



Università degli Studi di Trieste

PhD program in MOLECULAR BIOMEDICINE

PhD Thesis

**MECHANISMS OF MUSCLE ATROPHY
FOLLOWING INACTIVITY**

Francesco Agostini

UNIVERSITÀ DEGLI STUDI DI TRIESTE

Sede Amministrativa del Dottorato di Ricerca

UNIVERSITÀ DEGLI STUDI DI TRIESTE,
DIPARTIMENTO DI SCIENZE MEDICHE TECNOLOGICHE E TRASLAZIONALI

Posto di dottorato attivato grazie al contributo dell'Agenzia spaziale italiana.

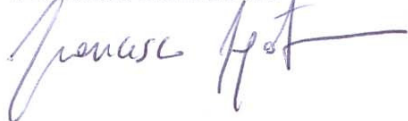
XXII ciclo del Dottorato di Ricerca in Biomedicina Molecolare

Titolo: MECHANISMS OF MUSCLE ATROPHY FOLLOWING INACTIVITY.

Settore scientifico disciplinare: Med 09/Medicina Interna

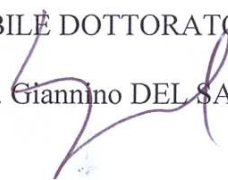
DOTTORANDO

Francesco AGOSTINI



RESPONSABILE DOTTORATO RICERCA

PROF. Giannino DEL SAL



RELATORE

PROF. Gianni BIOLO

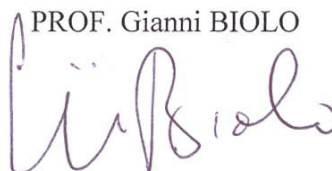


TABLE OF CONTENTS

ABSTRACT	4
INTRODUCTION	6
Why study physical inactivity?	6
Muscle atrophy.....	7
Inflammation.....	13
Oxidative stress.....	20
Homocysteine.....	32
Glutamine.....	35
Nutrition, inactivity and muscle atrophy.....	39
AIMS.....	49
MATERIALS AND METHODS	50
1) Short Term Bed Rest-Integrated Physiology (STBR-IP) at the Clinical Research Center of the DLR-German Aerospace Institute (Cologne, Germany)	50
2) Women International Space Simulation for Exploration (WISE) at MEDES Clinical Research Facility of the Rangueil University Hospital (Toulouse, France)	54
3) Valdoltra Bed Rest studies 2006 - 2007 - 2008, Valdoltra Orthopaedic Hospital, University of Koper (Capodistria, Slovenia).	59
RESULTS	74
Effect of bed rest on whole body inflammatory and oxidative stress status.....	74
Effects of bed rest on muscle atrophy and oxidative stress	83
Impact of nutrition on bed rest mediated alterations.....	91
DISCUSSION	106
Bed rest effects on inflammation and oxidative stress.....	106
Impact of physical inactivity on glutamine metabolism	113
Impact of nutrition on inflammation, oxidative stress and muscle atrophy.....	123
ACKNOWLEDGMENTS	132
REFERENCE LIST	133
LIST OF PAPERS INCLUDED	157

ABSTRACT

Background. Muscle atrophy is determined by specific molecular pathways controlling protein synthesis and degradation. Physical inactivity and muscle unloading are normally related to decreased muscle mass, but pathways controlling muscle atrophy during inactivity in humans are not presently clarified. Previous publications showed in animals a link between muscle atrophy and oxidative stress or inflammation. Inactivity was shown to be associated to inflammation and oxidative stress upregulation. Inflammation is controlled by several factors and polyunsaturated fatty acids (PUFA) of n-3 and n-6 series are known to play an anti-inflammatory and proinflammatory role, respectively: their relative content in cell membranes reflects the inflammatory condition at whole body level. Oxidative stress derives from reactive oxygen species (ROS) production in excess to the scavenging activity of antioxidant systems. Homocysteine is a non-proteinogenic amino acid: increased homocysteine concentration in plasma is directly linked to oxidative tissue damage. Glutathione (GSH) is an antioxidant tripeptide: upregulated GSH availability is considered a response to occurred ROS production. Glutamine, is synthesized by muscles and it is utilized by immune cells: its depletion occurs in critically ill patients. Nutrition can play a pivotal role during inactivity: dietary protein supplementation can ameliorate protein turnover while energy intake restriction was linked to enhanced muscle atrophy. Aims and experimental design. The aim of the present thesis is to investigate in human healthy volunteers: i) the impact of physical inactivity on inflammation and oxidative stress as potential mechanisms underlying muscle atrophy; ii) the impact of energy balance, and of protein supplementation during inactivity on muscle atrophy, oxidative stress and glutamine availability. Different metabolic tests were performed in five separate experimental bed rest campaigns: STBR-IP, WISE, Valdoltra Bed Rest 2006 – 2007 – 2008. Results. Eucaloric experimental bed rest at normal protein intake: decreased n-3 and increased n-6 PUFA in red blood cell membranes; increased arachidonic to eicosapentaenoic acid ratio (Valdoltra Bed Rest 2006 – 2007 – 2008); increased homocysteine plasma concentration lowering clearance by remethylation (WISE); reduced plasma glutamine concentrations lowering its *de novo* synthesis (STBR-IP); decreased muscle thickness and myofibres pennation angle; increased muscle protein carbonylation and GSH availability in direct correlation with atrophy progression (Valdoltra Bed Rest 2006 – 2007). High protein intake during bed rest significantly lowered homocysteine concentrations by increased clearance by transsulfuration. Calorie restriction (20% reduction) during muscle unloading failed to affect changes of glutamine kinetics and availability mediated by inactivity (STBR-IP). During bed

rest, excessive fat mass gain, when matched to stable fat mass maintenance, significantly: reduced vastus lateralis muscle thickness; increased plasma leptin, myeloperoxidase and C-reactive protein levels; enhanced, in red blood cells, activity and availability of GSH (Valdoltra Bed Rest 2006 – 2007). Discussion. The experimental bed rest mediated increase in n-6 relatively to n-3 polyunsaturated fatty acids of erythrocytes membranes confirm bed rest can play a pro-inflammatory role at whole body level. Upregulated levels of homocysteine plasma concentrations after bed rest underline that immobility can induce whole body oxidative stress and cardiovascular risk. Significant correlation between changes in protein carbonylation, as marker of oxidative damage, or antioxidant GSH availability, with muscle atrophy induced by bed rest, strongly suggest that physical inactivity can induce muscle atrophy by pathways involving oxidative stress. Carbonylated proteins are, in fact, directly and rapidly degraded by the proteasome system. Moreover, dietary protein supplementation during physical inactivity potentially reduces oxidative damage lowering plasma homocysteine concentration. This evidence is confirmed by publications showing, in animals, that high protein diet can increase homocysteine transsulfuration rate. Interestingly, during inactivity, positive energy balance, when matched to fat mass maintenance, was here shown to induce greater muscle atrophy together with greater upregulation of whole body inflammation and oxidative stress. Thus, such observations further suggest that inactivity mediated atrophy can be triggered and regulated by oxidative stress and inflammation. Calorie restriction probably failed to affect glutamine kinetics due to the low extent of energy intake reduction, but glutamine metabolism can be hypothesized to be independent from energy balance during inactivity.

INTRODUCTION

Why study physical inactivity?

The impact of physical exercise on human physiology has been studied during the last fifty years, yielding almost 185200 published works. Consequences deriving from low activity or from immobility were studied in humans over the last five decades and results were published in nearly 4000 articles. This emphasizes the scientific relevance of investigating the relationship between physical activity level and human health. Moderate exercise was demonstrated to play a positive role on health, such that the World Health Organization (WHO) is developing recommendations regarding required amount of physical activity in relation to age and gender ("Global recommendations on Physical Activity for Health" – WHO). On the contrary inactivity is considered to play a strongly negative role on human health, contributing to billions of deaths from chronic diseases, and increasing prevalence of physical disabilities, especially in elderly people (Fontana, 2009a). Immobility, or low activity, are frequent conditions, differentially associated to physiologic and pathologic states. Notably, social changes occurring in the last years has led to the onset of the so called "sedentary lifestyle", a severe reduction of average physical activity level, with undesired consequences in health (Chaput and Tremblay, 2009). Moreover, variably prolonged periods of inactivity can be due to several illness conditions: serious trauma and neurological diseases can preclude the possibility to walk or move, possibly leading to long term immobility. Equally, diseases belonging to internal medicine and cardiology, as well as all surgical interventions, often require prolonged recovery periods in the horizontal position. Finally, muscle inactivity also characterizes the spaceflight microgravity environment where physical effort for initiating movements in the environment is drastically reduced. All these conditions underline the importance of fully characterizing net effects of inactivity on human physiology. The impact of physical inactivity on metabolism is difficult to assessed in hospitalized patients due to the significant contribution of the underlying pathology. Studies associating activity level and parameters characterizing selected diseases can contribute to the elucidation of the consequences of a sedentary lifestyle on human health (Davy et al., 1998). To clearly elucidate the net effect of inactivity, different experimental models can be applied. Beside lowerlimb casting, experimental bed rest is the most accepted model of physical inactivity. During bed rest studies human healthy volunteers lay in bed for different periods, performing all activities in the horizontal position. Bed rest studies, thus, allow to investigate causes of metabolic and morphologic modifications occurring as a net consequence of physical inactivity.

Besides muscle atrophy, which is the most evident effect of immobility (Alkner and Tesch, 2004; Berry et al., 1993), unloading was shown to significantly modify the endocrine milieu (Biolo et al., 2005a).

Data reported and discussed in the present thesis were collected during five different bed rest campaigns: 1) Short term bed rest-integrated physiology (STBR-IP) at the Clinical Research Center of the DLR-German Aerospace Institute (Cologne, Germany); 2) Women International Space Simulation for Exploration (WISE) at MEDES Clinical Research Facility of the Rangueil University Hospital (Toulouse, France); 3) Valdoltra Bed Rest 2006; 4) Valdoltra Bed Rest 2007; 5) Valdoltra Bed Rest 2008, Valdoltra Orthopaedic Hospital (Ankaran, Capodistria, Slovenia). Within these study campaigns, the following bed rest mediated changes were evaluated: a) on factors reflecting oxidative stress and inflammation levels, b) on antioxidant system activation and c) on muscle atrophy. Obtained results elucidated a possible cause of muscle atrophy related to physical inactivity. Additionally, the impact of experimental changes of energy or protein intake during bed rest, on muscle atrophy, inflammation and oxidative stress were also evaluated.

Muscle atrophy

Muscle atrophy is defined as a decrease of muscle mass. The maintenance of skeletal muscle mass is determined by the balance between protein synthesis and protein degradation. Skeletal mass is increased when there is a net gain in protein synthesis which can occur following exercise training (Biolo et al., 1997). In contrast, skeletal muscle mass is reduced when degradation overcomes protein synthesis rate. Atrophy is linked to muscle weakness, as force development potential is almost directly related to muscle mass or volume (Seynnes et al., 2008). Muscle wasting (cachexia) is a co-morbidity feature frequently occurring in several common diseases, including e.g. cancer (Fearon and Moses, 2002), Acquired Immune Deficiency Syndrome (AIDS) (Dalakas and Pezeshkpour, 1988), congestive heart disease (Lunde et al., 2001), chronic obstructive pulmonary disease (COPD) (Couillard and Prefaut, 2005), renal failure (Mitch, 2006) and severe burns (Pereira et al., 2005). Clinical importance of muscle atrophy is underlined by poor prognosis characterizing cachectic patients in these setting of diseases (Dimitriu et al., 2005).

Molecular pathways regulating protein synthesis and degradation.

Protein synthesis

Studies performed in murine and cell models revealed the importance of Akt (also called protein kinase B) family factors (Akt-1, Akt-2, Akt-3) in the regulation of general protein synthesis in skeletal muscle. Akt factors are, in fact, serine/threonine-specific protein kinases, playing a pivotal role in muscle hypertrophy (Bodine et al., 2001b). Activation of signaling cascades involving insulin-like growth factor 1 (IGF-1) and phosphatidylinositol 3-kinase (PI3K) induces Akt phosphorylation and activation (Bodine et al., 2001b). Akt, in turn, was shown to activate eukaryotic translation initiation factor 2B (eIF2B) by stimulation of glycogen synthase kinase-3 β (GSK-3 β) (Rhoads, 1999). In parallel, Akt can activate initiation of protein translation stimulating p70S6 kinase (p70S6K) by mammalian target of rapamycin (mTOR) (Terada et al., 1994). Interestingly, a period of resistance training exercise induces muscle hypertrophy associated with increases in phosphorylated Akt, GSK-3b and mTOR (Leger et al., 2009).

Proteolytic pathways

Several proteolytic systems, can contribute to the degradation of muscle proteins. *In vitro* and animal studies, showed Ca²⁺-activated proteases (Calpain) and the proteasome system play important roles in muscle protein breakdown during muscle atrophy (Furuno and Goldberg, 1986; Ikemoto et al., 2001; Purintrapiban et al., 2003). Moreover, caspase-3 may also contribute to selected forms of muscle atrophy (Du et al., 2004). Actomyosin complexes represent 50–70% of muscle proteins (Tidball and Spencer, 2002) and, as the proteasome system can degrade only monomeric contractile proteins (i.e. actin and myosin) (Goll et al., 2003), monomeric myofilaments must be released from actomyosin complexes to be degraded by the proteasome (Goll et al., 2003). Both calpain and caspase-3 can play a key role in producing actomyosin disassociation (Du et al., 2004; Goll et al., 2003; Tidball and Spencer, 2002). Calpain activity is increased by elevations of cytosolic calcium concentrations (Goll et al., 2003) and caspases are cascade activated proteases triggered by several signaling pathways (Primeau et al., 2002): their activation can result in protein breakdown as well as in apoptosis. In the proteasome system, degradation can occur by the 26S subunit (Grune et al., 2003; Grune and Davies, 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 and Ordway, 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). Interestingly, two unique ubiquitin E3 ligases, atrogin1 and muscle ring

finger-1 (MuRF-1), were shown to be involved in skeletal muscle atrophy (Bodine et al., 2001a; Gomes et al., 2001). MuRF-1 was shown to be directly and indirectly upregulated by Forkhead family of transcription factors (FoXO) (Stitt et al., 2004) and by the NF- κ B transcription factor (Sandri 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 et al., 2003).

Figure 1

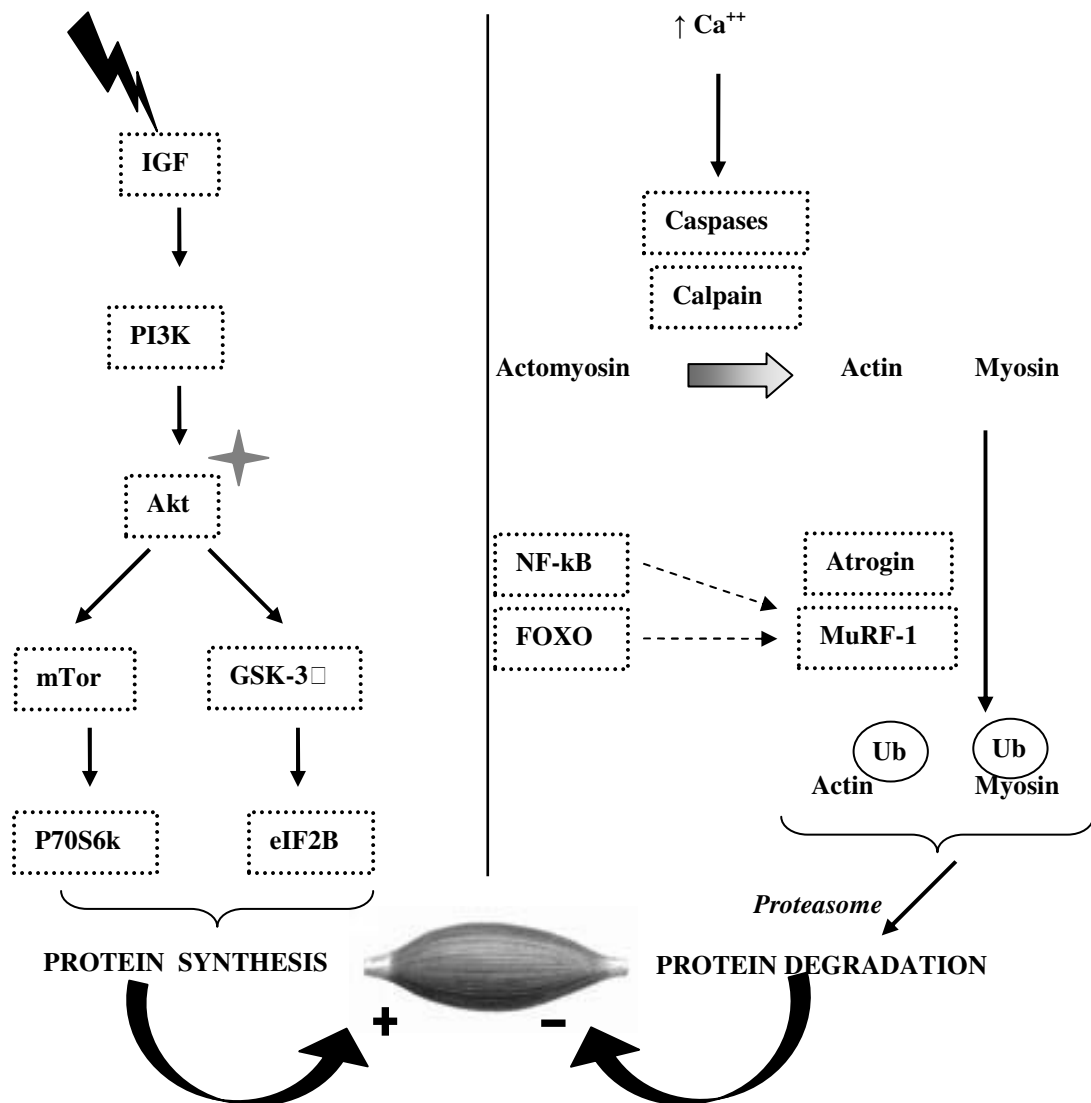


Figure 1 summarizes molecular pathways controlling muscle mass maintenance by modulation of protein synthesis and degradation.

IGF, Insulin-like growth factor; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; p70S6K, p70S6 kinase; GSK-3 β , synthase kinase-3 β ; eIF2B, eukaryotic translation initiation factor; NF- κ B, nuclear factor kappa B, FoXO, Forkhead family of transcription factors; MuRF-1, muscle ring finger-1.

Physical inactivity and muscle atrophy

Several previous works showed that physical inactivity can significantly induce muscle atrophy. A bed rest period of 20 days significantly induced 9 or 10 % thickness reduction in postural muscles (Kawakami et al., 2000) (Akima et al., 2005) . Equally, during one month of bed rest (Berry et al., 1993) quadriceps muscles uniformly reduced their cross sectional area. Specific morphology changes occurring as a consequence of one month of bed rest were demonstrated also on selected muscles. Vastus intermedius was, in fact, shown to be reduced by 12-16 % and a 9 % atrophy was assessed in knee extensors after seven weeks of experimental bed rest (Ellis et al., 1993). Vastus lateralis showed a 17 % decrease in cross sectional area as a consequence of an equal period of inactivity (Ferretti et al., 1997), in this way demonstrating this muscle is particularly affected by unloading. More prolonged periods of bed rest induced similar changes in human muscle atrophy: eight weeks of bed rest reduced the cross sectional area of thigh extensors by 14-17 % (Ferretti et al., 2001) and knee extensor muscles showed a similar cross sectional area reduction after the same experimental period (Berg et al., 1997). After 54 days of muscle unloading (Mulder et al., 2006) a linear decrease of 14 % in quadriceps femoris cross sectional area over the experimental period was demonstrated and a similar reduction was shown after a 120 day bed rest period (Leblanc et al., 1992) in thigh muscle mass. Thickness of postural calf muscles (soleus, flexor hallucis longus, and tibialis posterior) decreased by 9-12 % after 30 days of bed rest (Ellis et al., 1993). A previously published study demonstrated a 13 % and a 9 % cross sectional area decrease in soleus and in gastrocnemius after 1 month bed rest, respectively (Berry et al., 1993). An equal experimental period induced a 10 % calf volume and a 5 % cross sectional area reduction (Convertino et al., 1989).

In another study (Leblanc et al., 1988) 35 days of bed rest induced a 12 % decrease in muscle cross sectional area of postural plantar flexors muscles (gastrocnemius and soleus), whereas postural dorsiflexor muscles were not significantly affected. In a recently published study, 5 weeks bed rest were demonstrated to induce a 12 % reduction in cross sectional area of the ankle plantar flexors (Berg et al., 2007). Greater atrophy of calf muscles was demonstrated during a 5 week bed rest study in which soleus and gastrocnemius muscles were shown to lose 25 % cross sectional area (Gogia et al., 1988). After extremely long term experimental periods (120 days) a 30 % decrease in calf muscle mass was measured (Leblanc et al., 1992).

Thus, leg muscles are principally affected by bed rest, with thigh and calf muscles being almost equally sensitive to unloading.

These and other published data demonstrate that the loss of lean body mass (LBM) during prolonged periods of experimental inactivity in healthy volunteers, occurs at an average rate of 3-4% per week. This value is linearly maintained for the initial stages of the bed rest period, reaching a plateau after five or six weeks.

Nitrogen balance and whole body protein synthesis rate, as parameters affecting muscle mass maintenance, were measured in the crew of two Spacelab Life Sciences (SLS) shuttle missions before, during, and after spaceflight (Stein et al., 1996). In this condition of muscle unloading, a rapid weight decline during the first 5 days was observed, followed by stabilization. In addition the daily mean nitrogen balance decreased by almost 70% during flight, corresponding to a loss of approximately 1 kg of LBM over 14 days.

Methods for quantification of muscle mass changes.

Both muscle thickness and cross-sectional-area changes are reliable and direct markers of variations in muscle volume (Mathur et al., 2008) and force development (Akagi et al., 2008). Magnetic Resonance Imaging (MRI), is considered to be the gold standard for reliable detection of muscle volume and size changes: by this technique even little variations of muscle size can be accurately detected. It does not apply ionizing radiation, but uses a powerful magnetic field to align the nuclear magnetization of hydrogen atoms in body water. Radio frequency fields are used to systematically alter the alignment of this magnetization, causing the hydrogen nuclei to produce a rotating magnetic field detectable by the scanner. This signal can be modified by other magnetic fields in order to construct an image of the body providing contrast between the different soft tissues (Burghart and Finn Carol, 2010).

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 bulk alterations. Reliability of ultrasound imaging was validated *versus* MRI as a gold standard (Arbeille et al., 2009) and moreover, this technique allows a proper determination of muscle fibre orientation with respect to aponeurosis, i.e. pennation angle (Narici and Cerretelli, 1998). Pennation angle, is a reliable marker of muscle maintenance. Reduction of pennation angle was previously published to be associated to increased muscle atrophy. Aging (Morse et al., 2005) and immobilization (Narici and Cerretelli, 1998; Reeves et al., 2002), in fact, were shown to negatively affect muscle architecture leading to a sarcomere orientation remodelling and to a concomitantly enhanced muscle atrophy (de Boer et al., 2007). Interestingly, reductions in pennation angle were shown to be directly related to muscle thickness decreases (Ichinose et al., 1998).

Figure 2

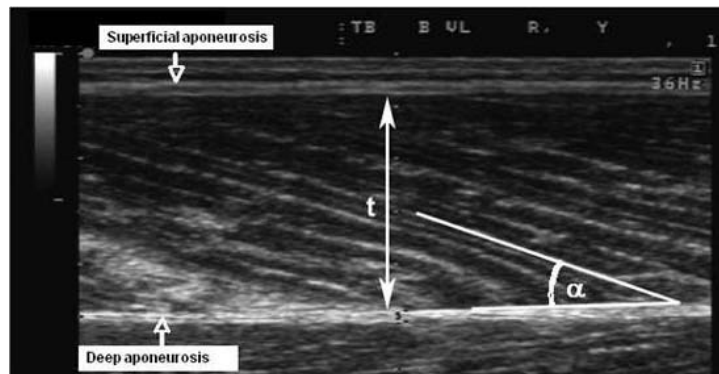


Figure 2 shows an ultrasound image of the human *vastus lateralis* muscle obtained in the sagittal plane. Muscle fibres fascicles are clearly visible as the structures stretching from the superficial and deep aponeuroses. t , muscle thickness; α , pennation angle.

Whole body bioimpedance analysis is also considered a reliable method to assess changes of total muscle mass (Lim et al., 2009). Bioimpedance analysis determines the opposition of body tissues to an external and low intensity electric current flow: this values can be used to calculate total body water which in turn allows estimation of fat-free body. This parameter is directly associated to muscle mass changes and allows determination, by difference, of body fat (Lim et al., 2009). Thus, bioimpedance analysis is a suitable method also for the assessment of body fat, and of body composition changes.

Measurements of body composition can be reliably performed by dual energy X-ray absorptiometry (DXA). This technique can measure body fat and lean masses, by emission of X-rays at two energies: the separate absorption of the two X-ray emission allows estimation of tissue distribution (fat and LBM). The approach is based on the assumption that fat mass hydration is kept constant at 73 %. Measurement can be performed in specific body regions, such as the arms, legs, and trunk (Laskey, 1996).

Inflammation

Inflammation is a multifactorial response occurring when the immune system is acutely activated against potentially harmful antigens, as pathogens or dead cells. In this phase, macrophages, dendritic cells and mastocytes resident in the infected or damaged tissue are activated to release inflammatory mediators, cytokines, which are the main actors accounting for clinical signs of inflammation (Walter and Talbot, 1996).

Cytokines and markers of inflammation.

Cytokines are known to play a pro- and anti-inflammatory role. Among several known proinflammatory cytokines, interleukin 1 (IL-1) is one of the mostly studied: it is composed of two subunits, and it plays a pleiotropic role in activating processes linked to inflammation as fever, lymphocytes and bone marrow cell proliferation (Dinarello, 1994). Interleukin-6 (IL-6) is a cytokine that acts as both a pro-inflammatory and anti-inflammatory agent. It is also known as “myokine”, as it is released by contracting muscle and is upregulated after high intensity physical exercise (Ostrowski et al., 1998). IL-6 upregulation after muscle contractions previously suggested that physical exercise can have anti-inflammatory properties (Petersen and Pedersen, 2006). Finally, interleukin 10 (IL-10) is an example of anti-inflammatory cytokine, produced by monocytes and lymphocytes. It is an immunomodulator decreasing expression of pro-inflammatory cytokines and downregulating the activation of factors involved in intracellular inflammatory response as NF- κ B and related tissue injury (Yoshidome et al., 1999). Physical exercise was shown to increase circulating IL-10 concentrations, thus suggesting muscle activity can exert a beneficial anti-inflammatory action (Nunes et al., 2008). Tumor necrosis factor alpha is an additional important cytokine involved in systemic inflammation, especially during the acute phase. It is mainly synthesized by macrophages, but it is also produced by other cells as adipocytes (Winkler et al., 2003). Tumor necrosis factor alpha was shown to play crucial roles in the onset and maintenance of inflammation process, stimulating acute phase reaction factors, in liver, macrophage phagocytosis and chemoattraction of neutrophils (Tracey and Cerami, 1990; Tracey and Cerami, 1994). Through the action on two specific cell membrane receptors, tumor necrosis factor alpha can activate several biological responses as activation of NF- κ B (Bouwmeester et al., 2004) and of intracellular pathways leading to cell differentiation or to apoptosis (Gaur and Aggarwal, 2003). Prolonged elevation of circulating tumor necrosis factor alpha can lead to muscle mass reduction: a wasting condition linked to poor prognosis of patients (Kandarian and Jackman, 2006). Synthesis of interleukins and tumor necrosis factor alpha can trigger the production of a known marker of inflammation called C-reactive protein (CRP). CRP is a factor mediating activation of a crucial immune response process as the complement system (Lee et al., 2002). In clinical practice it is used as marker of inflammation onset, both in chronic and acute conditions (Ho and Lipman, 2009). Mild elevation of CRP concentrations characterize metabolic and cardiovascular pathologies. Diabetes was associated to increased circulating CRP concentrations, and evidence indicates that inflammation can play a causative role in the pathology (Mugabo et al., 2009). Moreover, CRP levels over a threshold of 2 mg/l

were shown to be associated with an increased cardiovascular risk and mortality (Yang et al., 2009).

Myopathies and inflammation

Prolonged or chronic inflammation is associated not only to cardiovascular risk, but also to other pathologies linked to muscle atrophy. Experiments performed in a mouse model of myositis, a severe pathology leading to muscle weakening and wasting, showed that artificial induction of inflammation upregulated, especially at long term, specific molecular markers of such pathology (tau, Abeta) (Kitazawa et al., 2008). Duchenne muscular dystrophy, a genetic disease characterized by marked muscle wasting due to the lack of the dystrophin gene, is strictly associated to chronic muscle inflammation: NF-kB pathway was shown to be strongly involved in muscle wasting progression of subjects affected by such pathology (Acharyya et al., 2007). Finally, idiopathic myopathies are known to be associated with enhanced release of proinflammatory cytokines such as CCL3, CCL4, CCL5, and of their specific receptors (Civatte et al., 2005). Previously published studies underlined that inflammation can be a possible cause of cancer cachexia, a condition of muscle wasting associated to poor prognosis in patients: as abovementioned tumor necrosis factor alpha, known also as cachectin, was shown to be one of the factors triggering hypercatabolic processes in tumor bearing subjects (Tracey and Cerami, 1994).

Inflammation was shown to play a crucial role also in muscle mass wasting normally occurring in healthy aging subjects (sarcopenia) (Jensen, 2008). In particular IL-1, IL-6 and tumor necrosis factor-alpha were previously shown to potentially trigger muscle sarcopenia in elderly subjects (Yende et al., 2006). In addition, an interesting study based on DNA microarray analysis showed that a subgroup of genes involved in promotion of inflammation is upregulated in sarcopenic elderly subjects (Giresi et al., 2005). Interestingly, physical exercise training programs tailored to elderly subjects were shown to reduce muscle mass wasting and to reduce synthesis of proinflammatory cytokines such as interleukin-1 and tumor necrosis factor (Nicklas and Brinkley, 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 et al., 2005).

Inactivity and inflammation

It is well established that inactivity is linked to enhanced risk of cardiovascular events. In an epidemiologic study performed in female adolescents, sedentary lifestyle was associated, with increased concentrations of plasma markers of inflammation such as tumor necrosis factor

alpha (Ischander et al., 2007). Similarly, an observational study performed in a large sample of male and female subjects showed that persons adopting an unhealthy sedentary lifestyle were characterized by mildly increased levels of CRP, a marker of low grade inflammation. Interestingly, appropriate moderate training programs and the so called “mediterranean diet” reduced the observed CRP increase (Pitsavos et al., 2007). Another epidemiologic study confirmed that, regardless of the degree of obesity, sedentary lifestyle is associated with increased levels of interleukin-6 and of CRP (Fischer et al., 2007). 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 et al., 2008).

Inflammation and polyunsaturated fatty acids.

The proinflammatory role of n-6 polyunsaturated fatty acids

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 et al., 1990). Cell availability of polyunsaturated fatty acids (PUFA) of the n-6 series affects production of eicosanoids. Eicosanoids are, in fact, 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 $\Delta 6$ -desaturase, elongase and $\Delta 5$ -desaturase (Figure 3). Linoleic acid is an essential FA and many vegetable oils (corn, sunflower, soybean and margarines) contain elevated fractions of linoleic acid (Bozan and Temelli, 2008): availability of n-6 PUFA is determined by linoleic acid intake and by endogenous synthesis (Wertz, 2009). Elevated availability of n-6 PUFA in cell membranes was previously linked to inflammatory diseases (Ueda et al., 2008). Increased n-6 PUFA concentrations, moreover, can enhance gene expression of proinflammatory cytokines and transcriptional activity of NF-kB (Weaver et al., 2009). These and other published evidence demonstrate the key role of circulating and membrane n-6 PUFA in the stimulation of pro-inflammatory processes.

Anti-inflammatory effects of n-3 PUFA.

Apart from n-6 PUFA series, another family of fatty acids is deeply involved in the control of inflammation: the n-3 PUFA. They are synthesized from the n-3 PUFA alpha-linolenic acid in a metabolic pathway sharing the same enzymes promoting n-6 series synthesis. In n-3 PUFA

synthesis, $\Delta 5$ -desaturase leads to eicosapentaenoic acid, that in turn is converted to docosahexaenoic acid in the peroxisome by the action of $\Delta 6$ -desaturase. n-3 PUFA are well known to play an anti-inflammatory action (Figure 3). In analogy with n-6 PUFA, alpha-linolenic acid and n-3 PUFA availability is strongly conditioned by dietary intake of alpha-linolenic acid from vegetable oils (Wertz, 2009), but, in addition, fish oil is known to contain high amount of eicosapentaenoic and docosahexaenoic acids (Pickova, 2009): thus, dietary fish intake strongly affects availability of these two compounds. Fatty acids of the n-3 series are known to have an anti-inflammatory action. Increased intake of eicosapentaenoic and docosahexaenoic acids can affect the cell membrane content of these fatty acids (Lee et al., 1985), thus reducing the fraction of the pro-inflammatory arachidonic acid. Decreased production of selected prostaglandins as well as of interleukin-1 was demonstrated as a consequence of n-3 PUFA supplementation (Lee et al., 1985; Endres et al., 1989). Additionally, eicosapentaenoic acid can lead, as substrate for cyclooxygenase, to slightly different isoforms of prostaglandins, characterized by lower proinflammatory potential (Bagga et al., 2003). Synthesis of subclasses of anti-inflammatory molecules called resolvins was shown to be based on eicosapentaenoic and docosahexaenoic acids as precursors (Hong et al., 2003). *In vitro* cell studies showed eicosapentaenoic and docosahexaenoic acids can minimize the expression of interleukin-1 and of tumor necrosis factor alpha (Babcock et al., 2002) and such evidences were confirmed by studies demonstrating, in animals, anti-inflammatory effects of fish oil supplementation (Billiar et al., 1988).

Finally, n-3 PUFA can interfere with genetic regulation of proinflammatory factors principally downregulating the transactivation activity of NF-kB (Ross et al., 1999) that, in turn, can reveal the anti catabolic potential of such class of anti-inflammatory fatty acids. 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 et al., 2009)

Membrane fatty acid composition

Phospholipid content in red blood cell membranes can be considered a reliable marker of fatty acid availability in plasma, and of cell membrane composition of the whole body (Harris and Von Schacky, 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 et al., 2002). Moreover, the fraction of n-3 PUFA in cell membranes was directly associated to reduced incidence of cardiovascular diseases: this effect was associated to the anti-inflammatory role of n-3 PUFA and to other changes induced on cardiovascular system physiology by this class of fatty acids (Harris and Von Schacky, 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 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.

Physical exercise and membrane fatty acid composition.

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 et al., 1993). Another study emphasised n-6 PUFA content changes mediated by physical exercise: 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 et al., 1998). Regular exercise was shown, in rats, to decrease Δ -5 desaturase activity and arachidonic acid content; in addition, docosahexaenoic acid proportion in cell membrane was decreased while linoleic acid was increased (Helge et al., 1999). Observed effects on membrane composition mediated by regular exercise were hypothesized to be dependent on energy substrate utilization during training (Helge et al., 1999). Still, a previously published study showed in humans that fractions of oleic acid and docosahexaenoic acid were significantly higher in trained muscles when matched to untrained

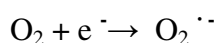
(Helge et al., 2001). Similarly, physical exercise significantly lowered the ratio between n-6 and n-3 PUFA in the trained muscle when compared to the untrained (Helge et al., 2001): also in this study regular physical exercise was confirmed to reduce whole body and muscle inflammation.

Oxidative stress

Oxidative stress stems from an unbalanced production of free radicals which is not sufficiently scavenged by the activity of antioxidant defenses of the organism. Interestingly, increased activity of antioxidant and cell damage repair systems can be evidenced after free radical production and oxidative stress onset (Tchou et al., 1991; Hardmeier et al., 1997).

Reactive oxygen species

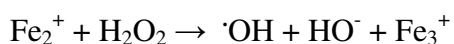
Free radicals are, in general, chemical compounds characterized by an unpaired free electron in the external orbit. Reactive oxygen species (ROS) are the most frequent free radicals in the organism. They are principally derived from the following reaction of molecular oxygen reduction, leading to highly reactive species such as superoxide anion and hydrogen peroxide:



or



H_2O_2 is in turn converted, by metal catalysis, into other highly reactive species (as oxydri radical) by the following reaction:



Other reactive radical species are derived from nitric oxide (NO^\cdot): this chemical compound can rapidly react with ROS leading to the formation of peroxynitrite (ONOO^\cdot), which is a potent pro-oxidant species (Ischiropoulos et al., 1992).

Free radical sources

Different biochemical reactions can cause the production of highly reactive species. Mitochondrial electron transport chain is deeply involved in this process: four complexes are

involved in the transport of electrons. Electrons derived from intermediates of tricarboxylic acid cycle and from reduced nicotinamide adenine dinucleotide (NADH) are transported to NADH dehydrogenase (Complex I) and to succinate dehydrogenase (Complex II). Intermediate carriers transport electrons to ubiquinon-cytochrome C reductase (complex III) and to cytochrome C oxidase (Complex IV) (Halliwell and Gutteridge, 1986). Afterwards electrons are transferred to O₂, leading to the production of hydrogen peroxide and of superoxide anion (Beckman and Ames, 1998).

NADPH oxidase activity can also lead to the production of free radicals and this process can be stimulated by increased availability of calcium through protein kinase C-ERK1/2 pathway (Javesghani et al., 2002).

Xanthine oxidase is an enzyme involved in purine catabolism. Xanthine oxidase can also cause the production of free radicals oxidizing hypoxanthine to xanthine, which in turn can be converted to uric acid (Whidden et al., 2009).

Microsomal complexes of cytochrome P450 are also involved in direct production of superoxide anions, while performing their detoxifying activity against xenobiotics (Goepfert et al., 1995).

Lipids β -oxidation can also determine the production of hydrogen peroxide in the peroxisomes, but the actual contribution of this process to free radical production is difficult to be assessed due to the presence of catalase enzyme (Beckman and Ames, 1998).

Highly reactive species are also significantly released by activated phagocytes during inflammation. After phagocytosis, in fact, this class of cells produces superoxide anions, hydrogen peroxide and nitric oxide in order to neutralize pathogenic agents (Moslen, 1994).

Finally, nitric oxide is rapidly produced by specific isoenzymes (nitric oxide synthetase) in order to control vasodilation and immune responses (Liu and Hotchkiss, 1995).

Molecular targets of free radicals action.

Several classes of macromolecules can be modified by the action of free radicals.

Peroxydation of cell membrane lipids can alter membrane fluidity, in turn affecting action of protein receptors exposed on the cell surface (Ghosh et al., 1993). Unsaturated aldehydes derived from lipid peroxydation can play a negative role on cell physiology potentially inactivating selected enzymes (Chen and Yu, 1994) and affecting, as mutagenic factors, DNA integrity (Marnett et al., 1985).

Nucleic acids can be directly modified by free radicals and this can induce DNA coiled strand breaks and formation of complexes with other molecules (Beckman and Ames, 1998).

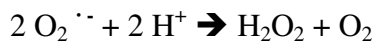
Beside lipid membranes and nucleic acids, proteins are one of the most important targets of free radical action. Proteins vary regarding their susceptibility to the action of free radicals by virtue of their sequence and structure (Beckman and Ames, 1998). Principally, oxidation of sulphhydryl groups and oxidation catalyzed by presence of metals in specific protein sites can affect protein structure, which in turn impairs polypeptide function and leads to disposal by fragmentation (Stadtman, 1990; Starke-Reed and Oliver, 1989).

Antioxidant systems.

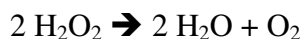
The ability of the organism to counteract the action of free radicals is exerted by enzymic and non-enzymic factors.

Enzymic antioxidants

The most known enzymic antioxidants are superoxide dismutase, catalase and thioredoxin reductase. The conversion of superoxide anion to hydrogen peroxide is performed by superoxide dismutase by the following reaction.

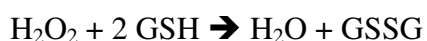


The conversion of hydrogen peroxide to water is achieved by the following reaction catalyzed by catalase:



Thioredoxin is a NADPH dependent enzyme involved in maintenance of the reduced condition of proteins: thioredoxin reductase restores functional (reduced) thioredoxin pool (Beckman and Ames, 1998).

Finally, glutathione (GSH) peroxidase catalyzes hydrogen peroxide reduction to water by the dimerization of glutathione:



Glutathione reductase is a NADPH dependent enzyme restoring monomeric reduced glutathione from the dimeric oxidized form (GSSG).



Non-enzymic antioxidants.

Several molecules were previously reported to scavenge free radicals thus inhibiting their action. They are: ascorbic acid, tocoferols (Vitamin E), flavonoids, carotenoids and ubiquinol (Beckman and Ames, 1998). Availability of these molecules is principally dependent on dietary intake of fruits, vegetables or of appropriate supplements (Beckman and Ames, 1998).

The glutathione system

The most important non-enzymic antioxidant in the organism is glutathione: it is a thiolic tripeptide formed by glutamic acid, cysteine and glycine (Pastore et al., 2003). Synthesis of this tripeptide is independent of mRNA. Glutathione synthesis is, in fact, achieved by the action of two ATP dependent enzymes γ -glutamyl cysteine synthetase (catalyzing the bond between glutamic acid and cysteine) and glutathione synthetase. The reaction catalyzed by the first enzyme is the rate limiting step in the synthesis of this antioxidant factor (Lu, 1999). The following reaction is rapid and occurs immediately after the first, leading to the final formation of reduced glutathione (Majerus et al., 1971). γ -glutamyl cysteine synthetase action is regulated by negative feed-back action of synthesized glutathione; the enzyme is a dimer constituted of a catalytic and a regulatory (modifier) subunit: availability of both these polypeptides can affect glutathione synthesis (Lu, 1999). Glutathione synthetase is not specifically regulated and fails to significantly influence glutathione synthesis rate.

Glutathione is further processed, within the γ -glutamate cycle, by γ -glutamyl transpeptidase leading to the formation of a γ -glutamyl amino acid and to the dipeptide cysteinylglycine (Pastore et al., 2003). γ -glutamyl amino acid is then transformed to 5-oxoprolin and glutamic acid by actions of γ -glutamyl cyclotransferase and oxoprolinase. Glutamic acid is then utilized to synthesize again glutathione (Pastore et al., 2003). Cysteinylglycine is then catabolized by a dipeptidase to provide 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 the synthesis rate of this antioxidant tripeptide (Bannai, 1984).

Glutathione availability.

Glutathione is synthesized in almost all human cells: intracellular concentrations are normally in the range of 0.5-10 mM, while urine and plasma concentrations are extremely lower (50-100 μM). Within the cell, glutathione can be detected in cytoplasm (90%), while the remaining part is imported in mitochondria from cytoplasm (Meredith and Reed, 1982).

Figure 4

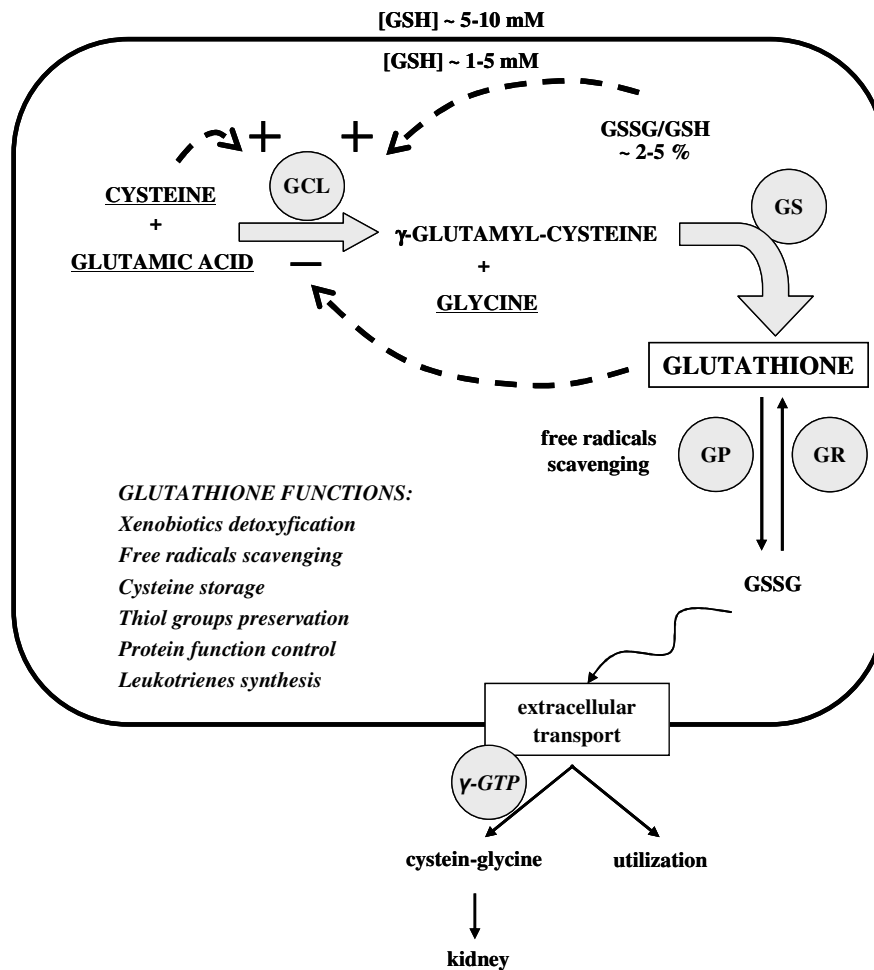


Figure 4 shows pathways of glutathione synthesis and utilization as well as enzymatic regulation.

GCL, glutamate-cysteine ligase; GR, glutathione reductase; GP, glutathione peroxidase; GS, glutathione synthetase, γ -GTP: γ -glutamyl transpeptidase

Biological functions of glutathione.

Antioxidant activity.

The abovementioned antioxidant activity of glutathione is primarily based on the action of glutathione peroxidase that catalyzes hydrogen peroxide conversion to water. This enzyme involves the oxidation of 2 molecules of reduced glutathione to form the glutathione dimer (Pastore et al., 2003). This important action allows the maintenance of protein sulphhydryl groups in a reduced state, thus contributing to preserve protein structure and function. Maintenance of reduced sulphhydryl groups can occur both by enzymic and non enzymic processes (Mannervik et al., 1983). Such thiol-disulphide balance is known to directly affect

enzyme activity and transport efficiency (DeLeve and Kaplowitz, 1991). Glutathione reductase converts oxidized glutathione in two molecules of reduced glutathione utilizing NADPH as cofactor: in this way the pool of available reduced glutathione can be reconstituted.

Effects on protein stability and function.

Glutathione can affect protein function by a process called glutathionylation that leads to the formation of a chemical bond between glutathione and thiolic group of the protein. This process affects protein stability of the cytoskeleton (Cotgreave and Gerdes, 1998) or transactivation capacity of selected transcription factors (Pastore et al., 2003).

Leukotriene synthesis.

Glutathione is required for synthesis of leukotrienes, important factors involved in promotion of inflammatory processes. Glutathione transferase leads to the formation of leucotriene C4: additional modifications mediated by selected peptidases can lead to the formation of leucotriene D₄ and E₄ (Anderson et al., 1982).

Glutathione and health

Aging

Aging is known to be associated to enhanced free radicals production as well as to lowered antioxidant system efficacy (Dogru-Abbasoglu et al., 1997). In aging subjects glutathione availability was shown to significantly decrease and this effect was demonstrated to be independent from gender (Lang et al., 1992). Moreover the decrease in glutathione availability is related to pathologic features that can be ascribed to enhanced free radicals production, as alterations of cognition and of immune system functionality (Weber et al., 1997).

Glutathione involvement in diseases

Previously published works demonstrated that glutathione depletion could interfere with immune system function. In particular, glutathione function is required for activation of lymphocytes (Droge et al., 1986) and NF- κ B can be hypothesized to be the molecular mechanism underlying this process (Staal et al., 1990).

Additionally, different pathologies of the central nervous system, such as Alzheimer and Parkinson's diseases or schizophrenia, are associated to decreased availability of glutathione (Schulz et al., 2000). Human Immunodeficiency Virus infected patients display an average increase of oxidative stress damage coupled with a depletion of glutathione both in plasma

and in red blood cells: this effect was hypothesized to be related to increased sensitivity to infections as well as to the muscle atrophy, which is characteristic of these patients (Staal et al., 1990). In addition glutathione depletion is frequently encountered in pathologies known to be associated to oxidative damage such as liver cyrrhosis (Altomare et al., 1988), chronic obstructive pulmonary disease or acute respiratory distress syndrome (Anderson, 1997) and cardiovascular pathologies (Morrison et al., 1999). This evidence of glutathione depletion in such illnesses demonstrates that glutathione synthesis rate and availability can be considered as a marker of previously occurred oxidative damage.

Glutathione and physical exercise

Physical exercise is related to enhanced fuel utilization through pathways of energy metabolism as well as to increased oxygen flux (100 folds), when compared to resting conditions (Sen et al., 1992). Concomitantly increased mitochondrial activity significantly enhances free radical production (Reid et al., 1992) and paves the way to oxidative stress onset which is strictly related to muscle fatigue and efficiency (Dillard et al., 1978; Reid et al., 1992). Regular exercise was previously demonstrated to enhance total and reduced glutathione availability (Sen and Packer, 2000). Moreover, the ratio between reduced and oxidized glutathione, a reliable marker of glutathione system activation is increased by exercise (Ji, 1995). Physical training, moreover, was shown to induce glutathione incorporation in muscle from blood (Leeuwenburgh and Ji, 1995). In the same work, enhanced *de novo* synthesis and release of glutathione from liver was observed as a consequence of regular physical activity (Leeuwenburgh and Ji, 1995). Activity of enzymes related to glutathione metabolism as glutathione peroxidase and glutathione reductase were shown to be coherently upregulated by moderate exercise (Ortenblad et al., 1997): this shows that glutathione system is activated by muscle contractions. Moreover, long term training was shown to decrease glutathione oxidation rate, when compared to untrained subjects performing a single bout of exercise (Michelet et al., 1995). Otherwise, intermediate intensity of exercise can transiently lower the reduced-to-oxidized glutathione ratio (Sen and Packer, 2000), while short term intensive exercise fails to affect glutathione redox balance (Sen, 1999). Several bouts of exercise, instead, can upregulate oxidized glutathione levels, especially in trained subjects (Sastre et al., 1992). Summation effect of such oxidative damage observed after acute exercise can account for upregulation of glutathione availability and enzymatic efficiency of glutathione system in trained subjects (Kretzschmar et al., 1991). Thus, increased availability of glutathione tripeptide, can be considered, in healthy subjects, as an active response of the organism to a previous release of free radicals.

Stable isotopes for the assessment of fractional synthesis rate of glutathione in human muscles.

Fractional synthesis rate of a small peptide can be assessed in humans by coupling the infusion of stable isotopic amino acid precursors and the analysis (performed by gas chromatography-mass spectrometry) of changes in the enrichment of isotopic product in seriated biological samples after precursor steady state achievement (see Methods section). This approach was, in fact, previously adopted to measure glutathione synthesis in red blood cells (Badaloo et al., 2002; Jahoor et al., 1995; Lyons et al., 2001).

In principle, at least two biological samples are required to assess changes in precursor incorporation after achievement of steady state. Nevertheless, the requirement of multiple sampling is a limitation of the method for application to muscles: repeated muscle biopsies are, in fact, difficult to perform due to ethical reasons, and due to possible local inflammatory processes potentially plaguing reliability of measurements. In this work, to assess muscle glutathione synthesis rate in healthy humans a new validated approach is used. This method involves two parallel infusions of different isotopes of the same amino acid precursor of glutathione: infusions are started with a calculated time shift and are terminated together concomitantly with muscle sampling. Measurement of enrichments of the two different isotopic products, as reflecting different times of precursor infusion and incorporation, allows the determination of glutathione synthesis rate in muscle (see Methods section).

Markers of oxidative stress

Previously published works assessing oxidative stress changes in a living organisms allowed the validation of several biomarkers as useful tools to assess activity of pro-oxidant and anti-oxidant system. In general, the biochemical modifications mediated by free radicals on selected molecules are utilized to quantify oxidative stress induction.

Malondialdehyde

Malondialdehyde is an end-product of lipid peroxidation and their concentration can be assessed by spectrophotometry after reaction with thiobarbituric acids (TBARS). Possible cross reactivity with aldehydes can reduce reliability of this assay (Mayne, 2003).

Specific anti-oxidized low density lipoproteins (LDL) antibodies

Oxidized LDL can induce the production of specific antibodies aimed to limit endothelium permeability for its protection (Roth et al., 2004). Quantification of concentration of such antibodies is considered to be a reliable marker of oxidative stress.

F2-Isoprostanes

Isoprostanes are product of peroxidation of arachidonic acids that are released from cell membranes (Roberts and Morrow, 1997). Increased release of isoprostanes was clearly shown in subject affected by pathologies correlated to oxidative stress (Lawson et al., 1999).

Protein carbonylation

Action of free radicals on proteins is known to induce the modification of selected amino acid (proline, arginine, lysine, and threonine) by stable addition of carbonyl groups (Roth et al., 2004). Protein carbonylation, can lead to altered enzyme structure and activity (Stadtman, 2001). When compared to other oxidative modifications, carbonyls are obtained by relatively complex mechanisms and in contrast to, for example, methionine sulfoxide and cysteine disulfide bond formation, carbonylation is an irreversible oxidative process (Dalle-Donne et al., 2003). In addition, carbonyl modifications on lysine, cysteine, and histidine can be achieved by secondary reactions with carbonyl compounds of carbohydrates (glycoxidation products), lipids, and advanced glycation/lipoxidation end products. (Requena et al., 2003). Increased levels of protein carbonylation were shown in patients affected by neurological diseases such as Alzheimer and Parkinson's diseases, as well as on myopathies as Duchenne muscular dystrophy or amyotrophic lateral sclerosis (Stadtman, 2001). Carbonylation level was, previously demonstrated to be a reliable marker of oxidative stress occurrence (Greilberger et al., 2008).

Oxidative stress and proteolysis

Oxidative stress is known to be involved in the regulation of complex pathways leading to protein and muscle wasting. Oxidative stress can, in fact, induce perturbations of intracellular ionic homeostasis (Kondo et al., 1994), as reactive aldehydes (i.e. 4-hydroxy-2,3-trans-nonenal) can decrease ATPase dependent Ca^{2+} removal from the cell (Siems et al., 2003): thus interactions between oxidative stress and calcium availability changes can effectively trigger calpains action (Primeau et al., 2002). This event can activate other calcium-activated proteases augmenting proteolysis of cytoskeletal proteins and release of myofilaments for degradation by proteasome (Tidball and Spencer, 2002; Goll et al., 2003). Moreover, oxidative stress can induce skeletal muscle atrophy, controlling factors in turn inducing

caspase-3 activity (Primeau et al., 2002). Oxidative stress can directly affect muscle protein degradation at proteasome level (Bettters *et al.*, 2004): it, in fact, has been shown to upregulate the expression of muscle atrophy F-box/atrogin1 and MuRF-1 in myotubes (Li et al., 2003). Increased expression of such E3 ubiquitin ligases in skeletal muscle can enhance proteolysis and muscle atrophy (Bodine et al., 2001a). Furthermore, there is evidence that the aforementioned oxidatively modified proteins by carbonylation, can be selectively degraded by the 20S core proteasome without ubiquitination (Grune et al., 2003; Grune and Davies, 2003). In addition, to avoid accumulation of damaged peptides, carbonylated proteins were shown to be more efficiently scavenged by proteolytic degradation than their nonoxidized counterparts (Dukan et al., 2000; Bota and Davies, 2002; Grune et al., 2003). Moreover, changes in protein carbonylation are significantly associated with disease progression in muscle wasting patients with leukemia (Ahmad *et al.*, 2008), as well as to myopathies as Duchenne muscular dystrophy or amyotrophic lateral sclerosis (Stadtman, 2001). Thus, such evidence directly correlates muscle protein carbonylation with rate of protein degradation and muscle atrophy.

Inactivity and oxidative stress: possible impact on proteolysis.

Low levels of physical activity can promote oxidative stress onset: studies performed in animals showed that regular housing, when compared to constant training, increased lipid peroxidation and ROS release (Laufs et al., 2005). This condition, in parallel with enhanced low grade inflammation (Pradhan et al., 2001; Hansson, 2005; Alberti et al., 2006) can be considered as a risk factor for cardiometabolic diseases such as type 2 diabetes, arteriosclerosis and metabolic syndrome (Hopps et al., 2009; Lastra and Manrique, 2007). It is noteworthy, that inactive lifestyle is known to be *per se* the primary and underlying cause of metabolic syndrome (Zhu et al., 2004).

Nevertheless, evidence suggests that inactivity mediated oxidative stress can promote muscle atrophy, as a typical consequence of immobility. Previously published evidence showed, in an animal model of muscle unloading, that inactivity can increase muscle oxidative stress with concomitantly impaired antioxidant defenses. In particular, experimental unloading increased, in soleus, lipid hydroperoxide levels and oxidation of selected target substrates (Lawler et al., 2003) In addition, in unloaded soleus Cu,Zn-superoxide dismutase increased while, catalase and glutathione peroxidase, significantly decreased, together with non-enzymatic antioxidant capacity (Kondo et al., 1994; Lawler et al., 2003; Kondo et al., 1992). Moreover, muscle unloading was shown to induce a decrease in antioxidant heat shock proteins as well as in glutathione peroxidase activity (Lawler et al., 2006). Otherwise, other published data showed

that muscle unloading can upregulate heme-oxygenase response in virtue of a previously occurred oxidative damage (Hunter et al., 2001). An important work investigating, by microarray approach, alterations in gene expression of unloaded muscle demonstrated that factors promoting oxidative stress as well as ubiquitination and protein degradation are significantly upregulated by immobility (Stevenson et al., 2003). As reviewed by Scott K. Powers (Powers et al., 2005; Powers et al., 2007), oxidative stress can effectively induce muscle atrophy activating specific intracellular pathways triggering upregulation of specific proteases and of apoptosis processes. Muscle inactivity is, in fact, associated with oxidative stress mediated calcium deregulation and calpain activation (Kourie, 1998), factors known to induce muscle wasting. Interestingly, lowered oxidative stress induction in unloaded muscles, achieved by supplementation of antioxidant vitamin E, can reduce protein wasting and muscle atrophy (Appell *et al.*, 1997). Muscle wasting is an important clinical feature of several chronic diseases and oxidative stress has been suggested as pathogenetic factor in such process (Moyle and Reid, 2007). Unfortunately, knowledge of direct links between oxidative stress and muscle inactivity is currently incomplete, but it seems plausible that inactivity can trigger oxidative stress (e.g. protein carbonylation) in muscle by interaction of at least five different oxidant production pathways (Kondo et al., 1993): 1) generation of ROS by the xanthine oxidase pathway (Whidden et al., 2009); 2) production of NO via increased NOS activity (Kondo et al., 1993); 3) formation of ROS by increased cellular levels of reactive iron (Kondo et al., 1992) ; 4) potential activation of NADPH oxidase by increased availability of calcium through protein kinase C-ERK1/2 pathway (Javesghani et al., 2002) and 5) contribution of mitochondrial production of superoxide radicals (Muller et al., 2007).

Figure 5

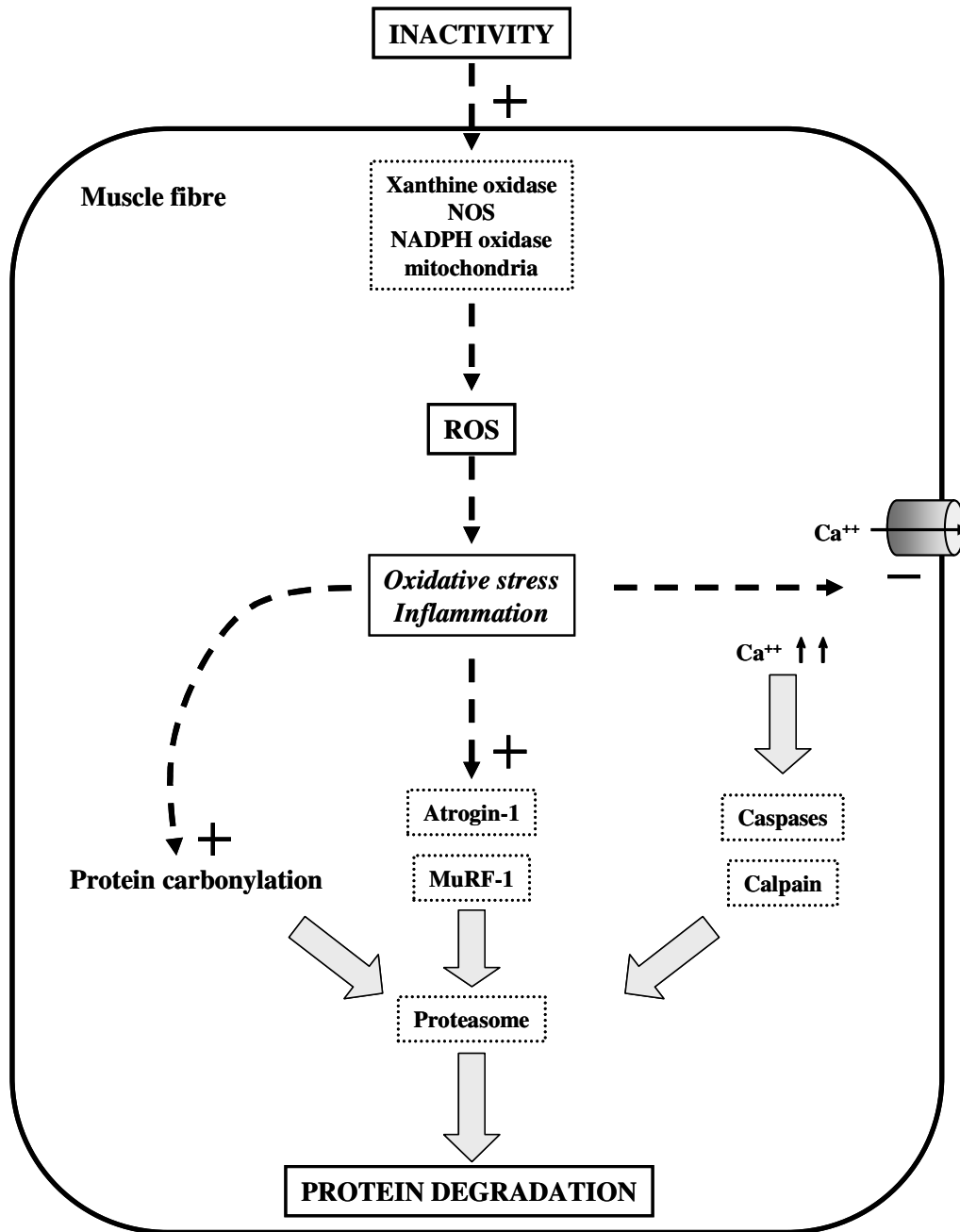


Figure 5 summarizes experimental evidences obtained in animal models suggesting relationships between inactivity mediated oxidative stress and regulation of protein degradation.

Homocysteine

Synthesis and metabolism

Homocysteine is a non proteinogenic sulfur amino acid, intermediary in the metabolism of cysteine and methionine. Methionine transmethylation is the reaction leading to homocysteine synthesis. This reaction is divided into different steps: methionine is transformed in S-adenosylmethionine by methionine adenosyl transferase. S-adenosylmethionine is in turn converted to S-adenosylhomocysteine by methyltransferase or by glycine N-methyltransferase (coupled to conversion of glycine to sarcosine). S-adenosylhomocysteine is then hydrolyzed to homocysteine with the release of homocysteine (Loenen, 2006). Specific mechanisms transport homocysteine from the intracellular pool to plasma, where concentration reach the average level of $10 \mu\text{mol} \times \text{L}^{-1}$. Homocysteine degradation occurs by two catabolic pathways, transsulfuration and remethylation, leading respectively to cysteine synthesis and to methionine recycling. Transsulfuration occurs by the action of cystathionine β -synthetase and by the following action of α -cystathioninase, converting cystathionine to cysteine. Remethylation occur by two parallel pathways. The first and more frequent, occurs by methionine synthase involving vitamin B₁₂ as cofactor and converting N⁵-methyltetrahydrofolate to tetrahydrofolate: this is transformed to N⁵-N¹⁰ methylenetetrahydrofolate and then recycled to N⁵-methyltetrahydrofolate by methylenetetrahydrofolate reductase (Lee and Frenkel, 2003). The second reaction leading to methionine from homocysteine is catalyzed by betaine-homocysteine methyltransferase, requiring betaine conversion to dimethylglycine. This minor pathway is required to preserve methionine concentrations in low folate conditions (Durand et al., 2001).

Regulation of homocysteine metabolism:

S-adenosylmethionine is a key regulator of homocysteine metabolism: it regulates transmethylation reaction and when it is sufficiently available it can reduce remethylation rate inhibiting methylenetetrahydrofolate reductase (Finkelstein, 2000). Moreover, to limit the cytotoxic effects of homocysteine, S-adenosylmethionine promotes transsulfuration by stimulation of related enzymes (Finkelstein, 2000). For such reasons high protein and amino acid diet, enhancing availability of S-adenosylmethionine can promote homocysteine disposal by transsulfuration route: otherwise, remethylation is favoured in conditions of normal or low protein dietary intake (Ueland and Refsum, 1989).

Dietary intake of folates is also a crucial regulator of homocysteine metabolism, by provision of N⁵-methyltetrahydrofolate: lowered folates availability reduces remethylation and

accelerate transmethylation rate by influencing S-adenosylmethione concentrations. Low levels of S-adenosylmethione, in turn, can inhibit transsulfuration pathway. Thus low folate intake can cause blood accumulation of homocysteine enhancing its cytotoxic effect (Lee and Frenkel, 2003).

Physiologic factors determining homocysteine availability.

Gender

Previously published studies assessed that gender can be considered as a crucial factor determining homocysteine availability. In particular, efficient remethylation pathway accounts for a 21% lower homocysteine concentration in blood of female subjects when compared to males (Fukagawa et al., 2000). Moreover, homocysteine concentration seem to be related to age and race (Nygard et al., 1995)

Genetic factors

Specific genetic factors can alterate homocysteine availability in blood. Mutations of cystationine β -synthetase can, expecially in homozygosity condition, induce severe hyperhomocysteinemia through inhibition of transsulfuration pathway (Crowther and Kelton, 2003). Additionally, another genetic mutation, affecting remethylation pathway, was found in position 677 of methylentetrahydrofolatereductase (MTHFR) gene: this leads to a unstable form of protein associated to lowered remethylation efficiency (Deloughery et al., 1996).

Physical activity

A relationship between physical exercise and homocysteine availability in blood was recently demonstrated. Acute physical exercise was shown to induce an immediate increase in plasma homocysteine concentration, while regular and moderate training lowered resting plasma concentrations of homocysteine when compared to untrained subjects (Duncan et al., 2004). Moreover, particularly fatiguing endurance exercises can upregulate homocysteine concentrations and related cardiovascular risk (Herrmann et al., 2003). Reasons explaining such observed effect are presently unknown. Additional studies showed no association between physical activity level and homocysteine concentrations in moderately training young humans (Di Santolo et al., 2009). Other reported evidences showed increased homocysteinemia following submaximal acute aerobic exercise but not after submaximal aerobic training, independently from duration or intensity (Gelecek et al., 2007).

A previously published epidemiological study showed that sedentary lifestyle was associated with increased concentrations of plasma homocysteine (Metsios et al., 2009), but mechanistic

explanations for this effect were not provided. To fully address the humans kinetic reasons underlying this effect, metabolic investigation by isotopic tracers must be applied to a model of physical inactivity.

Homocysteine forms

Homocysteine is found in human blood in two forms: the reduced one (homocysteine) and the oxidized and dimerized one called homocystine. Homocystine represents almost the 90% of total homocysteine in plasma (Jacobsen, 1998).

Mechanisms of cytotoxic effects of blood homocysteine.

Several studies and metaanalyses associated hyperhomocysteinemia to coronary diseases (Clarke et al., 1991; Graham et al., 1997; Boushey et al., 1995) and to “unhealthy lifestyle” (Cleophas et al., 2000). Moreover, homocysteine level was demonstrated to be a predictive value of mortality in patient’s with established coronary artery disease (Nygard *et al.*, 1997).

Hyperhomocysteinemia is, in fact, known to be associated to atherogenic and thrombotic effects: multifactorial causes can lead to this effect as a rise in procoagulant and proinflammatory substances in blood. Mild hyperhomocysteinemia in young patients, in fact, was associated to endothelial dysfunction and to increased levels of von Willebrand factor, of thrombomodulin as well as to impaired endothelial dependent vasodilation (van den et al., 1995). Oxidative stress is considered to be the principal effector of homocysteine mediated endothelial damage. Plasma homocysteine, in fact, rapidly auto-oxidize to homocystine, in turn leading to ROS production. Previous evidences (Harker et al., 1974) showed that homocysteine can induce endothelial damage exposing vessel matrix and smooth muscle cells to an increased concentration of hydrogen peroxide that promotes cell proliferation, platelet activation and lipidic peroxidation. Additionally, homocysteine was shown to promote oxidative damage inhibiting function of antioxidant systems as glutathione peroxidase (Upchurch, Jr. *et al.*, 1997). Despite this direct pro-oxidant effect, inhibition of glutathione peroxidase by hyperhomocysteinemia can lower the capacity of endothelial cells to synthesize the S-nitrous-homocysteine: as S-nitrous-homocysteine reduces homocysteine cytotoxicity, this is another way by which hyperhomocysteinemia can enhance oxidative tissue damage (Mezzano et al., 1999).

Hyperhomocysteinemia was, moreover, shown to enhance the pro-inflammatory condition by promoting arachidonic acid metabolism that, in turn, was shown to increase proinflammatory and procoagulant thromboxane A₂ release (Di Minno et al., 1993).

Figure 6

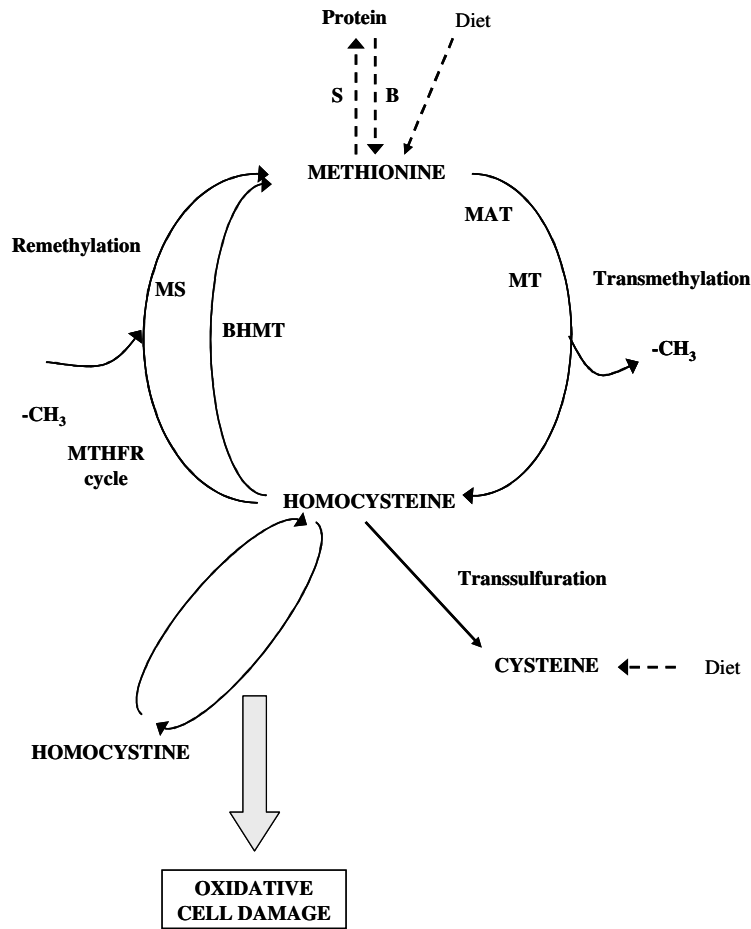


Figure 6 displays regulation of homocysteine metabolism and the related biological role.

S, synthesis; B, breakdown; MAT, methionine adenosil transferase; MT, methyltransferase; CBS, cystationine β -synthetase; MS, methionine synthase; BHMT, betaine-homocysteine methyltransferase; MTHFR, methylentetrahydrofolatereductase.

Glutamine

Glutamine turnover and regulation

Glutamine is an highly abundant amino acid, largely synthesized by skeletal muscle and then released in plasma. Liver, adipose tissue and lung also contribute to glutamine production. In human healthy subjects, glutamine concentration in muscle is about 20 mM, being 200-500 higher than all other essential amino acids (Biolo et al., 1995a). On the contrary, in plasma, glutamine is normally around 0.6 mM. Tracer kinetic studies in healthy subject estimated that

approximately 80 g of glutamine appear every day in the bloodstream from body tissues, while 5-10 g are derived from nutrient intake (Biolo et al., 1995a). Glutamine is endogenously synthesized from glutamate and ammonia by glutamine synthase (Krebs, 1935). Activity of such enzyme is affected by several hormones. Glucocorticoids (Max et al., 1988; Darmaun et al., 1988) promote glutamine synthesis while growth hormone down-regulates this process (Biolo et al., 2000). Moreover, glutamate, as a precursor of glutamine, is synthesized by transamination of the branched chain amino acids leucine, isoleucine and valine. So, adequate activation of pyruvate dehydrogenase and of the tricarboxylic acid (TCA) cycle is required to provide anaplerotic precursors for amination of alpha-ketoglutarate leading to glutamate synthesis (Gibala et al., 1998). This suggests glutamine synthesis is linked to substrate utilization for energy production. Additionally, glutamine availability can be affected by nutrients (Darmaun et al., 1994; Biolo et al., 2006). On the contrary, glutaminase is the key enzyme for glutamine utilization which leads to glutamate, aspartate, alanine, and lactate production (Curthoys and Watford, 1995).

Glutamine as immunomodulator

Glutamine involvement in cell function and regulation was firstly stated by Krebs (Krebs, 1935). In particular, glutamine is utilized by rapidly dividing cells as enterocytes (McCauley et al., 1998) and immune cells, especially lymphocytes or monocytes (Ardawi and Newsholme, 1982). For such reasons, glutamine was considered as conditionally essential and named as “fuel of immune system” (Ardawi and Newsholme, 1982). The importance of its role in activation of the immune system was investigated in different models. Glutaminase, a key enzyme for glutamine utilization, is expressed on the cell membrane of human neutrophils (Castell et al., 2004) and it is specifically activated in immune cells to provide useful substrates for proliferation (Newsholme and Calder, 1997). The conversion of glutamine to glutamate, aspartate, alanine and lactate represents the 85 % of the total amino acid utilized by cultured neutrophils (Curi et al., 1997). In this way glutamine metabolism provides precursors and intermediates needed for immune cell proliferation as well as protein synthesis (Newsholme et al., 1985). In vitro studies, underlined the regulative role of glutamine availability on immune cells. Evidence showed that cultured immune cells mostly utilizes glutamine among other amino acids culture medium (Sakagami et al., 2009). Artificial reduction of glutamine concentrations in culture medium below physiological levels decreased cell proliferation rate (Parry-Billings et al., 1990).

Glutamine and physical activity

Physical exercise leads to changes in energetic substrate utilization (Willis and Jackman, 1994), insulin sensitivity (Colberg and Grieco, 2009) and protein turnover (Biolo et al., 1995b). Changed plasma amino acid pattern can be evidenced after exercise. Interestingly, concentrations of two amino acids related to glutamine metabolism are known to be affected by exercise: alanine was demonstrated to be enhanced while glutamate was reduced (Blomstrand and Essen-Gustavsson, 2008). In particular, exercise can differentially affect glutamine availability in plasma and in muscles. Previously published studies assessed the impact of physical activity on glutamine in untrained healthy volunteers. Plasma concentrations measured after an acute session of eccentric resistance exercise (Miles et al., 1999) as well as after intermittent high intensity bouts (Walsh et al., 1998a) were decreased when compared to baseline values. Moreover, exhausting bouts of aerobic exercise induced, in male volunteers, a significant decrease in plasma glutamine (Brodan et al., 1976) and, interestingly, prolonged exhausting exercise sessions were displayed to induce a major negative effect on glutamine concentrations when compared to short exercise sessions (Robson et al., 1999). Such decreased glutamine availability following exhaustive exercise can be hypothesized to be due to enhanced glutamine uptake for anaplerotic processes and gluconeogenesis (Henriksson, 1991) as well as to reduced synthesis, as evidenced by lowered synthetase activity (Dos Santos et al., 2009). In contrast, three hours of moderate aerobic exercise were shown not to acutely affect glutamine rate of appearance into blood but to significantly increase alanine, as determined by a tracer kinetic study performed in fasting state (Williams et al., 1998). Indeed, selected kind of sports differently affected glutamine concentrations (Hiscock and Mackinnon, 1998): endurance and resistance sports characterized by prolonged and intense energy expenditure were associated to the worst decreases in glutamine availability. Sessions of moderate exercise were not usually associated with significant changes in circulating glutamine.

Glutamine uptake from activated immune system cells can contribute to glutamine depletion following intense exercise. Physical exercise was, in fact, previously suggested to increase the uptake of glutamine from plasma for utilization in immune system (Newsholme and Calder, 1997). Studies regarding glutamine depletion in blood and after heavy physical training periods showed reduced glutamine concentrations for one or two weeks after the last exercise session (Keast et al., 1995). For such reasons a decrease in plasma glutamine concentrations, below 500 microM as arbitrary threshold (Walsh et al., 1998b), can be considered as marker of the so called “overtraining syndrome” (Rowbottom et al., 1996). In contrast to exhaustive

exercise, evidence strongly suggests that optimal, moderate training can increase glutamine availability. Volunteers trained at low intensity showed lower plasma glutamine decreases after a physical exercise session, when matched to untrained control group or to exhaustively trained group (Kargotich et al., 2005; Santos et al., 2007). Strikingly, in moderately trained animals, short-term or endurance exercise could even enhance resting plasma glutamine concentrations (Hood and Terjung, 1994). Finally, in humans athletes, progressive increase of training intensity over a medium term period (6 weeks) significantly ameliorated resting plasma glutamine concentrations (Kargotich et al., 2007). This suggest that physiological adaptation, which is likely to occur during moderate training, is needed to protect the organism from glutamine depletion. So, summation of transiently enhanced glutamine concentrations following repeated and non-fatiguing exercise sessions (moderate training) could play a protective role on glutamine concentrations maintenance (Dos Santos Cunha et al., 2004). Repeated muscle contractions were, in fact, demonstrated to be linked to enhanced intermediates flux in TCA cycle (Gibala et al., 1998) thus potentially leading to higher glutamine synthesis and release (van Hall et al., 1999). Training levels thresholds or specific programs aimed to maximally improve glutamine turnover in blood were still not defined.

Figure 7

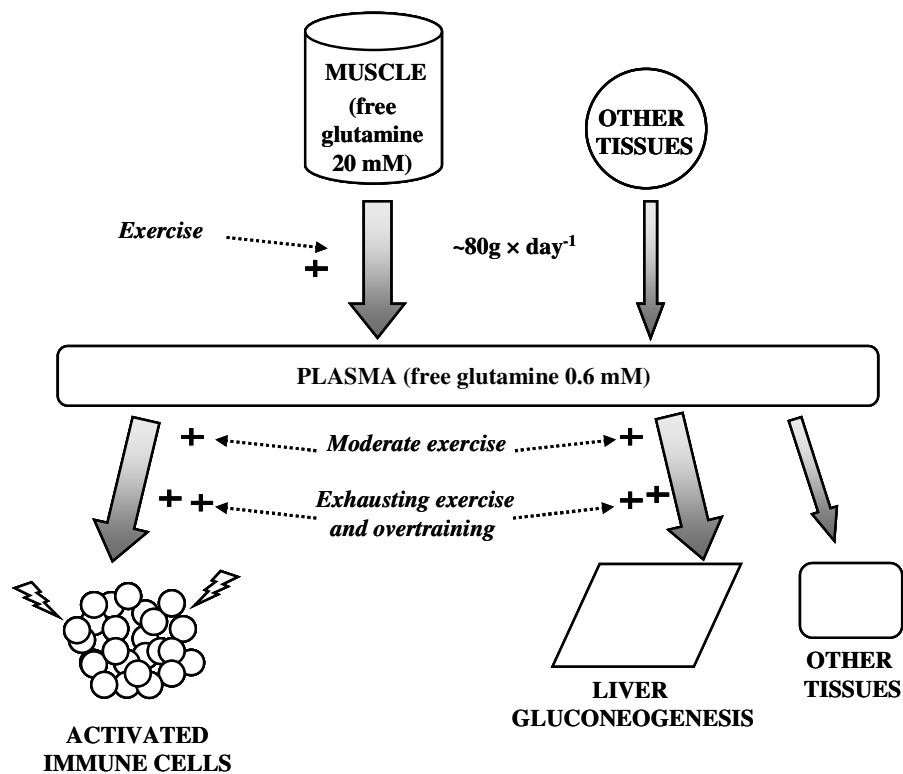


Figure 7 shows mechanisms underlying changes in glutamine availability during physical exercise.

Glutamine and antioxidant systems.

Expression profile analysis by microarray demonstrated that glutamine dietary supplementation can upregulate synthesis of genes involved in antioxidant response in the animal intestine (Wang et al., 2008). Moreover, in the same work, glutamine administration was shown to increase by almost 30% glutathione synthesis, thus demonstrating the actual impact of this nutritional treatment on intestinal antioxidant potential. Studies testing the efficacy of glutamine supplementation in patients undergoing surgery demonstrated a significant correlation between glutamine delivery and glutathione levels in blood (Engel et al., 2009). Other works performed in a mouse model of colitis demonstrated that enhanced glutamine availability was able to lower expression of genes involved in oxidative stress (NF- κ B) and inflammation (tumor necrosis factor) induction, thus ameliorating intestinal tissue damage (Kretzmann et al., 2008). These and other published works strongly suggest that glutamine availability is important to determine antioxidant response potential in tissues. The stimulatory effect of glutamine on antioxidant systems as well as on cell proliferation of intestine and immune system provide indications for glutamine supplementation in clinical practice with critically ill patients (Wernerman, 2008).

Nutrition, inactivity and muscle atrophy

Energy balance

Dietary energy balance can be defined as the difference between energy intake and expenditure. Energy expenditure for physical activity, beside resting energy expenditure, strongly affects total body energy requirements (Muller et al., 2004). For this reason, when physical activity is reduced, energy intake should be modified in order to become adequate to new requirements. Under ambulatory conditions, energy balance is universally accepted to be a determining factor for body weight and composition, i.e. the relative content of fat and lean mass of the body. Under strict physical inactivity a different situation may be encountered.

Physical inactivity and energy metabolism

In a previously published work (Krebs et al., 1990a) the effect of physical inactivity and diet on body weight and composition was analysed. Volunteers were fed with conventional foods on average daily caloric content of 2,600 kcal. Comparing 5 weeks of continuous bed rest with 6 weeks of ambulation no change in energy absorption or total body weight during bed rest were observed, but a significant decrease in LBM and a significant increase in total body fat was demonstrated. Other published evidences (Gretebeck et al., 1995) used doubly labeled

water to measure total energy expenditure in nine healthy adult men before and during a bed rest period of 10 days. Authors showed that mean energy expenditure was 21% lessened by bed rest and displayed that resting energy expenditures failed to change. This implies that the reduced energy expenditure is linked to lowered physical activity. During the bed rest period, energy intake was higher than energy expenditure but body weight did not change. This suggests that body weight alone is not a reliable marker of energy balance during bed rest as a mass compensation occurs when the reduction in LBM is replenished by fat mass. Other authors (Stein and Leskiw, 2000), in fact, observed after 17 days of bed rest only a slight reduction of food intake ($250 \text{ kcal} \times \text{day}^{-1}$) and confirmed that LBM was reduced (-1.4 kg) while fat mass was increased (+1.4 kg) determining a stable maintenance of body mass.

In a previously published work (Murgatroyd et al., 1999) the relationship between inactivity, diet composition and energy balance was investigated. Authors analyzed two groups of subjects fed with ad libitum diets characterized by respectively 35% and 60% energy as fat. Measurements of energy expenditure and substrate oxidation were performed: inactivity was shown to induce a more strongly positive energy balance in subjects fed by 60% fat diet versus 35% fat. Thus, in this work lack of adaptation to energy requirements during bed rest was further demonstrated. Nevertheless, in a previously published study (Blanc et al., 1998) a 12% reduction in energy intake after 42 days of simulated weightlessness was observed. Intakes of proteins, lipids, and carbohydrates decreased coherently with energy intake: this suggests that such changes could raise from the metabolic effects of bed rest and not from a voluntary change in subjects eating behaviour. As the reduction in energy intake occurred in parallel with a decrease of energy expenditure, subjects failed to display any change in fat mass. Nevertheless a loss of body weight was observed as the result of LBM reduction without significant changes in fat mass.

These evidence demonstrate that loss of body weight alone does not always accurately reflect the subject's energy state, and that spontaneous adaptation to energy requirements is a rare condition during experimental inactivity. So, during inactivity, energy intake should be artificially adapted to new metabolic requirements: fat mass gain is the most evident result of positive energy balance during inactivity. Muscle mass wasting due to inactivity could compensate fat mass changes leaving unaffected total body mass.

Overfeeding and related consequences

Results derived from bed rest campaigns are in perfect accordance with epidemiologic studies showing sedentary lifestyle is strictly associated to obesity (Chaput and Tremblay, 2009).

Lowered energy expenditure characterizing inactive lifestyle, especially when combined with overfeeding, in fact, can rapidly induce a significant fat gain. Previous publications showed, in fact, that reduced energy expenditure for daily activities can cause a significant increase in abdominal fat mass, despite controlled energy intake (Olsen et al., 2008). Overfeeding and fat accumulation leads to the condition of overweight (body mass index $> 25 \text{ kg} \times \text{m}^{-2}$) or of obesity (body mass index $> 30 \text{ kg} \times \text{m}^{-2}$). Physical activity, enhancing metabolic processes of energy substrate consumption is considered, in parallel with adjusted dietary energy intake, as an optimal choice to manage obesity and overweight states (Veiga et al., 2009). Obesity is strictly related to cardiovascular risk (Wei et al., 1999), insulin resistance (Karakelides et al., 2010) and type 2 diabetes (Venables and Jeukendrup, 2009). In obese subjects affected by type 2 diabetes, exercise significantly ameliorates glucose disposal by insulin action (O'Gorman et al., 2006; Krook et al., 2003). Moreover, enhanced lipid oxidation secondary to low intensity exercise training can decrease triglycerides and low density lipoproteins in plasma of obese subjects, thus reducing coronary disease risk and body mass (Despres and Lamarche, 1994).

Obesity and inflammation: influences on sarcopenia

Obesity was shown, both in animals and humans, to be associated to an increased level of inflammation (Elmarakby and Imig, 2009) and such effect was shown to be associated with increased cardiovascular risk (Police et al., 2009). Obesity can, in fact, be associated with upregulated inflammation as fat mass accumulation enhances synthesis of selected cytokines from adipose tissue. As released cytokines are directly synthesized by fat mass, these factors are called “adipokines” and for the same reasons, fat mass can be considered as an “endocrine organ”.

Fat mass accumulation characterizing severely obese subjects can be coupled to muscle mass reduction, leading to the so called “sarcopenic obesity”, a metabolic status linked to increased mortality in chronic diseases (Honda et al., 2007). Increased inflammation characterizing obese subjects can be associated to observed sarcopenia, due to the potential of several pro-inflammatory factors to induce muscle wasting (Acharyya et al., 2007; Civatte et al., 2005; Tracey and Cerami, 1994).

Proinflammatory adipokines

Leptin

Leptin is a protein encoded by the Obese (Ob) gene, synthesized by adipose tissue and aimed to control food intake and energy expenditure (Yadav et al., 2009). Plasma leptin

concentrations are positively correlated to adipose tissue accumulation (Baratta, 2002) and in addition, leptin was demonstrated to trigger pro-inflammatory factors as tumor necrosis factor alpha (Zhao et al., 2005), IL-6 (Fenton et al., 2006) and to activate the release of ROS (Yamagishi et al., 2001). Thus, leptin can be considered not only as marker of adiposity but also as index of inflammation (Cai et al., 2009). Leptin resistance is considered to be a possible link between chronic inflammation and cardiovascular risk or muscle tissue damage in obese subjects (Martin et al., 2008). Morphology of skeletal muscle of Ob knock-out mice resembles muscular architecture of wild type aged animals (Warmington et al., 2000). Leptin is considered to be the antagonist hormone of ghrelin, an hunger stimulating hormone secreted mainly by the specific cells of stomach: it revealed anti-cachectic properties, through regulation of energy intake and expenditure, adiposity and growth hormone secretion (Balasubramaniam et al., 2006). After a meal consumption ghrelin levels are known to decrease, but in obese subjects, ghrelin availability is not lowered in postprandial state, suggesting obesity can be linked to ghrelin resistance (Mittelman et al., 2010).

Tumor necrosis factor alpha

Another proinflammatory cytokine released by fat mass is tumor necrosis factor alpha. Upregulation of its expression was shown to be associated to obesity both in humans and in animal models (Hotamisligil et al., 1993). Tumor necrosis factor alpha, even though released in plasma to exert its biological role at whole body, can play a localized action in fat mass. Mice characterized by lowered affinity for tumor necrosis factor alpha in fat mass receptors are less susceptible to the development of experimental diabetes (Uysal et al., 1997). Tumor necrosis factor upregulation was shown to be crucial for the increase of proinflammatory leptin characterizing sarcopenic obese subjects (Kirchgesner et al., 1997).

Interleukin-6

Interleukin-6 is synthesized in fat mass by the stimulatory effect of tumor necrosis factor alpha (Rotter et al., 2003) and plays both pro- and anti-inflammatory effects at whole body. Exogenous interleukin-6 injection in obese rodents was associated to a decrease in fat mass, suggesting the beneficial lipolytic effect of this molecule to contrast obesity (Wallenius et al., 2002). Emerging evidences showed that interleukin-6 can be released in muscle: for such reasons this molecule can be defined as “miokine”. In particular, contracting muscles, in opposition to resting miofibres, were shown to release interleukin-6: this effect was related to

increased lytic activity on muscle glycogen stores (Keller et al., 2001). Thus, fat and muscle mass can be hypothesized to actively cooperate in regulation of metabolic processes.

Anti-inflammatory adipokines

Adiponectin

Adiponectin is secreted by fat tissue and is characterized by a collagenous domain, and by a globular trimerization domain. Adiponectin plays a crucial role in glucose and lipids metabolism.

Adiponectin is secreted by fat tissue cell types and exists as a trimer of the full-length protein or as a proteolytic cleavage fragment (globular). The trimer can further dimerize leading to an hexamer which can in turn create a polymer with different biological activities (Scherer et al., 1995). Adiponectin is recognized by at least two cell membrane receptors (ADIPOR1 and ADIPOR2) in this way regulating different pro- and anti-inflammatory cytokines. The principal anti-inflammatory function is exerted by reduction of tumour-necrosis factor synthesis (Maeda et al., 2005) and of interferon- γ (Yokota et al., 2000). Additionally, adiponectin can induce, in human leukocytes, the expression of anti-inflammatory factors as interleukin-10 and interleukin-1 receptor antagonist (Wolf et al., 2004). Interestingly, reduced adiponectin levels characterize subjects with diagnosed type 2 diabetes and are considered as maker of predisposition to insulin resistance (Spranger et al., 2003). Exogenous injections of recombinant adiponectin in diabetic mice reduces glucose production from liver, thus ameliorating hyperglycemia without rising plasma insulin concentrations (Berg et al., 2001). Physical exercise was shown to upregulate plasma adiponectin availability in female adolescents (Ischander et al., 2007). A significant decreased level of plasma adiponectin was reported in bedridden female patients, when compared to age matched healthy controls in ambulatory conditions (Kanda et al., 2005).

Figure 8

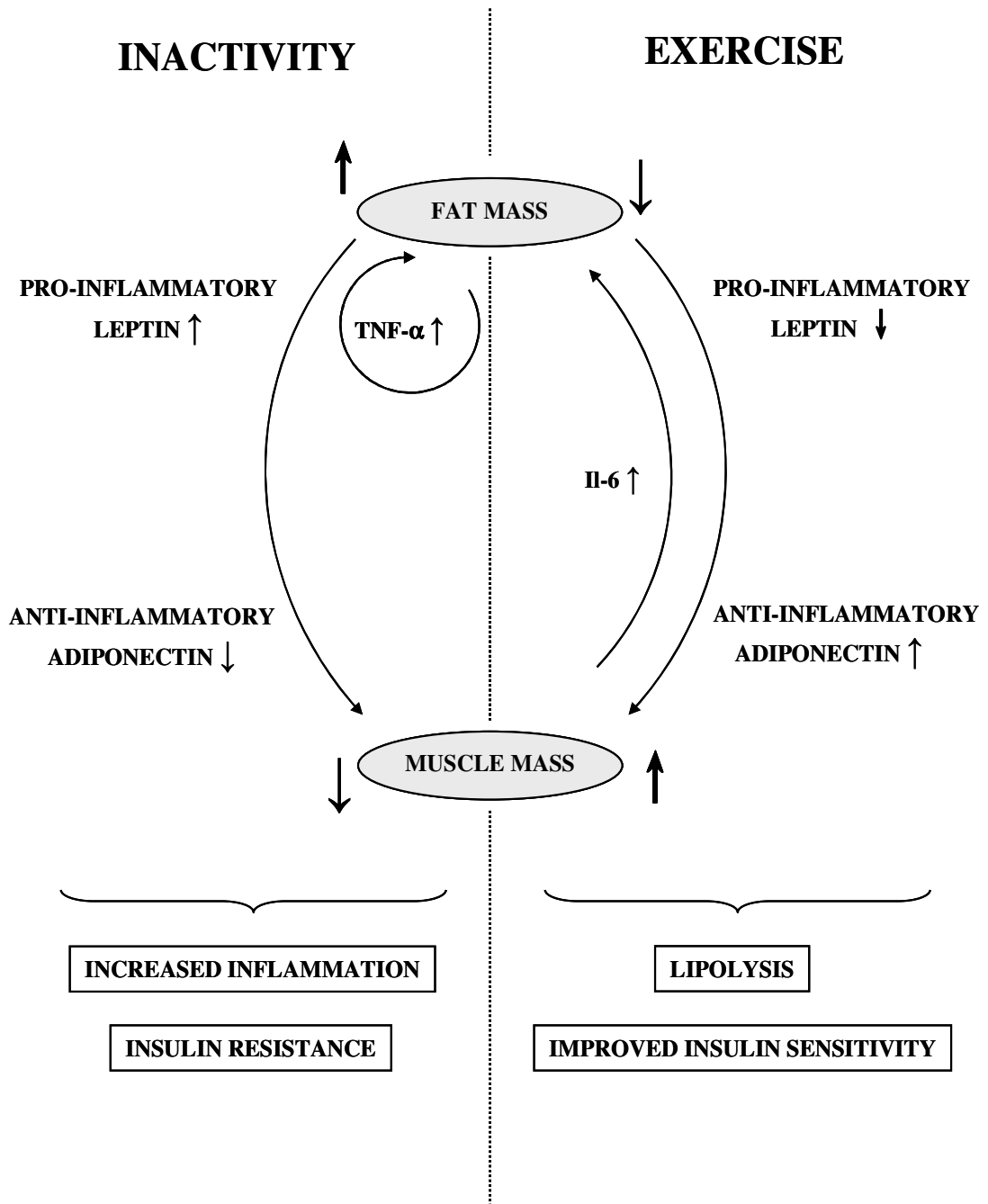


Figure 8 displays the endocrine cross-talk between muscle and fat mass potentially occurring in conditions of physical inactivity or exercise.

Macrophage infiltration in fat mass.

Inflammatory condition linked to obesity might derive from adipokines mediated infiltration of macrophages in increasing fat mass (Xu et al., 2003). Previous publications in animal models, in fact, suggested that in obese subjects, both cell number and size of macrophages in fat mass are significantly higher when compared to lean subjects (Weisberg et al., 2003). Tumor necrosis factor alpha was shown to play crucial role in macrophage recruitment into adipose tissue by stimulation of monocyte chemoattractant protein-1, as factor promoting migration of macrophage precursor cell into fat mass (Wellen and Hotamisligil, 2003).

Calorie restriction

Dietary calorie restriction is a frequent nutritional approach in hospitalized patients due to illness conditions or to surgery interventions. In addition, dietary energy intake reduction, normally associated with physical exercise programs, is a well established therapy for weight loss in obese subjects (Larson-Meyer et al., 2010). Moreover, in the same work, combined energy restriction and physical exercise were demonstrated to ameliorate insulin sensitivity and to reduce LDL cholesterol (Larson-Meyer et al., 2010). Long term calorie restriction *per se* was shown to ameliorate insulin sensitivity indexes measured in fasting human subjects (Fontana et al., 2009). Interestingly, calorie restriction was associated to improved life expectancy in virtue of reduced incidence of cardiovascular diseases (Fontana, 2009b). In animals, alternate-day fasting was shown to protect from left ventricle fibrosis mediated by oxidative stress, as typical feature of aging, and to reduce activation of pro-inflammatory NF- κ B factor (Castello et al., 2009).

Additionally, lowered cancer incidence was also associated to anti-inflammatory action and to lowered release of growth factors mediated by long term calorie restriction (Hursting et al., 2010).

Inactivity and calorie restriction

The potential of calorie restriction to ameliorate metabolic disorders related to physical inactivity was previously investigated in a previously published work (Bosutti et al., 2008). As above mentioned, authors described that experimental inactivity in eucaloric conditions upregulated plasma CRP and the ratio between plasma pro- and anti-inflammatory cytokines in plasma and white blood cells. Interestingly in the same work, a mild calorie restriction was shown to blunt bed rest mediated changes in CRP and pro- or anti-inflammatory cytokines:

this suggests that calorie restriction could significantly ameliorate metabolic changes mediated by physical inactivity. Such results were obtained in subjects involved within the STBR-IP study. Additional published results (Biolo et al., 2007b) obtained within the same experimental frame showed that calorie restriction significantly affected muscle mass metabolism. In particular, low energy intake significantly interacted with inactivity to increase leucine oxidation, as marker of protein wasting. Moreover, the catabolism of lean mass, known to normally occur during physical immobility, was shown to be particularly upregulated when low energy diet was associated to physical inactivity. This effect seems not to involve inflammation as energy restriction was shown to prevent inactivity-induced inflammation. Nevertheless, these data suggest that calorie restriction could exacerbate detrimental effects of physical inactivity on muscle mass and metabolism: this is a common condition in immobilized patients characterized by underfeeding and malnutrition.

Amino acid intake and protein synthesis: exercise and inactivity.

Protein intake and amino acid availability are the key regulators of muscle protein synthesis. Amino acid administration acutely upregulates muscle protein synthesis and such effect is greatly enhanced when resistance or aerobic exercise is performed (Biolo et al., 1997). Amino acid administration displays a major anabolic effect than insulin and glucose administration both in the resting state and after exercise (Biolo et al., 1999). While endurance exercise increases the oxidation of essential amino acids, thus raising the requirement for dietary protein (Tarnopolsky, 2004), resistance exercise results in a decrease in nitrogen excretion, lowering dietary protein needs (Phillips, 2004). 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, in fact, shown to be reduced (-20%) when compared to normal physical activity (Biolo et al., 2004). In addition, the rate of protein turnover in the fasting state was decreased both at muscle and whole body levels during bed rest (Biolo et al., 2004). Other results indicated that dietary protein restriction (i.e., $0.6 \text{ g protein} \times \text{kg}^{-1} \times \text{d}^{-1}$) led to 23% suppression of whole body protein turnover in the fasting state when compared to adequate (i.e., $1.0 \text{ g protein} \times \text{kg}^{-1} \times \text{d}^{-1}$) protein intake levels (Stuart et al., 1990). Further increases in dietary protein content ($1.5 \text{ g protein} \times \text{kg}^{-1} \times \text{d}^{-1}$) during bed rest periods were shown to better maintain myocardial mass (Dorfman et al., 2007). In an additional study, supplementation with about 50 g of essential amino acids during 4 weeks bed rest maintained leg mass and ameliorated the muscle strength decrease (Paddon-Jones et al., 2004).

Amino acid supplementation: cardiovascular risk, inflammation and oxidative stress.

Amino acid supplementation is known to influence cardiovascular risk affecting an important metabolic parameter as insulin resistance. Interestingly oral administration of L-arginine to cardiopathic patients was shown to decrease endothelial dysfunction, as assessed by changes of nitric oxide availability, and insulin sensitivity as evidenced by glucose tolerance tests (Lucotti et al., 2009). Nevertheless, published metanalysis taking into account trials testing arginine efficacy cardiovascular diseases, showed that supplementation displayed only a tendency to reduce of 7% mortality of treated patients (Sun et al., 2009).

Interestingly, a high dietary content of casein was previously shown in an animal model to increase homocysteine disposal via transsulfuration pathway, thus potentially reducing cardiovascular risk condition (Ohuchi et al., 2009). Interestingly, protein supplementation was shown to reduce the production of reactive oxygen species and apoptosis in mice embryos (Esfandiari et al., 2005). In parallel, glutamine supplementation was shown to ameliorate inflammatory markers and to ameliorate the expression of the antioxidant glutathione in postoperative cardiac patients (Engel et al., 2009). Thus, the ability of amino acid supplementation to downregulate inflammatory processes or oxidative stress onset could account for the beneficial role exerted on cardiovascular system.

Nutritional proteins: whey and casein

The concept of nutritional protein quality includes ability to provide specific amino acid patterns and digestibility. The major milk proteins are casein and whey: these two proteins differ in amino acid composition and digestion rate.

Whey is a fast-digesting protein and casein is a slow-digesting protein (Dangin et al., 2003). Casein is an excellent source of all the essential amino acids in balanced proportion relatively to individual requirements in healthy subjects. Whey is characterized by higher content of leucine and cysteine: leucine interacts with the insulin signaling pathway to stimulate downstream signal control of protein synthesis (Anthony et al., 2007) and cysteine is rate limiting for synthesis of glutathione, the main intracellular antioxidant system (Bannai, 1984). Whey ingestion was shown to promote protein anabolism in the elderly (Dangin et al., 2003) and in bed resting young subjects (Antonione et al., 2008) better than casein. Different mechanisms could be responsible for a greater whey anabolic efficiency during inactivity. The slow rate of amino acid absorption from casein may limit protein synthesis stimulation during inactivity, whereas a higher and short-term increase of aminoacidemia after whey ingestion could counteract inactivity-induced inhibition of protein anabolism (Biolo et al., 2004).

Moreover, increased leucine availability after whey ingestion could enhance, during inactivity, the intracellular signaling of muscle protein synthesis stimulation (Anthony et al., 2007). Finally, whey-mediated increased cysteine availability could activate the glutathione system thereby improving muscle redox balance and decreasing catabolism (Powers et al., 2007).

AIMS

The present thesis investigates the effects of physical inactivity on:

1. oxidative stress and inflammation
2. muscle atrophy
3. glutamine metabolism

Additionally, this work focusses on the impact of dietary energy balance and protein intake control during inactivity on above mentioned metabolic changes.

MATERIALS AND METHODS

The present thesis is principally intended to elucidate the effects of inactivity on oxidative stress, inflammation, muscle atrophy. The bed rest model is known to be a reliable approach to study the impact of muscle unloading on human metabolism (Biolo et al., 2005a). In parallel, the same experimental setting is a suitable tool to investigate, on earth, the impact of microgravity on human physiology during space flights (Biolo et al., 2005a).

Results reported in the present thesis were collected during five different bed rest campaigns: 1) Short term bed rest-integrated physiology (STBR-IP) at the Clinical Research Center of the DLR-German Aerospace Institute (Cologne, Germany); 2) Women International Space Simulation for Exploration (WISE) at MEDES Clinical Research Facility of the Rangueil University Hospital (Toulouse, France); 3) Valdoltra Bed Rest 2006; 4) Valdoltra Bed Rest 2007; 5) Valdoltra Bed Rest 2008, Valdoltra Orthopaedic Hospital, (Ankaran, Capodistria, Slovenia).

1) Short Term Bed Rest-Integrated Physiology (STBR-IP) at the Clinical Research Center of the DLR-German Aerospace Institute (Cologne, Germany)

The Short Term Bed Rest-Integrated Physiology (STBR-IP) study was organized at the Clinical Research Center of the German Aerospace Institute (DLR - Cologne, Germany) as a complex study involving different international scientific groups. In the frame of STBR-IP, the impact of physical inactivity and microgravity on glutamine turnover was assessed. The experimental protocol was in accordance with the local ethical standards on human experimentation and approval was obtained from the Ethics Committee of the Ärztekammer Nordrhein (Düsseldorf, Germany). The study was performed in accordance with the Declaration of Helsinki for human studies and relative amendments. Each subject signed an informed consent form upon admission.

As previously published (Biolo et al., 2007b) nine healthy volunteers (age: 24 ± 1 year; body mass index: $23 \pm 1 \text{ kg} \times \text{m}^{-2}$) participated in a four phase study. During the first and second phases (July–August 2001 and February–March 2002, respectively), subjects received, eucaloric diets in either bed rest or ambulatory conditions in order to keep a constant body weight. In contrast, during the third and fourth phases (July–August 2002 and February–March 2003, respectively), subjects received diets characterized by lowered calorie intake containing almost 80% of total energy requirement in either bed rest or ambulatory conditions.

Calorie restriction was achieved by reduction fat intake. The cross over design was applied as follows: four subjects were studied in ambulatory conditions during the first and third phases and in bed rest conditions during the second and fourth phases; the other five subjects were studied in bed rest conditions during the first and third phases and in ambulatory conditions during the second and fourth phases. During the 14 days of bed rest, participants were exposed to 6° head-down-tilt bed rest for 24 hours per day. Some activities (ie, food intake, using the toilet, showering, and weighing) were performed in near-horizontal position.

During the ambulatory periods, participants were in the normal upright position during the day and were allowed to walk around in the ward. During the ambulatory periods subjects performed light muscular workload (including bicycle ergometry for 10 min 3 times/d).

Diet control

During the entire study period diet was strictly controlled. Before each study period, resting energy expenditure (REE) of each subject was estimated according to the FAO/WHO equations (Muller et al., 2004). Dietary energy content was reduced during bed rest and hypocaloric periods: within the eucaloric phases, participants received a diet containing 1.4 and 1.1 times their calculated REE during the ambulatory and the bed rest periods, respectively; otherwise, within the hypocaloric phases, participants received a diet containing 1.1 and 0.9 times their REE during the ambulatory and the bed rest periods, respectively. Total energy intake was almost 20% lower during the hypocaloric phases when compared to the correspondent eucaloric phases both in bed rest or ambulatory conditions. Subjects received a constant protein intake of $1 \text{ g protein} \times \text{kg}^{-1} \times \text{d}^{-1}$ in all study phases. Fatty acid intake was composed by both saturated and polyunsaturated fatty acids: during the hypocaloric periods, energy restriction was achieved by decreasing fat intake to a minimum of $60 \text{ g} \times \text{d}^{-1}$. The remaining energy was composed of carbohydrates. Daily intakes of water ($50 \text{ mL} \times \text{kg}^{-1}$), sodium ($2.5 \text{ mmol} \times \text{kg}^{-1}$), calcium (1000 mg) and vitamin D (400 IU) were monitored during the experimental periods. No caffeine, methylxanthine, or alcohol was allowed. Six meals were given daily (breakfast, lunch, dinner and 3 snacks). All foods were exactly weighed for each participant, and volunteers were asked to consume the complete meal.

Body composition

The body composition of all subjects was measured by DXA at the end of the adaptation period and at the beginning of the recovery period with the use of the Hologic QDR-2000 (Waltham, MA). The enhanced whole-body scans were analyzed for lean tissue mass.

Metabolic test

In order to assess the impact of inactivity on glutamine metabolism, on the morning of the last day of bed rest or ambulatory periods in eucaloric or hypocaloric conditions, after a 12 h overnight fast, a metabolic study involving infusion of stable isotopes was performed: a polyethylene catheter was inserted into an antecubital vein for infusion of all test substances. A second polyethylene catheter was inserted in a wrist vein of the opposite hand, which was heated (+50°C) to obtain arterialized venous blood. After baseline determinations of α -[1-¹³C]ketoisocaproic ([¹³C]KIC) acid and L-[5-¹⁵N]glutamine ([¹⁵N]glutamine) enrichments in arterialized plasma, a primed ($5.4 \mu\text{mol} \times \text{kg}^{-1}$) continuous ($0.09 \mu\text{mol} \times \text{kg}^{-1} \times \text{min}^{-1}$) infusion of L-[5-¹⁵N]glutamine (Cambridge Isotope Laboratories, Andover, MA, USA) was started and continued for 8 h. Two hours later, a L-[1-¹³C]leucine ([¹³C]leucine) (Cambridge Isotope Laboratories) primed ($5.4 \mu\text{mol} \times \text{kg}^{-1}$) continuous ($0.09 \mu\text{mol} \times \text{kg}^{-1} \times \text{min}^{-1}$) infusion was started and continued for 6 h. Blood samples were obtained at 280, 290 and 300 min to determine [¹³C]KIC and [¹⁵N]glutamine enrichments as well as amino acid concentrations in arterialized plasma. After the fifth hour, a primed-constant intravenous infusion of a glutamine-free amino acid solution (Freamine III 8.5%; Clintec, Milan, Italy) was initiated and continued for 3 h (prime dose: $0.13 \text{ g} \times \text{kg LBM}^{-1}$, constant infusion rate: $0.13 \text{ g} \times \text{kg LBM}^{-1} \times \text{h}^{-1}$). The amino acid concentrations reported by the manufacturer (expressed as mg per 100 ml) were as follows: isoleucine 590; leucine 770; lysine 870; methionine 450; phenylalanine 480; threonine 340; tryptophan 130; valine 560; alanine 600, arginine 810; histidine 240; serine 500; cysteine 18; glycine 1190. Plasma amino acid concentrations and isotopic enrichments were determined at minutes 460, 470 and 480.

Analytical procedures

Plasma amino acid concentrations

Plasma glutamine, glutamate, leucine, valine and isoleucine concentrations were determined by high pressure liquid chromatography (Beckman Instruments Inc, San Ramon, CA).

A plasma volume of 1ml was deproteinized by addition of sulfosalicylic acid (SSA, 100 μl , 30%) in ice for 60 min. After centrifugation 50 μl of the upper phase were added to 25 μl of internal standard solution (containing known homoserine concentrations in appropriated borate buffer), and diluted to the final volume of 1250 μl by borate buffer and bidistilled water. Solutions containing known amounts of amino acids and homoserine in borate buffer were prepared for standard analyses. Derivatization was performed by o-phthaldialdehyde (OPA) (Sigma Chemical Co, St Louis, MO) in methanol, borate buffer and β -

mercaptoethanol. For analysis appropriate fractions of a Phase A solution composed by 50 ml sodium acetate (1 M) in 1000 ml bidistilled water and of a Phase B composed by methanol were used. Concentrations were calculated from peak areas according to internal standard concentrations and after normalization by homoserine peak area.

Plasma isotopic enrichments

[¹³C]KIC isotopic enrichments were measured by gas chromatography mass spectrometry (Agilent – HP 5973 Mass Spectrometer, Albertville, MN, USA) as follows: 200 µl of plasma were deproteinated with ethanol (1 ml), and after evaporation under N₂ flux and dissolution in water *p*-phenyldiamine (Sigma Chemical Co, St Louis, MO) (200 µl, 2% in 4N HCl) was added. After ethylacetate extraction and evaporation under N₂ flux, N,O-bis (trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (Pierce Chemical, Rockford, IL) (50 µl) was added to form the silylquinoxalinol derivative of KIC. Analyses were performed in single ion monitoring mode at $m \times z^{-1}$ 232 and 233 (Wolfe, 2004).

[¹⁵N]glutamine enrichments

A plasma aliquot of 300 µl was treated with SSA (300 µl, 30%). After centrifugation, amino acids were purified by a cationic resin column (AG50W-X8; Bio-Rad, Hercules, CA) and elution with NH₄OH (3M). After evaporation under N₂ flux and lyophilization, 50 µl acetonitrile + 50 µl N-Methyl-N-(Tert-Butyldimethylsilyl)trifluoroacetamide (MTBSTFA) (45 min, 90° C) were added for derivatization. Analyses were performed in single ion monitoring mode at mass-to-charge ratio ($m \times z^{-1}$) 431 and 432 (Wolfe, 2004).

Calculations

Glutamine total rate of appearance (Ra) was calculated by standard equation, dividing [¹⁵N]glutamine infusion rate by [¹⁵N]glutamine isotopic enrichment (Wolfe, 2004).

Glutamine Ra from proteolysis was determined on the base of leucine Ra from proteolysis multiplied by 0.47, i.e. a constant factor representing the average ratio between glutamine and leucine in mixed human muscle protein (Biolo et al., 2006). Glutamine de novo synthesis was assessed as difference between total glutamine Ra and Ra from proteolysis.

During 8 h primed-continuous infusion of [¹⁵N]glutamine no label can be detected in alanine (Biolo et al., 1995a): this suggests that nitrogen derived from glutamine amide deamination is diluted into the large ammonia pool and does not significantly recycle back to amino acids.

Glutamine de novo synthesis can be, therefore, reliably measured by this approach. In steady state conditions total glutamine Ra equals the rate of disappearance (Rd). Glutamine clearance was assessed as ratio between total glutamine Rd and plasma glutamine concentration. All kinetic data were expressed as ratio to LBM as detected by DXA.

Statistical analysis

All data were expressed as means±S.E.M. Results in the four different experimental conditions (ambulatory with eucaloric diet, bed rest with eucaloric diet, ambulatory with hypocaloric diet, and bed rest with hypocaloric diet) were analyzed using a repeated measures analysis of variance (ANOVA) with activity (ambulatory or bed rest) and diet (eucaloric or hypocaloric) as the two factors. Post hoc analysis was performed, when appropriate, by using Student's t test with Bonferroni's adjustment. Amino acid-mediated changes from the postabsorptive state in the ambulatory and bed rest conditions with eucaloric and hypocaloric diets were compared by using Student's paired t test. Statistical analysis was performed with SPSS software (version 12; SPSS Inc., Chicago, IL, USA). P-values <0.05 were considered as indicating significant differences.

2) Women International Space Simulation for Exploration (WISE) at MEDES Clinical Research Facility of the Rangueil University Hospital (Toulouse, France)

Sixteen healthy female subjects (age: 32.1 ± 4 years, BMI: $21 \pm 2 \text{ kg} \times \text{m}^{-2}$) were enrolled for the present study at MEDES Clinical Research Facility of the Rangueil University Hospital (Toulouse, France). All subjects were physically active before the study beginning. The study was performed in accordance with the Declaration of Helsinki for human studies and relative amendments. Each subject signed an informed consent form upon admission. All sixteen subjects performed a 60 days period of strict 6° head-down-tilt bed rest. All subjects remained in the HDT position during the whole experimental period except for meals assumption when they were allowed to elevate on one elbow.

Dietary control

Subjects were divided in two groups: eight subjects (standard protein intake) received a tailored diet containing a standard protein content of $1 \text{ g} \times \text{kg}^{-1} \times \text{d}^{-1}$, while the other eight subjects (high protein intake) received a diet containing an increased protein intake ($1.45 \text{ g} \times \text{kg}^{-1} \times \text{d}^{-1}$) and a fixed supplementation of free branched-chain amino acids (BCAA) ($3.6 \text{ g} \times \text{d}$ free leucine, $1.8 \text{ g} \times \text{d}$ free isoleucine, and $1.8 \text{ g} \times \text{d}$ free valine) (Friliver, Bracco, Italy) at the three main meals. Energy intake was tailored on each subject on the base of REE, which was

determined before bed rest by indirect calorimetry and body composition assessment and adjusted every 15 days. The maximum liquid intake of the standard and high protein intake groups was $60 \text{ ml} \times \text{kg}^{-1} \times \text{d}^{-1}$. For all subjects, sodium intake was 1.2 to 1.6 $\text{mmol} \times \text{kg}^{-1} \times \text{d}^{-1}$, potassium intake was 0.9 to 1.1 $\text{mmol} \times \text{kg}^{-1} \times \text{d}^{-1}$, calcium intake was $1 \text{ g} \times \text{d}^{-1}$, and phosphorus intake was 1.2 to 1.6 $\text{mmol} \times \text{kg}^{-1} \times \text{d}^{-1}$. Dietary folate intake was strictly kept constant during all study phases at $400 \mu\text{g} \times \text{day}^{-1}$.

Body composition

Body composition was measured before and every 15 days using bioimpedance analysis (Human IM Plus; DS Dietosystem, Milan, Italy) accordingly to manufacturer's instructions.

Metabolic test

In order to assess the impact of inactivity on homocysteine metabolism, on the morning of the last day of bed rest periods both in high or standard energy intake conditions, after a 12 h overnight fast, a metabolic study involving infusion of stable isotopes was performed: a polyethylene catheter was inserted into an antecubital vein for infusion of all test substances. A second polyethylene catheter was inserted in a wrist vein of the opposite hand, which was heated ($+50^\circ\text{C}$) to obtain arterialized venous blood. After baseline determinations of [ring- $^2\text{H}_5$]phenylalanine, [ring- $^2\text{H}_4$]tyrosine, [3,3- $^2\text{H}_2$]tyrosine, [methyl- $^2\text{H}_3,1\text{-}^{13}\text{C}$]methionine (i.e. m+4) and of [$1\text{-}^{13}\text{C}$]methionine (i.e. m+1) enrichments in arterialized plasma, primed continuous infusions of [ring- $^2\text{H}_5$]phenylalanine (infusion rate $4.8 \mu\text{mol} \times \text{kg}^{-1} \times \text{h}^{-1}$, priming dose $4.8 \mu\text{mol} \times \text{kg}^{-1}$), [3,3- $^2\text{H}_2$]tyrosine (infusion rate $1.2 \mu\text{mol} \times \text{kg}^{-1} \times \text{h}^{-1}$, priming dose $1.2 \mu\text{mol} \times \text{kg}^{-1}$), [methyl- $^2\text{H}_3,1\text{-}^{13}\text{C}$]methionine (infusion rate $4.4 \mu\text{mol} \times \text{kg}^{-1} \times \text{h}^{-1}$, priming dose $4.4 \mu\text{mol} \times \text{kg}^{-1}$) and a single bolus of [ring- $^2\text{H}_4$]tyrosine ($0.45 \mu\text{mol} \times \text{kg}^{-1}$) were started. Isotopic amino acids (Cambridge Isotope Laboratories, Andover, MA, USA) infusion was maintained for 8 hours. After the fifth hour an hyperinsulinemic-euglycemic clamp was performed administering $1\text{mU} \times \text{kg}^{-1} \times \text{min}^{-1}$ of insulin and an amino acid mixture at an infusion rate of $0.128 \text{ g} \times \text{kg}^{-1} \times \text{h}^{-1}$ (Vintene, Baxter, Deerfield, IL, USA). To assess enrichment changes over time, blood samples were taken 280, 290, 300 and 460, 470, 480 minutes after onset of infusion.

Analytical procedures

To assess plasma enrichments of isotopic tyrosine and phenylalanine the same technical procedure applied for glutamine was used. Phenylalanine and [ring-²H₅]phenylalanine were measured at $m \times z^{-1}$ 336 and 341, respectively. Tyrosine, [3,3-²H₂]tyrosine and [ring-²H₄]tyrosine were measured at $m \times z^{-1}$ 466, 468 and 470, respectively.

To analyze enrichments of sulphur amino acids, after treatment with 2-mercaptoethanol (3 µl) samples were evaporated under N₂ flux and treated with SSA (200 µl, 15%). After centrifugation, amino acids were purified from the upper phase by a cationic resin (AG50W-X8; Bio-Rad, Hercules, CA) and eluted by NH₄OH (3M). After ammonia elimination under gentle N₂ flux and liophilization, 50 µl acetonitrile + 50 µl MTBSTFA (45 min, 90° C) were added for derivatization. To assess homocysteine concentrations, a known amount of [²H₈]-homocysteine was added as internal standard to 200 µl of plasma before analysis. To assess methionine and cysteine concentrations known amount of L-[1-¹³C, methyl-²H₃]methionine, and L-[3,3-²H₂]cysteine (Cambridge Isotope Laboratories) were added. Measurements were performed in single ion monitoring mode at $m \times z^{-1}$ 496 and 497 and 500 for natural [¹²C] homocysteine, for [¹³C] homocysteine and for the internal standard. For methionine and cysteine concentrations $m \times z^{-1}$ ratios 320/324 and 406/408 were analyzed respectively (Wolfe, 2004).

Calculations

Homocysteine kinetics were calculated adapting a previously published model (Storch et al., 1988). Principally, the method is based on isotopic methyl release from [methyl-²H₃,1-¹³C]methionine tracer ($m + 4$) during transmethylation reaction, while isotopic carbon ($m + 1$) is maintained during remethylation reaction.

Thus, turnover rate of methionine labeled with isotopic methyl and carbon can be calculated as

$$Q_m = I_{met} / E_m + 4$$

while, turnover rate of methionine labeled with isotopic carbon can be calculated as

$$Q_c = I_{met} / (E_m + 4 + E_m + 1)$$

where Q_m and Q_c are turnover rates of methyl group of methionine and carbon backbone of methionine, respectively. I_{met} is [methyl- $2H_3,1-^{13}C$]methionine tracer infusion rate and E_{m+4} and E_{m+1} are [methyl- $2H_3,1-^{13}C$]methionine and [$1-^{13}C$]methionine enrichments, respectively. E_{m+4} must be taken into account in Q_c formula as this isotope includes an $m + 1$ mass.

In a steady state condition, methionine production equals methionine output. Thus

$$N + B + RM = Q_m = S + TM$$

and

$$N + B = Q_c = S + TS$$

where N is the contribution of diet, B of protein breakdown, RM of remethylation, TM of transmethylation and TS of transsulfuration. Q_c is not affected by RM as isotopic carbon is maintained along with homocysteine metabolism.

In the postabsorptive state, $N=0$: thus rate of remethylation (RM) can be calculated as

$$RM = Q_m - Q_c.$$

Methionine transmethylation (TM) can be calculated as

$$TM = RM + TS$$

In postabsorptive state, B can be calculated as

$$B = Q_c$$

and in simulated postprandial state (pp) as

$$B(pp) = Q_c - IAA$$

where IAA is exogenous methionine infusion rate.

TS can be calculated as

$$TS = Qc - S$$

S can be calculated as

$$S = \text{PheS} \times 0.63$$

where PheS is phenylalanine utilization for protein synthesis determined by [ring-²H₅]phenylalanine and [3,3-²H₂]tyrosine infusion and 0.63 is the molar ratio between methionine and phenylalanine.

In particular,

$$\text{PheS} = \text{PheB} - \text{PheOx}$$

PheB can be calculated as

$$\text{PheB} = \text{IPheD5} \times \text{EPheD5}$$

where IPheD5 is the infusion rate of [ring-²H₅]phenylalanine and EPheD5 is the enrichment of [ring-²H₅]phenylalanine

PheOx is the rate of phenylalanine hydroxylation to tyrosine and can be calculated as follows

$$\text{PheOx} = (\text{IRTyrD2} \times \text{ETyrD4}) \times (\text{ETyrD2} \times \text{EPheD5})$$

where IRTyrD2 is the infusion rate of [3,3-²H₂]tyrosine, ETyrD4 is the enrichment of [ring-²H₄]tyrosine, ETyrD2 is the enrichment of [3,3-²H₂]tyrosine and EPheD5 is the enrichment of [ring-²H₅]phenylalanine.

Thus, TS and TM can be calculated as

$$TM = Qm - S$$

or

$$TM = RM + TS$$

The assessment of homocysteine concentrations was performed taking into account the concentration of the internal standard.

$$[\text{Homocysteine}] = [\text{I.S.}] \times E_{\text{HomocysteineD4}}^{-1} \times \text{Pl.Vol.}^{-1}$$

where [I.S.] is the concentration of the added internal standard, $E_{\text{HomocysteineD7}}$ is the enrichment of D4 homocysteine and Pl.Vol. is the volume of analyzed plasma.

3) Valdoltra Bed Rest studies 2006 - 2007 - 2008, Valdoltra Orthopaedic Hospital, University of Koper (Capodistria, Slovenia).

Thirty healthy young male subjects (mean \pm S.E.M.; age 23.3 ± 0.4 years; body mass index $23.6 \pm 0.4 \text{ kg m}^{-2}$) were selected for three separated 35-day bed rest studies performed at the Valdoltra Hospital, University of Primorska, Ankaran-Capodistria, Slovenia, in July–August 2006 (A), 2007 (B) and 2008.

The projects were approved by the ethical committee of the University of Ljubljana and the experimental protocol was in accordance to the Declaration of Helsinki (2002). A written informed consent was obtained by each subject upon enrolment. All subjects were physically active before the admission to the Valdoltra Hospital (Slovenia). None of the subjects was under medication and their body weight was stable during the previous month. 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 period (Ambulatory period). At the end of the ambulatory period, each subject underwent 35 days of strict bed rest in which all daily activities were performed in horizontal clinostatic conditions. Subjects involved in 2008 study were in a 6°-head-down-tilt condition. Subjects were under periodical medical control and constant nursing assistance.

Dietary control: studies A, B and 2008

During the ambulatory and bed rest periods, diet composition and energy intake were daily monitored by a dietician. Individual diet composition reflected previous dietary habits.

Individual dietary preferences were collected by appropriate questionnaires received by each subject upon study enrolment and implemented during the ambulatory and bed rest periods in order to maintain individual dietary habits. Six meals were administered daily: 3 main meals (breakfast, lunch, and dinner) and 3 snacks. The three bedrest periods were preceded by an ambulatory adaptation period of 1 week, in which each subject received a weight-maintaining diet containing 1.4 times his resting energy expenditure, calculated by using the FAO/WHO equations (Muller et al., 2004). The diet contained almost 60% of energy as carbohydrate, 25% as fat, and 15% as protein. During the 35 days bed rest period of study A, a diet with the same energy, frequency, and relative macronutrient content of that planned in the ambulatory adaptation period was offered to subjects. Subjects were not required to consume all served food, but they were allowed to adapt spontaneously to decreased energy requirements during inactivity. Leftover food was monitored semiquantitatively by a dietitian to assess the relative macronutrient intake. Otherwise, during the 35 days bed rest period of studies B and 2008, each subject received an activity adjusted diet containing 1.2 times his resting energy expenditure and with the same meal frequency and relative macronutrient content of that planned in study A. Subjects were required to consume all served food.

Body composition

During A and B studies, fat mass and fat-free mass were monitored by multifrequency bioelectrical impedance (Human IM Plus; DS Dietosystem, Milan, Italy). Before and after studies A and B, the thickness of the vastus lateralis muscle was measured while the subject was in the supine position, by using ultrasound imaging on a portable ultrasound device (MyLab25; ESAOTE, Genoa, Italy) fitted with a 10–15-MHz linear probe (Reeves et al., 2004). Sagittal ultrasound images were obtained at 50% of muscle length measured along the midsagittal axis, after identification of the proximal and medial bone insertions of muscle. To ensure that all scanning measurements were taken in the same anatomical location, the ultrasound probe was positioned in the midsagittal plane, orthogonal to the mediolateral axis and its positioning was marked on acetate paper using moles and small angiomas (which may be assumed to maintain a fixed position) as reference points. Muscle thickness was measured in 8 subjects in Study B. Values were expressed in cm, as the vertical distance between muscle superficial and deep aponeuroses at an equidistant point from right and left borders of the image. Only in study A, in addition to the thickness of the vastus lateralis, the thicknesses of the gastrocnemius medialis, tibialis anterior, and biceps brachii muscles were measured. Then, a mean value of the thickness of representative postural and nonpostural muscles was calculated.

Vastus lateralis muscle architecture was measured in the supine position using realtime B-mode ultrasonography (ATL-HDI 3000, Bothell) with a 40 mm, 7.5 MHz linear-array probe. Scans were taken before and after bed rest with knee joint in anatomical position (passively fully extended). Measurements were performed at 50% of muscle length (previously estimated with ultrasound), in the midsagittal plane. To ensure that all scanning measurements were taken in the same anatomical location, the ultrasound probe was positioned in the midsagittal plane, orthogonal to the mediolateral axis and its positioning was marked on acetate paper using moles and small angiomas (which may be assumed to maintain a fixed position) as reference points. In each ultrasound image obtained at rest, the fascicular path was determined as the interspaces between echoes coming from the perimysial tissue surrounding the fascicle. Pennation angle was measured using Matlab (Matlab, The MathWorks Inc., S. Natick, MA, USA). Pennation angle was calculated as the angle between the fascicle and the deep aponeurosis of the muscle. In each scan, the average length and pennation angle of three fascicles was used for analysis (de Boer et al., 2007). Ultrasonographic analyses could be performed only on 9 subjects in study B for technical problems occurred during baseline measurements.

Metabolic test in study A: erythrocyte glutathione turnover rate

Glutathione turnover rates were determined during the postabsorptive state at the end of both the ambulatory adaptation and the bed-rest periods in study A. After background blood sampling, a primed continuous infusion of L-[3,3-²H₂]cysteine (Cambridge Isotope Laboratories, Andover, MA) (priming dose: 150 µmol; continuous infusion rate: 150 µmol/h) was started at 07.00 or 08.00 AM and continued for 5 h. A total of 10 mL arterialized venous blood was obtained at times 3, 4, and 5 h to measure hematocrit and the concentration and ²H₂ enrichment of erythrocyte-free cysteine and glutathione (Lyons et al., 2001). Whole blood was immediately centrifuged, and plasma and leukocytes were removed and replaced with an equal volume of cold distilled water. Both plasma and erythrocyte solutions were frozen at -80 °C for later analysis. Plasma and erythrocyte concentrations of glutathione and of glutathione amino acid precursors were measured in the background sample. At 1200 or 1300, isotope infusion was discontinued.

Markers of inflammatory and enzymic-antioxidant response in study A.

During metabolic tests, selected plasma hormone, mediator, and metabolite concentrations were measured in plasma. Glutathione peroxidase activity and protein concentrations in the

catalytic and modulatory subunits of glutamate cysteine ligase were measured in erythrocytes. Moreover, fatty acid membrane composition was assessed.

Analytical procedures (study A)

Glutathione synthesis rate in erythrocytes

The procedure for analysis of erythrocyte glutathione and cysteine isotopic enrichments was adapted from Lyons et al (Lyons et al., 2001). Erythrocyte suspension obtained from 400 μL whole blood was placed into pre-chilled tubes containing 1 mL ice-cold dithiothreitol (20 mmol \times L in 1 M acetic acid). Proteins were precipitated with 400 μL sulfosalicylic acid (30%) and centrifuged for 15 min at 10 000 \times g at 4 $^{\circ}\text{C}$. The supernatant fluid was transferred to a column containing 2 mL of a cation-exchange resin (AG50W-X8; Bio-Rad, Hercules, CA). After washing with Milli-Q water (5 mL \times 2; Millipore, Bedford, MA), glutathione was eluted from the column by using NH_4OH (3 mol/L) and collected into derivatization tubes (10 mL). Ammonia was eliminated under gentle nitrogen flow at room temperature. Samples were frozen, lyophilized, and reacted with 500 μL dithiothreitol solution (20 mM in 0.5 M acetic acid) at 100 $^{\circ}\text{C}$ for 1 h to reduce any dimerized glutathione and then dried again in nitrogen flow. Each sample was reacted with 300 μL HCl/methanol solution (250 μL 36% HCl in 7.5 mL methanol), incubated for 30 min at 80 $^{\circ}\text{C}$, and dried in nitrogen flow at 65 $^{\circ}\text{C}$ before being further reacted with 50 μL MTBSTFA and 50 μL acetonitrile for 40 min at 90 $^{\circ}\text{C}$. Afterward the sample was injected into a gas chromatograph–mass spectrometer [(GC-MS) HP 5890; Agilent Technologies, Santa Clara, CA]. The derivative was measured under electron-impact ionization by selective ion monitoring at a nominal $m \times z^{-1}$ of 363/365 for glutathione enrichment and of 406/408 for cysteine enrichments.

Glutathione concentrations in plasma and erythrocytes

Total glutathione concentrations in plasma and erythrocytes obtained from background samples were measured by using GC-MS and the internal standard technique. Briefly, known amounts of [$^{13}\text{C}_2$ - ^{15}N -glycine]glutathione (Cambridge Isotope Laboratories, Andover, MA) were added as internal standard to erythrocyte suspension obtained from 400 μL whole blood and to 200 μL plasma. Samples were processed as described above. The derivative was measured under electron-impact ionization by selective ion monitoring at a nominal $m \times z^{-1}$ of 363/366. Plasma and erythrocyte concentrations of glutathione precursor amino acids (glycine, glutamine, glutamate, methionine, homocysteine, and cysteine in plasma; glycine, glutamine, glutamate, methionine, and cysteine in erythrocytes) were measured in background

samples by using GC-MS and the internal standard technique. Briefly, known amounts of L-[¹⁵N]glycine, L-[¹⁵N]glutamate, L-[¹⁵N]glutamine, DL-[3,3,3',3',4,4,4',4'-²H₈]-homocysteine, L-[1-¹³C, methyl-²H₃]methionine, and L-[3,3-²H₂]cysteine (Cambridge Isotope Laboratories) were added as internal standards to an erythrocyte suspension obtained from 400 μL whole blood and to 200 μL plasma. Samples were processed as described. The derivative was measured under electron-impact ionization by selective ion monitoring at a nominal $m \times z^{-1}$ of 218/219 for glycine, 432/433 for glutamate, 431/432 for glutamine, 496/500 for homocysteine, 320/324 for methionine, and 406/408 for cysteine.

Plasma markers

Plasma leptin concentrations were measured by using an enzyme-linked immunosorbent assay (human Leptin Quantikine kit; R&D Systems, Minneapolis, MN). Plasma C-reactive protein (CRP) concentrations were measured by using a high-sensitivity enzyme-linked immunosorbent assay kit (Diagnostics Biochem, London, Canada). Plasma insulin concentrations were measured by using a radioimmunoassay (Adaltis insulin kit; Adaltis Inc, Montreal, Canada). Total plasma ghrelin concentrations were measured by using a radioimmunoassay (Total Human Ghrelin, GHRT-89HK; Linco, St Charles, MO). Plasma glucose, total cholesterol, HDLcholesterol, and triglyceride concentrations were measured by commercially available kits (Olympus System Reagents; Olympus Diagnostica GmbH, Hamburg, Germany) by using an autoanalyzer (Olympus AU400 System; Olympus, Tokyo, Japan). With antibodies obtained from rabbits and guinea pigs, neutrophil myeloperoxidase plasma concentrations were measured with the use of an enzyme-linked immunosorbent assay (Franck et al., 2005). Serum immunoglobulin G was isolated by affinity chromatography after immunization with purified human neutrophil myeloperoxidase. A reference curve was performed with purified human myeloperoxidase. Total -SH (thiol) functions (free -SH or bound to proteins) were measured in plasma by using spectrophotometry (at 412 nm) after the reaction of -SH with 5,5' dithiobis-2-nitrobenzoic acid (Sedlak and Lindsay, 1968). A reference curve was performed with reduced glutathione. Total -SH values were expressed as mol/g protein. Total proteins were measured by using the Bio-Rad Protein Assay reagent and expressed in $\text{mg} \times \text{mL}^{-1}$.

Red blood cell markers

Glutathione peroxidase activity was determined in erythrocytes according to Paglia and Valentine (Paglia and Valentine, 1967) and expressed as μmol metabolized NADPH $\times \text{min}^{-1} \times$

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 in erythrocytes was measured by Western blot analysis (Thompson 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× mL⁻¹, 0.1 mmol NaF × mL⁻¹, and 0.1 mM Na₃VO₄; all: Sigma-Aldrich). After centrifugation (10 min, RT, 16 00 × 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).

Calculations (study A)

LDL cholesterol was calculated by using the Friedewald equation:

$$\text{LDLcholesterol} = \text{total cholesterol} - \text{HDLcholesterol} - (\text{triglycerides} \times 5).$$

Metabolic test in study B: muscle glutathione turnover rate

During 2007 study, in the morning of the last day of the ambulatory period (baseline) and of the thirty-third day of bed rest (day 33), a metabolic test with stable isotope tracer infusions and a single *vastus lateralis* muscle biopsy was performed in order to assess muscle and red blood cells glutathione synthesis rate. In the morning of the seventh bed rest day (day 7) a metabolic study with the only stable isotope tracer infusions was performed to detect glutathione kinetics in red blood cells. In each study day, two subjects were analyzed. After a

12-h over-night fast (postabsorptive state) blood samples were taken before starting infusions, in order to assess in erythrocytes by gas chromatography and mass spectrometry (GC-MS) baseline natural enrichments of [$^2\text{H}_2$]glycine, [^{15}N]glycine, L-[$^2\text{H}_2$]glutathione and L-[^{15}N]glutathione. At 07.00 or 08.00 (t_0), a polyethylene catheter was inserted into an antecubital vein and a [$^2\text{H}_2$]glycine primed constant infusion (priming dose $26.5 \mu\text{mol} \times \text{kg}^{-1}$; infusion $26.5 \mu\text{mol} \times \text{kg}^{-1} \times \text{h}^{-1}$) was started and maintained throughout 7 hour, i.e. until 14.00 or 15.00. Four hours after the beginning of [$^2\text{H}_2$]glycine infusion (1100 or 1200), a [^{15}N]glycine primed constant infusion (priming dose $26.5 \mu\text{mol} \times \text{kg}^{-1}$; infusion $26.5 \mu\text{mol} \times \text{kg}^{-1} \times \text{h}^{-1}$) was started and maintained for 3 hours. Arterialized blood samples were collected from a second polyethylene catheter inserted in a controlateral heated wrist vein after 3 (t_3) and 7 (t_7) hours from the beginning of [$^2\text{H}_2$]glycine infusion. The last blood sample (t_7) was taken at the end of [$^2\text{H}_2$]glycine (7 hours) and [^{15}N]glycine (3 hours) infusions.

Infusions performed on day 7 in one volunteer were stopped for technical reasons.

Moreover, during metabolic tests performed before and after bed rest, fatty acid membrane composition was assessed.

Muscle sampling (Study B)

At baseline and day 33, after local anesthesia, at t_7 (i.e. at 1400 or 1500), a muscle biopsy was taken from the *vastus lateralis* in sterile conditions using a conchotome forceps according to standard techniques; muscle fibres were immediately cleaned from visible fat or connective tissue and accurately dried to remove blood with a sterile gauze. Quality of the procedure was monitored by microdissection microscope. Samples (averaging in mass 120 mg) were immediately frozen in liquid nitrogen and stored at -80°C . At the end of muscle biopsy procedure, both infusions were stopped and catheters removed. Muscle sampling in one subject failed to be completely performed for technical problems at day 33.

Analytical procedures (study B)

Glutathione kinetics and concentration evaluations in muscle could be performed only on nine subjects. The procedure for gas chromatography and mass spectrometry (GC-MS) analysis of muscle glutathione and glycine isotopic enrichments was adapted from abovementioned protocol (Lyons et al., 2001). Total glutathione concentrations were determined using GC-MS and the internal standard technique. Defrosted biopsies were weighted, homogenized in 500 μl SSA (6.5%) and known amounts (6.4 nmol final) of [glycine- $^{13}\text{C}_2$ ^{15}N]glutathione (Cambridge Isotope Laboratories, Andover, MA) were added as internal standard. After centrifugation, 1 ml of ice-cold dithiothreitol (DTT, 20 mM in 1 M acetic acid) was added to supernatant.

Proteins were precipitated with 400 μ l of sulphosalicylic acid (SSA) 30% and 15 min centrifugation at 10,000 g at 4°C. The supernatant was transferred to an ion exchange column (AG 50W-X8 cation exchange resin, Bio-Rad, Hercules, CA), washed with Milli-Q water (5 ml x2). Glutathione was eluted using NH_4OH (3 M, 4 ml). Frozen, lyophilized samples reacted with 500 μ l of DTT solution (20 mM in 0.5 M acetic acid) at 100°C and dried again in nitrogen flow. After reaction with 300 μ l of HCl/methanol solution (250 μ l of 36% HCl in 7.5 ml methanol), incubation for 30 min at 80 °C and drying under nitrogen flow, samples further reacted with 50 μ l MTBSTFA and 50 μ l of acetonitrile for 40 min at 90°C before injection into a gas chromatography-mass spectrometer (GC-MS) (HP 5890, Agilent Technologies, Santa Clara , CA, USA). The derivative was measured under an electron impact ionization by selective ion monitoring at a nominal mass-to-charge ratio ($m \times z^{-1}$) of 363, 364 and 365 for glutathione enrichment, and of 218, 219 and 220 for glycine enrichment. To measure [glycine- $^{13}\text{C}_2$ ^{15}N]glutathione (internal standard) enrichments the derivative was measured at a nominal mass-to-charge ratio ($m \times z^{-1}$) of 363 and 366.

A direct assessment of natural (before infusion beginning) isotopic enrichments in muscle could not be performed for reasons related to the study design. To estimate values of natural isotopic enrichments in muscle biopsies, red blood cells samples collected at t_0 in each subject were analyzed. Repeated measures were performed on specific amounts of red blood cells selected in order to provide glutathione chromatographic peak areas comparable with those obtained in muscle biopsies (t_7). Mean values were chosen as representing baseline natural isotopic enrichments in muscle. We adopted such approach because background glycine isotopic enrichments in circulating erythrocytes were previously demonstrated, in animals, to approximate enrichments measured in other tissues (bone marrow)(Hibbert et al., 2001). To assess glutathione concentrations in muscles a standard calibration curve was generated. A known amount of [glycine- $^{13}\text{C}_2$ ^{15}N]glutathione (internal standard) was added to prepared solutions containing different concentrations (serial dilution) of unlabelled glutathione (Sigma-Aldrich, Inc, MO, US). Glutathione and [glycine- $^{13}\text{C}_2$ ^{15}N]glutathione isotopic enrichments were measured monitoring appropriate mass-to-charge ratios following the same extraction and derivatization procedure adopted for muscle biopsies. Ratios between measured enrichments ($m \times z^{-1}$ 363 and 366) allowed standard calibration curve assessment. By interpolation analysis unlabelled glutathione concentrations could be measured in muscle biopsy samples.

Glutathione synthesis rate measurements in red blood cells were performed in ten subjects before and after bed rest while analyses at day 7 could be performed only in nine subjects. The

procedure to determine glutathione kinetics in red blood cells was adapted from abovementioned protocol used for study A with appropriate mass analysis.

Protein carbonylation. To directly assess muscle protein oxidation level, about 20 cryosections (12 μm) of each biopsy were solubilized at 4°C in 0.01% tetrafluoroacetic acid containing protease inhibitors, 5 mM EDTA and 2% beta-mercaptoethanol. Oxyblot can detect carbonyl groups formed in protein side chains as a consequence of oxidation. The Oxyblot Oxidized Protein Detection Kit (Chemicon – Millipore; Billerica, MA 01821). Derivatization using 2,4-dinitrophenylhydrazine was performed for 15 min following manufacturer’s instruction on 6 μg of protein (Vescovo et al., 2008). Protein final mixture was separated by electrophoresis on 10% SDS polyacrylamide gel. Proteins transferred to nitrocellulose membranes were stained by Red Ponceau and scanned. Specific proteins were detected by blots incubation with anti-4-dinitro phenyl hydrazine antibody followed by chemiluminescent development. Densitometry was performed on scanned autoradiographic films using an NIH image system. To allow the comparison of oxidation level between different samples the oxidative index (Oxy RP⁻¹) was defined as ratio between densitometric values of the oxyblot bands (oxidation level) and Red Ponceau stained bands (protein content). This value directly reflects the degree of myofibrillar protein oxidation. On each gel two standard samples (one positive and one negative control) were always loaded. The Oxyblot analysis was performed at day 33 in only 9 subjects because, as above mentioned, muscle sampling in one subject failed for technical reasons.

Calculations (study B)

In red blood cells, enrichments of [¹⁵N]glycine were calculated as tracer-to-tracee ratios as follows:

(Eq.1)

$$E^{15}\text{N-Gly}(t_i) = [\text{PAR}(219/218)_{t_i} - \text{PAR}(219/218)_{t_0}]$$

where E¹⁵N-Gly is [¹⁵N]glycine enrichment, PAR is peak areas ratio between areas measured for $m \times z^{-1}$ values indicated in brackets, t_i is one of the sampling times after steady state achievement (t_3 or t_7) and t_0 is the time of sampling before isotope infusion beginning (for natural enrichments).

To calculate [²H₂]glycine enrichment in red blood cells, correcting the influence of [¹⁵N]glycine infusion, the following equation was applied:

(Eq. 2)

$$ED_2\text{-Gly}(t_i) = [\text{PAR}(220/218)_{t_i} - \text{PAR}(220/218)_{t_0}] - [E^{15}\text{N-Gly}(t_i) \times \text{PAR}(219/218)_{t_0}]$$

where ED₂-Gly is enrichment of [²H₂]glycine and the other definitions are the same as in Eq. 1.

In muscle biopsies, enrichments of [¹⁵N]glycine were calculated as follows:

(Eq. 3)

$$E^{15}\text{N-Gly}_{\text{Muscle}} = [\text{PAR}(219/218)_{\text{Muscle}} - \text{PAR}(219/218)_{t_0}]$$

where _{Muscle} refers to measurements performed in muscle biopsies and the other definitions are the same as in Eq. 1 and 2.

Similarly, in muscle biopsies, enrichments of [²H₂]glycine were calculated as follows:

(Eq. 4)

$$ED_2\text{-Gly}(t_i) = [\text{PAR}(220/218)_{\text{Muscle}} - \text{PAR}(220/218)_{t_0}] - [E^{15}\text{N-Gly}(t_i) \times \text{PAR}(219/218)_{t_0}]$$

where definitions are the same as in equation 1, 2 and 3. Noteworthy, in Eq. 3 and 4, t₀ measurements were performed in red blood cells (see Eq. 2 and Eq. 1).

Enrichments of [¹⁵N]glutathione were assessed as follows:

(Eq. 5)

$$E^{15}\text{N-GSH}(t_i) = [\text{PAR}(364/363)_{t_i} - \text{PAR}(364/363)_{t_0}]$$

where E¹⁵N-GSH is enrichment of [¹⁵N]glutathione and other definitions are the same as in previous equations. Enrichments of [²H₂]glutathione were assessed as follows:

(Eq. 6)

$$ED_2\text{-GSH}(t_i) = [\text{PAR}(365/363)_{t_i} - \text{PAR}(365/363)_{t_0}] - [E^{15}\text{N-GSH}_{t_i} \times (\text{PAR} (364/363)_{t_0})]$$

where $ED_2\text{-GSH}$ is enrichment of $[^2\text{H}_2]\text{glutathione}$ and other definitions are the same as in previous equations. To calculate glutathione fractional synthesis rate (FSR) by stable isotope tracers and using enrichment data obtained in a single tissue sample we have developed the following equation:

(Eq. 7)

$$\text{FSR}_{\text{one-sample, double-tracer}} = \frac{[ED_2\text{-GSH}(t_7) / ED_2\text{-Gly}(t_7)] - [E^{15}\text{N-GSH}(t_7) / E^{15}\text{N-Gly}(t_7)]}{D_2\text{-Gly inf. duration} - {}^{15}\text{N-Gly inf. duration}} \times 100 \times 24$$

where $ED_2\text{GSH}(t_7)$ and $E^{15}\text{N-GSH}(t_7)$ are respectively $L\text{-}[^2\text{H}_2]$ and $L\text{-}[^{15}\text{N}]\text{glutathione}$ enrichments at the seventh hour of the metabolic study; $ED_2\text{-Gly}(t_7)$ and $E^{15}\text{N-Gly}(t_7)$ are respectively precursor enrichments of $L\text{-}[^2\text{H}_2]$ and $L\text{-}[^{15}\text{N}]\text{glycine}$ at the seventh hour of the metabolic study; $D_2\text{-Gly inf. duration}$ and ${}^{15}\text{N-Gly inf. duration}$ are respectively duration of $[^2\text{H}_2]$ and $[^{15}\text{N}]\text{glycine}$ tracer infusions. To express FSR as $\% \text{ day}^{-1}$, 100 and 24 coefficients were applied. The present equation relies on two parallel and separate infusions of different isotopes of the same precursor, $[^2\text{H}_2]\text{glycine}$ and $[^{15}\text{N}]\text{glycine}$, started at the beginning of the metabolic study (t_0) and four hours later, respectively. Precursor enrichments at steady-state ($[^2\text{H}_2]\text{glycine}$ and $[^{15}\text{N}]\text{glycine}$) and product enrichments after 3 ($[^{15}\text{N}]\text{glutathione}$) and 7 hours ($[^2\text{H}_2]\text{ glutathione}$) of infusion were measured in a single biological sample taken at the end of the metabolic study period (7 hours). So, the evaluation of product enrichment changes over time (the core parameter for FSR assessment) was obtained as difference between two single differently labeled product enrichments measured within only one biological sample. In this method $L\text{-}[^{15}\text{N}]\text{glutathione}$ enrichment, in fact, reflects short term tracer incorporation as $[^{15}\text{N}]\text{glycine}$ infusion is started three hours before the single final biological sampling. On the contrary, $L\text{-}[^2\text{H}_2]\text{glutathione}$ enrichment reflects long term tracer incorporation as $[^2\text{H}_2]\text{glycine}$ infusion is started seven hours before the single final biological sampling. By this approach,

glutathione FSR can be calculated over a net four hours incorporation period, as difference between differently labeled product enrichments measured at the same time point. Additionally, each product enrichment is normalized by precursor isotopic tracer enrichment at steady state. To validate the new equation, the traditional approach was applied to calculate glutathione FSR within the same experimental frame.

Glutathione FSR in red blood cells was calculated taking into account isotopic enrichments of L-[²H₂]glutathione measured after achievement of the steady state condition for [²H₂]glycine precursor enrichment in three different biological samples taken 3 (t₃) and 7 (t₇) hours after metabolic study beginning.

(Eq. 8)

$$FSR_{\text{multiple-sample, single-tracer}} = \frac{\text{Slope ED}_2\text{-GSH (t}_3 \rightarrow \text{t}_7)}{\text{Steady-state ED}_2\text{-Gly (t}_3 \rightarrow \text{t}_7)} \times 100 \times 24$$

where Slope ED₂-GSH (t₃→t₇) is the slope of L-[²H₂]glutathione product enrichments measured at t₃ and t₇, Steady-state ED₂-Gly (t₃→t₇) is the enrichment of [²H₂]glycine precursor at steady state. To express FSR as %/day, 100 and 24 coefficients were applied.

The absolute synthesis rate (ASR) was calculated as the product between FSR and glutathione concentration ([GSH]) values in studied tissues:

$$ASR = FSR \times [GSH]$$

Statistical analysis (Study A and B).

Data are presented as mean±SEM. In order to detect significant changes mediated by 5-weeks bed rest we applied the Student's *t* test for paired samples. p-values lower than 0.05 were chosen as threshold for statistical significance. Relationships between variables were analyzed by bivariate correlation using the Spearman's or Pearson's test where appropriate. Statistical analysis was performed using SPSS statistical software (version 12; SPSS, Inc., Chicago, IL).

Evaluated parameters in bed rest study 2008

Fatty acid membrane composition in red blood cells as well as plasma insulin and glucose concentrations were analyzed from single blood drawings taken before and after the bed rest period.

Analytical procedures (Bed rest 2008)

Membrane composition

Whole blood was immediately centrifuged after drawing: plasma and erythrocyte aliquots were stored at -80 °C until analysis. Fatty acid membrane compositions of red blood cells were analyzed modifying a previously published method (Burdge et al., 2002). Organic solvents and buffering salts were purchased from Sigma–Aldrich, Inc, MO, US. Erythrocytes (200 mL) were washed six 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 (Sigma–Aldrich, Inc, MO, US) as antioxidant, and 1 mL of 1 M NaCl solution. After centrifugation, the lower lipid phase was collected and dried under nitrogen flux at 40 °C. Pellets were dissolved in toluene (500 mL) and, after the addition of 1 mL methanol solution containing 2% of H₂SO₄, samples were heated at 50 °C for 2 h. A neutralizing solution (1.0 ml, 0.25 M KHCO₃ and 0.5 M K₂CO₃ in deionised H₂O) and hexane (1 mL) were added and, after centrifugation, the hexane layer, containing fatty acid methyl esters (FAMES), was collected; organic solvents were removed by nitrogen flux. Extracted lipids were resuspended in hexane (150 mL) and analyzed by gas-chromatography–flame ionization detection (GC-FID; GC 6850 Agilent Technologies, Santa Clara, CA, USA). Helium was used as carrier gas. Detector temperature as well as injector temperature were set at 300 °C. Column oven temperature started at 115 °C (constant for 2 min) and increased afterwards by a gradient ramping of 10 °C/min until 200 °C. Temperature remained constant at 115 °C for 11.5 min and reached 245 °C by a gradient ramping of 60 °C/min. Temperature remained constant at 245 °C for 8 min. Specific FA standards were used to identify FAMES by retention times in erythrocyte samples. A commercial mixture of purified fish oil FAs (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), a-linolenic acid (18:3, n-3), eicosaenoic acid (20:1, n-9), eicosadienoic acid (20:2, n-6), dihom-

γ -linolenic acid (20:3, n-6) as well as arachidonic acid (20:4, n-6) were identified by commercial standards purchased from Sigma–Aldrich, Inc, MO, US. Adrenic acid (22:4, n-6) and docosapentaenoic acid (22:5, n-6) were identified by commercial standards purchased from Nu-Check Prep, Inc, MN, US. 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).

Plasma markers

Plasma insulin concentrations were measured by using a radioimmunoassay (Adaltis insulin kit; Adaltis Inc, Montreal, Canada). Plasma glucose, total cholesterol, HDL cholesterol, and triglyceride concentrations were measured by commercially available kits (Olympus System Reagents; Olympus Diagnostica GmbH, Hamburg, Germany) by using an autoanalyzer (Olympus AU400 System; Olympus, Tokyo, Japan).

Calculations (Bed rest 2008)

Insulin sensitivity

Insulin sensitivity was determined according to the homeostasis assessment model (HOMA), i.e., $[\text{fasting insulin (mU} \times \text{L}^{-1}) \times \text{fasting glucose (mg} \times \text{dL}^{-1})] \times 405^{-1}$.

Membrane fatty acid composition

Red blood cell membrane level of each enlisted fatty acid was expressed as percent ratio between area-under-the-curve of each selected FAME peak and the sum of all measured FAME peaks. Total saturated fatty acids content was calculated as the sum of myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0) membrane levels. Results of elaidic acid are not reported because of uncompleted chromatographic separation from oleic acid. Results of elaidic acid were included in the monounsaturated FAs sum. Monounsaturated total content was calculated as the sum of palmitoleic acid (16:1, n-7), oleic acid (18:1, n-9), elaidic acid (trans 18:1, n-9) and eicosaenoic acid (20:1, n-9) membrane levels. n-6 Polyunsaturated total content was defined as the sum of linoleic acid (18:2, n-6), eicosadienoic acid (20:2, n-6), dihomo- γ -linolenic acid (20:3, n-6), arachidonic acid (20:4, n-6), adrenic acid (22:4, n-6) and docosapentaenoic acid (22:5, n-6) membrane level. D5 desaturase index was defined as ratio between arachidonic acid (20:4, n-6) and dihomo- γ -linolenic acid (20:3, n-6) membrane levels. n-3 Polyunsaturated sum was calculated as the sum of α -linolenic acid (18:3, n-3), eicosapentaenoic acid (20:5, n-3), docosapentaenoic acid (22:5, n-3) and docosahexaenoic

acid (22:6, n-3) membrane levels. D9 desaturase index was calculated as ratio between oleic (18:1, n-9) and stearic (18:0) acid contents. Arachidonic-to-eicosapentaenoic acid ratio was calculated as ratio between arachidonic acid (20:4, n-6) and eicosapentaenoic acid (20:5, n-3) membrane levels.

Statistical analysis

Wilcoxon test was applied to determine significant changes between the ambulatory and the experimental period in fatty acids membrane composition (Studies A, B and 2008). p values lower than 0.05 were considered statistically significant.

RESULTS

Effect of bed rest on whole body inflammatory and oxidative stress status.

Effect of bed rest on erythrocyte membrane composition.

Valdoltra Bed Rest studies 2006 (A), 2007 (B) and 2008.

Body composition (pooled subjects)

In pooled subjects, free-fat mass, as determined by bio-impedance, significantly decreased following bed rest as compared to the baseline ambulatory condition. Body fat mass significantly but slightly increased during the experimental period. (Table 1).

Table 1 effect of bed rest on body composition in pooled subjects

	Ambulatory	Bed rest	P ¹
Body weight	74.8±1.7	72.7±1.7	<0.01
Free-fat mass	63.2±1.2	60.4±1.1	<0.01
Fat mass	11.7±1.0	12.3±1.0	0.01

Values (kg) are expressed as mean±SEM. n=30. Body composition was assessed by bioimpedance (see Methods). ¹, Wilcoxon statistical test performed on absolute values of ambulatory vs bed rest conditions

Insulin resistance

Plasma insulin concentration significantly increased (+50.1±10.0%; p<0.01) while glucose level did not significantly change (-0.1±1.2; p=0.8) during the experimental period. Consequently, the HOMA index for insulin resistance significantly (p <0.01) increased from 1.4±0.1 at baseline to 2.0±0.2 after the experimental period.

Alterations of red blood cell membrane composition.

In order to investigate the effect of physical inactivity on whole body inflammatory status, the distribution of fatty acids in red blood cell membranes was analyzed. Table 2 displays the

relative content of selected fatty acids in erythrocyte membrane before and after 35 days of bed rest.

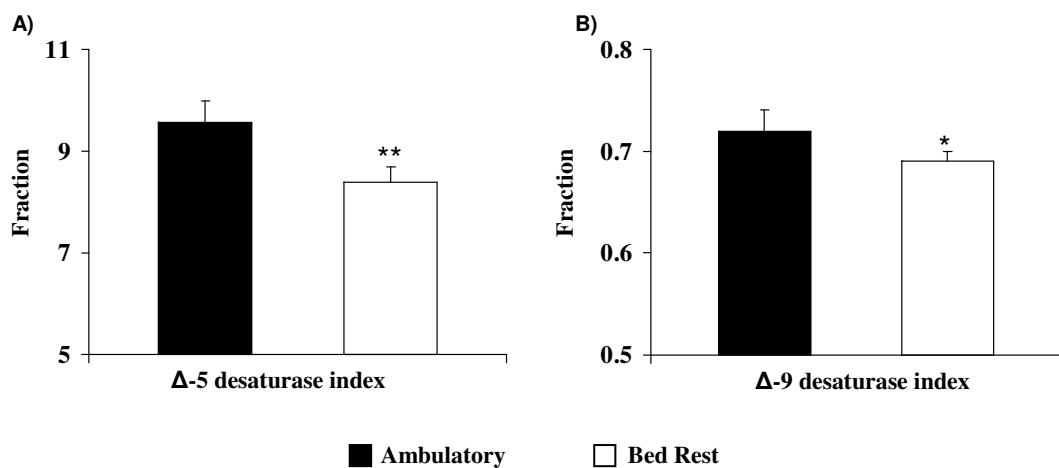
Table 2 Effects of 35 days bed rest on major fatty acid composition (%) in erythrocyte membranes.

	Ambulatory	Bed rest	P ¹
<i>Saturated FAs</i>			
Myristic 14:0	0.33±0.2	0.33±0.2	0.75
Palmitic 16:0	22.32±0.62	21.68±0.67	0.06
Stearic	19.46±0.31	19.19±0.33	0.14
Sum	42.31±0.92	41.48±1.02	0.10
<i>Monounsaturated FAs</i>			
Palmitoleic 16:1 n-7	0.25±0.02	0.24±0.01	0.56
Oleic 18:1 n-9	13.97±0.36	13.31±0.32	0.002
Eicosaenoic 20:1 n-9	0.24±0.01	0.23±0.01	0.04
Sum	15.57±0.40	14.87±0.34	0.003
<i>n-3 Polyunsaturated FAs</i>			
α-Linolenic acid 18:3 n-3	0.31±0.04	0.29±0.05	0.05
Eicosapentaenoic 20:5 n-3	0.39±0.02	0.35±0.01	0.05
Docosapentaenoic acid 22:5 n-3	3.91±0.20	4.15±0.15	0.01
Sum	7.12±0.19	7.34±0.17	0.06
<i>n-6 Polyunsaturated FAs</i>			
Linoleic acid 18:2 n-6	12.10±0.24	11.60±0.30	0.02
Eicosadienoic acid 20:2 n-6	0.39±0.03	0.38±0.02	0.36
Dihomo-γ-linolenic 20:3 n-6	1.72±0.07	2.08±0.08	<0.001
Arachidonic acid 20:4 n-6	16.20±0.63	17.18±0.59	0.01
Adrenic 22:4 n-6	4.20±0.19	4.47±0.19	0.01
Docosapentaenoic 22:5 n-6	0.80±0.04	0.93±0.09	0.003
Sum	35.45±0.90	37.16±0.42	0.005

FA, fatty acid; values are percent of total FAs (mean±SEM); measurements were performed on 30 subjects. ¹, Wilcoxon statistical test, performed on absolute values ambulatory vs bed rest conditions

Saturated fatty acids total content was unaffected by unloading. On the contrary, monounsaturated fatty acids total content was significantly reduced. In particular, oleic and eicosaenoic acid were significantly decreased at the end of the bed rest period. Unloading failed to significantly affect n-3 PUFAs total content, but selected n-3 fatty acids levels were differently influenced by physical inactivity: α -linolenic and eicosapentaenoic acid levels were significantly decreased, whereas docosahexaenoic acid level was significantly increased. Otherwise, bed rest enhanced n-6 polyunsaturated fatty acids total content. In particular, levels of dihomo- γ -linolenic, arachidonic as well as adrenic and docosapentaenoic acids were increased after 35 days of bed rest. Notably, linoleic acid levels were significantly decreased by bed rest.

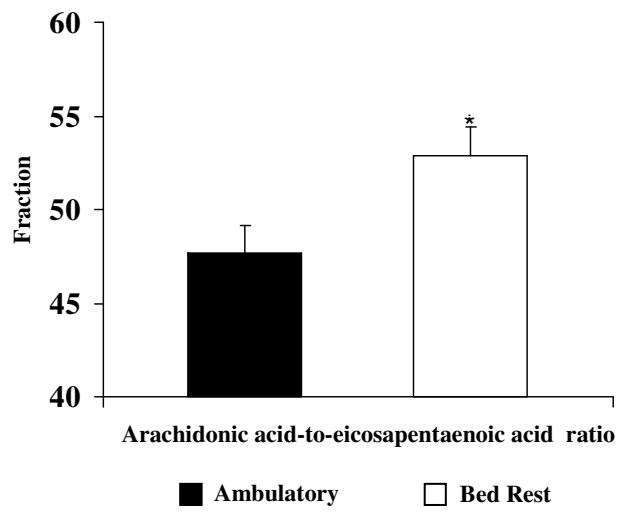
Figure 9



Estimated Δ 5 (A) and Δ 9 (B) desaturase activities before and after 35 days bed rest. n = 30, **p<0.001, *p<0.05. Wilcoxon statistical test performed on absolute values of ambulatory vs bed rest conditions

Δ 5 (Figure 9A) and Δ 9 (Figure 9B) desaturase indexes, as estimated from product-to-precursor ratio, significantly diminished following the experimental period. Additionally, arachidonic-to-eicosapentaenoic acid ratio was significantly increased after bedrest (Figure 10).

Figure 10



Arachidonic to eicosapentaenoic acid ratio before and after 35 days of bed rest. n=28, *p<0.01, Wilcoxon statistical test performed on absolute values of ambulatory vs bed rest conditions.

Effect of bed rest on homocysteine metabolism

Women's International Space Simulation for Exploration (WISE) study; MEDES clinic, Toulouse, (France)

Effect on homocysteine and on selected plasma amino acids concentrations

Table 3 bed rest effects on plasma methionine, cysteine and homocysteine concentrations

		Adequate protein diet (n = 8)		P ¹
		Ambulatory	Bed rest	
Methionine	Baseline	21±1	24±1	0.07
	Infusion	119±6 ²	142±6 ²	0.02
Cysteine	Baseline	215±5	208±4	0.08
	Infusion	194±5 ²	201±4	0.13
Homocysteine	Baseline	8.6±1.3	10.3±1.0	0.01
	Infusion	12.0±2.0	11.3±0.9 ²	0.70

Table 3 displays methionine, cysteine and homocysteine plasma concentrations ($\mu\text{mol} \times \text{L}^{-1}$) measured at baseline postabsorptive state (Baseline) and after intravenous infusions of amino acid mixture, insulin and glucose (Infusion). All measurements were performed before (Ambulatory) and after an experimental bed rest period (Bed rest) performed on subjects respectively fed by adequate protein levels. Data are shown as mean±SEM. ¹, “Ambulatory” vs “Bed rest”, paired Student's t-test; ², p<0.05 vs “Baseline”, paired Student's t-test

At baseline postabsorptive state, methionine and cysteine plasma concentrations were not significantly affected by bed rest (Table 3). Counterwise, homocysteine concentration were shown to be significantly upregulated by bed rest. Intravenous amino acid, insulin and glucose infusion significantly increased plasma methionine concentrations both in ambulatory and bed rest conditions (Table 3). The same infusions significantly downregulated cysteine availability only in ambulatory condition. Homocysteine concentrations were significantly upregulated by

amino acid, insulin and glucose infusions only after bed rest. Physical inactivity significantly elevated the increase of methionine concentrations induced by intravenous infusions. Nevertheless, physical activity level did not display significant effects on plasma cysteine or homocysteine concentrations after amino acid, insulin and glucose intravenous infusions (Table 3).

Effects on homocysteine kinetics parameters

Bed rest significantly upregulated baseline rates of homocysteine transsulfuration, while remethylation was reduced. Transmethylation rate was unaffected by unloading (Table 4).

Table 4. Plasma homocysteine kinetics measured at baseline postabsorptive state and after intravenous amino acid, insulin and glucose infusions.

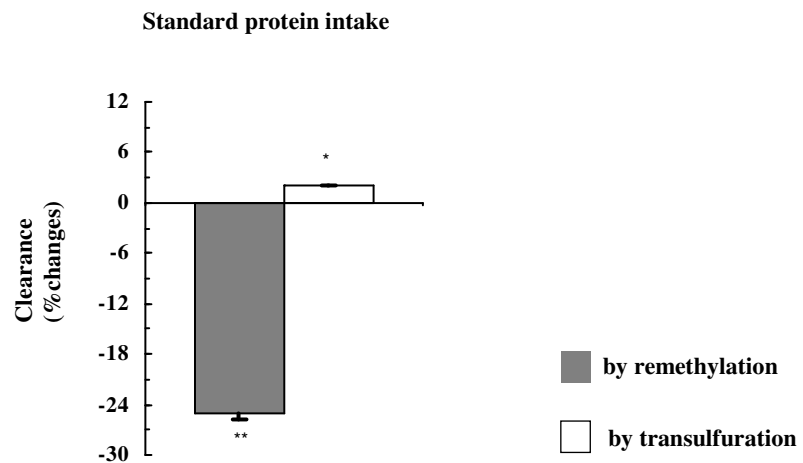
		Adequate protein intake (n = 8)		P ¹
		Ambulatory	Bed rest	
Transmethylation	Baseline	4.1±0.2	4.3±0.3	0.34
	Infusion	40.7±1.7	38.2±1.6	0.13
Remethylation	Baseline	2.5±0.1	2.3±0.2	0.03
	Infusion	6.5±0.7	4.7±0.7	<0.01
Transsulfuration	Baseline	1.7±0.1	2.0±0.1	0.03
	Infusion	34.2±1.9	33.5±1.3	0.60

Table 4 displays homocysteine kinetics values ($\mu\text{mol} \times \text{min}^{-1}$) measured at baseline postabsorptive state (Baseline) and after intravenous infusions of amino acid mixture, insulin and glucose (Infusion). All measurements were performed before (Ambulatory) and after an experimental bed rest period (Bed rest) on eight subjects fed by standard protein levels (see Methods). Rates of metabolic reactions were assessed by primed constant infusions of isotopic tracers coupled with gas chromatography-mass spectrometry analyses (see Methods). ¹, Data were analyzed by paired Student's t-test. Data are shown as mean±SEM. Kinetic values measured after intravenous infusions are all significantly different from baseline.

Intravenous amino acid, insulin and glucose infusion significantly increased homocysteine transmethylation, remethylation and transsulfuration rate before and after bed rest ($p < 0.05$,

paired Student's t-test). Bed rest decreased remethylation rates changes mediated by intravenous infusions (Table 4).

Figure 11



Homocysteine clearance changes by remethylation and transulfuration, mediated by bed rest at different protein intake.

*, $p < 0.02$ vs clearance by remethylation; **, $p < 0.05$ different from zero.

Bed rest, in association to normal protein intake, sharply reduced homocysteine clearance by remethylation, leaving unaffected clearance by transulfuration pathway (Figure 11).

Effect of bed rest on glutamine metabolism.

Short-Term Bed Rest-Integrated Physiology (STBR-IP) study; Clinical Research Center of the German Aerospace Institute (Cologne, Germany).

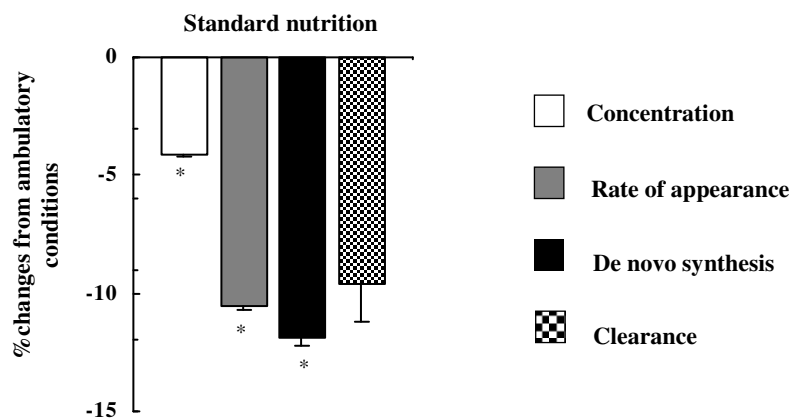
Effect of bed rest on body composition

Data describing changes in body composition were previously published (Biolo et al., 2007b).

Effect of bed rest on glutamine concentrations and kinetics

Figure 12 shows the effect of bed rest on glutamine concentrations and on selected glutamine kinetic parameters in normal eucaloric dietary conditions.

Figure 12



Percent changes of concentrations and metabolic parameters of glutamine mediated by bed rest; *, significant ($p < 0.05$) bed rest effect. Data were analyzed as explained in table 10 and in table 11.

Bed rest significantly decreased glutamine de novo synthesis, rate of appearance and concentrations. Glutamine clearance was not affected by bed rest.

Effects of bed rest on muscle atrophy and oxidative stress

Effects on muscle thickness, protein carbonylation and glutathione availability.

Bed rest Valdoltra 2007

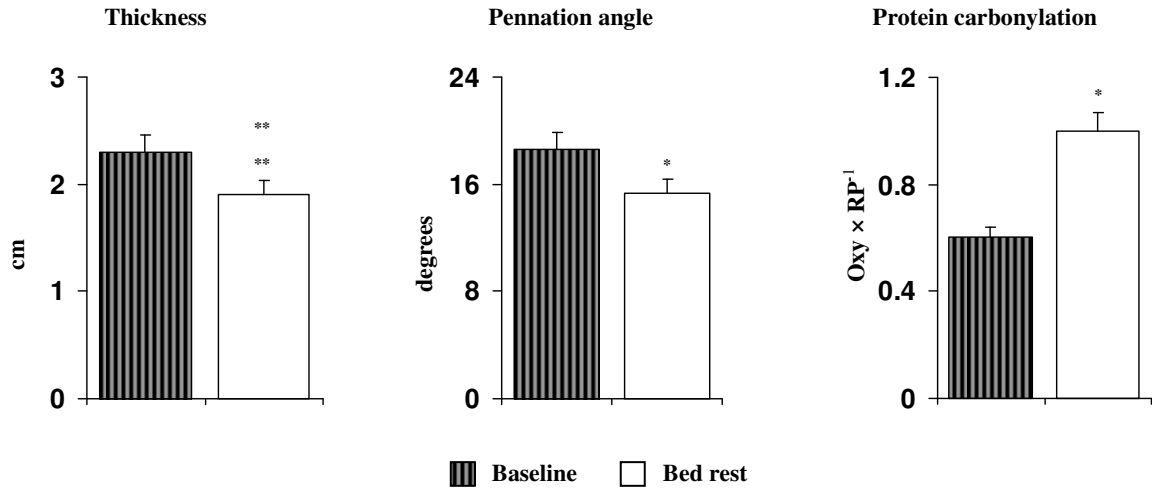
Effect of bed rest on body composition

Baseline body weight (72.8 ± 3.3 kg) displayed a bed rest mediated significant reduction (-2.3 ± 0.2 kg, $p=0.005$) after 33 days. As assessed by bioimpedance, baseline fat free mass (59.6 ± 2.0 kg) was significantly ($p < 0.001$) reduced after 33 days of bed rest (-2.9 ± 0.4 kg). In contrast, fat mass measured before bed rest failed to change significantly during the experimental period (from 13.8 ± 2.0 kg at baseline to 13.1 ± 1.8 kg at day 33, $p=0.06$).

Bed rest induced atrophy and oxidative stress in skeletal muscle.

In *vastus lateralis* both muscle thickness as well as fibre pennation angle were found to be significantly decreased from baseline ambulatory condition at bed rest day 33 (Figure 13).

Figure 13



Values of *vastus lateralis* thickness, fibre pennation angle and protein carbonylation measured before (Baseline) and after 33 days of bed rest (Bed rest) are shown. Muscle thickness and fibre pennation angle were determined in supine position by ultrasonography approaches. Protein carbonylation was determined by Oxyblot analysis. Oxy × RP⁻¹, ratio between quantified oxidized proteins and Red Ponceau stained total protein. **, p<0.001 vs Baseline; *, p<0.05 vs Baseline. Statistical analysis was performed by Student's *t* test.

In addition, muscle protein carbonylation levels were shown to be significantly increased after 33 days of bed rest by Oxyblot analysis (Figure 13) (Dalla et al., 2009). Muscle protein carbonylation changes mediated by 33 days of bed rest were inversely correlated to *Vastus lateralis* thickness changes induced during the same experimental period (Table 5).

Table 5. Correlations between bed rest mediated changes in muscle atrophy markers and selected redox parameters in vastus lateralis.

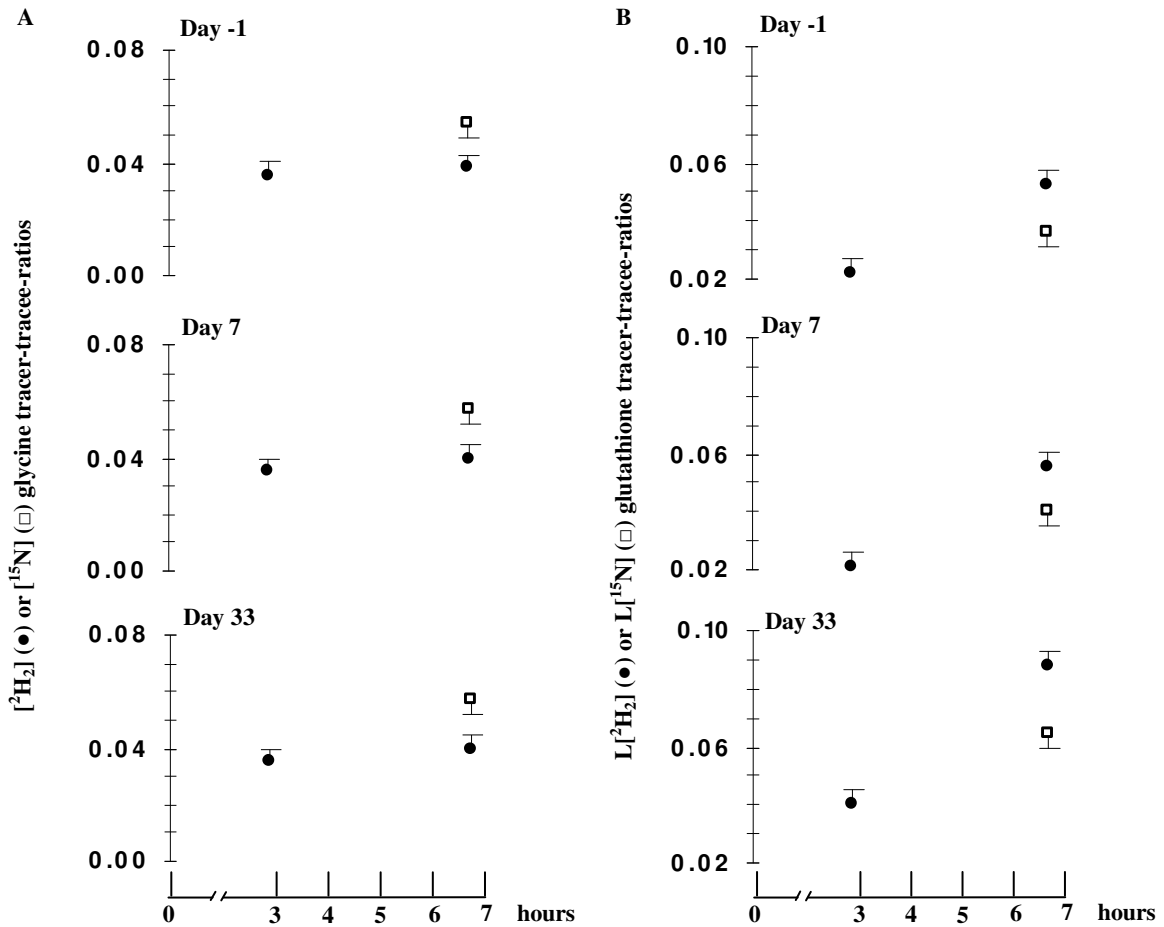
	Changes in	
	muscle thickness	fibre pennation angle
Protein carbonylation changes	-0.74 ¹ (n=8)	-0.06 (n=9)
Glutathione FSR change	0.14 (n=8)	-0.67 ¹ (n=9)
Glutathione concentration changes	0.05 (n=9)	0.45 (n=10)
Glutathione ASR changes	-0.14 (n=8)	-0.51 (n=9)

Data are values of correlation coefficients (R) between bed rest mediated changes in *vastus lateralis* muscle atrophy markers (Muscle thickness and Fibre pennation angle) both with changes in muscle protein oxidative stress (Protein carbonylation) and with muscle glutathione kinetics parameters. FSR, fractional synthesis rate; ASR, absolute synthesis rate. Relationships between variables were analyzed by bivariate correlation using the Spearman's or Pearson's test where appropriate. ¹, p< 0.05

Glutathione synthesis in erythrocytes: method validation.

Steady state of [²H₂]glycine enrichment in red blood cells was achieved after 3 hours of primed-continuous infusion and maintained for the following 4 hours (Figure 14).

Figure 14

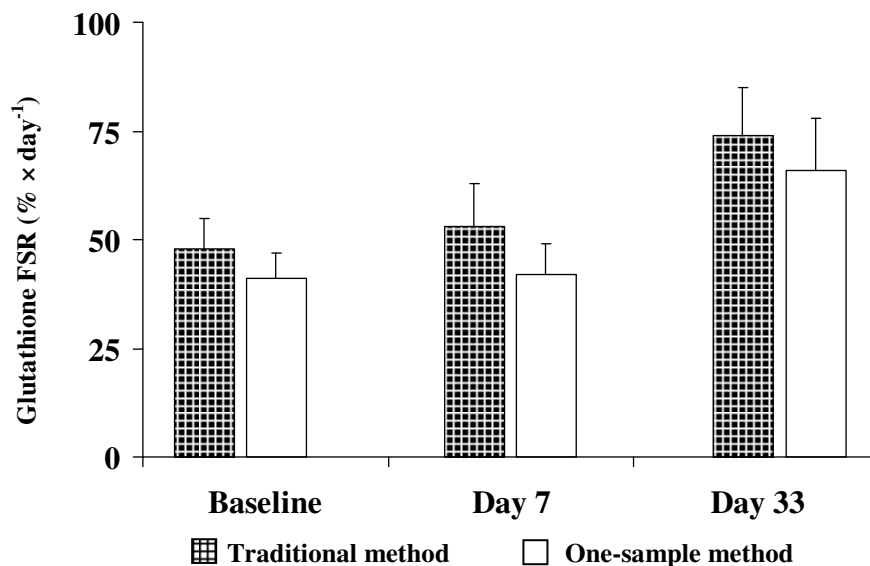


Red blood cells enrichments of isotopic tracers ($[^2\text{H}_2]$ and $[^{15}\text{N}]$ glycine) and products ($[^2\text{H}_2]$ and $[^{15}\text{N}]$ glutathione). **(A)** Steady state for $[^2\text{H}_2]$ glycine (●) precursor pool is shown while steady state condition for $[^{15}\text{N}]$ glycine (□) pool is assumed. Diversity between tracer isotopes steady state values are due to intrinsic metabolic differences between $^2\text{H}_2$ and ^{15}N isotopes. **(B)** $[^2\text{H}_2]$ glutathione (●) enrichment slope increase reflects linear tracer incorporation into glutathione products. Diversity between $[^2\text{H}_2]$ glutathione and $[^{15}\text{N}]$ glutathione (□) product enrichments measured at the end of infusions (7th hour) is the result of different tracer incorporation times. $[^2\text{H}_2]$ glycine was, in fact infused for 7 hours while $[^{15}\text{N}]$ glycine was infused for 3 hours.

The mean value of steady state- $[^2\text{H}_2]$ glycine enrichment at the end of bed rest was significantly greater when compared to the pre bed rest period ($p < 0.05$). In all three phases

(baseline, day 7 and day 33) [^{15}N]glycine enrichment mean values measured at the end of the infusion (7th hour) were greater ($p < 0.05$) than the corresponding [$^2\text{H}_2$]glycine enrichment values. To validate the single biopsy method against the traditional one to assess muscle glutathione synthesis rate, calculations were performed by both Eq. 7 and 8 (see “Methods”) in blood samples drawn during three metabolic tests (baseline, day 7 and day 33 of bed rest). Glutathione FSR ($\% \times \text{day}^{-1}$) values measured in red blood cells at baseline, day 7 and day 33 by “traditional” approach (Eq. 8) were comparable (no statistical difference) to values measured by “one-sample” method (Eq 7) (Figure 15).

Figure 15

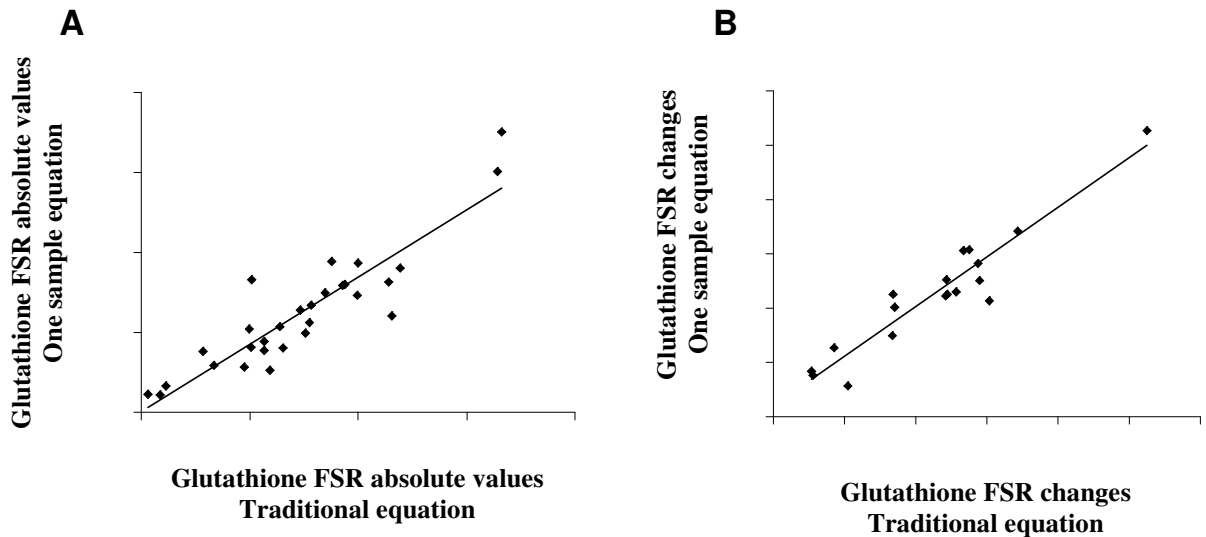


Red blood cells glutathione fractional synthesis rate (FSR) values measured both by traditional (empty bars) and one-sample (solid bars) approaches at selected study phases (Baseline, Day 7 and Day 33). Significant differences between FSR measured by traditional and novel one-sample approaches failed to be displayed in each study phase. No significant influence of bed rest on FSR values was shown, independently from adopted equation.

Independently from used calculations, no significant bed rest mediated changes in glutathione FSR were observed in red blood cells, either at early time points (day 7) or at the end of the experimental period (day 33) (Figure 15). Pooled absolute values of glutathione FSR measured in each study phase by “traditional” and “one-sample” approaches were displayed to be in significant ($p < 0.001$) and high direct correlation ($r = 0.84$; $n = 28$) (Figure 16A). Equally,

pooled FSR changes from baseline to day 7 as well as from day 7 to day 33 measured by “traditional” and “one-sample” approaches were shown to be in high significant ($p < 0.001$) correlation ($r = 0.91$, $n = 18$) (Figure 16B).

Figure 16.



Linear correlation between glutathione kinetics measurements in erythrocytes by traditional and by one-sample approach. (A) Glutathione fractional synthesis rate (FSR) absolute values measured in red blood cells by “traditional” and by “one-sample” approach are in positive and significant ($r = 0.84$, $p < 0.001$) linear correlation ($n = 29$). (B) Pooled changes of glutathione fractional synthesis rate from baseline to day 7 as well as from day 7 to day 33 measured by “standard” approach are in significant positive ($r = 0.91$, $p < 0.001$) correlation with analogous changes in glutathione FSR measured by “one-sample” approach ($n = 18$).

Bed rest effect on muscle glutathione synthesis.

Values of tracer-to-tracee ratios (TTR) for $[^{15}\text{N}]$ and $[^2\text{H}_2]$ glycine as well as for L- $[^{15}\text{N}]$ and L- $[^2\text{H}_2]$ glutathione measured in muscle biopsy (see project schedule) at the end of isotopic infusions are shown Table 6.

Table 6. Muscle glycine and glutathione tracer-tracee ratios at the end of infusions.

	Baseline	Bed rest
[¹⁵ N]glycine	0.0571±0.0040	0.0581±0.0062
L[¹⁵ N]glutathione	0.0138±0.0025	0.0103±0.0036
[² H ₂]glycine	0.0421±0.0027	0.0411±0.0027
L[² H ₂]glutathione	0.0273±0.0021	0.0317±0.0052

Muscle glycine and glutathione tracer-to-tracee ratios measured in *vastus lateralis* muscle are shown. Data were derived from isotopic enrichments measured by gas-chromatography and mass-spectrometry analyses (see “Methods”) performed in muscle biopsies taken at the end of metabolic tests performed in ambulatory conditions (Baseline) and after 33 days of bed rest (Bed rest) on nine subjects. When matched to baseline, bed rest values of tracer-to-tracee ratios failed to display significant differences. Data are presented as mean±SEM. Statistical analysis was performed by Student’s *t* test.

Bed rest failed to significantly affect the enrichment of each precursor ([¹⁵N] and [²H₂]glycine) as well as the enrichment of each isotopic product (L-[¹⁵N] and L-[²H₂]glutathione). In each study phase, [¹⁵N]glycine enrichment was found to be significantly higher than [²H₂]glycine. Glutathione fractional synthesis rate (FSR) displayed a tendency to be increased during bed rest (Table 7).

Table 7. Bed rest effect on *vastus lateralis* muscle glutathione kinetics parameters.

	Ambulatory	Bed rest	P ¹
<i>Glutathione</i>			
FSR (% × day ⁻¹) (n=9)	268±61	408±47	0.07
Concentration (mmol × kg wet tissue ⁻¹) (n=10)	2.3±0.2	2.7±0.1	0.30
ASR (mmol × kg wet tissue ⁻¹ × day ⁻¹) (n=9)	5.5±1.1	11.0±1.5	0.02

Glutathione fractional synthesis rate (FSR), concentration and absolute synthesis rate (ASR) measured in *vastus lateralis* by metabolic tests in ambulatory conditions (Baseline) and after 33 days of bed rest (Bed rest). Values were derived from isotopic enrichments measured by gas-chromatography and mass-spectrometry analyses performed in muscle biopsies taken at the end of metabolic tests. Concentration values were assessed by internal standard approach, while FSR and ASR were calculated by the novel one-sample, double-tracer approach. Data are presented as mean±SEM. ¹, Statistical analysis was performed by Student's t test.

Glutathione concentrations did not change significantly during bed rest. Nevertheless, glutathione absolute synthesis rate (ASR) was significantly upregulated by bed rest at day 33 when matched to baseline (Table 7). Glutathione FSR changes induced by 5 weeks of unloading were demonstrated to be significantly inversely associated to occurred pennation angle changes (Table 5).

Impact of nutrition on bed rest mediated alterations.

Effects of high protein diet on homocysteine metabolism

Women's International Space Simulation for Exploration (WISE) study; MEDES clinic, Toulouse, (France)

Table 8 Effect of bed rest at adequate or high protein intake on plasma methionine, cysteine and homocysteine concentrations

		Adequate protein diet (n = 8)		High protein diet (n = 8)		p ¹ (Effect)	
		Ambulatory	Bed rest	Ambulatory	Bed rest	Activity effect	Activity × diet interaction
Methionine	Baseline	21±1	24±1	22±1	23±1	0.08	0.35
	Infusion	119±6 ²	142±6 ²	129±5 ²	130±9 ²	0.06	0.08
Cysteine	Baseline	215±5	208±4	208±4	203±5	0.04	0.80
	Infusion	194±5 ²	201±4	187±6 ²	187±6 ²	0.24	0.28
Homocysteine	Baseline	8.6±1.3	10.3±1.0 ³	9.5±1.0	7.8±0.5 ³	0.92	0.001
	Infusion	12.0±2.0	11.3±0.9 ²	10.7±1.0 ²	8.6±0.6 ²	0.16	0.49

The table displays methionine, cysteine and homocysteine plasma concentrations ($\mu\text{mol} \times \text{L}^{-1}$) measured at baseline postabsorptive state (Baseline) and after intravenous infusions of amino acid mixture, insulin and glucose (Infusion). All measurements were performed before (Ambulatory) and after an experimental bed rest period (Bed rest) performed on two groups of subjects respectively fed by standard protein levels and by high protein and BCAA dietary intake.

Data are shown as mean±SEM. ¹, Data were analyzed with 2-factor (activity × diet) ANOVA with interaction; ², p< 0.05 vs “Baseline”; ³, p<0.05 vs “Ambulatory” condition (Wilcoxon test).

At baseline postabsorptive state, methionine plasma concentrations were not significantly affected by bed rest (Table 8). Moreover, changes in protein and BCAA dietary levels did not display significant effects on baseline methionine concentrations during bed rest. Baseline plasma cysteine concentrations were significantly reduced by bed rest, but changes in protein and BCAA dietary levels did not display significant effects on baseline cysteine

concentrations (Table 8). A significant diet × bed rest interaction was shown in affecting baseline plasma homocysteine concentrations: as confirmed by post hoc analysis, normal protein intake during bed rest induced a increase in plasma homocysteine concentrations, while high protein intake during bed rest was linked to a decrease in homocysteine plasma levels. Subjects supplemented by high protein diet, displayed significantly upregulated methionine and homocysteine but downregulated cysteine concentrations after intravenous amino acid, insulin and glucose infusions both in ambulatory and bed rest conditions (Table 8). Nevertheless, neither physical activity nor protein and BCAA dietary levels displayed significant effects on plasma methionine, cysteine or homocysteine changes secondary to amino acid, insulin and glucose intravenous infusions (Table 8).

Effects on homocysteine kinetics parameters

Bed rest significantly upregulated baseline rates of homocysteine transsulfuration and transmethylation, while remethylation rate was reduced by unloading (Table 9).

Table 9. Plasma homocysteine kinetics measured at baseline postabsorptive state and after intravenous amino acid, insulin and glucose infusions.

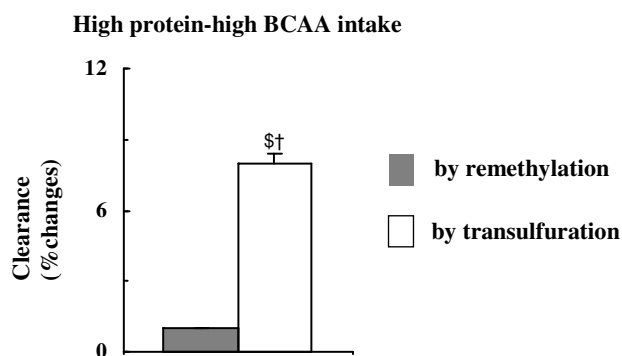
		Adequate protein intake (n = 8)		High protein-high BCAA intake (n = 8)		p ¹ (Effect)	
		Ambulatory	Bed rest	Ambulatory	Bed rest	Activity effect	Activity × diet interaction
Transmethylation	Baseline	4.1±0.2	4.3±0.3	4.2±0.2	4.6±0.3	0.02	0.25
	Infusion	40.7±1.7 ²	38.2±1.6 ²	39.9±1.8*	40.0±1.8 ²	0.46	0.42
Remethylation	Baseline	2.5±0.1	2.3±0.2	2.6±0.1	2.4±0.2	0.007	0.66
	Infusion	6.5±0.7 ²	4.7±0.7 ²	4.7±0.7 ²	3.9±0.7	0.04	0.42
Transsulfuration	Baseline	1.7±0.1	2.0±0.1	1.6±0.1	2.3±0.2	<0.001	<0.05
	Infusion	34.2±1.9 ²	33.5±1.3 ²	35.2±1.9 ²	36.1±1.2 ²	0.92	0.47

The table displays homocysteine kinetics values (μmol/min) measured at baseline postabsorptive state (Baseline) and after intravenous infusions of amino acid mixture, insulin and glucose (Infusion). All measurements were performed before (Ambulatory) and after an experimental bed rest period (Bed rest) performed on two groups of eight subjects respectively fed by standard protein levels and by an higher protein and BCAA dietary intake (see Methods). Rates of metabolic reactions were assessed by primed constant infusions of isotopic tracers coupled with gas chromatography-mass

spectrometry analyses (see Methods). ¹, ¹Data were analyzed with 2-factor (activity × diet) ANOVA with interaction. ², significantly different from baseline. Data are shown as mean±SEM.

Dietary supplementation with protein and BCAA significantly interacted with bed rest to enhance homocysteine transsulfuration rate, but transmethylation and remethylation rates failed to be affected by diet and activity interaction. In protein supplemented subjects, intravenous amino acid, insulin and glucose infusion significantly increased in ambulatory and bed rest conditions homocysteine transmethylation and transulfuration rates, while remethylation rate was significantly enhanced only in ambulatory conditions. Bed rest as well as diet control failed to affect increases in homocysteine transsulfuration and transmethylation rates induced by intravenous infusions (Table 9). Otherwise, bed rest negatively affected increases in remethylation rate induced by intravenous infusions.

Figure 17



Homocysteine clearance changes by remethylation and transulfuration, mediated by bed rest at different protein intake.

†, $p < 0.02$ vs clearance by remethylation; \$, $p < 0.05$ different from zero.

While bed rest, in association to normal protein intake, sharply reduced homocysteine clearance by remethylation, leaving unaffected clearance by transulfuration (Figure 11), protein and BCAA supplementation during experimental physical inactivity enhanced significantly homocysteine clearance by transulfuration, leaving unaffected clearance by remethylation (Figure 17).

Effect of bed rest and energy intake on glutamine metabolism.

Short-Term Bed Rest-Integrated Physiology (STBR-IP) study; Clinical Research Center of the German Aerospace Institute (Cologne, Germany).

Plasma amino acid concentrations

As shown in table 10, bed rest decreased baseline glutamine concentration in plasma. There were significant effects of both bed rest and hypocaloric diet to increase baseline plasma concentrations of all three branched-chain amino acids (BCAA), valine, leucine and isoleucine. No significant interaction was observed between the effects of bed rest and hypocaloric diet on BCAA concentrations. Plasma concentrations of all infused amino acids were increased as a consequence of the intervention (data not shown) (Biolo et al., 2007b). No significant effects of bed rest or hypocaloric diet was observed on amino acid infusion mediated changes of glutamine, glutamate and BCAA concentrations.

Table 10. Concentrations of plasma glutamine and glutamine precursor amino acid in postabsorptive state (Baseline) and percent increases after intravenous amino acid infusion (AA).

		Eucaloric Diet		Hypocaloric Diet		p ¹ (Effect)		
		Ambulatory	Bed Rest	Ambulatory	Bed Rest	Activity	Diet	Interaction
Glutamine	Baseline $\mu\text{mol} \times \text{L}^{-1}$	636±18	607±12	649±22	596±15 ²	0.03	0.96	0.33
	AA (% change)	3±1	7±2	7±1	9±3	0.20	0.18	0.66
Glutamate	Baseline $\mu\text{mol} \times \text{L}^{-1}$	54±4	52±5	60±5	57±6	0.40	0.13	0.72
	AA (% change)	9±5	9±5	10±5	6±5	0.71	0.86	0.66
Total BCAA	Baseline $\mu\text{mol} \times \text{L}^{-1}$	424±16	457±20 ²	494±24 ²	529±20 ²	<0.01	<0.01	0.90
	AA (% change)	142±4	142±13	143±6	142±5	0.97	0.86	0.93

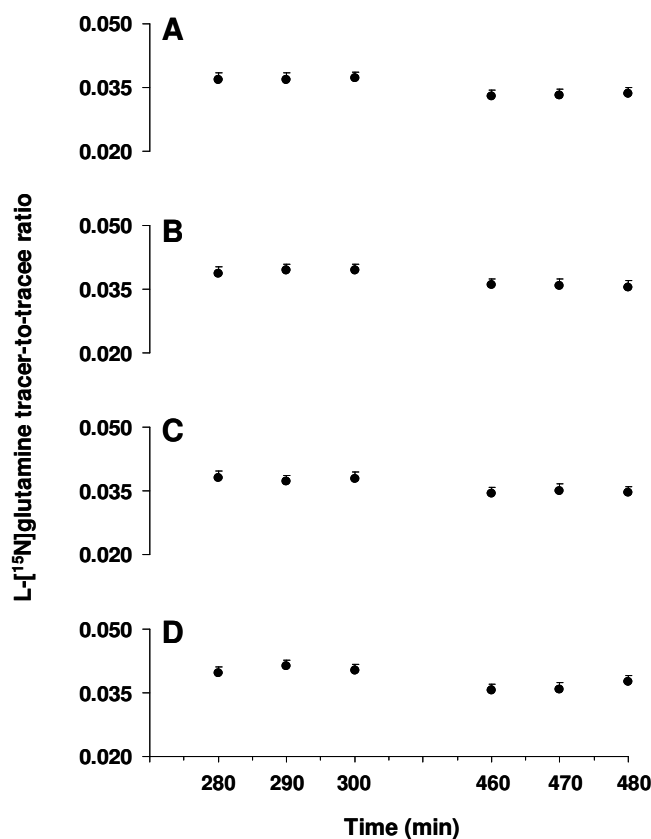
Data are mean±SEM; BCAA, branched-chain amino acids. Measurements were performed before (Ambulatory) and after (Bed Rest) a bed rest period of 14 days, both maintaining an energy intake balanced with activity determined requirements (Eucaloric Diet) or under dietary energy restriction (Hypocaloric Diet) calculated as 20% reduction of Eucaloric Diet. Amino acid infusion mediated changes from basal condition of selected amino acid concentrations are significantly different from zero (P<0.001, Student's t test). ¹, Data were analyzed with 2-factor (activity

× diet) ANOVA with interaction. ², p<0.01 versus Ambulatory Eucaloric Diet, based on Bonferroni's post hoc analysis.

Glutamine kinetics

Figure 18 shows L-[5-¹⁵N]glutamine tracer-to-tracee ratios at steady state in baseline postabsorptive state and during primed constant intravenous infusions of glutamine-free amino acid mixture in bed rest or ambulatory conditions during eucaloric or hypocaloric diets.

Figure 18



Steady state glutamine tracer-to-tracee ratios during baseline postabsorptive state (min. 280, 290 and 300) and during a primed constant intravenous infusion of glutamine free amino acid solution (min. 460, 470 and 480). A = Ambulatory, eucaloric diet; B = Bed rest, eucaloric diet; C = Ambulatory, hypocaloric diet; D = Bed rest, hypocaloric diet. In all four conditions, mean steady state tracer-to-tracee ratios significantly (p<0.001) decreased from baseline during amino acid infusions.

In all four conditions, mean steady state tracer-to-tracee ratios significantly (p<0.001) decreased from baseline during amino acid infusions. As evidenced in table 11, in both

eucaloric and hypocaloric conditions, bed rest induced a significant decrease of baseline glutamine total rate of appearance (Ra) and *de novo* synthesis.

Table 11. Whole body glutamine kinetics in the baseline postabsorptive state and percentage increase from baseline during intravenous amino acid infusion (AA).

			Eucaloric Diet		Hypocaloric Diet		P ¹ (Effect)		
			Ambulatory	Bed Rest	Ambulatory	Bed Rest	Activity	Diet	Interaction
Total Ra	Baseline	$\mu\text{mol} \times \text{kgLBM}^{-1} \times \text{min}^{-1}$	5.21±0.13	4.56±0.13 ²	4.82±0.17	4.48±0.11 ²	<0.001	0.16	0.28
	AA	% change	8±2	10±2	9±2	10±2	0.56	0.94	0.56
Ra from proteolysis	Baseline	$\mu\text{mol} \times \text{kgLBM}^{-1} \times \text{min}^{-1}$	1.05±0.02	1.00±0.02	1.03±0.05	0.99±0.03	0.20	0.69	0.91
	AA	% change	-15±2	-20±2	-12±4	-18±4	0.08	0.48	0.83
De novo synthesis	Baseline	$\mu\text{mol} \times \text{kgLBM}^{-1} \times \text{min}^{-1}$	4.17±0.14	3.56±0.13 ²	3.79±0.19	3.49±0.14 ²	<0.001	0.19	0.27
	AA	% change	14±3	19±3	15±2	18±3	0.18	0.96	0.65
Clearance	Baseline	$\mu\text{mol} \times \text{min}^{-1}$	8.3±0.4	7.5±0.3	7.5±0.3	7.6±0.3	0.15	0.15	0.37
	AA	% change	5±2	3±4	2±3	1±4	0.68	0.50	0.97

Data are mean±SEM. Ra, rate of appearance. AA, intravenous amino acid infusion. LBM, lean body mass. Measurements were performed before (Ambulatory) and after (Bed Rest) a bed rest period of 14 days, both maintaining an energy intake balanced with activity determined requirements (Eucaloric Diet) or under dietary energy restriction (Hypocaloric Diet) calculated as 20% reduction of Eucaloric Diet. Amino acid mediated changes from basal of total Ra, Ra from proteolysis and de novo synthesis are significantly different from zero (p<0.001, Student t test) in all conditions. ¹, data were analyzed with 2-factor (activity × diet) ANOVA with interaction. ², p<0.01 versus Ambulatory Eucaloric Diet, based on Bonferroni's post hoc analysis.

Bed rest did not significantly affect glutamine Ra from proteolysis as well as glutamine clearance. Significant effects of hypocaloric diet on glutamine kinetics were not observed. Intravenous infusion of glutamine-free amino acid solution significantly increased total Ra and *de novo* synthesis of glutamine in all conditions. Amino acid infusion significantly decreased glutamine Ra from proteolysis in all conditions. The effects of intravenous infusion of glutamine-free amino acid solution were not significantly different in bed rest or

ambulatory conditions during eucaloric or hypocaloric diets. Glutamine clearance was not affected by amino acid infusion.

Effect of bed rest and energy balance on inflammation oxidative stress and skeletal muscle atrophy

Bed rest Valdoltra 2006 (study A) and Valdoltra 2007 (study B)

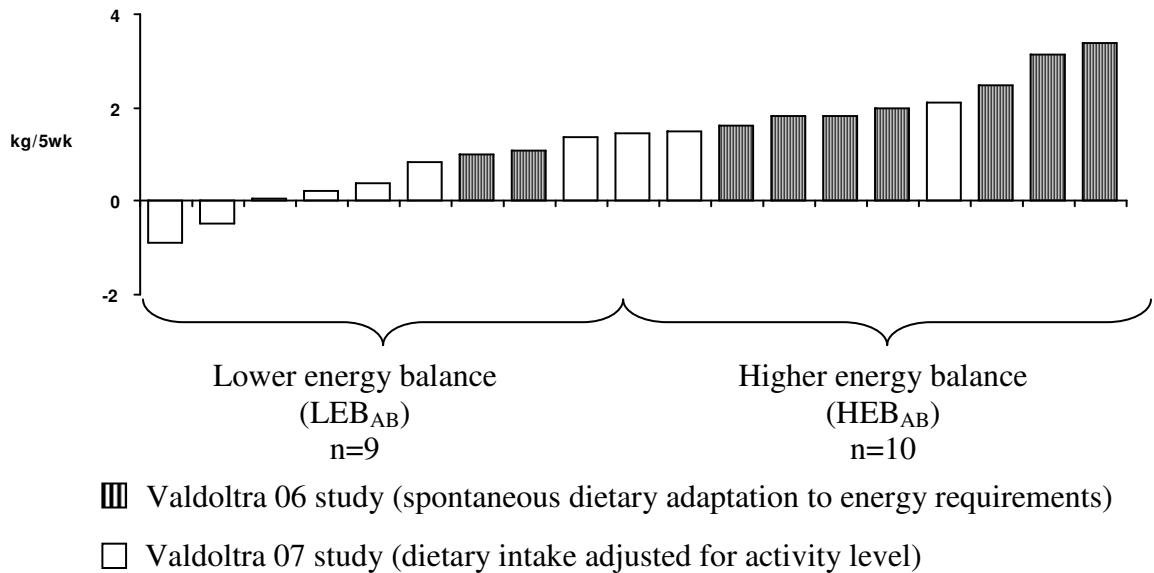
Effects on body composition

Baseline values of body weight, fat mass, fat-free mass, and vastus lateralis thickness did not differ significantly (unpaired t test) between subjects from Valdoltra 2006 (A) and 2007 (B) studies. During 5 wk of bed rest, body weight decreased ($p < 0.001$, repeated-measures ANOVA) from 76.5 ± 2.9 to 75.4 ± 2.8 kg in study A and from 76.3 ± 3.7 to 73.9 ± 3.5 kg in study B; fat mass increased ($P < 0.02$, repeated-measures ANOVA) from 10.2 ± 1.6 to 11.9 ± 1.6 kg in study A and from 13.6 ± 2.1 to 14.4 ± 2.3 in study B; fat-free mass decreased ($p < 0.001$, repeated-measures ANOVA) from 66.3 ± 2.1 to 63.4 ± 1.9 kg in study A and from 62.7 ± 2.2 to 59.4 ± 1.9 kg in B; thickness of vastus lateralis decreased ($p = 0.02$, repeated-measures ANOVA) from 2.39 ± 0.09 to 2.20 ± 0.09 cm in study A and from 2.09 ± 0.19 to 1.84 ± 0.17 cm in study B. There were no significant group (Study A or study B) \times bed rest interactions for body weight, fat mass, fat-free mass, or vastus lateralis thickness. In subjects from study B, the average thickness of the vastus lateralis, gastrocnemius medialis, tibialis anterior, and biceps brachii decreased significantly after bed rest (from 2.39 ± 0.09 to 2.20 ± 0.09 cm; $p < 0.001$, paired t test), and the decrease correlated directly with fat-free mass both before ($r=0.67$, $P<0.05$; $n=10$) and at the end of ($r=0.63$, $P<0.05$; $n=10$) the bed-rest period. Bed rest-induced percentage changes in fat-free mass and average muscle thickness were directly correlated ($R = 0.63$, $p < 0.05$; $n=10$).

Effects on muscle atrophy

To investigate the influence of energy balance on fat-free mass and muscle atrophy progression during 35 d of bed rest, pooled subjects from studies A and B were *a posteriori* divided into 2 groups accordingly to the median value of individual changes in fat mass ($+ 1.4$ kg) as threshold.

Figure 19



A posteriori subject stratification on the base of fat mass changes induced by 35 days of bed rest. Groups were identified across the median value of individual changes in fat mass (+1.4 kg)

The 10 subjects (7 from study A and 3 from study B) showing a fat mass gain greater than the median value were assigned to a group defined as higher energy balance (HEB_{AB}). Conversely, the 9 subjects (3 from study A and 6 from study B) with fat mass changes lower than the median value were assigned to a group defined as lower energy balance (LEB_{AB}) (Figure 19). Baseline body weight, fat-free mass, and vastus lateralis thickness did not differ significantly between the 2 groups (Table 12).

Table 12. Effects of bed rest at different energy intakes on body weight, fat-free mass, and vastus lateralis thickness

	HEB _{AB} (n =10)		LEB _{AB} (n =9)		p ¹ (Effect)		
	Ambulatory	Bed Rest	Ambulatory	Bed Rest	Diet	Activity	Interaction
Body weight (kg)	80.0±3.5	78.4±3.3	72.4±2.3	70.5±2.2	0.08	<0.01	0.66
Fat-free mass (kg)	67.1±1.8	63.4±1.8 ²	61.8±2.3	59.5±2.1 ²	0.11	<0.01	0.03
Vastus lateralis (cm)	2.43±0.13	2.10±0.13 ²	2.04±0.15	1.95±0.15	0.19	<0.001	0.01

HEB_{AB}, higher energy balance; LEB_{AB}, lower energy balance, as identified according to the median value of inactivity-induced fat mass changes (increase of 1.4 kg) measured in subjects of study A (spontaneous dietary adaptation to energy requirements) and study B (dietary intake adjusted for activity level). Fat-free mass and vastus lateralis thickness were measured by using bioelectrical impedance analysis and ultrasound imaging, respectively. All values are expressed as mean±S.E.M. ¹, data were analyzed with the use of a 2-factor (group × activity) ANOVA. ² Significantly different from the ambulatory adaptation condition, p<0.025 (Bonferroni's post hoc analysis).

There were significant bed-rest effects and group × bed rest interactions for fat-free mass and vastus lateralis thickness (Table 12). Bed rest–induced decreases in fat-free mass were greater in HEB_{AB} (-3.8 ± 0.4 kg) than in LEB_{AB} (-2.3 ± 0.5 kg) . In analogy, bed rest–induced decreases in thickness of vastus lateralis were greater in HEB_{AB} (-0.32 ± 0.04 cm; i.e., -14±2%) than in LEB_{AB} (-0.09 ± 0.04 cm; i.e., -6 ± 5%)

Baseline values of fat mass did not differ significantly between HEB_{AB} (12.9 ± 2.3 kg) and LEB_{AB} (10.6 ± 1.2 kg) groups. Changes in body fat in HEB_{AB} and LEB_{AB} groups averaged 2.1 ± 0.4 and 0.4 ± 0.3 kg, respectively. There were significant bed-rest effects (p < 0.001) and group × bed rest interactions (p = 0.01) for intracellular water, as determined by multifrequency bioimpedance analysis. Intracellular water decreased in HEB_{AB} from 32.4 ± 1.0 to 30.6 ± 1.0 kg and in LEB_{AB} from 30.6 ± 1.0 to 29.6 ± 0.9 kg. Bed rest–induced decreases in intracellular water were greater in HEB_{AB} (1.9 ± 0.2 kg) than in LEB_{AB} (1.0 ± 0.2 kg).

Effects of energy balance on plasma inflammatory markers and erythrocyte glutathione synthesis

Valdoltra 2006 study (study A)

To compare the influence of bed rest at different levels of energy balance on inflammatory markers and antioxidant defenses, subjects (n=10) from study A were considered. During this study volunteers were allowed to spontaneously adapt to decreased energy requirements. Similarly to abovementioned stratification, subjects were *a posteriori* divided in two groups accordingly to fat gain during the experimental period. The 5 subjects showing a fat mass accumulation greater than the median value (increase of 1.8 kg) were assigned to a group defined as HEB_A. Conversely, the 5 subjects with fat mass changes lower than the median value were assigned to a group defined as LEB_A. In pooled subjects, absolute values of fat mass measured before bed rest directly correlated with plasma leptin concentrations ($r = 0.72$, $p < 0.05$; $n=10$). Baseline values of fat mass did not differ significantly between HEB_A (11.6 ± 2.6 kg) and LEB_A (8.8 ± 1.6 kg) groups. Changes in body fat in the HEB_A and LEB_A groups averaged 2.6 ± 0.3 and 1.0 ± 0.5 kg, respectively.

Table 13. Effects of bed rest at different energy intakes on plasma hormone, mediator, and substrate concentrations

	HEB _A (n =5)		LEB _A (n =5)		P (Effect) ¹		
	Ambulatory	Bed Rest	Ambulatory	Bed Rest	Group	Activity	Interaction
Leptin (ng × mL ⁻¹)	3.4±1.2	6.9±2.6 ²	2.1±0.6	3.2±1.2	0.51	0.01	0.04
Ghrelin (ng × mL ⁻¹)	986±79	766±39	1083±212	929±119	0.52	0.004	0.21
Insulin (μU × mL ⁻¹)	8.1±1.2	12.0±1.7	8.1±0.8	11.2±2.3	0.77	0.05	0.65
Glucose (mg × dL ⁻¹)	88±1.2	89±2	92±3	87±3	0.59	0.41	0.32
CRP (μg × mL ⁻¹)	0.4±0.2	0.8±0.5	1.5±0.6	0.7±0.2	0.32	0.35	0.02
Myeloperoxidase (ng × mL ⁻¹)	327±18	381±25	345±25	326±22	0.54	0.21	0.02
Total -SH (pmol × g protein ⁻¹)	3.7±1.0	3.2±0.5	4.1±0.7	4.30±0.8	0.41	0.79	0.60
Triglycerides (mg × dL ⁻¹)	71±10	100±37	98±18	158±30	0.15	0.01	0.27
HDL cholesterol (mg × dL ⁻¹)	43±2	40±2	45±3	40±2	0.78	0.09	0.58
LDL cholesterol (mg × dL ⁻¹)	94±6	120±7	104±7	126±8	0.27	0.007	0.61

HEB_A, higher energy balance; LEB_A, lower energy balance; CRP, C-reactive protein; -SH, thiol function. ¹, Data were analyzed after log-transformation by using a 2-factor (group × activity) ANOVA with interaction. ², significantly different from the ambulatory adaptation condition, p<0.025 (Bonferroni's post hoc analysis). All values are expressed as mean±S.E.M.

Bed rest significantly increased plasma ghrelin and insulin concentrations, but there were no group × bed rest interactions for ghrelin and insulin. Plasma glucose did not change significantly after bed rest in either group. Plasma total thiol group concentrations did not change significantly after bed rest in either group. Finally, there was a significant bed-rest effect in increasing triglyceride and LDL-cholesterol concentrations (Table 13).

There was a significant group × bed rest interaction and bed-rest effect for plasma leptin concentration and, as shown by post hoc analysis, leptin concentrations significantly increased after bed rest in HEB_A group (Table 13). Moreover, there were significant group × bed rest interactions for plasma CRP and myeloperoxidase concentrations (Table 13).

Bed rest-mediated changes in CRP and myeloperoxidase concentrations in pooled subjects were directly correlated (r = 0.94, p < 0.001, n = 10).

Effects on whole body glutathione (red blood cells)

Table 14. Bed-rest effects at different energy intake levels on plasma and erythrocyte glutathione and glutathione precursor amino acid concentrations.

	HEB _A (n=5)		LEB _A (n=5)		P (Effect) ¹		
	Ambulatory	Bed Rest	Ambulatory	Bed Rest	Group	Activity	Interaction
Plasma ($\mu\text{mol} \times \text{L}^{-1}$)							
Glutathione	24±3	23±4	29±2	27±1	0.16	0.61	0.76
Glycine	240±23	240±18	242±16	217±12	0.63	0.40	0.42
Glutamine	501±33	562±17	514±35	487±31	0.25	0.64	0.24
Glutamate	88±6	86±9	82±4	85±15	0.75	0.99	0.80
Methionine	25±2	27±2	27±2	25±2	0.93	0.90	0.15
Homocysteine	18±2	18±1	13±2	12±2	0.06	0.17	0.69
Cysteine	27±1	26±1	27±2	25±2	0.96	0.22	0.47
Erythrocyte ($\mu\text{mol} \times \text{L}^{-1}$)							
Glutathione	2152±209	2254±115	2160±85	2394±131	0.70	0.06	0.42
Glycine	404±22	396±16	451±29	363±23	0.78	0.06	0.11
Glutamine	475±33	483±10	496±22	451±32	0.88	0.23	0.09
Glutamate	458±74	443±75	420±17	462±57	0.90	0.67	0.37
Methionine	25±3	29±3	28±2	29±2	0.62	0.24	0.52
Cysteine	67±13	75±13	69±16	66±11	0.82	0.80	0.64

HEB_A, higher energy balance; LEB_A, lower energy balance; ¹, data were analyzed by using a 2-factor (group × activity) ANOVA with interaction. All values are expressed as mean±S.E.M.

Plasma glutathione and glutathione precursor amino acid concentrations did not change significantly after bed rest in the HEB_A or LEB_A group (Table 14). We found a tendency ($p = 0.06$) toward an effect of bed rest on glutathione and glycine concentrations in erythrocytes, but no significant changes were observed in the other amino acids.

There was a significant ($p < 0.01$) effect of bed rest in increasing hematocrit both in the HEB_A (from $48 \pm 2\%$ to $50 \pm 2\%$) and LEB_A (from $48 \pm 2\%$ to $51 \pm 2\%$) groups. However, there was no group × bed rest interaction for hematocrit.

Precursor $^2\text{H}_2$ -cysteine enrichment was measured in erythrocytes and reached steady state in all groups and conditions by the end of the third hour of isotope infusion, whereas the $^2\text{H}_2$ -glutathione enrichment in erythrocyte increased linearly with time (Figure 20).

Figure 20

Fig. 1

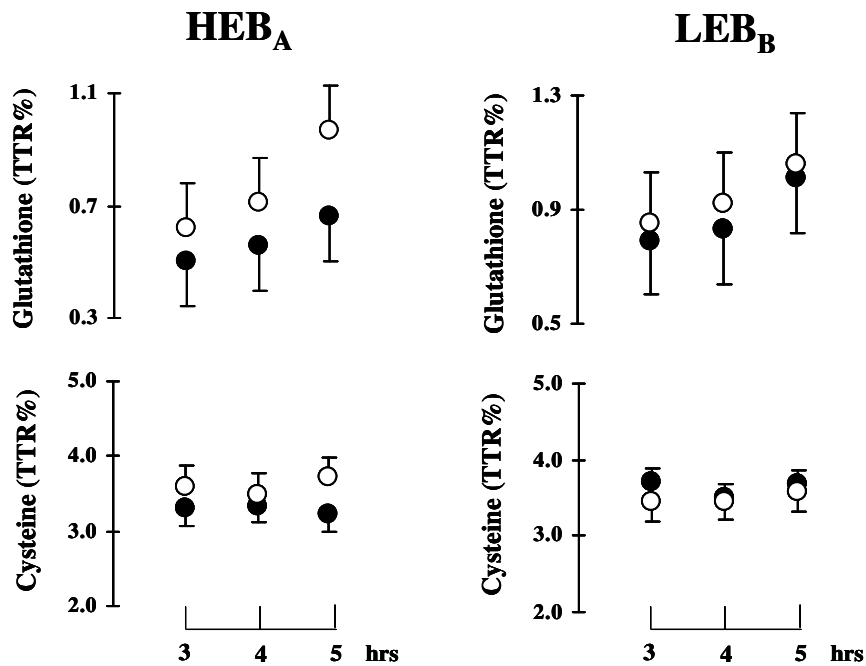


Figure 20 shows enrichments of $^2\text{H}_2$ -cysteine (precursor) and $^2\text{H}_2$ -glutathione (product) in red blood cell during primed continuous intravenous infusions of $^2\text{H}_2$ -cysteine.

Data are mean \pm SEM; TTRs, tracer to tracee ratios; ●, before bed rest; ○ after bed rest; HEB₀₆, higher energy balance in Valdoltra 2006 study; LEB₀₆, lower energy balance in Valdoltra 2006 study

The effects of bed rest at different energy intake levels on the erythrocyte glutathione system are shown in Table 15.

Table 15. Effects of bed rest at different energy intake levels on the erythrocyte glutathione system

	HEB _A (n =5)		LEB _A (n =5)		p (Effect) ¹		
	Ambulatory	Bed Rest	Ambulatory	Bed Rest	Group	Activity	Interaction
Glutathione FSR (% × day ⁻¹)	70±19	164±29 ²	103±23	84±27	0.43	0.13	0.03
Glutathione ASR (mmol × L ⁻¹ · day ⁻¹)	160±58	376±75 ²	217±43	197±62	0.40	0.09	0.05
GCL-C (fraction of GAPDH)	0.95±0.23	1.36±0.15	1.73±0.57	1.01±0.28	0.58	0.63	0.10
GCL-M (fraction of GAPDH)	0.42±0.11	0.48±0.13	0.57±0.10	0.63±0.21	0.36	0.68	0.99
GPX activity (μmol × min ⁻¹ × g protein ⁻¹)	1.04±0.13	1.01±0.31	1.18±0.54	1.46±0.32	0.48	0.71	0.65

HEB_A, higher energy balance; LEB_A, lower energy balance; FSR, fractional synthesis rate; ASR, absolute synthesis rate; GCL-C, glutamate cysteine ligase-catalytic subunit; GCL-M, glutamate cysteine ligase-modifier subunit, GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GPX, glutathione peroxidase. ¹, Data were analyzed by using a 2-factor (group × activity) ANOVA with interaction. ², Significantly different from the ambulatory adaptation condition, $P < 0.025$ (Bonferroni's post hoc analysis). All values are expressed as mean±S.E.M.

Before bed rest, glutathione absolute synthesis rates directly correlated with cysteine concentrations in erythrocytes of pooled subjects ($r = 0.74$, $p = 0.05$; $n = 10$). No significant correlations were found between absolute glutathione synthesis rates and glutamate ($r = -0.13$; $n = 10$) or glycine ($r = 0.50$; $n = 10$) concentrations. Bed rest tended to increase glutathione fractional and absolute synthesis rates in erythrocytes. There were significant group × bed rest interactions for glutathione fractional and absolute synthesis rates in erythrocytes (Table 15). Bed rest-mediated changes in glutathione fractional and absolute synthesis rates in erythrocytes were greater in the HEB_A group (+ 94 ± 18% and + 215 ± 42%, respectively) than in the LEB_A group (- 28 ± 37% and -40 ± 88%, respectively).

There was a tendency toward group × bed rest interaction for the effects of bed rest at different energy intake levels on the catalytic subunit for glutamate cysteine ligase, the key enzyme for glutathione synthesis; however, this trend was not statistically significant (Table 15). Bed rest-mediated changes in the catalytic subunit for glutamate cysteine ligase tended to

be greater in the HEB_A group ($96 \pm 65\%$) than in the LEB_A group ($-10 \pm 35\%$). Bed rest at different energy intake levels did not significantly affect glutathione peroxidase activity.

DISCUSSION

In this thesis, the impact of inactivity on inflammation, oxidative stress, and glutamine kinetics was investigated; in parallel, effects of dietary energy or protein intake changes on selected inactivity mediated alterations were studied. For this purpose, 5 bed rest campaigns (STBR-IP, WISE, Valdoltra 2006, 2007 and 2008 study) with different nutritional control approaches (see “Experimental design” section) and primary outcomes were organized.

Bed rest effects on inflammation and oxidative stress

Previously published evidences showed that chronic low grade inflammation (i.e. slight increase of proinflammatory cytokines) and oxidative stress are pathogenic factor of cardiometabolic diseases (Pradhan et al., 2001; Hansson, 2005; Alberti et al., 2006; Hopps et al., 2009; Lastra and Manrique, 2007). Inactive lifestyle is a demonstrated cause of cardiometabolic diseases (Zhu et al., 2004), and animal studies suggested unloading can enhance inflammation and oxidative stress (Fischer et al., 2007; Laufs et al., 2005). Thus, experimental inactivity can be hypothesized to induce in humans inflammation and oxidative stress, with potential metabolic and cardiovascular consequences.

Effect of bed rest on membrane composition.

Analysis of changes in membrane composition performed within Valdoltra bed rest 2006, 2007 and 2008 studies display experimental physical inactivity can exert a pro-inflammatory role at whole body level in humans.

The polyunsaturated fatty acids (PUFA) of the n-6 and n-3 series, are involved in up-regulation and down-regulation of the inflammatory response, respectively. Red blood cell membrane composition reliably reflects average cell membrane composition in different tissues and the whole body inflammatory condition (Harris and Von Schacky, 2004). Bed rest mediated changes in erythrocyte membrane composition was investigated and a significant shift toward a proinflammatory pattern was shown. Bed rest, in fact, led to a significant increase in pro-inflammatory n-6 PUFA content, including arachidonic acid. Interestingly, bed rest increased long-chain n-6 PUFA in erythrocyte membrane, whereas n-6 precursor content was significantly decreased, suggesting an active role of bed rest in enhancing n-6 turnover. Arachidonic acid is the most important pro-inflammatory n-6 PUFA and, as previously

reported, its level is negatively influenced by exercise (Helge et al., 2001). Bed rest associated increases of arachidonic acid content can lead, in turn, to increased pro-inflammatory eicosanoid production. On the contrary, bed rest induced a significant decrease in n-3 PUFA eicosapentaenoic acid which is known to play a key role as anti-inflammatory agent (Babcock *et al.*, 2000). Thus, these evidences show bed rest can directly affect n-6 and n-3 PUFAs metabolism, increasing inflammation level. The arachidonic-to-eicosapentaenoic acid ratio, calculated to monitor the competitive roles of arachidonic acid and eicosapentaenoic acid on inflammatory processes, was significantly increased after bed rest. This is an important bed rest mediated alteration as the balance between pro-inflammatory eicosanoids, principally produced from arachidonic acid, and anti-inflammatory molecules, mainly deriving from eicosapentaenoic acid, is a critical condition to maintain cell membrane functions (Serhan et al., 1996; Kelley, 2001). Additionally, n-3 and n-6 PUFAs are competitively metabolized by the same enzymes (Arterburn *et al.*, 2006). Consequently, increased availability of n-6 PUFA could have negatively affected n-3 PUFA metabolism leading to decreased content of the n-3 linolenic and eicosapentaenoic fatty acids in cell membranes. Interestingly, other stress conditions were associated with altered balance between n-3 fatty acids (Gudbjarnason, 1989). In contrast to linolenic and eicosapentaenoic fatty acids, docosahexaenoic acid proportion increased after bed rest: active eicosapentaenoic acid retrotransformation to docosahexaenoic acid (Conquer and Holub, 1997) can provide a mechanistic explanation for this effect. The proinflammatory effect of bed rest is further confirmed by significantly diminished $\Delta 9$ desaturase index, as estimated from product-to-precursor ratio. Such impairment of $\Delta 9$ desaturase activity led to significant decreases in monounsaturated fatty acids and a tendency towards increases in the saturated fatty acids. $\Delta 9$ desaturase, in fact, converts stearic and palmitic acids to monounsaturated oleic and palmitoleic acid, respectively (Ntambi and Miyazaki, 2004). Decreased monounsaturated relatively to unsaturated FAs can contribute to a pro-inflammatory status mediated by decreased clearance of pro-oxidant metabolites of saturated FAs, such as diacylglycerol and ceramides (Peter *et al.*, 2009).

Inactivity and enhanced inflammation lie at the base of metabolic syndrome and other insulin resistance conditions as type 2 diabetes (Zhu et al., 2004; Fischer et al., 2007). In this work, insulin sensitivity alterations mediated by experimental inactivity were assessed as changes in HOMA index: significant insulin resistance induction was demonstrated. Insulin resistance is known to rapidly develop following physical inactivity (Hamburg *et al.*, 2007) and this effect is consistent with the proinflammatory effect of bed rest evidenced by changes in membrane fatty acids composition. Moreover, published data indicate that activities of $\Delta 5$, $\Delta 6$ and $\Delta 9$

desaturases are affected by insulin action (Brenner, 2003). In addition, decreased long-chain PUFA content, due to low $\Delta 5$ desaturase activity, alters cell membrane physical properties, potentially leading to altered receptor binding capacities and further impairment of insulin sensitivity (Borkman *et al.*, 1993). A decrease of $\Delta 5$ desaturase activity was presently observed and can be considered to be caused by lowered insulin sensitivity.

Previous studies have investigated the influence of exercise training on muscle skeletal membrane fatty acids content showing, coherently, an opposite pattern as compared to that we have observed in erythrocytes following inactivity. Exercise training was associated with decreased saturated fatty acids, (e.g., palmitic acid) and n-6 PUFAs as well as increased monounsaturated fatty acids (e.g., oleic and stearic acids) and n-3 PUFAs. (Andersson *et al.*, 2000; Helge *et al.*, 2001).

Interestingly, $\Delta 5$ desaturase activity was shown to be improved by exercise training in parallel with insulin sensitivity (Andersson *et al.*, 2000). Thus, the decrease in $\Delta 5$ desaturase activity presently observed can be considered both as a consequence of inactivity and of increased insulin resistance.

In conclusion, several changes in fatty acid composition of erythrocyte membranes following long-term bed rest in healthy volunteers were described and these alterations are consistent with a proinflammatory metabolic pattern induced by inactivity. Interestingly, inactivity-mediated changes in insulin action have potentially affected desaturase activities leading to the observed relative changes in membrane fatty acids.

Impact of inactivity on plasma leptin concentrations

In the present work, other results contributed to underline the proinflammatory role of inactivity in humans: measurements performed in subjects enrolled in Valdoltra bed rest 2006 study, demonstrated that bed rest can significantly upregulate plasma leptin levels. Leptin is a protein encoded by the Obese (*Ob*) gene, synthesized by adipose tissue and aimed to control food intake and energy expenditure (Yadav *et al.*, 2009). Plasma leptin concentrations are positively correlated to adipose tissue accumulation (Baratta, 2002; Zhao *et al.*, 2005), IL-6 (Fenton *et al.*, 2006) and to activate the release of ROS (Yamagishi *et al.*, 2001). Thus, leptin can be considered not only as marker of adiposity but also as index of inflammation. Present results further show experimental bed rest in healthy volunteers can enhance the inflammation

status, significantly upregulating plasma leptin in parallel to increases in adipose tissue. Previously published results showed leptin concentrations can be upregulated by bed rest, independently from stress responses, changes in fat mass, body composition, energy intake or gender (Blanc et al., 2000a). Present results showing enhanced bed rest mediated inflammation as changes in membrane composition and increased leptin levels, further confirm previously published evidences obtained within the WISE study (Bosutti et al., 2008): this work showed a bed rest mediated upregulation of two related acute-phase reactants as PTX-3 and CRP, as well as of ratios between IL-6 and IL-10 further underlining that inactivity can effectively enhance plasma markers of inflammation.

Influences of bed rest on homocysteine kinetics and availability.

Inactivity effects on homocysteine metabolism and availability were investigated within the WISE study. Homocysteine is a non proteinogenic sulfur amino acid, and several studies associated hyperhomocysteinemia to coronary diseases (Clarke et al., 1991; Graham et al., 1997; Boushey et al., 1995) and to “unhealthy lifestyle” (Cleophas et al., 2000). Hyperhomocysteinemia is, in fact, associated to endothelial damage and to related atherogenic and trombotic effects enhancing blood procoagulant and proinflammatory factors (van den et al., 1995). Oxidative stress is considered to be the principal effector of homocysteine mediated endothelial damage (Harker et al., 1974). Dietary intake of proteins and of micronutrients as vitamins, is known to affect homocysteine metabolism (Lee and Frenkel, 2003). Physical exercise is known to downregulate homocysteine concentrations: this confirms the positive effect of moderate training on cardiovascular risk reduction (Duncan et al., 2004). Nevertheless, the impact of physical inactivity on homocysteine kinetics and availability was poorly investigated.

Primed continuous infusions of isotopic tracers, performed in women within the WISE study, allowed to assess kinetic changes leading to bed rest mediated effects on homocysteine availability. Bed rest was shown to significantly increase homocysteine concentrations in plasma. Epidemiological data show that in male human subjects average homocysteine concentrations reach an average value of 5-15 $\mu\text{mol} \times \text{L}^{-1}$ and that risk of coronary disease can be evidenced for values over 10 $\mu\text{mol} \times \text{L}^{-1}$ (Aguilar et al., 2004). Noteworthy, gender is a known factor influencing average plasma homocysteine concentrations: women display almost 20% lower homocysteine concentrations when compared to male subjects (Fukagawa et al., 2000). Thus, upregulation of homocysteine concentrations presently observed in female volunteers can be considered as a really relevant alteration. Analyses performed by isotopic

tracers approach, allowed to observe that bed rest significantly decreased remethylation and increased transsulfuration rates, while transmethylation was unaffected. To fully address metabolic alterations leading to accumulation of homocysteine, clearance changes by each disposal pathway of were analyzed. Interestingly, clearance by remethylation was significantly reduced by 60 days of bed rest, while clearance by transsulfuration was unaffected. Thus, reduction of homocysteine catabolism, coupled with unaltered rate of homocysteine synthesis can be considered as an appropriate explanation of enhanced plasma homocysteine availability observed after bed rest. This is the first study showing the effect of physical inactivity on homocysteine availability and kinetics: interestingly, observed changes can be ascribed to the net effect of bed rest because nutrition, a key factor controlling homocysteine metabolism, was strictly controlled during the experimental period. Dietary levels of protein and folic acid, two pivotal regulators of homocysteine metabolism, were, in fact, tailored and monitored to be maintained constant during the experimental period. In this way, contribution of dietary macro and micro-nutrient availability changes can be excluded. Even though, contribution of specific genetic polymorphisms to observed changes in homocysteine availability and metabolism could not be assessed, present data strongly suggest that bed rest can effectively upregulate blood homocysteine concentrations.

The impact of physical inactivity on homocysteine metabolism was never investigated in human healthy subjects, and reliable explanations about mechanisms leading to observed kinetic changes are not available. In conditions of normal or reduced protein availability, homocysteine is preferentially catabolized by MTHFR dependent remethylation (Ueland and Refsum, 1989): bed rest can be hypothesized to downregulate activity or availability of this enzyme, thus leading to the observed decrease in homocysteine clearance by remethylation. Further studies are needed to fully clarify molecular mechanisms underlining this process. Nevertheless, present results showing hyperhomocysteinemia in female healthy volunteers after a period of bed rest, suggest that physical inactivity can enhance oxidative stress induction at systemic level. Hyperhomocysteinemia, in fact, can potentially increase endothelial dysfunction (van den et al., 1995) reducing vasodilation (Tawakol et al., 1997): free radicals production secondary to homocysteine auto-oxidation is considered to be the mechanistic cause of vessel matrix and smooth muscle cell damage occurring in hyperhomocysteinemic patients (Harker et al., 1974). Moreover, lipid peroxidation linked to this process could contribute to atheromatose plaque induction (Huang et al., 2001). Thus, presently observed elevation of homocysteine availability due to bed rest can be related to an enhanced systemic oxidative stress condition that could also indicate increased cardiovascular

risk secondary to inactivity. Interestingly, bed rest mediated cardiac atrophy (Dorfman et al., 2007) occurred in parallel in volunteers enrolled in WISE study can underline reliability of hyperhomocysteinemia as marker of cardiovascular risk.

Previously published evidences suggested that plasma homocysteine levels are directly related not only to oxidative stress, but also to inflammation increase in coronary heart disease patients (Jonasson et al., 2005): this data are in accordance with results discussed above, showing enhanced inflammation after prolonged bed rest period.

Published evidences showing sedentary lifestyle is associated to an upregulation of homocysteine concentrations in plasma, independently from genetic factors, sex and age or from dietary intake of folates (Dankner et al., 2007; Nygard et al., 1995) support and confirm present results.

Accordingly, a program of six months resistance training reduced blood homocysteine levels in both lean and obese adult subjects (Vincent et al., 2006). Noteworthy, published data showed physical exercise can reduce cardiovascular risk lowering endothelial damage performed by inflammation, and oxidative stress (Zoppini *et al.*, 2006).

Effects of infusion of amino acid, insulin and glucose mixture on homocysteine metabolism.

In WISE study design, effect of intravenous infusion of amino acid mixture, glucose and insulin (see methods) was assessed before and after the experimental bed rest period. As expected, methionine concentrations significantly increased both before and after bed rest as a consequence of amino acid mixture intravenous administration as methionine was contained at relatively high concentrations in the infused solution: bed rest mediated increase of the upregulation of methionine concentration after intravenous infusions, can be ascribed to lowered stimulation of protein synthesis due to insulin resistance mediated by inactivity (Biolo et al., 2004).

On the contrary, intravenous glucose, insulin and amino acid infusion decreased cysteine concentrations in the ambulatory condition, but not after bed rest. Cysteine concentrations in infused mixture were low, so effect of infused insulin can explain observed results. Insulin mediated enhancement of plasma amino acid uptake for protein synthesis could explain observed decrease of cysteine concentrations in baseline conditions: low cysteine concentration in administered amino acid mixture possibly failed to sufficiently compensate amino acid uptake mediated by insulin. Otherwise, reduced uptake for protein synthesis linked

to inactivity mediated insulin resistance could explain why cysteine concentrations were not significantly reduced after intravenous infusions performed after bed rest.

Upregulation of homocysteine availability can be considered as a consequence of increased methionine concentrations. Homocysteine upregulation occurred only after bed rest probably because immobility was shown to significantly impair homocysteine remethylation and clearance by remethylation. Moreover, bed rest significantly reduced remethylation changes induced by intravenous infusions.

The proinflammatory and pro-oxidant effect of bed rest: conclusions

Results collected in Valdoltra 2006, 2007 and 2008 studies as well as in WISE study demonstrate and confirm that physical inactivity can increase inflammation and oxidative stress. Inflammation was shown to be increased in terms of changes in fatty acid membrane composition, while systemic oxidative stress was shown to be upregulated by increased availability of plasma homocysteine. Inflammation and oxidative stress are normally linked together to play a negative role in pathogenesis of several diseases. As already mentioned, they are both considered as mechanistic causes of metabolic syndrome, cardiovascular diseases and diabetes (Pradhan *et al.*, 2001; Hansson, 2005; Alberti *et al.*, 2006). Oxidative stress has been, moreover, recently recognized to be involved in selected diseases as for example cancer or chronic obstructive pulmonary disease (Moyle and Reid, 2007). Thus, bed rest, a frequent care approach in several pathologies, could play a worsening role in disease outcome. Otherwise, physical exercise can play a beneficial effect on both inflammation and oxidative stress induction. Evidences derived from diabetic animal models show that, independently from changes in glycemia, moderate exercise can reduce activities of factors triggered by oxidative stress as caspase-3 and 8 as well as the expression of proinflammatory markers as tumor necrosis factor alpha (Ghosh *et al.*, 2009). Moreover, the same work showed that exercise increased scavenging efficacy of antioxidant systems, thus reducing oxidative stress occurrence. Other evidences (Di Francescomarino *et al.*, 2009) demonstrated that regular moderate exercise can protect endothelial system from the damaging actions of oxidative factors and of inflammation. A previously published study showed that regular and moderate physical exercise was linked to cardioprotection as evidenced by improved hemodynamic parameters and by analysis of coagulation factors: such positive effect was shown to be dependent from reduced inflammation mediated by exercise (Abramson and Vaccarino, 2002). Thus, an early patient remobilization can be hypothesized as an optimal choice in care strategy of different pathologies.

Impact of physical inactivity on glutamine metabolism

Glutamine is a conditionally essential amino acid released from skeletal muscle and taken up by different tissues. Glutamine provides fuel for rapidly dividing cells as well as precursors for gluconeogenesis, DNA synthesis and urinary ammonia (Curthoys and Watford, 1995). Its availability is important for sustaining immune function (Ardawi and Newsholme, 1982) and depletion of free glutamine pools often occurs in pathological conditions as a consequence of accelerated whole body uptake and/or insufficient appearance from skeletal muscle (Biolo et al., 2000; Biolo et al., 2005b; Bongers et al., 2007). In critically ill patients, glutamine supplementation can improve clinical outcome (Bongers et al., 2007). Many illnesses are inevitably associated to bed rest and muscle unloading. In animal models muscle free glutamine concentration was shown to decrease following unloading (Jaspers et al., 1989).

Impact of bed rest on glutamine kinetics

The impact of physical inactivity on glutamine kinetics was assessed within the STBR-IP study. Obtained results principally demonstrate a down-regulation of glutamine kinetics occurring in short-term bed rest independently from changes in whole body protein turnover. Results regarding whole body protein turnover were previously published (Biolo et al., 2004): kinetics were determined by the by $[1-^{13}\text{C}]$ leucine tracer data and the reciprocal pool model. In the basal postabsorptive state, leucine kinetics, as index of whole body protein turnover was shown to be not substantially affected by bed rest. Interestingly, bed rest mediated a reduction of glutamine rate of appearance and *de novo* synthesis, while glutamine release from proteolysis and glutamine clearance were left unaffected. The parallel decrease displayed by plasma glutamine concentrations further confirms the effect of inactivity on glutamine appearance from tissues. These are novel results adding new insights to glutamine metabolism during the inactive state. The relationship between immobility and glutamine metabolism has not been investigated in humans before. In animals, it is known that hind limb suspension or muscle denervation decreases glutamine and ammonia levels (Jaspers et al., 1989) as well as glutamine synthetase activity (Hundal et al., 1990). In addition, free muscle glutamine levels of rats exposed to 7 days of weightlessness were decreased (Steffen and Musacchia, 1986). Mechanisms involved in the suppression of glutamine production in human or animal inactive muscle may include reduced availability of precursors. Glutamine is synthesized from glutamate and free ammonia. Glutamate carbon skeleton derives from the tricarboxylic acid cycle intermediate α -ketoglutarate, while nitrogen mainly derives from BCAA transamination. During bed rest muscle energy requirements are reduced as a consequence of lowered energy

expenditure for physical exercise. Status of tricarboxylic acid cycle intermediates in unloaded muscle was found to be down-regulated (Berg et al., 1993) leading to lowered α -ketoglutarate conversion to glutamate. Intensive muscle contractions are known to produce free ammonia (Meyer and Terjung, 1979) as a result of adenylate deaminase action on an actively synthesized adenosine monophosphate (AMP). For such reasons, during physical inactivity lowered production of AMP and free ammonia can be hypothesized. Free ammonia released as a consequence of ATP conversion to AMP is partially utilized in the glutamine synthetase reaction that leads to glutamine synthesis from glutamate. Consequently, lowered ammonia appearance could contribute to the reduction of glutamine synthesis rate observed after 14 days of bed rest. Glutamine synthetase is an important factor controlling glutamine *de novo* synthesis and the regulation of this enzyme was found to be lowered in an animal model of unloading (Hundal et al., 1990). In present results, bed rest impact on glutamine *de novo* synthesis was assessed at the whole body level without distinguishing between different source tissues. Nonetheless, glutamine rate of appearance has been consistently demonstrated to derive in its major part from skeletal muscle (Jaspers et al., 1989; Steffen and Musacchia, 1986; Berg et al., 1993). In a previous study (Biolo et al., 1995a), the relative contribution of skeletal muscle to whole body glutamine appearance was assessed by combining tracer infusion with the leg arteriovenous balance technique in healthy young volunteers. Results showed that skeletal muscle accounts for 40–50% of total glutamine appearance in physiological conditions (Biolo et al., 1995a). Other tissues contributing to whole body glutamine appearance include liver, lung, adipose tissue and kidney (Frayn et al., 1991; Abcouwer et al., 1995; van de Poll et al., 2004). Regarding the present study, skeletal muscle contribution to whole body glutamine appearance may be speculated to play a key role in glutamine decrease mediated by prolonged inactivity.

Inactivity as well as exposure to microgravity (Sonnenfeld et al., 2003) is known to be associated with alterations in the immune system (Kanikowska *et al.*, 2008) potentially leading to lowered immune cell proliferation and reactivity. Lowered glutamine availability presently observed as a consequence of inactivity, is in accordance with such published data, and could be speculated to participate in mechanisms leading to such immunosuppressant effect. Severe glutamine depletion can occur also in the critically ill patient (Biolo *et al.* 2000; Biolo *et al.* 2005; Bongers *et al.* 2007) and bed rest is a common situation during severe illness: this underlines the importance of understanding glutamine turnover regulation during physical inactivity itself. This study suggests that inactivity could worsen illness condition in

critical patients (Biolo *et al.* 2000) contributing to glutamine depletion by impairing *de novo* synthesis.

Differential effect of physical exercise and inactivity on glutamine metabolism.

In contrast to inactivity, well trained athletes showed higher plasma glutamine concentration at rest when matched to sedentary people (Rowbottom *et al.* 1997; Kargotich *et al.* 2007) and are characterized by ameliorated immune defences. Several publications show the relationship existing between glutamine concentrations and immune functions in athletes (Newsholme & Parry-Billings, 1990). As previously reported (Nieman & Pedersen, 1999), beneficial effects on immune system linked to moderate exercise training could derive from summation of positive effects of each single exercise bout. Significantly higher glutamine concentrations were, in fact, observed after single exercise bouts (Poortmans *et al.* 1974; Parry-Billings *et al.* 1992) as a consequence of higher synthesis rate and release from muscle (Bergström *et al.* 1985). So, physical inactivity reduces glutamine *de novo* synthesis, while optimal exercise training ameliorates plasma glutamine status in virtue of a positive glutamine balance instauration. In contrast to well trained athletes, overtraining is characterized by decreased plasma glutamine and systemic inflammatory response (Rowbottom *et al.* 1996). In this condition, an accelerated glutamine clearance by immune cells could be hypothesized (Rowbottom *et al.* 1996) (see Figure 7).

Principle and technique.

The main objective of this study was to assess whole body glutamine *de novo* synthesis. ¹⁵N-amide glutamine was used therefore to trace glutamine synthesis from glutamate. The first step of glutamine catabolism involves loss of amide nitrogen into the ammonia pool leading to glutamate synthesis. This latter amino acid can be transformed into α -ketoglutarate to enter the tricarboxylic acid cycle or reaminated to glutamine. ¹⁵N-amide tracer recycling among amino acids during prolonged L-[5-¹⁵N]glutamine infusion was previously shown to be not significant (Biolo *et al.*, 1995a). Oxidative glutamine utilization can be assessed by labelling the carbon skeleton of the amino acid. In postabsorptive humans, about 40% of glutamine appearing in the bloodstream is oxidized. This proportion increased to about 60% when the labelled amino acid was infused via the nasogastric route (Bourreille *et al.*, 2004). The physiological significance of plasma glutamine kinetic assessment based on isotopic tracers has been the subject of recent debate. Different experimental conditions led to contradictory conclusions about the potential of plasma glutamine turnover to reflect intracellular metabolism (Biolo *et al.*, 1995a) or simply transmembrane exchange (Darmaun *et al.*, 1986;

van Acker et al., 1998). Reasonably present kinetic results can reliably reflect intracellular glutamine metabolism for the following reasons. First, this approach was previously shown to allow the achievement of tracer steady state condition not only in plasma (see Figure. 18) but also in skeletal muscle cytoplasm (Biolo et al., 1995a). Second, bed rest mediated decreases in glutamine Ra were paralleled and confirmed by decreases in plasma glutamine concentrations. Finally, infusion of amino acid mixture not containing glutamine influenced glutamine rate of appearance, despite potential uncertainties in intracellular steady state achievement during short term metabolic perturbation. This indicates that the isotopic glutamine tracer approach can be considered as a suitable tool to assess changes in intracellular glutamine kinetics.

Impact of experimental bed rest on muscle atrophy

Several previous works showed that physical inactivity can significantly induce postural muscle atrophy (Kawakami et al., 2000; Akima et al., 2005) especially of lower limb (Alkner and Tesch, 2004; Berry et al., 1993). Experimental evidence obtained in animal models demonstrate sharp induction of oxidative stress during unloading, as assessed by upregulation of oxidative stress markers and by activation of antioxidant system (Kondo et al., 1994; Lawler et al., 2003; Kondo et al., 1992) aimed to scavenge oxidized substrates (Lawler et al., 2003). As reviewed by Scott K. Powers (Powers et al., 2005; Powers et al., 2007), oxidative stress was shown, in animal models, to induce muscle atrophy: upregulation of intracellular pathways triggering upregulation of specific proteases and of apoptosis were demonstrated to determine such outcome. Muscle wasting is an important clinical feature of several chronic diseases and oxidative stress has been recognized as pathogenetic factor in such process (Moylean and Reid, 2007). Immobility is frequently linked to acute and chronic diseases. The role of oxidative stress in inactivity mediated induction of muscle atrophy was not previously investigated in humans.

Results obtained within Valdoltra bed rest studies 2006 and 2007, confirm experimental bed rest can induce muscle atrophy in terms of changes in fat free mass determined by bioimpedance and in muscle thickness measured by ultrasonography. Bioimpedance is a validated approach to assess changes in body composition (Lim et al., 2009). Muscle thickness reduction is a direct index of muscle atrophy, validated also in other model of muscle wasting (Pinet *et al.*, 2004). As previously published (Tauler et al., 2006), present results show enhanced atrophy of the antigravity muscle *vastus lateralis* after prolonged bed rest in terms of decreased *vastus lateralis* thickness. Moreover, additional muscle

ultrasonography results show bed rest reduces pennation angle, i.e. orientation of muscle fibres with respect to aponeurosis. Pennation angle was previously demonstrated to be inversely correlated to muscle atrophy (Morse et al., 2005; Narici and Cerretelli, 1998; Reeves et al., 2002; de Boer et al., 2007). For such reasons, the decrease in pennation angle we observed can be considered as a strong marker of muscle atrophy occurrence. Thus, muscle atrophy induction mediated by 35 days of experimental bed rest was presently confirmed by analysis of two related indexes of muscle shape and architecture. Interestingly, additional results obtained by other research groups within the same experimental frame confirmed bed rest mediates atrophy in antigravity muscles as *gastrocnemius medialis*, while non-antigravity *tibialis anterior* and *biceps brachii* were not significantly affected (de Boer et al., 2008). Muscle atrophy induction was additionally assessed within the same experimental design as changes of another reliable index: cross sectional area of muscle fibres (Dalla et al., 2009). In particular, authors described a 18% reduction in fibre cross sectional area after 35 days of bed rest. In relation to these results showing muscle bulk reduction after bed rest, additional publications involving subjects of Valdoltra studies showed that contractile parameters (velocity of contraction) of selected muscles were significantly impaired by bed rest (Pisot et al., 2008). Moreover, similar data obtained by tensiomyography demonstrated that muscle stiffness was reduced after 35 days of bed rest (Pisot et al., 2008).

Mechanisms of muscle atrophy induction triggered by inactivity

In the present work, relationships between muscle atrophy and changes in oxidative status were investigated. An increase in protein carbonylation in atrophying muscles was observed by oxyblot analyses as a consequence of 35 days of bed rest: this reveals enhanced muscle fiber damage mediated by oxidative stress in *vastus lateralis*.

Carbonyl derivatives are formed by an oxidative amino acid modification (Requena et al., 2003). As protein carbonylation occurs following ROS action on carbon groups altering enzyme structure and activity (Stadtman, 2001), this posttranslational modification was, previously demonstrated to be a reliable marker of oxidative stress occurrence (Greilberger et al., 2008). Carbonylation is an irreversible oxidative process (Dalle-Donne et al., 2003): to avoid accumulation of damaged peptides, carbonylated proteins are efficiently scavenged by proteolytic degradation (Dukan et al., 2000; Bota and Davies, 2002; Grune et al., 2003): this suggests carbonylation can trigger or sustain muscle atrophy. Noteworthy, in this thesis a linear inverse relationship between changes in protein carbonylation and *vastus lateralis* thickness was shown, strongly suggesting that oxidative stress induced carbonylation in human muscle is one of the possible pathways leading to muscle atrophy. Other mechanisms

triggering protein degradation and subsequent muscle atrophy involve caspases, calpain (Du et al., 2004; Goll et al., 2003; Tidball and Spencer, 2002) and ubiquitin proteasome system regulation ((Furuno and Goldberg, 1986; Ikemoto et al., 2001; Purintrapiban et al., 2003). ROS production is known to affect activity of caspases and calpain, principally influencing calcium availability (Siems et al., 2003; Primeau et al., 2002). Moreover, oxidative stress, can upregulate E3 ubiquitin ligases as muscle atrophy F-box/atrogen1 and muscle ring finger-1 in myotubes (Li et al., 2003): these alterations could enhance skeletal muscle proteolysis and atrophy (Bodine et al., 2001a). Inactivity can trigger oxidative stress in muscle by interaction of at least five different oxidant production pathways (Kondo et al., 1993) involving xanthine oxidase, NOS activity (Kondo et al., 1993), reactive iron (Kondo et al., 1992), NADPH oxidase (Javesghani et al., 2002) and minor contribution of mitochondrial superoxide radicals (Muller et al., 2007). In the present experimental design, factors involved in all different pathways linking inactivity to muscle atrophy through oxidative damage could not be measured due to sampling limitations characterizing studies performed in human healthy volunteers. Nevertheless, data obtained from muscle biopsy showed that enhanced carbonylation, as peculiar oxidative damage leading to protein wasting, was increased in unloaded muscles. This event was directly related to muscle atrophy induction as measured by ultrasonography. Additionally, whole body oxidative stress was shown to be induced by inactivity in terms of upregulated homocysteine availability: even though this alteration failed to be related to worsened muscle atrophy, a possible contribution can not be excluded. Complexity of pathways linking inactivity to oxidative stress induction and to subsequent upregulation of proteolysis rate requires additional human studies to completely understand mechanisms underlining this process.

Antioxidant defense activation in atrophying muscles during experimental bed rest.

When excessive ROS production occur, antioxidant system activation is triggered (Pastore et al., 2003) in order to limit the final damage on biological substrates. Between several enzymic and non-enzymic mechanisms activated to reduce oxidative damage, the glutathione system is quantitatively the most important antioxidant in muscle (Dobrowolny *et al.*, 2008). This tripeptide is synthesized in order to scavenge hydroperoxides by self-oxidation and dimerization. Physiological conditions associated to increased ROS production lead to increased glutathione availability (Biolo et al., 2008; Ji et al., 1992). Thus, kinetic assessment of glutathione pool effectively monitors oxidative stress onset.

By the novel and validated (see below) one-sample and double isotopic tracer infusion approach we assessed bed rest impact on muscle glutathione synthesis rate. Our data

demonstrate that after 33 days of bed rest, muscle glutathione synthesis is significantly increased. This underlines that in humans, unloading can enhance muscle glutathione antioxidant system activity: such effect can be strongly hypothesized to be a response to an increased reactive oxygen species production. Previous animal studies yielded conflicting results about physical inactivity effect on muscle glutathione regulation. Glutathione concentrations were, in fact, shown to be negatively affected in rat unloaded muscle (Ikemoto *et al.*, 2002b) and activities of glutathione system key enzymes as glutathione reductase and glutathione peroxidase were demonstrated to be increased (Sen *et al.*, 1992), unaltered or downregulated (Tauler *et al.*, 2006). Reasons leading to such a wide range of results can be ascribed to model and experimental design differences. Glutathione assays in muscle biopsies can be theoretically biased by presence of red blood cells within explanted fibres. This is an intrinsic limitation for muscle studies but, in this work, its impact was strongly minimized by accurate cleaning of each biopsy.

In the present results, interestingly, an inverse correlation existing between glutathione synthesis rate and muscle pennation angle was observed: this suggests a link between muscle antioxidant potential and atrophy degree. Taking together this observation and the correlation existing between bed rest mediated changes in muscle oxidation level and muscle thickness, we can strongly suggest that high glutathione synthesis rate in human muscle can protect from atrophy by reduction of protein oxidation levels. So, maintenance of glutathione availability in muscle fibres of healthy inactive volunteers should be considered as a homeostatic response to counteract, at least partially, muscle atrophy (Gherghel *et al.*, 2005). Decreased glutathione level and increased oxidative stress are implied in a wide range of critical illness (Biolo *et al.*, 2007a) influencing clinical outcome (Crimi *et al.*, 2006), symptoms and severity (Najim *et al.*, 2007). Diseases downregulating muscle antioxidant systems are associated to cachexia (Laviano *et al.*, 2007). Conversely, an increased bioavailability of glutathione precursors as cysteine or N-acetyl-cysteine was previously published to ameliorate glutathione system scavenging action (Badaloo *et al.*, 2002) and to reduce, in an animal model, muscle protein ubiquitination (Ikemoto *et al.*, 2002a). Thus, dietary glutathione precursor supplementation is likely to ameliorate clinical conditions of muscle wasted critically ill patients.

Involvement of inflammation in inactivity mediated atrophy.

Oxidative stress is not the only mechanism leading to muscle atrophy. Inflammation, in fact, is known to play an active role in this process. Tumor necrosis factor alpha, a known mediator of inflammation, was shown in cellular model to increase total protein loss, total ubiquitination and activation of NF- κ B: the process was demonstrated to be triggered by

endogenous production of ROS (Li et al., 1998; Kramer and Goodyear, 2007). Similar evidences derive from other published works showing that diabetes induction by streptozotocin in rats, is paralleled by soleus muscle atrophy with activation of NF- κ B. Noteworthy, inflammation is considered to be a pathogenetic factor of diabetes (Duncan and Schmidt, 2006) and streptozotocin induces inflammation and oxidative stress (Rossi *et al.*, 2006): thus NF- κ B mediated muscle atrophy observed in diabetic rats is likely to be triggered by inflammation and oxidative stress. Additionally, sarcopenia and tissue damage associated to chronic obstructive pulmonary disease was linked to increased levels of circulating tumor necrosis factor alpha (Remels et al., 2007; Langen and Schols, 2007). Interestingly, another circulating proinflammatory factor - leptin, was shown to induce ROS production increasing fatty acid oxidation: this could contribute to insulin resistance and enhanced inflammation characterizing hyperleptinemic and sarcopenic subjects (Yamagishi et al., 2001). Implication of inflammation in muscle atrophy progression described in different models of diseases, allow to hypothesize that increased levels of proinflammatory markers mediated by bed rest, as discussed above and previously published (Bosutti et al., 2008), can be considered as an additional mechanism leading to enhanced muscle atrophy.

Role of physical exercise on muscle morphology and on regulation of inflammation and oxidative stress.

Abovementioned evidences showing muscle atrophy secondary to inactivity can be caused by oxidative stress and inflammation are further confirmed by data deriving from measurements performed in physically active subjects and athletes.

Strength and resistive training are known to increase muscle mass especially in male athletes, when compared to females (Melnyk *et al.*, 2009). Additionally, in elderly patients affected by sarcopenia, resistive training was shown to ameliorate, locomotor efficiency and changes in pennation angle of fibres (Narici and Maganaris, 2006). In parallel, other scientific evidences show that moderate training in rats can reduce muscle oxidative damage (Greathouse *et al.*, 2005). Moreover, in rats, ameliorated antioxidant efficiency was induced by exercise in selected muscles and as function of activity duration (Powers *et al.*, 1994). Other evidences showed prolonged moderate exercise can reduce plasma markers of systemic inflammation (Abramson and Vaccarino, 2002). Additionally, recent data obtained in animal models show that a period of moderate intensity aerobic training downregulates skeletal muscle production of cytokines involved in the onset, maintenance and regulation of inflammation (Lira et al., 2009). Thus, such published data strongly suggest that moderate exercise can protect from muscle atrophy by reduced inflammation and oxidative stress: this strengthen present results

demonstrating, in humans, the role of inflammatory and oxidative stress in muscle atrophy induction after experimental physical inactivity.

Novel single-sample method to determine peptide synthesis by constant tracer infusions: validation.

The core parameter for traditional determination of peptide FSR is the assessment of product enrichment changes over time during continuous infusion of isotopic precursor tracer (Wolfe, 2004). When precursor incorporation into product is linear, FSR can be determined evaluating at least two single product enrichments in two separate biological samples taken at different times. Muscle glutathione synthesis rate was measured in a single tissue sample applying a modified precursor-product approach. Isotopic glycine was chosen as tracer (Jahoor et al., 1995) and our novel method was based on performing two parallel and separate infusions of different isotopes, [$^2\text{H}_2$]glycine and [^{15}N]glycine, starting at different times (four hours shift). In this way, as shown in “Calculations” section, the difference between L-[^{15}N]glutathione and L-[$^2\text{H}_2$]glutathione enrichments measured in a single final biological sample, allowed FSR assessment. Linearity of precursor incorporation into the final product was previously (Biolo et al., 2008) shown. To directly validate in human red blood cells the “one-sample-double tracer” approach to measure glutathione kinetics in human muscle, several blood samples were drawn throughout each metabolic study. Glutathione FSR in red blood cells was assessed in two different ways: by the traditional approach (Eq. 8) and by the novel approach (Eq. 7) designed for muscle assay thus involving both tracer infusions and only the final blood sample (see “Calculations”). Comparing results obtained in red blood cells by both approaches, “traditional” and “one-sample” methods yielded comparable results. Additionally, validation of the present new method is confirmed by the high correlation existing between FSR values obtained by traditional and novel approaches. Reliability of this new approach is further confirmed by analyses shown in Figure 16 that underline the correlation between FSR changes from baseline to day 7 and from day 7 to day 33 measured by traditional approach with the same changes measured by one-sample equation. Finally, results fully demonstrate the reliability of this method to assess *in vivo* FSR and changes from baseline status.

Absolute values of tracers steady-state enrichments were different: [^{15}N]glycine enrichment was displayed to be higher than [$^2\text{H}_2$]glycine. Such a tendency for ^{15}N isotopes to reach higher steady state enrichments in comparison to ^2H isotopes was previously published for alanine tracer (Yang *et al.*, 1984). Alanine shares metabolic pathways with glycine, so that published evidences can explain obtained differences in tracer steady state enrichments. However, this peculiar feature characterizing [^{15}N]glycine, fails to affect reliability of our FSR estimation as

ratios between isotopic product and related tracer enrichments were introduced to exclude direct influences of precursor kinetics. In addition, as explained above, our method could be validated against the well assessed traditional one by separate correlation analyses. Two assumption were necessary to build our new equation. (i) While steady-state condition for [$^2\text{H}_2$]glycine enrichments was directly assessed three hours after infusion beginning (see Figure 14), it was assumed for [^{15}N]glycine after the same time of an equal infusion. This seems to be acceptable by itself but also as [^{15}N]glycine was previously published to reach steady state in plasma within, or even before, the third hour of infusion in similar conditions (Cryer *et al.*, 1986). (ii) Tracer incorporation into the final product can be considered as not affected by isotopic labelling as this chemical feature of precursors is known not to influence product tracer incorporation itself (Wolfe, 2004). Principally for such reasons, differences in precursor isotopic labelling should not reasonably affect respective tracer uptake into the final product.

The utility to assess protein or peptide FSR in a single sample stems from drawbacks linked to multiple sample collection. During investigations on small animals, the first tissue biopsy can determine animal's death, while in bigger animals or in humans multiple tissue sampling can lead to inflammatory process activation. For the same reasons and for clear ethical implications, complex metabolic studies requiring multiple tissue sampling are impossible to be performed during surgery in human subjects. Approaches aimed to measure peptide FSR in a single biological sample were previously published. Dudley *et al.* employed for the first time a multiple-tracer and single-sample method (Dudley *et al.*, 1998). The protocol was based on six staggered and overlapping isotopomer infusions. FSR was obtained designing *a posteriori* an enrichment curve. Protocol validation was indirectly performed by comparing plasma free amino acid turnover rates. An analogous technique aimed to measure muscle protein fractional breakdown rate (FBR) and FSR in a single muscle biopsy, has been also proposed by Zhang *et al.* (Zhang *et al.*, 2002). In this method, three pulse tracer injections of three different isotopic amino acid precursors were staggered at different time points. Authors demonstrated that three different enrichment assessed in a unique final muscle biopsy allowed FBR evaluation. Presently, a new single sample method was directly validated in human subjects involving only two separate isotopic infusions to reliably assess a peptide FSR: this approach simplifies calculations and reduces technical workloads as well as economical expenses.

Impact of nutrition on inflammation, oxidative stress and muscle atrophy.

Results shown in this work confirmed physical inactivity coherently upregulate oxidative stress at whole body level as well as in muscle: in particular, oxidative stress, together with inflammation, was strongly suggested to play a causative role on atrophy induction. Upregulated concentrations of homocysteine were, in fact, considered as marker of enhanced whole body oxidative stress. Moreover, enhanced muscle protein carbonylation and glutathione synthesis were strictly associated to muscle atrophy induction following experimental inactivity. Physical inactivity was also shown to upregulate whole body inflammation as displayed by changes in n-3 and n-6 fatty acid composition of red blood cell membrane: a link between whole body inflammation and muscle atrophy can be reliably hypothesized.

Sedentary lifestyle and immobility are associated to lowered energy requirements (Ritz et al., 1998). Excessive energy intake during long term period of inactivity can lead to obesity and to related drawbacks (Chaput and Tremblay, 2009). This suggest that dietary energy intake control during muscle unloading can be crucially important to compensate physical inactivity related consequences. Published evidences showed, in fact, that calorie restriction during experimental bed rest can worsen muscle atrophy progression (Biolo et al., 2007b): on the contrary, the impact of fat gain on inactivity mediated alterations was poorly investigated.

Dietary macronutrient composition can also play a role during inactivity. The role of protein supplementation on muscle atrophy during bed rest in healthy volunteers was previously investigated. Results displayed that supplementation with branched chain amino acids can attenuate protein loss during bed rest (Stein et al., 1999c), even though this intervention is not sufficient to ameliorate muscle atrophy (Brooks *et al.*, 2008). Nevertheless, evidences showed amino acid supplementation can protect muscle function when catabolism is enhanced by stressful conditions (Fitts *et al.*, 2007). Interestingly, protein and amino acid supplementation were, previously shown to ameliorate myocardial remodeling mediated by experimental bed rest (Dorfman et al., 2007).

Impact of protein supplementation on homocysteine metabolism.

The impact of bed rest and dietary protein supplementation on homocysteine kinetics was presently investigated. As shown in results obtained within the WISE study, a diet enriched in protein and amino acids during inactivity can blunt and reverse detrimental effects of muscle unloading on plasma concentrations and kinetics of homocysteine. While, in fact, bed rest in association with an adequate protein intake diet, increased homocysteine concentrations, the administration of a diet enriched in protein and amino acid content during bed rest,

significantly reduced homocysteine concentration at the end of the experimental period. This strongly suggests that amino acid supplementation during bed rest can effectively reduce cardiometabolic risk connected to inactivity, preventing whole body oxidative stress mediated by high levels of plasma homocysteine. Noteworthy, transsulfuration rate was shown to be significantly enhanced by bed rest both in subjects fed by a standard diet and by a high protein and amino acid intake diet; nevertheless, dietary supplementation significantly interacted with inactivity to induce a major increase in transsulfuration rate when matched to standard diet. In parallel to observed changes in transsulfuration kinetics, rates of remethylation and transmethylation were respectively decreased and increased by bed rest but no interaction with diet could be demonstrated: such evidences provide a convincing metabolic explanation of observed changes in plasma homocysteine concentrations secondary to dietary intervention. Analysis of relative changes in homocysteine clearance mediated by bed rest in association to the different dietary regimens further confirms such observations: high dietary protein and amino acid intake, in fact, significantly enhanced clearance by transsulfuration while failed to affect clearance by remethylation.

Previously published evidences suggest that in ambulatory conditions, artificially induced increase of animal proteins or methionine, even though determining a transient increase in homocysteine concentration, can stimulate transsulfuration pathway (Ueland and Refsum, 1989). A recently published work showed in caged rats that nutritional supplementation with casein and amino acids determined a significant decrease of plasma homocysteine concentrations and that such effect was mediated by a stimulation of transsulfuration pathway, as evidenced by enhanced CBS activity (Ohuchi et al., 2009). Amino acid intake is, in fact, known to differentially affect homocysteine metabolism, principally modulating a key regulator as S-adenosylmethionine. S-adenosylmethionine is a crucial factor in transmethylation reaction as methyl donor (Loenen, 2006) and when this pathway is activated S-adenosylmethionine can inhibit remethylation rate reducing the activity of MTHFR. To prevent accumulation of homocysteine S-adenosylmethionine can stimulate transsulfuration enhancing the activity of cystathionine β -synthetase and γ -cystathionase (Finkelstein, 1998; Finkelstein and Martin, 1984). On the contrary, in conditions of normal or reduced protein availability, homocysteine is preferentially catabolized by MTHFR dependent remethylation (Ueland and Refsum, 1989). Such published data could provide a mechanistic explanation for present results showing enhanced homocysteine clearance by transsulfuration after prolonged administration of high protein and amino acid diet. Present results, moreover, strongly suggest that high protein and amino acid feeding can interact with bed rest condition to blunt

hyperhomocysteinemia determined by physical inactivity itself. Thus, this dietary treatment can be considered as a countermeasure to a condition linked to hyperhomocysteinemia as physical inactivity: such intervention, moreover, could potentially reduce whole body oxidative stress and cardiovascular risk. As abovementioned, in this study the impact of folate levels on homocysteine metabolism can be excluded as folate intake was strictly controlled and maintained constant (see “Methods”): this strongly suggest that observed effects are actually mediated by bed rest and dietary protein control. A recently published work demonstrated that supplementation of sulphur amino acids in healthy subjects can reduce protein carbonylation and can ameliorate antioxidant response both at muscle and plasma level (Zembron-Lacny et al., 2009): this further confirm that protein supplementation can interfere with oxidative stress induction at whole body and muscle level.

Other results (unpublished data) deriving from WISE study show that amino acid and protein enriched diet ameliorated another alteration induced by physical inactivity as insulin resistance. Strikingly, in fact, protein and amino acid supplemented volunteers showed, after bed rest, almost unaltered insulin sensitivity values when compared to pre-bed rest assessments. This strongly suggest that high protein and amino acid content in diet can fully prevent insulin resistance induction mediated by physical inactivity, confirming the beneficial role of such dietary treatment on cardiovascular risk control. Finally, as previously evidenced within the WISE study, the protective effect of high protein and amino acid diet on cardiac atrophy mediated by bed rest, provides and additional evidence of cardioprotection linked to this nutritional regimen (Dorfman et al., 2007).

Effect of energy balance

The effect of energy balance control on selected alterations mediated by physical inactivity, was presently investigated: whole body composition and thickness of the vastus lateralis muscle were monitored in healthy volunteers during Valdoltra Bed Rest 2006 (Study A) and 2007 (Study B) at different energy intake levels. To assess changes of skeletal muscle and fat-free mass two independent methods were used. Bioimpedance analysis is a validated method for the assessment of fat and fat-free mass in healthy persons; the latter measure is a close marker of whole-body muscle mass (Kyle *et al.*, 2004). In parallel, muscle atrophy assessment was directly performed by ultrasonography measurement of muscle thickness. This is a validated technique for assessing muscle size (Reeves et al., 2004; Miyatani et al., 2002). The average thickness of representative postural (vastus lateralis, gastrocnemius medialis, and tibialis anterior) and nonpostural (biceps brachii) muscles correlated directly with whole body fat-free mass, as determined by bioimpedance analysis, both before and at the end of the bed-

rest period. Bioimpedance analysis, moreover, showed that most of the subjects accumulated body fat to various degrees. Results displayed that those who gained more fat suffered the greatest loss of skeletal muscle and fat-free mass. The decrease in thickness of the vastus lateralis, a key postural and locomotor muscle, and in total-body fat-free mass after bed rest, in subjects with greater increases in body fat, was greater than the decrease measured in volunteers with smaller changes in body fat. Additionally, positive energy balance and muscle atrophy were associated with activation of the systemic inflammatory response and antioxidant defenses. To investigate effects of bed rest and energy balance on antioxidant defenses, changes in glutathione metabolism were assessed within the study A. Glutathione is highly concentrated in erythrocytes where provides both local and systemic antioxidant protection (Richards *et al.*, 1998). Its synthetic capacity, as marker of activation of antioxidant response, was assessed by Western blot determination of expression levels of catalytic and modulatory subunits of the glutamate-cysteine ligase enzyme, which catalyzes the rate-limiting step in glutathione biosynthesis (Thompson et al., 2000). The actual rate of glutathione turnover was determined by a standard approach involving primed continuous infusion of deuterated cysteine and by monitoring, by GC-MS analysis in separate sequential blood samples, the incorporation of labeled precursor into glutathione (standard approach) (Lyons et al., 2001). Glutathione function was evaluated through measurement of glutathione peroxidase activity (Paglia and Valentine, 1967). As described in results, subjects were stratified a posteriori and divided into two groups accordingly to the median value of individual changes in fat mass: subjects showing a fat mass gain greater than the median value were assigned to a group defined as higher energy balance (HEB_A). Conversely, subjects with fat mass changes lower than the median value were assigned to a group defined as lower energy balance (LEB_A)

Significant group × bed rest interactions were found for the catalytic subunit of the glutamate-cysteine ligase enzyme and the rate of glutathione turnover leading to greater bed rest-mediated increases in glutathione synthesis in the HEB_A than in the LEB_A group. Glutathione peroxidase activity did not change in the 2 groups. Present results indicate that the erythrocyte glutathione system is greatly activated during bed rest at positive energy balance, whereas bed rest at near-neutral balance is not associated with significant changes in glutathione synthesis. Evidence indicates that glutathione synthesis is up-regulated by proinflammatory mediators and oxidative stress (Lu, 1999). Significant group × bed rest interactions were found for plasma CRP and myeloperoxidase concentrations, which led to greater bed rest-mediated increases of these mediators in the HEB_A group than in the LEB_A group. Plasma CRP and

myeloperoxidase are suitable markers of activation of systemic inflammation (Podrez et al., 2000). These results strongly suggest that an activation of the erythrocyte glutathione system is part of the systemic inflammatory and oxidative stress reaction to bed rest at positive energy balance. Such inflammatory and oxidative stress response can potentially upregulate molecular pathways leading to protein degradation (Kandarian and Jackman, 2006; Powers et al., 2007) during inactivity. Moreover, as discussed above, data derived from muscle biopsies performed in Valdoltra 2007 study (study B) showed that inactivity mediated inflammation and oxidative stress in muscle can induce muscle wasting. This further confirm that changes on systemic inflammation and oxidative stress mediated by fat gain during inactivity can effectively worsen the reduction of muscle mass and fat-free mass. Interestingly, published data showed in an animal model that overfeeding can depress muscle protein synthesis (Glick et al., 1982). Thus, enhanced inflammation and oxidative stress induced, during unloading, by excessive energy intake could provide reliable explanations for worsened muscle atrophy observed in HEB group. Furthermore, an increased ghrelin response after a period of overfeeding (Robertson et al., 2004) could contribute to an enhancement of muscle catabolism (Nagaya et al., 2005).

One limitation of the present study is that, typically, only a small number of persons can be enrolled in bed-rest studies. The effect of positive energy balance on inactivity-induced muscle atrophy was assessed by combining the results of 2 bed rest studies, performed under the same experimental conditions and with similar primary outcomes. In contrast, energy level \times bed rest interactions for inflammatory markers and glutathione kinetics were assessed in 2 groups of only 5 subjects. Although extending these observations in a larger population would reinforce the present findings, it was possible to detect significant differences in key markers of inflammatory response and glutathione kinetics.

Positive energy balance (ie, energy intake in excess of requirements) leads to fat deposition, and changes in body fat mass closely reflect energy balance over the long term, even in the presence of atrophying muscles. In fact, energy density of fat tissue is 9 times that of muscle and fat-free mass (FAO/WHO/UNU, 1985). Study A subjects were allowed to spontaneously adapt to decreased energy requirement during bed rest, whereas study B subjects were provided an activity-adjusted diet (1.2 times their calculated resting energy expenditure). At the end of the bed-rest periods, 9 of 10 study A subjects and 5 of 9 study B participants had gained >0.5 kg fat mass. Thus, study A subjects failed to spontaneously adapt to a lower energy requirement, whereas, in study B, an activity adjusted diet based on traditional predictive equations, overestimated the true requirement for most subjects. Achievement of

energy balance during experimental bed rest is a difficult task that involves constant monitoring of individual energy requirements and intakes and that goal has rarely been attained in previous investigations. Results obtained in the present and in 9 previous bed-rest studies reporting positive mean changes in fat mass (Blanc et al., 2000b; Krebs et al., 1990b; Gretebeck et al., 1995; Ferrando et al., 1996; Barbe et al., 1999; Lovejoy et al., 1999; Stein et al., 1999b; Scheld et al., 2001; Shackelford et al., 2004; Olsen et al., 2008) are shown in Figure 21.

Figure 21

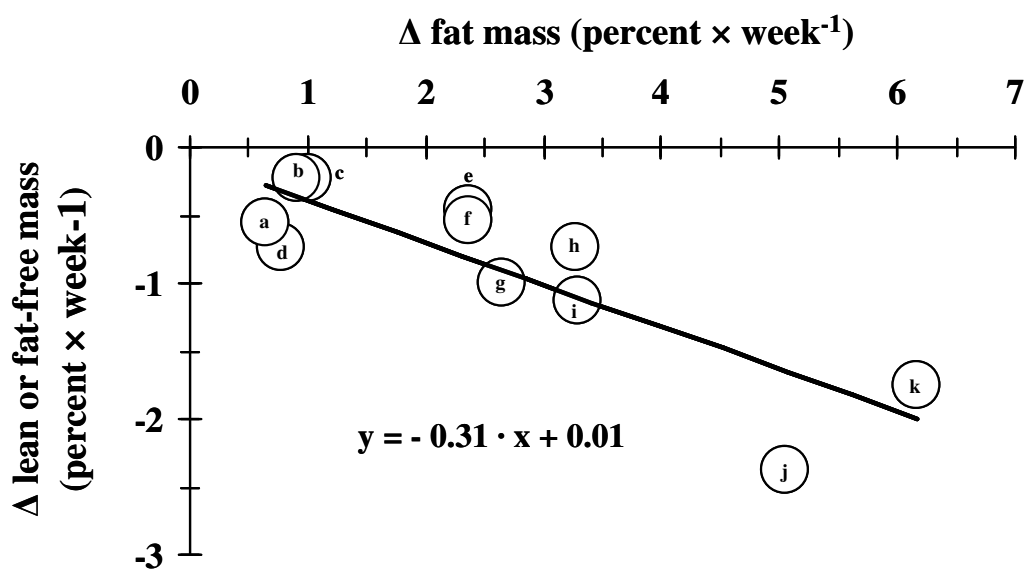


Figure 21 shows the relation between average values of absolute changes in fat mass and lean mass or fat-free mass measured in previous studies and in the present work. Letters inside (or just outside of) circles represent values from these studies: a, (Barbe et al., 1999); b, (Scheld et al., 2001); c, (Stein et al., 1999a); d, present work, lower energy balance group; e, (Blanc et al., 2000b); f, (Krebs et al., 1990b); g, (Lovejoy et al., 1999); h, (Gretebeck et al., 1995); i, present study, higher energy balance group; j, (Ferrando et al., 1996); and k, (Olsen et al., 2008).

$R=-0.85$, $p=0.001$; $n=11$

Changes in fat mass are related to changes in LBM (by dual-energy X-ray absorptiometry) or fat-free mass (by bioimpedance analysis). Gain of body fat or loss of lean (fat free) mass is expressed as weekly percentage changes from baseline. Linear regression analysis indicates an indirect relation between fat gain and lean (fat free) mass loss, regardless of the duration of

inactivity. Similar results are obtained by excluding the present study from the analysis ($r=-0.97$). Thus, there is excellent agreement between the retrospective analysis and the results from the present study, which suggest that, when energy intake is not strictly controlled during inactivity, fat mass tends to increase in parallel with LBM catabolism. The present study shows that excess fat deposition during physical inactivity is associated with greater muscle loss and greater activation of systemic inflammation and antioxidant defenses. Moreover, whole body or muscle inflammation and oxidative stress induction can potentially contribute to long-term changes in body composition and to the cardiometabolic risk observed in obese sarcopenic persons (Honda et al., 2007; Klein et al., 2007). In accordance with presently discussed stimulatory effect of positive energy balance on inflammation and oxidative stress during inactivity, previously published results obtained within STBR-IP study showed that calorie restriction could effectively reduce the concentration of proinflammatory markers (Bosutti et al., 2008). In particular, physical inactivity effectively interacted with calorie restriction to blunt and reverse inactivity mediated increases in CRP, IL-6 and IL-6 to IL-10 ratio. Coherently, inactivity and calorie restriction interacted to increase levels of the anti-inflammatory IL-10 (Bosutti et al., 2008). Thus mild reduction of calorie intake seems to play a positive and protective role on systemic inflammation. This could lead to hypothesize a beneficial effect also on muscle mass maintenance, but evidences collected in the same study design showed that hypocaloric diet during bed rest determines a significantly increased muscle atrophy associated to a greater rate of leucine oxidation, as a marker of net protein catabolism, and to slightly lowered protein synthesis, in the postabsorptive state (Biolo et al., 2007b). This effect is strictly dependent on calorie intake as protein administration was maintained constant during all study phases.

Effects of calorie restriction on glutamine and BCAA metabolism.

In the present study, bed rest suppressed glutamine synthesis despite slightly increased baseline plasma BCAA concentrations: this suggests an active role of muscle unloading in control of amino acid metabolism. In parallel, calorie restriction failed to affect bed rest mediated decreases in glutamine availability and kinetics. A significant effect of moderate energy restriction was shown only on plasma BCAA concentrations. This may be due to the fact that a 20% energy restriction obtained by reduction of fat and carbohydrate intake is not strong enough to have detectable results on glutamine kinetics. A potential effect of calorie restriction on splanchnic glutamine synthesis (Arola et al., 1981) was not evidenced in this study. At present, no comparable previous studies assessed the effect of moderate calorie

restriction on glutamine turnover. Nevertheless, previous publications demonstrated that the fasting condition can accelerate BCAA release from muscle (Fryburg et al., 1990) and reduce their metabolic clearance (Sherwin, 1978). Intravenous infusion of a glutamine-free amino acid solution, providing about 32 g of amino acids, increased glutamine *de novo* synthesis by about 14–19% in both ambulatory and bed rest conditions at eucaloric or hypocaloric energy intake. This effect can be explained by anaplerotic processes involving carbon skeletons of selected amino acids (e.g. alanine, arginine, asparagine, etc.) and by increased nitrogen availability from the BCAA. Animal studies showed that hyperaminoacidaemia can, in fact, directly increase glutamine turnover (Garber et al., 1976). In addition, a previously published work showed that muscle glutamine synthesis can be greatly stimulated in postsurgical cancer patients by infusion of a BCAA-enriched amino acid mixture (Biolo et al., 2006).

Effects of nutrition: conclusions

In this work, results collected from different studies show effects of modulation of energy balance and protein intake level on separate parameters affected by bed rest. A protein and amino acid enriched diet was shown to blunt physical inactivity mediated increases of plasma homocysteine concentrations, as marker of whole body oxidative stress, preferentially stimulating transsulfuration as catabolic pathway. This confirms that elevation of dietary protein intake during inactivity can reduce the impact on whole body oxidative damage and on cardiovascular risk, potentially lowering endothelial damage. Such evidence could be explained by animal studies showing increased amino acid and protein intake can stimulate the activity of enzymes involved in transsulfuration pathway (Ohuchi et al., 2009).

In parallel, energy balance was shown to actively affect muscle mass maintenance during inactivity. While previous publications showed that caloric restriction can enhance inactivity induced protein wasting and muscle atrophy in virtue of a reduction of energy intake (Biolo et al., 2007b), present data show a novel and unexpected achievement: positive energy balance itself can determine a significant reduction of muscle mass during bed rest. Worsened inflammatory and oxidative stress condition associated to positive energy balance during inactivity could explain the detrimental effect of fat gain on muscle mass maintenance. Interestingly, caloric restriction during bed rest was previously shown to attenuate inflammatory processes affecting the pattern of selected cytokine concentrations (Bosutti et al., 2008): as this dietary regimen worsened sarcopenia progression of volunteers (Biolo *et al.*, 2007b), involvement of additional factors in muscle mass control during inactivity can be hypothesized. Merging together such observations, dietary control of immobilized subjects

aimed to maintain neutral energy balance seems to be an eligible choice. This approach could, in fact, contemporarily minimize inactivity mediated muscle wasting and the pro-inflammatory effect of muscle unloading, leaving other parameters unaltered. The lack of effect, in fact, of calorie restriction on glutamine availability and kinetics during bed rest, suggest that energy balance should not affect the metabolism of such important amino acid. Additionally, evidences reported in this work strongly suggest that a safe supplementation of dietary proteins or amino acids can successfully blunt inactivity mediated alterations of the oxidative stress and inflammation conditions in humans, potentially contributing to counteract muscle atrophy and cardiovascular risk induction. Protein intake level could potentially interact with changes of energy balance during inactivity: at present, studies investigating the effect of a parallel control of these two nutritional parameters during experimental bed rest are lacking.

ACKNOWLEDGMENTS

Ringrazio il prof. Gianfranco Guarnieri, direttore del Dipartimento di Scienze Mediche Tecnologiche e Traslazionali, che mi ha seguito nel corso del dottorato di ricerca.

Desidero ringraziare sentitamente il prof. Gianni Biolo che nel corso di questi anni di lavoro e di studio mi ha guidato e consigliato con saggezza. Affidandomi, con fiducia, incarichi di notevole responsabilità pratica ed organizzativa ha saputo stimolare in me capacità scientifiche e professionali che difficilmente avrei potuto maturare in altri centri di ricerca.

Di seguito voglio ringraziare il prof. Igor B. Mekjavic, che mi ha seguito durante tutto il periodo di studio con interessanti scambi culturali e collaborazioni scientifiche.

Un sentito ringraziamento va indirizzato alla prof. Luisa Gorza ed il dott. Luciano Dalla Libera per la concreta collaborazione scientifica che ha contribuito alla realizzazione di questa tesi.

Un pensiero va rivolto a tutti i colleghi italiani ed stranieri con i quali ho cooperato durante questo intenso periodo di studio, con particolare riguardo a quelli “del laboratorio”: Sara, Mariella, Rossella, Barbara, Daniele, Pasquale, Cristina, Barbara, Lara, Annamaria, Paola, Anna, Gianluca e Giuseppe. Grazie per avermi supportato nei momenti difficili e “sopportato” nei momenti più ilari.

REFERENCE LIST

- Abramson, J.L. and Vaccarino, V. (2002). Relationship between physical activity and inflammation among apparently healthy middle-aged and older US adults. *Arch. Intern. Med.* 162, 1286-1292.
- Acharyya, S., Villalta, S.A., Bakkar, N., Bupha-Intr, T., Janssen, P.M., Carathers, M., Li, Z.W., Beg, A.A., Ghosh, S., Sahenk, Z., Weinstein, M., Gardner, K.L., Rafael-Fortney, J.A., Karin, M., Tidball, J.G., Baldwin, A.S., and Guttridge, D.C. (2007). Interplay of IKK/NF-kappaB signaling in macrophages and myofibers promotes muscle degeneration in Duchenne muscular dystrophy. *J. Clin. Invest* 117, 889-901.
- Aguiar, B., Rojas, J.C., and Collados, M.T. (2004). Metabolism of homocysteine and its relationship with cardiovascular disease. *J. Thromb. Thrombolysis.* 18, 75-87.
- Ahmad, R., Tripathi, A.K., Tripathi, P., Singh, S., Singh, R., and Singh, R.K. (2008). Malondialdehyde and protein carbonyl as biomarkers for oxidative stress and disease progression in patients with chronic myeloid leukemia. *In Vivo* 22, 525-528.
- Akagi, R., Kanehisa, H., Kawakami, Y., and Fukunaga, T. (2008). Establishing a new index of muscle cross-sectional area and its relationship with isometric muscle strength. *J. Strength. Cond. Res.* 22, 82-87.
- Akima, H., Katayama, K., Sato, K., Ishida, K., Masuda, K., Takada, H., Watanabe, Y., and Iwase, S. (2005). Intensive cycle training with artificial gravity maintains muscle size during bed rest. *Aviat. Space Environ. Med.* 76, 923-929.
- Alberti, K.G., Zimmet, P., and Shaw, J. (2006). Metabolic syndrome--a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet. Med.* 23, 469-480.
- Alkner, B.A. and Tesch, P.A. (2004). Efficacy of a gravity-independent resistance exercise device as a countermeasure to muscle atrophy during 29-day bed rest. *Acta Physiol Scand.* 181, 345-357.
- Altomare, E., Vendemiale, G., and Albano, O. (1988). Hepatic glutathione content in patients with alcoholic and non alcoholic liver diseases. *Life Sci.* 43, 991-998.
- Anderson, M.E. (1997). Glutathione and glutathione delivery compounds. *Adv. Pharmacol.* 38, 65-78.
- Anderson, M.E., Allison, R.D., and Meister, A. (1982). Interconversion of leukotrienes catalyzed by purified gamma-glutamyl transpeptidase: concomitant formation of leukotriene D4 and gamma-glutamyl amino acids. *Proc. Natl. Acad. Sci. U. S. A* 79, 1088-1091.
- Andersson, A., Sjodin, A., Hedman, A., Olsson, R., and Vessby, B. (2000). Fatty acid profile of skeletal muscle phospholipids in trained and untrained young men. *Am. J. Physiol Endocrinol. Metab* 279, E744-E751.
- Andersson, A., Sjodin, A., Olsson, R., and Vessby, B. (1998). Effects of physical exercise on phospholipid fatty acid composition in skeletal muscle. *Am. J. Physiol* 274, E432-E438.
- Anthony, T.G., McDaniel, B.J., Knoll, P., Bunpo, P., Paul, G.L., and McNurlan, M.A. (2007). Feeding meals containing soy or whey protein after exercise stimulates protein synthesis and translation initiation in the skeletal muscle of male rats. *J. Nutr.* 137, 357-362.
- Antonione, R., Caliendo, E., Zorat, F., Guarnieri, G., Heer, M., and Biolo, G. (2008). Whey protein ingestion enhances postprandial anabolism during short-term bed rest in young men. *J. Nutr.* 138, 2212-2216.
- Appell, H.J., Duarte, J.A., and Soares, J.M. (1997). Supplementation of vitamin E may attenuate skeletal muscle immobilization atrophy. *Int. J. Sports Med.* 18, 157-160.

- Arbeille,P., Kerbeci,P., Capri,A., Dannaud,C., Trappe,S.W., and Trappe,T.A. (2009). Quantification of muscle volume by echography: comparison with MRI data on subjects in long-term bed rest. *Ultrasound Med. Biol.* 35, 1092-1097.
- Ardawi,M.S. and Newsholme,E.A. (1982). Maximum activities of some enzymes of glycolysis, the tricarboxylic acid cycle and ketone-body and glutamine utilization pathways in lymphocytes of the rat. *Biochem. J.* 208, 743-748.
- Arola,L., Palou,A., Remesar,X., and Alemany,M. (1981). Glutamine synthetase activity in the organs of fed and 24-hours fasted rats. *Horm. Metab Res.* 13, 199-202.
- Arterburn,L.M., Hall,E.B., and Oken,H. (2006). Distribution, interconversion, and dose response of n-3 fatty acids in humans. *Am. J. Clin. Nutr.* 83, 1467S-1476S.
- Babcock,T., Helton,W.S., and Espat,N.J. (2000). Eicosapentaenoic acid (EPA): an antiinflammatory omega-3 fat with potential clinical applications. *Nutrition* 16, 1116-1118.
- Babcock,T.A., Novak,T., Ong,E., Jho,D.H., Helton,W.S., and Espat,N.J. (2002). Modulation of lipopolysaccharide-stimulated macrophage tumor necrosis factor-alpha production by omega-3 fatty acid is associated with differential cyclooxygenase-2 protein expression and is independent of interleukin-10. *J. Surg. Res.* 107, 135-139.
- Badaloo,A., Reid,M., Forrester,T., Heird,W.C., and Jahoor,F. (2002). Cysteine supplementation improves the erythrocyte glutathione synthesis rate in children with severe edematous malnutrition. *Am. J. Clin. Nutr.* 76, 646-652.
- Bagga,D., Wang,L., Farias-Eisner,R., Glaspy,J.A., and Reddy,S.T. (2003). Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. *Proc. Natl. Acad. Sci. U. S. A* 100, 1751-1756.
- Balasubramaniam,A., Wood,S., Joshi,R., Su,C., Friend,L.A., Sheriff,S., and James,J.H. (2006). Ghrelin stimulates food intake and growth hormone release in rats with thermal injury: synthesis of ghrelin. *Peptides* 27, 1624-1631.
- Bannai,S. (1984). Transport of cystine and cysteine in mammalian cells. *Biochim. Biophys. Acta* 779, 289-306.
- Baratta,M. (2002). Leptin--from a signal of adiposity to a hormonal mediator in peripheral tissues. *Med. Sci. Monit.* 8, RA282-RA292.
- Barbe,P., Galitzky,J., Thalamas,C., Langin,D., Lafontan,M., Senard,J.M., and Berlan,M. (1999). Increase in epinephrine-induced responsiveness during microgravity simulated by head-down bed rest in humans. *J. Appl. Physiol* 87, 1614-1620.
- Bautmans,I., Njemini,R., Vasseur,S., Chabert,H., Moens,L., Demanet,C., and Mets,T. (2005). Biochemical changes in response to intensive resistance exercise training in the elderly. *Gerontology* 51, 253-265.
- Beckman,K.B. and Ames,B.N. (1998). The free radical theory of aging matures. *Physiol Rev.* 78, 547-581.
- Berg,A.H., Combs,T.P., Du,X., Brownlee,M., and Scherer,P.E. (2001). The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat. Med.* 7, 947-953.
- Berg,H.E., Dudley,G.A., Hather,B., and Tesch,P.A. (1993). Work capacity and metabolic and morphologic characteristics of the human quadriceps muscle in response to unloading. *Clin. Physiol* 13, 337-347.
- Berg,H.E., Eiken,O., Miklavcic,L., and Mekjavic,I.B. (2007). Hip, thigh and calf muscle atrophy and bone loss after 5-week bedrest inactivity. *Eur. J Appl. Physiol* 99, 283-289.
- Berg,H.E., Larsson,L., and Tesch,P.A. (1997). Lower limb skeletal muscle function after 6 wk of bed rest. *J. Appl. Physiol* 82, 182-188.

- Berry,P., Berry,I., and Manelfe,C. (1993). Magnetic resonance imaging evaluation of lower limb muscles during bed rest--a microgravity simulation model. *Aviat. Space Environ. Med.* *64*, 212-218.
- Bettters,J.L., Criswell,D.S., Shanely,R.A., Van Gammeren,D., Falk,D., DeRuisseau,K.C., Deering,M., Yimlamai,T., and Powers,S.K. (2004). Trolox attenuates mechanical ventilation-induced diaphragmatic dysfunction and proteolysis. *Am. J. Respir. Crit Care Med.* *170*, 1179-1184.
- Billiar,T.R., Bankey,P.E., Svingen,B.A., Curran,R.D., West,M.A., Holman,R.T., Simmons,R.L., and Cerra,F.B. (1988). Fatty acid intake and Kupffer cell function: fish oil alters eicosanoid and monokine production to endotoxin stimulation. *Surgery* *104*, 343-349.
- Biolo,G., Agostini,F., Simunic,B., Sturma,M., Torelli,L., Preiser,J.C., Deby-Dupont,G., Magni,P., Strollo,F., di Prampero,P., Guarnieri,G., Mekjavic,I.B., Pisot,R., and Narici,M.V. (2008). Positive energy balance is associated with accelerated muscle atrophy and increased erythrocyte glutathione turnover during 5 wk of bed rest. *Am. J. Clin. Nutr.* *88*, 950-958.
- Biolo,G., Antonione,R., and De Cicco,M. (2007a). Glutathione metabolism in sepsis. *Crit Care Med.* *35*, S591-S595.
- Biolo,G., Ciocchi,B., Lebenstedt,M., Barazzoni,R., Zanetti,M., Platen,P., Heer,M., and Guarnieri,G. (2004). Short-term bed rest impairs amino acid-induced protein anabolism in humans. *J. Physiol* *558*, 381-388.
- Biolo,G., Ciocchi,B., Stulle,M., Bosutti,A., Barazzoni,R., Zanetti,M., Antonione,R., Lebenstedt,M., Platen,P., Heer,M., and Guarnieri,G. (2007b). Calorie restriction accelerates the catabolism of lean body mass during 2 wk of bed rest. *Am. J. Clin. Nutr.* *86*, 366-372.
- Biolo,G., Ciocchi,B., Stulle,M., Piccoli,A., Lorenzon,S., Dal,M., V, Barazzoni,R., Zanetti,M., and Guarnieri,G. (2005a). Metabolic consequences of physical inactivity. *J. Ren Nutr.* *15*, 49-53.
- Biolo,G., De Cicco,M., Dal,M., V, Lorenzon,S., Antonione,R., Ciocchi,B., Barazzoni,R., Zanetti,M., Dore,F., and Guarnieri,G. (2006). Response of muscle protein and glutamine kinetics to branched-chain-enriched amino acids in intensive care patients after radical cancer surgery. *Nutrition* *22*, 475-482.
- Biolo,G., Fleming,R.Y., Maggi,S.P., and Wolfe,R.R. (1995a). Transmembrane transport and intracellular kinetics of amino acids in human skeletal muscle. *Am. J. Physiol* *268*, E75-E84.
- Biolo,G., Iskra,F., Bosutti,A., Toigo,G., Ciocchi,B., Geatti,O., Gullo,A., and Guarnieri,G. (2000). Growth hormone decreases muscle glutamine production and stimulates protein synthesis in hypercatabolic patients. *Am. J. Physiol Endocrinol. Metab* *279*, E323-E332.
- Biolo,G., Maggi,S.P., Williams,B.D., Tipton,K.D., and Wolfe,R.R. (1995b). Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *Am. J. Physiol* *268*, E514-E520.
- Biolo,G., Tipton,K.D., Klein,S., and Wolfe,R.R. (1997). An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *Am. J. Physiol* *273*, E122-E129.
- Biolo,G., Williams,B.D., Fleming,R.Y., and Wolfe,R.R. (1999). Insulin action on muscle protein kinetics and amino acid transport during recovery after resistance exercise. *Diabetes* *48*, 949-957.
- Biolo,G., Zorat,F., Antonione,R., and Ciocchi,B. (2005b). Muscle glutamine depletion in the intensive care unit. *Int. J. Biochem. Cell Biol.* *37*, 2169-2179.
- Blanc,S., Normand,S., Pachiardi,C., Duvareille,M., and Gharib,C. (2000a). Leptin responses to physical inactivity induced by simulated weightlessness. *Am. J. Physiol Regul. Integr. Comp Physiol* *279*, R891-R898.
- Blanc,S., Normand,S., Pachiardi,C., Fortrat,J.O., Laville,M., and Gharib,C. (2000b). Fuel homeostasis during physical inactivity induced by bed rest. *J. Clin. Endocrinol. Metab* *85*, 2223-2233.
- Blanc,S., Normand,S., Ritz,P., Pachiardi,C., Vico,L., Gharib,C., and Gauquelin-Koch,G. (1998). Energy and water metabolism, body composition, and hormonal changes induced by 42 days of enforced inactivity and simulated weightlessness. *J. Clin. Endocrinol. Metab* *83*, 4289-4297.

- Blomstrand,E. and Essen-Gustavsson,B. (2008). Changes in amino acid concentration in plasma and type I and type II fibres during resistance exercise and recovery in human subjects. *Amino. Acids*.
- Bodine,S.C., Latres,E., Baumhueter,S., Lai,V.K., Nunez,L., Clarke,B.A., Poueymirou,W.T., Panaro,F.J., Na,E., Dharmarajan,K., Pan,Z.Q., Valenzuela,D.M., DeChiara,T.M., Stitt,T.N., Yancopoulos,G.D., and Glass,D.J. (2001a). Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 294, 1704-1708.
- Bodine,S.C., Stitt,T.N., Gonzalez,M., Kline,W.O., Stover,G.L., Bauerlein,R., Zlotchenko,E., Scrimgeour,A., Lawrence,J.C., Glass,D.J., and Yancopoulos,G.D. (2001b). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat. Cell Biol.* 3, 1014-1019.
- Bongers,T., Griffiths,R.D., and McArdle,A. (2007). Exogenous glutamine: the clinical evidence. *Crit Care Med.* 35, S545-S552.
- Borkman,M., Storlien,L.H., Pan,D.A., Jenkins,A.B., Chisholm,D.J., and Campbell,L.V. (1993). The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. *N. Engl. J. Med.* 328, 238-244.
- Bosutti,A., Malaponte,G., Zanetti,M., Castellino,P., Heer,M., Guarnieri,G., and Biolo,G. (2008). Calorie restriction modulates inactivity-induced changes in the inflammatory markers C-reactive protein and pentraxin-3. *J. Clin. Endocrinol. Metab* 93, 3226-3229.
- Bota,D.A. and Davies,K.J. (2002). Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. *Nat. Cell Biol.* 4, 674-680.
- Bourreille,A., Humbert,B., Maugere,P., Galmiche,J.P., and Darmaun,D. (2004). Glutamine metabolism in Crohn's disease: a stable isotope study. *Clin. Nutr.* 23, 1167-1175.
- Boushey,C.J., Beresford,S.A., Omenn,G.S., and Motulsky,A.G. (1995). A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *JAMA* 274, 1049-1057.
- Bouwmeester,T., Bauch,A., Ruffner,H., Angrand,P.O., Bergamini,G., Croughton,K., Cruciat,C., Eberhard,D., Gagneur,J., Ghidelli,S., Hopf,C., Huhse,B., Mangano,R., Michon,A.M., Schirle,M., Schlegl,J., Schwab,M., Stein,M.A., Bauer,A., Casari,G., Drewes,G., Gavin,A.C., Jackson,D.B., Joberty,G., Neubauer,G., Rick,J., Kuster,B., and Superti-Furga,G. (2004). A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. *Nat. Cell Biol.* 6, 97-105.
- Bozan,B. and Temelli,F. (2008). Chemical composition and oxidative stability of flax, safflower and poppy seed and seed oils. *Bioresour. Technol.* 99, 6354-6359.
- Brenner,R.R. (2003). Hormonal modulation of delta6 and delta5 desaturases: case of diabetes. *Prostaglandins Leukot. Essent. Fatty Acids* 68, 151-162.
- Brodan,V., Kuhn,E., Pechar,J., and Tomkova,D. (1976). Changes of free amino acids in plasma of healthy subjects induced by physical exercise. *Eur. J. Appl. Physiol Occup. Physiol* 35, 69-77.
- Brooks,N., Cloutier,G.J., Cadena,S.M., Layne,J.E., Nelsen,C.A., Freed,A.M., Roubenoff,R., and Castaneda-Sceppa,C. (2008). Resistance training and timed essential amino acids protect against the loss of muscle mass and strength during 28 days of bed rest and energy deficit. *J. Appl. Physiol* 105, 241-248.
- Burdge,G.C., Jones,A.E., and Wootton,S.A. (2002). Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men*. *Br. J. Nutr.* 88, 355-363.
- Burghart G. and Finn Carol A. (2010). *Handbook of MRI Scanning*. Mosby).
- Cai,C., Hahn,B.H., Matarese,G., and La Cava,A. (2009). Leptin in non-autoimmune inflammation. *Inflamm. Allergy Drug Targets.* 8, 285-291.

- Castell,L., Vance,C., Abbott,R., Marquez,J., and Eggleton,P. (2004). Granule localization of glutaminase in human neutrophils and the consequence of glutamine utilization for neutrophil activity. *J. Biol. Chem.* 279, 13305-13310.
- Castello,L., Froio,T., Maina,M., Cavallini,G., Biasi,F., Leonarduzzi,G., Donati,A., Bergamini,E., Poli,G., and Chiarpotto,E. (2009). Alternate-day fasting protects the rat heart against age-induced inflammation and fibrosis by inhibiting oxidative damage and NF-kB activation. *Free Radic. Biol. Med.*
- Chaput,J.P. and Tremblay,A. (2009). Obesity and Physical Inactivity: The Relevance of Reconsidering the Notion of Sedentariness. *Obes. Facts.* 2, 249-254.
- Chen,J.J. and Yu,B.P. (1994). Alterations in mitochondrial membrane fluidity by lipid peroxidation products. *Free Radic. Biol. Med.* 17, 411-418.
- Civatte,M., Bartoli,C., Schleinitz,N., Chetaille,B., Pellissier,J.F., and Figarella-Branger,D. (2005). Expression of the beta chemokines CCL3, CCL4, CCL5 and their receptors in idiopathic inflammatory myopathies. *Neuropathol. Appl. Neurobiol.* 31, 70-79.
- Clarke,R., Daly,L., Robinson,K., Naughten,E., Cahalane,S., Fowler,B., and Graham,I. (1991). Hyperhomocysteinemia: an independent risk factor for vascular disease. *N. Engl. J. Med.* 324, 1149-1155.
- Cleophas,T.J., Hornstra,N., van Hoogstraten,B., and van der,M.J. (2000). Homocysteine, a risk factor for coronary artery disease or not? A meta-analysis. *Am. J. Cardiol.* 86, 1005-9, A8.
- Colberg,S.R. and Grieco,C.R. (2009). Exercise in the treatment and prevention of diabetes. *Curr. Sports Med. Rep.* 8, 169-175.
- Conquer,J.A. and Holub,B.J. (1997). Dietary docosahexaenoic acid as a source of eicosapentaenoic acid in vegetarians and omnivores. *Lipids* 32, 341-345.
- Convertino,V.A., Doerr,D.F., and Stein,S.L. (1989). Changes in size and compliance of the calf after 30 days of simulated microgravity. *J. Appl. Physiol* 66, 1509-1512.
- Cotgreave,I.A. and Gerdes,R.G. (1998). Recent trends in glutathione biochemistry--glutathione-protein interactions: a molecular link between oxidative stress and cell proliferation? *Biochem. Biophys. Res. Commun.* 242, 1-9.
- Couillard,A. and Prefaut,C. (2005). From muscle disuse to myopathy in COPD: potential contribution of oxidative stress. *Eur. Respir. J.* 26, 703-719.
- Crimi,E., Sica,V., Williams-Ignarro,S., Zhang,H., Slutsky,A.S., Ignarro,L.J., and Napoli,C. (2006). The role of oxidative stress in adult critical care. *Free Radic. Biol. Med.* 40, 398-406.
- Crowther,M.A. and Kelton,J.G. (2003). Congenital thrombophilic states associated with venous thrombosis: a qualitative overview and proposed classification system. *Ann. Intern. Med.* 138, 128-134.
- Cryer,D.R., Matsushima,T., Marsh,J.B., Yudkoff,M., Coates,P.M., and Cortner,J.A. (1986). Direct measurement of apolipoprotein B synthesis in human very low density lipoprotein using stable isotopes and mass spectrometry. *J. Lipid Res.* 27, 508-516.
- Curi,T.C., De Melo,M.P., De Azevedo,R.B., Zorn,T.M., and Curi,R. (1997). Glutamine utilization by rat neutrophils: presence of phosphate-dependent glutaminase. *Am. J. Physiol* 273, C1124-C1129.
- Curthoys,N.P. and Watford,M. (1995). Regulation of glutaminase activity and glutamine metabolism. *Annu. Rev. Nutr.* 15, 133-159.
- Dalakas,M.C. and Pezeshkpour,G.H. (1988). Neuromuscular diseases associated with human immunodeficiency virus infection. *Ann. Neurol.* 23 *Suppl.*, S38-S48.

- Dalla,L.L., Ravara,B., Gobbo,V., Tarricone,E., Vitadello,M., Biolo,G., Vescovo,G., and Gorza,L. (2009). A transient antioxidant stress response accompanies the onset of disuse atrophy in human skeletal muscle. *J. Appl. Physiol* 107, 549-557.
- Dalle-Donne,I., Giustarini,D., Colombo,R., Rossi,R., and Milzani,A. (2003). Protein carbonylation in human diseases. *Trends Mol. Med.* 9, 169-176.
- Dangin,M., Guillet,C., Garcia-Rodenas,C., Gachon,P., Bouteloup-Demange,C., Reiffers-Magnani,K., Fauquant,J., Balleve,O., and Beaufriere,B. (2003). The rate of protein digestion affects protein gain differently during aging in humans. *J. Physiol* 549, 635-644.
- Dankner,R., Chetrit,A., Ken,D.G., and Sela,B.A. (2007). Physical activity is inversely associated with total homocysteine levels, independent of C677T MTHFR genotype and plasma B vitamins. *Age (Dordr.)* 29, 219-227.
- Darmaun,D., Just,B., Messing,B., Rongier,M., Thuillier,F., Koziat,J., and Grasset,E. (1994). Glutamine metabolism in healthy adult men: response to enteral and intravenous feeding. *Am. J. Clin. Nutr.* 59, 1395-1402.
- Darmaun,D., Matthews,D.E., and Bier,D.M. (1986). Glutamine and glutamate kinetics in humans. *Am. J. Physiol* 251, E117-E126.
- Darmaun,D., Matthews,D.E., and Bier,D.M. (1988). Physiological hypercortisolemia increases proteolysis, glutamine, and alanine production. *Am. J. Physiol* 255, E366-E373.
- Davy,K.P., Desouza,C.A., Jones,P.P., and Seals,D.R. (1998). Elevated heart rate variability in physically active young and older adult women. *Clin. Sci. (Lond)* 94, 579-584.
- de Boer,M.D., Maganaris,C.N., Seynnes,O.R., Rennie,M.J., and Narici,M.V. (2007). Time course of muscular, neural and tendinous adaptations to 23 day unilateral lower-limb suspension in young men. *J. Physiol* 583, 1079-1091.
- de Boer,M.D., Seynnes,O.R., di Prampero,P.E., Pisot,R., Mekjavic,I.B., Biolo,G., and Narici,M.V. (2008). Effect of 5 weeks horizontal bed rest on human muscle thickness and architecture of weight bearing and non-weight bearing muscles. *Eur. J. Appl. Physiol* 104, 401-407.
- DeLeve,L.D. and Kaplowitz,N. (1991). Glutathione metabolism and its role in hepatotoxicity. *Pharmacol. Ther.* 52, 287-305.
- Deloughery,T.G., Evans,A., Sadeghi,A., McWilliams,J., Henner,W.D., Taylor,L.M., Jr., and Press,R.D. (1996). Common mutation in methylenetetrahydrofolate reductase. Correlation with homocysteine metabolism and late-onset vascular disease. *Circulation* 94, 3074-3078.
- DeMartino,G.N. and Ordway,G.A. (1998). Ubiquitin-proteasome pathway of intracellular protein degradation: implications for muscle atrophy during unloading. *Exerc. Sport Sci. Rev.* 26, 219-252.
- Despres,J.P. and Lamarche,B. (1994). Low-intensity endurance exercise training, plasma lipoproteins and the risk of coronary heart disease. *J. Intern. Med.* 236, 7-22.
- Di Francescomarino,S., Sciartilli,A., Di,V., V, Di Baldassarre,A., and Gallina,S. (2009). The effect of physical exercise on endothelial function. *Sports Med.* 39, 797-812.
- Di Minno,G., Davi,G., Margaglione,M., Cirillo,F., Grandone,E., Ciabattini,G., Catalano,I., Strisciuglio,P., Andria,G., Patrono,C., and . (1993). Abnormally high thromboxane biosynthesis in homozygous homocystinuria. Evidence for platelet involvement and probucol-sensitive mechanism. *J. Clin. Invest* 92, 1400-1406.
- Di Santolo,M., Banfi,G., Stel,G., and Cauci,S. (2009). Association of recreational physical activity with homocysteine, folate and lipid markers in young women. *Eur. J. Appl. Physiol* 105, 111-118.
- Dillard,C.J., Litov,R.E., Savin,W.M., Dumelin,E.E., and Tappel,A.L. (1978). Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. *J. Appl. Physiol* 45, 927-932.

- Dimitriu,C., Martignoni,M.E., Bachmann,J., Frohlich,B., Tintarescu,G., Buliga,T., Lica,I., Constantinescu,G., Beuran,M., and Friess,H. (2005). Clinical impact of cachexia on survival and outcome of cancer patients. *Rom. J. Intern. Med.* 43, 173-185.
- Dinareello,C.A. (1994). The interleukin-1 family: 10 years of discovery. *FASEB J.* 8, 1314-1325.
- Dobrowolny,G., Aucello,M., Rizzuto,E., Beccafico,S., Mammucari,C., Boncompagni,S., Belia,S., Wannenes,F., Nicoletti,C., Del Prete,Z., Rosenthal,N., Molinaro,M., Protasi,F., Fano,G., Sandri,M., and Musaro,A. (2008). Skeletal muscle is a primary target of SOD1G93A-mediated toxicity. *Cell Metab* 8, 425-436.
- Dogru-Abbasoglu,S., Tamer-Toptani,S., Ugurnal,B., Kocak-Toker,N., Aykac-Toker,G., and Uysal,M. (1997). Lipid peroxidation and antioxidant enzymes in livers and brains of aged rats. *Mech. Ageing Dev.* 98, 177-180.
- Dorfman,T.A., Levine,B.D., Tillery,T., Peshock,R.M., Hastings,J.L., Schneider,S.M., Macias,B.R., Biolo,G., and Hargens,A.R. (2007). Cardiac atrophy in women following bed rest. *J. Appl. Physiol* 103, 8-16.
- Dos Santos Cunha,W.D., Giampietro,M.V., De Souza,D.F., Vaisberg,M., Seelaender,M.C., and Rosa,L.F. (2004). Exercise restores immune cell function in energy-restricted rats. *Med. Sci. Sports Exerc.* 36, 2059-2064.
- Dos Santos,R.V., Caperuto,E.C., de Mello,M.T., Batista Jr,M.L., and Rosa,L.F. (2009). Effect of exercise upon glutamine synthesis and transport in skeletal muscle from rats. *Clin. Exp. Pharmacol. Physiol.*
- Droge,W., Pottmeyer-Gerber,C., Schmidt,H., and Nick,S. (1986). Glutathione augments the activation of cytotoxic T lymphocytes in vivo. *Immunobiology* 172, 151-156.
- Du,J., Wang,X., Miereles,C., Bailey,J.L., Debigare,R., Zheng,B., Price,S.R., and Mitch,W.E. (2004). Activation of caspase-3 is an initial step triggering accelerated muscle proteolysis in catabolic conditions. *J. Clin. Invest* 113, 115-123.
- Dudley,M.A., Burrin,D.G., Wykes,L.J., Toffolo,G., Cobelli,C., Nichols,B.L., Rosenberger,J., Jahoor,F., and Reeds,P.J. (1998). Protein kinetics determined in vivo with a multiple-tracer, single-sample protocol: application to lactase synthesis. *Am. J. Physiol* 274, G591-G598.
- Dukan,S., Farewell,A., Ballesteros,M., Taddei,F., Radman,M., and Nystrom,T. (2000). Protein oxidation in response to increased transcriptional or translational errors. *Proc. Natl. Acad. Sci. U. S. A* 97, 5746-5749.
- Duncan,B.B. and Schmidt,M.I. (2006). The epidemiology of low-grade chronic systemic inflammation and type 2 diabetes. *Diabetes Technol. Ther.* 8, 7-17.
- Duncan,G.E., Perri,M.G., Anton,S.D., Limacher,M.C., Martin,A.D., Lowenthal,D.T., Arning,E., Bottiglieri,T., and Stacpoole,P.W. (2004). Effects of exercise on emerging and traditional cardiovascular risk factors. *Prev. Med.* 39, 894-902.
- Durand,P., Prost,M., Loreau,N., Lussier-Cacan,S., and Blache,D. (2001). Impaired homocysteine metabolism and atherothrombotic disease. *Lab Invest* 81, 645-672.
- Ellis,S., Kirby,L.C., and Greenleaf,J.E. (1993). Lower extremity muscle thickness during 30-day 6 degrees head-down bed rest with isotonic and isokinetic exercise training. *Aviat. Space Environ. Med.* 64, 1011-1015.
- Elmarakby,A.A. and Imig,J.D. (2009). Obesity is the major contributor to vascular dysfunction and inflammation in high fat diet hypertensive rats. *Clin. Sci. (Lond).*
- Endres,S., Ghorbani,R., Kelley,V.E., Georgilis,K., Lonnemann,G., van der Meer,J.W., Cannon,J.G., Rogers,T.S., Klempner,M.S., Weber,P.C., and . (1989). The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *N. Engl. J. Med.* 320, 265-271.
- Engel,J.M., Muhling,J., Kwapisz,M., and Heidt,M. (2009). Glutamine administration in patients undergoing cardiac surgery and the influence on blood glutathione levels. *Acta Anaesthesiol. Scand.* 53, 1317-1323.

- Esfandiari,N., Falcone,T., Agarwal,A., Attaran,M., Nelson,D.R., and Sharma,R.K. (2005). Protein supplementation and the incidence of apoptosis and oxidative stress in mouse embryos. *Obstet. Gynecol.* *105*, 653-660.
- FAO/WHO/UNU (1985). Energy and protein requirements. Report of a joint FAO/WHO/UNU Expert Consultation. *World Health Org Tech Rep Ser*), pp. 71-112.
- Fearon,K.C. and Moses,A.G. (2002). Cancer cachexia. *Int. J. Cardiol.* *85*, 73-81.
- Fenton,J.I., Hursting,S.D., Perkins,S.N., and Hord,N.G. (2006). Interleukin-6 production induced by leptin treatment promotes cell proliferation in an Apc (Min/+) colon epithelial cell line. *Carcinogenesis* *27*, 1507-1515.
- Ferrando,A.A., Lane,H.W., Stuart,C.A., Davis-Street,J., and Wolfe,R.R. (1996). Prolonged bed rest decreases skeletal muscle and whole body protein synthesis. *Am. J. Physiol* *270*, E627-E633.
- Ferretti,G., Antonutto,G., Denis,C., Hoppeler,H., Minetti,A.E., Narici,M.V., and Desplanches,D. (1997). The interplay of central and peripheral factors in limiting maximal O₂ consumption in man after prolonged bed rest. *J. Physiol* *501 (Pt 3)*, 677-686.
- Ferretti,G., Berg,H.E., Minetti,A.E., Moia,C., Rampichini,S., and Narici,M.V. (2001). Maximal instantaneous muscular power after prolonged bed rest in humans. *J. Appl. Physiol* *90*, 431-435.
- Finkelstein,J.D. (1998). The metabolism of homocysteine: pathways and regulation. *Eur. J. Pediatr.* *157 Suppl 2*, S40-S44.
- Finkelstein,J.D. (2000). Pathways and regulation of homocysteine metabolism in mammals. *Semin. Thromb. Hemost.* *26*, 219-225.
- Finkelstein,J.D. and Martin,J.J. (1984). Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. *J. Biol. Chem.* *259*, 9508-9513.
- Fischer,C.P., Berntsen,A., Perstrup,L.B., Eskildsen,P., and Pedersen,B.K. (2007). Plasma levels of interleukin-6 and C-reactive protein are associated with physical inactivity independent of obesity. *Scand. J. Med. Sci. Sports* *17*, 580-587.
- Fitts,R.H., Romatowski,J.G., Peters,J.R., Paddon-Jones,D., Wolfe,R.R., and Ferrando,A.A. (2007). The deleterious effects of bed rest on human skeletal muscle fibers are exacerbated by hypercortisolemia and ameliorated by dietary supplementation. *Am. J. Physiol Cell Physiol* *293*, C313-C320.
- Fontana,L. (2009a). Modulating human aging and age-associated diseases. *Biochim. Biophys. Acta* *1790*, 1133-1138.
- Fontana,L. (2009b). The scientific basis of caloric restriction leading to longer life. *Curr. Opin. Gastroenterol.* *25*, 144-150.
- Fontana,L., Klein,S., and Holloszy,J.O. (2009). Effects of long-term calorie restriction and endurance exercise on glucose tolerance, insulin action, and adipokine production. *Age (Dordr.)*.
- Franck,T., Grulke,S., Deby-Dupont,G., Deby,C., Duvivier,H., Peters,F., and Serteyn,D. (2005). Development of an enzyme-linked immunosorbent assay for specific equine neutrophil myeloperoxidase measurement in blood. *J. Vet. Diagn. Invest* *17*, 412-419.
- Frayn,K.N., Khan,K., Coppack,S.W., and Elia,M. (1991). Amino acid metabolism in human subcutaneous adipose tissue in vivo. *Clin. Sci. (Lond)* *80*, 471-474.
- Fryburg,D.A., Barrett,E.J., Louard,R.J., and Gelfand,R.A. (1990). Effect of starvation on human muscle protein metabolism and its response to insulin. *Am. J. Physiol* *259*, E477-E482.
- Fukagawa,N.K., Martin,J.M., Wurthmann,A., Prue,A.H., Ebenstein,D., and O'Rourke,B. (2000). Sex-related differences in methionine metabolism and plasma homocysteine concentrations. *Am. J. Clin. Nutr.* *72*, 22-29.

- Furuno,K. and Goldberg,A.L. (1986). The activation of protein degradation in muscle by Ca²⁺ or muscle injury does not involve a lysosomal mechanism. *Biochem. J.* 237, 859-864.
- Garber,A.J., Karl,I.E., and Kipnis,D.M. (1976). Alanine and glutamine synthesis and release from skeletal muscle. II. The precursor role of amino acids in alanine and glutamine synthesis. *J. Biol. Chem.* 251, 836-843.
- Gaur,U. and Aggarwal,B.B. (2003). Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. *Biochem. Pharmacol.* 66, 1403-1408.
- Gelecek,N., Teoman,N., Ozdirenc,M., Pinar,L., Akan,P., Bediz,C., and Kozan,O. (2007). Influences of acute and chronic aerobic exercise on the plasma homocysteine level. *Ann. Nutr. Metab* 51, 53-58.
- Gherghel,D., Griffiths,H.R., Hilton,E.J., Cunliffe,I.A., and Hosking,S.L. (2005). Systemic reduction in glutathione levels occurs in patients with primary open-angle glaucoma. *Invest Ophthalmol. Vis. Sci.* 46, 877-883.
- Ghosh,C., Dick,R.M., and Ali,S.F. (1993). Iron/ascorbate-induced lipid peroxidation changes membrane fluidity and muscarinic cholinergic receptor binding in rat frontal cortex. *Neurochem. Int.* 23, 479-484.
- Ghosh,S., Khazaei,M., Moien-Afshari,F., Ang,L.S., Granville,D.J., Verchere,C.B., Dunn,S.R., McCue,P., Mizisin,A., Sharma,K., and Laher,I. (2009). Moderate exercise attenuates caspase-3 activity, oxidative stress, and inhibits progression of diabetic renal disease in db/db mice. *Am. J. Physiol Renal Physiol* 296, F700-F708.
- Gibala,M.J., MacLean,D.A., Graham,T.E., and Saltin,B. (1998). Tricarboxylic acid cycle intermediate pool size and estimated cycle flux in human muscle during exercise. *Am. J. Physiol* 275, E235-E242.
- Giresi,P.G., Stevenson,E.J., Theilhaber,J., Koncarevic,A., Parkington,J., Fielding,R.A., and Kandarian,S.C. (2005). Identification of a molecular signature of sarcopenia. *Physiol Genomics* 21, 253-263.
- Glick,Z., McNurlan,M.A., and Garlick,P.J. (1982). Protein synthesis rate in liver and muscle of rats following four days of overfeeding. *J. Nutr.* 112, 391-397.
- Goepfert,A.R., Scheerens,H., and Vermeulen,N.P. (1995). Oxygen and xenobiotic reductase activities of cytochrome P450. *Crit Rev. Toxicol.* 25, 25-65.
- Gogia,P., Schneider,V.S., LeBlanc,A.D., Krebs,J., Kasson,C., and Pientok,C. (1988). Bed rest effect on extremity muscle torque in healthy men. *Arch. Phys. Med. Rehabil.* 69, 1030-1032.
- Goll,D.E., Thompson,V.F., Li,H., Wei,W., and Cong,J. (2003). The calpain system. *Physiol Rev.* 83, 731-801.
- Gomes,M.D., Lecker,S.H., Jagoe,R.T., Navon,A., and Goldberg,A.L. (2001). Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc. Natl. Acad. Sci. U. S. A* 98, 14440-14445.
- Graham,I.M., Daly,L.E., Refsum,H.M., Robinson,K., Brattstrom,L.E., Ueland,P.M., Palma-Reis,R.J., Boers,G.H., Sheahan,R.G., Israelsson,B., Uiterwaal,C.S., Meleady,R., McMaster,D., Verhoef,P., Witteman,J., Rubba,P., Bellet,H., Wautrecht,J.C., de Valk,H.W., Sales Luis,A.C., Parrot-Rouland,F.M., Tan,K.S., Higgins,I., Garcon,D., Andria,G., and . (1997). Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. *JAMA* 277, 1775-1781.
- Greathouse,K.L., Samuels,M., DiMarco,N.M., and Criswell,D.S. (2005). Effects of increased dietary fat and exercise on skeletal muscle lipid peroxidation and antioxidant capacity in male rats. *Eur. J. Nutr.* 44, 429-435.
- Greilberger,J., Koidl,C., Greilberger,M., Lamprecht,M., Schroecksnadel,K., Leblhuber,F., Fuchs,D., and Oetzl,K. (2008). Malondialdehyde, carbonyl proteins and albumin-disulphide as useful oxidative markers in mild cognitive impairment and Alzheimer's disease. *Free Radic. Res.* 42, 633-638.
- Gretebeck,R.J., Schoeller,D.A., Gibson,E.K., and Lane,H.W. (1995). Energy expenditure during antiorthostatic bed rest (simulated microgravity). *J. Appl. Physiol* 78, 2207-2211.
- Grune,T. and Davies,K.J. (2003). The proteasomal system and HNE-modified proteins. *Mol. Aspects Med.* 24, 195-204.

- Grune,T., Merker,K., Sandig,G., and Davies,K.J. (2003). Selective degradation of oxidatively modified protein substrates by the proteasome. *Biochem. Biophys. Res. Commun.* 305, 709-718.
- Gudbjarnason,S. (1989). Dynamics of n-3 and n-6 fatty acids in phospholipids of heart muscle. *J. Intern. Med. Suppl* 731, 117-128.
- Halliwell,B. and Gutteridge,J.M. (1986). Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* 246, 501-514.
- Hamburg,N.M., McMackin,C.J., Huang,A.L., Shenouda,S.M., Widlansky,M.E., Schulz,E., Gokce,N., Ruderman,N.B., Keaney,J.F., Jr., and Vita,J.A. (2007). Physical inactivity rapidly induces insulin resistance and microvascular dysfunction in healthy volunteers. *Arterioscler. Thromb. Vasc. Biol.* 27, 2650-2656.
- Hansson,G.K. (2005). Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med.* 352, 1685-1695.
- Hardmeier,R., Hoeger,H., Fang-Kircher,S., Khoschorur,A., and Lubec,G. (1997). Transcription and activity of antioxidant enzymes after ionizing irradiation in radiation-resistant and radiation-sensitive mice. *Proc. Natl. Acad. Sci. U. S. A* 94, 7572-7576.
- Harker,L.A., Slichter,S.J., Scott,C.R., and Ross,R. (1974). Homocystinemia. Vascular injury and arterial thrombosis. *N. Engl. J. Med.* 291, 537-543.
- Harris,W.S. and Von Schacky,C. (2004). The Omega-3 Index: a new risk factor for death from coronary heart disease? *Prev. Med.* 39, 212-220.
- Helge,J.W., Ayre,K.J., Hulbert,A.J., Kiens,B., and Storlien,L.H. (1999). Regular exercise modulates muscle membrane phospholipid profile in rats. *J. Nutr.* 129, 1636-1642.
- Helge,J.W., Wu,B.J., Willer,M., Daugaard,J.R., Storlien,L.H., and Kiens,B. (2001). Training affects muscle phospholipid fatty acid composition in humans. *J. Appl. Physiol* 90, 670-677.
- Henriksson,J. (1991). Effect of exercise on amino acid concentrations in skeletal muscle and plasma. *J. Exp. Biol.* 160, 149-165.
- Herrmann,M., Schorr,H., Obeid,R., Scharhag,J., Urhausen,A., Kindermann,W., and Herrmann,W. (2003). Homocysteine increases during endurance exercise. *Clin. Chem. Lab Med.* 41, 1518-1524.
- Hibbert,J.M., Sutherland,G.B., Jr., Wright,L.L., Jr., Wolfe,L.G., Wolfe,K.A., Gao,S.P., Gore,D.C., and Abd-Elfattah,A.S. (2001). Measurement of hemoglobin synthesis rate in vivo using a stable isotope method. *Anal. Biochem.* 291, 118-123.
- Hiscock,N. and Mackinnon,L.T. (1998). A comparison of plasma glutamine concentration in athletes from different sports. *Med. Sci. Sports Exerc.* 30, 1693-1696.
- Ho,K.M. and Lipman,J. (2009). An update on C-reactive protein for intensivists. *Anaesth. Intensive Care* 37, 234-241.
- Honda,H., Qureshi,A.R., Axelsson,J., Heimbürger,O., Suliman,M.E., Barany,P., Stenvinkel,P., and Lindholm,B. (2007). Obese sarcopenia in patients with end-stage renal disease is associated with inflammation and increased mortality. *Am. J. Clin. Nutr.* 86, 633-638.
- Hong,S., Gronert,K., Devchand,P.R., Moussignac,R.L., and Serhan,C.N. (2003). Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. *J. Biol. Chem.* 278, 14677-14687.
- Hood,D.A. and Terjung,R.L. (1994). Endurance training alters alanine and glutamine release from muscle during contractions. *FEBS Lett.* 340, 287-290.
- Hopps,E., Noto,D., Caimi,G., and Averna,M.R. (2009). A novel component of the metabolic syndrome: The oxidative stress. *Nutr. Metab Cardiovasc. Dis.*

- Hotamisligil,G.S., Shargill,N.S., and Spiegelman,B.M. (1993). Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science* 259, 87-91.
- Huang,R.F., Hsu,Y.C., Lin,H.L., and Yang,F.L. (2001). Folate depletion and elevated plasma homocysteine promote oxidative stress in rat livers. *J. Nutr.* 131, 33-38.
- Hundal,H.S., Babij,P., Watt,P.W., Ward,M.R., and Rennie,M.J. (1990). Glutamine transport and metabolism in denervated rat skeletal muscle. *Am. J. Physiol* 259, E148-E154.
- Hunter,R.B., Mitchell-Felton,H., Essig,D.A., and Kandarian,S.C. (2001). Expression of endoplasmic reticulum stress proteins during skeletal muscle disuse atrophy. *Am. J. Physiol Cell Physiol* 281, C1285-C1290.
- Hursting,S.D., Smith,S.M., Lashinger,L.M., Harvey,A.E., and Perkins,S.N. (2010). Calories and carcinogenesis: lessons learned from 30 years of calorie restriction research. *Carcinogenesis* 31, 83-89.
- Ichinose,Y., Kanehisa,H., Ito,M., Kawakami,Y., and Fukunaga,T. (1998). Relationship between muscle fiber pennation and force generation capability in Olympic athletes. *Int. J. Sports Med.* 19, 541-546.
- Ikemoto,M., Nikawa,T., Kano,M., Hirasaka,K., Kitano,T., Watanabe,C., Tanaka,R., Yamamoto,T., Kamada,M., and Kishi,K. (2002a). Cysteine supplementation prevents unweighting-induced ubiquitination in association with redox regulation in rat skeletal muscle. *Biol. Chem.* 383, 715-721.
- Ikemoto,M., Nikawa,T., Takeda,S., Watanabe,C., Kitano,T., Baldwin,K.M., Izumi,R., Nonaka,I., Towatari,T., Teshima,S., Rokutan,K., and Kishi,K. (2001). Space shuttle flight (STS-90) enhances degradation of rat myosin heavy chain in association with activation of ubiquitin-proteasome pathway. *FASEB J.* 15, 1279-1281.
- Ikemoto,M., Okamura,Y., Kano,M., Hirasaka,K., Tanaka,R., Yamamoto,T., Sasa,T., Ogawa,T., Sairyo,K., Kishi,K., and Nikawa,T. (2002b). A relative high dose of vitamin E does not attenuate unweighting-induced oxidative stress and ubiquitination in rat skeletal muscle. *J. Physiol Anthropol. Appl. Human Sci.* 21, 257-263.
- Ischander,M., Zaldivar,F., Jr., Eliakim,A., Nussbaum,E., Dunton,G., Leu,S.Y., Cooper,D.M., and Schneider,M. (2007). Physical activity, growth, and inflammatory mediators in BMI-matched female adolescents. *Med. Sci. Sports Exerc.* 39, 1131-1138.
- Ischiropoulos,H., Zhu,L., and Beckman,J.S. (1992). Peroxynitrite formation from macrophage-derived nitric oxide. *Arch. Biochem. Biophys.* 298, 446-451.
- Jacobsen,D.W. (1998). Homocysteine and vitamins in cardiovascular disease. *Clin. Chem.* 44, 1833-1843.
- Jahoor,F., Wykes,L.J., Reeds,P.J., Henry,J.F., del Rosario,M.P., and Frazer,M.E. (1995). Protein-deficient pigs cannot maintain reduced glutathione homeostasis when subjected to the stress of inflammation. *J. Nutr.* 125, 1462-1472.
- Jaspers,S.R., Henriksen,E.J., Satarug,S., and Tischler,M.E. (1989). Effects of stretching and disuse on amino acids in muscles of rat hind limbs. *Metabolism* 38, 303-310.
- Javesghani,D., Magder,S.A., Barreiro,E., Quinn,M.T., and Hussain,S.N. (2002). Molecular characterization of a superoxide-generating NAD(P)H oxidase in the ventilatory muscles. *Am. J. Respir. Crit Care Med.* 165, 412-418.
- Jensen,G.L. (2008). Inflammation: roles in aging and sarcopenia. *JPEN J. Parenter. Enteral Nutr.* 32, 656-659.
- Ji,L.L. (1995). Exercise and oxidative stress: role of the cellular antioxidant systems. *Exerc. Sport Sci. Rev.* 23, 135-166.
- Ji,L.L., Fu,R., and Mitchell,E.W. (1992). Glutathione and antioxidant enzymes in skeletal muscle: effects of fiber type and exercise intensity. *J. Appl. Physiol* 73, 1854-1859.
- Jonasson,T., Ohlin,A.K., Gottsater,A., Hultberg,B., and Ohlin,H. (2005). Plasma homocysteine and markers for oxidative stress and inflammation in patients with coronary artery disease--a prospective randomized study of vitamin supplementation. *Clin. Chem. Lab Med.* 43, 628-634.

- Kanda,T., Takahashi,T., Sumino,H., Nakahashi,T., Iwai,K., Morimoto,S., and Matsumoto,M. (2005). Hypoadiponectinemia in bedridden female patients younger than 75. *J. Am. Geriatr. Soc.* 53, 2039-2041.
- Kandarian,S.C. and Jackman,R.W. (2006). Intracellular signaling during skeletal muscle atrophy. *Muscle Nerve* 33, 155-165.
- Kanikowska,D., Sato,M., Iwase,S., Shimizu,Y., Inukai,Y., Nishimura,N., and Sugenoja,J. (2008). Immune and neuroendocrine responses to head-down rest and countermeasures. *Aviat. Space Environ. Med.* 79, 1091-1095.
- Karakelides,H., Irving,B.A., Short,K.R., O'Brien,P., and Nair,K.S. (2010). Age, obesity, and sex effects on insulin sensitivity and skeletal muscle mitochondrial function. *Diabetes* 59, 89-97.
- Kargotich,S., Goodman,C., Dawson,B., Morton,A.R., Keast,D., and Joske,D.J. (2005). Plasma glutamine responses to high-intensity exercise before and after endurance training. *Res. Sports Med.* 13, 287-300.
- Kargotich,S., Keast,D., Goodman,C., Bhagat,C.I., Joske,D.J., Dawson,B., and Morton,A.R. (2007). Monitoring 6 weeks of progressive endurance training with plasma glutamine. *Int. J. Sports Med.* 28, 211-216.
- Kawakami,Y., Muraoka,Y., Kubo,K., Suzuki,Y., and Fukunaga,T. (2000). Changes in muscle size and architecture following 20 days of bed rest. *J. Gravit. Physiol* 7, 53-59.
- Keast,D., Arstein,D., Harper,W., Fry,R.W., and Morton,A.R. (1995). Depression of plasma glutamine concentration after exercise stress and its possible influence on the immune system. *Med. J. Aust.* 162, 15-18.
- Keller,C., Steensberg,A., Pilegaard,H., Osada,T., Saltin,B., Pedersen,B.K., and Neufer,P.D. (2001). Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content. *FASEB J.* 15, 2748-2750.
- Kelley,D.S. (2001). Modulation of human immune and inflammatory responses by dietary fatty acids. *Nutrition* 17, 669-673.
- Kirchgesner,T.G., Uysal,K.T., Wiesbrock,S.M., Marino,M.W., and Hotamisligil,G.S. (1997). Tumor necrosis factor-alpha contributes to obesity-related hyperleptinemia by regulating leptin release from adipocytes. *J. Clin. Invest* 100, 2777-2782.
- Kitazawa,M., Trinh,D.N., and LaFerla,F.M. (2008). Inflammation induces tau pathology in inclusion body myositis model via glycogen synthase kinase-3beta. *Ann. Neurol.* 64, 15-24.
- Klein,S., Allison,D.B., Heymsfield,S.B., Kelley,D.E., Leibel,R.L., Nonas,C., and Kahn,R. (2007). Waist Circumference and Cardiometabolic Risk: a Consensus Statement from Shaping America's Health: Association for Weight Management and Obesity Prevention; NAASO, the Obesity Society; the American Society for Nutrition; and the American Diabetes Association. *Obesity.* (Silver. Spring) 15, 1061-1067.
- Kondo,H., Miura,M., Kodama,J., Ahmed,S.M., and Itokawa,Y. (1992). Role of iron in oxidative stress in skeletal muscle atrophied by immobilization. *Pflugers Arch.* 421, 295-297.
- Kondo,H., Nakagaki,I., Sasaki,S., Hori,S., and Itokawa,Y. (1993). Mechanism of oxidative stress in skeletal muscle atrophied by immobilization. *Am. J. Physiol* 265, E839-E844.
- Kondo,H., Nishino,K., and Itokawa,Y. (1994). Hydroxyl radical generation in skeletal muscle atrophied by immobilization. *FEBS Lett.* 349, 169-172.
- Kourie,J.I. (1998). Interaction of reactive oxygen species with ion transport mechanisms. *Am. J. Physiol* 275, C1-24.
- Kramer,H.F. and Goodyear,L.J. (2007). Exercise, MAPK, and NF-kappaB signaling in skeletal muscle. *J. Appl. Physiol* 103, 388-395.
- Krebs,H.A. (1935). Metabolism of amino-acids: The synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues. *Biochem. J.* 29, 1951-1969.

- Krebs, J.M., Schneider, V.S., Evans, H., Kuo, M.C., and LeBlanc, A.D. (1990a). Energy absorption, lean body mass, and total body fat changes during 5 weeks of continuous bed rest. *Aviat. Space Environ. Med.* *61*, 314-318.
- Krebs, J.M., Schneider, V.S., Evans, H., Kuo, M.C., and LeBlanc, A.D. (1990b). Energy absorption, lean body mass, and total body fat changes during 5 weeks of continuous bed rest. *Aviat. Space Environ. Med.* *61*, 314-318.
- Kretzmann, N.A., Fillmann, H., Mauriz, J.L., Marroni, C.A., Marroni, N., Gonzalez-Gallego, J., and Tunon, M.J. (2008). Effects of glutamine on proinflammatory gene expression and activation of nuclear factor kappa B and signal transducers and activators of transcription in TNBS-induced colitis. *Inflamm. Bowel. Dis.* *14*, 1504-1513.
- Kretzschmar, M., Muller, D., Hubscher, J., Marin, E., and Klinger, W. (1991). Influence of aging, training and acute physical exercise on plasma glutathione and lipid peroxides in man. *Int. J. Sports Med.* *12*, 218-222.
- Krook, A., Holm, I., Pettersson, S., and Wallberg-Henriksson, H. (2003). Reduction of risk factors following lifestyle modification programme in subjects with type 2 (non-insulin dependent) diabetes mellitus. *Clin. Physiol. Imaging.* *23*, 21-30.
- Kyle, U.G., Bosaeus, I., De Lorenzo, A.D., Deurenberg, P., Elia, M., Manuel, G.J., Lilienthal, H.B., Kent-Smith, L., Melchior, J.C., Pirlich, M., Scharfetter, H., Schols, W.J., and Pichard, C. (2004). Bioelectrical impedance analysis-part II: utilization in clinical practice. *Clin. Nutr.* *23*, 1430-1453.
- Lang, C.A., Naryshkin, S., Schneider, D.L., Mills, B.J., and Lindeman, R.D. (1992). Low blood glutathione levels in healthy aging adults. *J. Lab Clin. Med.* *120*, 720-725.
- Langen, R.C. and Schols, A.M. (2007). Inflammation: friend or foe of muscle remodelling in COPD? *Eur. Respir. J.* *30*, 605-607.
- Larson-Meyer, D.E., Redman, L., Heilbronn, L.K., Martin, C.K., and Ravussin, E. (2010). Caloric restriction with or without exercise: the fitness versus fatness debate. *Med. Sci. Sports Exerc.* *42*, 152-159.
- Laskey, M.A. (1996). Dual-energy X-ray absorptiometry and body composition. *Nutrition* *12*, 45-51.
- Lastra, G. and Manrique, C. (2007). The expanding role of oxidative stress, renin angiotensin system, and beta-cell dysfunction in the cardiometabolic syndrome and Type 2 diabetes mellitus. *Antioxid. Redox. Signal.* *9*, 943-954.
- Laufs, U., Wassmann, S., Czech, T., Munzel, T., Eisenhauer, M., Bohm, M., and Nickenig, G. (2005). Physical inactivity increases oxidative stress, endothelial dysfunction, and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* *25*, 809-814.
- Laviano, A., Meguid, M.M., Preziosa, I., and Rossi, F.F. (2007). Oxidative stress and wasting in cancer. *Curr. Opin. Clin. Nutr. Metab Care* *10*, 449-456.
- Lawler, J.M., Song, W., and Demaree, S.R. (2003). Hindlimb unloading increases oxidative stress and disrupts antioxidant capacity in skeletal muscle. *Free Radic. Biol. Med.* *35*, 9-16.
- Lawler, J.M., Song, W., and Kwak, H.B. (2006). Differential response of heat shock proteins to hindlimb unloading and reloading in the soleus. *Muscle Nerve* *33*, 200-207.
- Lawson, J.A., Rokach, J., and Fitzgerald, G.A. (1999). Isoprostanes: formation, analysis and use as indices of lipid peroxidation in vivo. *J. Biol. Chem.* *274*, 24441-24444.
- Leblanc, A., Gogia, P., Schneider, V., Krebs, J., Schonfeld, E., and Evans, H. (1988). Calf muscle area and strength changes after five weeks of horizontal bed rest. *Am. J. Sports Med.* *16*, 624-629.
- Leblanc, A.D., Schneider, V.S., Evans, H.J., Pientok, C., Rowe, R., and Spector, E. (1992). Regional changes in muscle mass following 17 weeks of bed rest. *J. Appl. Physiol.* *73*, 2172-2178.
- Lee, R. and Frenkel, E.P. (2003). Hyperhomocysteinemia and thrombosis. *Hematol. Oncol. Clin. North Am.* *17*, 85-102.

- Lee,R.T., Takagahara,I., and Lee,Y.C. (2002). Mapping the binding areas of human C-reactive protein for phosphorylcholine and polycationic compounds. Relationship between the two types of binding sites. *J. Biol. Chem.* 277, 225-232.
- Lee,T.H., Hoover,R.L., Williams,J.D., Sperling,R.I., Ravalese,J., III, Spur,B.W., Robinson,D.R., Corey,E.J., Lewis,R.A., and Austen,K.F. (1985). Effect of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. *N. Engl. J. Med.* 312, 1217-1224.
- Leeuwenburgh,C. and Ji,L.L. (1995). Glutathione depletion in rested and exercised mice: biochemical consequence and adaptation. *Arch. Biochem. Biophys.* 316, 941-949.
- Leger,B., Senese,R., Al Khodairy,A.W., Deriaz,O., Gobelet,C., Giacobino,J.P., and Russell,A.P. (2009). Atrogin-1, MuRF1, and FoXO, as well as phosphorylated GSK-3beta and 4E-BP1 are reduced in skeletal muscle of chronic spinal cord-injured patients. *Muscle Nerve* 40, 69-78.
- Lewis,R.A., Austen,K.F., and Soberman,R.J. (1990). Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. *N. Engl. J. Med.* 323, 645-655.
- Li,Y.P., Chen,Y., Li,A.S., and Reid,M.B. (2003). Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes. *Am. J. Physiol Cell Physiol* 285, C806-C812.
- Li,Y.P., Schwartz,R.J., Waddell,I.D., Holloway,B.R., and Reid,M.B. (1998). Skeletal muscle myocytes undergo protein loss and reactive oxygen-mediated NF-kappaB activation in response to tumor necrosis factor alpha. *FASEB J.* 12, 871-880.
- Lim,J.S., Hwang,J.S., Lee,J.A., Kim,D.H., Park,K.D., Jeong,J.S., and Cheon,G.J. (2009). Cross-calibration of multi-frequency bioelectrical impedance analysis with eight-point tactile electrodes and dual-energy X-ray absorptiometry for assessment of body composition in healthy children aged 6-18 years. *Pediatr. Int.* 51, 263-268.
- Lira,F.S., Koyama,C.H., Yamashita,A.S., Rosa,J.C., Zanchi,N.E., Batista,M.L., Jr., and Seelaender,M.C. (2009). Chronic exercise decreases cytokine production in healthy rat skeletal muscle. *Cell Biochem. Funct.* 27, 458-461.
- Liu,R.H. and Hotchkiss,J.H. (1995). Potential genotoxicity of chronically elevated nitric oxide: a review. *Mutat. Res.* 339, 73-89.
- Loenen,W.A. (2006). S-adenosylmethionine: jack of all trades and master of everything? *Biochem. Soc. Trans.* 34, 330-333.
- Lovejoy,J.C., Smith,S.R., Zachwieja,J.J., Bray,G.A., Windhauser,M.M., Wickersham,P.J., Veldhuis,J.D., Tulley,R., and de la Bretonne,J.A. (1999). Low-dose T(3) improves the bed rest model of simulated weightlessness in men and women. *Am. J. Physiol* 277, E370-E379.
- Lu,S.C. (1999). Regulation of hepatic glutathione synthesis: current concepts and controversies. *FASEB J.* 13, 1169-1183.
- Lucotti,P., Monti,L., Setola,E., La Canna,G., Castiglioni,A., Rossodivita,A., Pala,M.G., Formica,F., Paolini,G., Catapano,A.L., Bosi,E., Alfieri,O., and Piatti,P. (2009). Oral L-arginine supplementation improves endothelial function and ameliorates insulin sensitivity and inflammation in cardiopathic nondiabetic patients after an aortocoronary bypass. *Metabolism* 58, 1270-1276.
- Lunde,P.K., Sjaastad,I., Schiøtz Thorud,H.M., and Sejersted,O.M. (2001). Skeletal muscle disorders in heart failure. *Acta Physiol Scand.* 171, 277-294.
- Lyons,J., Rauh-Pfeiffer,A., Ming-Yu,Y., Lu,X.M., Zurakowski,D., Curley,M., Collier,S., Duggan,C., Nurko,S., Thompson,J., Ajami,A., Borgonha,S., Young,V.R., and Castillo,L. (2001). Cysteine metabolism and whole blood glutathione synthesis in septic pediatric patients. *Crit Care Med.* 29, 870-877.

- Maeda,K., Cao,H., Kono,K., Gorgun,C.Z., Furuhashi,M., Uysal,K.T., Cao,Q., Atsumi,G., Malone,H., Krishnan,B., Minokoshi,Y., Kahn,B.B., Parker,R.A., and Hotamisligil,G.S. (2005). Adipocyte/macrophage fatty acid binding proteins control integrated metabolic responses in obesity and diabetes. *Cell Metab* 1, 107-119.
- Majerus,P.W., Brauner,M.J., Smith,M.B., and Minnich,V. (1971). Glutathione synthesis in human erythrocytes. II. Purification and properties of the enzymes of glutathione biosynthesis. *J. Clin. Invest* 50, 1637-1643.
- Mannervik,B., Axelsson,K., Sundewall,A.C., and Holmgren,A. (1983). Relative contributions of thioltransferase- and thioredoxin-dependent systems in reduction of low-molecular-mass and protein disulphides. *Biochem. J.* 213, 519-523.
- Marnett,L.J., Hurd,H.K., Hollstein,M.C., Levin,D.E., Esterbauer,H., and Ames,B.N. (1985). Naturally occurring carbonyl compounds are mutagens in *Salmonella* tester strain TA104. *Mutat. Res.* 148, 25-34.
- Martin,S.S., Qasim,A., and Reilly,M.P. (2008). Leptin resistance: a possible interface of inflammation and metabolism in obesity-related cardiovascular disease. *J. Am. Coll. Cardiol.* 52, 1201-1210.
- Mathur,S., Takai,K.P., Macintyre,D.L., and Reid,D. (2008). Estimation of thigh muscle mass with magnetic resonance imaging in older adults and people with chronic obstructive pulmonary disease. *Phys. Ther.* 88, 219-230.
- Max,S.R., Mill,J., Mearow,K., Konagaya,M., Konagaya,Y., Thomas,J.W., Banner,C., and Vitkovic,L. (1988). Dexamethasone regulates glutamine synthetase expression in rat skeletal muscles. *Am. J. Physiol* 255, E397-E402.
- Mayne,S.T. (2003). Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research. *J. Nutr.* 133 Suppl 3, 933S-940S.
- McCauley,R., Kong,S.E., and Hall,J. (1998). Glutamine and nucleotide metabolism within enterocytes. *JPEN J. Parenter. Enteral Nutr.* 22, 105-111.
- Melnyk,J.A., Rogers,M.A., and Hurley,B.F. (2009). Effects of strength training and detraining on regional muscle in young and older men and women. *Eur. J. Appl. Physiol* 105, 929-938.
- Meredith,M.J. and Reed,D.J. (1982). Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J. Biol. Chem.* 257, 3747-3753.
- Metsios,G.S., Stavropoulos-Kalinoglou,A., Panoulas,V.F., Wilson,M., Nevill,A.M., Koutedakis,Y., and Kitas,G.D. (2009). Association of physical inactivity with increased cardiovascular risk in patients with rheumatoid arthritis. *Eur. J. Cardiovasc. Prev. Rehabil.* 16, 188-194.
- Meyer,R.A. and Terjung,R.L. (1979). Differences in ammonia and adenylate metabolism in contracting fast and slow muscle. *Am. J. Physiol* 237, C111-C118.
- Mezzano,D., Munoz,X., Martinez,C., Cuevas,A., Panes,O., Aranda,E., Guasch,V., Strobel,P., Munoz,B., Rodriguez,S., Pereira,J., and Leighton,F. (1999). Vegetarians and cardiovascular risk factors: hemostasis, inflammatory markers and plasma homocysteine. *Thromb. Haemost.* 81, 913-917.
- Michelet,F., Gueguen,R., Leroy,P., Wellman,M., Nicolas,A., and Siest,G. (1995). Blood and plasma glutathione measured in healthy subjects by HPLC: relation to sex, aging, biological variables, and life habits. *Clin. Chem.* 41, 1509-1517.
- Miles,M.P., Naukam,R.J., Hackney,A.C., and Clarkson,P.M. (1999). Blood leukocyte and glutamine fluctuations after eccentric exercise. *Int. J. Sports Med.* 20, 322-327.
- Mitch,W.E. (2006). Proteolytic mechanisms, not malnutrition, cause loss of muscle mass in kidney failure. *J. Ren Nutr.* 16, 208-211.
- Mittelman,S.D., Klier,K., Braun,S., Azen,C., Geffner,M.E., and Buchanan,T.A. (2010). Obese Adolescents Show Impaired Meal Responses of the Appetite-Regulating Hormones Ghrelin and PYY. *Obesity.* (Silver. Spring).

- Miyatani,M., Kanehisa,H., Kuno,S., Nishijima,T., and Fukunaga,T. (2002). Validity of ultrasonograph muscle thickness measurements for estimating muscle volume of knee extensors in humans. *Eur. J. Appl. Physiol* 86, 203-208.
- Morrison,J.A., Jacobsen,D.W., Sprecher,D.L., Robinson,K., Khoury,P., and Daniels,S.R. (1999). Serum glutathione in adolescent males predicts parental coronary heart disease. *Circulation* 100, 2244-2247.
- Morse,C.I., Thom,J.M., Reeves,N.D., Birch,K.M., and Narici,M.V. (2005). In vivo physiological cross-sectional area and specific force are reduced in the gastrocnemius of elderly men. *J. Appl. Physiol* 99, 1050-1055.
- Moslen,M.T. (1994). Reactive oxygen species in normal physiology, cell injury and phagocytosis. *Adv. Exp. Med. Biol.* 366, 17-27.
- Moylan,J.S. and Reid,M.B. (2007). Oxidative stress, chronic disease, and muscle wasting. *Muscle Nerve* 35, 411-429.
- Mugabo,Y., Li,L., and Renier,G. (2009). The Connection Between C-reactive Protein (CRP) and Diabetic Vasculopathy. Focus on Preclinical Findings. *Curr. Diabetes Rev.*
- Mulder,E.R., Stegeman,D.F., Gerrits,K.H., Paalman,M.I., Rittweger,J., Felsenberg,D., and de Haan,A. (2006). Strength, size and activation of knee extensors followed during 8 weeks of horizontal bed rest and the influence of a countermeasure. *Eur. J. Appl. Physiol* 97, 706-715.
- Muller,F.L., Song,W., Jang,Y.C., Liu,Y., Sabia,M., Richardson,A., and Van Remmen,H. (2007). Denervation-induced skeletal muscle atrophy is associated with increased mitochondrial ROS production. *Am. J. Physiol Regul. Integr. Comp Physiol* 293, R1159-R1168.
- Muller,M.J., Bosity-Westphal,A., Klaus,S., Kreymann,G., Luhrmann,P.M., Neuhauser-Berthold,M., Noack,R., Pirke,K.M., Platte,P., Selberg,O., and Steiniger,J. (2004). World Health Organization equations have shortcomings for predicting resting energy expenditure in persons from a modern, affluent population: generation of a new reference standard from a retrospective analysis of a German database of resting energy expenditure. *Am. J. Clin. Nutr.* 80, 1379-1390.
- Murgatroyd,P.R., Goldberg,G.R., Leahy,F.E., Gilsenan,M.B., and Prentice,A.M. (1999). Effects of inactivity and diet composition on human energy balance. *Int J. Obes. Relat Metab Disord.* 23, 1269-1275.
- Nagaya,N., Itoh,T., Murakami,S., Oya,H., Uematsu,M., Miyatake,K., and Kangawa,K. (2005). Treatment of cachexia with ghrelin in patients with COPD. *Chest* 128, 1187-1193.
- Najim,R.A., Sharquie,K.E., and Abu-Raghif,A.R. (2007). Oxidative stress in patients with Behcet's disease: I correlation with severity and clinical parameters. *J. Dermatol.* 34, 308-314.
- Narici,M. and Cerretelli,P. (1998). Changes in human muscle architecture in disuse-atrophy evaluated by ultrasound imaging. *J. Gravit. Physiol* 5, 73-74.
- Narici,M.V. and Maganaris,C.N. (2006). Adaptability of elderly human muscles and tendons to increased loading. *J. Anat.* 208, 433-443.
- Newsholme,E.A. and Calder,P.C. (1997). The proposed role of glutamine in some cells of the immune system and speculative consequences for the whole animal. *Nutrition* 13, 728-730.
- Newsholme,E.A., Crabtree,B., and Ardawi,M.S. (1985). Glutamine metabolism in lymphocytes: its biochemical, physiological and clinical importance. *Q. J. Exp. Physiol* 70, 473-489.
- Nicklas,B.J. and Brinkley,T.E. (2009). Exercise training as a treatment for chronic inflammation in the elderly. *Exerc. Sport Sci. Rev.* 37, 165-170.
- Ntambi,J.M. and Miyazaki,M. (2004). Regulation of stearoyl-CoA desaturases and role in metabolism. *Prog. Lipid Res.* 43, 91-104.

- Nunes,R.B., Tonetto,M., Machado,N., Chazan,M., Heck,T.G., Veiga,A.B., and Dall'Ago,P. (2008). Physical exercise improves plasmatic levels of IL-10, left ventricular end-diastolic pressure, and muscle lipid peroxidation in chronic heart failure rats. *J. Appl. Physiol* *104*, 1641-1647.
- Nygaard,O., Nordrehaug,J.E., Refsum,H., Ueland,P.M., Farstad,M., and Vollset,S.E. (1997). Plasma homocysteine levels and mortality in patients with coronary artery disease. *N. Engl. J. Med.* *337*, 230-236.
- Nygaard,O., Vollset,S.E., Refsum,H., Stensvold,I., Tverdal,A., Nordrehaug,J.E., Ueland,M., and Kvale,G. (1995). Total plasma homocysteine and cardiovascular risk profile. The Hordaland Homocysteine Study. *JAMA* *274*, 1526-1533.
- O'Gorman,D.J., Karlsson,H.K., McQuaid,S., Yousif,O., Rahman,Y., Gasparro,D., Glund,S., Chibalin,A.V., Zierath,J.R., and Nolan,J.J. (2006). Exercise training increases insulin-stimulated glucose disposal and GLUT4 (SLC2A4) protein content in patients with type 2 diabetes. *Diabetologia* *49*, 2983-2992.
- Ohuchi,S., Matsumoto,Y., Morita,T., and Sugiyama,K. (2009). High casein diet decreases plasma homocysteine concentration in rats. *J. Nutr. Sci. Vitaminol. (Tokyo)* *55*, 22-30.
- Olsen,R.H., Krogh-Madsen,R., Thomsen,C., Booth,F.W., and Pedersen,B.K. (2008). Metabolic responses to reduced daily steps in healthy nonexercising men. *JAMA* *299*, 1261-1263.
- Ortenblad,N., Madsen,K., and Djurhuus,M.S. (1997). Antioxidant status and lipid peroxidation after short-term maximal exercise in trained and untrained humans. *Am. J. Physiol* *272*, R1258-R1263.
- Ostrowski,K., Rohde,T., Zacho,M., Asp,S., and Pedersen,B.K. (1998). Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. *J. Physiol* *508 (Pt 3)*, 949-953.
- Paddon-Jones,D., Sheffield-Moore,M., Urban,R.J., Sanford,A.P., Aarsland,A., Wolfe,R.R., and Ferrando,A.A. (2004). Essential amino acid and carbohydrate supplementation ameliorates muscle protein loss in humans during 28 days bedrest. *J. Clin. Endocrinol. Metab* *89*, 4351-4358.
- Paglia,D.E. and Valentine,W.N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab Clin. Med.* *70*, 158-169.
- Parry-Billings,M., Evans,J., Calder,P.C., and Newsholme,E.A. (1990). Does glutamine contribute to immunosuppression after major burns? *Lancet* *336*, 523-525.
- Pastore,A., Federici,G., Bertini,E., and Piemonte,F. (2003). Analysis of glutathione: implication in redox and detoxification. *Clin. Chim. Acta* *333*, 19-39.
- Pereira,C., Murphy,K., Jeschke,M., and Herndon,D.N. (2005). Post burn muscle wasting and the effects of treatments. *Int. J. Biochem. Cell Biol.* *37*, 1948-1961.
- Peter,A., Weigert,C., Staiger,H., Machicao,F., Schick,F., Machann,J., Stefan,N., Thamer,C., Haring,H.U., and Schleicher,E. (2009). Individual stearyl-coa desaturase 1 expression modulates endoplasmic reticulum stress and inflammation in human myotubes and is associated with skeletal muscle lipid storage and insulin sensitivity in vivo. *Diabetes* *58*, 1757-1765.
- Petersen,A.M. and Pedersen,B.K. (2006). The role of IL-6 in mediating the anti-inflammatory effects of exercise. *J. Physiol Pharmacol.* *57 Suppl 10*, 43-51.
- Phillips,S.M. (2004). Protein requirements and supplementation in strength sports. *Nutrition* *20*, 689-695.
- Pickova,J. (2009). Importance of knowledge on lipid composition of foods to support development towards consumption of higher levels of n-3 fatty acids via freshwater fish. *Physiol Res.* *58 Suppl 1*, S39-S45.
- Pinet,C., Scillia,P., Cassart,M., Lamotte,M., Knoop,C., Melot,C., and Estenne,M. (2004). Preferential reduction of quadriceps over respiratory muscle strength and bulk after lung transplantation for cystic fibrosis. *Thorax* *59*, 783-789.

- Pisot,R., Narici,M.V., Simunic,B., De Boer,M., Seynnes,O., Jurdana,M., Biolo,G., and Mekjavic,I.B. (2008). Whole muscle contractile parameters and thickness loss during 35-day bed rest. *Eur. J. Appl. Physiol* 104, 409-414.
- Pitsavos,C., Panagiotakos,D.B., Tzima,N., Lentzas,Y., Chrysohoou,C., Das,U.N., and Stefanadis,C. (2007). Diet, exercise, and C-reactive protein levels in people with abdominal obesity: the ATTICA epidemiological study. *Angiology* 58, 225-233.
- Podrez,E.A., Abu-Soud,H.M., and Hazen,S.L. (2000). Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radic. Biol. Med.* 28, 1717-1725.
- Police,S.B., Thatcher,S.E., Charnigo,R., Daugherty,A., and Cassis,L.A. (2009). Obesity promotes inflammation in periaortic adipose tissue and angiotensin II-induced abdominal aortic aneurysm formation. *Arterioscler. Thromb. Vasc. Biol.* 29, 1458-1464.
- Powers,S.K., Criswell,D., Lawler,J., Ji,L.L., Martin,D., Herb,R.A., and Dudley,G. (1994). Influence of exercise and fiber type on antioxidant enzyme activity in rat skeletal muscle. *Am. J. Physiol* 266, R375-R380.
- Powers,S.K., Kavazis,A.N., and DeRuisseau,K.C. (2005). Mechanisms of disuse muscle atrophy: role of oxidative stress. *Am. J. Physiol Regul. Integr. Comp Physiol* 288, R337-R344.
- Powers,S.K., Kavazis,A.N., and McClung,J.M. (2007). Oxidative stress and disuse muscle atrophy. *J. Appl. Physiol* 102, 2389-2397.
- Pradhan,A.D., Manson,J.E., Rifai,N., Buring,J.E., and Ridker,P.M. (2001). C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* 286, 327-334.
- Primeau,A.J., Adhihetty,P.J., and Hood,D.A. (2002). Apoptosis in heart and skeletal muscle. *Can. J. Appl. Physiol* 27, 349-395.
- Purintrapiban,J., Wang,M.C., and Forsberg,N.E. (2003). Degradation of sarcomeric and cytoskeletal proteins in cultured skeletal muscle cells. *Comp Biochem. Physiol B Biochem. Mol. Biol.* 136, 393-401.
- Reeves,N.D., Maganaris,C.N., and Narici,M.V. (2004). Ultrasonographic assessment of human skeletal muscle size. *Eur. J. Appl. Physiol* 91, 116-118.
- Reeves,N.J., Maganaris,C.N., Ferretti,G., and Narici,M.V. (2002). Influence of simulated microgravity on human skeletal muscle architecture and function. *J. Gravit. Physiol* 9, 153-154.
- Reid,M.B., Haack,K.E., Franchek,K.M., Valberg,P.A., Kobzik,L., and West,M.S. (1992). Reactive oxygen in skeletal muscle. I. Intracellular oxidant kinetics and fatigue in vitro. *J. Appl. Physiol* 73, 1797-1804.
- Remels,A.H., Gosker,H.R., van,d., V, Langen,R.C., and Schols,A.M. (2007). Systemic inflammation and skeletal muscle dysfunction in chronic obstructive pulmonary disease: state of the art and novel insights in regulation of muscle plasticity. *Clin. Chest Med.* 28, 537-52, vi.
- Requena,J.R., Levine,R.L., and Stadtman,E.R. (2003). Recent advances in the analysis of oxidized proteins. *Amino. Acids* 25, 221-226.
- Rhoads,R.E. (1999). Signal transduction pathways that regulate eukaryotic protein synthesis. *J. Biol. Chem.* 274, 30337-30340.
- Richards,R.S., Roberts,T.K., Dunstan,R.H., McGregor,N.R., and Butt,H.L. (1998). Erythrocyte antioxidant systems protect cultured endothelial cells against oxidant damage. *Biochem. Mol. Biol. Int.* 46, 857-865.
- Ritz,P., Acheson,K.J., Gachon,P., Vico,L., Bernard,J.J., Alexandre,C., and Beaufreere,B. (1998). Energy and substrate metabolism during a 42-day bed-rest in a head-down tilt position in humans. *Eur. J. Appl. Physiol Occup. Physiol* 78, 308-314.
- Roberts,L.J. and Morrow,J.D. (1997). The generation and actions of isoprostanes. *Biochim. Biophys. Acta* 1345, 121-135.

- Robertson,M.D., Henderson,R.A., Vist,G.E., and Rumsey,R.D. (2004). Plasma ghrelin response following a period of acute overfeeding in normal weight men. *Int. J. Obes. Relat Metab Disord.* 28, 727-733.
- Robson,P.J., Blannin,A.K., Walsh,N.P., Castell,L.M., and Gleeson,M. (1999). Effects of exercise intensity, duration and recovery on in vitro neutrophil function in male athletes. *Int. J. Sports Med.* 20, 128-135.
- Ross,J.A., Moses,A.G., and Fearon,K.C. (1999). The anti-catabolic effects of n-3 fatty acids. *Curr. Opin. Clin. Nutr. Metab Care* 2, 219-226.
- Rossi,S., D'Amico,M., Capuano,A., Romano,M., Petronella,P., and Di Filippo,C. (2006). Hyperglycemia in streptozotocin-induced diabetes leads to persistent inflammation and tissue damage following uveitis due to reduced levels of ciliary body heme oxygenase-1. *Mediators. Inflamm.* 2006, 60285.
- Roth,E., Manhart,N., and Wessner,B. (2004). Assessing the antioxidative status in critically ill patients. *Curr. Opin. Clin. Nutr. Metab Care* 7, 161-168.
- Rotter,V., Nagaev,I., and Smith,U. (2003). Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. *J. Biol. Chem.* 278, 45777-45784.
- Rowbottom,D.G., Keast,D., and Morton,A.R. (1996). The emerging role of glutamine as an indicator of exercise stress and overtraining. *Sports Med.* 21, 80-97.
- Sakagami,H., Kishino,K., Amano,O., Kanda,Y., Kunii,S., Yokote,Y., Oizumi,H., and Oizumi,T. (2009). Cell death induced by nutritional starvation in mouse macrophage-like RAW264.7 cells. *Anticancer Res.* 29, 343-347.
- Sandri,M., Sandri,C., Gilbert,A., Skurk,C., Calabria,E., Picard,A., Walsh,K., Schiaffino,S., Lecker,S.H., and Goldberg,A.L. (2004). Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* 117, 399-412.
- Santos,R.V., Caperuto,E.C., and Costa Rosa,L.F. (2007). Effects of acute exhaustive physical exercise upon glutamine metabolism of lymphocytes from trained rats. *Life Sci.* 80, 573-578.
- Sastre,J., Asensi,M., Gasco,E., Pallardo,F.V., Ferrero,J.A., Furukawa,T., and Vina,J. (1992). Exhaustive physical exercise causes oxidation of glutathione status in blood: prevention by antioxidant administration. *Am. J. Physiol* 263, R992-R995.
- Scheld,K., Zittermann,A., Heer,M., Herzog,B., Mika,C., Drummer,C., and Stehle,P. (2001). Nitrogen metabolism and bone metabolism markers in healthy adults during 16 weeks of bed rest. *Clin. Chem.* 47, 1688-1695.
- Scherer,P.E., Williams,S., Fogliano,M., Baldini,G., and Lodish,H.F. (1995). A novel serum protein similar to C1q, produced exclusively in adipocytes. *J. Biol. Chem.* 270, 26746-26749.
- Schulz,J.B., Lindenau,J., Seyfried,J., and Dichgans,J. (2000). Glutathione, oxidative stress and neurodegeneration. *Eur. J. Biochem.* 267, 4904-4911.
- Sedlak,J. and Lindsay,R.H. (1968). Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal. Biochem.* 25, 192-205.
- Sen,C.K. (1999). Glutathione homeostasis in response to exercise training and nutritional supplements. *Mol. Cell Biochem.* 196, 31-42.
- Sen,C.K., Marin,E., Kretschmar,M., and Hanninen,O. (1992). Skeletal muscle and liver glutathione homeostasis in response to training, exercise, and immobilization. *J. Appl. Physiol* 73, 1265-1272.
- Sen,C.K. and Packer,L. (2000). Thiol homeostasis and supplements in physical exercise. *Am. J. Clin. Nutr.* 72, 653S-669S.
- Serhan,C.N., Haeggstrom,J.Z., and Leslie,C.C. (1996). Lipid mediator networks in cell signaling: update and impact of cytokines. *FASEB J.* 10, 1147-1158.

- Seynnes,O.R., Maganaris,C.N., de Boer,M.D., di Prampero,P.E., and Narici,M.V. (2008). Early structural adaptations to unloading in the human calf muscles. *Acta Physiol (Oxf)* 193, 265-274.
- Shackelford,L.C., Leblanc,A.D., Driscoll,T.B., Evans,H.J., Rianon,N.J., Smith,S.M., Spector,E., Feeback,D.L., and Lai,D. (2004). Resistance exercise as a countermeasure to disuse-induced bone loss. *J. Appl. Physiol* 97, 119-129.
- Sherwin,R.S. (1978). Effect of starvation on the turnover and metabolic response to leucine. *J. Clin. Invest* 61, 1471-1481.
- Siems,W., Capuozzo,E., Lucano,A., Salerno,C., and Crifo,C. (2003). High sensitivity of plasma membrane ion transport ATPases from human neutrophils towards 4-hydroxy-2,3-trans-nonenal. *Life Sci.* 73, 2583-2590.
- Sonnenfeld,G., Butel,J.S., and Shearer,W.T. (2003). Effects of the space flight environment on the immune system. *Rev. Environ. Health* 18, 1-17.
- Spranger,J., Kroke,A., Mohlig,M., Bergmann,M.M., Ristow,M., Boeing,H., and Pfeiffer,A.F. (2003). Adiponectin and protection against type 2 diabetes mellitus. *Lancet* 361, 226-228.
- Staal,F.J., Roederer,M., Herzenberg,L.A., and Herzenberg,L.A. (1990). Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. *Proc. Natl. Acad. Sci. U. S. A* 87, 9943-9947.
- Stadtman,E.R. (1990). Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radic. Biol. Med.* 9, 315-325.
- Stadtman,E.R. (2001). Protein oxidation in aging and age-related diseases. *Ann. N. Y. Acad. Sci.* 928, 22-38.
- Starke-Reed,P.E. and Oliver,C.N. (1989). Protein oxidation and proteolysis during aging and oxidative stress. *Arch. Biochem. Biophys.* 275, 559-567.
- Steffen,J.M. and Musacchia,X.J. (1986). Spaceflight effects on adult rat muscle protein, nucleic acids, and amino acids. *Am. J. Physiol* 251, R1059-R1063.
- Stein,T.P. and Leskiw,M.J. (2000). Oxidant damage during and after spaceflight. *Am. J. Physiol Endocrinol. Metab* 278, E375-E382.
- Stein,T.P., Leskiw,M.J., and Schluter,M.D. (1996). Diet and nitrogen metabolism during spaceflight on the shuttle. *J Appl. Physiol* 81, 82-97.
- Stein,T.P., Leskiw,M.J., Schluter,M.D., Donaldson,M.R., and Larina,I. (1999a). Protein kinetics during and after long-duration spaceflight on MIR. *Am. J. Physiol* 276, E1014-E1021.
- Stein,T.P., Leskiw,M.J., Schluter,M.D., Hoyt,R.W., Lane,H.W., Gretebeck,R.E., and Leblanc,A.D. (1999b). Energy expenditure and balance during spaceflight on the space shuttle. *Am. J. Physiol* 276, R1739-R1748.
- Stein,T.P., Schluter,M.D., Leskiw,M.J., and Boden,G. (1999c). Attenuation of the protein wasting associated with bed rest by branched-chain amino acids. *Nutrition* 15, 656-660.
- Stevenson,E.J., Giresi,P.G., Koncarevic,A., and Kandarian,S.C. (2003). Global analysis of gene expression patterns during disuse atrophy in rat skeletal muscle. *J. Physiol* 551, 33-48.
- Stitt,T.N., Drujan,D., Clarke,B.A., Panaro,F., Timofeyva,Y., Kline,W.O., Gonzalez,M., Yancopoulos,G.D., and Glass,D.J. (2004). The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol. Cell* 14, 395-403.
- Storch,K.J., Wagner,D.A., Burke,J.F., and Young,V.R. (1988). Quantitative study in vivo of methionine cycle in humans using [methyl-2H3]- and [1-13C]methionine. *Am. J. Physiol* 255, E322-E331.
- Stuart,C.A., Shangraw,R.E., Peters,E.J., and Wolfe,R.R. (1990). Effect of dietary protein on bed-rest-related changes in whole-body-protein synthesis. *Am. J. Clin. Nutr.* 52, 509-514.

- Sumikawa,K., Mu,Z., Inoue,T., Okochi,T., Yoshida,T., and Adachi,K. (1993). Changes in erythrocyte membrane phospholipid composition induced by physical training and physical exercise. *Eur. J. Appl. Physiol Occup. Physiol* 67, 132-137.
- Sun,T., Zhou,W.B., Luo,X.P., Tang,Y.L., and Shi,H.M. (2009). Oral L-arginine supplementation in acute myocardial infarction therapy: a meta-analysis of randomized controlled trials. *Clin. Cardiol.* 32, 649-652.
- Taouis,M., Dagou,C., Ster,C., Durand,G., Pinault,M., and Delarue,J. (2002). N-3 polyunsaturated fatty acids prevent the defect of insulin receptor signaling in muscle. *Am. J. Physiol Endocrinol. Metab* 282, E664-E671.
- Tarnopolsky,M. (2004). Protein requirements for endurance athletes. *Nutrition* 20, 662-668.
- Tauler,P., Aguilo,A., Gimeno,I., Fuentespina,E., Tur,J.A., and Pons,A. (2006). Response of blood cell antioxidant enzyme defences to antioxidant diet supplementation and to intense exercise. *Eur. J. Nutr.* 45, 187-195.
- Tawakol,A., Omland,T., Gerhard,M., Wu,J.T., and Creager,M.A. (1997). Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans. *Circulation* 95, 1119-1121.
- Tchou,J., Kasai,H., Shibutani,S., Chung,M.H., Laval,J., Grollman,A.P., and Nishimura,S. (1991). 8-oxoguanine (8-hydroxyguanine) DNA glycosylase and its substrate specificity. *Proc. Natl. Acad. Sci. U. S. A* 88, 4690-4694.
- Terada,N., Patel,H.R., Takase,K., Kohno,K., Nairn,A.C., and Gelfand,E.W. (1994). Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. *Proc. Natl. Acad. Sci. U. S. A* 91, 11477-11481.
- Thompson,S.A., White,C.C., Krejsa,C.M., Eaton,D.L., and Kavanagh,T.J. (2000). Modulation of glutathione and glutamate-L-cysteine ligase by methylmercury during mouse development. *Toxicol. Sci.* 57, 141-146.
- Tidball,J.G. and Spencer,M.J. (2002). Expression of a calpastatin transgene slows muscle wasting and obviates changes in myosin isoform expression during murine muscle disuse. *J. Physiol* 545, 819-828.
- Tracey,K.J. and Cerami,A. (1990). Metabolic responses to cachectin/TNF. A brief review. *Ann. N. Y. Acad. Sci.* 587, 325-331.
- Tracey,K.J. and Cerami,A. (1994). Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu. Rev. Med.* 45, 491-503.
- Ueda,Y., Kawakami,Y., Kunii,D., Okada,H., Azuma,M., Le,D.S., and Yamamoto,S. (2008). Elevated concentrations of linoleic acid in erythrocyte membrane phospholipids in patients with inflammatory bowel disease. *Nutr. Res.* 28, 239-244.
- Ueland,P.M. and Refsum,H. (1989). Plasma homocysteine, a risk factor for vascular disease: plasma levels in health, disease, and drug therapy. *J. Lab Clin. Med.* 114, 473-501.
- Upchurch,G.R., Jr., Welch,G.N., Fabian,A.J., Freedman,J.E., Johnson,J.L., Keaney,J.F., Jr., and Loscalzo,J. (1997). Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. *J. Biol. Chem.* 272, 17012-17017.
- Uysal,K.T., Wiesbrock,S.M., Marino,M.W., and Hotamisligil,G.S. (1997). Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 389, 610-614.
- van Acker,B.A., Hulsewe,K.W., Wagenmakers,A.J., Deutz,N.E., Van Kreef,B.K., Halliday,D., Matthews,D.E., Soeters,P.B., and Von Meyenfeldt,M.F. (1998). Absence of glutamine isotopic steady state: implications for the assessment of whole-body glutamine production rate. *Clin. Sci. (Lond)* 95, 339-346.
- van de Poll,M.C., Soeters,P.B., Deutz,N.E., Fearon,K.C., and Dejong,C.H. (2004). Renal metabolism of amino acids: its role in interorgan amino acid exchange. *Am. J. Clin. Nutr.* 79, 185-197.

- van den, B.M., Boers, G.H., Franken, D.G., Blom, H.J., Van Kamp, G.J., Jakobs, C., Rauwerda, J.A., Kluft, C., and Stehouwert, C.D. (1995). Hyperhomocysteinaemia and endothelial dysfunction in young patients with peripheral arterial occlusive disease. *Eur. J. Clin. Invest* 25, 176-181.
- van Hall, G., Saltin, B., and Wagenmakers, A.J. (1999). Muscle protein degradation and amino acid metabolism during prolonged knee-extensor exercise in humans. *Clin. Sci. (Lond)* 97, 557-567.
- Veiga, O.L., Gomez-Martinez, S., Martinez-Gomez, D., Villagra, A., Calle, M.E., Marcos, A., and Study Group (2009). Physical activity as a preventive measure against overweight, obesity, infections, allergies and cardiovascular disease risk factors in adolescents: AFINOS Study protocol. *BMC. Public Health* 9, 475.
- Venables, M.C. and Jeukendrup, A.E. (2009). Physical inactivity and obesity: links with insulin resistance and type 2 diabetes mellitus. *Diabetes Metab Res. Rev.* 25 *Suppl 1*, S18-S23.
- Vescovo, G., Ravara, B., and Dalla, L.L. (2008). Skeletal muscle myofibrillar protein oxidation and exercise capacity in heart failure. *Basic Res. Cardiol.* 103, 285-290.
- Vincent, H.K., Bourguignon, C., and Vincent, K.R. (2006). Resistance training lowers exercise-induced oxidative stress and homocysteine levels in overweight and obese older adults. *Obesity. (Silver. Spring)* 14, 1921-1930.
- Wallenius, V., Wallenius, K., Ahren, B., Rudling, M., Carlsten, H., Dickson, S.L., Ohlsson, C., and Jansson, J.O. (2002). Interleukin-6-deficient mice develop mature-onset obesity. *Nat. Med.* 8, 75-79.
- Walsh, N.P., Blannin, A.K., Clark, A.M., Cook, L., Robson, P.J., and Gleeson, M. (1998a). The effects of high-intensity intermittent exercise on the plasma concentrations of glutamine and organic acids. *Eur. J. Appl. Physiol Occup. Physiol* 77, 434-438.
- Walsh, N.P., Blannin, A.K., Robson, P.J., and Gleeson, M. (1998b). Glutamine, exercise and immune function. Links and possible mechanisms. *Sports Med.* 26, 177-191.
- Walter, J.B. and Talbot, I.C. (1996). *General pathology.* (Edinburgh: Churchill Livingstone).
- Wang, J., Chen, L., Li, P., Li, X., Zhou, H., Wang, F., Li, D., Yin, Y., and Wu, G. (2008). Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. *J. Nutr.* 138, 1025-1032.
- Warmington, S.A., Tolan, R., and McBennett, S. (2000). Functional and histological characteristics of skeletal muscle and the effects of leptin in the genetically obese (ob/ob) mouse. *Int. J. Obes. Relat Metab Disord.* 24, 1040-1050.
- Weaver, K.L., Ivester, P., Seeds, M., Case, L.D., Arm, J.P., and Chilton, F.H. (2009). Effect of dietary fatty acids on inflammatory gene expression in healthy humans. *J. Biol. Chem.* 284, 15400-15407.
- Weber, G.F., Mirza, N.M., Yunis, E.J., Dubey, D., and Cantor, H. (1997). Localization and treatment of an oxidation-sensitive defect within the TCR-coupled signalling pathway that is associated with normal and premature immunologic aging. *Growth Dev. Aging* 61, 191-207.
- Wei, M., Kampert, J.B., Barlow, C.E., Nichaman, M.Z., Gibbons, L.W., Paffenbarger, R.S., Jr., and Blair, S.N. (1999). Relationship between low cardiorespiratory fitness and mortality in normal-weight, overweight, and obese men. *JAMA* 282, 1547-1553.
- Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L., and Ferrante, A.W., Jr. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest* 112, 1796-1808.
- Wellen, K.E. and Hotamisligil, G.S. (2003). Obesity-induced inflammatory changes in adipose tissue. *J. Clin. Invest* 112, 1785-1788.
- Wernerman, J. (2008). Clinical use of glutamine supplementation. *J. Nutr.* 138, 2040S-2044S.
- Wertz, P.W. (2009). Essential fatty acids and dietary stress. *Toxicol. Ind. Health* 25, 279-283.

- Whidden, M.A., McClung, J.M., Falk, D.J., Hudson, M.B., Smuder, A.J., Nelson, W.B., and Powers, S.K. (2009). Xanthine oxidase contributes to mechanical ventilation-induced diaphragmatic oxidative stress and contractile dysfunction. *J. Appl. Physiol* *106*, 385-394.
- Williams, B.D., Chinkes, D.L., and Wolfe, R.R. (1998). Alanine and glutamine kinetics at rest and during exercise in humans. *Med. Sci. Sports Exerc.* *30*, 1053-1058.
- Willis, W.T. and Jackman, M.R. (1994). Mitochondrial function during heavy exercise. *Med. Sci. Sports Exerc.* *26*, 1347-1353.
- Winkler, G., Kiss, S., Keszthelyi, L., Sapi, Z., Ory, I., Salamon, F., Kovacs, M., Vargha, P., Szekeres, O., Speer, G., Karadi, I., Sikter, M., Kaszas, E., Dworak, O., Gero, G., and Cseh, K. (2003). Expression of tumor necrosis factor (TNF)-alpha protein in the subcutaneous and visceral adipose tissue in correlation with adipocyte cell volume, serum TNF-alpha, soluble serum TNF-receptor-2 concentrations and C-peptide level. *Eur. J. Endocrinol.* *149*, 129-135.
- Wolf, A.M., Wolf, D., Rumpold, H., Enrich, B., and Tilg, H. (2004). Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes. *Biochem. Biophys. Res. Commun.* *323*, 630-635.
- Wolfe, R.R. (2004). *Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis*. (New York, United States: *Wiley-IEEE, Inc*).
- Xu, H., Barnes, G.T., Yang, Q., Tan, G., Yang, D., Chou, C.J., Sole, J., Nichols, A., Ross, J.S., Tartaglia, L.A., and Chen, H. (2003). Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest* *112*, 1821-1830.
- Yadav, V.K., Oury, F., Suda, N., Liu, Z.W., Gao, X.B., Confavreux, C., Klemenhausen, K.C., Tanaka, K.F., Gingrich, J.A., Guo, X.E., Tecott, L.H., Mann, J.J., Hen, R., Horvath, T.L., and Karsenty, G. (2009). A serotonin-dependent mechanism explains the leptin regulation of bone mass, appetite, and energy expenditure. *Cell* *138*, 976-989.
- Yamagishi, S.I., Edelstein, D., Du, X.L., Kaneda, Y., Guzman, M., and Brownlee, M. (2001). Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. *J. Biol. Chem.* *276*, 25096-25100.
- Yang, E.Y., Nambi, V., Tang, Z., Virani, S.S., Boerwinkle, E., Hoogeveen, R.C., Astor, B.C., Mosley, T.H., Coresh, J., Chambless, L., and Ballantyne, C.M. (2009). Clinical implications of JUPITER (Justification for the Use of statins in Prevention: an Intervention Trial Evaluating Rosuvastatin) in a U.S. population insights from the ARIC (Atherosclerosis Risk in Communities) study. *J. Am. Coll. Cardiol.* *54*, 2388-2395.
- Yang, R.D., Matthews, D.E., Bier, D.M., Lo, C., and Young, V.R. (1984). Alanine kinetics in humans: influence of different isotopic tracers. *Am. J. Physiol* *247*, E634-E638.
- Yende, S., Waterer, G.W., Tolley, E.A., Newman, A.B., Bauer, D.C., Taaffe, D.R., Jensen, R., Crapo, R., Rubin, S., Nevitt, M., Simonsick, E.M., Satterfield, S., Harris, T., and Kritchevsky, S.B. (2006). Inflammatory markers are associated with ventilatory limitation and muscle dysfunction in obstructive lung disease in well functioning elderly subjects. *Thorax* *61*, 10-16.
- Yokota, T., Oritani, K., Takahashi, I., Ishikawa, J., Matsuyama, A., Ouchi, N., Kihara, S., Funahashi, T., Tenner, A.J., Tomiyama, Y., and Matsuzawa, Y. (2000). Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. *Blood* *96*, 1723-1732.
- Yoshidome, H., Kato, A., Edwards, M.J., and Lentsch, A.B. (1999). Interleukin-10 inhibits pulmonary NF-kappaB activation and lung injury induced by hepatic ischemia-reperfusion. *Am. J. Physiol* *277*, L919-L923.
- Zembron-Lacny, A., Ostapiuk, J., and Szyszka, K. (2009). Effects of sulphur-containing compounds on plasma redox status in muscle-damaging exercise. *Chin J. Physiol* *52*, 289-294.
- Zhang, L., Geng, Y., Yin, M., Mao, L., Zhang, S., and Pan, J. (2009). Low omega-6/omega-3 polyunsaturated fatty acid ratios reduce hepatic C-reactive protein expression in apolipoprotein E-null mice. *Nutrition*.

Zhang,X.J., Chinkes,D.L., and Wolfe,R.R. (2002). Measurement of muscle protein fractional synthesis and breakdown rates from a pulse tracer injection. *Am. J. Physiol Endocrinol. Metab* 283, E753-E764.

Zhao,T., Hou,M., Xia,M., Wang,Q., Zhu,H., Xiao,Y., Tang,Z., Ma,J., and Ling,W. (2005). Globular adiponectin decreases leptin-induced tumor necrosis factor-alpha expression by murine macrophages: involvement of cAMP-PKA and MAPK pathways. *Cell Immunol.* 238, 19-30.

Zhu,S., St Onge,M.P., Heshka,S., and Heymsfield,S.B. (2004). Lifestyle behaviors associated with lower risk of having the metabolic syndrome. *Metabolism* 53, 1503-1511.

Zoppini,G., Targher,G., Zamboni,C., Venturi,C., Cacciatori,V., Moghetti,P., and Muggeo,M. (2006). Effects of moderate-intensity exercise training on plasma biomarkers of inflammation and endothelial dysfunction in older patients with type 2 diabetes. *Nutr. Metab Cardiovasc. Dis.* 16, 543-549.

LIST OF PAPERS INCLUDED

- 1: Inactivity-mediated insulin resistance is associated with upregulated pro-inflammatory fatty acids in human cell membranes. Mazzucco S, Agostini F, Biolo G. Clin Nutr. 2009 Oct 27.
- 2: Regulation of muscle disuse atrophy by energy and protein intake in humans. Insights from bed rest studies. Biolo G, Antonione R, Caliandro E, Piccoli A, Mazzucco S, Massolino B, Zurlo M, Pastò A, Agostini F. Basic Applied Myology. 2009, 19 (2&3): 113-116.
- 3: Effect of physical activity on glutamine metabolism. Agostini F, Biolo G. Curr Opin Clin Nutr Metab Care. 2010 Jan;13(1):58-64.
- 4: Positive energy balance is associated with accelerated muscle atrophy and increased erythrocyte glutathione turnover during 5 wk of bed rest. Biolo G, Agostini F, Simunic B, Sturma M, Torelli L, Preiser JC, Deby-Dupont G, Magni P, Strollo F, di Prampero P, Guarnieri G, Mekjavic IB, Pisot R, Narici MV. Am J Clin Nutr. 2008 Oct;88(4):950-8.
- 5: Physical inactivity decreases whole body glutamine turnover independently from changes in proteolysis. Agostini F, Heer M, Guarnieri G, Biolo G. J Physiol. 2008 Oct 1;586(Pt 19):4775-81. Epub 2008 Jul 31.