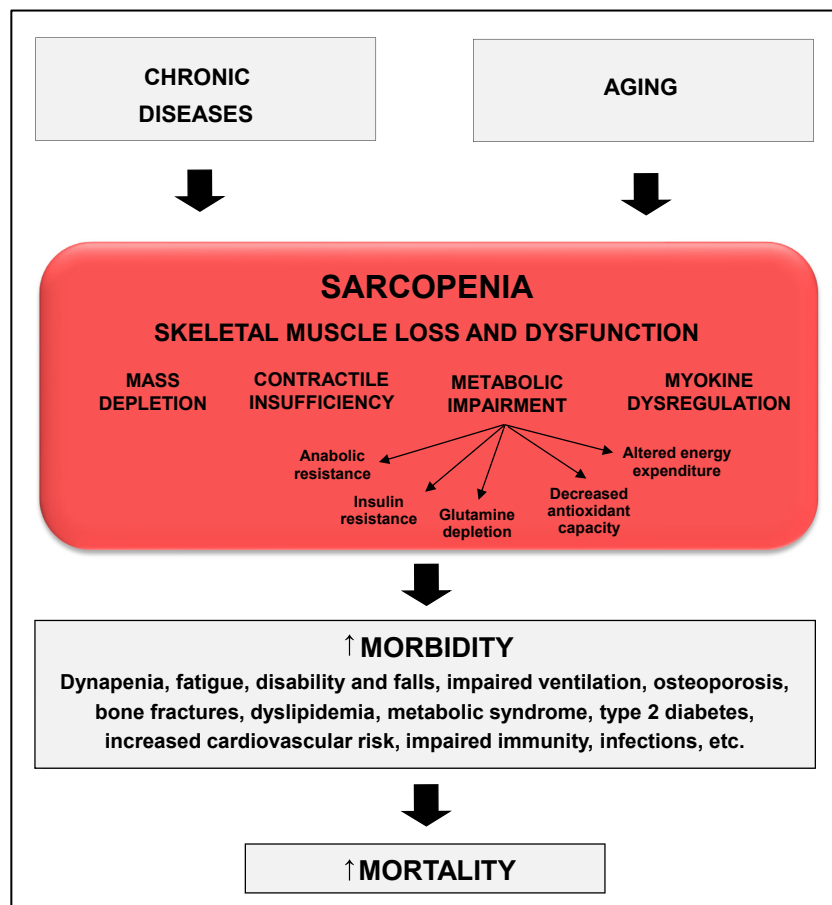


Figure 5. Loss of skeletal muscle mass and function characterize sarcopenia of both aging and diseases.

From Biolo et al 2014

Besides amino acid availability, physical activity, with muscle loading, also exerts anabolic effects, both in muscles and in bones. Inactivity is common in the elderly, caused by loss of strength, overweight, balance and locomotion disturbances, fear of falls, low motivation and presence of diseases (Biolo G et al. 2014). This sedentary lifestyle may contribute to the decline of mass and function in muscle and bone tissues. Bed rest, that is a condition of prolonged and total muscle unloading, in experimental condition, causes a loss of 3-5% of lean body mass in young healthy subjects (Biolo G et al. 2014).

The loss of muscle mass in sarcopenic subjects causes a reduction in the basal metabolic rate and in physical activity energy requirements (Biolo G et al. 2014). These changes, if not compensated by lower energy intake, or by a heightened energy expenditure, through increased physical activity, as often is the case with ageing, may lead to an increased fat deposition (Biolo G et al. 2014). Furthermore physical inactivity itself leads within a two weeks, short term period to an accumulation of visceral fat, and insulin resistance, as shown in young subjects who reduced experimentally their level of physical activity to about 15% of the basal level (Biolo G et al. 2014). Sarcopenia therefore is generally associated with an increase in the percentage of body fat which can lead to a condition called *sarcopenic obesity* (Biolo G et al. 2014). This rise in fat mass is observed in both sexes and at any given BMI, being present even in normal or underweight individuals. Two different pathways can lead to sarcopenic obesity, the first is that associated to conditions of positive energy balance, from overfeeding, or long-term inactivity, in otherwise healthy individuals (Biolo G et al. 2014), the second is observed in selected inflammatory states such as COPD, rheumatoid arthritis, chronic kidney disease or chronic heart failure (Prado CM et al. 2012). In these conditions the increased level of cytokines and the hormonal changes are not associated with appetite loss, thus fat mass is preserved or even increased (Biolo G et al. 2014). The so called “obesity paradox” may be explained on the basis of a larger skeletal muscle mass in overweight subjects induced by their higher body mass (Kalantar-Zadeh K et al. 2005). Fat tissue is mostly expanded at the visceral or abdominal level, with an increase in waist circumference, observed more frequently in females. Furthermore there is an age related deposition of ectopic fat at the intra-muscular, intra-hepatic and intra-pancreatic levels. Enlarged visceral adipocytes and activated macrophages, attracted in the adipose tissue by adipocyte secreted chemokines, release hormones and cytokines such as adiponectin, leptin, tumor necrosis factor and interleukin 6 (IL-6), giving rise to an inflammatory response and to insulin resistance. A higher release of free fatty acids

further contributes to the insulin resistance. TNF- α has direct inhibitory effects on insulin signaling and also increases the release of free fatty acids (FFA) from adipose tissue. These changes increase the risk of developing type II diabetes, metabolic syndrome and cardiovascular complications (Biolo G et al. 2014).

Recently it has been shown that, besides fat tissue, also skeletal muscle can be considered an endocrine organ, producing peptides both anabolic (insulin-like growth factor, IL-15) and catabolic (myostatin) and cytokines, called myokines including IL-6, IL-8 and IL-15 (produced during muscle contraction) (Biolo G et al. 2014). Physical activity increases the muscle synthesis of IL-6 with useful metabolic consequences such as an increased local glucose uptake and fat oxidation, a higher gluconeogenesis in liver and lipolysis in adipose tissue. IL-15 plays anabolic activity in skeletal muscle and has a role in lipid metabolism.

Sarcopenia, by reducing strength and function, leads to poorer balance and higher risk of falls and fractures, decreased autonomy and lower quality of life, metabolic complications and higher morbidity and mortality. Therefore adequate countermeasures need to be taken (Biolo G et al. 2014).

Currently proposed criteria for sarcopenia assessment in a clinical setting include: evaluation of muscle mass, determination of strength and assessment of physical performance (Miller MD et al 2002; Cruz-Jentoft AJ et al. 2010; Malmstrom TK & Morley JE. 2013). Muscle mass can be measured by many methods including anthropometry (Miller MD et al 2002), bioimpedance analysis (BIA) (Cruz-Jentoft AJ et al. 2010; Vermeeren MA et al. 2006; Norman K et al. 2012; Mijnders DM et al 2013), dual energy X-ray absorptiometry (DXA) (Fearon K et al. 2011; Coin A et al. 2012), computed tomography (CT) scan (Mourtzakis M et al. 2008; Fearon K et al. 2011), and magnetic resonance imaging (MRI). BIA cannot reliably assess skeletal muscle mass in patients with body fluid abnormalities, as liver cirrhosis (LC), chronic kidney disease (CKD), chronic heart failure (CHF) or some cancer (Norman K et al 2012). Ultrasonography is another reliable imaging method that can be applied to the analysis of the musculoskeletal system, based on computerized analysis of ultrasonic acoustic echoes. Ultrasound imaging can allow proper estimation of muscle thickness changes as direct evidence of muscle alterations. Reliability of ultrasound imaging was validated versus MRI as a gold standard (Arbeille P et al., 2009) and moreover, this technique allows a proper determination of muscle fiber orientation, with respect to aponeurosis, i.e. pennation angle (Narici M & Cerretelli P 1998). For measurement of muscle strength the hand-held dynamometer is a reliable tool to assess strength in upper

extremities (Mijnarends DM et al 2013). This method is widely used and has been validated in many physiological and pathological conditions. Several tests of physical performance are available such as the gait speed, the Timed Up and Go (TUG) and the Short Physical Performance Battery (SPPB) which includes standing balance, gait speed, and chair rises (sit-to-stand) (Mijnarends DM et al 2013). The EWGSOP criteria for sarcopenia diagnosis are described in figure 6.

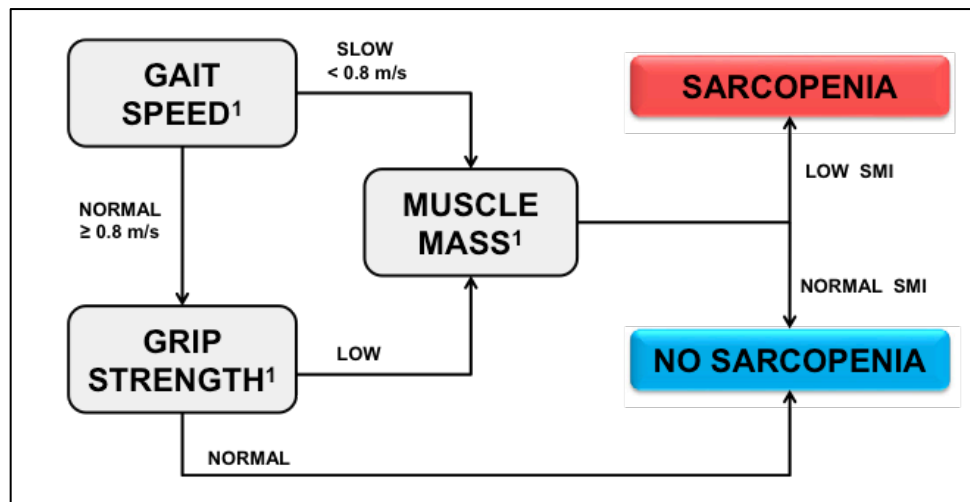


Figure 6. EWGSOP criteria for sarcopenia diagnosis

The diagnosis requires: the presence of low muscle mass by BIA, plus low muscle strength, or low physical performance; conversely, the presence of low muscle mass, normal muscle strength, and physical performance is defined as pre-sarcopenia. From Biolo G et al. 2014

1.3.1.1. Anabolic resistance

The reduced response to an anabolic stimulus, such as dietary protein intake, is defined “anabolic resistance” (Burd NA et al., 2013). With the onset of this condition, the MPS response to hyperaminoacidemia is impaired, independently from the insulin availability (Burd NA et al., 2013).

In physiologic condition, in the post-prandial state, protein synthesis overcome the protein breakdown in order to equalize the protein loss of the fasting state. The anabolic resistance impairs the post-prandial protein synthesis and causes muscle mass loss (Deutz NE et al., 2011). In a previous study (Biolo G et al., 2004), our research group investigated the effects of 14-d of experimental immobilization in nine healthy young subjects. The study consisted in a constant infusion of both the stable isotopes L[1-¹³C]-leucine, to assess the anabolic response, and of an amino acid solution to simulate the post-prandial condition. Our results showed a significant reduction of protein synthesis in the fed state, due to reduced anabolic sensitivity to the amino acids stimulation. Thus anabolic resistance is one of the major mechanisms of muscle atrophy during inactivity (Biolo G et al., 2004). On the other hand, a bout of exercise before the dietary protein intake, determines an improved amino acid utilization, allowing a post-prandial muscle mass gain (Burd G et al, 2013).

Physical exercise associated to an adequate dietary protein intake showed an anabolic effect on muscle protein balance (Biolo G et al., 2005). The combined effect of physical exercise and increased availability of amino acids on the regulation of muscle protein kinetics was evaluated on healthy young subjects through an intravenous infusion of a balanced amino acids mixture, during rest and after a resistance exercise (Biolo G et al., 1997). The results obtained, from muscle protein kinetics with the infusion of stable isotopes, suggested that the MPS amino acids stimulatory effects is increased by the exercise session. These evidences (Figure 7) are probably due to the augmented blood flow promoted by physical activity. Accordingly, the amino acids derived from dietary proteins may have a greater anabolic effect if administered immediately after physical activity (Biolo G et al., 1997).

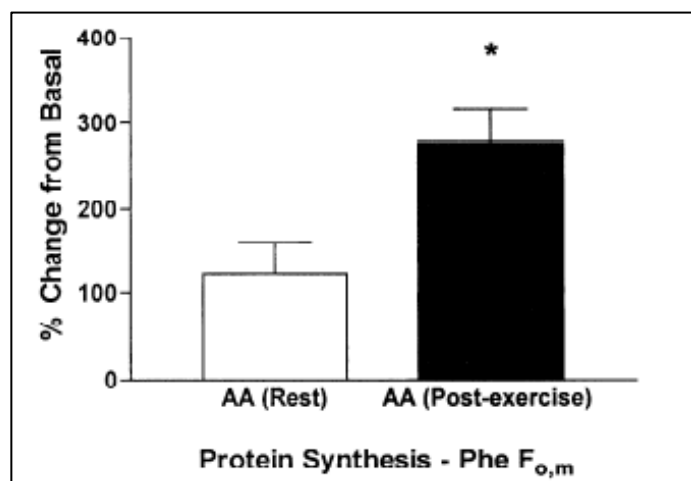


Figure 7. Muscle protein synthesis level ($F_{(o,m)}$) assessed during an amino acid infusion in conditions of rest and after resistance training.

MPS is expressed as percent variation compared to basal values (*, $p < 0,05$) (Biolo G et al., 1997).

In elderly there is a reduced dose-response relationship between the EAA availability and the myofibrillar protein synthesis (Burd NA et al, 2013). Moreover, the anabolic resistance in aging involves also the insulin anabolic effect, with a reduction of the proteolysis inhibitory effect promoted by insulin after a meal (Rennie MJ 2009), and the exercise anabolic effect (Russ DW et al., 2012). This resistance to anabolic stimuli, such as nutrition, exercise or hormones (e.g. insulin), is the basis of the impaired aging muscle protein turnover (Russ DW et al., 2012).

Cuthbertson and colleagues were the first to compare the levels of protein synthesis, following the oral administration of EAA, in young and elderly individuals. They showed that the basal levels of MPS in fasting state, were comparable in both young and elderly groups, but after the assumption of EAA the MPS response in elderly individuals was lower than that showed by the young subjects (Cuthbertson D et al., 2005). Another study demonstrated that in elderly individuals the ability of insulin and amino acids, especially branched (BCAA), to stimulate the protein transduction processes is reduced compared to the young subjects (Guillet C et al., 2004).

These results were sustained by other findings showing that the reduced vasodilator response to insulin, in aging muscles, can play a key role in the onset of the anabolic resistance, possibly because of a lower muscle availability of nutrients (Rasmussen BB et al., 2006). Breen et al. also confirms the detrimental effect of aging on anabolic sensitivity. They have demonstrated that after a single bout of endurance training, the physiological acute alterations of the MPS are compromised (Breen L. & Phillips SM 2011). Moreover it was observed that even after 3/6 h after the physical activity, an

elderly subject does not reach the normal level of MPS stimulation (Drummond MJ et al. 2008). The detrimental effects of physical inactivity on the levels of protein synthesis are confirmed by a recent study showing that two weeks of sedentary lifestyle is associated with loss of gravitational muscle mass, involving mainly the lower limbs (Krogh-Madsen R et al.,2010). An active lifestyle in aging can be critical to maintain adequate sensitivity to an anabolic stimulus (Burd NA et al. 2013). The anabolic resistance in aged is represented in figure 8.

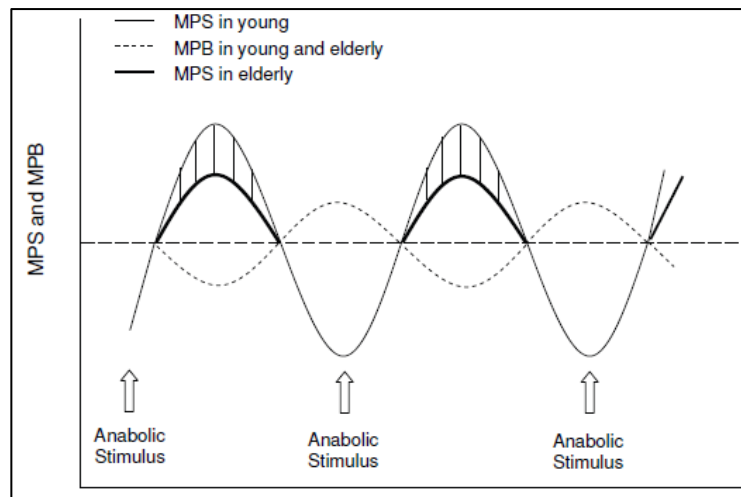


Figure 8. Protein metabolism trend after an anabolic stimulus (physical activity and/or amino acids intake) in young and elderly subjects.

The shaded sections mark the difference between elderly and young people in the MPS after an anabolic stimulus (from Breen & Phillips, 2011).

The age-related sarcopenia has a multifactorial etiology and the mechanisms associated to the anabolic resistance development require further investigation, however, the reduction of physical activity levels (i.e. sedentary lifestyles) and/or the potential presence of acute or chronic diseases could have a role.

Accordingly, some studies showed that both protein turnover and anabolic sensitivity could be altered by the development of an inflammatory condition, associated with aging (Breen L & Phillips SM, 2011). The role of inflammation on the alteration of protein metabolism has been extensively studied. In humans an association was found between MPS levels and plasma concentrations of several inflammation markers (Toth MJ et al., 2005).

Many cytokines, and particularly the TNF- α could alter MPS though the inhibition of the phosphorylation of the proteins involved in the mTOR pathway. It was

demonstrated that mTOR signaling is crucial to stimulate the MPS after endurance training and furthermore, the phosphorylation of the proteins involved in this pathway seems to be enhanced by the availability of amino acids (Breen L & Phillips SM, 2011). Consequently, the muscle loss and the reduced sensitivity to anabolic stimuli, observed during inflammation could be associated with an altered protein phosphorylations of the mTOR signaling pathway. This mechanism could affect the elderly individuals. The development of a low-grade inflammation may reduce their sensitivity of this signaling pathway to a load of amino acids, thus determining anabolic resistance and reduced levels of MPS (Breen L & Phillips SM, 2011)

Recent studies suggest an impact of the oxidative stress on the inactivity-related muscle mass loss, probably determining an unbalance between protein synthesis and degradation (Pellegrino MA et al., 2011). However further investigation are needed to better understand the pivotal mechanism of such influence.

1.3.1.2. Insulin resistance

Insulin is a peptide hormone secreted by the β -cells of the pancreatic islets of Langerhans that maintains normal blood glucose levels by facilitating cellular glucose uptake. Besides carbohydrate metabolism, insulin regulates also lipid and protein metabolism and cell division and growth (Bailey CJ et al 2010).

The term “insulin resistance” refers to a condition in which, insulin is correctly produced by the pancreas, but the target tissues (i.e. muscle, liver and adipose tissue) show a reduced sensitivity to its actions (Berg JM et al., 2012). Consequently, the tissue uptake of the circulating glucose is impaired in both fasting and fed state, leading to hyperglycemia and to detrimental effects in lipid and glucose metabolism (Silverthorn DU, 2010). Insulin resistance is an important T2DM risk factor and may be detectable even years before the appearance of hyperglycemia and T2DM. Essentially, the first stages are characterized by a hyperinsulinemic response to glucose that ensures a proper glucose metabolism (Hunter SJ & Garvey WT, 1998). In advanced stages, on the contrary, the even greater amount of insulin produced by the β -cells is insufficient to compensate the insulin resistance of the tissues, leading to hyperglycemia and T2DM (Reaven G, 2004). This mechanism could be one of the reasons for the development of T2DM during aging (Silverthorn DU, 2010).

The cellular mechanisms responsible of the altered insulin functions may involve detrimental modifications for the insulin receptor, signal transduction and glucose transport. The major cause of the altered insulin sensitivity seems to be the missed or reduced translocation of the GLUT4 transporter on the cellular membrane of the skeletal muscle and adipose tissue, in response to the hormone; consequently, glucose absorption by these cells is lowered (Hunter SJ & Garvey WT, 1998). The insulin resistance effects vary in relation to the type of tissue and its insulin dependence for metabolic process regulation. The organs and tissues most affected by the altered insulin sensitivity are: skeletal muscle, adipose tissue, liver, endothelium, brain, pancreas, pituitary gland, kidney, gonads and bone (Wilcox G, 2005). The impaired glucose tolerance and the reduced insulin sensitivity are two common phenomena in the elderly population. The association between aging and insulin resistance was deeply investigate, since there is an increased prevalence of T2DM with ageing (Karakelides H et al., 2010). The progressive age-related glucose tolerance derangement depends mainly on the decreased tissue sensitivity to insulin and the resulting reduced ability of tissues to metabolize glucose (DeFronzo RA, 1981) (Figure 9).

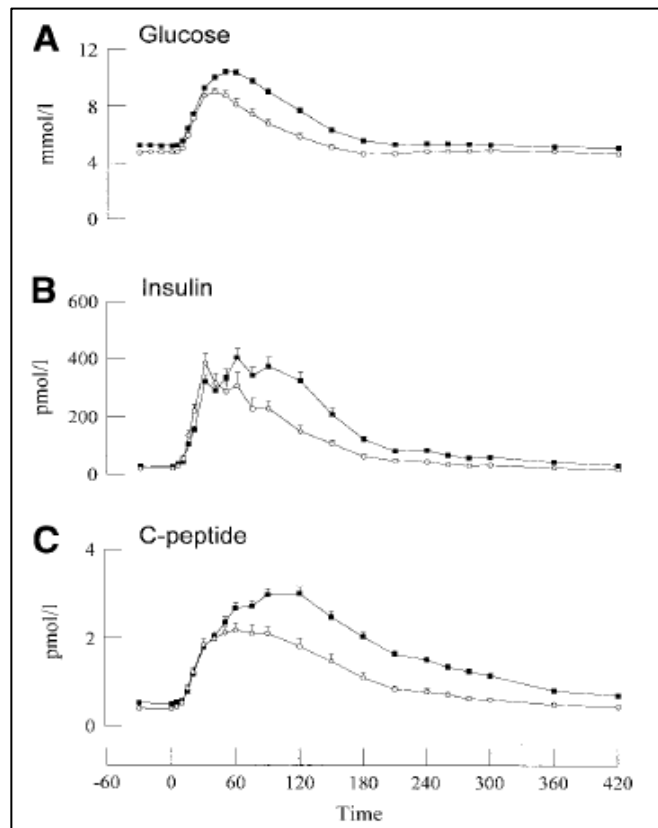


Figure 9. Plasma glucose (A), insulin (B) and C peptide (C) concentrations in young (○) and elderly (■) subjects in fed state.

from Basu R et al, 2003.

This altered glucose tolerance has a multifactorial etiology and it involves mainly the skeletal muscle (Jackson RA, 1990). Among other factors, the reduced physical activity and body composition changes are critical contributors to this progressive condition during aging. In the elderly subjects there is a significant modification of the body composition with a reduction of the lean mass in favor of enhanced fat mass. Since one of the main actions of insulin is promoting the muscle glucose uptake, a reduced muscle mass could lead to insulin resistance (DeFronzo RA, 1981). An inverse relation was found (Srikanthan P et al. 2011) between skeletal muscle mass and insulin resistance and the risk of developing pre-diabetes, while an increased muscle mass was associated with additional protection against insulin resistance and pre-diabetes. The protective association was stronger in individuals with overt diabetes (Srikanthan P et al. 2011). Moreover the reduced physical activity associated to aging could contribute to the impaired glucose metabolism (DeFronzo RA, 1981). Some authors argue that aging per se has a harmful effect on tissue sensitivity to insulin and consequently on glucose metabolism (DeFronzo RA, 1981). On the other side, recent studies suggest that the

age-related insulin resistance is associated to body composition changes and the lowered physical activity rather than aging per se (Karakelides H et al., 2010). For example, the increased abdominal fat mass is a key factor in the onset of insulin resistance (Karakelides H et al., 2010).

Aging is also associated to a low-grade systemic inflammation and to an increased reactive oxygen species production (i.e. oxidative stress) (Karakelides H et al., 2010; Csiszar A et al., 2008). Inflammation and oxidative stress are considered two of the major mechanisms in the onset of the insulin resistance most likely due also to the higher cardiovascular risk associated to a sedentary lifestyle. The individuals affected by T2DM or metabolic syndrome, frequently show a low-grade systemic inflammation and are characterized by higher concentration of proinflammatory fatty acids in the cell membranes (Mazzucco S et al., 2009). Oxidative stress and low-grade systemic inflammation are common alterations in obese or diabetics but in aged persons they seems independent from body composition and may directly affect the onset of T2DM and cardiovascular diseases (CVD) (Karakelides H et al., 2010; Csiszar A et al., 2008).

A good way to prevent or at least improve insulin resistance is physical activity. A study aiming to analyze the distinct effects of age, physical activity and fat accumulation on glucose tolerance and insulin sensitivity, demonstrated that regular physical activity can prevent the age-related insulin resistance (Seals DR et al., 1984). Insulin sensitivity decreases in sedentary subjects, while it is enhanced in trained individuals (DeFronzo RA, 1981). The detrimental effect of inactivity on insulin sensitivity was showed in many studies. Furthermore, experimental immobilization in healthy young subjects (Figure 10) caused an altered glucose tolerance and a higher insulin secretion. Stuart et al. showed an impaired glucose metabolism after just 7 days of bed rest in healthy subjects (Stuart CA et al, 1988)

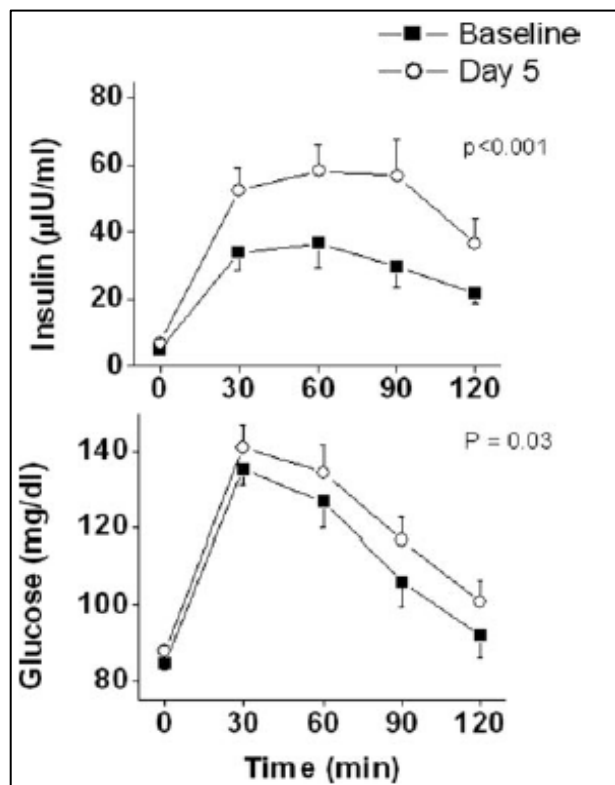


Figure 10. Effect of 5 days of bed rest insulinaemia (upper figure) and glycaemia (lower figure) levels after an oral glucose load in 20 healthy subjects.

Bed rest is associated to a significant augmented insulin ($p < 0.001$) and glucose ($p = 0.03$) response and thus to insulin resistance (from Hamburg NM et al., 2007).

Another study (Lipman RL et al., 1972) showed that 3 days of experimental immobilization are sufficient to determine an impairment of about 50% of the glucose uptake from the tissue and an augmented level of insulinaemia and glycaemia after an oral glucose load. The same authors have observed that physical exercise during immobilization can improve insulin resistance. The bed rest state has different effects on trained and inactive individuals. Smorawiński and colleagues have observed the effects of three days of experimental immobilization on endurance and power athletes and sedentary subjects. After an oral glucose load, the insulin resistance-derived hyperinsulinemia affect both groups but the athletes (especially those performing resistance exercise) were more responsive. This result is probably due to a faster compensatory response to insulin resistance (Smorawiński J et al., 2000). Recently, a study has shown the association between five days of bed rest on healthy volunteers with dyslipidemia and altered blood pressure and vascular function (Hamburg NM et al., 2007). The authors propose a direct connection among the vascular dysfunction and the onset of insulin resistance suggesting that a lower peripheral blood flow, especially

in the lower limbs, could determine a reduced tissues glucose uptake (Hamburg NM et al., 2007). Another study comparing athletes and subjects inactive for 7-10 days, showed an association between physical inactivity and both an impaired glucose tolerance and the energy intake, while the trained subjects (controls) maintained a suitable glucose tolerance. This positive effect in athletes is associated to an increased peripheral blood flow favoring the insulin-mediated glucose uptake by the skeletal muscle (Arciero PJ et al., 1998; Hayashi T et al., 1997). It was also demonstrated (Vukovich MD et al., 1996) that the impaired insulin action evidenced after 6 days of physical activity in runners is associated to a reduced levels of GLUT4, the skeletal muscle tissues glucose transporter. These data were recently confirmed (Henriksen EJ, 2002), however, further studies are required to verify that such mechanisms (i.e. impaired blood flow and GLUT4 levels) are responsible of the inactivity-related insulin resistance. In addition, preliminary data of our research group, showed a development of insulin resistance after 7 days of bed rest in healthy subjects. We have observed that after 35 days of experimental inactivity the insulin resistance levels are comparable to those measured after a week of bed rest, suggesting a rapid and full expression of this condition maintained in the long-term.

Increased levels of physical activity could have a fast and healthy effect on the metabolic alterations determined by a sedentary life style or resting from hospitalization. Recently (Heer M et al., 2014) it was demonstrated that 4 days of regular physical activity are not sufficient for counteract the insulin resistance determined by 21 days of experimental inactivity. In healthy individuals 5 to 14 days of specific training are needed to reach again a suitable glucose metabolism. Moreover insulin resistance decreases after a single bout of physical exercise, in young subjects while in middle aged or elderly individuals several workout sections are needed to reach the same metabolic improvement (Henriksson J, 1995). Nonetheless further investigations are necessary to select the correct categories and levels of physical activity that could improve insulin resistance (Heer M et al., 2014).

Insulin resistance is also associated to the so called “metabolic syndrome”, a disorder characterized by at least three of the following conditions: abdominal obesity, elevated blood pressure, hyperglycaemia, high serum triglyceride levels and low plasma HDL concentrations. Metabolic syndrome increases the risk of developing cardiovascular disease and diabetes (Kaur J. 2014). Besides the high plasma glucose levels, several studies suggest a direct influence of the insulin resistance on augmented VLDL hepatic levels, hypertension and atherosclerosis (Biolo G et al., 2005). Together these risk

factors are defined “insulin resistance syndrome” (Hunter SJ & Garvey WT, 1998). Skeletal muscle insulin resistance directly contributes to cardiovascular risk through induction of dyslipidemia (i.e., decreased HDL cholesterol and increased triglycerides), as shown in experimental bed rest studies (Mazzucco S, et al. 2010). Prolonged obesity leads to ectopic lipid accumulation in non-adipose tissues, particularly in skeletal muscles, inducing metabolic dysfunctions (reduced glucose uptake, mitochondria dysfunction, etc.) (Masgrau A et al. 2012). The accumulation of lipids within skeletal muscle, due to a blunted muscle capacity to oxidize fatty acids, is a relevant factor in the pathogenesis of insulin resistance. Fat infiltration is also associated with muscle fiber modification, decrease in muscle mass and impairment in muscle strength. Thus, obesity causes quantitative and qualitative alterations in skeletal muscle. This obesity-related insulin resistance not only causes defective insulin-stimulated glucose disposal but has also detrimental consequences on muscle protein metabolism with a reduced post-prandial anabolic response. Moreover, the fat tissue release pro-inflammatory cytokines, including Tumor Necrosis Factor- α , interleukines and PAI-1, associated with the development of insulin resistance (Wilcox G, 2005). The best way to reduce the detrimental effect of the obesity-related insulin resistance is the weight loss through physical activity.

1.3.1.3. Inflammation

Inflammation is a multifactorial response mediated by the activity of cytokines, which can have pro-inflammatory (as interleukin 1, IL-1), anti-inflammatory roles (as interleukin 10, IL-10) or both (as interleukin-6, IL-6). IL-6 is also known as “myokine”, as it is released by contracting muscle and is up-regulated after high intensity physical exercise (Petersen AM & Pedersen BK, 2006). Furthermore it has an immunomodulatory role, decreasing expression of pro-inflammatory cytokines and down-regulating the activation of factors involved in intracellular inflammatory response, as NF- κ B and related tissue injury (Yoshidome H et al., 1999). Physical exercise was shown to increase also circulating IL-10 concentrations, thus suggesting that muscle activity can exert a beneficial anti-inflammatory action (Nunes RB et al., 2008). Tumor necrosis factor alpha (TNF- α) is an additional important cytokine that plays a crucial role in the onset and maintenance of the inflammation process, by stimulating acute phase reaction factors, in liver, macrophage phagocytosis and chemoattraction of neutrophils (Tracey KJ & Cerami A, 1990; Tracey KJ & Cerami A 1994). Through the action on two specific cell membrane receptors, TNF- α can activate several biological responses as activation of NF- κ B (Bouwmeester T et al., 2004) and of intracellular pathways leading to cell differentiation or to apoptosis (Gaur U & Aggarwal BB 2003). Prolonged elevation of circulating TNF- α can lead to muscle mass reduction: a wasting condition linked to poor prognosis of patients (Kandarian SC & Jackman RW 2006). Synthesis of interleukins and TNF- α can trigger the production of a known marker of inflammation called C-reactive protein (CRP), a factor used in clinical practice as marker of inflammation (Ho KM & Lipman J 2009).

Inflammation was shown to play a crucial role in muscle mass wasting, occurring in healthy aging subjects (Jensen GL 2008). In particular IL-1, IL-6 and TNF- α were previously shown to potentially trigger muscle sarcopenia in elderly subjects (Yende S et al., 2006). Interestingly, physical exercise training in elderly subjects was shown to limit the muscle mass wasting and the synthesis of proinflammatory cytokines (Nicklas BJ & Brinkley TE 2009). Moreover, strength training programs in older volunteers ameliorated muscle strength and performance and, in parallel, increased levels of anti-inflammatory interleukin-6 and 10 (Bautmans I et al., 2005).

A previously published work showed in humans that experimental bed rest upregulated plasma CRP (+143%), the ratio between plasma IL-6 and IL-10 (4 times) and, in white blood cell, the ratio between IL-6 and IL-10 mRNAs (5 times) (Bosutti A et al., 2008).

Cytokines are direct modulators of inflammatory pathways, but other factors are also deeply involved in the control of inflammation. Eicosanoids, including prostaglandines tromboxanes and leukotrienes are key mediators and regulators of inflammation (Lewis RA et al., 1990). Cell availability of polyunsaturated fatty acids (PUFA) of the n-6 series affects production of eicosanoids. Eicosanoids are synthesized from the n-6 PUFA arachidonic acid by the enzymic action of cyclooxygenase. Arachidonic acid is, in turn, synthesized in separate biochemical steps from the n-6 PUFA linoleic acid, principally by the action of Δ 6-desaturase, elongase and Δ 5-desaturase (Figure 11) (Mazzucco S et al. 2009).

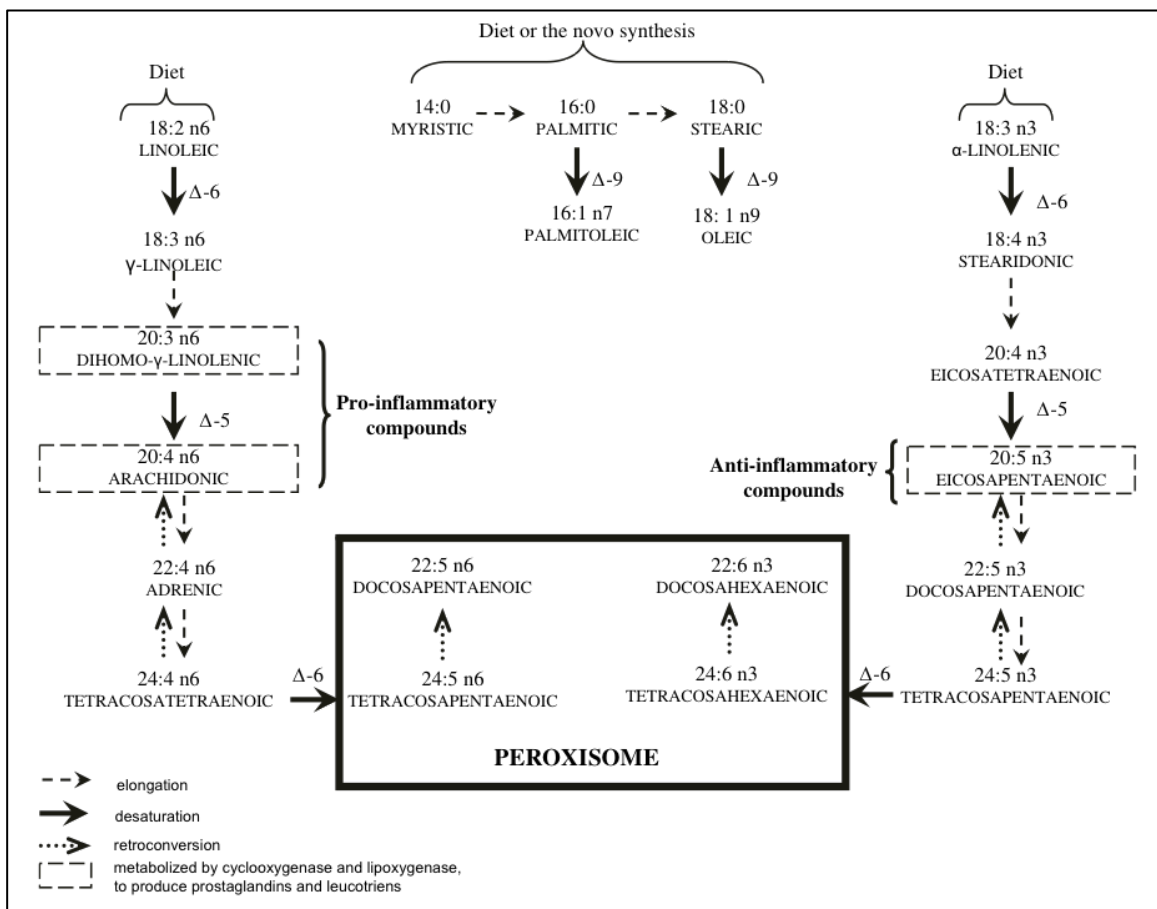


Figure 11. Fatty acid biosynthesis.

Fatty acids are metabolized by desaturases and elongases in the endoplasmic reticulum; only 24:5 n-6 and 24:6 n-3 are β -oxidized into the peroxisome. From Mazzucco S et al 2010

Linoleic acid is an essential FA, contained in many vegetable oils (Bozan B & Temelli F 2008), which determines, together with the endogenous synthesis, the availability of n-6 PUFA (Wertz PW 2009). Elevated availability of n-6 PUFA in cell membranes was linked to inflammation (Ueda Y et al., 2008) and enhanced gene expression of proinflammatory cytokines and transcriptional activity of NF- κ B (Weaver KL et al.,

2009). Thus the n-6 PUFAs have a key role in the stimulation of pro-inflammatory processes.

The n-3 PUFAs are synthesized from the n-3 PUFA alpha-linolenic acid in a pathway sharing the same enzymes involved in the n-6 series synthesis, the $\Delta 5$ -desaturase. This enzyme leads to eicosapentaenoic acid (EPA) that is converted, by $\Delta 6$ -desaturase, to docosahexaenoic acid (DHA) in the peroxisome. n-3 PUFA are well known to play an anti-inflammatory action (Figure 11). In analogy with n-6 PUFAs, alpha-linolenic acid and n-3 PUFAs availability are strongly conditioned by dietary intake of alpha-linolenic acid from vegetable oils (Wertz PW 2009), or EPA and DHA from fish (Pickova J 2009). Fatty acids of the n-3 series are known to have an anti-inflammatory action. Increased intake of EPA and DHA can affect the cell membrane content of these PUFAs (Lee TH et al., 1985), thus reducing the fraction of the pro-inflammatory arachidonic acid. Increased dietary n-6 to n-3 PUFA ratio was shown to increase the expression of CRP and of other proinflammatory agents, as tumor necrosis factor (Zhang L et al., 2009).

Phospholipids content in red blood cell membranes can be considered reliable markers of fatty acid availability in plasma, and of cell membrane composition in the whole body (Harris WS & Von Schacky C 2004). Interestingly, fatty acid membrane composition affects the activity of surface membrane receptors, influencing the activation of downstream intracellular pathways. For example, increased levels of n-3 PUFA can enhance the expression and the signaling activity of glucose receptor (GLUT-4) on muscle cell membrane, thus potentially ameliorating insulin sensitivity (Taouis M et al., 2002). Moreover, the fraction of n-3 PUFAs in cell membranes was directly associated to reduced incidence of cardiovascular diseases: this effect was associated with the anti-inflammatory role of n-3 PUFAs and to other changes induced on cardiovascular system physiology by this class of fatty acids (Harris WS & Von Schacky C 2004). Published evidence proved that in human neutrophil membranes, increases in n-6 to n-3 PUFA ratios are directly associated with the ability of synthesizing pro-inflammatory mediators (Zhang L et al., 2009). Such evidence confirms that cell membrane relative content of total n-6 and n-3 fatty acids can be considered as a marker of whole body inflammatory condition.

The impact of physical exercise on membrane fatty acid composition was investigated in several studies. Constant moderate training and acute exercise were shown to decrease both phosphatidylserine and polyunsaturated fatty acids in erythrocyte membranes: this effect was hypothesized to be caused by increased lipid peroxidation

due to muscle contraction (Sumikawa K et al., 1993). Another study emphasised n-6 PUFA content changes mediated by physical exercise. The authors showed that linoleic acid and the sum of n-6 fatty acids were decreased in trained skeletal muscle phospholipids. This reveals that physical exercise can directly exert an anti-inflammatory role at muscle level, in this way potentially ameliorating insulin sensitivity (Andersson A et al., 1998). Regular exercise was shown, in rats, to decrease Δ -5 desaturase activity and arachidonic acid content; in addition, docosahesanoic acid proportion in cell membrane was decreased while linoleic acid was increased (Helge JW et al., 1999). Observed effects on membrane composition mediated by regular exercise were hypothesized to be dependent on energy substrate utilization during training (Helge JW et al., 1999). Still, a previously published study showed in humans that fractions of oleic acid and docosahexanoic acid were significantly higher in trained muscles when matched to untrained (Helge JW et al., 2001). Similarly, physical exercise significantly lowered the ratio between n-6 and n-3 PUFA in the trained muscle vs those untrained (Helge JW et al., 2001). This study confirmed also that regular physical exercise can reduce whole body and muscle inflammation.

1.3.1.4. Oxidative stress

Oxidative stress is the result of an unbalance between free radicals and the activity of the antioxidant defense system of the body (Tchou J et al. 1991; Hardmeier R et al., 1997) (Figure 12).

The anti-oxidant defense includes non enzymatic and enzymatic systems.

- *Enzymatic systems.* There are several enzyme systems, part of the endogenous defense mechanisms, which catalyze reactions able to neutralize free radicals and ROS, thus preventing cell damage. The major systems are: superoxide dismutase (SOD), catalases, glutathione peroxidase and glutathione reductase, the last two being part of the glutathione system. Metal ions, including iron, selenium, copper, zinc and manganese, act as co-factors in these reactions.
- *Non-enzymatic systems.* This category includes: GSH, ascorbic acid (Vitamin C), tocopherols (Vitamin E), carotenoids, flavonoids, and ubiquinol.

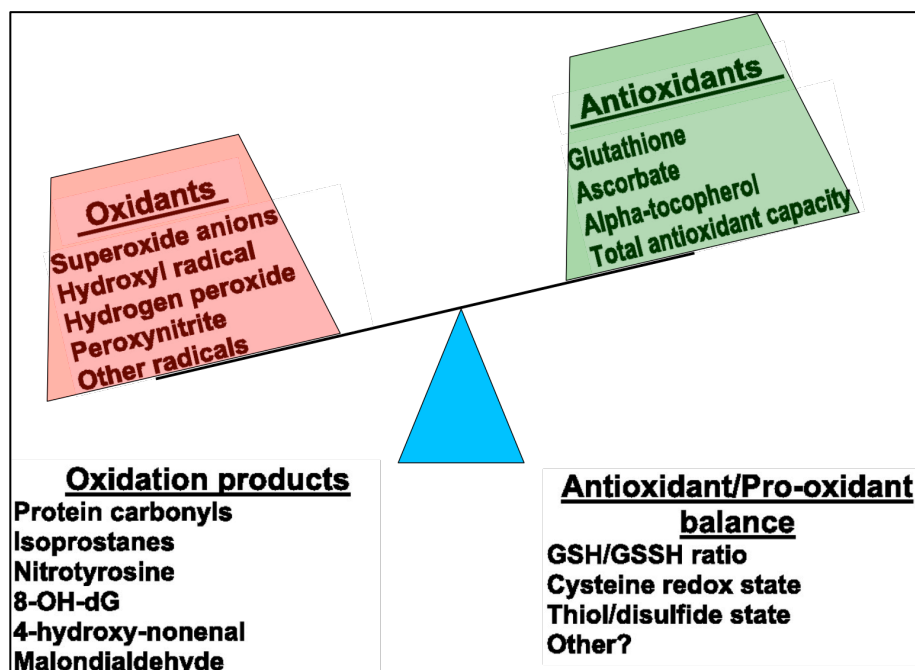


Figure 12. Oxidants and antioxidant agents in the human body

Glutathione is the most important non-enzymic antioxidant in the organism (Pastore A et al., 2003).

Glutathione synthesis is achieved by the action of two ATP dependent enzymes γ -glutamyl cysteine synthetase (catalyzing the bond between glutamic acid and cysteine) and glutathione synthetase, leading to the final formation of reduced glutathione (Majerus PW et al., 1971); the first reaction being the rate-limiting step (Lu, SC 1999) (figure 13).

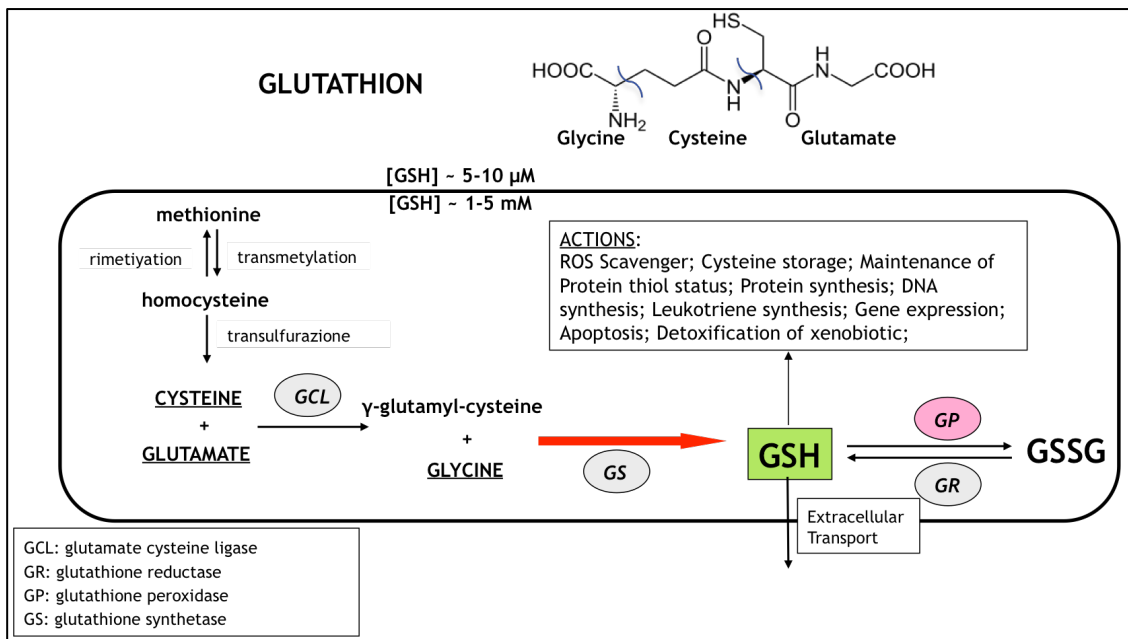


Figure 13. Glutathione biosynthetic pathway

Glutathione is further processed (figure 14), within the γ -glutamate cycle, by γ -glutamyl transpeptidase leading to the formation of a γ -glutamyl amino acid and to the dipeptide cisteinglycine (Pastore A et al., 2003). γ -glutammil amino acid is then transformed to 5-oxoprolin and glutamic acid by γ -glutamyl cyclotransferase and oxoprolinase. Glutamic acid is re-utilized to synthesize again glutathione (Pastore A et al., 2003). Cysteinglycine is then catabolized to free cysteine and glycine amino acids. Cysteine is a limiting substrate for glutathione synthesis, but also glutamate and glycine, as direct glutathione precursors, can affect its synthesis rate.

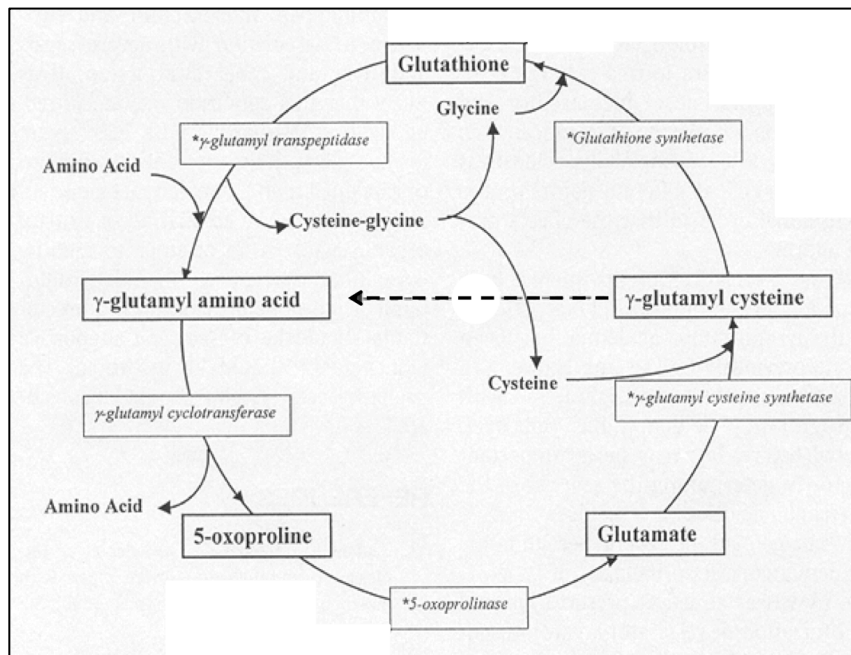


Figure 14. The metabolic glutathione pathway or the γ -glutamyl cycle

Physical exercise increases oxidative stress; Regular physical exercise enhance total and reduced glutathione availability (Sen CK & Packer L 2000), thus counteracting the effect of free radical formation. The ratio between reduced and oxidized glutathione, a reliable marker of glutathione system activation is increased by exercise (Ji LL 1995). Thus, increased availability of GSH levels, can be considered, in healthy subjects, as an active response of the organism to a previous release of free radicals.

Exercise training results in an elevation in the activities of both superoxide dismutase (SOD) and glutathione peroxidase (GPx), along with increased cellular concentrations of glutathione (GSH) in skeletal muscles. In contrast, inactivity leads to redox unbalance both in skeletal muscle (Agostini F et al 2010) and at the systemic level (Biolo G et al 2008).

Low levels of physical activity also promote oxidative stress, which, through the activation of specific proteases and of apoptosis (Powers SK et al., 2005; Powers SK et al., 2007), is involved in the processes leading to muscle atrophy (Laufs U et al., 2005). Our research group has investigated, for the first time, the association (Brocca L et al 2012) in healthy subjects between immobilization and oxidative stress through human bed rest. The development of muscle atrophy is associated with impaired anti-oxidant defense systems (de Boer MD et al 2008), protein oxidation and/or lipid peroxidation (indices of redox imbalance), suggesting a major role of oxidative stress in disuse atrophy (Pellegrino MA et al 2011; Dalla Libera L et al 2009).

In our previous data, we observed that bed rest caused an early and persistent down-regulation of myofibrillar protein content, impairment of antioxidant defense systems and redox imbalance, followed, at later stages, by muscle fiber atrophy (de Boer MD et al 2008). The experimental inactivity caused an early down-regulation (after 8 days) of the antioxidant defense system, including: superoxide dismutase (SOD - which converts the superoxide ion into hydrogen peroxide), peroxiredoxine (such as catalases, which work downstream of SOD, converting hydrogen peroxide into water), α - β -crystallin and some heat shock proteins (such as HspB1 and Hsp70 – which remove products induced by free radicals and have been shown to protect cells against oxidative damage). Only few components i.e. heme-oxygenase-1 (HO-1) and glucose regulated protein-75 (Grp75), of the antioxidant system showed a transient early, 8 days, post-BR up-regulation (Mazzucco S et al 2010). After a prolonged period of inactivity (24 days) the mRNA for NRF2, the major transcription factor for the expression of the antioxidant defense system, such as heme oxygenase-1 (HO-1), catalase, SOD, peroxiredoxins, and genes, involved in glutathione synthesis and function, was found to be up-regulated, possibly in response to the ongoing redox imbalance. In our study we observed an increased protein carbonylation (an index of oxidative stress) at 35-days post-BR, in muscle biopsy samples (Mazzucco S et al 2010; Lawler JM et al 2003), which correlated positively with the decreased muscle fiber, cross sectional areas (CSA) associated with muscle atrophy and reduced protein synthesis (Mazzucco S et al 2010). 35 days of experimental inactivity determined also an increased muscle glutathione synthesis. These data correlated with the levels of protein carbonylation, suggesting the relevance of maintaining adequate GSH levels. Glutathione depletion can influence bed rest outcomes (Sastre J et al. 1989), while an increased bioavailability of glutathione precursors, such as cysteine or N-acetyl-cysteine, can improve glutathione system scavenging action (Khamaisi M et al. 2000) and reduce, in an animal model, muscle protein catabolism (Ling PR et al. 2007). Thus, dietary glutathione precursor supplementation may ameliorate immobilization outcomes.

Inactivity leads to insulin resistance and low-grade systemic inflammation (Lawler JM et al. 2003). Indeed, during bed rest there is an activation of the inflammatory response (Lawler JM et al. 2003; Biolo G et al 2007) that is related, with reciprocal influences, to the increased oxidative stress and insulin resistance. We have evaluated in a 14-days experimental bed rest (Biolo G et al 2007) the effects of immobilization on inflammatory response by assessing the levels of pro-inflammatory cytokines (Interleukine-6, IL-6) and anti-inflammatory cytokines (Interleukine-10, IL-10) and of

two acute-phase proteins (C-Reactive Protein, CRP or short pentraxin and long pentraxin-3, PTX3). Bed rest, on eucaloric conditions, significantly increased CRP and IL-6 concentrations and decreased mRNA transcript levels for IL-10.

Notably oxidative stress, through the glutathione system, and changes in the reduced glutathione (GSH)/oxidized glutathione disulfide (GSSG) ratio is related to the inflammatory response. Indeed ROS modulate the transcription of IL-4, IL-6, IL-8 and tumor necrosis factor- α (TNF- α), through thiol-dependent mechanisms, while cytokines can mediate ROS signaling (Laviano, A et al 2007). Our preliminary data suggest that oxidative stress and changes in glutathione levels are associated with insulin resistance conditions (such as type II diabetes and obesity) (Badaloo, A et al. 2002; Ikemoto M et al. 2002). Decreased intracellular glutathione levels have been suggested to play a direct causative role in the development of impaired insulin action in adipose tissue and skeletal muscle (Agostini F et al 2010). Insulin resistance and deficiency are often associated with hyperglycemia in diabetes and critical illness and hyperglycemia seems to be related with generation of ROS, increased oxidative stress and decreased liver glutathione concentrations (Bosutti A et al. 2008). Indeed liver is the major glutathione storage organ and the major source of plasma glutathione; circulating erythrocytes may reflect the synthetic capacity of the liver in the inter-organ glutathione homeostasis (Haddada JJ & Harbb HL 2005).

Cross-sectional studies identified strong relationships between insulin resistance, systemic inflammatory response and alterations in fatty acid (FA) composition of cell membrane phospholipids (Das UN 2004; Vessby B et al. 2002). The poly-unsaturated FAs (PUFAs) of the n-6 and n-3 series are involved in up-regulation and down-regulation of the inflammatory response, respectively.

We have already demonstrated that bed rest prolonged for 35 days causes increased insulin resistance (Lawler JM et al. 2003). These metabolic alterations were associated with changes in erythrocyte membrane fatty acid composition. We observed increased levels of pro-inflammatory n-6 PUFAs and fractional content of pro-inflammatory arachidonic acid, decreased levels of anti-inflammatory omega 3 PUFAs (alpha-linolenic and eicosapentaenoic, EPA) and of monounsaturated fatty acids. These changes were associated with altered activity of the enzymes $\Delta 5$ and $\Delta 9$ desaturase, affected by insulin action (Lawler JM et al. 2003). Low $\Delta 5$ desaturase activity decreased long-chain PUFA content. This may cause changes in the cell membrane physical properties, potentially leading to altered receptor binding capacities and further impairment of insulin sensitivity (Peter A et al. 2009).

1.3.1.5. Hypoxia

Sarcopenia is caused by multiple factors; recently a role has been attributed to hypoxia (Di Giulio C et al. 2009). Conditions associated with hypoxia are aging and chronic respiratory diseases (i.e. COPD), obesity-related obstructive sleep apnea syndrome (OSAS) and cardiorespiratory disorders. During aging there is a general increased production of ROS with possible negative effects on proteins, nucleic acids and lipids and on membrane functions (Di Giulio C et al. 2009; Cataldi A & Di Giulio C. 2009). This higher oxidative stress can be related, among other factors, to a reduction of the oxygen flow from the lungs to the tissues, leading to a lower cellular pO_2 . The reduced blood flow and oxygenation of skeletal muscle, and the higher ROS production together with a diminished mitochondrial density (Di Giulio C et al. 2009), can contribute to the loss of muscle mass of aging (Gunnarsson L et al. 1996; Muller FL et al. 2007; Porter MM et al. 1995).

Furthermore the normal ventilatory response to hypoxia characterized by increased volume and ventilatory frequency are attenuated with aging (Fukuda Y et al 1992).

Muscle atrophy has been shown also in subjects with recurrent obstructive sleep apnea syndrome (OSAS), cardiopulmonary disease and in COPD patient (Di Giulio C et al. 2009). Hypoxia is central to the pathogenesis of both OSAS and COPD, further sustained by inflammatory and oxidative stress pathways (Figure 15)

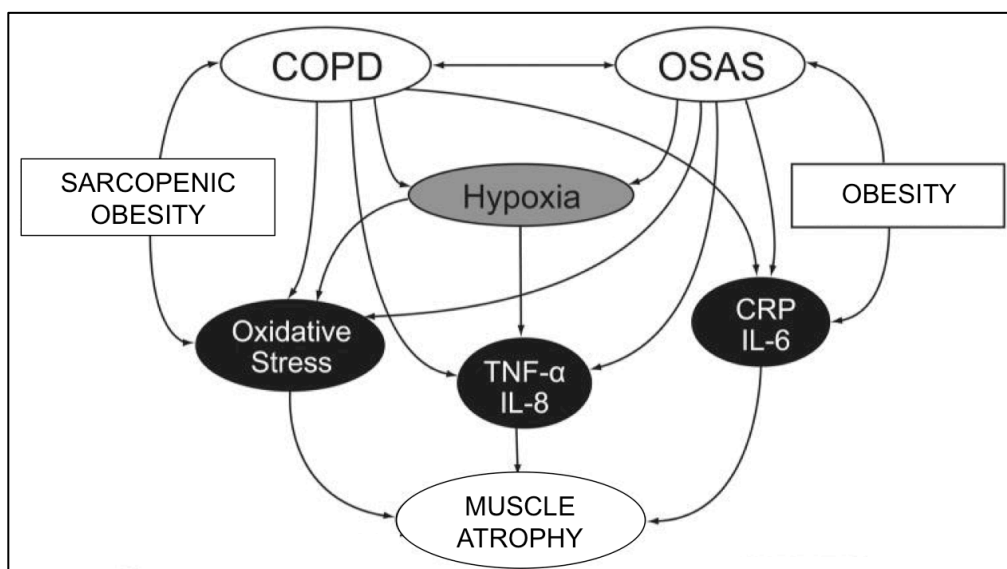


Figure 15. Hypoxia, the link among COPD, OSAS, systemic inflammation, and muscle atrophy.

Adapted from McNicholas WT. Am J Respir Crit Care Med. 2009

Patients with chronic heart failure or COPD during the course of the illness lose skeletal muscle mass leading to exercise limitations and lower quality of life. Epidemiological studies have shown that between 15-30% of COPD patients were found sarcopenic (Jones SE et al 2015; Biolo et al 2014; Koo HL et al 2014) with or without sarcopenic obesity. Sarcopenia and obesity were independent risk factors for respiratory complications and worsening of COPD, however, the respiratory loss was most severe in obese sarcopenic subjects (Koo HL et al 2014). Sarcopenic COPD patients had a greater airflow obstruction, were significantly older and compared to non-sarcopenic subjects, showed reduced quadriceps strength, functional performance and exercise capacity (Jones SE et al 2015).

2. AIM

The aim of the present thesis was to investigate in human healthy volunteers new biomarkers adequate to define optimal protein intake. We based our research on recent studies that have determined protein needs by measuring whole-body protein metabolism using stable labeled isotope-amino acids.

The present work includes two experimental protocols:

- 1) The PLANHAB study on the effects of hypoxia and inactivity isolated or combined on muscle mass and function and protein, glucose and lipid metabolism, oxidative stress and inflammation. Hypoxia and inactivity can be considered models, in humans, of clinical conditions such as COPD, CVD and OSAS characterized by loss of muscle mass and function and normal or expanded fat tissue (sarcopenia and sarcopenic obesity).
- 2) The INTERREG PANGeA on the effects of aging and immobilization on muscle mass and function, protein and glucose metabolism in elderly subjects compared to young controls

3. METHODS

The main goal of this thesis is to find new biomarkers to define optimal protein requirement in physio-pathological conditions. Specifically we have investigated the detrimental effects of physical inactivity with or without hypoxia and in aging, on muscle protein synthesis. Moreover the effects of these conditions on, oxidative stress, inflammation, lipid metabolism and insulin sensitivity were also evaluated as related conditions influencing MPS. The bed rest model is known to be a reliable approach to study the impact of muscle unloading on human metabolism (Biolo G et al., 2005). To investigate hypoxia, we utilized a special structure with areas at controlled oxygen levels. The results reported in the present thesis were collected during two different European funded experimental bed rest study: 1) the FP7 PLANHAB study (Planetary Habitat simulation). A 7 Framework program 2007-2013 and 2) the INTERREG PANGeA (Physical Activity and Nutrition for Quality Ageing). Italy-Slovenia European Program for Cross Border Cooperation 2011-2014.

3.1. Experimental procedures

3.1.1. *Whole body protein kinetics*

Whole body protein kinetics were determined in plasma by the tracer model of phenylalanine and tyrosine metabolism, as previously described (Antonione R et al., 2008).

Briefly, after an overnight fasting, two polyethylene catheters were inserted into a forearm vein of a subject for isotope infusion and into a wrist vein of the opposite arm, the latest was heated at 50°C, for arterialized venous blood collection. Before the start of the infusion, at time 0, a background blood sample was collected to assess the natural occurring isotopic enrichments of [ring-²H₅]-phenylalanine, [ring-²H₄]-tyrosine, ²H₂-tyrosine in plasma. After background blood collection, 7-hour primed-continuous infusions of [ring-²H₅]-phenylalanine (3.3 μmol×kg⁻¹×h⁻¹) and ²H₂-tyrosine (1.0 μmol×kg⁻¹×h⁻¹) in parallel with a single bolus of [ring-²H₄]-tyrosine (1.1 μmol×kg⁻¹) (Cambridge Isotope Laboratories, Andover, MA). Blood samples were drawn 180, 300 and 420 minutes after isotope infusion, to assess the plasma enrichments of [ring-²H₄]-tyrosine and [ring-²H₅]-phenylalanine. Blood was collected in EDTA tubes, immediately centrifuged at 3000 g at 4°C for 10 minutes; plasma and erythrocytes were immediately stored at -80°C for further analysis.

Isotopic enrichments of phenylalanine and tyrosine, in plasma, were determined by gas chromatography–mass spectrometry (GC-MS) (HP 5890; Agilent Technologies, Santa Clara, CA) as previously described (Biolo G et al., 2008; Antonione R et al., 2008). Isotopic enrichments were assessed considering the following mass-to-charge ratios ($m \times z^{-1}$): phenylalanine $m \times z^{-1}$ 234-239; tyrosine $m \times z^{-1}$ 466-470.

Phenylalanine appearance from protein proteolysis (Phe Ra from protein proteolysis) was calculated multiplying the infusion rate of [ring- $^2\text{H}_5$]-phenylalanine for the enrichment of [ring- $^2\text{H}_5$]-phenylalanine. The rate of phenylalanine disappearance through hydroxylation to tyrosine (Phe Rd to hydroxylation), an index of net protein catabolism, was defined as follows:

$$\text{Phe Rd to hydroxylation} = (\text{IRD}_2\text{Tyr} \times \text{ED}_2\text{Tyr}^{-1}) \times (\text{ED}_4\text{Tyr} \times \text{ED}_5\text{Phe}^{-1})$$

where IRD_2Tyr is the infusion rate of $^2\text{H}_2$ -tyrosine; ED_2Tyr is the enrichment of $^2\text{H}_2$ -tyrosine; ED_4Tyr is the enrichment of [ring- $^2\text{H}_4$]-tyrosine; and ED_5Phe is the enrichment of [ring- $^2\text{H}_5$]-phenylalanine. Phe Rd to protein synthesis was calculated as the difference between Phe Ra from protein proteolysis and Phe Rd to hydroxylation (Figure 16).

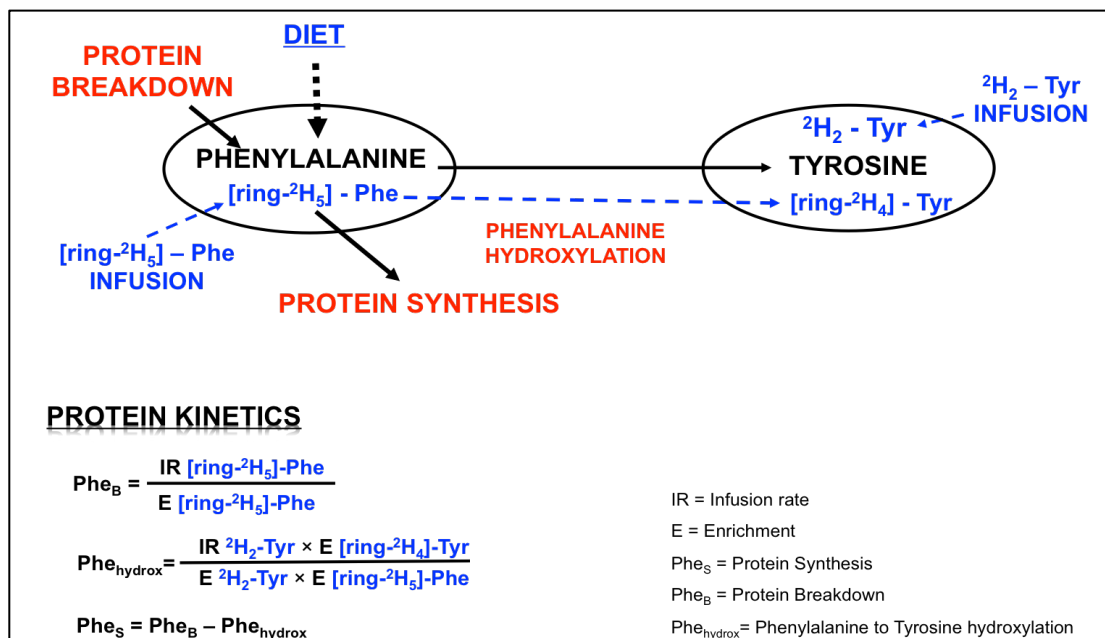


Figure 16. Measurement of whole body protein kinetics Humans

In the postabsorptive state there is no entry of amino acids from dietary sources, and the flux of phenylalanine in the body is derived from entry of phenylalanine released from protein breakdown. That input is matched by phenylalanine removal via protein synthesis and via metabolic disposal by conversion to tyrosine. Therefore, the measurement of the whole body rate of appearance of phenylalanine in the postabsorptive state is a measure of the whole body rate of proteolysis (Matthews DE 2007).

3.1.2. Post-prandial anabolic resistance

In the morning a polyethylene catheter was inserted into a forearm vein of subjects, fasted overnight, for blood collection. After basal blood sampling, at time 0 (t_0), an *oral load* of 0.3g of $^2\text{H}_5$ -phenylalanine, dissolved in 15-20mL of water, was administered to the volunteers to be drunk in 5 minutes. Blood samples were drawn at 30, 60, 120, 180, 240, 300 and 360 minutes after the the loading, in EDTA tubes then immediately centrifuged at 3000g at 4°C for 10 minutes. Plasma was immediately stored at -80°C. Plasma samples were used to assess the concentration of $^2\text{H}_5$ -phenylalanine (D5-Phe) and $^2\text{H}_4$ -tyrosine (D4-Phe), the product of phenylalanine hydroxylation.

Isotopic enrichments of plasma phenylalanine and tyrosine, derived by phenylalanine hydroxylation, were determined by gas chromatography–mass spectrometry (GC-MS) (HP 5890; Agilent Technologies, Santa Clara, CA) as t-butyldimethylsilyl derivatives (Biolo G et al., 2008). Plasma concentrations of leucine, phenylalanine and tyrosine were assessed in all samples by GC-MS, using the internal standard technique, as previously described (Biolo G et al., 2008). Known amounts of ^{13}C -leucine, ^{13}C -phenylalanine and $^2\text{H}_2$ -tyrosine (Cambridge Isotope Laboratories) were added as internal standards. Isotopic enrichments were assessed considering the following mass-to-charge ratios ($m \times z^{-1}$): phenylalanine $m \times z^{-1}$ 234-239; tyrosine $m \times z^{-1}$ 466-470. Amino acids in plasma were monitored using the following $m \times z^{-1}$: leucine $m \times z^{-1}$ 302-303, phenylalanine $m \times z^{-1}$ 336-337 and tyrosine $m \times z^{-1}$ 466-468.

Amino acid concentrations $[aa]$ was determined by the internal standard technique as follows:

$$[aa] = a \times \text{TTR}$$

where a is the concentration of the internal standard added to plasma samples and TTR (Tracer-to-Tracee Ratio) is the isotopic enrichment of the internal standard.

Phe-D₅ and Tyr-D₄ concentrations, $[aa^*]$, was calculated as follows:

$$[aa^*] = [aa] \times \text{TTR}^*$$

where $[aa]$ is plasma concentration of the tracee (phenylalanine or tyrosine amino acid)

and TTR* is the isotopic enrichment of Phe-D₅ or Tyr-D₄.

The area-under-the curve (AUC), i.e., the area under the plasma amino acid concentration versus time curve, was estimated using the linear trapezoidal method.

Blood was collected every 60 minutes for 6 hours. Thus the index of anabolic resistance was calculated as ratio between AUC Tyr-D₄ and AUC Phe-D₅, assessed over 6h meal test.

$$\text{AUC Tyr-D}_4/\text{AUC Phe-D}_5$$

A new method

After the data analysis, we calculated the same index, using only the blood collections of the first two 2 hours from meal and [ring-²H₅]-phenylalanine load, requiring 2 blood draws, over the 7 planned, and 2h, over 6 of observation.

A simplify index of anabolic resistance was calculated as ratio between Tyr-D₄ concentration and Phe-D₅ concentration measured after 2 h from meal and Phe-D₅ load.

$$[\text{Tyr-D}_4]_{\text{T120}}/[\text{Phe-D}_5]_{\text{T120}}$$

The new, simplify index displayed similar results compared with the (bed-rest effect $p<0.05$; bed-rest×group interaction $p<0.05$) ($R=0.75$; $p<0.001$).

3.1.3. Post-prandial insulin resistance

Post-prandial state

In the morning, a polyethylene catheter was inserted into a forearm vein of a subject, fasted overnight, for blood collection. After basal blood sampling, at time 0 (t_0), a standardized *test meal* (500mL, 500Kcal, 55% of carbohydrate 15% of protein and 30% of fat; vanilla flavour, Nutricomp ®, B.Braun), was administered to the volunteers to be drank in 5 minutes. Blood samples were drawn at 30, 60, 120, 180, 240, 300 and 360 minutes after the the loading, in EDTA tubes then immediately centrifuged at 3000g at 4°C for 10 minutes. Plasma was immediately stored at -80°C. Plasma samples were used to assess insulinaemia and glycaemia in the fasting and fed state.

Insulin and glucose levels were measured with standard procedures by a certified external laboratory (Synlab Italia Srl, Italy) and their values were used to calculate:

The Area Under the Curve (AUC) of post-load insulinemia and glycemia.

The Matsuda index as parameter of insulin sensitivity in fed state.

$$\text{Matsuda Index} = 10000 / \sqrt{(\text{glyc}_{T0} \times \text{ins}_{T0} \times \text{ins}_{\text{average OGTT}} \times \text{glyc}_{\text{average OGTT}})}$$

Where (ins) and (glyc) indicate insulin and glucose plasma levels, respectively.

Fasting state

The HOmeostatic Model Assessment, as index of insulin resistance (HOMA-IR).

$$\text{HOMA index} = (\text{fasting glucose} \times \text{fasting insulinemia}) / 405$$

3.1.4. Amino acids

A known amount of internal standards were added to plasma samples (200 μL) to determinate the concentration of unlabeled AAs. The plasma samples were deproteinized adding sulfosalicylic acid (200 μL , 10%). After centrifugation (4000 rpm per 20 min at 4°C) the supernatants were purified in a cationic resin (AG50W-X8; Bio-Rad, Hercules, CA) using NH_4OH (4N) as eluent. The NH_4OH excesses were evaporated under N_2 flux. Samples were then lyophilized and the powders obtained were derivatized by the addition of 50 μL acetonitrile and 50 μL MTBSTFA and by heating at 90°C for 45 min. After derivatization, samples were injected into a gas chromatography-mass spectrometer (GC-MS) (HP 5890, Agilent Technologies, Santa Clara, CA, USA). Gas chromatographic measurements were performed in single ion monitoring mode, using the following mass-to-charge ratio (m/z): phenylalanine m/z -1 336; [ring-2H5]-phenylalanine m/z -1 341; tyrosine m/z -1 466; [3,3-2H2]-tyrosine m/z -1 468; [ring-2H4]-tyrosine m/z -1 470; homocysteine m/z -1 496; [13C]-homocysteine m/z -1 497; [2H8]-homocysteine m/z -1 500; methionine m/z -1 320; [1-13C, methyl-2H3]-methionine m/z -1 324; cysteine m/z -1 406; [3,3-2H2]-cysteine m/z -1 408.

3.1.5. Oxidative stress

Glutathione synthesis rate

In the morning, after an overnight fasting, two polyethylene catheters were inserted: one into a forearm vein for isotope infusion, and the other into a wrist vein of the opposite arm, heated at 50°C, for arterialized venous blood collection. Before the beginning of the infusion (time 0), a background blood sample was collected to assess the natural isotopic enrichments of $^2\text{H}_2$ -glycine and $^2\text{H}_2$ -glutathione in red blood cells (RBC). After that, a 7-hour primed-continuous infusion of $^2\text{H}_2$ -glycine ($26.5 \mu\text{mol}\times\text{kg}^{-1}\times\text{h}^{-1}$) was started (Cambridge Isotope Laboratories, Andover, MA). Blood samples were drawn at 180, 300 and 420 minutes after the start of isotope infusion, to assess enrichments of $^2\text{H}_2$ -glycine and [$^2\text{H}_2$ -glycine]-glutathione in RBC. Blood, collected in EDTA tubes, was immediately centrifuged at 3000 g at 4°C for 10 minutes; plasma and erythrocytes were immediately stored at -80°C for further analysis.

Isotopic enrichments of glycine and glutathione, in RBC, were determined by gas chromatography–mass spectrometry (GC-MS) (HP 5890; Agilent Technologies, Santa Clara, CA) as previously described (Biolo G et al., 2008; Antonione R et al., 2008). Isotopic enrichments were assessed considering the following mass-to-charge ratios ($m\times z^{-1}$): glycine $m\times z^{-1}$ 218-220; glutathione $m\times z^{-1}$ 363-366. Erythrocyte total glutathione concentrations were determined in background blood samples using the internal standard approach, through the addition of known amount of [$^{13}\text{C}_2$ - ^{15}N -glycine]-glutathione (Cambridge Isotope Laboratories, Andover, MA).

Glutathione fractional turnover rate (FTR; in $\% \times \text{d}^{-1}$) was calculated as:

$$[\text{E}(^2\text{H}_2\text{-glutathione})\times\text{t}^{-1}]\times\text{E}(^2\text{H}_2\text{-glycine})^{-1} \times 24 \times 100$$

where $\text{E}(^2\text{H}_2\text{-glutathione}) \times \text{t}^{-1}$ is the slope of the regression line describing the rise in erythrocyte $^2\text{H}_2$ -glutathione enrichment as a function of time (hours); $\text{E}(^2\text{H}_2\text{-glycine})$ is the mean glycine enrichment in erythrocytes after steady state achievement. Coefficients (i.e., 100 and 24) were applied to express glutathione fractional turnover rate as $\%\times\text{d}^{-1}$. The absolute turnover rate was calculated as the product of FTR and glutathione concentrations.

The ratio between the concentrations of reduced and oxidized forms of glutathione in erythrocytes (GSH/GSSG ratio) was assessed in the same background blood sample, by a commercially available kit (GT40, Oxford Biomedical Research; Oxford, MI).

Glutathione synthetic capacity.

Glutathione peroxidase activity was determined in erythrocytes according to Paglia and Valentine (Paglia DE & Valentine WN 1967) and expressed as μmol metabolized NADPH \times min⁻¹ \times g protein⁻¹ in the presence of an organic hydroperoxide (cumolhydroperoxide) and of reduced glutathione as enzyme cofactor.

Catalytic and modulator subunit expression of glutamatecysteine ligase (GLC) in erythrocytes was measured by Western blot analysis (Thompson SA et al., 2000). Briefly, proteins were extracted from red blood cells by using a lysis buffer (45 mmol/L Tris-HCl, 0.2% Nlaurylsarcosine; Sigma-Aldrich, St Louis, MO) containing proteinase and phosphatase inhibitors (0.2 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol, 2 μg aprotinin/mL, 2 μg pepstatin \times mL⁻¹, 0.1 mmol NaF \times mL⁻¹, and 0.1 mM Na₃VO₄; all: Sigma-Aldrich). After centrifugation (10 min, RT, 16 00 \times g), proteins were separated by sodium dodecyl sulfate–polacrylamide gel (12%) electrophoresis and transferred to a nitrocellulose membrane (Protran; Perkin Elmer, Boston, MA). Proteins were recognized by using commercial antibodies raised against the catalytic (GCLc: sc-22755) and modulator (GCLm: sc-22754) (both: Santa Cruz Biotechnology Inc, Santa Cruz, CA) subunits of the glutamate-cysteine ligase. Glyceraldehyde-3-phosphate dehydrogenase was recognized by commercial antibody (sc- 25778; Santa Cruz Biotechnology Inc). A goat anti-rabbit horseradish peroxidase–conjugated immunoglobulin G (Sigma-Aldrich) was used as secondary antibody. Protein complexes were detected by enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL) on photographic film (Kodak Biomax Light Film; Sigma-Aldrich). Protein concentrations in the catalytic and modulator subunits of glutamate-cysteine ligase were measured by band densitometry as a ratio with glyceraldehyde-3-phosphate dehydrogenase protein concentration (Model 45–700 Imaging Densitometer; Bio-Rad).

3.1.6. Lipid pattern

Total cholesterol, HDL cholesterol and triglycerides were measured with standard methods by a certified external laboratory (Synlab Italia Srl, Italy).

Commercially available kits were used to determine plasma concentrations of:

- Cholesterylester transfer protein (CETP, ALPCO, Salem, NH, USA),
- Lecithin cholesterol acyltransferase (LCAT, MyBioSource, CA, USA),
- Lipoprotein lipase (LPL, MyBioSource, CA, USA),
- Hepatic lipase (HL, MyBioSource, CA, USA),
- Serum amyloid A, (SAA, Abcam, UK),
- TNF-related apoptosis-inducing ligand (TRAIL, R&D Systems, Minneapolis, MN).

HDL2 and HDL3 concentrations were assessed using the precipitation technique (chemical reagents were purchased from Gesan Production srl (Italy) and Fitzgerald Industries International, MA, USA).

Plasma LDL cholesterol was measured by the Friedewald's formula:

$$\text{total cholesterol} - \text{HDL cholesterol} - (\text{triglycerides} \times 5 - 1).$$

3.1.7. Systemic inflammation

high-sensitivity C reactive protein, was measured with standard methods by a certified external laboratory (Synlab Italia Srl, Italy).

3.1.8. Membrane fatty acid composition

Fatty acid membrane composition of red blood cells was analyzed modifying a previously published method (Burdge GC et al. 2002). Erythrocytes (200 μ L) were washed five times with decreasing concentrations (10 mmol/L, 2.5 mmol/L; 1.25 mmol/L; 0.625 mmol/L; 0.312 mmol/L) of phosphate buffered saline (PBS). Total lipid extraction was performed in 5 mL of a chloroform–methanol (2:1) solution, containing 50 mg/L of butylhydroxytoluene as antioxidant, and 1mL of 1M NaCl solution. After centrifugation, the lower lipid phase was collected and dried under nitrogen flux at 40 °C. Pellets were dissolved in toluene (500 μ L), and after the addition of 1mL of a methanol solution containing 2% of H₂SO₄, were heated at 50 °C for 2 h. A neutralizing solution (1.0 ml, 0.25 M KHCO₃ and 0.5 M K₂CO₃ in deionized H₂O) and hexane (1 mL) was added. After centrifugation, the hexane layer, containing fatty acid methyl esters (FAMES), was collected and organic solvents were removed by N₂ flux. After the addition of hexane (150 μ L), samples were analyzed by gas-chromatography–flame ionization detection (GC- FID; GC 6850 Agilent Technologies, Santa Clara, CA, USA). Specific fatty acid standards were used to identify FAMES by retention times in erythrocyte samples. A commercial mixture of purified fish oil fatty acids (Menhaden oil, Sigma–Aldrich, Inc, MO, US) was used to detect: oleic acid (18:1, n-9), elaidic acid (trans 18:1, n-9), eicosapentaenoic acid (20:5, n-3), docosapentaenoic acid (22:5, n-3) and docosahexaenoic acid (22:6, n-3). Retention times of myristic acid (14:00), palmitic acid (16:00), palmitoleic acid (16:1, n-7), stearic acid (18:00), linoleic acid (18:2, n-6), α -linolenic acid (18:3, n-3), eicosaenoic acid (20:1, n-9), eicosadienoic acid (20:2, n-6), dihomogamma-linolenic acid (20:3, n-6) and arachidonic acid (20:4, n-6) were identified by commercial standards. Adrenic acid (22:4, n-6) and docosapentaenoic acid (22:5, n-6) were identified by commercial standards from Nu-Check Prep, Inc, MN, US. Organic solvents and buffering salts were purchased from Sigma–Aldrich, Inc, MO, US, if not differently specified.

Area-under-the-curve of each selected peak was determined by highly standardized hand integration performed using commercial software (HP Chem station; Agilent Technologies, Santa Clara, CA, USA).

Erythrocyte membrane fatty acid (EMFA) composition was assessed by gas-chromatography-flame ionization detection (GC-FID; GC 6850 Agilent Technologies, Santa Clara, CA, USA), as previously reported (Mazzucco et al. 2010).

Red blood cell membrane level of each enlisted FA was expressed as percent ratio between area-under-the-curve of each selected EMFA peak and the sum of all measured EMFA peaks.

The following indices were calculated:

- Δ -5 desaturase index, as index of insulin sensitivity, was defined as ratio between arachidonic acid (20:4, n-6) and dihomo- γ -linolenic acid (20:3, n-6) membrane levels.
- Δ -9 desaturase index, as index of insulin sensitivity, was calculated as ratio between oleic (18:1, n-9) and stearic (18:0) acid contents.
- Arachidonic-to-eicosapentaenoic acid ratio, as index of inflammation, was calculated as ratio between arachidonic acid (20:4, n-6) and eicosapentaenoic acid (20:5, n-3) membrane levels.

3.2. Human study protocols

3.2.1. *The PLANHAB study (experimental hypoxia and bed rest)*

This study was conducted at the Olympic Sport Center Planica, in Rateče, Slovenia. Two floors of the Olympic center are structured to simulate any altitude conditions in the all areas and in each room individually. Eleven male young subjects (mean \pm sem; age 24 ± 4 yr; BMI 22 ± 2 kg/m²) were selected to participate to the experimental study. The experimental protocol was approved by the ethical committee of the University of Ljubljana (Slovenia) and was in accordance with the Declaration of Helsinki and following amendments. A written informed consent was obtained from each individuals. Subjects were physically active before the study and none of them was under medication.

Subjects have been randomly assigned to three groups. Each group participated to three experimental campaigns: i) 10-day normoxic normoxia (21 kPa oxygen) bed rest, NBR ii) 10-day hypoxic (12.5 kPa oxygen, corresponding to 4000 m above sea level) bed rest, HBR iii) 10-day hypoxic ambulation HAMB. Each campaign was preceded by 5 days of dietary and environmental adaptation and was followed by 5 days of recovery (Figure 17). A 2-month wash-out period was allowed between each campaign. Dietary intake was controlled to maintain subjects in eucaloric condition; before the study, individual resting energy expenditure (REE) was calculated according to the FAO/WHO equations (Muller MJ et al. 2004).

Each subject received a dietary energy intake equals to 1.4 and 1.1 times his REE in the ambulatory (adaptation and recovery periods as well as hypoxic ambulation) and bed rest (normoxic and hypoxic bed rests) periods, respectively. Menu composition was adapted to individual dietary habits. Subjects received six meals daily (i.e., 3 main meals and 3 snacks). Each individual was tested at the baseline (normoxic ambulation) and at the end (day 10) of each experimental condition. All foods were weighed for each participant, and volunteers were asked to consume the complete meal. At the beginning and at the end of each experimental condition, body composition was assessed by DXA (Discovery W—QDr series, Hologic, Bedford USA). Whole body protein turnover as well as glutathione kinetics were determined by stable isotope infusion technique, as previously described (see experimental design section 2.1.1, 2.2.5).

Statistics

All data were expressed as mean \pm S.E.M. Hypoxia and bed rest effects were analyzed by repeated measure ANOVA with two levels of interaction, physical activity and oxygen partial pressure. If significant interaction was evidenced, post hoc analysis was performed using Student t-test with Bonferroni correction. Statistical significance was achieved with p-value <0.05 . Statistical analysis was performed using SPSS software (version 12; SPSS, Inc., Chicago, IL).

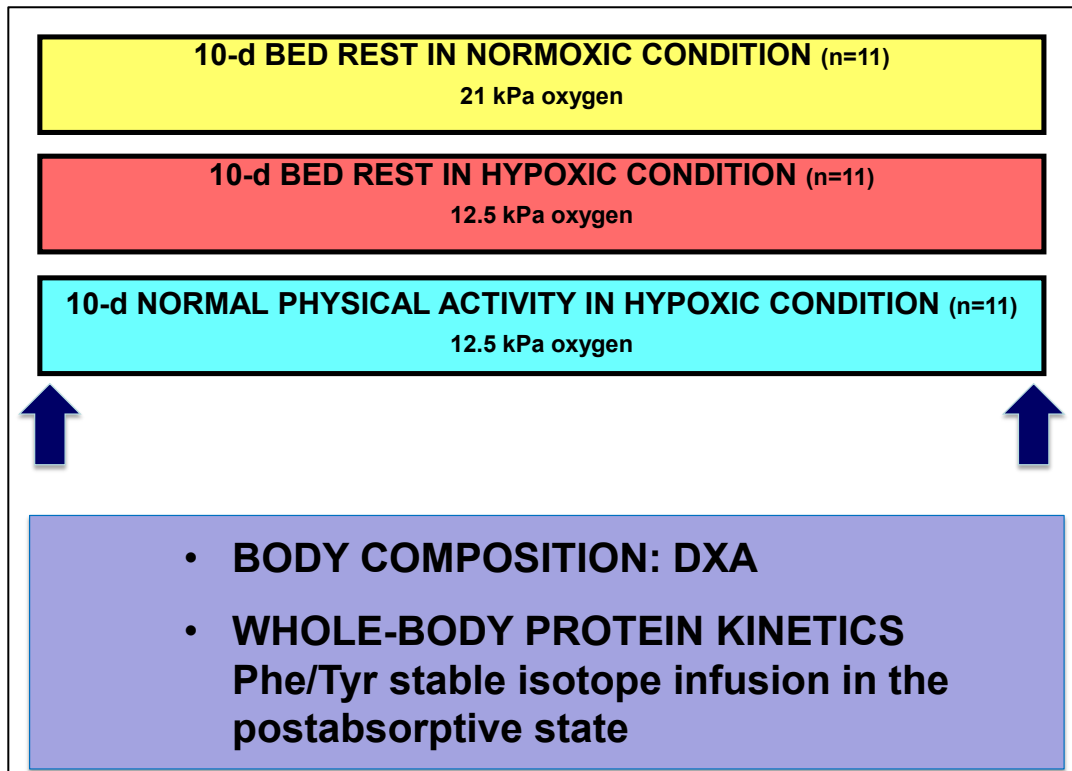


Figure 17. The PLANHHAB Study experimental design

Subjects were randomly assigned to three groups. Each group participated to three experimental campaigns: i) 10-day normoxic normoxia bed rest (yellow) ii) 10-day hypoxic bed rest (red) iii) 10-day hypoxic ambulation (blue). A 2-month wash-out period was allowed between each campaign.

3.2.2. *The PANGeA study (bed rest in the elderly)*

PANGeA is an European program (INTERREG) aiming to answer to some scientific questions that refer to the impact of motor inactivity on the elderly and, consequently, on their health.

The bed rest study was conducted at the Valdoltra Hospital (University of Primorska, Ankaran-Capodistria, Slovenia). Fifteen healthy male subjects were enrolled: 7 young males (mean \pm S.E.M.; age 23 ± 1 years; BMI 24.0 ± 0.9 kg/m²) and 8 elderly subjects (mean \pm S.E.M.; age 59 ± 1 years; BMI 26.8 ± 1.5 kg/m²). Body weight of all subjects had been stable from the previous 3 months. Preliminary standard anthropometric measures and routine medical screening were performed. Volunteers were admitted at the hospital one week before the bed rest period for dietary and environmental adaptation phase (Ambulatory period). At the end of this period, each subject underwent 14 days of bed rest in which all daily activities were performed in horizontal conditions.

The experimental protocol was approved by the ethical committee of the University of Ljubljana (Slovenia) and was in accordance with the Declaration of Helsinki and following amendments. A written informed consent was obtained from each persons. Subjects were physically active before the study and none of them was under medication, or had any acute or chronic illness at least from the three months preceding the protocol.

Dietary intake was controlled to maintain subjects in eucaloric condition; before the study, individual resting energy expenditure (REE) was calculated according to the FAO/WHO equations (Muller MJ et al. 2004). Participants received a diet containing 1.4 and 1.1 times their calculated REE during the ambulatory and the bed rest periods, respectively; All foods were weighed for each participant, and volunteers were asked to consume the complete meal.

Menu composition was adapted to individual dietary habits. Subjects received three meals daily (i.e., breakfast, lunch and dinner). Each individual was tested at the baseline (BR0) and at the end (BR+14) of the experimental condition. In these days all subjects underwent to a metabolic test in order to estimate both insulin sensitivity and anabolic sensitivity in fed state through a new, simple, safe and quick method, as previously described (see experimental design section - 2.2.2, 2.2.3)

At the beginning and at the end of each experimental condition, body composition was assessed by bioimpedentiometry (BIA 101, Akern Srl, Italy, following manufacture instructions) (Figure 18).

Statistics

Data are expressed as mean \pm SEM. In order to evaluate the effect of the bed rest and/or the effects of aging on the insulin resistance and on the anabolic resistance, multivariate ANCOVA statistical analysis was applied. Basal values were used as covariate. The changes induced by bed rest and/or aging, were assessed through a Student *T*-test.

Linear regression analyses were performed using Pearson's correlation. Data were logarithmic transformed when appropriate. P-values <0.05 were considered statistically significant. Statistical analysis was performed using SPSS software (version 12; SPSS, Inc., Chicago, IL).

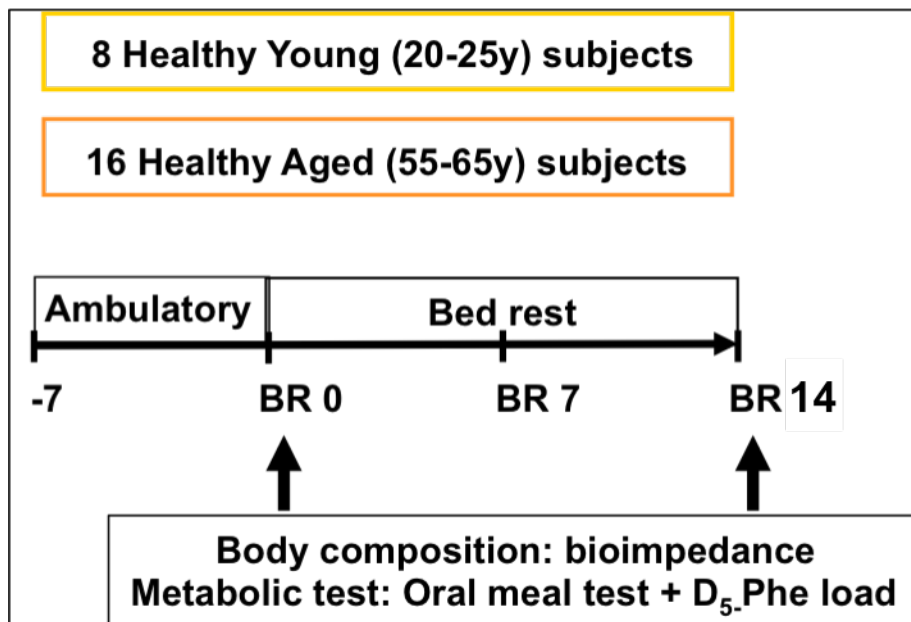


Figure 18. The PANGeA Study experimental design.

During each experimental day (BR0 and BR+14), after basal blood sampling, at time 0 (t_0), an oral load of 0.3g of 2H5-phenylalanine, dissolved in 15-20mL of water, was administered to the volunteers to be drunk in 5 minutes. Blood samples were drawn at 30, 60, 120, 180, 240, 300 and 360 minutes after the the loading.

4. RESULTS

4.1. The PLANHAB study (experimental hypoxia and bed rest).

4.1.1. Body composition

As reported elsewhere (Debevec T. et al. 2014) there was a significant reduction in body weight in all 3 conditions (-2.1% , -2.8% , and -2.0% for HAMB, HBR, and NBR, respectively; $p<0.05$), due to a significant decrease in lean body mass (-3.8% , -3.8% , -4.3% for HAMB, HBR, and NBR, respectively; figure 19A) with a slight but not significant increase in fat mass (Figure 19B).

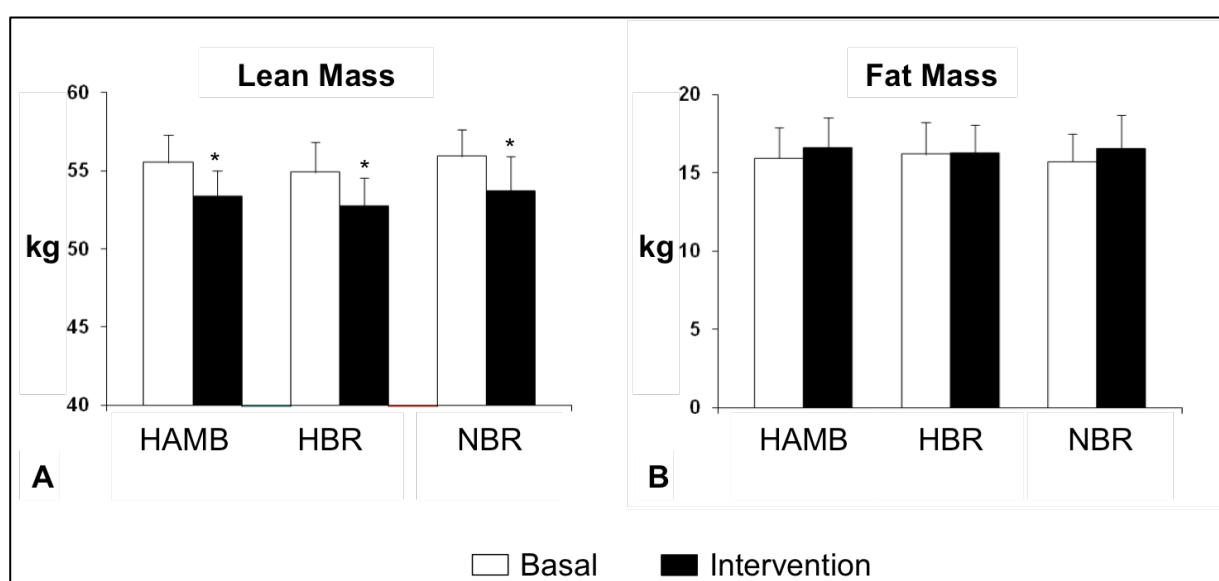


Figure 19. Effect of hypoxia in ambulatory conditions and during 10-d of bed rest on body composition.

N=11. Body composition changes before (basal, white columns) and after (intervention, black columns) ambulatory hypoxia (HAMB), bed rest Hypoxia (HBR) and bed rest normoxia (NBR) conditions * for $p<0.05$.

4.1.2. Whole body protein kinetics

As showed on table 3 Hypoxia significantly decreased whole body protein turnover (protein synthesis, $-8\pm 3\%$; protein degradation, $-9\pm 3\%$) in ambulatory conditions, while hypoxia tended to increase the rates of synthesis ($+4\pm 4\%$) and degradation ($+2\pm 4\%$) in bed rest. There were not significant effects of hypoxia or bed rest on the rates of phenylalanine hydroxylation to tyrosine (Figure 18), an index of net protein loss in the postabsorptive state (Basal 0.12 ± 0.01 , HAMB 0.11 ± 0.01 , NBR 0.12 ± 0.01 , HBR

0.11±0.01 mmol/min/kg LBM; bed rest effect p=0.98, hypoxia effect p=0.08, interaction 0.66).

Table 3 Effects of 10-d bed rest and hypoxia, per se or in combination, on protein metabolism.

	Ambulatory		Bed Rest		p [#]		
	Control	Hypoxia	Control	Hypoxia	Bed rest effect	Hypoxia effect	Interaction
Rd to protein synthesis (mmol/min/kg LBM)	0.96±0.03	0.88±0.03*	0.92±0.02	0.95±0.03	0.86	0.57	0.001
Ra from proteolysis (mmol/min/kg LBM)	1.08±0.03	0.99±0.04*	1.04±0.02	1.06±0.03	0.91	0.36	0.001
Rd to hydroxylation (mmol/min/kg LBM)	0.12±0.01	0.11±0.01	0.12±0.01	0.11±0.01	0.98	0.08	0.66

N=11. Data were expressed as mean±S.E.M. # Data were analyzed with the use of a 2-factor repeated measure ANOVA. Rd, rate of disappearance. Ra, rate of appearance. * p<0.05

On figure 20 are shown the effects of hypoxia and bed rest, alone or in combination, on the rates of whole body protein synthesis and degradation. There was significant hypoxia×bed rest interaction for the rates of protein synthesis and degradation.

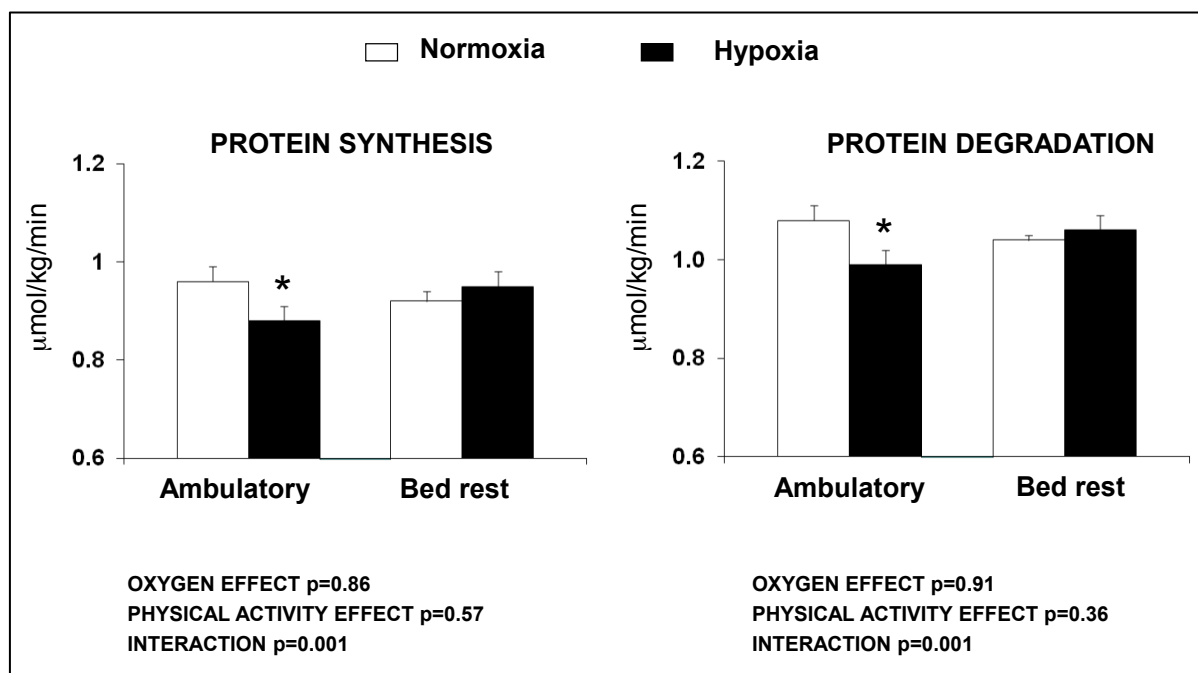


Figure 20. Effect of hypoxia in ambulatory conditions and during 10-d of bed rest on whole body protein kinetics.

N=11. Whole body protein kinetics changes in ambulatory and bed rest states during hypoxia (white columns), or normoxia (black columns) conditions * for p<0.05.

No changes were observed on phenylalanine hydroxylation an index of net protein loss (Figure 21).

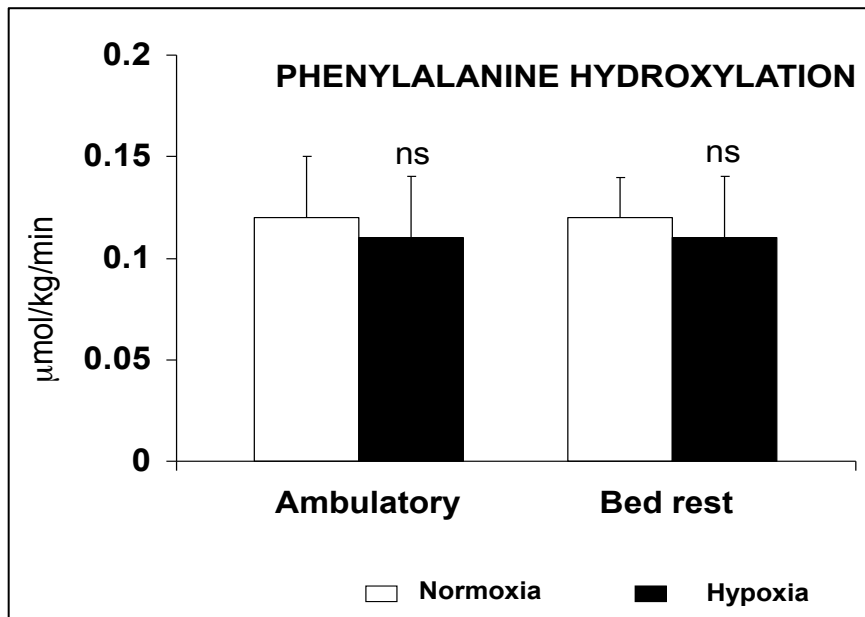


Figure 21. Effect of hypoxia in ambulatory conditions and during 10-d of bed rest on phenylalanine hydroxylation.

N=11. phenylalanine hydroxylation changes in ambulatory and bed rest states during hypoxia (white columns), or normoxia (black columns) conditions * for $p < 0.05$. ns = non significant

4.1.3. Plasma lipid profile

Effects of hypoxia per se and/or combined to bed rest lipid profile are reported on table 4.

Table 4. Effects of 10-d bed rest and hypoxia, per se or in combination on plasma lipid profile.

	Ambulatory		Bed Rest		p [#]		
	Normoxia	Hypoxia	Normoxia	Hypoxia	Bed rest effect	Hypoxia effect	Interaction
PLASMA LIPIDS							
Total cholesterol (mg·dL ⁻¹)	184±13	173±8	174±10	180±11	0.66	0.67	0.12
Tryglicerides (mg·dL ⁻¹)	94±14	113±14*	109±20	102±13	0.65	0.38	0.02
HDL cholesterol (mg·dL ⁻¹)	49±3	41±2*	42±2	39±3	<0.01	<0.001	0.02
LDL cholesterol (mg·dL ⁻¹)	117±12	110±7	110±7	121±9	0.57	0.70	0.14
HDL2-to-HDL3 ratio	0.48±0.08	0.43±0.07	0.55±0.09	0.38±0.05	0.93	0.26	0.55
ENZYMES INVOLVED IN LIPID METABOLISM							
Cholesteryl ester transfer protein (µg·mL ⁻¹)	2.91±0.23	3.00±0.16	2.86±0.20	3.01±0.19	0.87	0.31	0.85
Lecithin-cholesterol acyltransferase (ng·L ⁻¹)	21.8±4.2	20.3±4.3	21.7±4.7	23.8±6.3	0.66	0.99	0.45
Lipoprotein lipase (ng·L ⁻¹)	28.4±3.3	23.9±3.6	27.5±4.0	26.8±3.7	0.48	0.28	0.43
Hepatic lipase (U·mL ⁻¹)	0.72±0.10	0.79±0.09	0.80±0.09	0.90±0.08	0.15	0.04	0.81

N=11. Data were expressed as mean±S.E.M. # Data were analyzed with the use of a 2-factor repeated measure ANOVA. *p<0.05

HDL concentrations were significantly decreased both by bed rest and hypoxia with a significant negative bed rest×hypoxia interaction (Figure 20).

Furthermore the ratio between HDL and total cholesterol (Figure 20) was significantly decreased by physical inactivity (-8±4%, p=0.001) and hypoxia (-11±4%, p=0.003).

The ratio between HDL2 and HDL3 were not significantly changed. Plasma levels of total cholesterol, LDL and triglycerides were not significantly affected by 10-d of bed rest with or without hypoxia. On the other hand, the exposure to hypoxia in ambulatory conditions increased the triglycerides concentrations without changes in the other plasma lipid fractions (Figure 22).

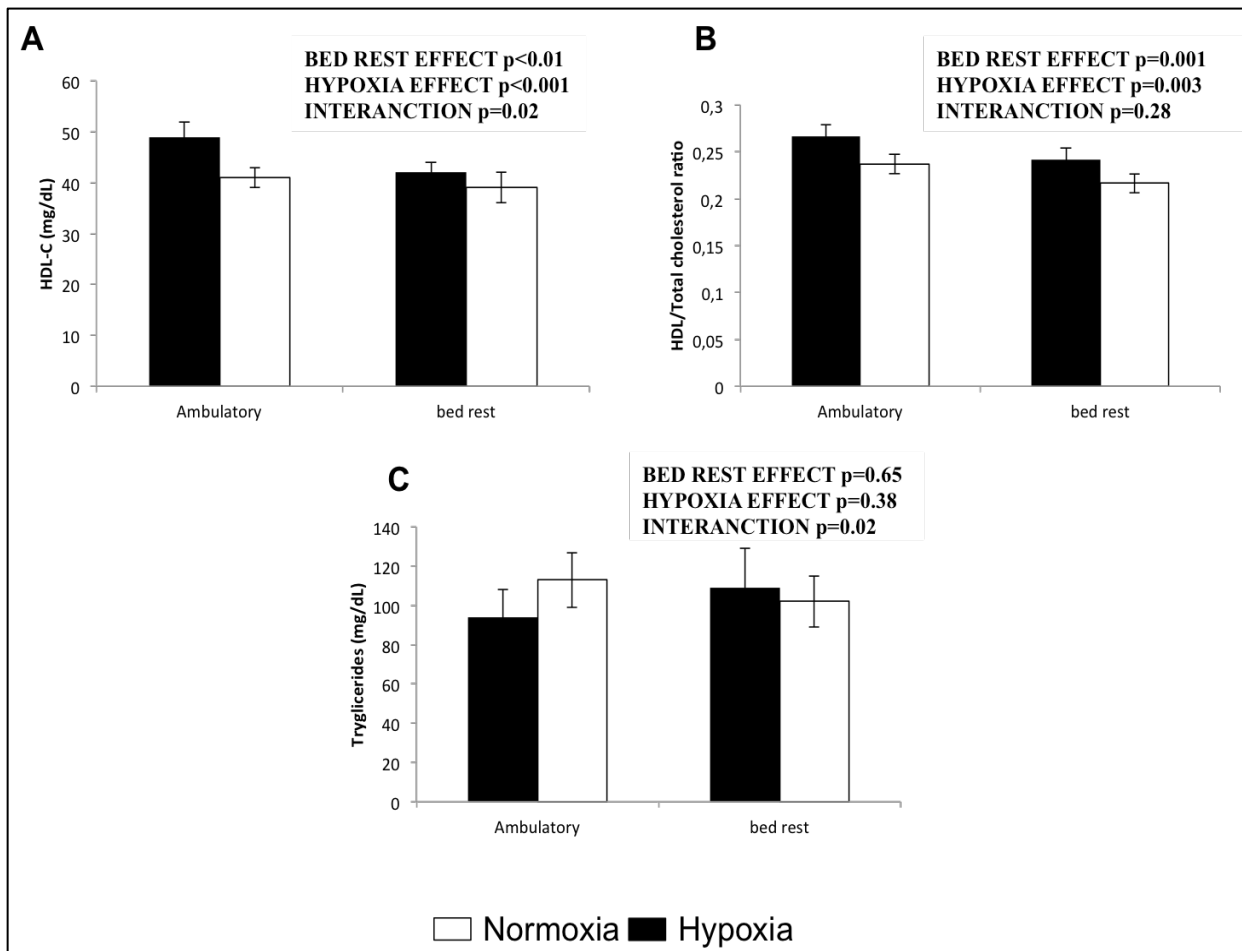


Figure 22. Effect of hypoxia in ambulatory conditions and during 10-d of bed rest on total and HDL cholesterol.

N=11. HDL cholesterol (A), Total Cholesterol (B) and HDL-to-total cholesterol ratio (C) modifications in ambulatory and bed rest states during hypoxia (black columns), or normoxia (white columns) conditions. Data were analyzed with the use of a 2-factor repeated measure ANOVA. Significance for $p < 0.05$.

Among the enzymes involved in lipid metabolism only HL was modified, with a significant increase in hypoxic conditions, together with a decreased LPL-to-HL ratio ($p=0.05$), independently from the physical activity levels (Figure 23).

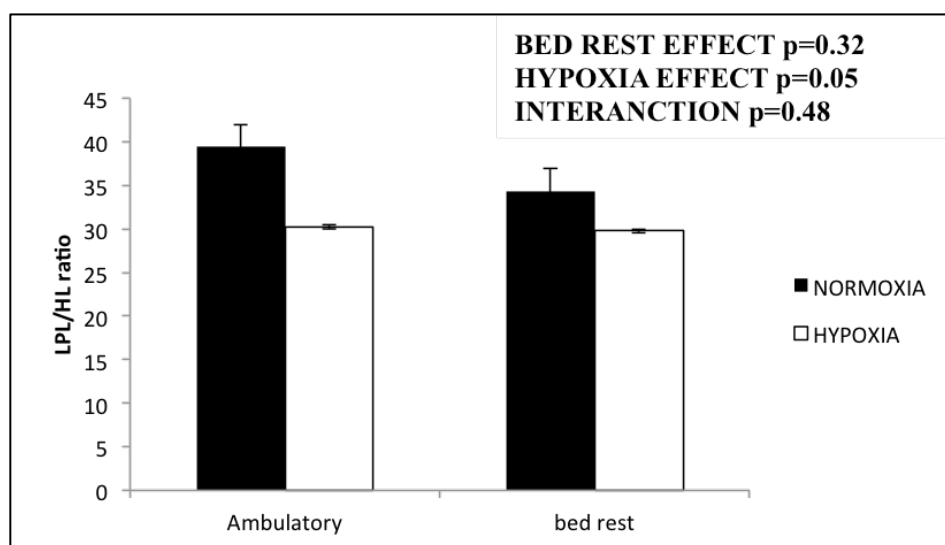


Figure 23. Effect of hypoxia in ambulatory conditions and during 10-d of bed rest on enzymes involved in lipid metabolism.

N=11. LPL-to-HL ratio modifications in ambulatory and bed rest states during hypoxia (black columns), or normoxia (white columns) conditions. Data were analyzed with the use of a 2-factor repeated measure ANOVA. Significance for $p<0.05$. LPL, Lipoprotein lipase. HL, Hepatic Lipase.

4.1.4. Insulin sensitivity.

There were no significant effects of hypoxia or bed rest on plasma glucose and insulin concentrations or on HOMA-IR index (Table 5). However the Δ -5 desaturase index was significantly reduced by both exposure to bed rest ($p=0.03$) and hypoxia ($p=0.02$). The index decreased by $5\pm 2\%$ after 10 days of physical inactivity and by $6\pm 2\%$ after 10 days of hypoxia, as compared to baseline conditions (Figure 24). A bed rest \times hypoxia interaction was not observed ($p=0.15$).

Table 5. Effects of 10-d bed rest and hypoxia, per se or in combination, on glucose metabolism.

INSULIN SENSITIVITY	Ambulatory		Bed Rest		$p^{\#}$		
	Normoxia	Hypoxia	Normoxia	Hypoxia	Bed rest effect	Hypoxia effect	Interaction
Fasting insulin ($\mu\text{U}\cdot\text{mL}^{-1}$)	7 \pm 1	7 \pm 1	7 \pm 1	6 \pm 1	0.85	0.93	0.27
Fasting glucose ($\text{mg}\cdot\text{dL}^{-1}$)	94 \pm 2	93 \pm 1	93 \pm 1	92 \pm 1	0.35	0.57	1.00
HOMA index	1.5 \pm 0.2	1.7 \pm 0.2	1.6 \pm 0.2	1.5 \pm 0.1	0.74	0.98	0.29

N=11. Data were expressed as mean \pm S.E.M. $\#$ Data were analyzed with the use of a 2-factor repeated measure ANOVA. * $p<0.05$

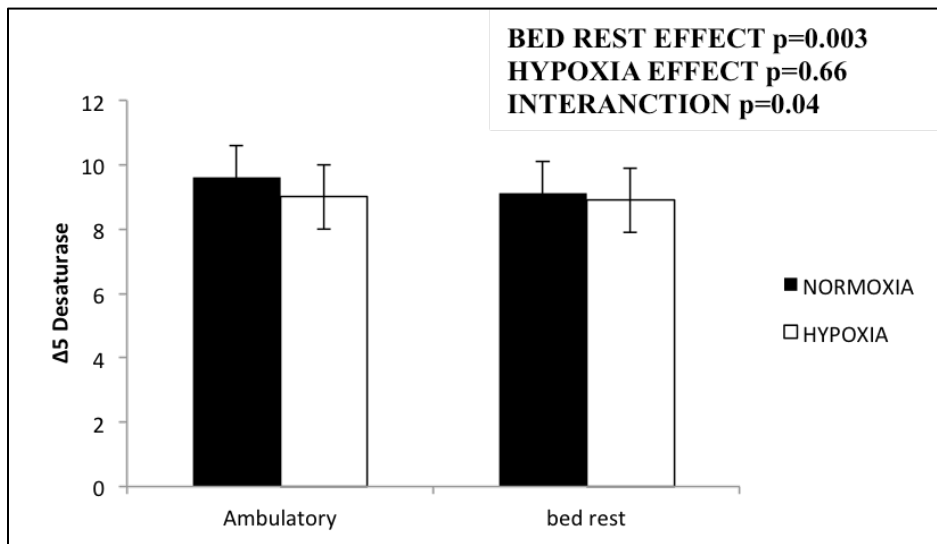


Figure 24. Effect of hypoxia in ambulatory conditions and during 10-d of bed rest on the activity of Δ -5 desaturase enzyme.

N=11. Δ -5 desaturase activity modifications in ambulatory and bed rest states during hypoxia (black columns), or normoxia (white columns) conditions. Data were analyzed with the use of a 2-factor repeated measure ANOVA. Significance for $p < 0.05$.

Tumor necrosis factor Related Apoptosis Induce Ligand (TRAIL)

Plasma TRAIL concentration was significantly increased by hypoxia ($+36 \pm 6\%$, $p=0.001$) and a significant bed rest \times hypoxia interaction was reported ($p=0.04$). A significant direct correlation between TRAIL and GSH-to-GSSG ratio was observed, as reported in figure 25.

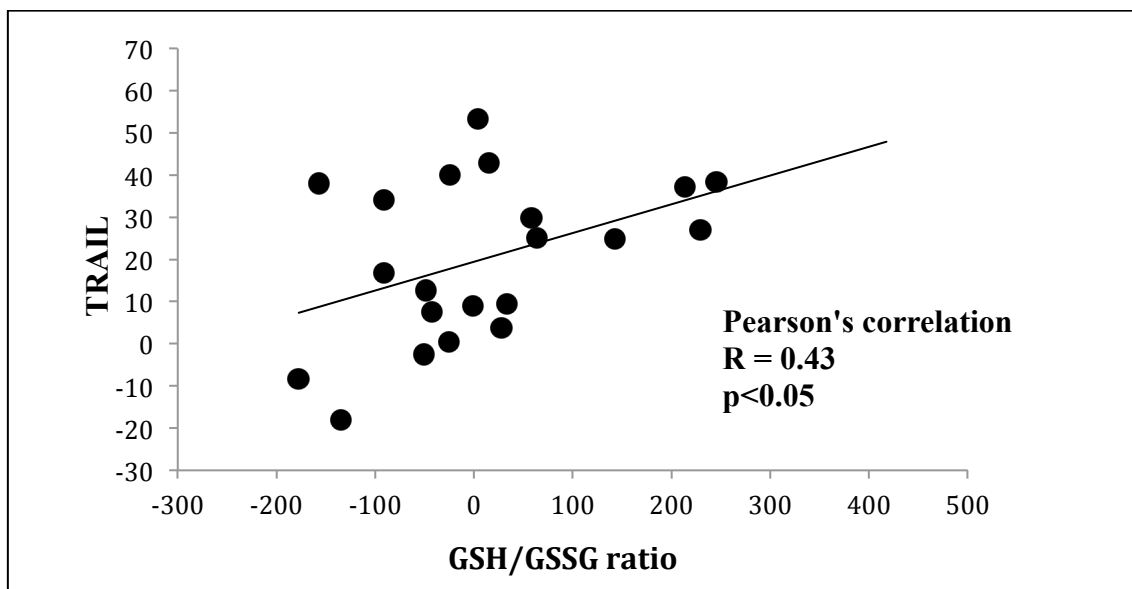


Figure 25. Correlation between TRAIL and GSH-to-GSSG ratio.

N=11. TRAIL and GSH-to-GSSG ratio positive relationship in hypoxic bed rest conditions. Significance for $p < 0.05$.

4.1.5. Inflammatory responses.

Hypoxia, but not bed rest, significantly increased the levels of C reactive protein (CRP) and serum amyloid A (SAA) indices of systemic inflammation, without a significant bed rest×hypoxia interaction (Figure 26).

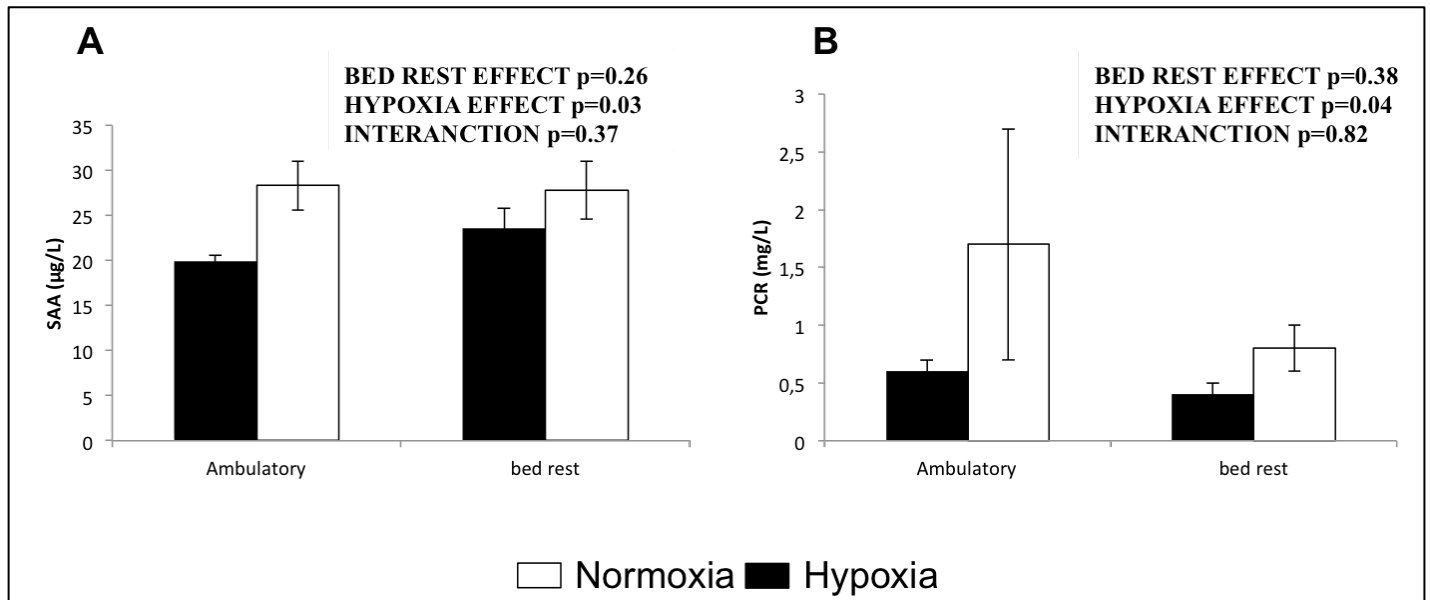


Figure 26. Effect of hypoxia in ambulatory conditions and during 10-d of bed rest on the activity of inflammatory status.

N=11. SAA (A) and PCR (B) modifications in ambulatory and bed rest states during hypoxia (black columns), or normoxia (white columns) conditions. Data were analyzed with the use of a 2-factor repeated measure ANOVA. Significance for $p < 0.05$. SAA, serum amyloid A. CRP, C reactive protein.

4.1.6. Oxidative stress

Glutathione redox capacity

Data are reported on table 6. Hypoxia conditions significantly increased the hematocrit values (Figure 27). Thus, the results of glutathione concentrations and glutathione absolute turnover rate (ATR) were normalized by whole blood volume, taking into account the hypoxia-induced hematocrit changes, and also by erythrocyte volume.

Table 6. Effects of 10-d bed rest and hypoxia, per se or in combination, on glutathione redox capacity in erythrocytes (RBC) and in whole blood (WB).

	Ambulatory		Bed Rest		p [#]		
	Normoxia	Hypoxia	Normoxia	Hypoxia	Bed rest effect	Hypoxia effect	Interaction
Hematocrit (%)	46±1	51±1	47±1	51±1	0.07	0.03	0.10
Total glutathione (mmol/L RBC)	2692±84	2620±79	2564±85	2547±86	0.03	0.22	0.42
Glutathione ATR (mmol/day/L RBC)	656±135	858±88	869±82	1250±263	0.05	0.06	0.53
Total glutathione (mmol/L WB)	1245±49	1332±51	1207±37	1302±45	0.21	<0.001	0.82
Glutathione ATR (mmol/day/L WB)	299±61	430±44	409±38	637±134	0.04	0.03	0.49
Glutathione FTR (percent/day)	25±5	33±3	35±4	50±10	0.04	0.05	0.58
GSH/GSSG (ratio)	137±26	237±43*	221±37	176±36	0.81	0.40	0.009

N=11. Data were expressed as mean±S.E.M. # Data were analyzed with the use of a 2-factor repeated measure ANOVA. ATR, absolute turnover rate. FTR, fractional turnover rate. GSH/GSSG, ratio between reduced and oxidised glutathione. RBC, red blood cell. WB, whole blood. Significance for p<0.05.

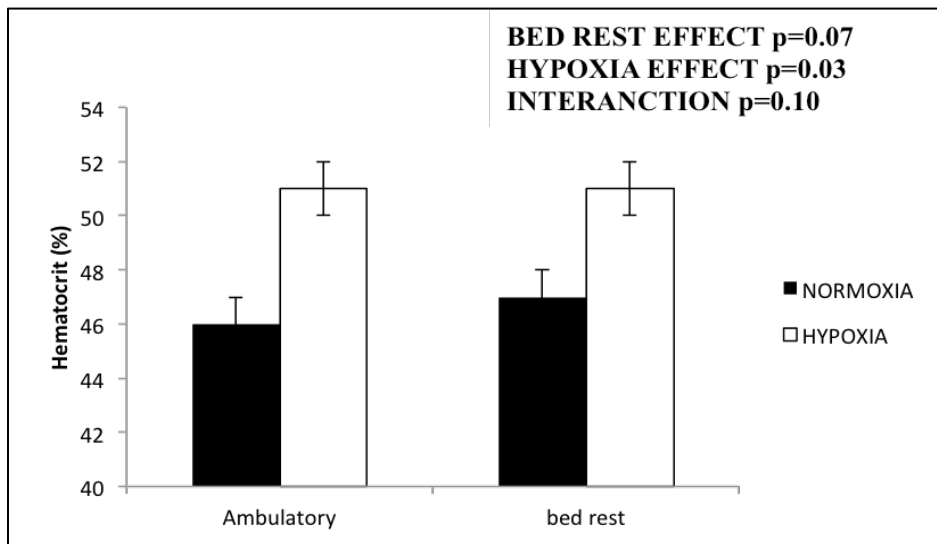


Figure 27. Effect of hypoxia in ambulatory conditions and during 10-d of bed rest on Hematocrit.

N=11. Hematocrit modifications in ambulatory and bed rest states during hypoxia (black columns), or normoxia (white columns) conditions. Data were analyzed with the use of a 2-factor repeated measure ANOVA. Significance for $p < 0.05$.

Total glutathione concentrations normalized by erythrocytes volume significantly decreased following bed rest in both normoxic or hypoxic conditions, with no significant bed rest \times hypoxia interaction (Figure 28A).

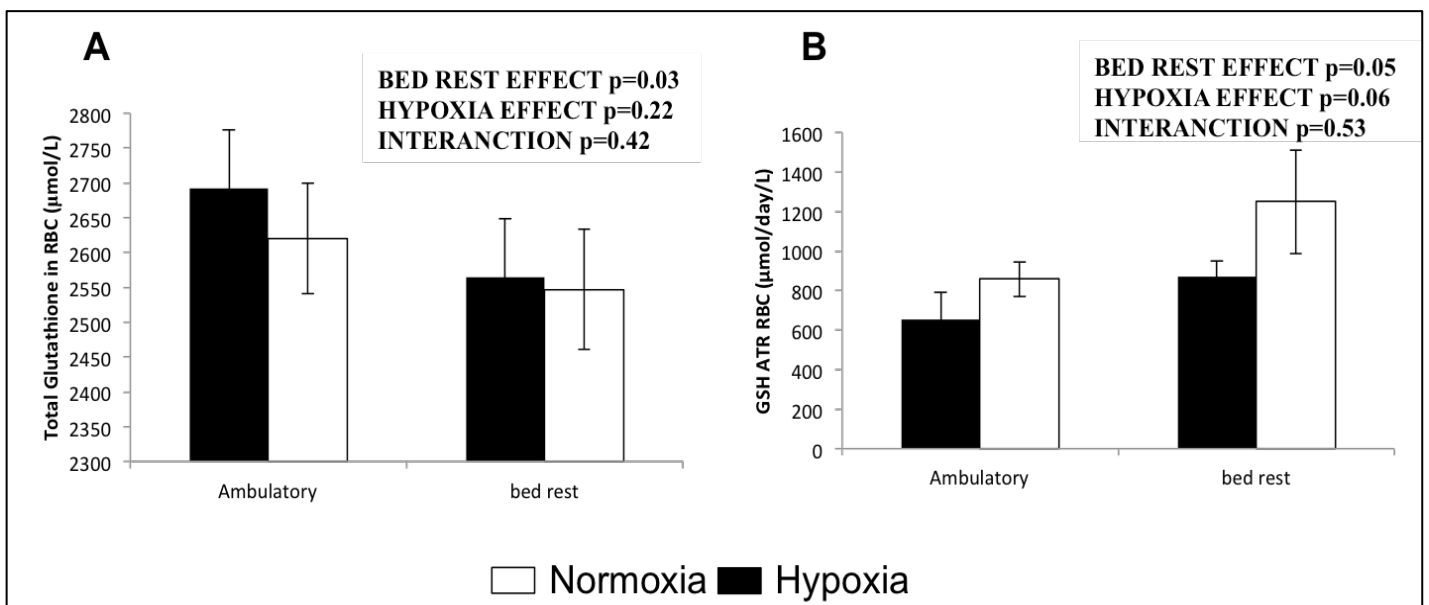


Figure 28. Effect of hypoxia in ambulatory conditions and during 10-d of bed rest on glutathione metabolism in erythrocytes.

N=11. Total glutathione concentration (A) and Glutathione Absolute synthesis rate (B) modifications in red blood cells during ambulatory and bed rest states in hypoxia (black columns), or normoxia (white columns) conditions. Data were analyzed with the use of a 2-factor repeated measure ANOVA. Significance for $p < 0.05$. GSH, glutathione. ATR, absolute synthesis rate. RBC, red blood cell.

The glutathione ATR normalized by RBC volume, shows a significant increase following 10-d of immobilization in both normoxic and hypoxic states (Figure 28B).

The total glutathione concentration normalized by whole blood volume shows a significant positive effect of hypoxia in both ambulatory and BR conditions (Figure 29A). The glutathione ATR normalized by whole blood volume shows an increased turnover rate influenced both by hypoxia and physical inactivity (Figure 29B).

The GSH/GSSG ratio, an index of the redox balance, indicates a significant hypoxia×bed rest interaction on the ratio. Hypoxia and BR conditions significantly increased the glutathione FTR (Figure 29C).

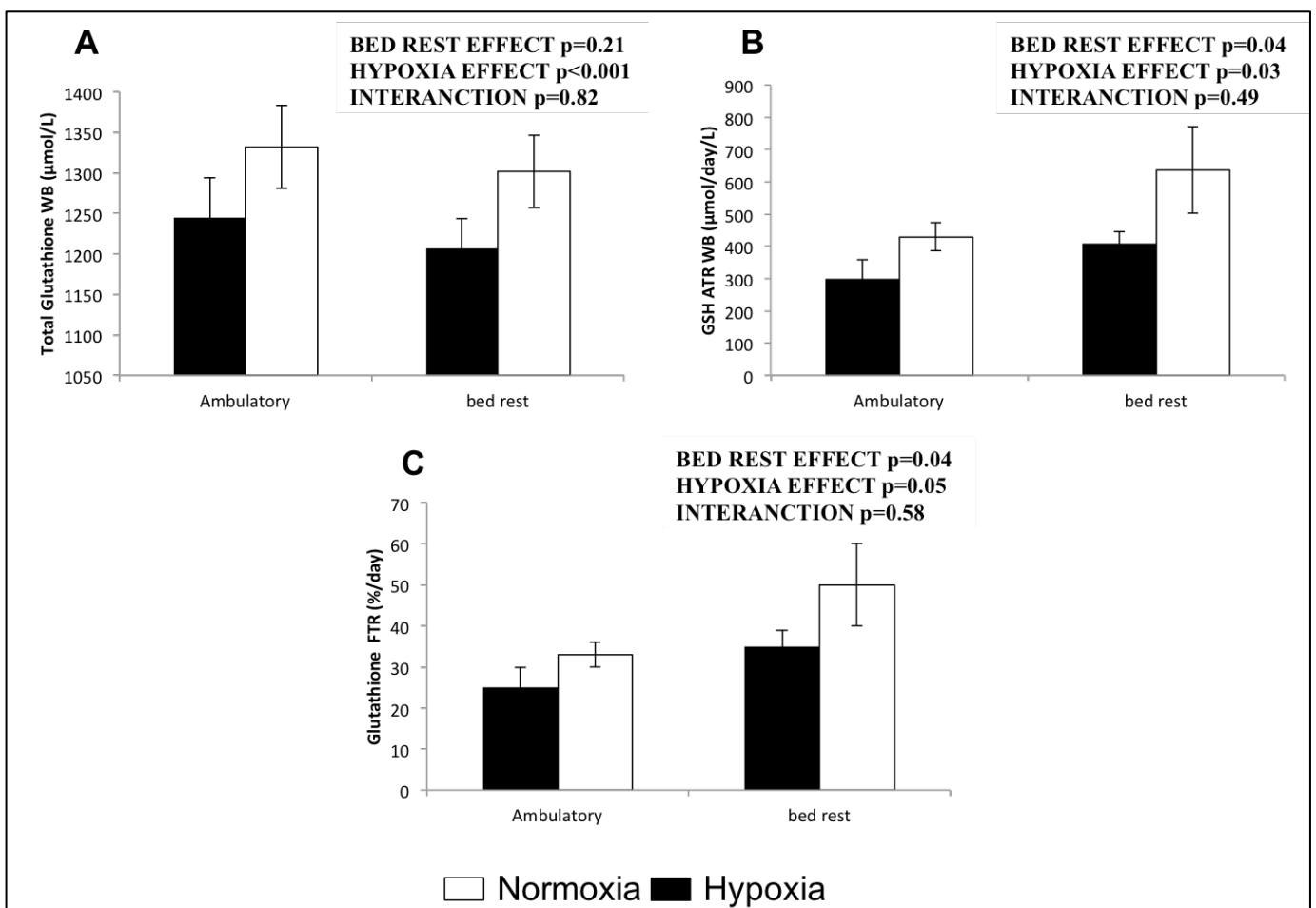


Figure 29. Effect of hypoxia in ambulatory conditions and during 10-d of bed rest on glutathione metabolism in whole blood.

N=11. Total glutathione concentration (A) and Glutathione Absolute synthesis rate (B) modifications in whole blood and glutathione fractional synthesis rate (C) changes, measured during ambulatory and bed rest states in hypoxia (black columns), or normoxia (white columns) conditions. Data were analyzed with the use of a 2-factor repeated measure ANOVA. Significance for $p<0.05$. GSH, glutathione. ATR, absolute synthesis rate. FTR fractional synthesis rate. WB, whole blood.

4.1.7. Plasma and erythrocyte GSH amino acid precursor

Plasma concentrations of glutamate and cysteine were increased due to hypoxia, for glycine plasma levels there is a bed rest×hypoxia interaction. In erythrocytes, hypoxia induced a statistically significant increase in glycine concentrations while no effects were observed on the concentrations of the other glutathione amino acid precursor (Table 7). Hypoxia inversely affected plasma concentrations of glutamate and glutamine. Glutamine decreased by about 4%, while glutamate increased by about 12%, both in ambulatory and bed rest conditions. There was a significant hypoxia-induced increase (+19±6%, $p<0.01$) on the glutamate-to-glutamine ratio. The 5-oxoproline plasma concentrations tended to decrease in hypoxic condition both in ambulatory and bed rest states

Table 7. Effects of 10-d bed rest and hypoxia, per se or in combination, on amino acid concentrations in plasma and red blood cells.

	Ambulatory		Bed Rest		p [#]		
	Normoxia	Hypoxia	Normoxia	Hypoxia	Bed rest effect	Hypoxia effect	Interaction
PLASMA AMINO ACID CONCENTRATIONS							
Cysteine ($\mu\text{mol}\times\text{L}^{-1}$)	294±12	299±11	277±10	304±11	0.45	0.005	0.27
Glycine ($\mu\text{mol}\times\text{L}^{-1}$)	172±9	185±10*	196±12	185±12	0.04	0.78	0.01
Glutamate ($\mu\text{mol}\times\text{L}^{-1}$)	61±4	69±5	62±3	67±3	0.81	0.03	0.59
Glutamine ($\mu\text{mol}\times\text{L}^{-1}$)	541±10	515±9	552±21	522±7	0.55	0.01	0.86
Homocysteine ($\mu\text{mol}\times\text{L}^{-1}$)	15.3±1.7	17.0±1.4	14.7±1.4	16.2±1.4	0.38	0.003	0.93
5-oxoproline ^a ($\mu\text{mol}\times\text{L}^{-1}$) ^a	1.01±0.08	0.91±0.06	1.00±0.07	0.91±0.06	0.92	0.08	0.90
ERYTHROCYTE AMINO ACID CONCENTRATIONS							
Cysteine ($\mu\text{mol}\times\text{L}^{-1}$)	70±3	79±3	72±4	70±3	0.20	0.33	0.07
Glycine ($\mu\text{mol}\times\text{L}^{-1}$)	368±11	389±13	372±12	393±12	0.49	<0.001	0.98
Glutamate ($\mu\text{mol}\times\text{L}^{-1}$)	477±24	481±18	448±16	478±17	0.33	0.16	0.37
Glutamine ($\mu\text{mol}\times\text{L}^{-1}$)	1053±147	1015±209	1042±141	1000±215	0.79	0.73	0.97

N=11. Data were expressed as mean±S.E.M. # Data were analyzed with the use of a 2-factor repeated measure ANOVA. a, 5-oxoproline concentrations have been estimated as ratio between 5-oxoproline and glutamate. Significance for $p<0.05$.

4.1.8. Red blood cell membrane lipid composition.

Data on lipid composition in RBC membranes are reported in Table 8. There are no changes in FAs concentrations in any of the study protocols.

Table 8. Effects of 10-d bed rest and hypoxia, per se or in combination on fatty acids composition (%) of erythrocyte membranes.

	Ambulatory		Bed Rest		p [#]		
	Normoxia	Hypoxia	Normoxia	Hypoxia	Bed rest effect	Hypoxia effect	Interaction
SATURATED FATTY ACIDS							
Myristic 14:00	0.20 ± 0.024	0.31 ± 0.32	0.3 ± 0.025	0.25 ± 0.020	0.45	0.08	0.01
Palmitic 16:00	20.61 ± 0.36	20.9 ± 0.31	20.62 ± 0.26	20.81 ± 0.29	0.71	0.15	0.76
Stearic 18:00	18.54 ± 0.20	18.42 ± 0.08	18.64 ± 0.17	18.45 ± 0.14	0.58	0.27	9.64
SUM	39.35 ± 0.45	39.63 ± 0.37	39.55 ± 0.32	39.52 ± 0.34			
MONOUNSATURATED FATTY ACIDS							
Palmitoleic 16:1 n7	0.25 ± 0.04	0.27 ± 0.04	0.28 ± 0.04	0.26 ± 0.03	0.72	1.0	0.26
Oleic 18:1 n9	13.34 ± 0.33	12.87 ± 0.33	12.81 ± 0.28	12.94 ± 0.34	0.08	0.1	0.06
Elaidic trans 18:1n-9	1.03 ± 0.04	1.02 ± 0.03	1.01 ± 0.05	1.05 ± 0.05	0.7	0.45	0.14
Eicosenoic 20:1n-9	0.31 ± 0.01	0.3 ± 0.01	0.3 ± 0.01	0.31 ± 0.01	0.86	0.75	0.1
SUM	14.92 ± 0.37	14.46 ± 0.34	14.39 ± 0.23	14.56 ± 0.37			
n-3 POLYUNSATURATED FATTY ACIDS							
α-Linolenic acid 18:3 n3	0.04 ± 0.02	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	ns	ns	ns
Eicosapentaenoic acid 20:5n-3	0.53 ± 0.04	0.58 ± 0.04	0.56 ± 0.03	0.54 ± 0.04	0.94	0.5	0.1
Docosapentaenoic 22:5n-3	2.50 ± 0.08	2.57 ± 0.10	2.55 ± 0.09	2.57 ± 0.09	0.43	0.02	0.41
Docosahexaenoic acid 22:6n-3	4.48 ± 0.23	4.69 ± 0.26	4.63 ± 0.25	4.80 ± 0.2	0.02	0.01	0.83
SUM	7.50 ± 0.25	7.84 ± 0.30	7.74 ± 0.26	7.91 ± 0.19			
n-6 POLYUNSATURATED FATTY ACIDS							
Linoleic acid 18:2 n6	11.87 ± 0.35	11.72 ± 0.32	11.6 ± 0.35	11.39 ± 0.29	0.08	0.21	0.83
Eicosadienoic acid 20:2n-6	1.09 ± 0.51	1.47 ± 0.56	1.13 ± 0.19	0.89 ± 0.24	0.6	0.63	0.5
Dihomo-γ-linolenic 20:3n-6	1.97 ± 0.13	2.04 ± 0.13	2.08 ± 0.12	2.16 ± 0.13	0.001	0.02	0.98
Arachidonic acid 20:4n-6	18.11 ± 0.28	17.7 ± 0.27	18.16 ± 0.21	18.39 ± 0.32	0.04	0.49	0.02
Adrenic 22:4n-6	4.15 ± 0.20	3.99 ± 0.18	4.12 ± 0.17	4.17 ± 0.18	0.07	0.02	0.02
Docosapentaenoic 22:5n-6	0.99 ± 0.08	1.10 ± 0.13	1.17 ± 0.17	0.98 ± 0.08	0.71	0.47	0.17
SUM	38.22 ± 0.79	38.08 ± 0.72	38.31 ± 0.65	38.01 ± 0.64			

N=11. Values are percent of total Fatty Acids. Data were expressed as mean±S.E.M. # Data were analyzed with the use of a 2-factor repeated measure ANOVA Significance for p<0.05.

4.2. The PANGEA STUDY (bed rest in the elderly)

4.2.1. Body composition

Body composition was significantly modified after 14 days of BR both in young and elderly subjects. As showed in table 9, body weight and BMI significantly decrease after 14-d of resting with a reduction in FFM (Figure 30A) and a slight but significant increase in FM (Figure 30B).

Table 9. Anthropometrical data before (BDC-1) and after (BR+14) bed rest in young and elderly subjects.

				p[#]	
	subjects	BDC-1	BR+14	Bed rest effect	bed rest × group interaction
Body weight (kg)	Young	74.84 ± 3.32	71.59 ± 3.14	<0.001	0.08
	Elderly	79.64 ± 3.71	77.58 ± 3.67		
BMI (kg/m²)	Young	23.96 ± 0.89	22.91 ± 0.81	<0.001	0.12
	Elderly	26.85 ± 1.47	26.15 ± 1.45		
FFM (kg)	Young	60.86 ± 1.46	56.36 ± 1.43	<0.001	0.18
	Elderly	59.79 ± 2.43	56.66 ± 2.41		
FM (kg)	Young	13.99 ± 2.34	15.33 ± 2.66	0.01	0.74
	Elderly	19.85 ± 1.75	20.91 ± 1.69		

N=7 young group; N=8 elderly group. Data were expressed as mean±S.E.M. #Data were analyzed with the use of a 2-factor repeated measure ANCOVA. BMI: Body Mass Index, FFM: Fat-Free Mass, FM: Fat Mass.

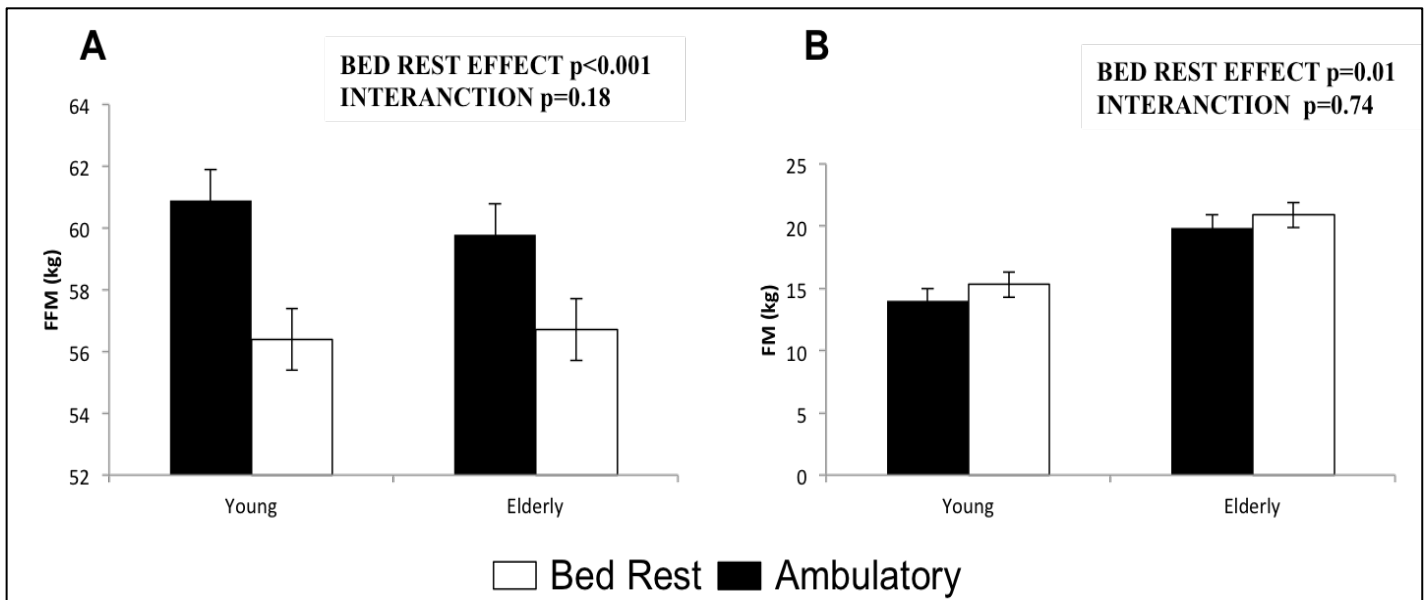


Figure 30. Body composition modifications after 14-d of bed rest in young and elderly subjects.

N=7 young group; N=8 elderly group. Body composition modifications in ambulatory conditions (black column) and after 14-d of bed rest (white column). in young and elderly subjects. Data were expressed as mean±S.E.M. Data were analyzed with the use of a 2-factor repeated measure ANCOVA. FFM, Fat-free Mass. FM Fat Mass.

4.2.2. Insulin sensitivity.

Glucose and insulin metabolism data, assessed in basal conditions and during load, before and after 14-d of experimental inactivity, are reported in table 10. The metabolic basal values were similar between the two groups. Plasma glucose concentration in fasting state were not significantly modified after 14-d of BR while the fasting insulin plasma concentration were augmented especially in young subjects (+74±21% and +16±12% in young and elderly individuals respectively).

The HOMA-IR, as index of insulin resistance in fasting state, significantly increase after BR in both young and elderly subjects, however, young individuals shown significantly ($p=0.04$) higher values (+80±29%) than elderly (+12±13%).

Table 10. Glucose metabolism data before (BDC-1) and after (BR+14) bed rest in young and elderly subjects.

	subjects	BDC-1	BR+14	Delta % (BR+14 – BDC-1)	p [#]	
					Bed rest effect	bed rest × group interaction
Fasting glycaemia (mg/dL)	Young	87 ± 4.30	86.57 ± 1.90	1.09 ± 5.74	0.45	0.54
	Elderly	96.38 ± 4.00	92.38 ± 2.39	3.29 ± 3.75		
Fasting insulinaemia (UI/L)	Young	5.14 ± 1.10	8.43 ± 1.54	74.32 ± 21.06	<0.01	<0.05
	Elderly	5.25 ± 0.77	5.88 ± 0.90	15.63 ± 11.52		
HOMA index	Young	1.11 ± 0.23	1.82 ± 0.35	79.67 ± 28.86	0.02	<0.05
	Elderly	1.27 ± 0.20	1.34 ± 0.21	12.44 ± 12.72		
Matsuda index	Young	26.93 ± 4.24	14.21 ± 2.38	-45.95 ± 4.51	<0.001	0.01
	Elderly	18.79 ± 1.50	14.95 ± 2.09	-20.34 ± 9.42		
AUCIns (UI h/L)	Young	99.86 ± 17.47	207.68 ± 37.96	113.29 ± 19.12	<0.01	0.02
	Elderly	133.84 ± 12.97	198.38 ± 26.82	48.78 ± 14.24		
AUCGluc (mg h/dL)	Young	311.61 ± 13.76	334.50 ± 13.82	7.84 ± 3.64	<0.05	0.94
	Elderly	372.13 ± 23.56	396.81 ± 24.47	7.58 ± 5.66		
AUCIns/AUCGluc	Young	0.33 ± 0.06	0.62 ± 0.11	98.31 ± 17.22	0.001	0.02
	Elderly	0.37 ± 0.04	0.51 ± 0.07	38.93 ± 12.73		

N=7 young group; N=8 elderly group. Data were expressed as mean±S.E.M. #Data were analyzed with 2-factor repeated measure

ANCOVA. data were logarithmic transformed when appropriate

Matsuda index, as parameter of insulin sensitivity in fed state (loading), showed a reduction due to the 14-d of BR (Figure 31) in both groups but it was doubled in young than in elderly ($-46\pm 5\%$ vs $-20\pm 9\%$, respectively, $p=0.04$).

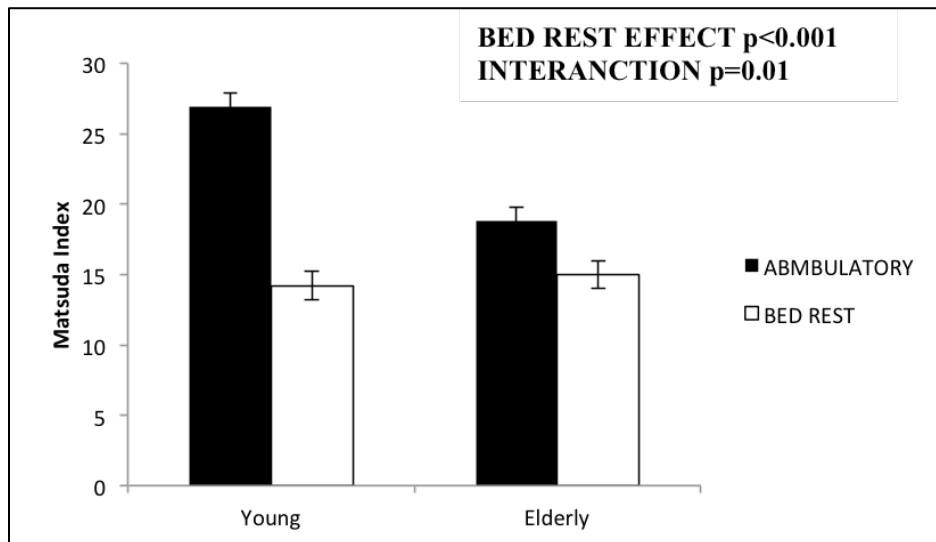


Figure 31. Matsuda index modifications after 14-d of bed rest in young and elderly subjects.

Interaction = bed rest \times group interaction. Data analyzed with the 2-factor repeated measure ANCOVA

The area-under-the curve (AUC), i.e., the area under of both plasma glucose (AUC_{Gly}) and plasma insulin (AUC_{Ins}) concentrations during the test meal versus time, was estimated using the linear trapezoidal method. In basal condition the AUC_{Ins} values were comparable between the two groups while the AUC_{Gly} values were greater in elderly than in young people about of 19% (Figure 32B and D). After BR the AUC_{Ins} was augmented in both groups with a significant ($p=0.02$) higher increase in the young population ($+113\pm 19\%$) if compared to the elderly group ($+49\pm 14\%$). (Figure 32A and C).

The AUC_{Ins}/AUC_{Gly} ratio, as index of insulin resistance after load, was augmented in both young and elderly subjects after bed rest however the young individuals showed a significant ($p=0.01$) higher increase of this value ($+98\pm 17\%$) than elderly population ($+39\pm 13\%$) (Table 9).

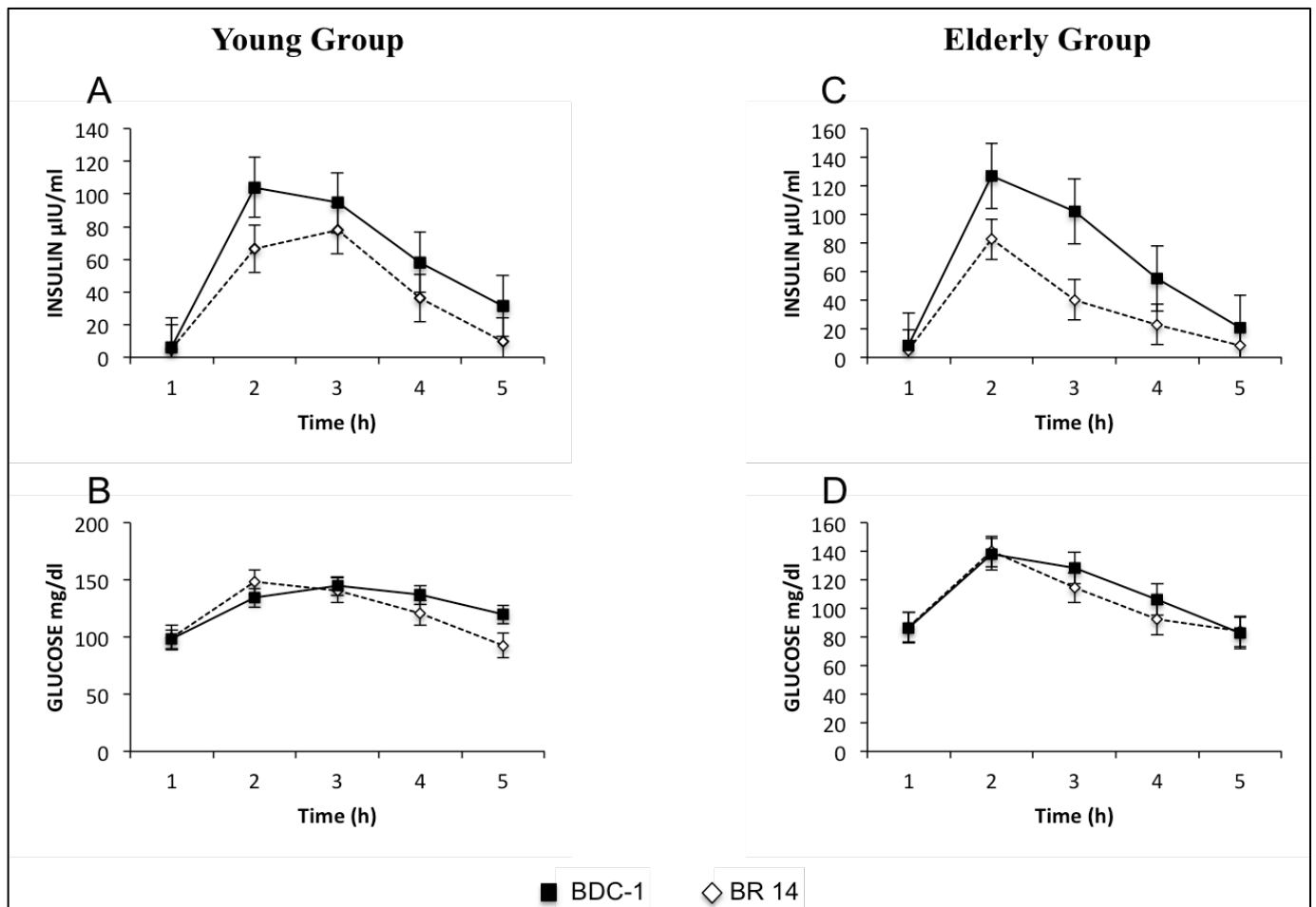


Figure 32. Glycaemia and insulinaemia modifications before (■) and after 14-d of bed rest (◇) in young (A and B) and elderly (C and D) subjects.

Interaction = bed rest × group interaction. Data analyzed with the 2-factor repeated measure ANCOVA

4.2.3. Anabolic resistance

The plasma AA concentrations as well as the isotopic enrichment of D5-Phe and D4-Tyr, before and after the bed rest period, were assessed by GCMS as previously reported (see methods section).

To evaluate the effect of ageing and bed rest on anabolic resistance the AUC i.e., the area under of both plasma D5-Phe (AUC_{D5-Phe}) and plasma D4-Tyr (AUC_{D4-Tyr}) concentrations during the test meal versus time, were calculated. Moreover the AUCs of unlabeled phenylalanine (AUC_{Phe}), tyrosine (AUC_{Tyr}) and leucine (AUC_{Leu}) were also assessed (Table 11).

As showed in table 10 AUC_{Phe} significantly increased after 14-d of bed rest (4%) with no time×group interaction. No significant changes were observed after 14-d of experimental inactivity on AUC_{Tyr} and AUC_{Leu} values. Leucine plasma concentrations are considered an index of the absorption of the amino acids from the protein administered with the *test meal*. We observed that the leucine peak at 60 minutes was comparable between the ambulatory and bed rest conditions both in young and elderly subjects, showing no interference from the amino acid absorption rate.

Table 11. Bed rest effect on labeled and unlabeled plasma amino acids concentration in young and elderly subjects

		BDC-1	BR+14	Delta % (BR+14 – BDC-1)	p [#]	
					Bed rest effect	bed rest × group interaction
AUC Phenylalanine	Young	413.14 ± 27.58	430.49 ± 19.37	6.10 ± 5.93	0.03	0.60
	Elderly	401.27 ± 11.51	415.51 ± 16.13	3.61 ± 3.19		
AUC Tyrosine	Young	516.02 ± 58.58	502.44 ± 44.51	0.47 ± 6.21	0.37	0.23
	Elderly	599.72 ± 44.09	614.95 ± 52.29	2.12 ± 2.92		
AUC Leucine	Young	818.32 ± 41.91	815.92 ± 58.18	-0.31 ± 4.77	0.55	0.93
	Elderly	793.23 ± 43.15	798.06 ± 37.71	1.06 ± 2.64		
AUC D5-Phe	Young	16.33 ± 1.67	16.44 ± 1.39	4.19 ± 9.41	0.14	0.54
	Elderly	16.13 ± 1.55	17.99 ± 2.29	13.24 ± 12.13		
AUC D4-Tyr	Young	3.38 ± 0.60	3.30 ± 0.36	9.06 ± 14.06	<0.01	0.05
	Elderly	3.84 ± 0.43	4.93 ± 0.66	33.32 ± 15.78		
AUC D4-Tyr/ AUC D5-Phe (T ₀ -T ₃₆₀)	Young	0.20 ± 0.03	0.20 ± 0.01	5.20 ± 9.36	<0.01	0.01
	Elderly	0.24 ± 0.02	0.28 ± 0.02	18.5 ± 7.27		
AUC D4-Tyr/ AUC D5-Phe (T ₁₂₀)	Young	0.19 ± 0.02	0.19 ± 0.02	-0.53 ± 1.85	<0.05	<0.05
	Elderly	0.21 ± 0.02	0.27 ± 0.03	6.79 ± 3.85		

N=7 young group; N=8 elderly group. Data were expressed as mean±S.E.M. #Data were analyzed with 2-factor repeated measure

ANCOVA. data were logarithmic transformed when appropriate. AUC, area under the curve.

On figure 33 were reported the D5-Phe and D4-Tyr plasma concentrations during the test meal before and after the BR period on both young and elderly subjects.

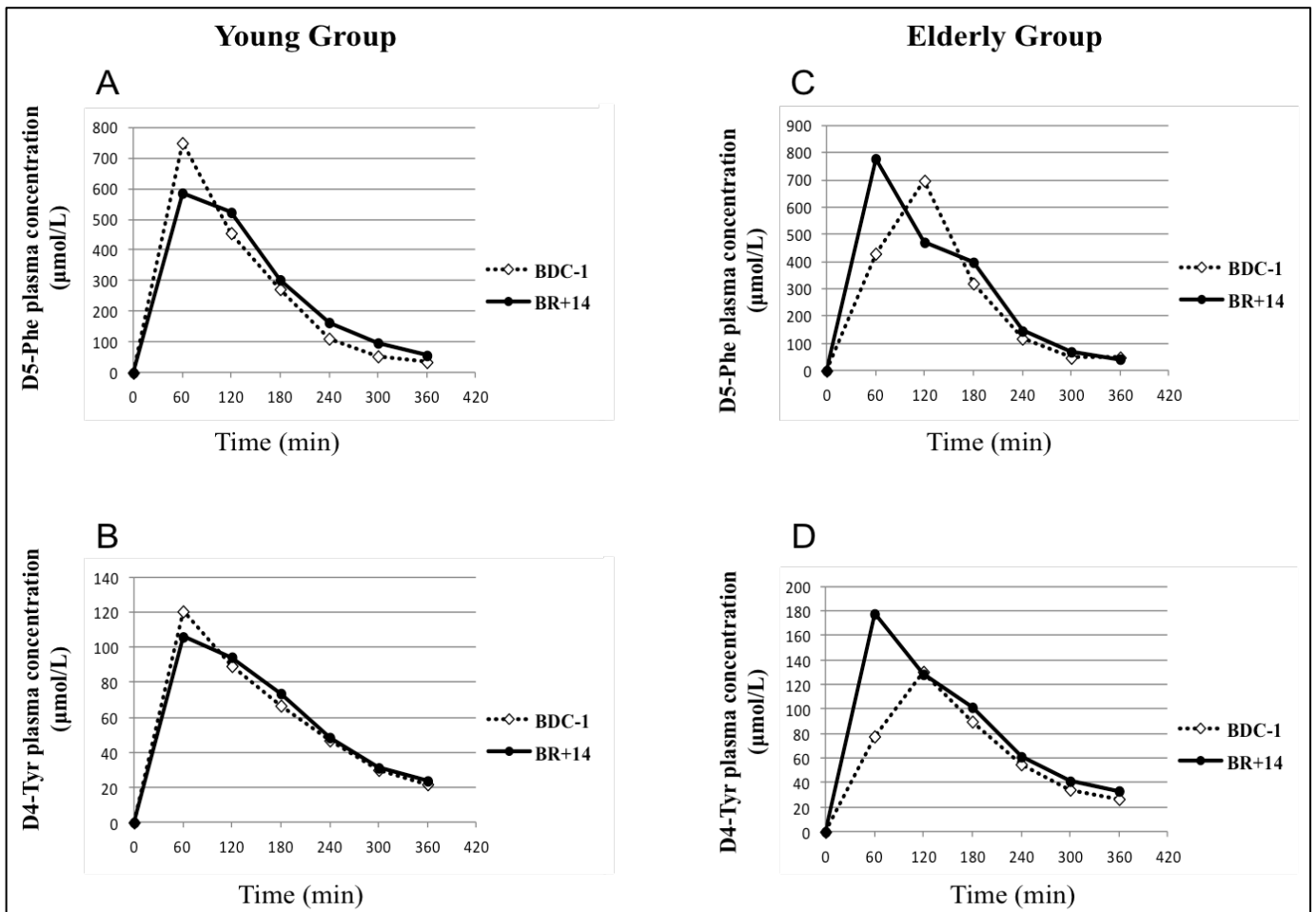


Figure 33. D5-Phe and D4-Tyr plasma concentration before (■) and after 14-d of bed rest (◇) in young (A and B) and elderly (C and D) subjects.

Interaction = bed rest × group interaction. Data analyzed with the 2-factor repeated measure ANCOVA

No significant changes were observed on AUC_{D5-Phe} after 14-d of experimental BR while the AUC_{D4-Tyr} values were significantly modified.

In basal condition, the $AUC_{D4-Tyr}/AUC_{D5-Phe} - T_{360}$ ratio, as index of anabolic resistance, was comparable between the two groups, after 14-d of BR. It appear increased in both young and elderly subjects, but with higher values in the elderly ($+19\pm 7\%$) than in the young ($+5\pm 9\%$) population, as confirmed by the significant $time \times group$ interaction (Table 10).

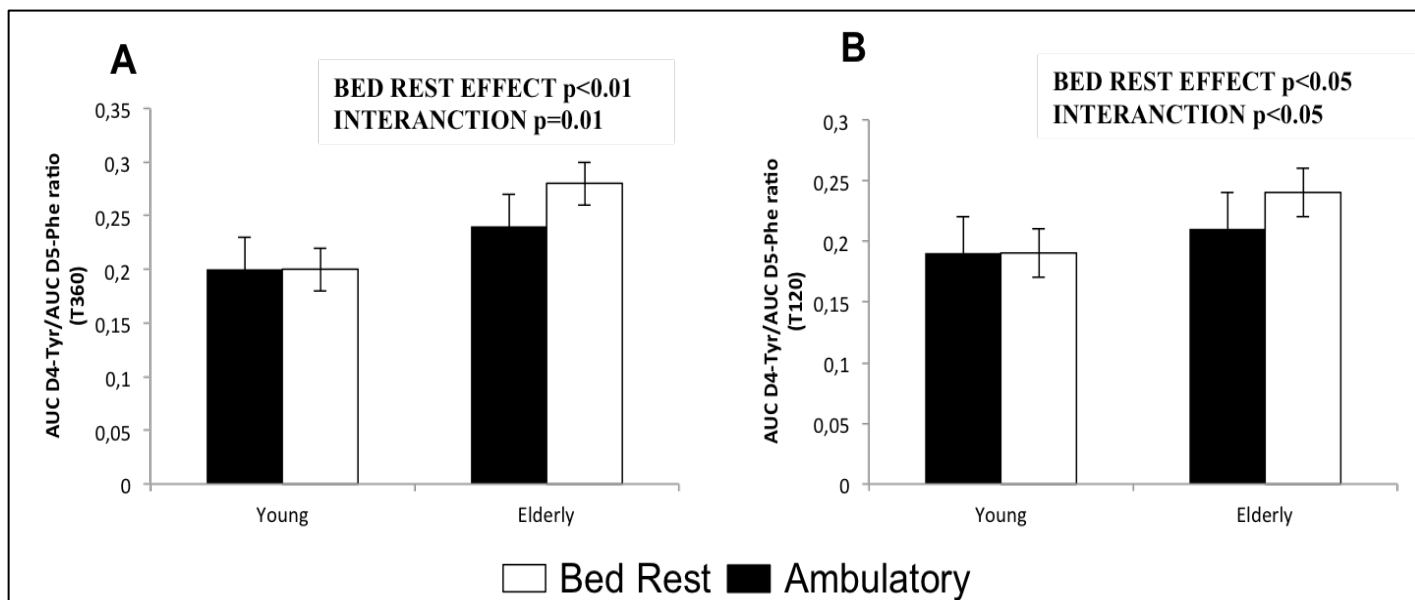


Figure 34. Bed rest effect on anabolic resistance expressed as $AUC_{D4-Tyr}/AUC_{D5-Phe}$ ratio in young and elderly subjects

Interaction = bed rest \times group interaction. Data analyzed with the 2-factor repeated measure ANCOVA

The $AUC_{D4-Tyr}/AUC_{D5-Phe} - T_{360}$ ratio was evaluated considering all the blood draw points as reported in the experimental design (Figure 18). The same index was also calculated using the blood draw points of the first two hours of the experimental protocol ($AUC_{D4-Tyr}/AUC_{D5-Phe} - T_{120}$). As reported on figure 34 (A and B) the $AUC_{D4-Tyr}/AUC_{D5-Phe} - T_{120}$ ratio showed the same trend observed with the $AUC_{D4-Tyr}/AUC_{D5-Phe} - T_{360}$ ratio, moreover, a significantly direct correlation ($R=0.75$; $p < 0.001$) was found comparing the two indices (Figure 35).

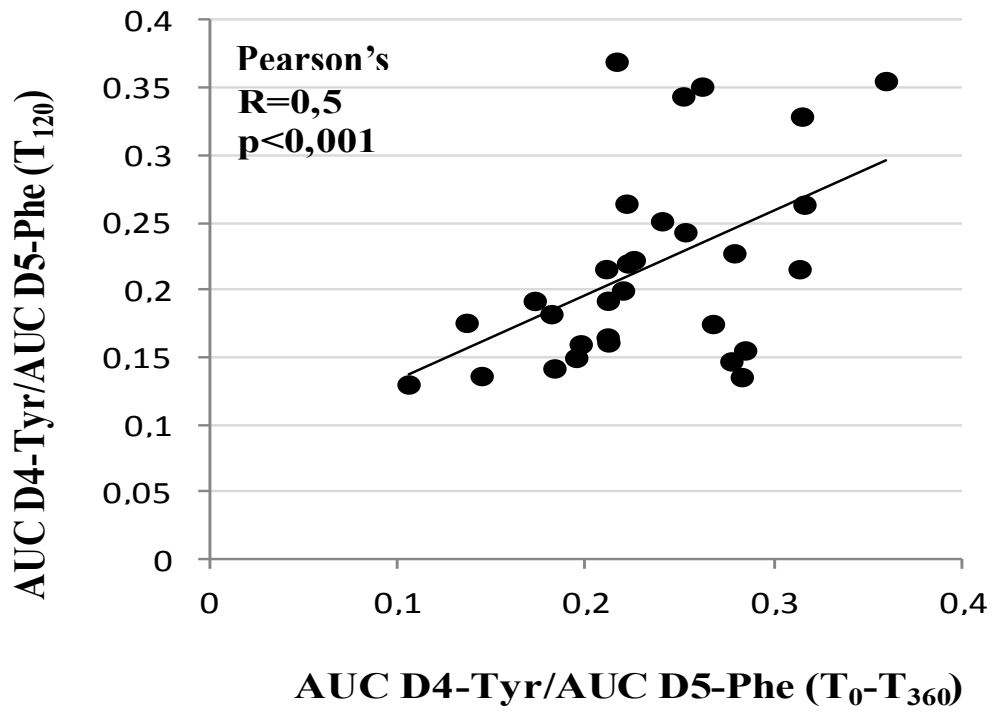


Figure 35. Correlation between the $AUC_{D4-Tyr}/AUC_{D5-Phe} - T_{360}$ ratio and the $AUC_{D4-Tyr}/AUC_{D5-Phe} - T_{120}$ ratio

5. DISCUSSION

The primary aim of the present work was to develop biomarkers useful to define, *in vivo*, in humans, the optimal protein intake in different physiological and pathological conditions. Within the frame of two studies sponsored by the European Community (i.e., the FP7 PLANHAB Study and the INTERREG ITA-SLO PANGAEA bed rest study), we applied such methodology to investigate protein requirement in conditions of inactivity, with or without hypoxia, as well as in a population of healthy elderly subjects assessed at different levels of physical activity, from ambulatory to bed rest.

The assessment of whole body protein kinetics is a standard methodology to detect conditions of impaired protein synthesis and anabolic resistance, in the fasting state and stimulation by protein nutrition. We applied this method in the PLANHAB Study to investigate protein requirement in hypoxic condition both in ambulatory and bed rested individuals. The PANGeA study was the second study in the world where aged healthy people were bed ridden and the first one in which healthy elderly subjects were compared to healthy young individuals (as control group), during a period of experimental inactivity. Moreover it was the first time that both glucose and protein metabolism were assessed in healthy elderly volunteers undergoing bed rest.

In the PANGEA study we developed a new method to assess the ability to utilize dietary protein during and after a mixed meal. Anabolic resistance is a condition characterized by reduced ability of meal protein to stimulate protein synthesis, thus increasing protein requirements. Our new method was used to compare anabolic resistance and protein requirement in elderly and young subjects either in ambulatory or in bed rest conditions.

Moreover this new method allowed evaluate at the same time anabolic and insulin resistance in the post-prandial state. We had therefore the opportunity to explore the relationships between changes in insulin and anabolic resistance in elderly and young subjects at different levels of physical activity. The quantity of protein intake, not only influences the rate of protein synthesis but has an impact also on other metabolic targets, such as the redox balance. In particular the glutathione system is influenced by the availability of the precursor amino acids. We have therefore investigated different aspects of the glutathione system in the PLANHAB study with the aim to define the effects of hypoxia, with or without bed rest.

The relative proportion of cell membrane fatty acids was determined in the PLANHAB study in order to detect changes induced by insulin resistance or systemic inflammation and oxidative stress.

The main results of our work can be summarized as follows:

- 5.1. Hypoxia decreases protein synthesis.
- 5.2. Aging is characterized by anabolic resistance.
- 5.3. Aging is a protective factor against inactivity mediated insulin resistances?
- 5.4. Hypoxia per se improves oxidative stress.
- 5.5. Hypoxia-bed rest interaction induces multiple metabolic changes.
- 5.6. Identification of a new biomarker to evaluate anabolic (and insulin) resistance to define optimal protein requirements.

5.1. Hypoxia decreases protein synthesis

Chronic hypoxia and unloading are well-known protein catabolic factors for skeletal muscle (Biolo et al 2014; Di Giulio C et al. 2009). Ten days of normobaric hypoxia in ambulatory conditions or in horizontal bed rest in healthy volunteers led to similar losses of lean body mass. Bed rest in normoxic conditions led to a similar muscle catabolic response. Our study shows that when the two factors are applied in combination for 10 days in healthy volunteers they do not exhibit additive effects. The hypertrophic response of skeletal muscle trained in chronic hypoxia conditions was significantly lower than that produced in normoxia (Narici MV et al. 1995). Muscle loss associated with unloading is characterized by decreased protein turnover with a resistance to amino acid-mediated stimulation of protein synthesis as key catabolic mechanism. Protein synthesis is an energy consuming process. Hypoxia, which limits energy production, has been shown to decrease muscle protein synthesis (Preedy VR et al. 1985) in animal models, in vitro and in vivo. Decreased muscle protein synthesis has been observed also in emphysema patients with chronic respiratory failure (Morrison WL et al. 1988). In parallel with the rate of protein synthesis, hypoxia in ambulatory conditions also decreased by about 8% the rate of whole body protein degradation, leading to a decreased protein turnover.

While normoxic subjects are characterized by accelerated post-exercise muscle protein turnover, in COPD patients with emphysema the rates of protein synthesis and degradation were suppressed after low-intensity exercise (Engelen MP et al. 2003). A blunted post-exercise suppression of muscle protein synthesis was also observed in experimental hypoxia (Etheridge T et al. 2011). Our results are in perfect agreement with these previous observations. Hypoxia in ambulatory conditions decreased by about 8% the rates of whole body protein synthesis and degradation. In contrast, hypoxia in bed rest conditions did not significantly affect whole body protein turnover. As expected the net protein loss in the post-absorptive state was not accelerated by bed rest. In fact, post-prandial resistance to amino acid-mediated stimulation of protein synthesis is the key catabolic mechanism during muscle unloading. Net protein loss in the post-absorptive state was not accelerated during hypoxia suggesting postprandial anabolic resistance also in this condition.

5.2. Aging is characterized by anabolic resistance

During the PANGeA study we tested a new, simple, safe and quick method to evaluate the anabolic resistance to stimuli inducing protein synthesis (e.g. exercise, amino acids, etc.), associated with ageing.

Our results confirm that both aging and physical inactivity impairs the sensitivity to anabolic stimuli (Kim IY et al. 2015; Biolo G et al. 2004).

The development of anabolic resistance following physical inactivity was higher in the elderly than in the young subjects, evidencing that aging is a detrimental factor in the risk of an impaired inactivity-mediated protein metabolism. There is a potential decline in the efficiency of muscle protein synthesis with advancing age when a small amount of EAAs are ingested (Katsanos CS et al. 2005). Moreover, it was previously demonstrated (Biolo G et al. 2004) that decreased levels of physical activity in healthy young subjects were associated to compromise anabolic sensitivity to an amino acidic stimulus. After 14 days of experimental bed rest, in fact, a reduction in protein synthesis after AA administration was observed (Biolo G et al. 2004). Consequently, after a period of inactivity, it seems necessary to increase the protein intake in order to improve the protein anabolism in the fed state and overcome the anabolic resistance. The reduction of the sensibility of the muscle protein synthesis to an anabolic stimulus (i.e. protein intake and/or physical activity) associated with aging (Kim IY et al. 2015), leads to a reduction in muscle mass and strength, to an increased frailty with a higher risk of falls and generally to a higher morbidity and mortality (Breen L & Phillips SM. 2011). Moreover, this resulting loss of skeletal muscle protein could be a key factor in the development and the progression of sarcopenia and loss of function (Volpi E et al 2000).

Thus anabolic resistance is the principal catabolic mechanism responsible of muscle atrophy after a period of physical inactivity or during aging (Biolo G et al. 2004). The decreased ability to use AA for the MPS observed either with aging or in bed ridden young subject, seems to require an increased protein intake with the diet, especially when the two condition are associated. Furthermore, a larger intake of EAAs (i.e., 15 g in the form of beef) resulted in a similar stimulation of muscle protein synthesis (MPS), in young and old adults (Symons TB et al. 2007).

However, a diet with high protein content could determine harmful effects in elderly subjects such as impairment of kidney function (Deutz NE et al. 2014). Nonetheless, a regular physical activity stimulates the MPS and contributes to maintain a positive protein balance, favoring the muscle mass gain both in young and elderly subjects

(Fiatarone MA et al.1990; Fiatarone MA et al. 1994). In healthy adults, both young and elderly, resistance and endurance exercise acutely stimulates muscle protein synthesis (Cuthbertson D et al. 2005; Short KR et al. 2004) and this effect is increased when physical activity is associated to protein administration (Breen L & Phillips SM. 2011). Our evidences suggest that during inactivity or aging there is an augmented protein need, and both exercise and proper nutrition could have intense effects on muscle mass and function. Therefore a correct lifestyle could counteract the consequences of aging. Specific nutritional programs can attenuate or even overcome the muscle loss associated to these conditions should be associated to specific training protocol. The hypothesis that an association of resistance training with higher protein intake from the diet or dietary supplements may have synergic effects has been confirmed in acute exercise conditions both in young and elderly subjects, (Biolo G et al. 1997; Rasmussen BB et al. 2006)

5.3. Aging is a protective factor against inactivity mediated insulin resistances?

Aging can determine a progressive reduction of the tissues sensitivity to the insulin stimulation and therefore an impaired capability of the tissues to metabolize glucose (DeFronzo RA. 1981). The same detrimental effect is observed after a period of physical inactivity. Bed rest studies (Stuart CA et al. 1988) conducted on healthy young subjects showed that reduced insulin sensitivity develops soon after few days.

Trained subjects show greater insulin sensitivity than individuals with a sedentary lifestyle. Both strength and resistance training improve insulin sensitivity and therefore glucose metabolism either in young or elderly subjects (Henriksen EJ. 2002), however if young individuals have an improved insulin sensitivity even after a single bout of exercise, in aged subjects more bouts are required to observe the same effect (Henriksen EJ. 2002). These data seem to show that in healthy young trained persons the glucose level control is more efficient. Smorawiński J and his group have shown how, after three days of bed rest, both trained and untrained subjects exhibit a higher insulin resistance. Surprisingly the values of insulin resistance were greater in active than in the sedentary lifestyle subjects (Smorawiński J et al. 2000). These findings suggest that in trained subjects there is a faster compensatory response than in sedentary lifestyle individuals.

The data observed in the PANGeA study confirm the findings of Smorawiński J and his group. The healthy young subjects, apparently more active than the elderly individuals, showed higher levels of insulin in fed state, after a period of 14-d of bed rest. The mechanisms of this peculiar insulin response require indeed further investigations. Probably aging determines a sort of adaptation to physical inactivity and consequently a higher resistance to alteration of the glucose metabolism.

In ambulatory condition no insulin sensitivity changes were observed in both young and elderly groups, neither in fasting or fed state.

5.4. Hypoxia per se improves oxidative stress

Glutathione is a critical factor in protecting cells against oxidative stress and in erythrocytes is critical for hemoglobin function (Dumaswala, UJ et al. 2001). In addition, erythrocyte glutathione contributes to the whole body redox equilibrium and is a marker of the glutathione status in other tissues, in particular in the liver (Biolo, G., et al. 2007). Glutathione redox capacity depends on the availability of total glutathione and by the ratio between reduced and oxidized glutathione (i.e., the GSH/GSSG ratio) (Biolo, G., et al. 2007).

We have observed that hypoxia directly increased the hematocrit and total whole blood erythrocyte availability, while glutathione concentration per erythrocyte volume was not significantly changed. Such increase in total glutathione availability was associated with a parallel acceleration in the rate of glutathione turnover, expressed either as Fractional and Absolute Turnover Rate.

The amino acid 5-oxoproline is an intermediate of the γ -glutamyl cycle and has been proposed as marker of glutathione status and turnover in cell systems and in animal models (Geenen S et al., 2013). In preliminary work we have tested the hypothesis that plasma 5-oxoproline level is a marker of in vivo glutathione synthesis and turnover in humans. We have developed a method to determine plasma 5-oxoproline levels in humans by stable isotopes, used as internal standard, and gas chromatography - mass spectrometry. The method has been validated in humans and first results have been published (Qi L et al. 2013). We have used the data obtained in the PLANHAB study to test the hypothesis that plasma 5-oxoproline levels could be suitable markers of glutathione turnover in red blood cells as determined by stable isotope infusions. The relationship between red blood cell glutathione synthesis rate and plasma 5-oxoproline levels, relative to the basal condition in healthy volunteers, are shown in figure 36.

According to these results we are using plasma 5-oxoproline in conjunction with expression of the key enzyme for the glutathione synthesis glutamate cysteine ligase to assess glutathione synthesis capacity at the whole body level in the PLANHAB study.

The 5-oxoproline in plasma tended to decrease suggesting an increased activity of the γ -glutamyl cycle and glutathione turnover, not only in erythrocytes but also at the whole body level. Moreover the glutamate-to-glutamine ratio in plasma was increased in hypoxic conditions both in ambulatory and bed rest state, suggesting a higher disposal of this glutathione precursor.

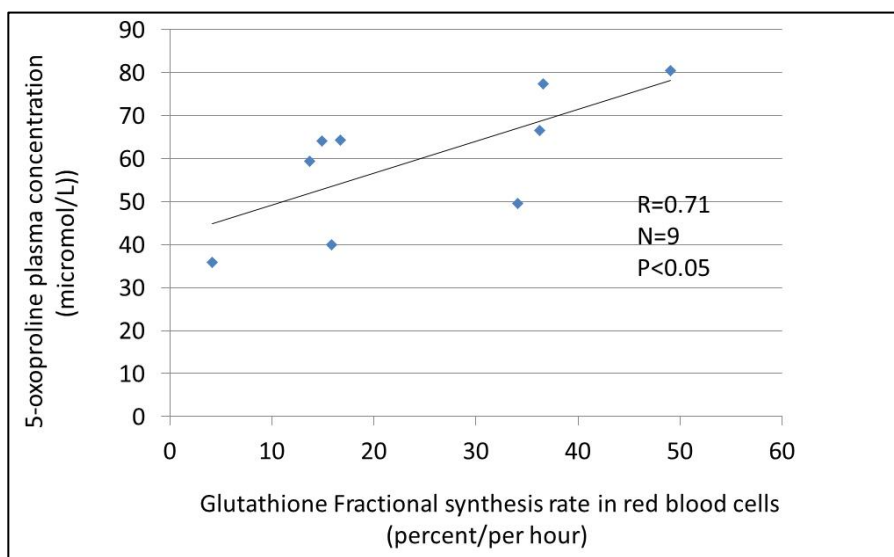


Figure 36. Relationship between glutathione fractional synthesis rate in red blood cells and plasma 5-Oxoprolinone concentration, in healthy volunteers in the postprandial state.

The hypoxia-mediated increase in glutathione turnover was accompanied by a rise in the plasma concentrations of precursor amino acids involved in the glutathione synthesis, i.e. glutamate and cysteine. Glycine plasma concentration is also increased by hypoxia but only in ambulatory conditions, however the erythrocyte concentration of this amino acid is increased by hypoxia in both ambulatory and bed rest condition.

Hypoxia in ambulatory conditions also improved the GSH/GSSG ratio, suggesting a decreased production of reactive oxygen species (ROS). Moderate hypoxia is known to limit the excess production of ROS as well as reduce the oxidative damage to cells (Lopez-Barneo J et al. 2001). In contrast to the ambulatory condition, hypoxia in bed rest did not change significantly the GSH/GSSG ratio. Erythrocyte GSSG levels increased by about 100% following hypoxia in bed rest conditions, while it tended to decrease in ambulatory conditions. Evidence in vivo indicates that muscle unloading is characterized by oxidative stress and increased oxidized glutathione in skeletal muscle. We have recently shown that bed rest combined with overfeeding was associated with activation of erythrocyte glutathione system and markers of oxidative stress in plasma (Biolo, G. et al. 2008). In the present study, hypoxia enhanced glutathione redox capacity in either bed rest or ambulatory conditions. An increased hypoxia-mediated oxidative stress was demonstrated only in bed rest condition.

Plasma glutamine concentration.

In our study, plasma glutamine concentrations decreased by about 4% following hypoxia. Glutamine depletion is often associated with stress conditions and systemic inflammatory response due to decreased muscle production and increased utilization by the immune system. Plasma glutamine concentrations have been found either normal or decreased in patients with COPD (Engelen MP et al. 2003). Pulmonary emphysema was associated with depletion of muscle glutamine (Engelen MP et al. 2000). Moreover, glutamine has been shown to upregulate glutathione recycling enzymes including 5-oxoprolinase (Kaufmann Y et al. 2008).

Tumor necrosis factor Related Apoptosis Induce Ligand (TRAIL)

TRAIL is a member of the TNF-ligand family emerging as protecting factor against atherosclerosis (Volpato S. et al. 2011). As previously reported, TRAIL is inversely related to the risk of mortality in patients affected by cardiovascular disease (Biolo G. et al. 2012; Secchiero P. et al. 2009; Volpato S. et al. 2011). In moderately active subjects, in hypoxic conditions, we observed an increased TRAIL concentration that is directly correlated to changes in the ratio between GSH and GSSG. These data suggest that, in hypoxic condition, the anti-atherosclerosis properties of TRAIL are strictly related to hypoxia-induced increase in glutathione antioxidant defenses. The hypoxia mediated protective effects of TRAIL are completely blunted by exposure to physical inactivity.

5.5. Hypoxia-bed rest interaction induces multiple metabolic changes.

Hypoxic diseases such as COPD and OSAS are associated with an accelerated atherogenesis and an increased cardiovascular morbidity and mortality (Schneider C et al., 2010; Ozkan Y et al., 2008). Hypoxic diseases are often associated with decreased physical activity till immobilization. Physical inactivity per se causes insulin resistance, dyslipidemia, hyperhomocysteinemia, flogosis and altered redox balance (Mazzucco S. et al., 2010; Biolo G. et al., 2005; Biolo G. et al., 2008). However, the separated and combined effects of hypoxia and/or inactivity on cardiovascular risk are not well defined (Drager L. F. et al., 2010; Taylor T. C. 2009). In the PLANHAB study we have investigated the net effects of normobaric hypoxia and/or physical inactivity, using the experimental model of bed rest, in healthy young volunteers.

Considering the relevance of the protective effect of HDLs against atherosclerosis, the significant reduction in the plasma levels of these lipoproteins, observed after 10 days of normobaric hypoxia in all conditions (HBR, HAMB), can contribute to the increased cardio vascular risk of hypoxia, which worsens the negative effects of physical inactivity. This relation between hypoxia and decreased levels of HDLs was recently confirmed by extensive a meta-analysis (Nadeem R. et al. 2014). Other studies, however, have shown contrasting results (Wee J et al. 2013) possibly from inhomogeneous research protocols.

In our study, hypoxia condition per se caused an increment of HL, a lipolytic enzyme synthesized and secreted by the liver, localized mostly at the surface of hepatic sinusoidal capillaries. HL is responsible for a faster hepatic HDL clearance, through various mechanisms, including the insulin resistance induced by both hypoxia and/or inactivity. The higher HL levels with a lower LPL/HL ratio correlated with the HDL plasmatic levels; HL and LPL being enzymes both involved in the clearance of these lipoproteins (Chatterjee C.& Sparks D. L. 2011; Annema W. & Tietge UJ. 2011; Blades B. et al., 1993). HL transforms HDL2 to smaller and denser HDL3 particles, which are probably cleared faster by the liver, causing a reduction of total HDL levels. In our study, the HDL2-to-HDL3 ratio did not significantly change suggesting an increased clearance of HDL3 possibly combined with a higher HDL3 formation from HDL2, from an enhanced HL activity. We may speculate that a higher HL concentration associated with reduced HDL plasma levels, may result in an increased atherosclerosis risk, through the of highly atherogenic, small, low-density-lipoproteins (LDL). Other enzymes, including LPL, LCAT and CETP can be involved in such alteration of lipid

metabolism (Mazzucco S. et al., 2010), nevertheless we did not observe changes in these enzyme levels, possibly because of the shorter protocol duration.

Our data showed that triglyceride (TG) levels were increased in hypoxic ambulatory conditions, but not after bed rest. Different studies have shown an association between hypoxia, from high altitude exposure (Barnholt KE et al. 2006; Farias JG et al. 2006; Young PM et al. 1989) or respiratory clinical disorders (Sharma SK et al. 2011; Drager LF et al. 2010), and increased TG plasma concentrations. The mechanisms underlying these changes, however, have not been completely defined (Adedayo AM et al. 2014; Jun JC et al. 2012).

Inflammation plays a key role in the pathogenesis of many clinical conditions. Biolo G. et al. have previously reported an activation of systemic inflammation following 5 weeks of physical inactivity, during bed rest (Biolo G. et al. 2008). Other studies (Siervo M et al. 2014; Regazzetti C. et al., 2008) have shown the role of hypoxic conditions on local and/or systemic inflammation. In our study short-term BR did not modify CRP and SAA levels but they were augmented in all hypoxic conditions. Our findings are consistent with reports showing that high altitude hypoxia stimulates the expression of pro-inflammatory markers, including CRP (Bailey DM et al. 2004; Hartmann G et al. 2000) and with studies on patients with severe OSAS, characterized by elevated SAA level.

SAA is a HDL-associated apolipoprotein, which is considered an acute-phase factor during inflammation (Kotani K et al. 2013) and a biomarker of CVD and COPD (Korita I et al. 2013; Bozinovski S et al. 2008). SAA may act as pro-atherogenic agent inducing HDL dysfunction by apoA-1 displacement (Kotani K et al. 2013; Van Lenten B et al. 2006; Yokoyama S et al 2006; Jahangiri A. et al. 2009; Tölle M et al. 2012; Alwaili K et al. 2012). Furthermore, SAA has been showed to affect cholesterol efflux from cells to HDL (Wroblewski J.M. et al. 2011). This could have contributed to the increased plasma HDL clearance leading to the decreased HDL plasma levels that we have observed. In our study the ratio between HDL and SAA was significantly reduced, more by hypoxia ($-37\pm 7\%$, $p<0.01$) than bed rest ($-28\pm 9\%$, $p<0.01$) suggesting that different mechanisms are involved in the combined effect of inactivity and hypoxia on HDL metabolism.

Homocysteine plasma level is a well-recognized marker of atherosclerosis development (Glueck CJ et al. 1995; Baszczuk A et al. 2014), it might be responsible, alone, for 10% of heart failures, with a rising of this percentage to 90% when this marker is associated with other classic risk factors, including reduced HDL plasma levels (Baszczuk A et al.

2014). Lately, evidence (Nunomiya K et al. 2013; Kai S et al. 2006; Seemungal TA et al. 2007) indicates an involvement of homocysteinemia in the pathogenesis of COPD (Seemungal TA et al. 2007). Furthermore an association between CRP and homocysteine plasma levels was reported in these patients (Seemungal TA et al. 2007), suggesting a possible contribution of hyperhomocysteinemia to the development of the systemic inflammation. We reported a significant increase in homocysteine plasma levels due to hypoxia per se in all conditions.

Activation of systemic inflammation is often observed during the development of insulin resistance. Ten days of bed rest and/or hypoxia did not significantly affected HOMA-IR index of insulin resistance (Blanc S. et al., 2000; Alibegovic A. C. et al., 2010; Bergouignan A. et al., 2011; Bienso R. S. et al., 2012), however, we found a significant negative effect of both hypoxia and bed rest on the $\Delta 5$ -desaturase activity index in erythrocyte membranes. As previously reported $\Delta 5$ desaturase activity, can be considered a reliable marker of insulin sensitivity (Mazzucco S. et al., 2010).

5.6. Identification of a new biomarker to evaluate anabolic (and insulin) resistance to define optimal protein requirement.

It is well documented that life expectancy in Europe is increasing. In the last years there has been a higher awareness of the metabolic alteration, associated to aging. The goal is to ensure a longer lifespan, together with an improved quality of life. Several metabolic pathologies are related to aging including: loss in muscle mass and strength (i.e. sarcopenia), T2DM, atherosclerosis and cardiovascular disease. These disorders are frequently associated to a reduced physical activity level, exercise being a key factor in the maintenance of the homeostasis of glucose and protein metabolism. Indeed, inactivity promotes the development of insulin resistance, T2DM and CVD, and can impair the muscle sensitivity to anabolic stimuli (e.g. amino acids, protein, etc.). On the contrary, higher levels of physical activity are associated with improved insulin sensitivity and enhanced protein synthesis in the fed state. Thus, both aging and physical inactivity have a negative impact on insulin and anabolic sensitivity. Moreover, because of the few available scientific evidences, one of the goals of the present work was to investigate the possible synergic effect of these two factors.

The evaluation of “insulin resistance” and “anabolic resistance” requires complex and invasive techniques. The “gold standard” procedure for insulin sensitivity in human is the euglycemic-hyperinsulinemic clamp. This measurement consists in an infusion of insulin and glucose in the blood stream in order to evaluate the glucose uptake capacity of the tissues. Despite the extreme precision of this technique, the procedure is very invasive, time consuming and expensive, so it is frequently replaced by the oral glucose tolerance test (OGTT). This method, commonly used in clinical diagnosis, allows an evaluation of the glycaemia levels during and after a glucose load. The procedure however still remain invasive requiring at least 3 blood draws. Other methods utilize mathematical models that calculate insulin sensitivity in fasting or fed states (e.g. HOMA-IR index and Matsuda index, respectively). These models require few blood samplings (one for the fasting state evaluation and at least two for the after load measurements), are the easiest to apply.

To assess anabolic resistance and protein metabolism the most effective technique is the infusion in the blood stream of stable-labeled isotopes (tracers) and the measurement of the isotopic enrichment at the steady state. One the most used tracer in the assessment of the protein metabolism is the L-[ring-²H₅]-phenylalanine (D₅-Phe). After administration, D₅-Phe is hydroxylated in [ring-²H₄]-tyrosine (D₄-Tyr), that represents

an index of net protein loss. For gold standard procedures this protocol has to be assessed during an anabolic stimulus, such as amino acids infusion.

Stable-labeled isotopes can be administered either orally or intravenously, but the latter way is the most used. The intravenous infusion protocol, however, is complex (it requires asepsis of the tracer solutions, two vein catheters etc.) and expensive and requires several blood samples, decreasing the compliance of the evaluated subjects. The oral administration protocol, on the other hand, consists in repeated intake of the tracer solution, at fixed period intervals. This is safer (just one vein catheter for the blood draws) and requires a simpler protocol (there is no need of an aseptic tracer solution). However, it still remains expensive, because of the quantity of stable-isotopes needed, and time consuming.

With the above-mentioned techniques it is not possible to assess “insulin resistance” and “anabolic resistance” at the same time or, at least, in the same experimental protocol. The present work proposes a new, simple, safe and quick method to evaluate simultaneously the “insulin resistance” and the “anabolic resistance” in fed state i.e. after a total meal (500 ml, 500 kcal, 55% carbohydrate, 15% protein, 30% fat) load with the administration of a single dose of tracer solution. This method was used to assess insulin and anabolic resistance after 14-d of bed rest in young and elderly subjects at different level of physical activity. The anabolic resistance was evaluated through a new index (i.e. $AUC_{D4-Tyr}/AUC_{D5-Phe-t_{120}}$ ratio), proposed in this work for the first time, which needs a two hours duration procedure (instead of the 6h often required) and just 2 blood draws versus the 7 samplings required in other protocols. This method does not allow to determine the protein kinetics (i.e. rate of synthesis and degradation) but it permits to calculate an important metabolic parameter, the anabolic sensitivity. The anabolic sensitivity, more than protein kinetics is an important index of the detrimental effect of aging or sedentary life style on muscle mass and protein metabolism. A safe, simple and quick method with limited cost is proposed. Moreover, as already said, this new method allows to evaluate simultaneously, and with the same procedure, anabolic and insulin resistance in post-prandial conditions, with an additional reduction in time and cost and with an improved compliance of the evaluated individuals.

6. CONCLUSIONS

- Aging and bed rest are characterized by a higher protein requirement, confirming data from other studies. Anabolic resistance and insulin resistance are shared biomarkers of these conditions associated with skeletal muscle mass and function loss. However, the effect of inactivity on insulin resistance is blunted in aging.
- We developed a new method to assess at the same time the two most relevant metabolic biomarkers (anabolic resistance and insulin resistance) of protein requirement in humans.
- Other biomarkers related to these sarcopenic conditions are inflammation and redox balance.
- Chronic exposure to experimental hypoxia decreases whole body protein synthesis in the post-absorptive state, suggesting an increased protein requirement also in this condition.
- In addition, chronic hypoxia has a strong impact on cardiovascular risk markers (insulin resistance, homocysteine, systemic inflammation), often additive to that of inactivity (HDL-cholesterol).
- Furthermore, hypoxia, in either ambulatory or bed rest conditions, significantly increased total glutathione concentrations and synthesis rate in erythrocytes. Hypoxia significantly increased redox glutathione capacity (i.e., GSH/GSSG ratio) in ambulatory conditions while this effect was blunted in bed rest.

7. ACKNOWLEDGMENT

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