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INACTIVITY AS A KEY FACTOR INDUCING INSULIN RESISTANCE AND METABOLIC SYNDROME

Sara Mazzucco

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DOTTORANDA

SARA MAZZUCCO

RESPONSABILE DOTTORATO DI RICERCA

PROF. *GIANNINO DEL SAL*

RELATORE

PROF. *GIANNI BIOLO - Università di Trieste*

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TABLE OF CONTENTS

ABSTRACT

Introduction. The metabolic syndrome is a cluster of alterations, including insulin resistance, dyslipidaemia, hypertension, hyperglycaemia, abdominal obesity, hyperhomocysteinemia, inflammation and oxidative stress, leading to type II diabetes and cardiovascular disease. The metabolic syndrome is usually associated with sedentary lifestyle and overweight, while regular physical activity and weight loss can counteract these alterations and prevent type II diabetes and cardiovascular disease.

Aim of the Thesis. In order to define the net role of inactivity as key factor inducing insulin resistance and metabolic syndrome independently from changes in body fat we have investigated the net impact of experimental bed rest on human metabolism. Experimental bed rest in healthy, young, lean subjects represents a suitable model to determine the effects of inactivity on physiology, avoiding potential interferences and confounding effects of diseases, ageing, energy unbalance and excess body fat. We have focused on inactivity-related development of insulin resistance, dyslipidaemia, hypertension as well as inflammation and oxidative stress. These aspects have been investigated during four different experimental bed rest protocols, lasting 2 months (WISE-Toulouse, France) and 5 weeks (Valdoltra, Slovenia 2006–2007–2008). Energy requirements and intakes were strictly controlled to avoid changes in fat mass.

Results and discussion. Muscle atrophy. Muscle atrophy was evidenced after three weeks of bed rest and was worsened by prolonged exposure to inactivity (WISE, Valdoltra studies). However, muscle loss rate was higher in the first 5 weeks of bed rest while it decreased in the second month of inactivity (WISE). Time-course analysis of insulin resistance development. Insulin resistance, measured by an oral glucose tolerance test, rapidly developed in the first week of inactivity and was maintained after 5 weeks of bed rest, as assessed by the ISI-Belfiore index of insulin sensitivity (Valdoltra 2008). Cardiovascular regulation. In the first week of bed rest, baroreflex sensitivity decreased indicating that, in an early phase, alterations in the sympatovagal balance paralleled changes in insulin resistance development. At the end of 5 week-bed rest, heart rate and heart rate variability as well as systolic blood pressure variability, indexes of cardiovascular regulation, were also impaired (Valdoltra 2008). Plasma lipids and lipid metabolism. Five weeks of bed rest induced a decrease in high-density lipoprotein (HDL) cholesterol. During inactivity, cholesteryl ester transfer protein (CETP), a key enzyme involved in HDL metabolism, was up-regulated and changes in CETP inversely correlated with changes in HDL-to-non-HDL cholesterol ratio. Conversely, changes in CETP and HDL were not directly correlated to insulin resistance (Valdoltra studies). Cell membrane lipids. Bed rest reduced monounsaturated FAs, enhanced n-6 polyunsaturated FA

total contents and affected activities of both Δ-5 and Δ-9 desaturases, enzymes involved in FA metabolism. These data further support that membrane FA composition and activities of Δ-5 and Δ-9 desaturases are predictive indicators of metabolic syndrome development. Moreover, arachidonic-to-eicosapentaenoic acid ratio, reflecting the competitive role of these FAs in the modulation of inflammatory processes, was shifted towards pro-inflammatory state (Valdoltra studies). Oxidative stress and glutathione kinetics. Bed rest induced oxidative stress as showed by enhanced muscle protein carbonylation, a marker of tissue exposure to oxidative damage, and increased muscle glutathione absolute synthesis, as assessed by a new one-sample, double-isotope tracers infusion method (Valdoltra 2007). Homocysteinemia and homocysteine kinetics. Plasma homocysteine level was increased by bed rest, due to a decrease in homocysteine clearance related to remethylation (WISE). Hyperhomocysteinemia is a further evidence of inactivity-mediated oxidative stress and increased cardiovascular risk.

Conclusions. Physical inactivity in healthy young subjects is a suitable model to define the net impact of physical inactivity on the development of metabolic alterations observed in patients with the metabolic syndrome. Our results indicate that inactivity is directly involved in insulin resistance development, low-grade systemic inflammation, dyslipidaemia, hyperhomocysteinemia, oxidative stress and autonomic-cardiovascular abnormalities.

INTRODUCTION

THE METABOLIC SYNDROME

The metabolic syndrome is a quite common pathological condition, defined as a cluster of metabolic alterations.
A recent report (Tentolouris, Argyrakopoulou, and Katsilambros 2008) indicates that the metabolic syndrome interests the 25% of the general population and the 70% of subjects suffering type 2 diabetes (Bianchi et al. 2008; Monami et al. 2007). Moreover, its frequency is increased with aging (Day 2007). Nevertheless, the absence of unified criteria for the metabolic syndrome diagnosis does not permit to precisely define its prevalence.
The central points of the metabolic syndrome are insulin resistance and visceral obesity (Chew, Gan, and Watts 2006). Both conditions are, in fact, strictly related to the other components of the metabolic syndrome (Reaven 1993; Maison et al. 2001; Reaven 2006). Additionally to the high prevalence of metabolic syndrome development in type 2 diabetes, epidemiological data demonstrate that diabetes usually precedes by many years the diagnosis for the metabolic syndrome (Bianchi et al. 2008; Monami et al. 2007).
The onset of the metabolic syndrome, in turn, significantly increases the risk of development of type 2 diabetes and of cardiovascular diseases.

CRITERIA USED FOR THE DIAGNOSIS OF METABOLIC SYNDROME

Unified criteria for metabolic syndrome diagnosis are still lacking. Presently, the most common and used definition of metabolic syndrome are four: the WHO (World Health Organization) criteria, defined in 1998; the EGIR (European Group for the Study of Insulin Resistance) definition, presented in 1999; the IDF (International Diabetes Federation) criteria presented in 2005 in parallel to the revised NCEP ATP III (National Cholesterol Education Program/Adult Treatment Panel III) definition.

WHO definition

The first definition of criteria for the metabolic syndrome diagnosis has been presented in 1998 by the World Health Organization (Alberti and Zimmet 1998).

Criteria for the metabolic syndrome.

1. Insulin resistance, is absolutely required for metabolic syndrome diagnosis and is defined as one of the following indexes:
    - fasting glucose exceeding 100 mg/dL;

- glucose level exceeding 140 mg/dL, 2 hours after glucose (75 g) load during an oral glucose tolerance test (OGTT);
- elevated homeostatic model assessment of insulin resistance (HOMA index);
- lowest quartile of insulin sensitivity as assessed during an euglycemic hyperinsulinemic clamp.

2. Obesity, defined as one of the following indexes:
    - waist-to-hip ratio over 0.90 (males) or 0.85 (females);
    - body mass index (BMI) over 30 kg/m^2.
3. Dyslipidaemia, defined as one of the following indexes:
    - plasma triglycerides level major or equal to 150 mg/dL;
    - plasma HDL cholesterol less than 35 mg/dL (males) or 39 mg/dL (females).
4. Hypertension, defined as systolic/diastolic pressure major or equal to140/90 mmHg.
5. Microalbuminuria, defined as one of the following indexes:
    - urinary albumin excretion major or equal to 20 μg/min;
    - albumin-to-creatinine ratio major or equal to 30 mg/g.

Metabolic syndrome definition.

The metabolic syndrome is defined by the co-presence of insulin resistance and at least 2 over the other 4 listed additional criteria.

Limitations.

The mandatory presence of insulin resistance for the diagnosis of the metabolic syndrome excludes subjects with normal insulin sensitivity and, on the other hand, all the other criteria of disease. Some analyses required by the WHO definition are not routinely provided. For such reason, the application of these criteria for metabolic syndrome assessment is not easily applied in clinic or in large epidemiological studies.

EGIR definition

In 1999 the European Group for the Study of Insulin Resistance presented a revision of the WHO definition of the metabolic syndrome (Balkau and Charles 1999).

Criteria for the metabolic syndrome.

1. Insulin resistance is a fundamental criterion for the metabolic syndrome diagnosis but, differently from the WHO definition, it is defined as hyperinsulinemia, i.e., fasting plasma insulin greater than the 75th percentile.
2. Obesity, defined as waist circumference major or equal to 94 cm (males) or 80 cm (females).

3. Dyslipidaemia, defined as one of the following indexes:
    - plasma triglycerides level major or equal to 177 mg/dL;
    - plasma HDL cholesterol less than 39 mg/dL.
4. Hypertension, defined following one of the above reported criteria:
    - systolic/diastolic pressure major or equal to140/90 mmHg;
    - reported pharmacological treatment for hypertension.

Metabolic syndrome definition.

The metabolic syndrome is defined by the co-presence of insulin resistance and at least 2 over the other 3 listed additional criteria.

Limitations.

Hyperinsulinemia is a simplification for insulin resistance determination. This measure is not a gold standard and could be insufficient for insulin resistance determination in some patient categories, such as subjects suffering type 2 diabetes. For this reason, the use of the EGIR definition could exclude from the diagnosis of metabolic syndrome patients affected by type 2 diabetes.

IDF definition

A new definition of the metabolic syndrome has been proposed in 2005 by the International Diabetes Foundation (Zimmet et al. 2005). Insulin resistance is not a fixed requirement for metabolic syndrome diagnosis whereas a fundamental condition is obesity.

Criteria for the metabolic syndrome.

1. Insulin resistance is defined as fasting plasma glucose major or equal to 100 mg/dL.
2. Obesity, is a fundamental criteria for the metabolic syndrome diagnosis and is determined using the waist circumference. Cut-points used to define obesity are specific for type of populations, since body weight and waist circumference display different distributions related to different populations, ethnicities and nationalities.
3. Hypertriglyceridemia, defined using one of the following criteria:
    - plasma triglycerides level major or equal to 150 mg/dL;
    - reported pharmacological treatment for hypertriglyceridemia.
4. Low HDL cholesterol level, defined using one of the following criteria:
    - values inferior to 40 mg/dL (males) or 50 mg/dL (females);
    - reported pharmacological treatment for dyslipidaemia.
5. Hypertension, defined following one of the above reported criteria:
    - systolic pressure higher than 130 mmHg;
    - diastolic pressure major than 85 mmHg;

- reported pharmacological treatment for hypertension.

Metabolic syndrome definition.

The metabolic syndrome is defined by the co-presence of obesity and at least 2 over the other 4 listed additional criteria.

Limitations.

The application of the IDF definition has been criticized for the central role attributed to obesity and the marginal role credited to insulin resistance in the development of the metabolic syndrome (Reaven 2006).

NCEP-ATP III definition

The NCEP-ATP III is the most widely used definition of metabolic syndrome. The first NCEP-ATP III definition has been presented by the National Cholesterol Education Program and Adult Treatment Panel III in 2001 (Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report2002) and updated in 2005 by the American Heart Association and the National Heart Lung and Blood Institute (Grundy et al. 2005).

Criteria for the metabolic syndrome.

1. Insulin resistance is not absolutely required for metabolic syndrome diagnosis and it is defined using one of the following criteria:
    - fasting plasma glucose major or equal to 100 mg/dL;
    - reported pharmacological treatment for diabetes.
2. Obesity is not a fundamental criteria for the metabolic syndrome diagnosis and is defined as a waist circumference over 40 inches (approximately 100 cm) for males or 35 inches (approximately 90 cm) for females.
3. Hypertriglyceridemia, defined using one of the following criteria:
    - plasma triglycerides level major or equal to 150 mg/dL;
    - reported pharmacological treatment for hypertriglyceridemia.
4. Low HDL cholesterol level, defined using one of the following criteria:
    - values inferior to 40 mg/dL (males) or 50 mg/dL (females);
    - reported pharmacological treatment for dyslipidaemia.
5. Hypertension, defined following one of the above reported criteria:
    - systolic pressure higher than 130 mmHg;
    - diastolic pressure major than 85 mmHg;
    - reported pharmacological treatment for hypertension.

Metabolic syndrome definition.

The metabolic syndrome is diagnosed when at least 3 over the 5 listed criteria are met.

Advantages.

The NCEP ATP III definition includes measurements as well as laboratory analyses that could be routinely provided and that can be easily used in clinical practice and in epidemiological studies. Additionally, no specific criterion needs to be met for metabolic syndrome diagnosis.

Unified metabolic syndrome definition.

The absence of unified criteria for metabolic syndrome diagnosis is a critical point for the proper identification of subjects at higher risk of development of type 2 diabetes and, primarily, cardiovascular diseases. Rationales for the development of a new world-wide definition of the metabolic syndrome include the problem that different formula usually employed display significant differences in the identification of the metabolic syndrome prevalence. In fact only the 30% of subjects could be diagnosed by most criteria whereas it has been estimated that the 35-40% of subjects could be diagnosed for the metabolic syndrome only using one of reported definition (The Decode Study Group and Qiao 2005).

For such reasons, literature data are often incomparable, leading to possible underestimation of the prevalence of this pathological condition. Moreover, these formula differ for the ability in predicting cardiovascular mortality or diabetes development, and these dissimilarities are also more marked considering distinct populations (Dunstan et al. 2002; Hu et al. 2004; Hunt et al. 2004; Lorenzo et al. 2003; Laaksonen et al. 2002).

CENTRAL FEATURES OF THE METABOLIC SYNDROME

The WHO, EGIR, IDF as well as the NCEP ATP III definition of the metabolic syndrome evidence the four central features of this disorder: insulin resistance, visceral obesity, dyslipidaemia and endothelial dysfunction. Among these, insulin resistance and obesity seem to play a central role in the development of abnormalities typically associated to the metabolic syndrome, in particular dyslipidaemia and endothelial dysfunction. Nevertheless, several other metabolic abnormalities have been associated to this pathological condition and could be involved in the pathophysiology of the syndrome. Among them, oxidative stress, systemic inflammation, altered autonomic regulation and hyperhomocysteinemia are typically observed.

INSULIN RESISTANCE

Insulin is a hormone primarily exhibiting anabolic properties at glucose but also protein and lipid levels. Nevertheless, insulin is also involved in endothelial function as well as in cellular growth and differentiation. Insulin resistance is defined as a reduction of responsiveness of peripheral tissues to physiological plasma insulin levels (Figure 1).

Insulin resistance is a central feature of the metabolic syndrome even though mechanisms linking impaired insulin sensitivity and metabolic abnormalities evidenced in this pathological condition are not completely elucidated. Correlations between insulin resistance and some features of the metabolic syndrome, such as hypertension and prothrombotic condition, need to be further investigated (Grundy et al. 2004). Nevertheless, the number of metabolic alterations of the metabolic syndrome is directly related to the degree of insulin resistance (Nesto 2003; Alberti, Zimmet, and Shaw 2006); additionally, strong correlations between altered insulin sensitivity and dyslipidaemia, pro-inflammatory condition as well as increased cardiovascular risk have been evidenced (Grundy et al. 2004).

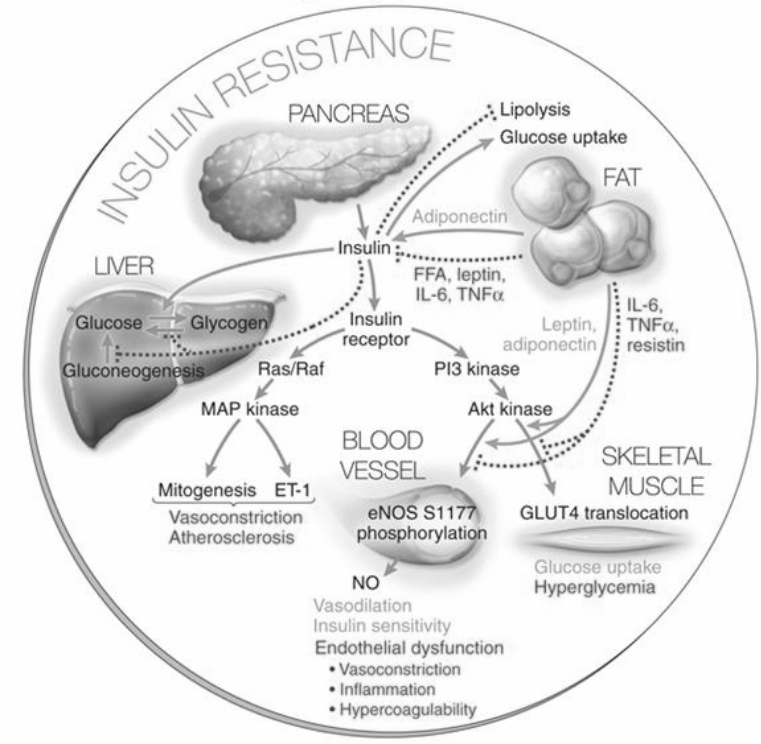

Figure 1. Effects of normal (light grey) or impaired (dark grey) insulin signalling and activities at different tissue targets. Dashed lines indicate insulin inhibitory effects whereas continuous thick lines indicates insulin stimulatory effects (Huang 2009). eNOS, nitric oxide synthase; ET-1, endothelin; FFA, free fatty acid; NO, nitric oxide.

INSULIN SIGNALLING

Insulin exerts its physiological activities through the bind with its specific cell-surface insulin receptor (IR). Insulin receptors are ligand-activated tyrosine kinases, composed by four subunits, the two extracellular α-subunits and the two cytoplasmic transmembrane β-subunits, linked together by disulphide bonds (Ullrich et al. 1985; Ebina et al. 1985). Insulin binds on α-subunit, inducing conformational changes that lead to the autophosphorylation of tyrosine residues in the β-subunit. Activated tyrosine kinase catalyses the phosphorylation of several intracellular substrates, including the Shc adaptor protein, as well as insulin receptor substrates (IRS) (Sun et al. 1995; Sesti et al. 2001), responsible for insulin activities.

The phosphorylation of IRSs and Shc adaptor protein represents the first step in the activation of the two main signalling pathways of insulin: the phosphatidylinositol-3'-kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway, respectively. The PI3K- and MAPK-dependent pathways are differently involved with insulin activities (Figure 2).

The PI3K pathway plays a crucial role in the metabolic actions of insulin at skeletal muscle and adipose tissue levels, being involved in the upstream of glucose transporters (GLUT) 4 translocation; moreover, the activation of the PI3K pathway is required also for glycogen and lipid metabolism, protein synthesis, vasodilatation processes as well as anti-inflammatory effects.

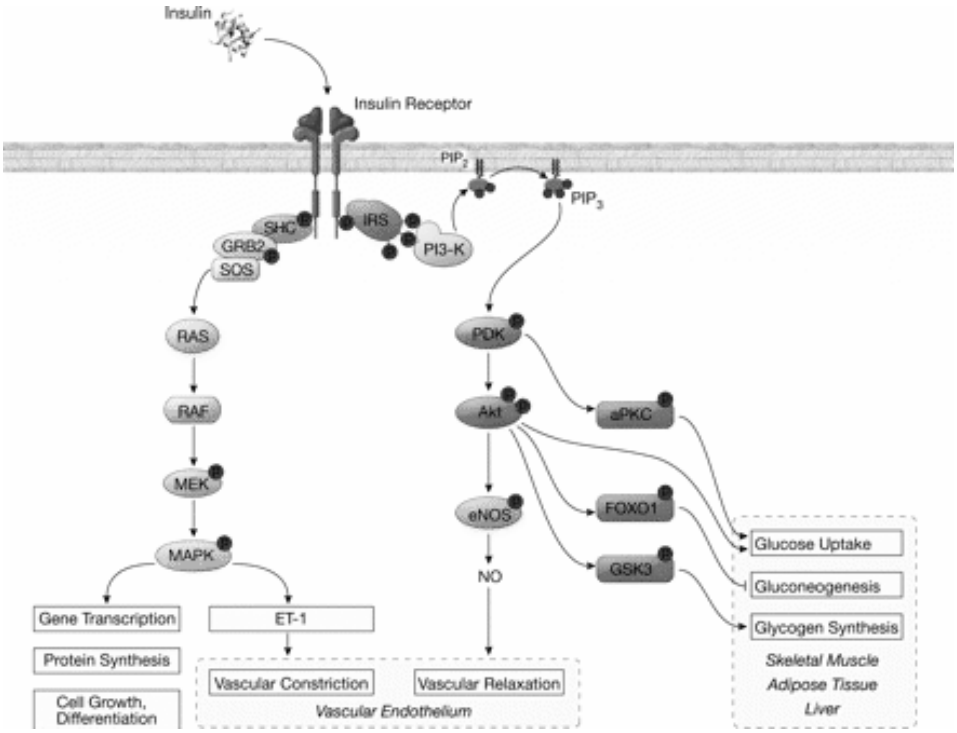

Figure 2. Schematic description of the two insulin signalling transduction pathways (Muniyappa et al. 2007).

This signalling pathway starts with the phosphorylation of IRS by tyrosine kinase. Nowadays, four different IRS have been identified, characterised by tissue-specific differences in mediating insulin action. Among them, IRS-1 is mainly involved with insulin action in skeletal muscle whereas IRS-2 is mainly associated to insulin activity in the liver.
Phosphorylated IRSs bind and activate several Src homology 2 (SH2) domain proteins, such as the p85 regulatory subunit of PI3K and the growth factor receptor-binding protein 2 (Grb-2) (Saltiel and Kahn 2001). The phosphorylation of PI3K, leading to activation of the 3-phosphoinositide-dependent protein kinase 1 (PDK1) that, subsequently, phosphorylates Akt (or protein kinase B) as well as atypical protein kinase C (PKC) isoforms. Akt mediates different metabolic effects of insulin, at different levels: in endothelium, Akt activates endothelial nitric oxide synthase; in the liver, Akt decreases gluconeogenesis and glucose output whereas in skeletal muscle and adipose tissue, Akt regulates GLUT 4 translocation on cell membrane, favouring glucose uptake. Finally, Akt enhances glycogen synthesis and than glucose storage in insulin target tissues (i.e., skeletal muscle, adipose tissue and liver) (Farese 2002).
The other main insulin signalling cascade involved the MAPK pathway. Differently from the PI3K one, the activation of MAPK pathway is associated to the regulation of insulin non-metabolic actions related to growth, mitogenesis, differentiation as well as decrease in nitric oxide production and procoagulant effects (Wang, Goalstone, and Draznin 2004). This signalling pathway starts with the phosphorylation of Shc protein by tyrosine kinase. The subsequent activation of factor Sos induces the MAPK pathway that involved molecules as Ras, Raf, MAPK kinase (MEK) and extracellular regulated kinase (ERK). The MAPK pathway stimulates endothelin-1 synthesis, vascular cell adhesion molecules VCAM-1 and E-selectin expressions as well as vascular smooth muscle cells growth and mitogenesis.

Alterations at different levels in insulin signalling pathways could cause insulin resistance. Insulin receptor is one of the potential site involved in insulin resistance, possibly to genetic alterations of its structure or down-regulation of its expression. Moreover, impaired insulin sensitivity could derive from an up-regulation of IR/IGF-IR hybrid receptor expression. IR/IGF-IR hybrid receptors display high affinity for IGF-1 and low affinity for insulin (Soos, Nave, and Siddle 1993). An increase in IR/IGF-IR hybrids, as observed in skeletal muscle and adipose tissue of subjects affected by type 2 diabetes (Federici et al. 1997) and in skeletal muscle of obese subjects (Federici et al. 1998), could reduce insulin sensitivity.

Additionally, insulin resistance development could also be related to abnormalities in insulin signalling cascade, such as altered insulin-induced phosphorylation of IR or IRS-1 as well as reduced PI3K activity (Sesti 2006).
Moreover, impaired GLUT4 translocation is an additional critical point potentially involved in whole-body glucose uptake (Zierath et al. 1996) and insulin resistance.
Finally, defects in insulin sensitivity could be associated to the up-regulation of proteins with inhibitory effects on insulin signalling pathway, such as PTPase (protein-tyrosine phosphatases) or PC-1. Studies in humans, in fact, demonstrate that improvement in insulin sensitivity in obese subjects after body weight loss is inversely correlated to the decrease in PTPase activity as well as in other proteins suppressing insulin activity (Ahmad et al. 1997). Additionally, PC-1 content is augmented in adipose tissue and skeletal muscle in insulin resistant subjects (Youngren et al. 1996; Frittitta et al. 1997).

TARGET TISSUES OF INSULIN

The first and main activity of insulin is to guarantee a correct glucose utilization and storage, removing exceeding glucose from the blood stream. In this case, main target tissues for insulin action are skeletal muscle, liver and adipose tissue. Additionally, insulin displays important vascular effects that are coupled with glucose homeostasis. Nevertheless, other tissues, like the brain, are interested by insulin activity.

Skeletal muscle.

The skeletal muscle is the most important organ for glucose metabolism, since it accounts for approximately the 75% of glucose disposal. At this level, insulin promotes glucose uptake, inducing GLUT4 translocation from intracellular vesicles to cell membrane (DeFronzo 1988; Shulman 2000), and stimulates glucose storage, promoting glycogenesis (Shulman 2000) and inhibiting glycogenolysis. Once entered cells, glucose could also be immediately oxidized to generate ATP (Shulman 2000).
Moreover, skeletal muscle is the principal tissue involved in insulin resistance development, through the reduction of glucose uptake and the increase of glucose synthesis (Thorell et al. 1999; Langouche and Van den Berghe 2006). Glycogen storage in skeletal muscle is decreased when insulin sensitivity is impaired. Even though mechanisms are still debated, it is widely suggested that the principal alteration occurring in insulin resistant skeletal muscle concerns down-regulation of GLUT4 transporter (Thorell et al. 1999; Thorell et al. 1999; Langouche and Van den Berghe 2006). This is in agreement with the impairment of PI3K insulin signalling pathway that typically occurs in

insulin resistance. Moreover, insulin resistance increases the expression of insulin-independent glucose transporters, i.e., GLUT1, 2 and 3; localised in neurons, renal cells, erythrocytes, exposing these tissues to the toxicity of hyperglycaemia (Langouche and Van den Berghe 2006).
Additionally, skeletal muscle presents a subpopulation of GLUT4 that are not responsive to insulin but to physical exercise. A reduction of physical activity could thus worsened insulin resistance due to a decreased expression of these transporters (Thorell et al. 1999). These data, associated to the increase in circulating levels of triglycerides and fatty acids typically observed in insulin resistance conditions (Straczkowski et al. 2007), suggest that glucose flux is redirected from skeletal muscle to adipose tissue.
Moreover, also in skeletal muscle impaired insulin sensitivity has been associated to enhanced intracellular lipid content and metabolism (Jacob et al. 1999). Particularly, in insulin resistant states, contents of triglycerides, long-chain saturated fatty acids, diacylglycerols, ceramides and other lipid intermediates are increased in skeletal muscle (Straczkowski et al. 2007). In turn, intracellular accumulation of lipid intermediates could *per se* affects insulin transduction pathway (Bruce et al. 2006), as demonstrated in skeletal muscle (Straczkowski et al. 2007; Chavez et al. 2003; Powell et al. 2004). Several mechanisms have been proposed for altered lipid metabolism in insulin resistant muscle, even though a complete description is still lacking. Insulin resistance increases muscular uptake of fatty acids that in turn is directly correlated to increased intracellular lipid intermediates (Bonen et al. 2004). It has been suggested that an increase in the expression of fatty acid transporters could be responsible for the augment in lipid influx in resistant muscle (Chabowski et al. 2006). On the contrary, intramuscular lipid accumulation could be associated to altered lipid degradation. In fact, abnormalities in mitochondrial content, function as well as oxidative activity have been reported in subjects with impaired insulin sensitivity (Schrauwen-Hinderling et al. 2007; Holloway et al. 2008), potentially contributing to further worsen insulin resistance, in a vicious cycle. Moreover, the reduction of muscular fatty acid oxidation, promoting the increase in circulating free fatty acids, could partly contribute to altered fatty acid metabolism in liver. Circulating ketone bodies, produced by the partial hepatic fatty acid oxidation, are elevated in obesity and type 2 diabetes (Turcotte, Hespel, and Richter 1995).
Moreover, accumulation of fatty acids or their metabolites inhibits PI3K activity, further reducing glucose uptake and promoting insulin resistance development, in a vicious cycle (Avramoglu, Basciano, and Adeli 2006).

Liver.

Insulin reduces hepatic glucose production, through the inhibition of genes encoding for enzymes involved in gluconeogenesis and glycogenolysis (Shulman 2000). Additionally, insulin stimulates hepatic glucose storage, promoting glycogenesis and hepatic lipid synthesis. These hepatic insulin actions prevent further increase in glucose into the blood stream (DeFronzo 1988; Shulman 2000). On the contrary, insulin resistance is associated to increased hepatic glucose production and enhanced hepatic glucose release. The liver then plays a key role in hyperglycaemia subsequent to insulin resistance development (Michael et al. 2000). Moreover, also in liver, altered insulin sensitivity is associated to altered fatty acid metabolism, characterised by intramyocytes lipid accumulation (Petersen et al. 1998) and enhanced ketone bodies production that could further worsen insulin resistance (Avramoglu, Basciano, and Adeli 2006).

Adipose tissue.

The effects of insulin are, also in adipose tissue, aimed to increase glucose uptake and storage (Kim et al., 2006). In fact, insulin stimulates GLUT4 translocation in adipocyte surface, promoting glucose uptake (DeFronzo 1988; Shulman 2000) and stimulates glucose storage as fat, enhancing fat synthesis (Shulman 2000). Additionally, in adipocytes, insulin inhibits lipolysis and fatty acid release in the blood stream (Shulman 2000). An important demonstration of the key role of adipose tissue in insulin sensitivity has been reported in transgenic mice, overexpressing lipoprotein lipase in adipose tissue. In this condition, the accumulation of triglycerides was associated to insulin resistance development (Ferreira et al. 2001; Kim et al. 2001).

Vascular endothelium.

Insulin displays different effects in the vascular endothelium, principally leading to vasodilation and increase in blood flow. In turn, insulin-induced hemodynamic changes further promote insulin as well as glucose delivery to skeletal muscle. The net effect is an increase in glucose disposal associated to insulin-induced increase in GLUT4 translocation at muscle level (Vincent et al. 2004). This underlines the couple between insulin-induced hemodynamic and metabolic effects.
Insulin actions in vascular endothelium are exploited through both insulin main signalling cascades, the PI3K and the MAPK pathways, with different effects.
The insulin-related activation of the PI3K cascade determines the stimulation of endothelial nitric oxide (NO) synthase (eNOS) activity, leading to enhanced NO production (Zeng and Quon 1996; Montagnani et al. 2002). eNOS, in fact, catalyses the conversion of L-arginine, whose endothelial uptake is stimulated by insulin (Sobrevia et al. 1996), to NO and L-citrulline. Classically, stimulators of eNOS activity activate eNOS through a calcium dependent pathway. On the contrary,

insulin induces eNOS activation through a distinct and independent mechanism, the PI3K pathway. Vasodilatory effect of insulin is a consequence also of insulin-induced production of prostacyclin (PGI2), an endothelium-derived vasodilatator synthesised from arachidonic acid. Interestingly, eNOS *per se* directly suppresses the activity of cyclooxygenase-1, enzyme that catalyses PGI2 synthesis; however, insulin stimulates PGI2 synthesis through an NO-independent pathway. Enhanced NO synthesis determines vasodilation that occurs in two phases (Vincent et al. 2002; Kim et al. 2006). Within few minutes, insulin induces dilation of terminal arterioles (capillary recruitment), whereas the insulin-induced increase in blood flow, secondary to relaxation of larger resistance vessels, occurs within 30 minutes and reaches its maximum after 2 hours from physiological insulin stimulation (Baron et al. 1996). Capillary recruitment and augmented blood flow favour insulin and glucose availability at skeletal muscle level, stimulating insulin-induced GLUT4 translocation and glucose uptake in skeletal muscle. Interestingly, it has been estimated that insulin vasodilatory effect is responsible, *per se*, for the 40% of insulin-mediated glucose uptake in skeletal muscle (Mather et al. 2000).

Nevertheless, insulin-related hemodynamic regulation depends also by the MAPK signalling pathway that leads to opposite effects to those induced by the activation of the PI3K cascade. In fact, insulin-induced activation of MAPK signalling pathway stimulates the secretion of endothelin-1, a vasoconstrictor peptide (Potenza et al. 2005), as well as the endothelial expression of VCAM-1 and E-selectin (Montagnani et al. 2002). Endothelial expression of VCAM-1 and E-selectin is a key point in modulating cell-cell interactions between circulating inflammatory cells and vascular endothelium. The complex hemodynamic regulation associated to insulin activity is further coupled to insulin-related activation of sympathetic nervous system.

Insulin resistance is characterised by the disruption of PI3K signalling pathway and by the maintenance of MAPK signalling branch. As a consequence, the reduction of insulin-induced vasodilatation is not balanced by a decrease in insulin-induced vasoconstrictor production, via the MAPK pathway.

Endothelial dysfunction in insulin resistance (Williams et al. 1996) is probably not only associated to impaired insulin sensitivity but could be the consequence of multiple abnormalities typically associated to this pathological condition, such as the development of a pro-atherogenic lipid pattern and of a low-grade systemic inflammation. Nevertheless, the role of insulin on the maintenance of endothelial function seems to be crucial.

DYSLIPIDAEMIA

Dyslipidaemia is a common feature of the metabolic syndrome and of insulin resistant conditions, typically characterised by an increase in plasma triglycerides and low-density lipoprotein (LDL) as well as by a reduction in plasma high-density lipoprotein (HDL) content (Alberti, Zimmet, and Shaw 2006; Avramoglu, Basciano, and Adeli 2006).

PLASMA LIPIDS: HIGH-DENSITY LIPOPROTEINS

HDL are the smallest and densest cholesterol-rich particles. HDL are composed by a hydrophobic core, presenting neutral lipids, such as cholesteryl esters and triglycerides, and by a surface monolayer, containing phospholipids, free cholesterol and apolipoproteins. The peculiarity of HDL is apolipoprotein pattern. Apolipoproteins constitute the 50% of HDL molecule weight and comprise apolipoprotein A-I, that accounts for the 90% of HDL apolipoproteins, apolipoprotein A-II and a little amount of exchangeable apolipoproteins (such as apolipoprotein C-1, C-II, C-III, E and A-IV). HDL are characterized by the absence of apolipoprotein B.

Physiological activities of HDL are mainly associated to its protective cardiovascular effects. First, HDL displays anti-atherogenic properties (Kontush and Chapman 2006), principally associated to HDL role in the removal of peripheral excess cholesterol, through the reverse cholesterol transport (Lewis and Rader 2005).

Moreover, HDL exhibits antioxidant property (Kontush and Chapman 2006; Navab et al. 2009); in fact, apolipoprotein A-1 stimulates lecithin:cholesterol acyltransferase (LCAT) activity, favouring the hydrolysis of LDL oxidized phospholipids (Navab et al. 2009).

Third, HDL inhibits the expression of adhesion molecules (Nicholls et al. 2005), induced by vascular injury and inflammatory conditions (Ridker et al. 1998), with consequent endothelial protective effects. HDL also prevents monocyte chemotaxis and infiltration at vascular level (Lawrence and Springer 1991), through a direct inhibitory effect on monocyte migration (Ansell et al. 2003). Moreover, in endothelium, HDL stimulates eNOS activity, through a PI3K-dependent signalling mechanism (Mineo and Shaul 2003), similarly to insulin (Vecchione et al. 2002).

HDL protective effects on endothelium depend also on HDL-induced inhibition of platelet activation (Mineo et al. 2006) and of coagulation cascade, though the inactivation of factors Va and VIIa (Mineo et al. 2006; Eitzman et al. 2005).

REVERSE CHOLESTEROL TRANSPORT

The reverse cholesterol transport (RCT) is the mechanism through with cholesterol excess is transported from peripheral tissues to the liver, to be removed (Figure 3). Extracellular acceptor of

free cholesterol is apolipoprotein A-I, that, as already mentioned, constitutes the 90% of apolipoproteins in HDL and its precursors (Lewis 2006).

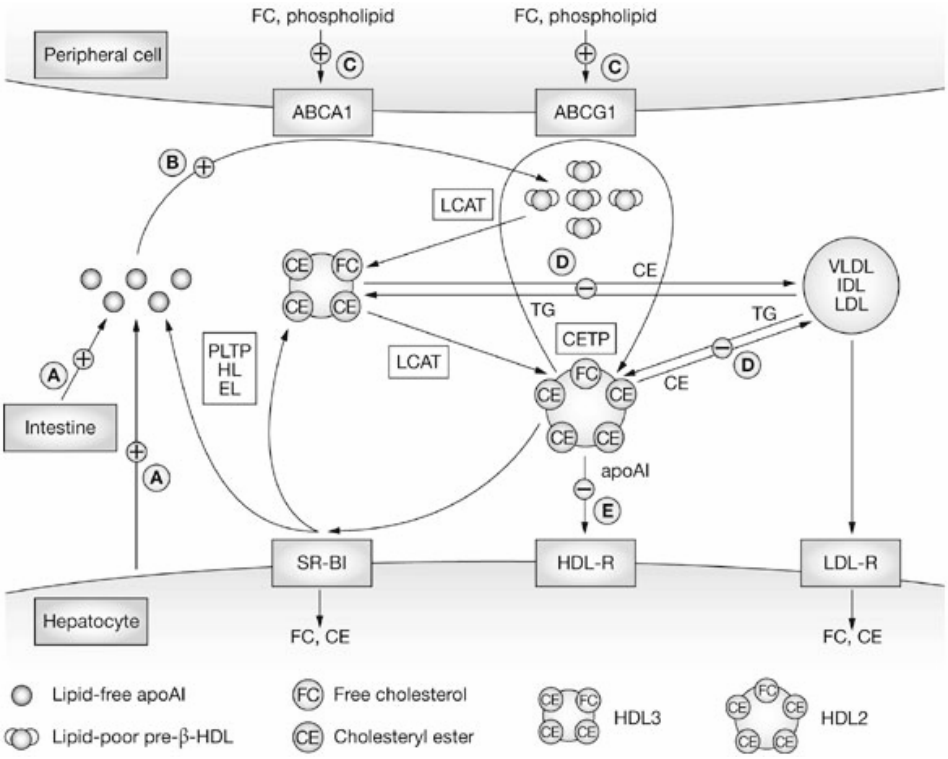

Figure 3. HDL metabolism and the reverse cholesterol transport (Kontush, Guerin, and Chapman 2008). ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; apoAI, apolipoprotein AI; apoB, apolipoprotein B; apoE, apolipoprotein E; CE, cholesteryl ester; EL, endothelial lipase; FC, free cholesterol; HDL-R, HDL receptor; HL, hepatic lipase; IDL, intermediate-density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL-R, LDL receptor; PLTP, phospholipid transfer protein; SR-BI, scavenger receptor type BI; TG, triglyceride.

The reverse cholesterol transport initiates with the cholesterol efflux, mediated by ATP binding cassette transporters (ABCs). In this phase, free cholesterol is transported from peripheral tissues to apolipoprotein A-I. ABCs transfer cholesterol in a unique direction and, in particular, ABCA1 mediates the cholesterol efflux to lipid-free apolipoprotein A-I and pre-β HDL (Wang and Tall 2003) whereas ABCG1 is responsible for cholesterol efflux to mature HDL (Kennedy et al. 2005). The pre-β HDL represents the nascent form of HDL, rich in apolipoprotein A-I and with a discoidal shape (Castro and Fielding 1988); pre-β HDL could be secreted *de novo* from the liver or intestines,

by lipoprotein lipase-induced dissociation of triglyceride-rich lipoproteins or could be produced as by-product of HDL interconversion (Tall 2008).
To avoid the transfer of free cholesterol back to peripheral tissues, once uptaken by apolipoprotein A-I molecules, free cholesterol is esterified by the LCAT (Subbaiah et al. 1994). Cholesteryl esters migrate in the core of the particle, transforming pre-β HDL in the spherical mature HDL.
Cholesteryl ester delivery can proceed through two different pathways. In liver, mature HDL selectively binds to scavenger receptor class B type 1 (SRB1) (Marguet and Chimini 2002), and cholesteryl esters are directly uptaken in hepatocytes through transcytosis (Silver et al. 2001), without HDL catabolism (Bultel-Brienne et al. 2002). In this case, cholesterol is transformed in bile acids and excreted in the biliary tract. Alternatively, the transfer of cholesteryl esters to the liver can proceed indirectly through a process of lipid exchange, mediated by the cholesteryl ester transfer protein (CETP). CETP exchanges cholesteryl esters with triglycerides from HDL to lipoproteins containing apolipoprotein B, principally LDL and very-low density lipoprotein (VLDL) (Tall 1993). LDL and VLDL, enriched in cholesteryl esters, enter hepatocytes via the LDL receptor and/or LDL receptor-related protein. On the contrary, HDL particles are transformed in smaller HDL3 and in apolipoprotein A-I by the actions of phospholipids transfer protein (PLTP) and hepatic lipase (HL), respectively involved in phospholipids transport and triglyceride hydrolysis. After this remodelling, HDL3 and apolipoprotein A-I re-enter the reverse cholesterol transport (Jin, Marchadier, and Rader 2002).

CHOLESTERYL ESTER TRANSFER PROTEIN

The cholesteryl ester transfer protein (CETP) is a hydrophobic glycoprotein (Bruce, Chouinard, Jr., and Tall 1998), composed by 476 amino acid residues. Liver and adipose tissue represent the main sites of CETP production, even though CETP is also synthesised by skeletal muscles, spleen, small intestines, adrenal gland, kidney, heart and can be secreted by different cell types, such as macrophages and B-lymphocytes (Tall 1993).
CETP displays structural similarities with other plasma proteins, as phospholipid transfer protein (PLTP) and C reactive protein (CRP) (Lepper et al. 2007; Masson et al. 2009). Interestingly, all these proteins are known to be involved, to various degrees, in atherosclerosis and inflammation (Lepper et al. 2007; Masson et al. 2009).
CETP plays a key role in cholesterol metabolism (Figure 4) and, particularly, in the reverse cholesterol transport, since it is responsible for the transfer of cholesteryl esters from HDL, mainly HDL2, to LDL and VLDL, exchanging triglycerides (Tall 1993). This lipid exchange determines an increase in pro-atherogenic particles, i.e., small HDL as well as dense LDL and VLDL. However,

CETP secretion is directly related to intracellular cholesteryl ester content and CETP expression (Kinoshita et al. 1996) as well as mass (Gaynor et al. 1994) are increased in response to high dietary intake of triglycerides and cholesterol, suggesting that physiological increase in CETP activity could occur to counteract excess cholesterol accumulation. Moreover, through VLDL and LDL, cholesteryl esters are removed from tissues with an excess of cholesterol and redistributed to other tissues requiring cholesterol or removed by the liver (Tall 1993). These evidences suggest that CETP is deeply involved in the maintenance of intracellular cholesterol homeostasis.

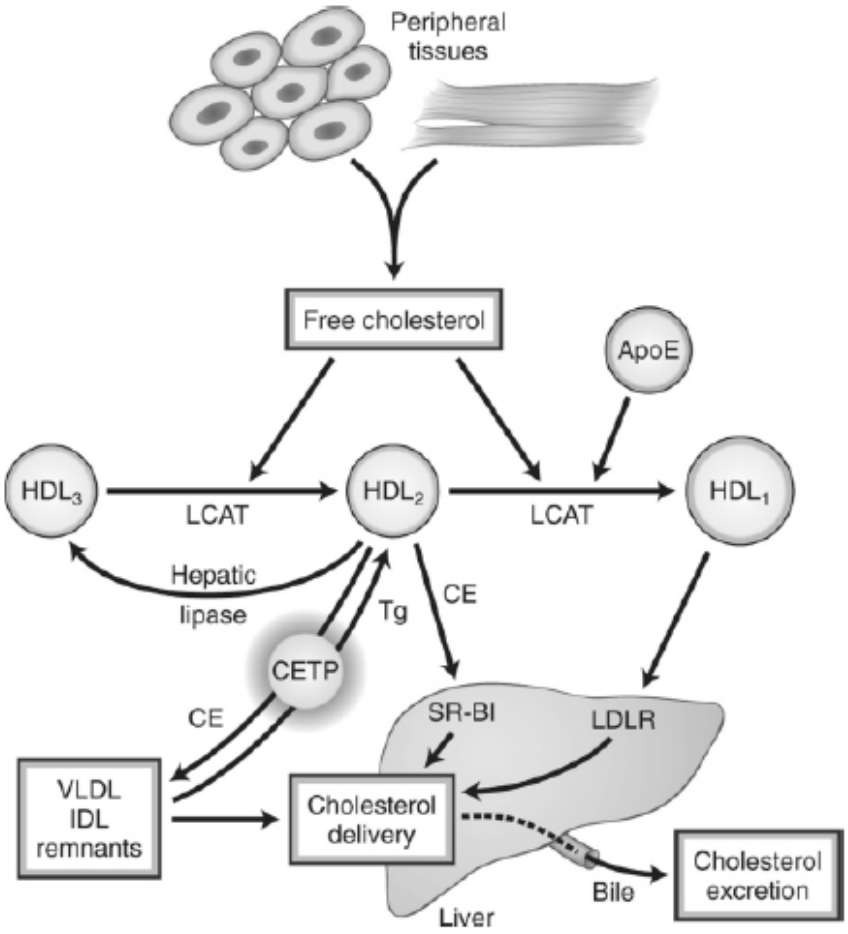

Figure 4. The role of CETP on the reverse cholesterol transport (Kronenberg and Williams 2008). CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; HDL-E, HDL with apolipoprotein E; IDL, intermediate-density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDLR, LDL receptor; PL, phospholipids; SR-B1, class B, type I scavenger receptor; Tg, triglyceride.

CETP in the metabolic syndrome.

CETP activity is up-regulated in subjects suffering the metabolic syndrome, possibly leading to a decrease in HDL and an increase in dense LDL (Sandhofer et al. 2006). Interestingly, the degree of increase in CETP is directly related to increasing number of the metabolic syndrome components (Sandhofer et al. 2006). In this context, CETP displays pro-atherogenic effects, counteracting the anti-atherogenic effects of HDL, i.e., removal of excess cholesterol from peripheral tissues (Brewer, Jr. 2004), protection of LDL from oxidation (Barter et al. 2004), decrease of endothelial cell adhesion molecules expression (Barter, Baker, and Rye 2002).

CETP in atherosclerosis.

The role of CETP in atherosclerosis is still controversial since in literature CETP has been demonstrated to be both pro (Quinet et al. 1991; Marotti et al. 1993; Agellon et al. 1991; Bhatnagar et al. 1993; Mabuchi et al. 1995) and anti-atherogenic (Hayek et al. 1995; Nigon et al. 1991; Hirano et al. 1995; Stein et al. 1985; Zhong et al. 1996; Sakai et al. 1995).

Overexpression of CETP in transgenic mice decreases HDL concentration (Agellon et al. 1991), even though the risk for atherosclerotic lesions is decreased (Hayek et al. 1995). Mutations in CETP gene, and consequent CETP deficiency, are associated to increased HDL and decreased atherosclerosis risk (Mabuchi et al. 1995); nevertheless the risk of coronary artery disease could be increased (Zhong et al. 1996). Additionally, in CETP deficiencies, the increase in HDL is mainly associated to the increase in large HDL2 particles (Hirano et al. 1995), characterised by a minor capability in cholesterol removal from peripheral cells, compared to the well-known anti-atherogenic small HDL3 particles (Yamashita et al. 1995). Moreover, in CETP deficiencies, LDL affinity for its own receptor is impaired leading to LDL accumulation (Sakai et al. 1995), whereas in physiological condition, CETP induces cholesteryl esters transfer to LDL that display an optimal affinity for LDL receptor (Nigon et al. 1991). Moreover, it has been suggested that due to its small molecular dimension, CETP enters the capsular interstitium and directly removes cholesteryl esters from the atherosclerotic lesion (Stein et al. 1985; Hennessy, Kunitake, and Kane 1993).

These apparent contradictory results concerning CETP role in atherosclerosis lead to hypothesis that CETP pro or anti-atherogenic effects are strictly related to the metabolic pattern considered (Stevenson 1998). For example, lipoprotein pattern influences CETP activity (Liinamaa et al. 1997; Tall 1995); particularly, an increase in plasma VLDL enhances cholesterol esters transfer from HDL to VLDL and LDL whereas an increase in HDL decreases this lipid exchange (Liinamaa et al. 1997). Moreover a further increase in HDL reverts lipid shift, promoting the accumulation of

cholesteryl esters in HDL and inducing triglyceride transfer from HDL to VLDL and LDL (Liinamaa et al. 1997). Dietary fat content also affects CETP activity as demonstrated in rabbits fed with eicosapentaenoic acid (EPA) (Sugano, Makino, and Yanaga 1997); in this condition, HDL content increases, independently from changes in CETP or LCAT, both in plasma and in vessel interstitium suggesting a qualitative modulation of HDL induced by EPA and a consequent modulation of modified HDL on CETP activity (Sugano, Makino, and Yanaga 1997). Finally, insulin *per se* inhibits CETP activity even though an insulin resistant condition, as observed in type 2 diabetes, could impair insulin-induced suppression in CETP (Stevenson 1998).

LIPOPROTEIN METABOLISM IN INSULIN RESISTANCE AND METABOLIC SYNDROME

As abovementioned, a common feature of insulin resistance and of the metabolic syndrome is the alteration of plasma lipid pattern, typically characterised by an increase in plasma triglycerides and LDL as well as by a reduction in plasma HDL content (Alberti, Zimmet, and Shaw 2006; Avramoglu, Basciano, and Adeli 2006) (Figure 5). These alterations in plasma lipid pattern represents also the key point in atherogenesis (Semenkovich 2006).

Even though mechanisms relating insulin resistance development and dyslipidaemia are not fully elucidated, it is widely suggested that insulin resistance precedes altered lipid metabolism (Funada et al. 2004; Annuzzi et al. 2004).

Insulin resistance and VLDL metabolism.

In physiological condition, insulin effects on lipid metabolism is quite complex. Insulin displays lipogenic effects due to its regulatory action on enzymes involved in triglycerides synthesis (Foufelle and Ferre 2002). However, insulin inhibits VLDL synthesis (Lewis and Steiner 1996), partly stimulating apolipoprotein B degradation (Taghibiglou et al. 2002). The consequences of altered insulin sensitivity on these mechanisms have not been completely elucidated. However, in insulin resistance, an increase in VLDL synthesis and release in the blood stream typically occurs. Several explanations have been proposed and possibly concur to hypertriglyceridemia in insulin resistance. First, altered insulin sensitivity is associated to increased availability of free fatty acids. In liver, increased free fatty acid availability determines an increase in hepatocytes fatty acid uptake, that leads to enhanced apolipoprotein B production and then to enhanced VLDL synthesis (Lewis et al. 1995). Moreover, insulin resistance directly stimulates VLDL production due to impaired PI3K signalling pathway that normally promotes apolipoprotein B degradation. Interestingly, in insulin resistance, the level of free fatty acid is directly related to the degree of fat accumulation in liver (Holt et al. 2006). The increase of free fatty acid availability in insulin

resistance is, at least in part, due to enhanced fatty acid release from adipose tissue (Yu and Ginsberg 2005) that is associated to the defect in insulin-induced inhibition of lipolysis in adipocytes.

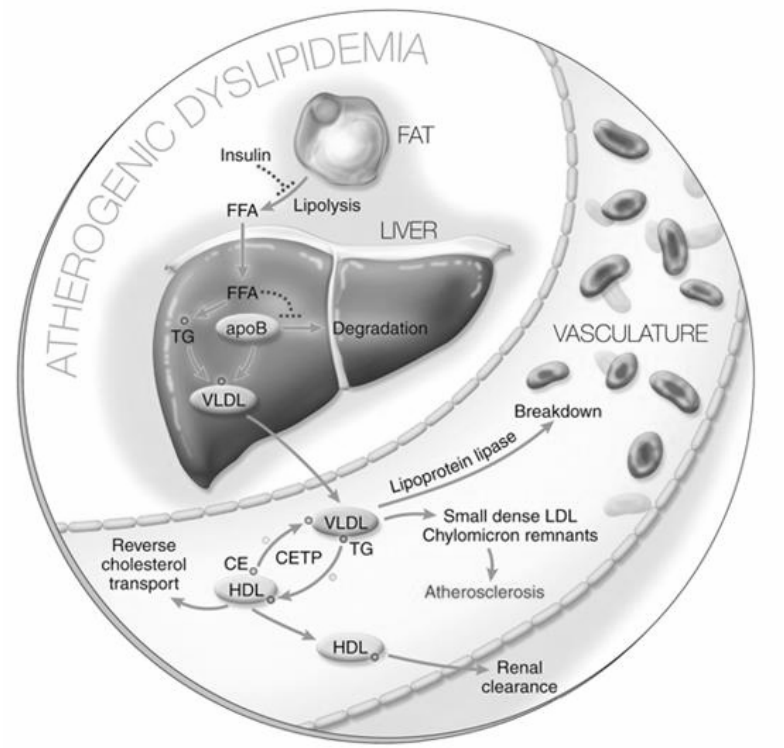

Figure 5. Normal (light grey) lipid metabolism and metabolic abnormalities typically occurring in the metabolic syndrome (dark grey) (Huang 2009). apoB, apolipoprotein B; CE, cholesteryl esters; FFA, free fatty acid, TG, tryglicerides.

Second, the augment of plasma VLDL is associated to decreased VLDL clearance, associated to altered activity of lipoprotein lipase, normally regulated by insulin (Eckel, Yost, and Jensen 1995). Third, in insulin resistance the shift from lipid oxidation to lipid storage could derive from altered activities of key enzymes involved in lipogenesis. In liver, in fact, insulin up-regulates the microsomal transfer proteins (MTP), that promote the formation of VLDL and chylomicron (Taskinen 2005); the sterol regulatory element-binding protein-1c (SREBP-1c), that activates enzymes involved in *de novo* lipogenesis, such as fatty acid synthase, acetyl-CoA carboxylase and stearoyl-CoA desaturase (Shimomura et al. 2000); as well as the xylulose 5-phosphate that promotes lipogenesis using as substrates the end products of glycolysis (Kathiresan et al. 2008). The increase in VLDL activates the MAPK/NFκB pathway, leading to a pro-inflammatory and a pro-thrombotic conditions (Norata et al. 2007) and contributing to endothelial dysfunction. The pro-thrombotic state is characterised by the activation of plasminogen activator inhibitor-1 and the cell

adhesion molecule P-selectin (Tushuizen, Diamant, and Heine 2005). Moreover, VLDL-related pro-inflammatory and pro-thrombotic states are associated to the increase in oxidative stress (Ceriello et al. 2002).

Insulin resistance and HDL metabolism.

Insulin resistance is associated to alteration in circulating HDL amount but also composition that determines, in a first phase, impairment in the reverse cholesterol transport.

In type 2 diabetes, modifications in HDL could derive from altered activities of enzymes involved in lipid metabolism. It is in fact demonstrated that in insulin resistance, lipoprotein lipase activity is decreased (Taskinen 2003) whereas hepatic lipase (Taskinen 2003) and CETP activities are increased. The unbalance between lipoprotein lipase and hepatic lipase activities could stimulate HDL catabolism (Taskinen 2003). The increase in CETP activity in insulin resistant condition could be stimulated by the enhanced availability of lipoproteins exposing apolipoprotein B (Borggreve, de, and Dullaart 2003). Enhanced CETP activity determines an increase in HDL enriched in triglycerides (Khovidhunkit et al. 2004; Lamarche, Rashid, and Lewis 1999); triglyceride-rich HDL are more instable and are rapidly degraded or hydrolysed by hepatic lipase (Lamarche, Rashid, and Lewis 1999). Moreover, the uptake of triglyceride-rich HDL through the hepatic SRB1 is reduced, further contributing to the decrease in the reverse cholesterol transport (Lamarche, Rashid, and Lewis 1999). Moreover, the reduction in pre-β HDL particles is not only associated to increased CETP activity but also to the parallel decrease in phospholipid transfer protein (PLTP) activity (Borggreve, de, and Dullaart 2003; de et al. 2008).

Additionally, in insulin resistance qualitative changes in HDL composition are evidenced. In fact, even though the main apolipoprotein constituting HDL is apolipoprotien A-I, HDL usually contains lower amounts of apolipoprotein A-II, A-V, C-II and C-III, that are typically recycled between HDL and the other triglyceride-rich lipoproteins. Alterations in HDL metabolism as well as in HDL-related cholesterol reverse transport determine modifications in HDL-containing apolipoproteins that could contribute in a vicious cycle to altered lipid metabolism. For example, in insulin resistant states, a decrease in apolipoprotein A-I is typically observed, mainly due to the increase in HDL catabolism (Duvillard et al. 2000). Furthermore, in type 2 diabetes, the increase in apolipoprotein C-III, that inhibits lipoprotein lipase activity, associated to a reduction in apolipoprotein C-II, that stimulates lipoprotein lipase activity, favours HDL hydrolysis (Taskinen 2005). Moreover, the increase in apolipoprotein C-III *per se* reduces hepatic uptake and than the clearance of triglyceride-rich lipoproteins, in type 2 diabetes (Taskinen 2005).

Insulin resistance and LDL metabolism.

In insulin resistance, plasma LDL concentration is usually unchanged whereas LDL composition is often altered (de Graaf et al. 1993), as a consequence of parallel changes in VLDL and HDL metabolism and composition (Sacks and Campos 2003). Possibly to increased depletion of cholesterol and phospholipids associated to increased or unchanged content of triglycerides, in the metabolic syndrome and in insulin resistance, circulating LDL particles are smaller, denser (Kwiterovich, Jr. 2002) and, consequently, more atherogenic (Krauss 1995) that the nascent LDL. Small and dense LDL in fact are unstable molecules and can be easily oxidized; moreover, small dense LDL display a lower affinity for LDL receptor and, consequently, exhibit a prolonged residence time in plasma and an high level of infiltration in endothelium.

VISCERAL ADIPOSITY

Obesity is a key factor in the pathogenesis of the metabolic syndrome as well as in the development of insulin resistance (Figure 6). In addition to fat accumulation, fat distribution has to be considered since visceral adiposity displays different metabolic effects than subcutaneous adipose tissue. Particularly, free fatty acids, released by visceral adipose tissue, could directly enter the liver, through the splanchnic circulation, potentially affecting hepatic glucose and lipid metabolism; on the contrary, free fatty acids released by subcutaneous adiposity could be directly discharge in the systemic circulation with potentially minor hepatic effects. For such reasons, in the criteria of the metabolic syndrome waist circumference, more than body mass index, is usually considered (Alberti and Zimmet 1998).

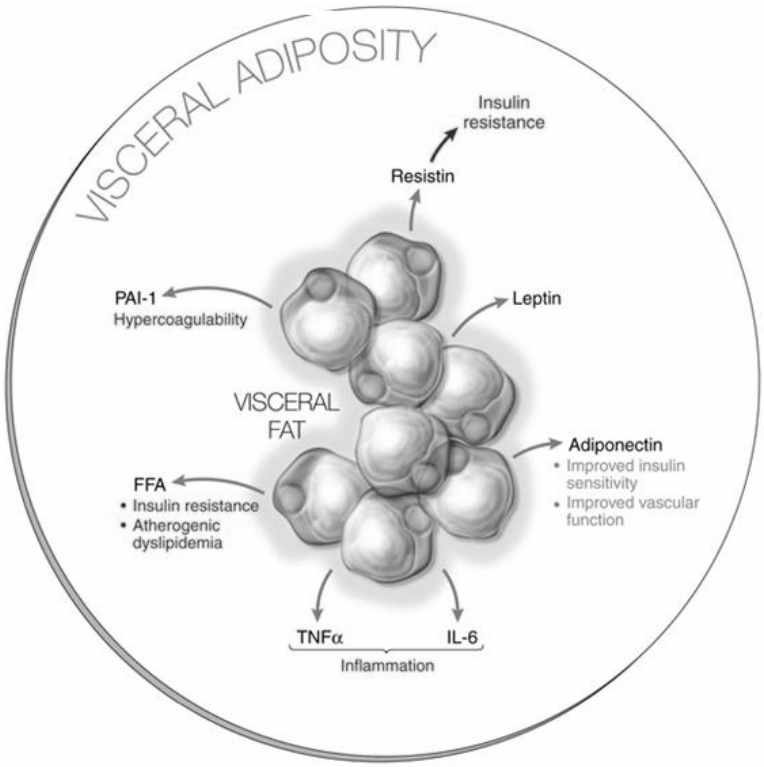

Figure 6. Normal (light grey) and impaired (dark grey) visceral adiposity metabolism occurring in the metabolic syndrome (Huang 2009). FFA, free fatty acid; PAI-1, plasminogen activator inhibitor 1.

Several studies evidence the strong association between visceral fat accumulation and other components of the metabolic syndrome, including primarily insulin resistance (Poirier and Eckel 2002) and pro-atherogenic dyslipidaemia (Despres et al. 1990; Avramoglu, Basciano, and Adeli 2006; Pouliot et al. 1992). Mechanisms underlying these associations are not completely

understood, nevertheless adipose tissue-secreted adipocytokines play a central role. The five adipocytokines mainly involved in metabolic regulation are leptin, resistin and adiponectin as well as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) (Kershaw and Flier 2004). The latter two exert also important roles as inflammatory mediators and have been discussed later.

Leptin.

Leptin is a protein mainly synthesised by adipocytes and is encoded by the ob gene. Leptin production in subcutaneous adiposity is greater than in visceral adiposity (Van Harmelen et al. 1998) and leptin secretion is strictly related to adipocyte lipid content (Zhang et al. 2002). This hormone is a modulator of energy status and metabolism and its expression as well as its level are increased in obesity, both in human and animal models (Girard 1997; Van Harmelen et al. 1998). Leptin exerts several effects on insulin activity. In adipocytes, prolonged leptin exposure impairs insulin-stimulated glucose uptake and glycogenesis, lipogenesis and inhibition of lipolysis as well as insulin effects on protein synthesis (Muller et al. 1997). In skeletal muscle (Muoio et al. 1997) as well as in hepatocytes (Nemecz et al. 1999), leptin inhibits insulin stimulatory action on glycogenesis. Finally, leptin stimulates insulin production and secretion in pancreatic β cells whereas it inhibits glucose-induced insulin secretion (Ceddia et al. 1999; Seufert, Kieffer, and Habener 1999). Mechanisms linking hyperleptinemia to impaired insulin sensitivity are unclear, even though it has been hypothesis that leptin indirectly interferes in insulin activities, possibly through changes in fat accumulation, rather than through direct effects on insulin signalling pathway.

Resistin.

Resistin is an adipocytokine characterised by an high cysteine content. Although further investigations are required to determine resistin physiological role, several evidences suggest the potential negative role of resistin in insulin sensitivity (Vidal-Puig and O'Rahilly 2001; Lee et al. 2003).

Adiponectin.

Adiponectin is an adipocytokine related to the maintenance of insulin sensitivity (Tschritter et al. 2003) and to the development of the metabolic syndrome (Medina et al. 2004). Adiponectin levels are reduced in obese (Milan et al. 2002) and type 2 diabetes subjects as well as in the metabolic syndrome (Facchini et al. 1991). Moreover, decreased adiponectin level has been related to increased cardiovascular risk (Pischon et al. 2004; Maahs et al. 2005). Adiponectin primarily acts in

skeletal muscle and in liver (Yamauchi et al. 2003), through the activation of AMP-activated protein kinase and peroxisome proliferator-activated receptor α (PPAR-α), leading to enhanced fatty acid and glucose metabolism. In fact, adiponectin reduces neoglucogenesis (Combs et al. 2001), increases glucose uptake in muscle (Fruebis et al. 2001) and favours insulin activity in liver (Berg et al. 2001). Moreover, through the activation of the AMP-activated protein kinase pathway, adiponectin ameliorates glucose utilization without inducing insulin secretion. Adiponectin also promotes fatty acid oxidation in both skeletal muscle (Fruebis et al. 2001) and liver (Xu et al. 2003), preventing fat mass deposition. Finally adiponectin decreases circulating free fatty acid level (Yamauchi et al. 2002). Adiponectin effects on glucose as well as lipid metabolism are responsible for adiponectin-induced reduction in fat mass and stimulation of insulin sensitivity.

OXIDATIVE STRESS

Oxidative stress is define as an unbalance between reactive oxygen species (ROS) production and antioxidant systems activity.

REACTIVE OXYGEN SPECIES

ROS are derivatives of oxygen metabolism and are characterised by high reactivity and very short life. ROS are ubiquitously produced and are strictly involved in several biological and phsyological processes, including intracellular signalling, cellular differentiation, apoptosis (Ghosh and Myers 1998), immunity (Tohyama, Takano, and Yamamura 2004), defense against microorganisms (Lee et al. 1998) as well as vascular tone maintenance.
Some of radical species normally involved in physiological processes are superoxide radical ($O2^{-}$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) as well as reactive nitrogen species, including nitric oxide (NO) and the peroxynitrite radical ($OONO^{-}$).

ANTIOXIDANT DEFENCES

Antioxidant systems are represented by several antioxidant enzymes, including glutathione peroxidase, superoxide dismutases and catalase, as well as several endogenous and exogenous molecules, such as glutathione, ascorbic acid, tocoferols, flavonoids, carotenoids and ubichinol (Beckman and Ames 1998), able to react and to neutralise ROS. The availability of non-enzymatic antioxidants is mainly related to adequate dietary intake of vitamins and microelements (Beckman and Ames 1998).

Superoxide dismutase catalyzes the conversion of superoxide anion to hydrogen peroxide (Fridovich 1995), through the following reaction:

$$2\ O_2^{\cdot -} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Catalase as well as the glutathione peroxidase catalyse the conversion of peroxides to water. Catalase catalyses the following reaction:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Glutathione peroxide, instead, is a selenium-containing tetrameric enzyme that neutralises peroxides using reduced glutathione as a hydrogen donor, as described by the following reaction:

$H_2O_2 + 2\ GSH \rightarrow H_2O + GSSG$

Glutathione peroxide reduces peroxides, lipoperoxides and other organic hydroperoxides, as well as peroxynitrite radicals (Sies et al. 1997).

THE GLUTATHIONE SYSTEM

Glutathione is the most important non-enzymic antioxidant and is synthesised in almost all human cells: intracellular concentrations normally range 0.5-10 mM, whereas plasma concentrations are extremely low (10 μM). Within the cell, the 90% of glutathione is present in cytoplasm (90%) and almost the remaining 10% is found in mitochondria, where it is transported from cytoplasm; a very small amount of glutathione can be detected in endoplasmic reticulum (Meredith and Reed 1982). In physiological condition, glutathione is mainly present in its active reduced form (GSH) characterised by reduced cysteine thiolic group; a smaller amount of glutathione is present in its oxidised form (GSSG), formed by two molecules of GSH, bonded by a disulphide link. GSH availability is maintained by the NADPH-dependent glutathione reductase that converts GSSG in two molecules of GSH, restoring the pool of glutathione in the active form.

Glutathione metabolism.

Glutathione is the most important non-enzymic antioxidant in humans. Glutathione is a tripeptide formed by glutammic acid, cysteine and glycine (Pastore et al. 2003), whose synthesis is mRNA-independent (Figure 7). Glutathione *de novo* synthesis is catalysed by two enzymes: γ-glutamil cysteine synthetase and glutathione synthetase. The first forms the dipeptide γ-glutamil cysteine, whereas the glutathione synthetase catalyses the bond with glycine (Majerus et al. 1971). Cysteine is a limiting substrate and γ-glutamil cysteine is the rate limiting step in glutathione synthesis; γ-glutamil cysteine is regulated by negative feed-back mechanism by glutathione itself (Lu 1999). Glutathione could then enter the γ-glutamate cycle leading to the production of γ-glutamil amino acid and cistein-glycine through γ-glutamyl transpeptidase activity (Pastore et al. 2003). γ-glutamil cyclotransferase and oxoprolinase transform γ-glutammil amino acid in 5-oxoprolin and glutamic acid. Glutamic acid as well as cysteine and glycine, obtained by the action of a dipeptidase on the cistein-glycine dipeptide, can then be utilized for glutathione synthesis (Pastore et al. 2003).

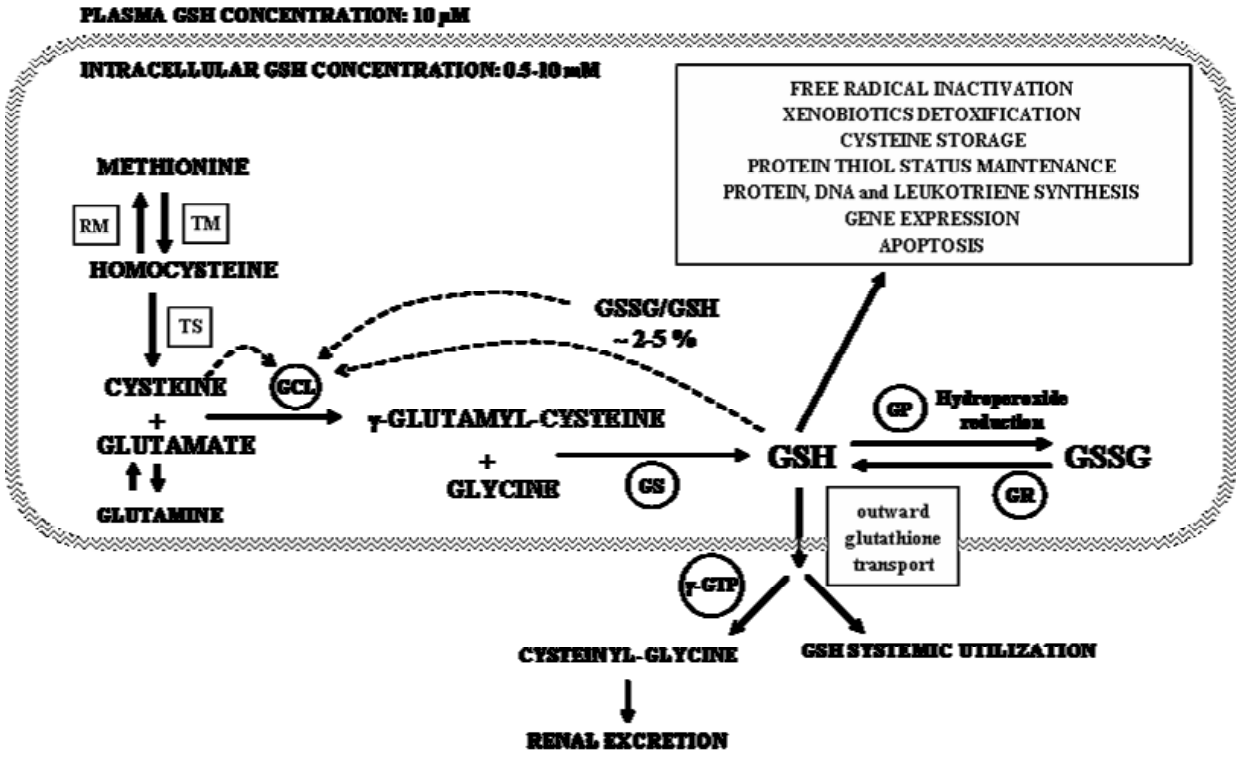

Figure 7. Glutathione synthesis and metabolism (continuous thick lines) and enzymatic regulation of glutathione synthesis (dashed lines). GCL, glutamate-cysteine ligase; GP, glutathione peroxidase; GR, glutathione reductase; GS, glutathione sinthetase, γ-GTP: γ-glutammil transpeptidase; RM, remethylation pathway; TM, transmethylation pathway; TS, transulfuration pathway.

Glutathione biological functions.

Antioxidant activity. The most important glutathione function is its antioxidant activity, obtained through both enzymatic and non-enzymatic mechanisms. Enzymatic antioxidant activity is related to the activity of glutathione peroxidase. This enzyme, in fact, while converting hydrogen peroxide in water, utilises two molecules of GSH to form the glutathione dimer or GSSG (Pastore et al. 2003). Glutathione non-enzymatic antioxidant defence is related to the ratio between GSH and GSSG; in physiological condition, the GSH/GSSG ratio is around 10 and this creates a reduced intracellular condition. Through these two mechansims, glutathione preserves protein sulphydril groups in a reduced state, preventing impairment in protein structure and function.

Leukotriene synthesis.

Glutatione is an important cofactor in leukotrienes synthesis. In particular, glutathione is involved in the synthesis of leucotriene C4, Dq and E4 (Anderson, Allison, and Meister 1982).

Protein function modification.

In addition to its, abovementioned, effects on sulphydril groups in proteins, glutathione could modulate protein stability and function through the formation of chemical bonds with the thiolic groups. This reaction, known as glutathionilation, is implied in protein stability, in the prevention of protein cysteine oxidation as well as in the regulation of enzymes activity and transcription (Pastore et al. 2003).

Cysteine pool maintenance. Glutathione represents a reserve of cysteine, that can be released through the γ-glutamate cycle. Cysteine is in fact highly unstable and undergoes auto-oxidation leading to cystine and ROS production. Cysteine availability is important for *de novo* protein synthesis and, mainly, for glutathione *de novo* production (Lu 1999).

OXIDATIVE STRESS AND THE METABOLIC SYNDROME

The increase in oxidative stress determines an excess in ROS that could react with cellular macromolecules leading to impaired protein structure and function, lipid peroxidation and DNA damage (Dean et al. 1997). Moreover, alterations in cellular signalling and metabolic regulation are also observed (Chopra and Wallace 1998), possibly contributing to the pathogenesis of several diseases (Halliwell 1997).

Oxidative stress is recognised as a key factor in the development of inflammatory conditions, in the progression of insulin resistance (Stump et al. 2006), obesity (Ford et al. 2003), hypertension, atherosclerosis (Ceriello and Motz 2004), liver steatosis and cardiovascular diseases. Moreover, oxidative stress is considered a component of the metabolic syndrome (Van Guilder et al. 2006). Subjects suffering the metabolic syndrome typically evidence a decrease in antioxidant enzyme activities as well as in antioxidant molecules, as vitamin C and E (Demircan et al. 2008; Ford et al. 2003). Interestingly, the degree of antioxidant status reduction is directly correlated to the number of clustered components of the syndrome (Demirbag et al. 2006).

Oxidative Stress and Insulin Resistance

Several studies evidence a strong association between insulin resistance and systemic oxidative stress (Meigs et al. 2007; Blendea et al. 2005; Ogihara et al. 2004), strongly suggesting a central role of oxidative stress in the pathogenesis of insulin resistance (Evans, Maddux, and Goldfine 2005; Houstis, Rosen, and Lander 2006).

Several interesting correlations have been demonstrated between levels of oxidative stress and the degree of glycemic control in type 2 diabetes (Davi, Falco, and Patrono 2005; Nourooz-Zadeh et al. 1997). Moreover, insulin resistant conditions are characterised by altered redox status. ROS production is stimulated in insulin target organs, i.e., skeletal muscle, adipose tissue and liver, in

animal model of metabolic syndrome (Furukawa et al. 2004). In addition, the levels of protein, lipid and DNA oxidation are increased in type 2 diabetes (Davi, Falco, and Patrono 2005) as well as plasma and tissue contents of glycoxidation and lipoxidation products. Further evidences of the central role of oxidative stress in insulin sensitivity impairment derive from interventional studies. Supplementations with antioxidants (Blendea et al. 2005; Davi et al. 1999), including, as an example, α-lipoic acid (Henriksen 2006) and vitamin E (Henriksen 2006), reduce ROS contents (Davi et al. 1999) and restore antioxidant systems in diabetes (Chander et al. 2004; Namikoshi et al. 2007). Antioxidant treatments ameliorate whole body glucose tolerance (Henriksen 2006; Blendea et al. 2005) as well as promote insulin sensitivity in metabolic syndrome models (Kunitomo et al. 2008; Furukawa et al. 2004) and in type 2 diabetes (Bakris et al. 2004; Dandona, Ghanim, and Brooks 2007).

Mechanisms through which oxidative stress could worsen insulin sensitivity are not completely elucidated. ROS interfere with several signalling pathways depending on type and amount of ROS as well as cell type and time of exposure. For example, ROS stimulate the expression of pro-inflammatory genes, as TNF-α and CRP (Nathan 2003), known to be involved in the pathogenesis of inflammation and insulin resistance (Willerson and Ridker 2004). Additionally, ROS affect PI3K and JNK pathways possibly contributing to insulin resistance (Kamata et al. 2005).

Moreover, ROS activate the NF-kB pathway, a stress-signalling pathway, possibly inducing endothelial dysfunction related to altered fatty acid flux, increased asymmetric dimethylarginine (ADMA) level and impaired NOS regulation (Davi et al. 1999). This effect on endothelium, associated to vasoconstriction induced by excess in ROS (Annuk, Zilmer, and Fellstrom 2003), could induce insulin resistance (Davi et al. 1999; Annuk, Zilmer, and Fellstrom 2003).

Oxidative stress and obesity

Obesity is associated to enhanced ROS content and weight reduction determines a decrease in ROS level (Vincent and Taylor 2006). Moreover, an observational study performed in approximately 3000 subjects evidences that systemic oxidative stress is highly associated to diabetes as well as body mass index (Keaney, Jr. et al. 2003). Fat mass deposition leads to increase in adipocytokines production, as TNF-α, and in circulating free fatty acids that could contribute to enhanced oxidative stress (Fujita 2008). The augment in free fatty acids, typically observed in obesity, activates the NADPH oxidase, contributing to oxidative stress (Furukawa et al. 2004). An increase in oxidative stress, induced by both increase in ROS production and decrease in antioxidant system activity, is observed both in animals (Roberts et al. 2006; Galili et al. 2007) and humans (Devaraj et al. 2008; Cardona et al. 2008) after fat load. In addition, in animals, the early phases of obesity determine a

local vascular oxidative stress that could be responsible for the observed impairment of endothelial function in obesity (Galili et al. 2007).
Oxidative stress occurring in visceral fat, more than in subcutaneous fat, is considered a precocious index of metabolic syndrome development.

Oxidative stress and inflammation

Oxidative stress *per se* stimulates inflammatory condition increasing the expression of plasminogen activator inhibitor-1, IL- 6 and monocyte chemoattractant protein-1 and decreasing adiponectin level (Furukawa et al. 2004). In contrast, treatments aimed to reduce ROS production restore physiological balance in the production of pro and anti-inflammatory cytokines; this effect then counteracts altered insulin sensitivity, altered plasma lipid and fat deposition in liver (Furukawa et al. 2004). The metabolic syndrome is characterised by increased indexes of oxidative stress, such as ROS and GSSG-to-GSH ratio, as well as increased markers of inflammatory condition, as CRP and fibrinogen (Skalicky et al. 2008).

Oxidative stress and cardiovascular diseases.

Oxidative stress has been suggested to be implied also in the development of cardiovascular diseases. Markers of oxidative stress as glutathione peroxidase (Blankenberg et al. 2003), myeloperoxidase (Brennan et al. 2003), oxidized LDL (Holvoet et al. 2001), isoprostanes (Patrono and FitzGerald 1997) and nitrotyrosine (Shishehbor et al. 2003) are also independent predictor of early risk for cardiovascular diseases and myocardial ischemia.
Moreover, the increase in oxidative stress, promoting the oxidation of lipoproteins containing apolipoprotein B, could be directly involved in atherosclerosis (Lusis 2000). Additionally, the balance between oxidant production and antioxidant defences modulates vascular function and structure (Taddei et al. 1998) and then arterial pressure. As a consequence, oxidative stress could also be involved in hypertension development. In agreement with these considerations, antioxidant supplementations ameliorates hypertension, while reducing oxidative stress (Plantinga et al. 2007; Cangemi et al. 2007).

INFLAMMATION

The activation of a low-grade systemic inflammation is a typical feature of the metabolic syndrome (Sutherland, McKinley, and Eckel 2004) as well as of insulin resistance development (Hotamisligil 2006). Nevertheless, the cause-effect relationship between metabolic alterations, primarily insulin resistance, and increased pro-inflammatory cytokines level has not been fully elucidated (Stump, Hamilton, and Sowers 2006; Wellen and Hotamisligil 2005; Shoelson, Lee, and Goldfine 2006) Several evidences support a central role of chronic inflammation in insulin resistance in skeletal muscle and, then, at systemic level, especially through the interference with the IRS-PI3K-Akt signalling pathway of insulin (Wellen and Hotamisligil 2005; Perreault and Marette 2001; Bruunsgaard 2005). Moreover, increase in adipose tissue, leading to pro-inflammatory cytokines overproduction (Trayhurn and Wood 2004), contributes, at local as well as at systemic level (Xu et al. 2003), to impair insulin sensitivity, worsening altered metabolic condition (Fernandez-Real and Ricart 2003).

Several cytokines have been recognised to be involved in insulin resistance development, such as tumor necrosis factor-α (TNF-α), C-reactive protein (CRP), several interleukins (IL) and chemokines. Interestingly, in obese subjects suffering for the metabolic syndrome, the enhance in CRP, TNF-α, IL-6, IL-18 and oxidized LDL (Van Guilder et al. 2006) is proportional to the number of clustered components of the metabolic syndrome (Girona et al. 2008).

Additionally, as already discussed, also adipocytokines such as leptin, resistin and adiponectin could interfere in insulin metabolism and signalling pathways.

Moreover, in addition to direct modulators of inflammatory pathways, other factors are also involved in the control of inflammation. Among them, polyunsaturated n-6 and n-3 fatty acid have to be take into account.

Polyunsaturated n-6 and n-3 fatty acids.

Polyunsaturated n-6 and n-3 fatty acids are involved at several levels with inflammatory condition and inflammatory response.

The arachidonic acid (a n-6 fatty acid) is the precursor of pro-inflammatory eicosanoids, synthesised by cyclooxygenase. Eicosanoids include prostaglandines, tromboxanes and leukotrienes, that are molecules involved in the activation and regulation of inflammation (Lewis, Austen, and Soberman 1990). Not only arachidonic acid, but n-6 fatty acid availability in general influences eicosanoid production. Increased membrane content of n-6 fatty acids has been associated to inflammatory diseases (Ueda et al. 2008) as well as to stimulated gene expression of pro-inflammatory cytokines and transcriptional activity of NF-kB (Weaver et al. 2009). The n-3

fatty acids, on the other side, are recognised to have anti-inflammatory properties. Eicosapentaenoic acid is a substrate for cyclooxygenase and is the precursor of prostaglandins, characterized by lower pro-inflammatory potential (Bagga et al. 2003). Moreover, docosahexaenoic (another n-3 fatty acid) as well as eicosapentaenoic acids are precursors of resolvins, subclasses of anti-inflammatory molecules (Hong et al. 2003). In vitro (Babcock et al. 2002) and in animal (Endres et al. 1989; Lee et al. 1985) studies evidence that eicosapentaenoic and docosahexaenoic acids down-regulate the expression and the production of IL-1 and of TNF-α. In general, n-3 fatty acids negatively modulate genetic regulation of pro-inflammatory factors, principally NF-kB (Ross, Moses, and Fearon 1999), that are involved also in the catabolism of n-3 anti-inflammatory fatty acids. Finally, the increase in eicosapentaenoic and docosahexaenoic acids intake is reflected in cell membrane fatty acid content (Lee et al. 1985), reducing, in turn, the fraction of the pro-inflammatory arachidonic acid. Finally, also the relative content of n-3 over n-6 fatty acids has to be considered. An high dietary n-6 to n-3 fatty acid ratio intake stimulates the expression of CRP and other pro-inflammatory agents, such as TNF-α (Zhang et al. 2010).

Polyunsaturated n-6 and n-3 fatty acids metabolism.

Polyunsaturated n-6 and n-3 fatty acids metabolism is schematically reported in figure 8.

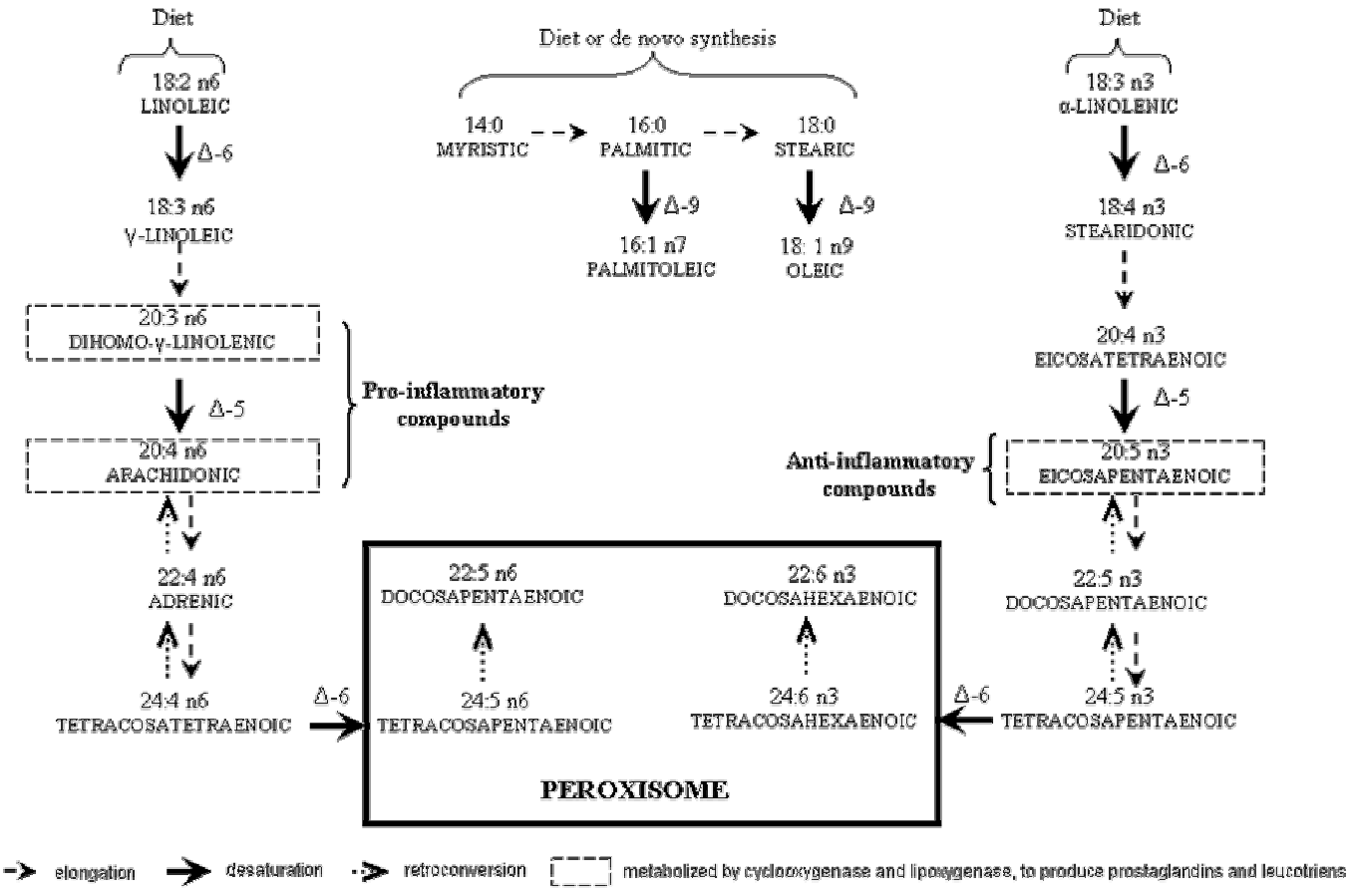

Figure 8. Synthetic pathways of saturated, monounsaturated and polyunsaturated (n-3 and n-6) fatty acids.

n-6 fatty acids availability is principally related to precursor availability and endogenous synthesis (Wertz 2009). n-6 fatty acid precursor is linoleic acid, an essential fatty acid, typically found in vegetable oils (Bozan and Temelli 2008). Otherwise, the precursor of n-3 fatty acids is the essential α-linolenic acid, contained in high amount in vegetable oils (Wertz 2009). Moreover, n-3 fatty acids availability is also strictly related to fish oil intake, rich in eicosapentaenoic and docosahexaenoic acids (Pickova 2009).

Interestingly, n-3 and n-6 fatty acids are metabolism by the same pattern of enzymes, composed by desaturases, like Δ-5 Δ-6 and Δ-9 desaturases, and elongases.

Membrane fatty acid composition.

A reliable marker of fatty acid availability in whole body and in tissues is fatty acid pattern in erythrocytes membrane phospholipids (Harris and Von 2004). Changes in membrane fatty acid composition are associated to several metabolic alterations; as an example, changes in relative fatty acid levels affect membrane fluidity and stability, altering signalling pathways and membrane permeability. It has been demonstrated that an high membrane content of n-3 fatty acids enhances GLUT-4 expression and signalling activity on muscle cell, possibly ameliorating insulin sensitivity (Taouis et al. 2002). In addition, membrane level of n-3 fatty acids is directly associated to reduced incidence of cardiovascular diseases (Harris and Von 2004). In human neutrophils, the increase in membrane ratio between n-6 and n-3 fatty acids is associated to enhanced pro-inflammatory molecule synthesis (Zhang et al. 2010).

TNF-α

TNF-α is a pleiotropic cytokine, that is mainly produced by macrophages; however, many other cells secret TNF-α including skeletal muscle of subjects suffering type 2 diabetes (Saghizadeh et al. 1996) and adipose tissue, especially during fat mass accumulation (Saghizadeh et al. 1996)

Several evidences indicate that TNF-α is a mediator of insulin resistance development in obesity (Hotamisligil, Shargill, and Spiegelman 1993), probably through the interaction with other cytokines (Bedard, Marcotte, and Marette 1997; Uysal et al. 1997). TNF-α has been demonstrated to affect the main tissue-targets of insulin action, i.e., skeletal muscle, adipose tissue and liver. In skeletal muscle of insulin resistant as well as type 2 diabetic subjects, TNF-α level is significantly increased (Hotamisligil et al. 1995; Lappas et al. 2005; Saghizadeh et al. 1996) and is inversely related to glucose availability (Saghizadeh et al. 1996). TNF-α impairs insulin-induced activation of insulin receptor and IRS-1 (Hotamisligil et al. 1994; del Aguila, Claffey, and Kirwan

1999(Bouzakri and Zierath 2007), in muscle (Li and Reid 2001), adipocytes (Hauner et al. 1995) as well as in liver (Hotamisligil et al. 1994).
TNF-α is also involved in free fatty acid metabolism; in muscle and in adipose tissue; TNF-α down-regulates lipoprotein lipase expression and suppresses fatty acid oxidation, leading to an increase in circulating free fatty acids and in intramuscular diacylglycerol content (Kroder et al. 1996).
Finally, TNF-α modulates the expressions of other cytokines, stimulating IL-6 (Fasshauer et al. 2003) and, possibly, leptin (Bullo et al. 2002) expressions and inhibiting adiponectin synthesis (Kern et al. 2003).

IL-6

IL-6 is a adipocytokine (Fried, Bunkin, and Greenberg 1998), exhibiting both pro- and anti-inflammatory actions, that is secreted by several tissues, including skeletal muscle (Hacham et al. 2004; Lopez-Soriano et al. 2006) and adipose tissue (Bastard et al. 2000). IL-6 concentration in fat mass increases in obese subjects, especially in visceral adiposity compared to subcutaneous fat deposition (Okuno et al. 1998). Several correlations have been reported between IL-6 content and risk or indexes of insulin resistance and type 2 diabetes (Bastard et al. 2000; Monzillo et al. 2003; Fernandez-Real et al. 2001). Nevertheless, it has been hypothesised that IL-6 role in insulin resistance development is mainly related to IL-6-mediated regulation of resistin and, possibly, other adipocytokines secretion (Kaser et al. 2003), rather than to a direct molecular interaction with insulin signalling pathway (Bastard et al. 2000; Kern et al. 2001).

IL-10

IL-10 is an anti-inflammatory cytokine, expressed both in skeletal muscle and adipose tissue. IL-10 is reported to modulate glucose metabolism in skeletal muscle (Hacham et al. 2004) and to prevent detrimental effects of high-fat diet and of IL-6 on hepatic fat accumulation (den Boer et al. 2006) and insulin signalling defects (Kim et al. 2004), respectively.

IL-1β

IL-1β is a pro-inflammatory cytokine, involved in pancreatic β cell destruction in type 1 diabetes. its effects on insulin signalling pathway and metabolism are presently unknown; nonetheless, IL-1β concentration is increased in β cells of subjects suffering also type 2 diabetes and its level is strictly related to the progression in β cell dysfunction and apoptosis, induced by hyperglycaemia (Maedler et al. 2002; Maedler and Donath 2004).

IL-15

IL-15, involved in weight regulation (Quinn et al. 2005), promotes insulin sensitivity. IL-15 in fact enhances glucose uptake, mainly through the stimulation of GLUT-4 expression (Busquets et al. 2006).

C-reactive protein

Plasma levels of CRP have been related to the degree of visceral adiposity and insulin resistance; however, great differences in CRP levels have been reported among different populations and ethnic groups, masking the effective potential predictive and/or causative role of CRP on diabetes or metabolic syndrome development (Chambers et al. 2001).

Chemokines

Chemokines are chemotactic cytokines, characterised by small molecular weight, potentially involved in insulin signalling pathway as well as in glucose metabolism. Among them, monocyte chemotactic protein-1 (MCP-1) has been related to impaired insulin sensitivity in adipose (Kamei et al. 2006; Kanda et al. 2006) and skeletal muscle tissues (Boyd et al. 2006; Torres et al. 2004) in type 2 diabetes.

HYPERHOMOCYSTEINEMIA

HOMOCYSTEINE METABOLISM

Homocysteine is a non-proteinogenic amino acid, that is present in human plasma in two different forms, homocysteine and homocystine. Homocystine is the dimerized form of the reduced homocysteine and accounts for almost the 90% of total homocsyetine in blood (Jacobsen 1998). Homocysteine is involved in methionine metabolism (Figure 9). Methionine is converted to homocysteine through the transmethylation pathway in three steps. First, methionine adenosyltransferase catalyses the conversion of methionine to S-adenosylmethionine (SAM). S-adenosylmethionine is the methyl donor virtually involved in all methylation reactions and is transformed in S-adenosylhomocysteine (SAH) by the action of several methyltransferase enzymes. Among these methyltransferases, glycine N-methyltransferase (GNMT) is responsible for the metabolism of excess methionine and, in fact, is only weakly inhibited by S-adenosylhomocysteine production. The third step is characterised by the hydrolysis of S-adenosylhomocysteine to homocysteine, by S-adenosylhomocysteine hydrolase (SAHH). Once synthesised, homocysteine can be converted back to methionine, through the remethylation pathway, or can be transformed to cysteine, through the transulfuration process. The remethylation of homocysteine to methionine is provided by two independent enzymes, methionine synthase and betaine:homocysteine methyltransferase (BHMT), using respectively N5-methyltetrahydrofolate and betaine as methyl donors (Torres et al. 2004). The transulfuration, instead, is an irreversible process transforming homocysteine to cysteine, in a two-step pathway. The cystathionine β-synthase (CBS) condensates homocysteine and serine in cystathionine and the cystathionine γ -lyase (CGL) hydrolyses cystathionine to cysteine, α-oxobutyrate and ammonium. The transulfuration pathway prevails over the remethylation when the requirement of methionine is decreased or the one of cysteine is increased (Wijekoon, Brosnan, and Brosnan 2007).

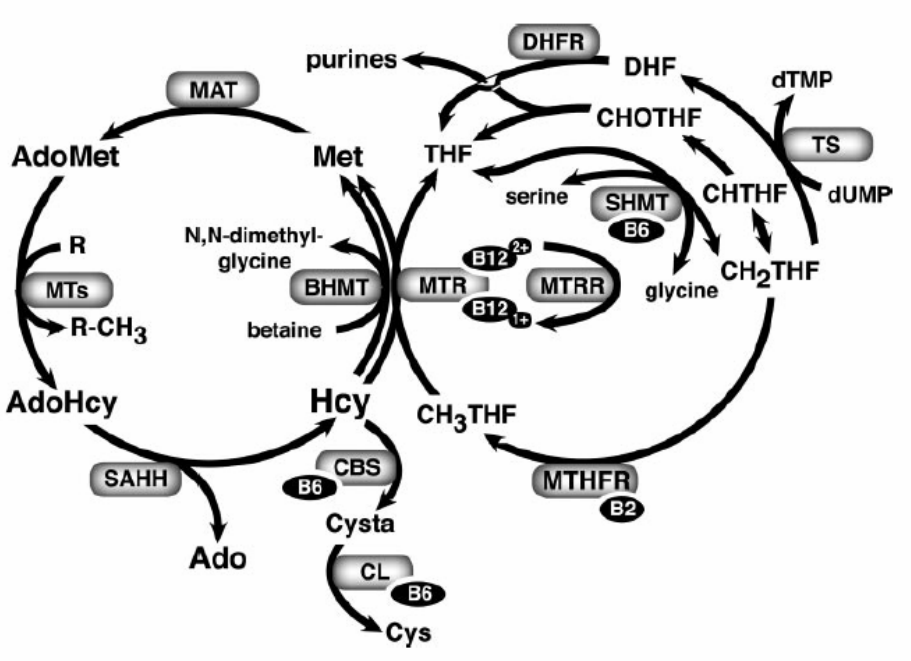

Figure 9. Homocysteine metabolic pathway (Refsum et al. 2004). Hcy, homocysteine; Met, methionine; Ado, adenosine; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; $B12^{+1,+2}$, oxidation state of cobalt atom in B12; BHMT, betaine-homocysteine S-methyltransferase; CH2THF, 5,10-methylenetetrahydrofolate;CH3THF, 5-methyltetrahydrofolate; CL, cystathionine γ-lyase; Cysta, cystathionine; DHF, dihydrofolate; DHFR, dihydrofolate reductase; MAT, methionine adenosyltransferase; MTR, methionine synthase; MTRR, methionine synthase reductase; MTs, AdoMet-dependent methyltransferases; R, methyl acceptor (e.g., phospholipids, proteins, DNA, RNA, amino acids, and neurotransmitters); SAHH, S-adenosylhomocysteine hydrolase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase.

FACTORS INFLUENCING HOMOCYSTEINE METABOLISM

Dietary factors.

The methyl donor S-adenosylmethionine is a key regulator of homocysteine metabolism, promoting the transmethylation and the transulfuration (Finkelstein 2000) as well as decreasing the remethylation, through the inhibition of the methylentetrahydrofolate reductase (Finkelstein 2000). Proteins and amino acids are a source of S-adenosylmethionine; as a consequence, if in normal diet conditions (normal or low protein intakes) homocysteine is mainly catabolized through the remethylation, in an high protein diet homocysteine is mainly removed through the transulfuration pathway (Ueland and Refsum 1989).

Folates are also key regulators of homocysteine metabolism. Low plasma folate levels determine an increase in plasma homocysteine due to a reduction of remethylation and an increase in transmethylation and transulfuration pathways (Lee and Frenkel 2003).
Moreover, nutritional deficiencies of cobalamin (Refsum et al. 2001) and pyridoxine (Slavik, Smith, and Blanc 1982) involved in the methionine synthesis and in the activity of methylenetetrahydrofolate reductase, respectively, are associated to hyperhomocysteinemia.

Gender.
Homocysteine concentration is related to age, race (Nygard et al. 1995) and gender (Fukagawa et al. 2000). Homocysteine level is lower in female compared to male subjects, matched for age and healthy condition, probably due to a more efficient remethylation process (Fukagawa et al. 2000).

Genetic factors.
Hyperhomocysteinemia has been associated primarily to two main genetic mutations: one involving the cystathionine β-synthase with consequent reduction in transulfuration (Crowther and Kelton 2003), and the second interesting the methylentetrahydrofolatereductase (MTHFR), inducing an impairment in remethylation (Deloughery et al. 1996).

Level of physical activity.
Homocysteine concentration is differently affected by physical activity level. Even though some discrepancies have been reported (Gelecek et al. 2007; Di et al. 2009; DiSantolo et al. 2009), acute exercise enhances homocysteine level (Herrmann et al. 2003; Duncan et al. 2004) whereas constant regular training decreases homocysteine concentration, compared to untrained subjects (Duncan et al. 2004). Moreover, low physical activity, as observed in sedentary lifestyle condition, is associated to increased plasma homocsyetine content (Metsios et al. 2009), independently from other confounding factors such as genetics, sex, age or folate dietary intake (Nygard et al. 1995). On the contrary, regular and constant physical activity decreases homocysteinemia in lean but also obese adults (Vincent, Bourguignon, and Vincent 2006). Nevertheless, mechanisms relating physical activity level and homocysteine metabolism are presently not elucidated.

HOMOCYSTEINE AND CARDIOVASCULAR RISK

Hyperhomocysteinemia is a marker of cardiovascular disease since elevated plasma levels of homocysteine are recognised to be involved in cardiovascular damages (Clarke et al. 1991; Graham et al. 1997; Boushey et al. 1995). Moreover, in recognised coronary heart disease patients

homocysteinemia is directly related to flogosis as well as oxidative stress (Jonasson et al. 2005) and can be considered a predictive index of mortality (Nygard et al. 1997). However, the molecular mechanism linking homocysteine and cardiovascular injury has not been completely elucidated. Nevertheless, several hypothesis have been proposed.

Several evidences indicate that a negative correlation exists between homocysteinemia and HDL level in subjects differently affected by cardiovascular diseases (Qujeq, Omran, and Hosini 2001; Glueck et al. 1995). It has been suggested that hyperhomocysteinemia induces cardiovascular diseases at least in part through the impairment of HDL and apolipoprotein A-I metabolism (Liao, Yang, and Wang 2007). In mice, high homocysteine levels are associated to dyslipidaemia, characterised by increased total cholesterol, triglycerides, LDL and VLDL levels as well as by decreased HDL cholesterol (Qujeq, Omran, and Hosini 2001; Glueck et al. 1995; Wang et al. 2003). It has been proposed that dyslipidaemia associated to hyperhomocysteinimia could be induced by homocysteine effects at hepatocyte level. In fact in hepatocytes, homocysteine determines endoplasmic reticulum stress, activates protective mechanisms such as the unfolded protein response that promotes protein degradation by the endoplasmic reticulum and inhibits protein synthesis, and activates the sterol regulatory element-binding proteins. These events, in turn, impair the sterol response pathway and induce cholesterol and triglyceride accumulation (Holven et al. 2003). Additionally, enhanced homocysteine levels have been associated to increase in extrahepatic scavenger receptors involved in the peripheral uptake of LDL and oxidised LDL (Wang et al. 2003; Holven et al. 2003).

Another hypothesis is that enhanced plasma homocysteine could affect vascular functions, at several levels. Through autoxidation, homocysteine could transform in homocystine and other reactive oxygen species, respectively in plasma and in cells, (Welch and Loscalzo 1998); moreover homocysteine inhibits glutathione peroxidase activity (Nishio and Watanabe 1997) and expression (Upchurch, Jr. et al. 1997), potentially determining oxidative damage. Homocysteine reacts with nitric oxide to counteract its potential oxidative properties; however, this determines a reduction of nitric oxide availability. Additionally, homocysteine could react with several factors of the coagulation, promoting a prothrombotic condition (Nishinaga, Ozawa, and Shimada 1993). Furthermore, homocysteine is a potent mitogen for vascular smooth-muscle cells (Harker, Harlan, and Ross 1983; Tsai et al. 1994) and could impair intracellular protein folding as well as determine endoplasmic reticulum stress (Outinen et al. 1999). Moreover, homocysteine promotes a pro-inflammatory condition through the stimulation of arachidonic acid metabolism and, consequently the increase in pro-inflammatory and procoagulant thromboxane A2 synthesis (Di Minno et al. 1993). Interestingly, inflammation *per se* could stimulate homocysteine level since the increase of

DNA, RNA and protein remethylation during tissue repair in inflammatory states enhances S-adenosylhomocysteine and than homocysteine synthesis (Dudman 1999). Finally, homocysteine can be transformed in cyclical products, such as homocysteine thiolactone that could be incorporated *per se* or in aggregate with LDL-cholesterol in vascular cells or atheromatous plaques, leading to protein dysfunction and contributing to the atherosclerosis process (Jakubowski 1997). Nevertheless, mechanisms linking hyperhomocysteinemia and cardiovascular alterations need further investigations.

Insulin resistance and homocysteine metabolism.

Type 2 diabetes is strongly associated to increased cardiovascular injuries and the 75% of deaths in subjects suffering for type 2 diabetes are due to cardiovascular diseases (Davies et al. 2001). Hyperhomocysteinemia and insulin resistance are considered two independent cardiovascular risk factors (Mayer, Jacobsen, and Robinson 1996), even though high homocysteine level is a stronger predictor for cardiovascular diseases (Hoogeveen et al. 1998) and death (Hoogeveen et al. 2000) in type 2 diabetic compared to non-diabetic subjects. However, biological mechanisms linking these metabolic alterations are still unknown. Nevertheless, homocysteine could contribute to the induction of some features typically observed in insulin resistant states such as increased oxidative stress and endothelial dysfunction (Bellamy et al. 1998; Chambers et al. 1999).

AUTONOMIC NERVOUS SYSTEM DYSREGULATION

ASSESSMENT OF AUTONOMIC NERVOUS SYSTEM ACTIVITY

In vivo, a non-invasive method used to estimate the role of autonomic nervous system control on cardiovascular system is the analysis of the heart rate or RR interval variability.

The power spectral analysis is based on the determination of heart rate and systolic blood pressure variabilities and permits to assess cardiac autonomic regulation (Pagani et al. 1986). The α index is usually used to define such relationship. The power spectral analysis reflects the degree of autonomic nervous system modulation at cardiac level, more than sympathetic or parasympathetic tone levels (Lipsitz et al. 1990).

Heart rate as well as its variability reflects chronotropic and inotropic actions of parasympathetic and sympathetic nervous system branches on the cardiac muscle and the sinus node (Malliani et al. 1991; (Pagani et al. 1986). An increase in heart rate is usually the consequence of the increase in sympathetic and the decrease in parasympathetic tones. On the contrary, an enhance in parasympathetic activity is usually the responsible for the reduction on heart rate (Aubert, Seps, and Beckers 2003; Rajendra et al. 2006).

Moreover, heart rate variability is an early index of health impairment. Heart rate variability in fact reflects the sensitivity to cardiac autonomic regulation. An appropriate adaptation to changes in environmental conditions is associated to an increase in heart rate variability whereas a low heart rate variability is often marker of impaired or inadequate autonomic response (Pumprla et al. 2002). A decreased heart rate variability has been observed in patients with heart failure and myocardial ischaemia (Dekker et al. 2000) as well as in young, middle-age and elderly subjects affected by the metabolic syndrome (Liao et al. 1998; Stein et al. 2007; Koskinen et al. 2009). Additionally, different metabolic alterations typically evidenced in the metabolic syndrome, such as hyperglycaemia (Singh et al. 2000), low physical activity level, high body mass index as well as increased plasma total cholesterol and blood pressure (Britton et al. 2007) have been associated to a reduction in heart rate variability.

The heart rate power spectrum is usually divided into two components: low- and high-frequency oscillations. The investigation of these two components of heart rate variability, through the frequency domain analysis, permits to define the role of each branch of the autonomic nervous system. The high-frequency component (HF), ranging between 0.15 and 0.5 hertz of the HR variability spectrum, reflects vagal modulation of the sinus node whereas the low-frequency component (LF), ranging between 0.04 and 0.14 hertz of the heart rate variability spectrum, reflects both the sympathetic modulation of vasomotor tone and the combination of sympathetic and

parasympathetic systems on heart rate (Heart rate variability. Standards of measurement, physiological interpretation, and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology1996; Cottin, Papelier, and Escourrou 1999). Low as well as high-frequencies may be expressed both in absolute values and in normalized units. If the evaluation of these components in absolute value provides measurements of the degree of autonomic nervous system modulations (Malik and Camm 1993), the expression of these components in normalised units stresses the balanced contribution and activity of both sympathetic and parasympathetic branches, minimizing the effect of overall total power changes.

The ratio between LF and HF components (LF-to-HF ratio) is calculated as index of influence of sympathovagal balance on heart rate control (Pagani et al. 1986). When this ratio is minor than 1 a parasympathetic predominance is evidenced whereas values of LF-to-HF ratio major than 1 indicate an higher sympathetic activation (Malliani 1999; Heart rate variability. Standards of measurement, physiological interpretation, and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology1996).

The assessment of systolic blood pressure variability furnishes other information useful to define autonomic nervous system regulation. Systolic blood pressure is reduced by parasympathetic whereas is enhanced by sympathetic nervous system activation. The LF component of the systolic blood pressure variability is an index of sympathetic-induced activation of α-adrenergic receptors at vascular level (Japundzic et al. 1990). The HF component, instead, is possibly associated to the mechanical effect of breathing (Cottin, Papelier, and Escourrou 1999).

Finally, the cross spectral analysis and the spontaneous sequence technique are usually utilised to define baroreflex sensitivity. Particularly, the linear spontaneous sequence technique is based on the occurrence of spontaneous fluctuations in blood pressure associated to concordant RR interval changes. Spontaneous sequences of three or more cycles allows to calculate the linear regression slope between blood pressure and RR interval changes, that represents the spontaneous baroreflex sensitivity. A decrease in baroreflex sensitivity reflects a depression of reflex vagal activity. Arterial baroreceptors, located in the adventitia of the carotid sinuses and aortic arch, are highly sensitive and they respond to changes of arterial flow without inducing measurable changes in pressure. Baroreceptors sense distortions and changes of dimension, but not changes in pressure. However, the degrees of pressure and distortion usually are related closely. Changes in dimensions alter firing of stretch-sensitive neurons that are located in arteries walls and cardiac chambers; the consequent modifications in baroreceptor input to the brain induces are reflected by changes of neural output from sympathetic and parasympathetic motoneurons, leading to cardiovascular adjustments aimed to counteract initial dimensions changes detected by beroreceptors. Baroreflex mechanisms are

strictly related to regulation of heart rate, atrioventricular node conduction, myocardial contractility and electrophysiologic properties, as well as peripheral resistances. In this way, baroreflex regulation reduces the effects of everyday living environmental perturbations (Eckberg et al. 1992).

AUTONOMIC NERVOUS SYSTEM AND THE METABOLIC SYNDROME

The metabolic syndrome has been associated to alterations of the autonomic nervous system, mainly characterised by an over-activation of the sympathetic system associated to a withdrawal of parasympathetic activity (Licht et al. 2010). A decrease in heart rate variability as well as in baroreflex sensitivity have been reported in subjects suffering for the metabolic syndrome. Moreover, the degree of these alterations is strictly related to the number of component of metabolic syndrome observed (Stein et al. 2007; (Lindgren et al. 2006) and an alteration in sympathetic activity is *per se* involved in the pathogenesis of several distinct components of the metabolic syndrome. In fact, an excessive and/or prolonged stimulation of the autonomic nervous system has been associated to several metabolic alterations, such as altered blood pressure, altered plasma lipid pattern, as in particular increased triglycerides and decreased HDL (Anagnostis et al. 2009; Tentolouris, Argyrakopoulou, and Katsilambros 2008) as well as insulin resistant condition (Berntson et al. 2008). Heart rate variability is inversely associated to the number of the metabolic syndrome disorders (Liao et al. 1998). Moreover, the concomitant presence of different features of the metabolic syndrome has been demonstrated to differently affect components of heart rate variability (Liao et al. 1998). In particular, hypertension *per se* is associated to decreased heart rate variability, and this alteration is further increase by the parallel presence of diabetes. On the contrary, the combination of hypertension and dyslipidaemia does not show additional adverse effects on heart rate. Finally, dyslipidaemia development has inverse multiplicative effects on heart rate alteration, when associated to diabetes (Liao et al. 1998). Nevertheless, sympathovagal unbalance evidenced in the metabolic syndrome is reversible, since metabolic syndrome resolution as well as changes in lifestyle aimed to counteract observed metabolic disorders ameliorate autonomic regulation (Emdin et al. 2001; Brunner et al. 2002).

Autonomic dysregulation and insulin resistance.

Insulin centrally stimulates the sympathetic nervous system (Muntzel et al. 1994), as evidenced during physiological and pharmacological hyperinsulinemia (Scherrer et al. 1993; Vollenweider et al. 1993). Hyperinsulinemia-induced activation of sympathetic nervous system has been observed both in obese (Reaven, Lithell, and Landsberg 1996) and lean healthy subjects (Hausberg et al. 1997; Paolisso et al. 2000). A decrease in parasympathetic nervous system activity occurs when

elevated but physiological plasma insulin levels are maintained at long time (36 hours), through glucose infusion. Moreover, exposure to insulin decreases mRNA expression of M2-muscarinic receptors in rat atrial cardiomyocytes, in a dose and time-dependent manner (Pathak et al. 2005). Interestingly, a decrease in baroreflex sensitivity has been previously reported in insulin resistant states (Kuusisto et al. 1994; Smith 1599-601;Pikkujamsa et al. 523-31). Additionally, altered insulin sensitivity is directly associated to the LF-to-HF ratio, index of sympathovagal balance, independently from body mass and, particularly, body fat (Emdin et al. 2001). Moreover, stimulated sympathetic activity directly inhibits insulin release, reducing thus glucose uptake in peripheral tissues and enhancing hepatic gluconeogenesis (Nonogaki 2000; Fehm, Kern, and Peters 2006). Through these mechanism, sympathetic activation could possibly favour insulin resistance development (Mancia et al. 2007).

Moreover, enhanced sympathetic nervous system activity could *per se* determine an increase in plasma glucose level, through the stimulation of lipolysis, by the activation of β-3 adrenergic receptors in visceral adipose tissue. This leads to an increase in plasma free fatty acids as well as in hepatic glucose production and to a reduction of glucose uptake in muscle. Moreover, sympathetic activity increases glucagons secretion that also contributes to enhanced hepatic glucose production. As previously suggested, enhanced gluconeogenesis associated to sympathetic-induced decrease in glucose utilization by muscle, could be responsible, at least in part, for alterations in insulin and glucose metabolism typically observed in subjects suffering for metabolic syndrome (Tentolouris, Argyrakopoulou, and Katsilambros 2008).

Nevertheless, mechanism relating insulin resistance and altered sympathetic nervous system activation has not been elucidated.

Autonomic dysregulation and obesity.

Obesity *per se* (Grassi 1998) is characterised by altered sympathetic activation that, in such condition, could also contribute to enhanced cardiovascular risk. In obesity, autonomic impairment depends on adiposity localization, and is more evidenced in subjects with greater visceral fat compared to subjects with lower visceral fat (Beske et al. 2002). It has been suggested that the increase in plasma free fatty acids, derived from stimulated lipolysis in visceral fat accumulation (Ostman et al. 1979), could be responsible for sympathetic nervous system activation in obese subjects (Paolisso et al. 2000; Gadegbeku et al. 2002).

Autonomic dysregulation and hypertension.

An increase in sympathetic nervous system has been also associated to the development and the degree of hypertension. Altered sympathovagal balance affects heart rate and cardiac output (Grassi 2006). Additionally, sympathetic nervous system activation stimulates smooth muscle cell proliferation, reducing peripheral vasculature compliance and increasing diastolic blood pressure. Finally, over-activated sympathetic nervous system contributes to hypertension development increasing sodium retention (Grassi 2006).

The development of hypertension has been demonstrated to be preceded by high-fat diet induced insulin resistance (Hall et al. 1993). Interestingly, modification of lifestyle risk factors for insulin resistance (Krotkiewski et al. 1979; Stamler et al. 1987) ameliorates not only insulin sensitivity itself but also hypertension development and autonomic dysregulation (Tuck 1992). Even though mechanisms relating insulin resistance and hypertension development are not elucidated, it has been suggested that an altered activation of sympathetic tone, especially in subjects affected by metabolic disorders, could affect vasodilatation normally induced by insulin (Anderson et al. 1991).

Autonomic dysregulation and dyslipidaemia.

Subjects suffering the metabolic syndrome often display altered plasma lipid profile, mainly characterised by decreased HDL cholesterol as well as increased triglycerides and free fatty acids. It has been demonstrated that the increase in free fatty acids activates sympathetic nervous system (Paolisso et al. 2000) and impairs, in a dose-dependent manner, baroreflex sensitivity (Gadegbeku et al. 2002).

PHYSICAL ACTIVITY AND AUTONOMIC REGULATION

Physical activity is known to directly influence autonomic regulation, in particular inducing a decrease in sympathetic and an increase in parasympathetic activities (Furlan 1993) (Iellamo et al. 2002). Regular physical activity, in fact, improves heart rate variability and reduces heart rate in healthy young and old subjects (Stein et al. 1999) as well as in patients suffering type 2 diabetes and hypertension (Sridhar et al. 2010). In type 2 diabetes, both systolic and diastolic blood pressures are reduced after one-year regular physical training (Sridhar et al. 2010). In healthy subjects, regular physical activity improves also the high and low-frequency components of heart rate variability (Achten and Jeukendrup 2003). Even though the mechanism underlining the relationship between physical activity and autonomic regulation needs to be fully elucidated, a physiological adaptation to the exercise-induced increase in myocardial work has been suggested.

CAUSES OF THE METABOLIC SYNDROME: THE ROLE OF PHYSICAL INACTIVITY

Lifestyle and genetic factors are the major determinants for the development of insulin resistance and of the metabolic syndrome. Due to the impossibility to modify genetic background, great interest has been applied in the assessment of the influence of different lifestyles as well as of changes in lifestyle on the development of metabolic alterations. The metabolic syndrome, and in particular insulin resistance, has been associated to low levels of physical activity, unbalanced diet, stress and smoking (Despres 2006). The main conequences of the metabolic syndrome are the development of type 2 diabetes and of cardiovascular diseases (Lakka et al. 2002; Resnick et al. 2003).

Physical inactivity.

Immobility, or low activity, are frequently observed in pathologic states, being associated to several illness conditions, such as serious trauma, neurological and cardiological diseases, as well as all surgical interventions. Inactivity also occurs during spaceflights, characterised by microgravity environments. Moreover, 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 (Chaput and Tremblay 2009). Low grade of physical activity negatively affects human health, contributing to deaths from chronic diseases, and increasing prevalence of physical disabilities, especially in elderly people (Fontana 2009). Moreover, physical inactivity leads to a low-grade systemic inflammation, as evidenced by the unbalance between pro and anti-inflammatory cytokines as well as the increase in acute-phase proteins such as C reactive protein and long pentraxin-3 (Bosutti et al. 2008).

On the contrary, regular physical activity has been demonstrated to reduce both the prevalence of the metabolic syndrome (Farrell, Cheng, and Blair 2004) and the mortality risk in such pathological condition (Katzmarzyk, Church, and Blair 2004).

The importance of physical activity on human health is also stressed by the increasing interest of the World Health Organization (WHO). Current recommendations indicates as correct level of activity at least 30 minutes of moderate-intensity physical activity on most, and preferably all, days of the week (Pate et al. 1995).

Nevertheless, the net impact of physical activity or inactivity on the development of the metabolic syndrome as well as related metabolic alterations has never been investigated.

The role of energy balance in physical inactivity conditions.

In association to sedentary lifestyle, altered energy intake, and particularly excess weight and fat accumulation, is another important determinant of metabolic alterations (Resnick et al. 2003); in fact, several nutritional and dietary strategies have been proposed to counteract, at least in part, the constant increase in the incidence of the metabolic syndrome. Nevertheless, the role of an appropriate nutritional intervention is still more precious in condition of inactivity or sedentary lifestyle (Bosutti et al. 2008; Biolo et al. 2007; Biolo et al. 2008).

Several pathological conditions as well as elderly are characterised by a decrease in lean body mass, due to both muscle atrophy or wasting and reduced physical activity level, that determine a reduction of energy requirement. However, in this condition, an inappropriate modulation of energy intake leads to a positive energy balance and fat accumulation (Honda et al. 2007). The increase in adipose tissue stimulates the production and the release of several pro-inflammatory adipocytokines that could worsen muscle atrophy development, as observed in the sarcopenic obesity. Moreover, positive energy balance in healthy subjects exposed to prolonged physical inactivity activates systemic inflammation and oxidative stress, further contributing to loss in muscle mass (Biolo et al. 2008) and, possibly, to other metabolic abnormalities observed in inactive conditions.

On the contrary, chronic pathologies are often characterised by reduced appetite and physical activity. In these subjects, that are frequently malnourished and underfed, muscle loss is paralleled by fat mass loss; however, in such conditions, muscle atrophy could develop in cachexia. As observed in healthy young subjects, the association between physical inactivity and calorie restriction prevents inactivity-induced increase in pro-inflammatory mediators (Bosutti et al. 2008); however physical inactivity combined to calorie restriction further worsen the degree of muscle atrophy, induced by inactivity (Biolo et al. 2007).

These evidences suggest that dietary control and neutral energy balance maintenance are key tools in subjects with reduced physical activity level, as in subjects affected by insulin resistance and metabolic syndrome.

AIMS

The present thesis is principally aimed to define the net role of physical inactivity in the development of insulin resistance and metabolic syndrome, avoiding potential interferences and confounding factors effects of ageing, energy unbalance and diseases. Experimental bed rest in healthy, young, lean subjects represents a reliable approach to determine the net effects of physical inactivity on human metabolism (Biolo et al. 2005).

Results discussed in the present thesis were collected during four different bed rest studies:

- Women International Space Simulation for Exploration (WISE), performed at the MEDES Clinical Research Facility of the Rangueil University Hospital (Toulouse, France);
- Valdoltra Bed Rest Study 2006;
- Valdoltra Bed Rest Study 2007;
- Valdoltra Bed Rest Study 2008, performed at the Valdoltra Orthopaedic Hospital, (Ankaran, Capodistria, Slovenia).

MATERIALS AND METHODS

EXPERIMENTAL BED REST STUDIES

Women International Space Simulation for Exploration (WISE)

Sixteen healthy female subjects (mean ± S.E.M; age: 32.1 ± 4 years, BMI: 21 ± 2 kg × m^{-2}) were recruited for the present study performed at MEDES Clinical Research Facility of the Rangueil University Hospital (Toulouse, France). A written informed consent was signed by each participant before the beginning of the study. The study was performed in accordance to the standards set by the Declaration of Helsinki (2002) and its amendments. All subjects were physically active before the admission to the institute and none of them was under medication. At admission, routine medical screening was performed. The study design consisted in 20 days of ambulatory adaptation to a standardized diet, during which diet and physical activity levels were strictly monitored; 60 days of 6° head-down tilt bed rest, during which all activities were performed in head-down tilt position, except meals assumption that was performed in horizontal position; 20 days of recovery. Periodical medical control and constant nursing assistance were ensured through all the experimental period.

Main examinations performed in the present study.

Body composition was monitored every 15 days, through all the experimental bed rest.

At the beginning as well as at the end of 60 days of the experimental bed rest, a metabolic test, aimed to assess homocysteine metabolism, was performed.

Valdoltra Bed Rest Studies 2006 -2007 -2008

Thirty healthy young male subjects (age 23.3 ± 0.4 years; BMI 23.6 ± 0.4 kg m^{-2}) were selected to participate to three separated bed rest studies, performed at the Valdoltra Hospital, University of Primorska, Ankaran-Capodistria, Slovenia, in July–August 2006, 2007 and 2008. A written informed consent was signed by each participant before the beginning of the study. All studies were approved by the Ethical Committee of the University of Ljubljana and were performed in accordance to the standards set by the Declaration of Helsinki (2002) and its amendments. All subjects were physically active before the admission to the institute and none of them was under medication. At admission, routine medical screening was performed. The study design consisted in 1 week of dietary and environmental adaptation in ambulatory condition; 35 days of bed rest; 1 week of recovery. In 2006 and 2007, bed rest was performed in horizontal clinostatic conditions

whereas in 2008 bed rest was performed in a 6°-head-down-tilt condition. Periodical medical control and constant nursing assistance were ensured through all the experimental periods.

Main examinations performed in the present studies.

Body composition was monitored in all Valdoltra studies.

In 2006 and 2007, a metabolic test with stable isotope tracers, aimed to assess glutathione metabolism in erythrocyte, was performed, before and at the end of the experimental bed rest (33-day bed rest).

Moreover, in 2007 glutathione metabolism was defined in muscle, through a new approach, based on double tracers infusion and single sample collection. This approach was validated in erythrocytes, using the traditional and new technique. This metabolic test was performed before, after 7 days as well as at the end of bed rest (33-day bed rest) . Muscle biopsies and muscle glutathione metabolism were obtained only at the beginning and at the end of bed rest. In addition, *vastus lateralis* thickness and architecture as well as muscle protein carbonylation were determined. *Vastus lateralis* thickness was also assessed in 2006 and 2008.

In 2008, before, after 7 days and at the end (33-day bed rest) of the bed rest, insulin sensitivity and autonomic nervous system regulation were assessed. An oral glucose tolerance test was used to measure insulin sensitivity.

Finally, in 2006-2007 and 2008 plasma insulin and glucose, plasma lipid pattern and cholesteryl ester transfer protein mass as well as erythrocyte membrane fatty acid composition were also determined before and after 33 days of bed rest.

DIETARY INTAKE

Women International Space Simulation for Exploration (WISE)

Energy intake was tailored on each subject on the base of resting energy expenditure (REE), which was determined before bed rest by indirect calorimetry and body composition assessment and adjusted every 15 days. In all study phases (i.e., ambulatory, bed rest and recovery) energy requirements were calculated for each individual according to the FAO/WHO equations. Each subject received a specifically prepared diet containing 1.4 or 1.1 times her REE during the ambulatory and bed rest periods, respectively, to balance energy intake in accordance with the level of physical activity. Ten per cent of the total kilocalories was added to account for dietary-induced thermogenesis. All subjects received 1 g protein $\times$ kg^{-1} $\times$ d^{-1} . The fat content of the diet was planned to provide about 30% of energy and included both saturated and polyunsaturated fatty acids. The remaining energy was supplied as carbohydrates. Daily intake of water, sodium, calcium

and vitamin D was also defined and monitored during the two periods. No caffeine, methylxanthine, or alcohol were allowed. Every day three main meals (breakfast, lunch, dinner) and three snacks, exactly weighed for each participant, were prepared. Subjects were required to completely consume prepared meal. Dietary folate intake was kept constant at 400 μg × day^{-1}.

Valdoltra Bed Rest Study 2006

Diet composition reflected subjects previous dietary habits, as assessed through appropriate questionnaires by an expert dietician. During the ambulatory period, energy intake was calculated for each subjects multiplying individual resting energy expenditure, determined by using the FAO/WHO equations, for a factor of 1.4 (Muller et al., 2004). The diet contained almost 60% of energy as carbohydrate, 25% as fat, and 15% as protein. Daily, subjects received 3 main meals (breakfast, lunch and dinner) and 3 snacks. The same diet was observed also during the experimental bed rest, with identical energy intake, frequency and food macronutrient composition than during the ambulatory period. Subjects were allowed to spontaneously adapt to decreased energy requirements during bed rest; for such reason, participants were not required to consume all served meal and leftover food was monitored semi-quantitatively by a dietitian to assess the relative macronutrient intake.

Valdoltra Bed Rest Studies 2007 and 2008

During the ambulatory period, diet control was identical to that followed in the Valdoltra Bed Rest Study 2006. On the contrary, energy intake during the experimental bed rest period was adjusted to consider the decreased level of physical activity. For this reason, during the bed rest period, each subjects received a diet containing 1.2 times his resting energy expenditure. Macronutrient relative content as well as food frequency were the same planned in Valdoltra Bed Rest Study 2006. In Valdoltra Bed Rest Studies 2007 and 2008 subjects were required to completely consume prepared meals.

BODY COMPOSITION

In the WISE study body composition was assessed at the beginning, every 15 days as well as at the end of 60-day bed rest using DXA (Dual energy X-ray Absorptiometry) scans. During all remaining studies, body composition was assessed at the beginning and at the end of each experimental bed rest by multifrequency bioelectrical impedance (Human IM Plus; DS Dietosystem, Milan, Italy), in accordance with manufacturer's instructions.
During the WISE as well as Valdoltra 2007 and 2008 studies body composition was also monitored through all the experimental bed rest to monitor and eventually properly calibrate energy intake, in order to maintain subjects in a near neutral energy balance.

Vastus lateralis thickness and architecture – Valdoltra Bed Rest Studies

Ultrasound imaging was used at the beginning and at the end of the bed rest periods to measure the thickness of the *vastus lateralis* muscle (Valdoltra Bed Rest Studies 2006-2007-2008). Measurements were performed in supine position, employing a portable ultrasound device (MyLab25; ESAOTE, Genoa, Italy) fitted with a 10–15-MHz linear probe (Reeves, Maganaris, and Narici 2004). After the identification of the midsagittal axis, defined as the middle axis between proximal and medial muscle insertions on bone, sagittal ultrasound images were acquired at 50% of muscle length, measured along this axis. The ultrasound probe was placed in the midsagittal plane, orthogonal to the mediolateral axis and its positioning was marked on acetate paper using moles and small angiomas as reference points. 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. For technical reasons, during the Valdoltra Bed Rest Study 2007 muscle thickness was measured in 8 subjects over 10.
Vastus lateralis muscle architecture was assessed (Valdoltra Bed Rest Study 2007) with knee joint in anatomical position (passively fully extended) by a real-time B-mode ultrasonography (ATL-HDI 3000, Bothell) with a 40 mm, 7.5 MHz linear-array probe. Analogously to ultrasound imaging, measurements were performed at 50% of muscle length, in the midsagittal plane, positioning the probe in the midsagittal plane, orthogonal to the mediolateral axis and marking its position on acetate paper. The fascicular path was defined as the interspaces between echoes deriving from the perimysial tissue surrounding the fascicle. The pennation angle of *vastus lateralis* was assessed by Matlab (Matlab, The MathWorks Inc., S. Natik, MA, USA) and was calculated as the angle between the fascicle and the deep aponeurosis of the muscle. For technical problems, these analyses were performed on 9 subjects over 10 during the Valdoltra Bed Rest Study 2007.

All these measures were performed in triplicate and the value considered for the analysis was the average of the three repetitions.

METABOLIC TESTS

Homocysteine kinetics – WISE study

Homocysteine metabolism was assessed using the approach of stable isotopes infusion.
After 12 h overnight fast, a polyethylene catheter was inserted into an antecubital vein for isotope infusion. Arterialized venous blood was obtained by a second polyethylene catheter inserted in a wrist vein of the opposite hand, heated at +50°C. Basal blood draw was performed at baseline to measure natural enrichments in arterialized plasma of [ring-$^{2}H_5$]-phenylalanine, [ring-$^{2}H_4$]-tyrosine, [3,3-$^{2}H_2$]-tyrosine, [methyl-$^{2}H_3$,1-^{13}C]-methionine and [1-^{13}C]-methionine. Immediately after basal blood collection, 8-hours of primed continuous infusions of [ring-$^{2}H_5$]-phenylalanine (infusion rate 4.8 μmol×kg^{-1}×h^{-1}, priming dose 4.8 μmol×kg^{-1}), [3,3-$^{2}H_2$]-tyrosine (infusion rate 1.2 μmol×kg^{-1}×h^{-1}, priming dose 1.2 μmol×kg^{-1}), [methyl-$^{2}H_3$,1-^{13}C]-methionine (infusion rate 4.4 μmol×kg^{-1}×h^{-1}, priming dose 4.4 μmol×kg^{-1}) and a single bolus of [ring-$^{2}H_4$]-tyrosine (0.45 μmol×kg^{-1}) were initiated. To measure isotopic enrichments through the infusion period, blood was collected 280, 290, 300, 460, 470, 480 minutes after infusion protocol start. After collection, blood was immediately centrifuged; plasma was stored at -80 °C for analysis.

Glutathione kinetics in erythrocytes- Valdoltra 2006

Glutathione turnover was assessed using the approach of stable isotopes infusion.
In the morning, after 12 h overnight fast, a polyethylene catheter was inserted into an antecubital vein for isotope infusion; a second polyethylene catheter was inserted in a wrist vein of the opposite hand that was heated at 50°C to obtain arterialized venous blood.
At baseline, a blood draw was performed to determine natural enrichments of [3,3-$^{2}H_2$]cysteine and [$^{2}H_2$-cysteine]-glutathione. Immediately after blood collection, a primed continuous infusion of [3,3-$^{2}H_2$]cysteine (Cambridge Isotope Laboratories, Andover, MA) (priming dose: 150 μmol; infusion rate: 150 μmol×kg^{-1}×h^{-1}) was started and maintained for 5 hours. To measure changes in erythrocytes [$^{2}H_2$]-cysteine and [$^{2}H_2$-cysteine]-glutathione enrichments through the infusion period, blood was collected 180, 240 and 300 minutes after infusion protocol start. After collection, whole blood was centrifuged; erythrocytes were resuspended in an equal volume of milliQ water and stored for analysis.

Glutathione kinetics in erythrocytes and muscle: double tracer-single sample approach – Valdoltra 2007

Muscle and erythrocytes glutathione turnover were assessed through stable isotope tracer infusions and a single *vastus lateralis* muscle biopsy.

In the morning, after 12 h overnight fast, a polyethylene catheter was inserted into an antecubital vein for isotope infusion; a second polyethylene catheter was inserted in a wrist vein of the opposite hand that was heated at 50°C to obtain arterialized venous blood.

At baseline, blood was collected to assess background enrichments of [$^2H_2$]-glycine, [^{15}N]-glycine, [$^2H_2$]-glutathione and L-[^{15}N]-glutathione. Immediately after blood draw, a primed constant infusion of [$^2H_2$]-glycine (priming dose 26.5 μmol×kg^{-1}; infusion rate 26.5 μmol×kg^{-1}×h^{-1}) was initiated and maintained for 7 hours. After 4 hours from the beginning of [$^2H_2$]-glycine infusion, a primed constant infusion of [^{15}N]-glycine (priming dose 26.5 μmol×kg^{-1}; infusion rate 26.5 μmol×kg^{-1}×h^{-1}) was started and continued for 3 hours. To measure enrichment changes, blood draws were performed 180 and 420 minutes after the beginning of [$^2H_2$]-glycine infusion. After collection, whole blood was centrifuged; erythrocytes were resuspended in an equal volume of milliQ water and stored for analysis.

Muscle biopsy.

At the end of [$^2H_2$]-glycine and [^{15}N]-glycine infusions (7 hours after the beginning of the infusion protocol), a muscle biopsy (averaging in mass 120 mg) was taken under local anesthesia from the *vastus lateralis*, using a conchotome forceps according to standard techniques. Muscle fibres were immediately cleaned from visible fat or connective tissues and accurately dried to remove blood. A microdissection microscope was employed to verify procedure quality. Samples were immediately frozen in liquid nitrogen and stored at -80°C. The protocol infusion was stopped at the end of muscle biopsy. At the end of the experimental bed rest, muscle sampling was performed on 9 subjects over 10 for technical reasons.

Oral glucose tolerance test – Valdoltra Bed Rest Study 2008

Insulin sensitivity was assessed through the oral glucose tolerance test (OGTT) method.

The OGTT was performed in accordance with the standard American Diabetes Association (ADA) guidelines. In the morning, after 12 h overnight fast, a polyethylene catheter was inserted into an antecubital vein for blood draw. After baseline blood draws (performed 30, 15 minutes and immediately before the metabolic test start), each subject received 75 g of glucose in 300 mL water. Blood was collected after 30, 60, 90 and 120 minutes from glucose load, to measure changes in

plasma insulin and glucose. Whole blood was immediately centrifuged and plasma aliquots were stored at -80°C until analysis.

Autonomic regulation assessment - Valdoltra Bed Rest Study 2008

Autonomic function regulation was measured during the OGTT, to define the impact of glucose load as well as of inactivity-induced insulin resistance on autonomic cardiovascular regulation.
All experiments were carried out in a thermostated room, at constant temperature of 24°C.
Autonomic function was assess before glucose load as well as during OGTT (after 30, 60, 90 and 120 minutes from glucose load), immediately after blood draw. Before and after each autonomic function test, systolic and diastolic pressures were measured by sphygmomanometer. During each autonomic function test, the following signals were recorded: (a) continuous arterial pressure at the finger level by a Portapres device (Finapres Medical System), (b) RR interval from ECG (1 channel from Portapres), and (c) a respiratory signal from an inductance plethysmograph. Recordings were obtained during 7 minutes of quiet rest, after instrumentation and after 5 min of adaptation to a quiet condition.
In addition, after 14 days of bed rest, in order to define the impact of water load on autonomic response, an OGTT test was simulated (Blank test). After 12 h overnight fast, subjects received 300 ml of aspartame sweetened water, to simulate the taste of glucose load. Autonomic function was measured before as well as after 30, 60, 90 and 120 minutes of aspartame-sweetened water load. Blood draws were not performed during the Blank test.

ANALYTICAL PROCEDURES

Homocysteine kinetics – WISE study

Sulfur amino acids analysis.

Plasma samples (200 µL) were treated with 2-mercaptoethanol (3 µl), evaporated under N_2 flux and added of SSA (200 µl, 15%). After centrifugation, amino acids were purified in a cationic resin (AG50W-X8; Bio-Rad, Hercules, CA) using as NH_4OH (3M) eluent. Ammonia was eliminated under N_2 flux; samples were lyophilized. Sulfur amino acids were derivatized by the addition of 50 µl acetonitrile and 50 µl MTBSTFA and by heating at 90°C for 45 min.

After derivatization, samples were injected into a gas chromatography-mass spectrometer (GC-MS) (HP 5890, Agilent Technologies, Santa Clara , CA, USA).

To assess homocysteine, methionine and cysteine concentrations a known amount of [$^2H_8$]-homocysteine, [1-^{13}C, methyl-$^2H_3$]-methionine and [3,3-$^2H_2$]-cysteine (Cambridge Isotope Laboratories) were respectively added as internal standard to 200 µl of plasma before analysis.

Gas chromatographic measurements were performed in single ion monitoring mode, using the following mass-to-charge ratio ($m\times z^{-1}$): phenylalanine $m\times z^{-1}$ 336; [ring-$^2H_5$]-phenylalanine $m\times z^{-1}$ 341; tyrosine $m\times z^{-1}$ 466; [3,3-$^2H_2$]-tyrosine $m\times z^{-1}$ 468; [ring-$^2H_4$]-tyrosine $m\times z^{-1}$ 470; homocysteine $m\times z^{-1}$ 496; [^{13}C]-homocysteine $m\times z^{-1}$ 497; [$^2H_8$]-homocysteine $m\times z^{-1}$ 500; methionine $m\times z^{-1}$ 320; [1-^{13}C, methyl-$^2H_3$]-methionine $m\times z^{-1}$ 324; cysteine $m\times z^{-1}$ 406; [3,3-$^2H_2$]-cysteine $m\times z^{-1}$ 408.

Glutathione kinetics in erythrocytes

Glutathione and cysteine were assessed in erythrocytes using an adaptation of previously proposed protocol (Lyons et al. 2001). Briefly, 400 µL of erythrocyte suspension (i.e., erythrocytes resuspended in an equal volume of milliQ water) was placed into pre-chilled tubes with 1 mL ice-cold dithiothreitol (DTT; 20 mmol×L in 1M acetic acid). SSA (400µL, 30%) was added to precipitate proteins. After centrifugation, the supernatant was transferred to a column with 2 mL of a cation-exchange resin (AG50W-X8; Bio-Rad, Hercules, CA). After washing twice with Milli-Q water (5mL; Millipore, Bedford, MA), glutathione was eluted with NH_4OH (3 mol/L). Ammonia was eliminated under N_2 flux and samples were lyophilized. Samples were added of 500 µL of DTT solution (20 mM in 0.5 M acetic acid) and heated at 100 °C for 1 h to reduce glutathione in oxidized form. Samples were dried again under N_2 flux. Samples reacted with 300µL HCl/methanol solution (250 µL 36% HCl in 7.5 mL methanol) for 30 min at 80 °C, and dried in nitrogen flow at 65 °C. Afterward samples were derivatized with 50 µL MTBSTFA and 50 µL 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).

To assess erythrocytes glutathione concentration a known amount of [$^{13}C_2$-^{15}N-glycine]-glutathione (Cambridge Isotope Laboratories, Andover, MA) was added as internal standard to 400 µl of erythrocyte suspension before analysis.

Gas chromatographic measurements were performed in single ion monitoring mode, using the following mass-to-charge ratio ($m \times z^{-1}$):

1. *Valdoltra Bed Rest Study 2006*: cysteine $m \times z^{-1}$ 406; [$^2H_2$]cysteine $m \times z^{-1}$ 408; glutathione $m \times z^{-1}$ 363; [$^2H_2$-cysteine]-glutathione $m \times z^{-1}$ 365; [$^{13}C_2$-^{15}N-glycine]-glutathione $m \times z^{-1}$ 366.
2. *Valdoltra Bed Rest Study 2007*: glycine $m \times z^{-1}$ 218; [$^2H_2$]-glycine $m \times z^{-1}$ 220; glutathione $m \times z^{-1}$ 363; [$^2H_2$-glycine]-glutathione $m \times z^{-1}$ 365; [$^{13}C_2$-^{15}N-glycine]-glutathione $m \times z^{-1}$ 366.

Glutathione kinetics in muscle – Valdoltra Bed Rest Study 2007

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 for the assessment of cysteine and glutathione enrichments in erythrocytes.

Muscle biopsies were defrosted, weighted and homogenized in 500 µl SSA (6.5%). After centrifugation, 1 ml of ice-cold DTT (20 mM in 1 M acetic acid) and SSA (400 µl, 30%; to precipitate proteins) were added. After centrifugation, the supernatant was transferred to a column with 2 mL of a cation-exchange resin (AG50W-X8; Bio-Rad, Hercules, CA), washed twice with Milli-Q water (5mL; Millipore, Bedford, MA) and eluted with NH_4OH (3 M, 4 ml). Samples were lyophilized and added of 500 µL of DTT solution (20 mM in 0.5 M acetic acid) at 100 °C for 1 h. Samples were dried again under N_2 flux and reacted with 300µL HCl/methanol solution (250 µL 36% HCl in 7.5 mL methanol) for 30 min at 80 °C, and dried in nitrogen flow at 65 °C. Afterward samples were derivatized with 50 µL MTBSTFA and 50 µL 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).

Muscle glutathione concentrations were determined through a standard calibration curve. A known amount of [$^{13}C_2$-^{15}N-glycine]-glutathione (used as internal standard) was added to serial dilutions of unlabelled glutathione (Sigma-Aldrich, Inc, MO, US). Glutathione and [$^{13}C_2$-^{15}N-glycine]-glutathione isotopic enrichments were measured monitoring appropriate mass-to-charge ratios, following the analysis procedure described for muscle biopsies. Standard calibration curve was

assessed using ratios between enrichments. By interpolation muscle unlabelled glutathione concentrations could be determined.

Gas chromatographic measurements were performed in single ion monitoring mode, using the following mass-to-charge ratio ($m \times z^{-1}$): glycine $m \times z^{-1}$ 218; [^{15}N]-glycine $m \times z^{-1}$ 219; [$^{2}H_2$]-glycine $m \times z^{-1}$ 220; glutathione $m \times z^{-1}$ 363; [^{15}N-glycine]-glutathione $m \times z^{-1}$ 364; [$^{2}H_2$-glycine]-glutathione $m \times z^{-1}$ 365; [$^{13}C_2$-^{15}N-glycine]glutathione $m \times z^{-1}$ 366.

Due to study design, a background assessment of natural isotopic enrichments in muscle could not be performed. For this reason, basal isotopic enrichments in muscle biopsies were estimated in erythrocytes samples, collected in each subjects before protocol infusion start. As already demonstrated, in fact, background glycine isotopic enrichments in circulating erythrocytes were comparable to those observed in other tissues (Hibbert et al. 2001). Estimation was performed obtaining from erythrocytes chromatographs with glutathione peak areas comparable with those observed in muscle. Since repeated measurements were performed, mean values were used as representing natural isotopic enrichments in muscle.

Protein carbonylation - Valdoltra Bed Rest Study 2007

About 20 cryosections (12 μm) of each biopsy were solubilized at 4°C in 0.01% tetrafluoroacetic acid, added of protease inhibitors, 5 mM EDTA and 2% β-mercaptoethanol. The Oxyblot (Chemicon – Millipore; Billerica, MA 01821) was used to detect carbonyl groups formed in protein side chains as a consequence of oxidation. Proteins (6 μg) were derivatized with 2,4-dinitrophenylhydrazine for 15 min following manufacturer's instruction and separated by electrophoresis on 10% SDS polyacrylamide gel. On each gel one positive and one negative control standards were always loaded. 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 ratio is a direct index of myofibrillar protein oxidation level.

Membrane fatty acid composition – Valdoltra Bed Rest Studies 2006-2007-2008

Fatty acid membrane compositions of red blood cells were analyzed modifying a previously published method (Burdge, Jones, and Wootton 2002).

Erythrocytes (200 µL) were washed five times with decreasing concentrations (10 mmol/L, 2.5 mmol/L; 1.25 mmol/L; 0.625 mmol/L; 0.312 mmol/L) of phosphate buffered saline (PBS). Total lipid extraction was performed in 5 mL of a chloroform–methanol (2:1) solution, containing 50 mg/L of butylhydroxytoluene as antioxidant, and 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 µL), added of 1 mL of a methanol solution containing 2% of H_2SO_4, and heated at 50 °C for 2 h. A neutralizing solution (1.0 ml, 0.25 M $KHCO_3$ and 0.5 M K_2CO_3 in deionized H2O) and hexane (1 mL) were added. After centrifugation, the hexane layer, containing fatty acid methyl esters (FAMEs), was collected and organic solvents were removed by N_2 flux. After the addition of hexane (150 µL), samples were analyzed by gas-chromatography–flame ionization detection (GC-FID; GC 6850 Agilent Technologies, Santa Clara, CA, USA). Specific fatty acid standards were used to identify FAMEs by retention times in erythrocyte samples. A commercial mixture of purified fish oil fatty acids (Menhaden oil, Sigma–Aldrich, Inc, MO, US) was used to detect: oleic acid (18:1, n-9), elaidic acid (trans 18:1, n-9), eicosapentaenoic acid (20:5, n-3), docosapentaenoic acid (22:5, n-3) and docosahexaenoic acid (22:6, n-3). Retention times of myristic acid (14:00), palmitic acid (16:00), palmitoleic acid (16:1, n-7), stearic acid (18:00), linoleic acid (18:2, n-6), a-linolenic acid (18:3, n-3), eicosaenoic acid (20:1, n-9), eicosadienoic acid (20:2, n-6), dihomo-γ-linolenic acid (20:3, n-6) as well as arachidonic acid (20:4, n-6) were identified by commercial standards. Adrenic acid (22:4, n-6) and docosapentaenoic acid (22:5, n-6) were identified by commercial standards purchased from Nu-Check Prep, Inc, MN, US.

Organic solvents and buffering salts were purchased from Sigma–Aldrich, Inc, MO, US, if not differently specified.

GC-FID conditions. 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.

Chromatograms analysis. 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 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) using an auto-analyzer (Olympus AU400 System; Olympus, Tokyo, Japan).

Erythrocyte marker

Concentrations of reduced and oxidized forms of glutathione in erythocytes were assessed by a commercially available kit (GT40, Oxford Biomedical Research; Oxford, MI).

CALCULATIONS

Protein kinetics

Whole body protein kinetics was assessed determining plasma phenylalanine (Phe) turnover.

$$PheS = PheB - PheOx$$

where PheS is Phe employment for protein synthesis; PheB is Phe appearance from protein breakdown; PheOx is the rate of Phe hydroxylation to tyrosine (Tyr).

PheB can be calculated as:

$$PheB = IPhe_{D5} \times EPhe_{D5}$$

where IPheD5 is the infusion rate of [ring-$^{2}H_5$]-Phe and EPheD5 is the enrichment of [ring-$^{2}H_5$]-Phe

PheOx is the rate of Phe hydroxylation to Tyr and can be calculated as follows:

$$PheOx = \left(IRTyr_{D2} \times ETyr_{D4}\right) \times \left(ETyr_{D2} \times EPhe_{D5}\right)$$

where IRTyrD2 is the infusion rate of [3,3-$^{2}H_2$]-Tyr; ETyrD4 is the enrichment of [ring-$^{2}H_4$]-Tyr; ETyrD2 is the enrichment of [3,3-$^{2}H_2$]-Tyr and EPheD5 is the enrichment of [ring-$^{2}H_5$]-Phe.

Homocysteine kinetics

Homocysteine kinetics were assessed modifying a previous approach (Storch et al. 1988).
The principle is that the [methyl-$^{2}H_3$,1-^{13}C]-methionine, entering the methionine-homocysteine cycle, definitively loses its isotopic methyl during transmethylation reaction, whereas the isotopic carbon is maintained during remethylation reaction, leading to isotopic homocysteine production.

Thus, the turnover rate of [methyl-$^{2}H_3$,1-^{13}C]-methionine (Qm) is as follows:

$$Q_m = \frac{I_{met}}{E_{m+4}}$$

where Imet is [methyl-$^{2}H_3$,1-^{13}C]-methionine tracer infusion rate; E_{m+4} is [methyl-$^{2}H_3$,1-^{13}C]-methionine enrichment.

The turnover rate of carbon backbone of methionine ([1-^{13}C]-methionine) (Qm) is as follows:

$$Q_m = \frac{I_{met}}{(E_{m+4} + E_{m+1})}$$

where Em_{+1} is [1-^{13}C]-methionine enrichments. E_{m+4} is considered as this isotope includes an m+1 mass.

In steady state, methionine input and out are equal. Thus:

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

and

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

where N is methionine derived from diet, B from protein breakdown, RM from remethylation, TM from transmethylation and TS from transulfuration.
S is defined as:

$$S = PheS \times 0.63$$

where PheS is phenylalanine utilization for protein synthesis (see above) and 0.63 is the molar ratio between methionine and phenylalanine.

Qc is not influenced by RM since the isotopic carbon is maintained along with homocysteine metabolism.

In fasting state, diet contribution to methionine pool is absent (N=0), thus B can be calculated as:

$$B = Q_c$$

Thus, remethylation rate is defined as:

$$RM = Q_m - Q_c$$

Methionine transmethylation is calculated as:

$$TM = RM + TS$$

TS can be calculated as:

$$TS = Q_c - S$$

Thus, TM can be calculated as:

$$TM = Q_m - S \quad \text{or} \quad TM = RM + TS$$

Homocysteine concentrations were calculated using the internal standard approach; thus:

$$[Homocysteine] = \frac{[I.S.]}{EHomocysteine_{D4} \times Vol}$$

where [I.S.] is the concentration of the added internal standard (m+8); EHomocysteineD4 is the enrichment of homocysteineD4 and Vol is the volume of analyzed plasma.

Glutathione kinetics in erythrocytes

Enrichments of labeled precursor (*Pre) were calculated as tracer-to-tracee ratios (TTR), following the general equation (Eq. 1):

$$E * Pre_{(t_i)} = TTR_{(t_i)} - TTR_{(t_0)}$$

where t_i indicates one of the sampling times after steady state achievement whereas t_0 is the time of sampling before isotope infusion beginning (for natural enrichments).

The glutathione fractional synthesis rate (FSR) was calculated as follows:

$$FSR_{(GSH)} = \frac{\frac{E_{*GSH}}{t}}{\frac{E_{*Pre_{(t_i)}}}{t}} \times 24 \times 100$$

where *GSH is isotopic GSH, obtained by the incorporation over time of *Pre; (E *GSH/t) is the slope of the regression line describing the rise in sample *GSH enrichment as a function of time (hours) after isotopic precursor steady state achievement; E *Pre (t_i) is the mean steady-state of *Pre enrichment in samples after steady state achievement.
FSR was expressed as % × day^{-1}.

Glutathione absolute synthesis rate (ASR) is calculated as follows (Eq. 2):

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

where [GSH] indicate glutathione concentration.
ASR was expressed as (µmol × L^{-1} × day^{-1}).

This approach permits to calculate glutathione turnover rate during the Valdoltra Bed Rest Study 2006 (in which [$^2H_2$]-cysteine was employed as glutathione isotopic precursor).

Glutathione kinetics in muscle

Enrichments of [^{15}N]-glycine ([^{15}N]-Gly) can be calculated using the general equation (Eq. 1) reported above.
Thus, in erythrocytes, [^{15}N]-glycine enrichments were defined as:

$$E\left(\left[^{15}N\right]Gly\right)_{(t_i)} = TTR\left(\left[^{15}N\right]Gly\right)_{(t_i)} - TTR\left(\left[^{15}N\right]Gly\right)_{(t_0)}$$

where TTR([^{15}N]-Gly) is peak areas ratio between areas measured for m×z^{-1} of 218 ([^{14}N]-Gly) and 219 ([^{15}N]-Gly).

To calculate [$^{2}H_2$]-glycine ([$^{2}H_2$]-Gly) enrichments in erythrocytes, the influence of [^{15}N]-glycine infusion has to be considered, as follows:

$$E\left(\left[^{2}H_2\right]Gly\right)_{(t_i)} = TTR\left(\left[^{2}H_2\right]Gly\right)_{(t_i)} - TTR\left(\left[^{2}H_2\right]Gly\right)_{(t_0)} - \left(E\left(\left[^{15}N\right]Gly\right)_{(t_i)} \times TTR\left(\left[^{15}N\right]Gly\right)_{(t_0)}\right)$$

The calculation of [^{15}N]-glycine enrichments in muscle biopsies (E [^{15}N]-Gly $_{muscle}$) was performed using the following equation:

$$E\left(\left[^{15}N\right]Gly\right)_{muscle} = TTR\left(\left[^{15}N\right]Gly\right)_{muscle} - TTR\left(\left[^{15}N\right]Gly\right)_{(t_0)}$$

where TTR([^{15}N]-Gly)(t_0) refers to erythrocytes natural enrichments, assessed in the blood draw performed before isotope infusion start.

In muscle biopsies [$^{2}H_2$]-glycine enrichments (E [$^{2}H_2$]-Gly $_{muscle}$) were calculated, similarly to E[$^{2}H_2$]-Gly in red blood cells, as follows:

$$E\left(\left[^{2}H_2\right]Gly\right)_{muscle} = TTR\left(\left[^{2}H_2\right]Gly\right)_{muscle} - TTR\left(\left[^{2}H_2\right]Gly\right)_{(t_0)} - \left(E\left(\left[^{15}N\right]Gly\right)_{muscle} \times TTR\left(\left[^{15}N\right]Gly\right)_{(t_0)}\right)$$

where TTR([$^{2}H_2$]-Gly)(t_0) and TTR([^{15}N]-Gly)(t_0) refer to erythrocytes natural enrichments, assessed in the blood draw performed before isotope infusion start.

[^{15}N]-glutathione ([^{15}N]-GSH) enrichments were calculated as follows:

$$E\left(\left[^{15}N\right]GSH\right)_{(t_i)} = TTR\left(\left[^{15}N\right]GSH\right)_{(t_i)} - TTR\left(\left[^{15}N\right]GSH\right)_{(t_0)}$$

Whereas, to calculate [$^2H_2$]-glutathione ([$^2H_2$]-GSH) enrichments, the influence of [^{15}N]-glycine infusion has to be considered, as follows:

$$E\left(\left[^2H_2\right]GSH\right)_{(t_i)} = TTR\left(\left[^2H_2\right]GSH\right)_{(t_i)} - TTR\left(\left[^2H_2\right]GSH\right)_{(t_0)} - \left(E\left(\left[^{15}N\right]GSH\right)_{(t_i)} \times TTR\left(\left[^{15}N\right]GSH\right)_{(t_0)}\right)$$

To calculate glutathione FSR through enrichment data obtained in a single tissue sample, we developed the following equation (Eq. 2):

$$FSR_{(\text{one sample-double tracers})} = \frac{\dfrac{E\left[^2H_2\right]GSH_{(t_7)}}{E\left[^2H_2\right]Gly_{(t_7)}} - \dfrac{E\left[^{15}N\right]GSH_{(t_7)}}{E\left[^{15}N\right]Gly_{(t_7)}}}{\left[^2H_2\right]GlyInf - \left[^{15}N\right]GlyInf} \times 24 \times 100$$

where (t_7) indicates that enrichments are determined at the seventh hour of the metabolic study; [$^2H_2$]-Gly Inf and [^{15}N]-Gly Inf indicate the duration of infusion of [$^2H_2$]-glycine and [^{15}N]-glycine, respectively.

This equation is based on two parallel and separate infusions of two isotopomers of the same precursor, glycine, started at the beginning and four hours later the metabolic study start (t_0). Precursor enrichments at steady-state ([$^2H_2$]-glycine and [^{15}N]-glycine) and product enrichments after 3 ([^{15}N]-glutathione) and 7 hours ([$^2H_2$]-glutathione) of infusion were measured in a single biological sample taken at the end of the metabolic study period (7 hours). So, product enrichment changes over time, necessary for FSR assessment, can be evaluated as difference between two single differently labeled product enrichments measured within only one final biological sample. Thus, [^{15}N]-glutathione enrichment reflects short term tracer incorporation, since [^{15}N]-glycine infusion started three hours before the single final muscle biopsy, whereas [$^2H_2$]-glutathione enrichment reflects long term tracer incorporation, since [$^2H_2$]-glycine infusion started at the beginning of the metabolic test and finished after seven hours, when final biopsy was collected. Moreover, each product enrichment has been normalized by precursor isotopic tracer enrichment at steady state.

This new equation was validated using the traditional approach, that has been applied to calculate glutathione FSR in erythrocytes within the same experimental condition.

Glutathione FSR in erythrocyte was calculated considering isotopic enrichments of [$^2H_2$]-glutathione measured after achievement of the steady state condition for [$^2H_2$]-glycine precursor enrichment, as follows (Eq.3):

$$FSR_{(\text{multiple samples-single tracer})} = \frac{slope E\left[{}^2H_2\right] GSH_{(t_3 \rightarrow t_7)}}{steady state E\left[{}^2H_2\right] Gly_{(t_3 \rightarrow t_7)}} \times 24 \times 100$$

where slope E[$^2H_2$]-GSH($t_3 \rightarrow_7$) is the slope of [$^2H_2$]-glutathione product enrichments measured at t_3 and t_7; steady state E[$^2H_2$]-Gly($t_3 \rightarrow_7$) is the enrichment of [$^2H_2$]-glycine precursor at steady state.

In erythrocytes as well as in muscle biopsies, ASR was calculated as abovementioned (Eq.2).

Membrane fatty acid composition

Erythrocytes membrane level of measured fatty acids 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) and 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. n-3 polyunsaturated sum was calculated as the sum of a-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. Δ-9 desaturase index was calculated as ratio between oleic (18:1, n-9) and stearic (18:0) acid contents whereas Δ-5 desaturase index was calculated as ratio between arachidonic acid (20:4, n-6) and dihomo-γ-linolenic acid (20:3, n-6) membrane levels. 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.

Insulin sensitivity

Homeostasis assessment model of insulin resistance

In Valdoltra Bed Rest Studies 2006, 2007 and 2008, in the fasting state, insulin sensitivity was determined in according to the homeostasis assessment model of insulin resistance (HOMA), as follows:

$$HOMA = \frac{FPI \times FPG}{22.5}$$

where FPI is fasting plasma insulin concentration (mU × L^{-1}); FPG is fasting plasma glucose level (mmol × L^{-1}).

OGTT-related assessment of insulin sensitivity

During OGTT, insulin sensitivity was measured by the composite insulin sensitivity index (ISI) as follows:

$$ISI = \frac{10000}{\sqrt{FPI \times FPG \times G \times I}}$$

where FPI is fasting plasma insulin concentration (mU × L^{-1}); FPG is fasting plasma glucose (mmol × L^{-1}) level; G is the mean plasma glucose concentration during OGTT; I is the mean plasma insulin concentration during OGTT.

In addition, insulin resistance was defined, during OGTT, by the insulin area-under-the curve index (InsAUC).

Autonomic regulation assessment

To assess autonomic function, a software (Heartscope, ver.1.6, A.M.P.S. llc, New York, USA) was used to identify the peak of R wave on ECG and systolic arterial pressure (SAP). The software constructs automatically time series of RR intervals and SAP, with low operator–analysis interaction. Spontaneous variability of RR interval and SAP was evaluated by means of power spectral analysis using an autoregressive algorithm on all recorded parameters, as previously described (Pagani et al. 1986). Briefly, from beat-to-beat variability series of adequate length and stationarity (usually 250– 350 beats), the software calculated simple statistics and the best autoregressive estimate of the power spectral density. The total power of RR and SAP variabilities,

corresponding to variance, was initially obtained. Subsequently, powers and frequencies of the low (0.03–0.14 Hz) and the high (0.15–0.5 Hz) frequency spectral components (low frequency – LF – and high frequency – HF, respectively), expressed in absolute and normalized units (nu), were computed as the ratio of the absolute power of either HF or LF to the total power, subtracting the very-low-frequency component, and multiplying this ratio by 100.

Baroreflex sensitivity was dynamically assessed by the sequence technique (Di Rienzo, Mancia, and Pedotti 1985; Bertinieri et al. 1988). Briefly, this procedure is based on automatic scanning of SAP and RR interval series, searching for sequences of three or more consecutive beats in which SAP and RR interval changed in the same direction, either increasing or decreasing. For each sequence, the regression line is computed between SAP and RR interval values, and the mean slope of this relationship, obtained by averaging all slopes computed within a given test period, reflects the average spontaneous baroreflex sensitivity for the tested period.

The α index is defined as the average of the square root of the ratio between cardiac period and systolic blood pressure spectral powers, both in its LF and HF components (Pagani et al. 1988).

Plasma markers

LDL cholesterol was calculated by using the Friedewald equation:

$$LDLcholesterol = total.cholesterol - HDLcholesterol - \frac{triglycerides}{5}$$

STATISICAL ANALYSIS

Data are presented as mean±SEM. Data were log-transformed when appropriate. Wilcoxon test was applied to determine significant changes between the ambulatory and the experimental period. p-values lower than 0.05 were chosen as threshold for statistical significance.

Results obtained during the Valdoltra Bed Rest Studies, in ambulatory or bed rest conditions, were analyzed using an repeated-measures ANOVA with activity (ambulatory or bed rest) as within-subject factor, experimental period (2006, 2007 and 2008) as between-subject factor and baseline ambulatory values as covariate. There was no significant experimental period × bed rest interaction for investigated variables. Thus, results obtained during the three experimental periods, in ambulatory or bed rest conditions, were pooled together and expressed as mean±SEM.

To validate the new double tracers-single sample method used to assess glutathione FSR in muscle (Valdoltra Bed Rest Study 2007), glutathione FSR absolute values or pooled changes from baseline to day 33 measured in erythrocytes by traditional (multiple samples-one tracer) approach were correlated to the same measurements performed in erythrocytes by the double tracers-single sample method. Regression line analysis of such correlations was performed by the Passing–Bablok test. The Altman–Bland plot was applied to validate the two methods. These analyses were performed by MedCalc (version 11.2.1.0; MedCalc Software; Mariakerke, Belgium).

To evaluate the effects of physical inactivity on insulin sensitivity, assessed during OGTT, and autonomic regulation before, after 7 days and at the end of the experimental bed rest, repeated measures ANOVA with interaction was used and the Bonferroni's post-hoc analysis was employed.

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), if not differently specified.

RESULTS

EFFECT OF BED REST ON BODY COMPOSITION

Bioimpedance analysis evidenced a significant decrease in free-fat mass (-4.3±0.4 kg; n=30; $p<0.01$) and a significant, but slightly (from 11.7±1.0 kg to 12.3±1.0 kg; n=30; $p=0.01$), increase in fat mass after 33 days of bed rest, in pooled subjects participating to the Valdoltra Bed Rest Studies 2006, 2007 and 2008.

DXA analysis, performed in women involved in the WISE Study, demonstrated that lean mass was significantly decreased after 60 days of bed rest (-7.6±0.3%; n=8; $p<0.01$), whereas no significant changes in fat mass occurred (from 14.6±1.4 kg to 14.3±1.2 kg; n=8; $p>0.05$) (Figure 10). Lean mass significantly decreased during the first 31 days of bed rest, whereas lean mass did not change significantly during the following 29 days.

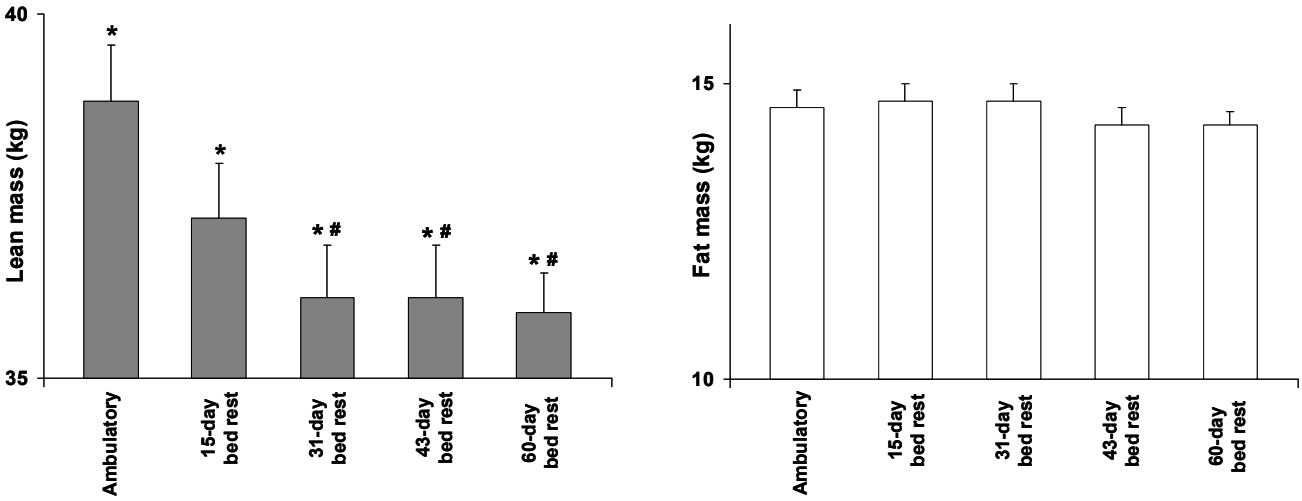

Figure 10. Absolute values of lean and fat mass measured before (Ambulatory), every 15 days (15-day bed rest; 31-day bed rest; 43-day bed rest) and at the end (60-day bed rest) of the WISE bed rest study are reported. Body composition was assessed by DXA. *, $p<0.01$ vs Ambulatory; #, $p<0.05$ vs 15-day bed rest. Statistical analysis was performed by repeated measures ANOVA.

EFFECT OF BED REST ON *VASTUS LATERALIS* THICKNESS AND ARCHITECTURE

Valdoltra Bed Rest Studies 2006, 2007 and 2008.

Vastus lateralis thickness, as assessed by ultrasound imaging, significantly decreased in the three bed rest studies, from 2.27±0.08 cm to 1.99±0.08 cm ($p<0.05$; paired t test). *Vastus lateralis* thickness decreased by 19.1±7.6% ($p<0.02$; paired t test) in the Valdoltra Study 2006, by 23.8±6.6% ($p<0.01$; paired t test) in the Valdoltra Study 2007. In the Valdoltra Study 2008, thickness of *vastus lateralis* was reduced from 2.31±0.16 cm to 1.93±0.13 cm ($p<0.01$; paired t test) after 33 days of bed rest.

Fiber pennation angle, measured in Valdoltra 2007 by ultrasonography, was significantly reduced after 33 days of bed rest, from 18.6±1.2 to 15.3±1.0 degrees ($p<0.05$; Student's *t* test).

EFFECT OF BED REST ON OXIDATIVE STRESS IN ERYTHROCYTES

Valdoltra Bed Rest Study 2006.

In the Valdoltra Bed Rest Study 2006, glutathione FSR increased after 33 days of bed rest, from 73.3±14.8 %×day^{-1} to 124.5±16.4 %×day^{-1} ($p<0.05$). Glutathione concentration tended to increase (from 2156.0±106.4 μmol×L^{-1} to 2323.9±85.2 μmol×L^{-1}) even though the statistical significance was not achieved. Glutathione ASR was increased from 157.5±33.3 mmol×L^{-1}day^{-1} to 289.2±37.8 mmol×L^{-1}day^{-1} ($p=0.01$). Nevertheless, when we excluded by the analysis subjects that failed to adapt to inactivity-induced changes in energy requirement and that were in positive energy balance, glutathione concentration as well as glutathione FSR were unchanges following bed rest (Biolo et al. 2008).

EFFECTS OF BED REST ON OXIDATIVE STRESS IN MUSCLE.

Valdoltra Bed Rest Study 2007.

Double tracer-single sample method validation: glutathione synthesis in erythrocytes

[$^{2}H_{2}$]-glycine enrichment in erythrocytes reached the steady state after 3 hours from the infusion protocol start. [$^{2}H_{2}$]-glycine enrichment steady state was maintained up to the end of the protocol infusion (Figure 11). The achievement of [^{15}N]-glycine enrichment steady state can only be assumed at the end of the infusion protocol (i.e., after 3 hours of [^{15}N]-glycine infusion start). [$^{2}H_{2}$]-glycine and [^{15}N]-glycine steady state values were different due to intrinsic metabolic differences between precursors. Differences in product ([$^{2}H_{2}$]-glutathione and [^{15}N]-glutathione) enrichments at the end of the metabolic test reflected the different tracer infusion and then incorporation times ([$^{2}H_{2}$]-glycine primed continuous infusion started at time 0 and was maintained for 7 hours, whereas [^{15}N]-glycine primed continuous infusion was started 4 hours later and maintained for 3 hours).

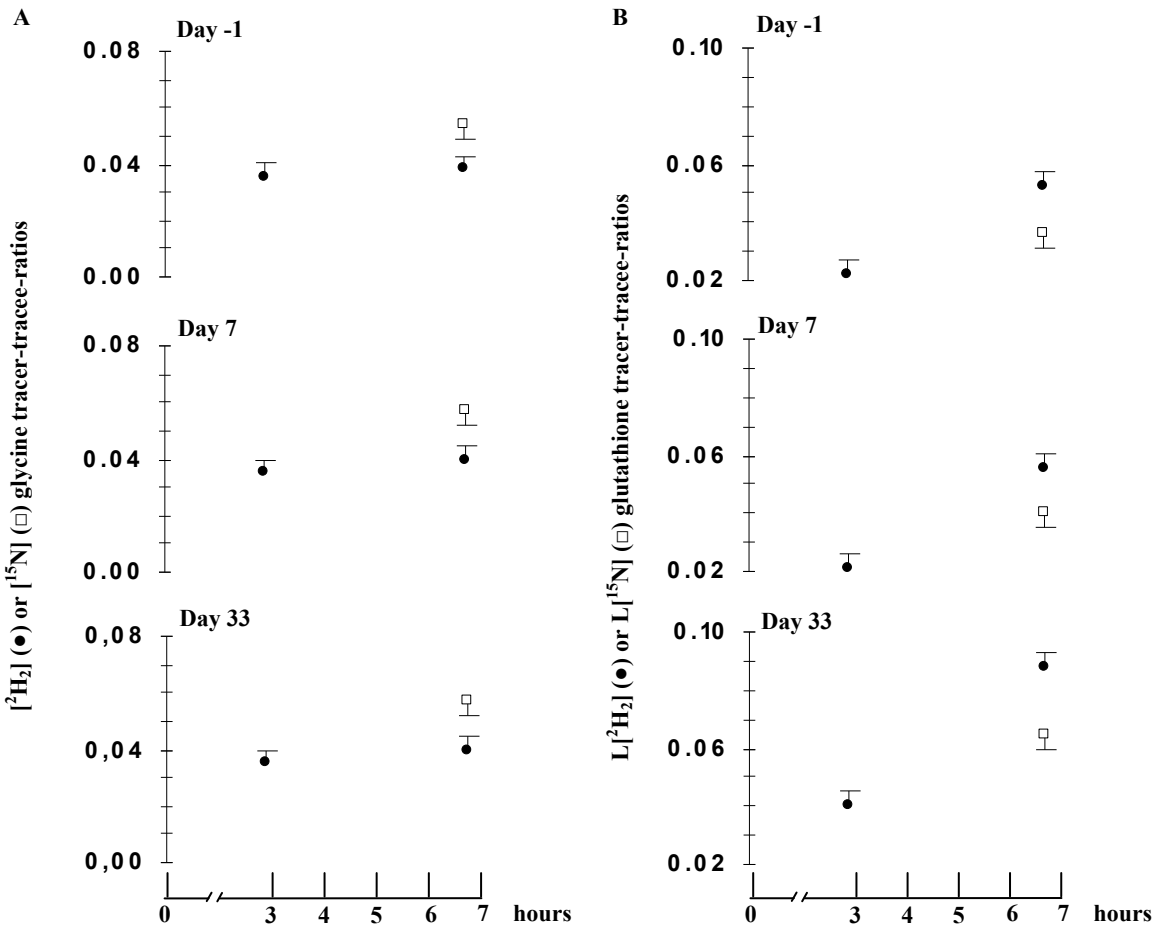

Figure 11. Enrichments of [$^2H_2$]-glycine and [^{15}N]-glycine as well as of [$^2H_2$]-glutathione and [^{15}N]-glutathione, during the 7-hour primed continuous isotope infusion. (**A**) Steady state for [$^2H_2$]-glycine (●) and [^{15}N]-glycine (□) precursors. Steady state for [$^2H_2$]-glycine enrichment was assessed through the infusion protocol and determined using a mass-spectrometry gas-chromatographer. Steady state for [^{15}N]-glycine (□) pool was assumed. (**B**) [$^2H_2$]-glutathione (●) and [^{15}N]-glutathione (□) enrichment slopes, reflecting linear tracer incorporation ([$^2H_2$]-glycine and [^{15}N]-glycine, respectively) into glutathione products.

[$^2H_2$]-glycine steady state mean value was significantly increased after 33 days of bed rest ($p<0.05$). [^{15}N]-glycine enrichment mean values measured at the end of the infusion were greater ($p<0.05$) than the corresponding [$^2H_2$]-glycine enrichment values.

The validation of the new double tracer-single sample approach versus the traditional single tracer-multiple sample method was performed in blood samples collected during the metabolic tests,

performed before, after 7 days and at the end of the experimental bed rest. Glutathione FSR was estimated using the equations Eq. 2 and Eq. 3, as reported in the section "Method". Glutathione FSR values assessed by the two equations were not statistical different. Pooled absolute values of glutathione FSR measured in the three study phase by the double tracer-single sample approach highly correlated (r=0.84; n=28; p<0.001) with those assessed by the traditional single tracer-multiple sample method (Figure 12). Moreover, pooled changes in glutathione FSR from baseline to day 7 as well as from day 7 to day 33 of bed rest measured by both approaches were highly correlated (r=0.91, n=18; p<0.001) (Figure 12).
Glutathione FSR in erythrocytes was unchanged after 7 and 33 days of bed rest.

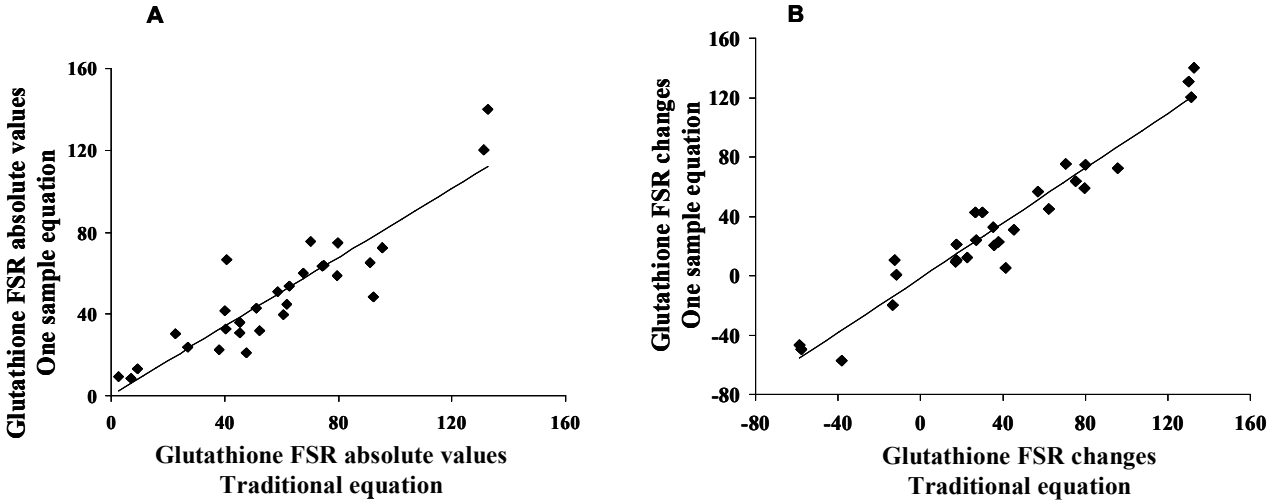

Figure 12. Linear correlations between glutathione fractional synthesis rate (FSR) measurements in erythrocytes by the traditional (single tracer-multiple sample) and by double tracer-single sample approaches. (**A**) Glutathione FSR absolute values (r=0.84; n=28; p<0.001). (**B**) Pooled changes of glutathione FSR from baseline to day 7 and from day 7 to day 33 (r=0.91; n=18; p<0.001).

Analysis of linear regression correlations between the double tracer-single sample approach and the traditional approach was performed by Passing–Bablok method (Figure 13). Glutathione FSR values measured in erythrocytes by the two methods were in positive linear correlation (R=0.90; n=29; p < 0.001) and line slope and intercept were contained in their relative confidence intervals (Table 1). A positive linear correlation (R=0.91; n=19; p < 0.001) was also evidence when pooled changes of glutathione FSR from baseline to day 7 and to day 33, assessed by the two methods,

were considered (Figure 13). Also in this case, line slope and intercept were contained in their relative confidence intervals (Table 1).

The Altman–Bland method was finally applied to validate the double tracer-single sample approach against the traditional one for glutathione kinetics assessment (Figure 13). Altman–Bland test evidenced data distribution across the mean for both glutathione FSR absolute values and glutathione FSR changes. Moreover, only two measurements for glutathione FSR absolute values and one measurement for glutathione FSR changes were outside the confidence interval.

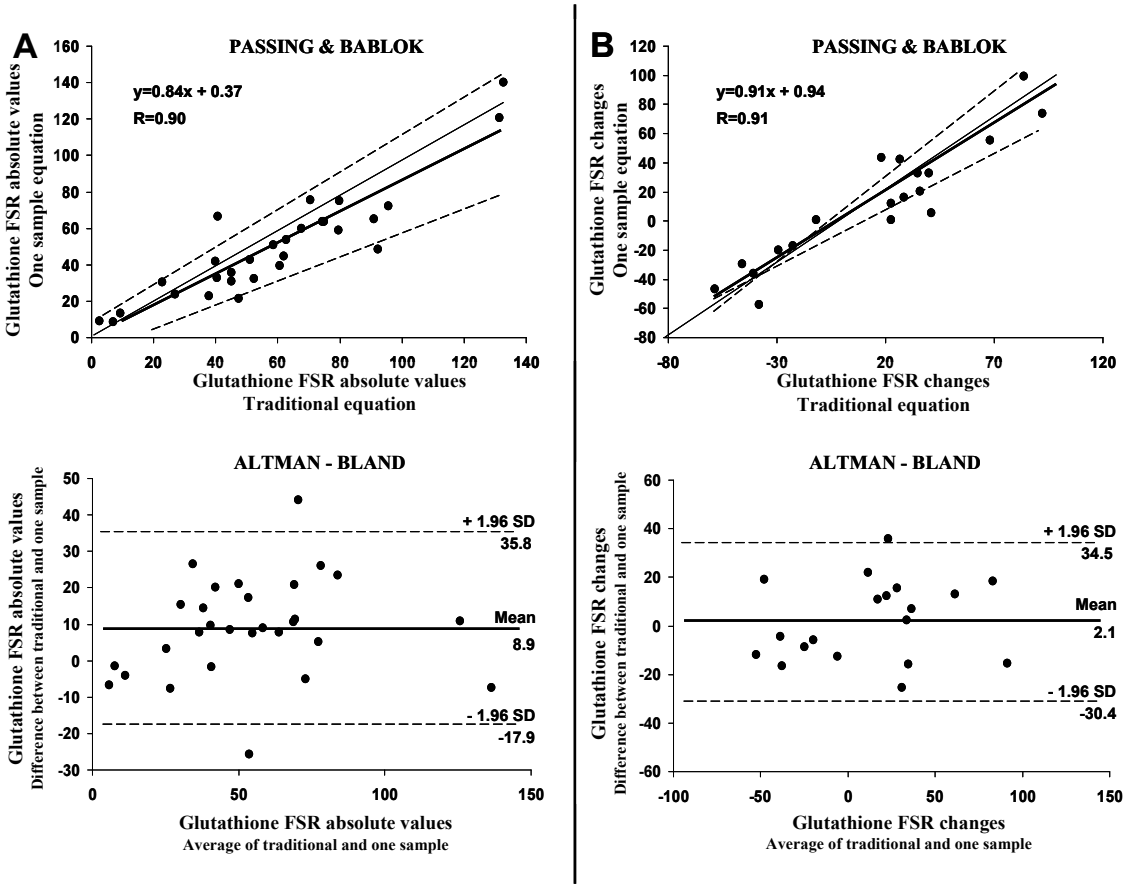

Figure 13. Validation of the double tracer-single sample method versus the traditional approach by analysis of linear regression of correlations by Passing–Bablok approach and by Altman–Bland test, applied to measurements of glutathione fractional synthesis rate in erythrocytes, expressed as absolute values and as changes from baseline to day 7 as well as from day 7 to day 33 of bed rest. In Passing–Bablok plots regression lines were drawn as continuous thick lines, identity lines (x=y) as continuous thin lines, limits of confidence intervals are dashed lines. In Altman–Bland plots the mean value line was represented by a continuous thick line and limits of confidence intervals were presented by dashed lines.

Table 1. Passing–Bablok regression analysis applied to compare the traditional and the double tracer-single sample approaches for glutathione FSR assessment in erythrocytes.

REGRESSION ANALYSIS 1		
VARIABLE X **Absolute glutathione FSR values** **(traditional equation)**		**VARIABLE Y** **Absolute glutathione FSR values** **(double tracer-single sample equation)**
	VALUE	95 % C.I.
Intercept A	−0.02	from −11.32 to 7.01
Slope B	0.86	from 0.68 to 1.04
Cusum test for linearity	No significant deviation from linearity (P > 0.05)	
REGRESSION ANALYSIS 2		
VARIABLE X **Glutathione FSR changes** **(traditional equation)**		**VARIABLE Y** **Absolute glutathione FSR values** **(double tracer-single sample equation)**
	VALUE	95 % C.I.
Intercept A	0.95	from -9.10 to 1.47
Slope B	0.91	from 0.75 to 1.20
Cusum test for linearity	No significant deviation from linearity (P > 0.05)	

Regression analysis 1 was performed to compare absolute glutathione FSR values measured by the traditional approach (X variable) with the same values measured by the double tracer-single sample (Y variable) approach. Regression analysis 2 was performed to compare changes from baseline to day 7 and to day 33 of glutathione FSR measured by the traditional equation (Variable X), with the same values measured by the double tracer-single sample equation (Variable Y).

Bed rest effect on muscle glutathione kinetics.

Values of [^{15}N] and [$^{2}H_2$] enrichments in glycine and glutathione pools in muscle, as assessed at the beginning as well as at the end of the experimental bed rest, were comparable (statistical significance, determined by Student's *t* test, was not achieved). Similarly to data obtained in erythrocytes, muscle [^{15}N]-glycine enrichments were significantly ($p<0.05$) higher than [$^{2}H_2$]-glycine (Table 2). Muscle glutathione FSR tended to be increased during bed rest, even though statistical significance was not achieved ($p=0.07$). Muscle glutathione concentrations were unaffected by experimental physical inactivity. Nonetheless, glutathione ASR was significantly enhanced after 33 days of bed rest, compared to basal condition (Table 2).

Table 2. Effects of bed rest on muscle *vastus lateralis* [$^2H_2$] and [^{15}N] precursors and products enrichments as well as in glutathione kinetics.

	AMBULATORY	33-day BED REST	p
MUSCLE ENRICHMENTS			
[^{15}N]-glycine	0.057±0.004	0.058±0.006	ns
[^{15}N]-glutathione	0.014±0.003	0.010±0.004	ns
[$^2H_2$]-glycine	0.042±0.003	0.041±0.003	ns
[$^2H_2$]-glutathione	0.027±0.002	0.032±0.005	ns
GLUTATHIONE KINETICS			
FSR (%×day^{-1})	268±61	408±47	ns
Concentration (mmol×kg wet tissue^{-1})	2.3±0.2	2.7±0.1	ns
ASR (mmol×kg wet tissue^{-1}×day^{-1})	5.5±1.1	11.0±1.5	0.02

Data are presented as mean±SEM. Statistical analysis was performed by Student's *t* test. Muscle isotopic enrichments on [$^2H_2$] and [^{15}N] precursors ([$^2H_2$]- and [^{15}N]-glycine) and products ([$^2H_2$]- and [^{15}N]-glutathione) were assessed by GC-MS analysis, on *vastus lateralis* biopsies performed on 9 subjects at the end of the infusion protocol at baseline (Ambulatory) and at the end (33-day bed rest) of experimental bed rest. Glutathione fractional synthesis rate (FSR), concentration and absolute synthesis rate (ASR) were measured in the same *vastus lateralis* biopsy. Glutathione FSR was calculated by the double tracer-single sample approach; glutathione concentrations were measured by the internal standard technique; ASR was defined as the product between glutathione FSR and concentration.

Bed rest effects on muscle atrophy and oxidative stress.

Experimental bed rest significantly impaired *vastus lateralis* thickness and fiber pennation angle (Figure 14). Inactivity-induced changes in *vastus lateralis* thickness were directly related to inactivity-related changes in fat-free mass ($R=0.63$; $n=10$; $p<0.05$). On the contrary, bed rest-induced changes in glutathione FSR inversely correlated to decreases in fiber pennation angle ($R=-0.67$; $n=9$; $p<0.05$).

Vastus lateralis protein carbonylation levels, assessed by Oxyblot analysis, were significantly increased after 33 days of bed rest (Dalla Libera et al. 2009) (Figure 14) and were inversely

correlated (R=-0.74; n=8; p<0.05) to changes in muscle thickness, observed in the same experimental period.

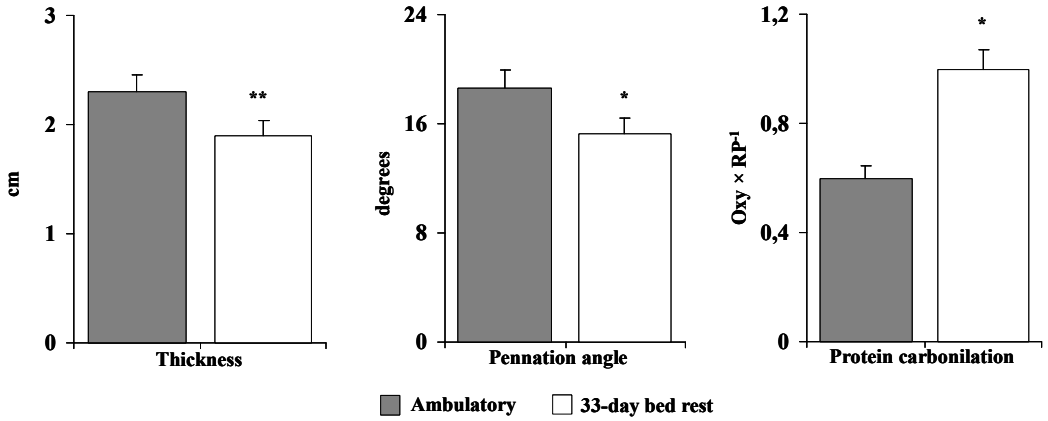

Figure 14. Values of *vastus lateralis* thickness, fiber pennation angle and protein carbonylation measured at the beginning (Ambulatory) and at the end (33-day bed rest) of the experimental bed rest period. Muscle thickness and fiber pennation angle were determined by ultrasonography. Protein carbonylation was measured by Oxyblot analysis. Oxy×RP^{-1}, ratio between quantified oxidized proteins and Red Ponceau stained total protein. **, p<0.001 vs Ambulatory; *, p<0.05 vs Ambulatory. Statistical analysis were performed by Student's *t* test.

EFFECT OF BED REST ON SYSTEMIC INFLAMMATION

Effect of bed rest on erythrocyte membrane composition.

Valdoltra Bed Rest Studies 2006, 2007 and 2008.

The impact of experimental bed rest on systemic inflammatory condition was investigated through the assessment of fatty acid composition in erythrocyte membranes.

Total membrane content of saturated fatty acids did not significantly change after bed rest, whereas the total monounsaturated fatty acids content was significantly reduced. Particularly, oleic and eicosaenoic acid levels were significantly reduced after 33-day bed rest. Bed rest did not influence n-3 polyunsaturated fatty acid total content, even though inactivity displayed different effects on selected n-3 fatty acids, leading to a statistical significant decrease in α-linolenic and eicosapentaenoic acid levels and to a significant increase in docosahexaenoic acid content. On the contrary, all detected n-6 polyunsaturated fatty acids content, with the exception of linoleic and eicosadienoic acid, and, consequently, the total content of n-6 polyunsaturated fatty acids were significantly enhanced during 5 weeks of bed rest. Interestingly, linoleic acid levels were significantly decreased at the end of the experimental period (Table 3). The Δ-5 and Δ-9 desaturase activities, as estimated from product-to-precursor ratio, were significantly reduced following 33 days of bed rest (Figure 15) whereas the arachidonic-to-eicosapentaenoic acid ratio was significantly increased after unloading (Figure 16).

Table 3. Effects of 5 weeks of bed rest on major fatty acids (%) in erythrocyte membranes.

	AMBULATORY	BED REST	p
SATURATED FATTY ACIDS			
Myristic 14:0	0.33 ± 0.02	0.33 ± 0.02	0.75
Palmitic 16:0	22.32 ± 0.62	21.68 ± 0.67	0.06
Stearic 18:0	19.46 ± 0.31	19.19 ± 0.33	0.14
SUM	42.31 ± 0.92	41.48 ± 1.02	0.10
MONOUNSATURATED FATTY ACIDS			
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 20:1n-9	0.24 ± 0.01	0.23 ± 0.01	0.04
SUM	15.57 ± 0.40	14.87 ± 0.34	0.003
n-3 POLYUNSATURATED FATTY ACIDS			
α-Linolenic acid 18:3 n-3	0.31 ± 0.04	0.29 ± 0.05	0.05
Eicosapentaenoic acid 20:5n-3	0.39 ± 0.02	0.35 ± 0.01	0.05
Docosapentaenoic acid 22:5n-3	2.35 ± 0.09	2.49 ± 0.05	0.13
Docosahexaenoic acid 22:6n-3	3.91 ± 0.20	4.15 ± 0.15	0.01
SUM	7.12 ± 0.19	7.34 ± 0.17	0.06
n-6 POLYUNSATURATED FATTY ACIDS			
Linoleic acid 18:2 n-6	12.10 ± 0.24	11.60 ± 0.30	0.02
Eicosadienoic acid 20:2n-6	0.39 ± 0.03	0.38 ± 0.02	0.36
Dihomo-γ-linolenic 20:3n-6	1.72 ± 0.07	2.08 ± 0.08	<0.001
Arachidonic acid 20:4n-6	16.20 ± 0.63	17.18 ± 0.59	0.01
Adrenic 22:4n-6	4.20 ± 0.19	4.47 ± 0.19	0.01
Docosapentaenoic 22:5n-6	0.80 ± 0.04	0.93 ± 0.09	0.003
SUM	35.45 ± 0.90	37.16 ± 0.42	0.005

Data are expressed as mean±SEM. n=30. Erythrocyte membrane fatty acid composition was assessed by gas-chromatographic analysis. Red blood cell membrane level of each enlisted fatty acid was expressed as percent ratio between area-under-the-curve of each selected fatty acid peak and the sum of all measured fatty acid peaks. Statistical analysis was performed by Wilcoxon statistical test.

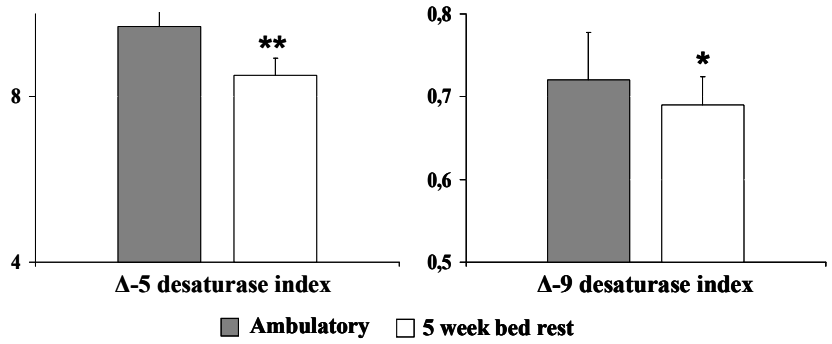

Figure 15. Estimated activities of Δ-5 and Δ-9 desaturases before and following 5 weeks of bed rest. Δ-5 desaturase activity was estimated as arachidonic acid-to- dihomo-γ-linolenic acid ratio.Δ-9 desaturase activity was estimated as oleic acid-to-stearic acid ratio. n=30. **p<0.001, *p<0.05. Statistical analysis was performed by Wilcoxon statistical test.

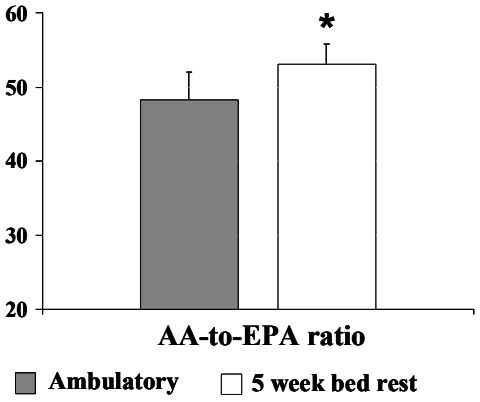

Figure 16. Arachidonic acid-to-eicosapentaenoic acid ratio before and at the end of 5 weeks of bed rest. n=28. *, p<0.01. Statistical analysis was performed by Wilcoxon statistical test.

EFFECT OF BED REST ON INSULIN SENSITIVITY

HOMA index of insulin resistance

Valdoltra Bed Rest Studies 2006, 2007 and 2008.

Bed rest induced a significant increase in plasma insulin concentration (from 6.5±0.6 $mU \times L^{-1}$ to 9.3±0.8 $mU \times L^{-1}$; n=30; $p<0.001$). On the contrary, plasma glucose concentrations were not significantly changed after 5 weeks of bed rest (from 4.90±0.06 $mmol \times L^{-1}$ to 4.88±0.05 $mmol \times L^{-1}$; n=30; p=0.8). Consequently, the HOMA index of insulin resistance significantly ($p<0.01$) increased from 1.4±0.1 at baseline to 2.0±0.2 after the experimental period.

Oral Glucose Tolerance Test

Valdoltra Bed Rest Study 2008.

Plasma insulin and glucose concentrations during OGTT at baseline as well as after 5 and 33 days of bed rest were reported in Figure 17.

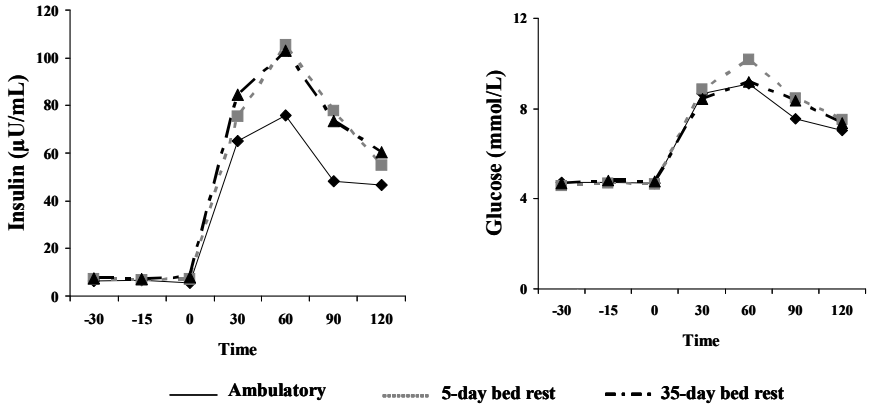

Figure 17. Plasma insulin and glucose concentrations during OGTT, at baseline (Ambulatory), after 5 days (5-day bed rest) and at the end (33-day bed rest) of the experimental period.

The composite ISI index of insulin sensitivity calculated during the OGTT significantly decreased after 5 days of bed rest (24±5 %; n=10; p=0.02) and after 33 days of bed rest (26±5 %; n=10; p=0.02; compared to ambulatory condition). Insulin sensitivity was similar following 5 and 33 days of bed rest and percent changes from the basal ambulatory condition were similar (p=ns; Wilcoxon test) at day 5 and at day 33 of bed rest (Figure 18).

The area under the curve (AUC) of plasma insulin concentrations following OGTT exhibited similar changes of the ISI index. Moreover, percent changes from the basal ambulatory condition

were not significantly different after 5 days (48±9%) and after 33 days (46±11%). On the contrary, the AUC of plasma glucose following OGTT did not change significantly during bed rest.

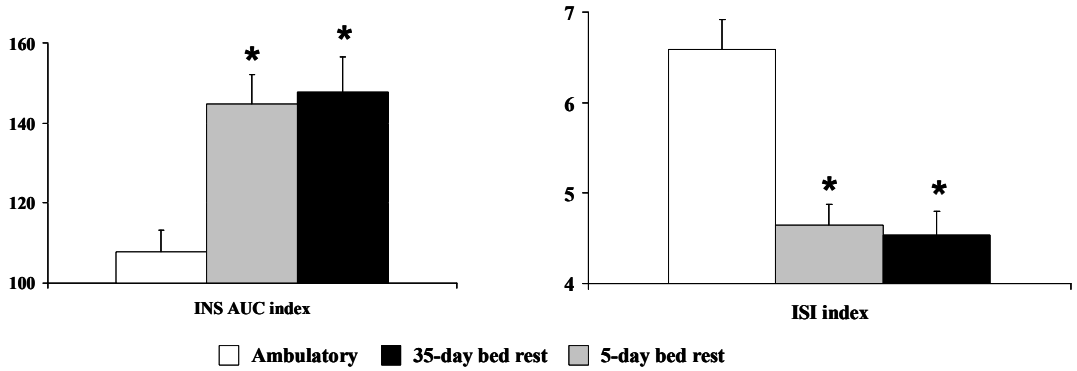

Figure 18. Insulin sensitivity, as assessed by the composite ISI index of insulin sensitivity during the OGTT performed in the three designed metabolic test sections (Ambulatory, 5-day bed rest and 33-day bed rest).

EFFECT OF BED REST AND GLUCOSE LOAD ON AUTONOMIC REGULATION

Effects of bed rest on autonomic nervous system regulation.

There was significant bed rest effect on brachial systolic, diastolic and mean arterial pressure. Diastolic arterial pressure significantly increased after 5 days of bed rest and further increased after 33 days of bed rest. Percent changes from the basal ambulatory condition were greater ($p=0.01$, Wilcoxon test) after 33 days (15±3%) than after 5 days (8±2%) of bed rest. Similarly results were observed considering systolic arterial pressure (changes after 5 days 10±6%; changes after 33 days 5±1%; $p<0.01$).

Heart rate did not change significantly at after 5 days while it increased significantly after 33 days of bed rest in the fasting state. Percent changes from the basal ambulatory condition were greater ($p<0.01$, Wilcoxon test) after 33 days than after 5 days.

RR interval did not change significantly after 1 week while it increased significantly at the end of the experimental period. Percent changes from baseline were not significantly different after one week (-34±9%) and at the end of bed rest (-49±6%).

There was a significant bed rest effect on LF of RR interval (RR-LF) expressed in normalized units (RR-LF(nu)). In the fasting state RR-LF(nu) did not change significantly after 5 days while it significantly increased at the end of the experimental period. Percent changes from the basal ambulatory condition were greater ($p=0.03$, Wilcoxon test) after 33 days (210±117%) than after 5 days of bed rest. Bed rest significantly decreased HF component of RR interval (RR-HF) and tended to decrease RR-HF in normalized units (RR-HF(nu)). RR-HF at the end of the experimental period was significantly lower than that after one week of unloading and at basal ambulatory condition. Similar trend was observed for RR-HF(nu). Bed rest mediated changes of RR-HF in the fasting state were significantly greater ($p=0.01$, Wilcoxon test) after 33 days (7±47%) than those at day 5 of bed rest (+60±17%).

There was significant bed rest effect on the ratio between RR-LF and RR-HF (LF-to-HF ratio). LF-to-HF ratio in the fasting state after 33 days of inactivity was significantly greater than that after 5 days of bed rest. Bed rest mediated changes of LF-to-HF ratio in the fasting state were significantly greater ($p=0.02$, Wilcoxon test) at the end of bed rest (-0.5±0.5) than those measured after one week of inactivity (2.1±1.2). SAP, as determined at the finger level by a Portapres device, tended to increase following bed rest. SAP variability significantly increased following bed rest. Bed rest mediated changes of SAP variability in the fasting state were significantly greater ($p=0.02$, Wilcoxon test) after 5 days (1.79±59%) than after 33 days (55±32%) of unloading.

Bed rest did not influence SAP-LF and SAP-LF at short (following 5 days of bed rest) nor at long time (following 33 days of inactivity).
Baroreflex sensitivity significantly decreased following bed rest. Fasting baroreflex sensitivity significantly decreased following 5 days of bed rest. Bed rest mediated decreases of fasting baroreflex sensitivity were significantly greater (p=0.01, Wilcoxon test) at day 5 (-41±10) than at day 33 (-23±16%) of inactivity. There was significant bed rest effect on α-index. α-index significantly decreased from the ambulatory condition after 5 days of unloading and remained significantly suppressed at the end of the experimental period.

Effects of bed rest and glucose load on autonomic nervous system regulation.
Effects of bed rest and glucose load on autonomic nervous system regulation were reported in Table 4.
During OGTT, heart rate did not change significantly after 5 days while it increased significantly after 33 days of bed rest. Percent changes from the basal ambulatory condition were greater ($p<0.01$, Wilcoxon test) after 33 days than after 5 days after OGTT. There was significant OGTT effect, but there was not significant bed rest × OGTT interaction on heart rate.
RR interval did not change significantly after 1 week while it increased significantly at the end of the experimental period after glucose load. Bed rest induced changes (calculated between consecutive measurements, i.e., ambulatory to day 5 of bed rest and from day 5 of bed rest to day 33 of bed rest) of insulin AUC and RR interval after OGTT were directly correlated (R=0.64; n=20;$p<0.01$; Spearman test) (Fig. 10). RR variability significantly decreased following bed rest but there was not significant OGTT effect nor bed rest × OGTT interaction. RR variability during OGTT was significantly decreased from the ambulatory condition at day 5 as well as at day 33 of bed rest. There was not significant OGTT effect or bed rest × OGTT interaction on RR-LF. The OGTT significantly increased RR-LF(nu). There was a significant bed rest × OGTT interaction for RR-LF(nu). OGTT mediated changes in RR-LF(nu) were greater ($p<0.05$, Wilcoxon test) after 33 days (68±27%) than after 5 days of inactivity (12±11%). RR-HF, assessed during the OGTT, at the end of the experimental period was significantly lower than that after one week of unloading and at basal ambulatory condition. OGTT significantly decreased RR-HF and RR-HF(nu). There was not significant bed rest × OGTT interaction for RR-HF and RR-HF(nu). OGTT significantly increased LH/HF, while there was not significant bed rest × OGTT interaction. There was significant OGTT effect on SAP, without significant bed rest × OGTT interaction. Bed rest induced changes of insulin AUC and SAP after OGTT (calculated between consecutive measurements, i.e., ambulatory to bed rest +5 and from bed rest +5 to bed rest +33) were directly correlated (R=0.67; n=20; p=0.01;

Spearman test) (Fig. 10). OGTT significantly increased SAP variability. There was significant bed rest × OGTT interaction for SAP variability. OGTT significantly increased the low-frequency component of SAP variability (SAP-LF) in ambulatory conditions, while this effect was not significant following 5 nor following 33 days of bed rest. OGTT mediated changes in SAP-LF were greater (p=0.01, Wilcoxon test) in the ambulatory conditions (314±73%) compared to OGTT performed on day 5 of bed rest (25±31%). There were not OGTT effects on SAP-HF. OGTT significantly decreases baroreflex sensitivity in ambulatory conditions by -27±6%; moreover such change was significantly greater (p=0.03, Wilcoxon test) than that at day 5 of bed rest (2±12%). There was not significant OGTT effect nor bed rest × OGTT interaction on α-index.

Table 4. Effects of short-term (5-day bed rest) and long-term (33-day bed rest) bed rest on autonomic system regulation in the fasting state and after oral glucose tolerance test (OGTT).

	Ambulatory	5-day bed rest	33-day bed rest	Bed rest effect	OGTT effect	Bed rest × OGTT interaction
Heart rate (b/min)						
Fasting	60±2	58±2	65±2[b]	0.01	0.01	0.19
OGTT	63±2	64±2[c]	73±2 [abc]			
RR interval (ms)						
Fasting	1007±38	1048±44	926±25[b]	0.02	0.01	0.24
OGTT	971±36	956±40[c]	837±29[abc]			
RR variability (ms^2)						
Fasting	5472±1798	3376±1019	3152±841	0.02	0.40	0.58
OGTT	4680±1067	2749±483[a]	2385±471[a]			
RR variability LF (a)						
Fasting	610±159	510±126	656±272	0.43	0.17	0.10
OGTT	1390±352	721±220	778±440			
RR variability LF (n.u.)						
Fasting	39.5±9.6	36.9±7.3	59.3±7.6 [b]	0.04	0.01	0.04
OGTT	54.9±6.5	55.3±8.8 [c]	63.7±7.6			
RR variability HF (a)						
Fasting	2181±1044	1109±339	359±87[ab]	0.01	0.02	0.37
OGTT	1616±530	592±189 [c]	328±114[a]			
RR variability HF (n.u.)						
Fasting	52.2±9.5	55.1±6.9	36.1±8.2	0.07	0.01	0.28
OGTT	38.4±5.7	36.7±8.3 [c]	31.1±7.6			
LF-to-HF ratio						
Fasting	1.6±0.5	1.1±0.4	3.6±1.1[b]	0.05	0.01	0.23
OGTT	2.7±0.6	3.4±1.0[c]	7.1±2.9			

SAP (mmHg)						
Fasting	120±3	126±4	126±2	0.07	0.04	0.81
OGTT	123±3	133±3	133±4			
SAP variability (mmHg)						
Fasting	21.7±3.1	48.5±7.4[a]	30.3±6.8	0.02	<0.01	0.01
OGTT	73.8±25.6	63.0±10.4	78.7±17.4[c]			
SAP variability LF (a)						
Fasting	4.6±1.8	16.7±7.3	5.6±1.4	0.08	0.01	0.04
OGTT	13.4±3.6 [c]	12.4±3.3	8.6±1.6			
SAP variability HF (a)						
Fasting	1.1±0.2	1.6±0.3	0.9±0.3	0.22	0.50	0.14
OGTT	1.9±0.5	1.0±0.1	1.0±0.2			
BRS (ms/mmHg)						
Fasting	25.8±3.4	12.7±1.0[a]	17.1±2.8	0.04	0.01	0.09
OGTT	18.3±2.8 [C]	12.6±1.4	12.0±1.6[a]			
α-index (ms/mmHg)						
Fasting	26.9±3.6	16.9±3.3[a]	18.1±3.1	0.01	0.15	0.56
OGTT	23.7±4.9	15.2±1.8[a]	16.0±3.9[a]			

Data are expressed as mean±SEM. n=10. Repeated measures ANOVA with interaction. [a], $p<0.05$ versus Amb; [b], $p<0.05$ versus BR+5; [c], $p<0.05$ OGTT versus Fasting (Bonferroni's post-hoc test). LF (a), low-frequency component as expressed in absolute values; LF (n.u.), low-frequency component as expressed in normalized units; HF (a), high-frequency component as expressed in absolute values; HF (n.u.), high-frequency component as expressed in normalized units; SAP, systolic arterial pressure; BRS, baroreflex sensitivity.

In order to assess the net impact of glucose load on autonomic nervous system regulation, on day 14 of bed rest a aspartame-sweetened water ingestion was performed. This blank test was used to investigate potential effects of 300 mL of water ingestion as well as of glucose taste on autonomic nervous system regulation. Effects of simulated glucose load on autonomic nervous system parameters were reported in Table 5. Aspartame-sweetened water ingestion induced a decrease in RR-HF expressed in absolute values whereas no effects on RR-HF expressed in normalized units were observed. In addition, SAP variability was significantly enhanced after aspartame-sweetened water ingestion.

Table 5. Effects of blank test (aspartame-sweetened water ingestion) on autonomic system regulation after 14-days of bed rest.

	BASELINE	BLANK TEST	p
Heart rate (b/min)	59±2	59±2	0,27
RR interval (ms)	1026±40	1044±43	0,16
RR variability (ms^2)	3226±672	4034±622	0,05
RR variability LF (a)	830±225	824±233	0,94
RR variability LF (nu)	54,3±9,3	45,9±8,6	0,05
RR variability HF (a)	752±268	1182±353	0,04
RR variability HF (nu)	42,6±8,9	49,8±8,3	0,06
LF-to-HF ratio	2,5±0,7	4,3±2,2	0,35
SAP (mmHg)	128±8	128±3	0,96
SAP variability (mmHg)	39,5±14,3	67,2±14,9	0,02
SAP variability LF (a)	9,8±4,9	8,5±3,7	0,83
SAP variability HF (a)	1,5±0,6	1,6±0,3	0,84
BRS (ms/mmHg)	14,4±1,6	15,2±1,9	0,63
α-index (ms/mmHg)	20,0±3,4	20,2±3,0	0,93

Data are expressed as mean±SEM. n=10. Statistical analysis was performed by Wilcoxon statistical test. Subjects ingested 300 ml of aspartame-sweetened water at room temperature to mimic volume ingestion and taste of the oral glucose tolerance test. LF (a), low-frequency component as expressed in absolute values; LF (n.u.), low-frequency component as expressed in normalized units; HF (a), high-frequency component as expressed in absolute values; HF (n.u.), high-frequency component as expressed in normalized units; SAP, systolic arterial pressure; BRS, baroreflex sensitivity.

EFFECT OF BED REST ON PLASMA LIPID PATTERN AND CETP

Valdoltra Bed Rest Studies 2006, 2007 and 2008.

To avoid the potential interference of changes in adipose tissue on cholesteryl ester transfer protein (CETP) production, subjects who did not maintain energy balance throughout the experimental period (Biolo et al. 2008) were excluded from the present analysis. Particularly, five subjects who participated in the bed rest study 2006 failed to spontaneously adapt to decreased energy requirement (Biolo et al. 2008) and gained 2.6±0.3 kg fat mass; these subjects were excluded from the analysis. In addition, one subject who participated in the bed rest study 2007 and gained 2.0 kg fat mass despite a tailored diet was also excluded. In the 24 subjects selected for this statistical analysis, fat mass did not significantly change throughout the experimental period whereas fat-free mass significantly decreased by 3.9±0.4% ($p<0.001$). Bed rest significantly affected plasma triglycerides and HDL levels. HDL decreased by 12±3% ($p<0.001$), while triglycerides increased by 51±10% ($p<0.001$). Physical inactivity did not change plasma total cholesterol and LDL concentrations. The ratio between HDL and non-HDL cholesterol (i.e., the sum of VLDL and LDL) significantly ($P<0.01$) decreased following bed rest from 0.44±0.04 to 0.35±0.04. CETP concentrations significantly increased by 27±9%. Inactivity mediated changes in plasma CETP inversely correlated with changes in HDL to non-HDL cholesterol ratio (Fig. 10A). In these subjects, insulin concentration increased by 46±10% ($p<0.001$) following the experimental bed rest and, consequently, the HOMA index of insulin resistance significantly increased by 47±11%. Inactivity mediated changes in plasma fasting insulin inversely correlated with changes in CETP (Fig. 10B).

Statistical analysis performed in all subjects (n=30), including those who gained fat mass at the end of the experimental period, indicated that CETP levels significantly increased ($P<0.05$) by 23±8% and that inactivity mediated changes in plasma CETP inversely correlated with changes in HDL to non-HDL cholesterol ratio ($R=-0.51$; n=30; $p<0.01$; Pearson test). Bed rest induced changes in fat mass did not significantly correlate with changes in CETP levels ($R=0.34$; n=30; $p=0.07$; Pearson test). In the 6 subjects who gained fat mass at the end of the bed rest period CETP levels did not change significantly (from 3.63±0.33 to 3.73±0.43 mg/L).

As abovementioned, inactivity-mediated changes in insulin concentrations were inversely correlated with changes in CETP concentrations (Fig. 10B). Subjects (n=24) were then stratified according to the median value of bed rest mediated changes in fasting insulin levels; the group (n=12) with lower changes in plasma insulin (+4±2 pmol/L) exhibited the greatest increases in CETP concentrations (+53±12%), whereas in the group (n=12) displaying higher insulin changes (+30±6 pmol/L), CETP levels did not change significantly following bed rest (0±9%) (repeated measured ANOVA, bed rest

effects: P=0.015, bed rest × insulin change interactions: P=0.003). There were not significant bed rest × insulin change interactions for the effects of inactivity on triglycerides (P=0.68). Plasma triglicerides significantly increased by 49±14 and 56±16 % in the groups displaying lower or greater changes in insulin concentrations, respectively.

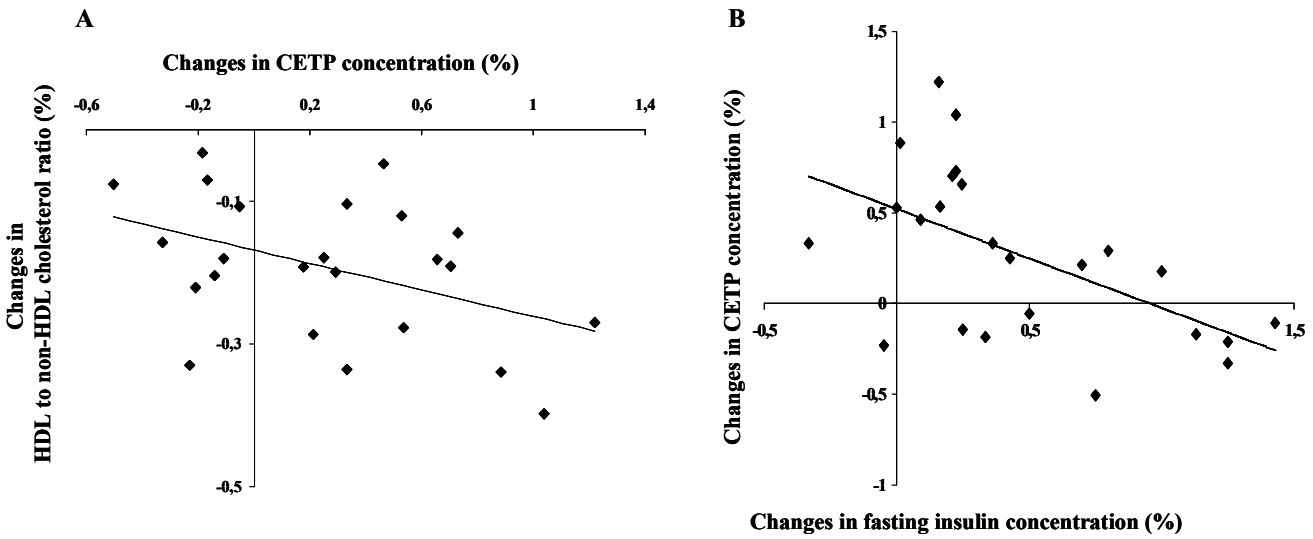

Fig. 10. Correlation between percent changes from ambulatory to bed rest of CETP levels and HDL to non-HDL cholesterol ratio (**A**); correlation between percent changes from ambulatory to bed rest of insulin and CETP levels (**B**); (n=24; statistical analysis was performed using Pearson test).

EFFECT OF BED REST ON HOMOCYSTEINE METABOLISM

WISE Study.

Plasma concentrations of amino acids involved in the homocysteine metabolic pathways were differently affected by 60 days of physical inactivity. Plasma methionine and cysteine levels were not significantly changed after prolonged bed rest, ranging, respectively, from 21±1 $\mu mol \times L^{-1}$ to 24±1 $\mu mol \times L^{-1}$ (n=8; p=0.07) and from 215±5 $\mu mol \times L^{-1}$ to 208±4 $\mu mol \times L^{-1}$ (n=8; p=0.08). On the contrary, plasma homocysteine concentration was significantly up-regulated after 60 days of bed rest, from 8.6±1.3 $\mu mol \times L^{-1}$ to 10.3±1.0 $\mu mol \times L^{-1}$ (n=8; p=0.01).

As reported in Table 6, bed rest significantly enhanced homocysteine transulfuration rate whereas reduced remethylation rate. No effects of prolonged inactivity were detected on transmethylation rate.

The clearance of homocysteine through the remethylation was significantly reduced by prolonged bed rest whereas the homocysteine clearance by transulfuration pathway was not significantly different after 6 days of bed rest, as compared to ambulatory condition (Fig. 11).

Table 6. Plasma homocysteine kinetics measured before (Ambulatory) and at the end (60-day bed rest) of the experimental period.

	Ambulatory	60-day bed rest	p
Transmethylation rate (μmol×min-1)	4.1±0.2	4.3±0.3	0.34
Remethylation rate (μmol×min-1)	2.5±0.1	2.3±0.2	0.03
Transulfuration rate (μmol×min-1)	1.7±0.1	2.0±0.1	0.03

Data are shown as mean±SEM. n=8. Statistical analysis was performed by paired Student's *t* test. Rates of metabolic pathways were assessed through the isotopic tracers infusion approach coupled with gas chromatography-mass spectrometry analyses.

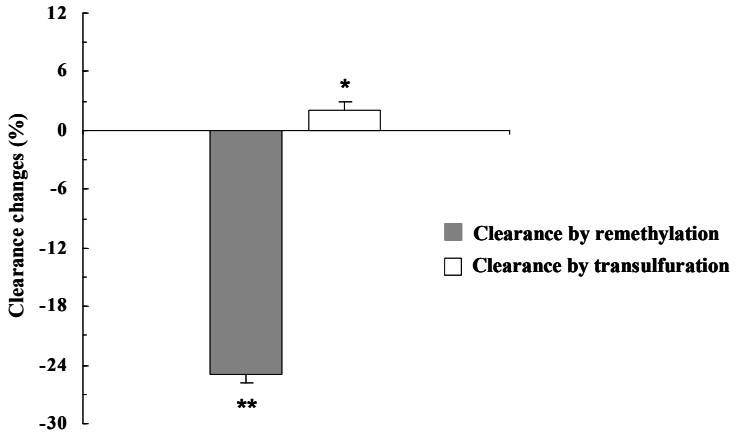

Fig. 11. Bed rest induced changes in homocysteine clearance by remethylation and transulfuration pathways. n=8. Statistical analysis was performed by Student'*t* test. *, $p<0.02$ vs clearance by remethylation; **, $p<0.05$ different from zero.

DISCUSSION

In the present thesis, the net impact of physical inactivity on several features characterising insulin resistance and metabolic syndrome has been investigated. In detail, the role of inactivity on insulin sensitivity, inflammation, oxidative stress, homocysteinemia, lipidemia as well as on autonomic nervous system regulation was assessed.

These parameters have been monitored during 4 different bed rest studies (Valdoltra Bed Rest Studies 2006, 2007 and 2008; WISE Study).

THE ROLE OF PHYSICAL INACTIVITY ON MUSCLE ATROPHY AND OXIDATIVE STRESS IN MUSCLE

Muscle atrophy following long term and very-long term bed rest

It is well known that exposure to inactivity leads to decreased postural muscle mass (Akima et al. 2005), especially of lower limb (Alkner and Tesch 2004). Moreover, bed rest is a suitable model to study the net effect of inactivity on muscle mass (Stein and Wade 2005). In all analyzed bed rest studies, both in men and women, unloading determined decreased in muscle. These alterations were confirmed by data of fat free mass, assessed through bioimpedance, and of muscle mass, determined by DXA. Muscle mass reduction was observed following 33 (Valdoltra Bed Rest Studies) and 60 (Wise Study) days of bed rest. Moreover, other research groups, participating to the same experimental frame, confirmed that bed rest mediates atrophy in antigravity muscles as *gastrocnemius medialis*, whereas inactivity does not significantly affect non-antigravity muscles, as *tibialis anterior* and *biceps brachii* (de Boer et al. 2008).

Alterations in body composition were observed after few days of unloading, however changes in whole body water distribution has to be considered and could account for the great loss in muscle mass detected by bioimpedance after few days of inactivity. Interestingly, data obtained in the long-term WISE bed rest evidenced that complete reduction in muscle mass occurred in the first month of inactivity, whereas continued exposure to inactivity (i.e., one month more) did not display additional negative effects on muscle mass. These results suggest that muscle atrophy completely developed after 4 weeks of bed rest and that this alteration is then maintained at long term.

In the Valdoltra Bed Rest Studies, muscle thickness and architecture were also measured. The decrease in muscle thickness is a direct index of muscle atrophy, validated also in other model of muscle wasting (Pinet et al. 2004). Moreover, pennation angle was previously demonstrated to be inversely correlated to muscle atrophy (de Boer et al. 2008; Morse et al. 2005). 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 deep reduction of fibre pennation angle and to a concomitantly enhanced muscle atrophy (de Boer et al. 2007). In agreement with these results, 33-day bed rest enhanced *vastus lateralis* muscle atrophy, assessed as reduction in *vastus lateralis* thickness and fibers pennation angle. Since pennation angle was previously demonstrated to be correlated to muscle shape, the observed decrease in pennation angle, following prolonged bed rest, could be considered to occur in parallel with muscle atrophy. These data are confirmed also by results obtained in the same experimental design by other researchers, demonstrating that bed rest significantly reduced (-18%) cross sectional area of muscle fibres, another recognised index of

muscle atrophy (Pisot et al. 2008; Dalla Libera et al. 2009). In the same bed rest project, tensiomyographic analysis evidenced a significant reduction in contractile parameters of *biceps brachii*, *vastus medialis*, *biceps femoris* and *gastrocnemius medialis* following bed rest (Pisot et al. 2008).

Validation of the new single sample-multiple tracer method for the assessment of peptide synthesis.
The traditional method used to determine peptide FSR is based on the assessment of product enrichment changes over time during continuous infusion of isotopic precursor tracer, when precursor enrichment reaches the steady state (Wolfe and Chinkes 2005). In fact, 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 applying a modified method, which allows measurement in a single sample, after a double tracer precursor infusion. Isotopic glycine was chosen as tracer (Jahoor et al. 1995) and this novel method was based on two parallel and separate infusions of different isotopes, [$^{2}H_2$]-glycine and [^{15}N]-glycine, started at different times (four hours shift). In this way, the difference between [^{15}N]-glutathione and [$^{2}H_2$]-glutathione enrichments measured in a single final biological sample, allowed FSR determination. Linearity of precursor incorporation into the final product was already demonstrated (Biolo et al. 2008). The new method was directly validated in human red blood cells through the comparison of results obtained through the traditional multiple samples-single tracer approach. During each metabolic test, several blood samples were drawn. Glutathione FSR in red blood cells was assessed using both the novel single sample-multiple tracers approach (Eq. 2) and the traditional multiple samples-single tracer method (Eq. 3). The novel approach has been specifically designed for muscle assay, and in erythrocytes has been applied considering both tracer infusions and only the final blood sample (see "Calculations"). Data obtained in red blood cells by both approaches yielded comparable results. Additionally, Passing–Bablok regression line analysis of correlations between absolute values of glutathione FSR obtained by traditional and novel methods, strengthens the validation of the new technical protocol. This statistical approach, in fact, quantitatively describes parameters validating a new method when matched to a standard one, with no assumptions regarding sample distribution (Passing and Bablok 1983). Inclusion of intercept A value in the confidence interval demonstrates that no constant difference between the two methods can be evidenced. Similarly, *B* slope value belonging to its relative confidence interval underlines that there are not significant proportional differences between the two methods. Data distribution fails to significantly deviate from linearity. Even though these results strongly suggest the reliability of the novel approach, also the Altman–

Bland test was performed on the analysed data set. The Altman–Bland confidence interval ranges from −17.9 to 35.8% day−1 and data are well distributed suggesting no proportional errors. Moreover, the presence of only two measurements outside the confidence interval can be considered as a satisfactory condition. A constant bias seems to appear from localization of the mean value line, but this effect can derive from the fact that the dataset was constituted by repeated measures during the bed rest period. Moreover, the reliability of this new single sample-multiple tracers approach was further assessed comparing pooled FSR changes from baseline to day 7 and to day 33 measured by the traditional approach, with the same changes measured by the novel equation. Similarly to data analysis performed on absolute values of glutathione FSR, Passing–Bablok and Altman–Bland tests were performed. The Passing–Bablok test confirms that the novel method applied to bed rest mediated changes is not plagued by a proportional or constant error. Additionally, no significant deviation from linearity of data distribution was observed. In parallel, the Altman–Bland plot shows only one measurement is outside a sufficiently narrow confidence interval including well distributed values. Thus, taken together, these statistical results suggest that the novel single sample-multiple tracers method can be reliably utilized *in vivo* to assess glutathione FSR.

Absolute values of tracers steady-state enrichments were different: [^{15}N]-glycine enrichment was displayed to be higher than [$^{2}H_2$]-glycine. Such a tendency for ^{15}N isotopes to reach higher steady state enrichments in comparison to ^{2}H isotopes was previously demonstrated for alanine tracer (Yang et al. 1984). Due to analogies between alanine and glycine metabolic pathways, it is possible to compare obtained differences in tracer steady state enrichments of glycine with those observed for alanine. Additionally, to exclude direct influences of precursor kinetics, ratios between isotopic product and related tracer enrichments were introduced, to prevent potential pitfall related to [^{15}N]-glycine peculiar feature.

The novel equation used to FSR determination requires two assumptions. The first is that while steady-state condition for [$^{2}H_2$]-glycine enrichments was directly assessed three hours after infusion beginning, it was assumed for [^{15}N]-glycine after the same time of an equal infusion. This seems to be acceptable by itself; however, in addition, [^{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). The second assumption is that 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 and Chinkes 2005). 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 and FSR in a single muscle biopsy, has been also proposed by Zhang et al. (Zhang, Chinkes, and Wolfe 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 fractional breakdown rate 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.

Muscle oxidative stress and muscle atrophy following one month bed rest.

Animal studies demonstrated that muscle unloading enhances reactive oxygen species production (Lawler, Song, and Demaree 2003). Additionally, during unloading the activation of antioxidant system (Kondo, Nishino, and Itokawa 1994; Lawler, Song, and Demaree 2003) aimed to scavenge oxidized substrates (Lawler, Song, and Demaree 2003) has also been detected. As a consequence of increased oxidative stress, proteolysis by the ubiquitin proteasome system is up-regulated (Bar-Shai et al. 2008; Powers and Lennon 1999). For such reasons, a link between physical inactivity, muscle oxidative stress induction and consequent atrophy has been strongly suggested (Powers, Kavazis, and DeRuisseau 2005; Bar-Shai et al. 2008). Moreover, oxidative stress is recognized as a pathogenic factor mediating muscle atrophy and wasting, in several pathological conditions (Moylan and Reid 2007). However, the role of oxidative stress in inactivity mediated induction of muscle atrophy in humans is poorly investigated. In the Valdoltra Bed Rest Study 2007, relationships between muscle atrophy and changes in oxidative status were investigated. Presented data demonstrate that in atrophied muscles, bed rest significantly enhanced glutathione availability as well as fiber oxidative damage, as assessed through muscle protein carbonylation determination.

After 33 days of inactivity, protein carbonylation in muscle was significantly increased and can be associated to enhanced muscle fiber damage secondary to oxidative stress induction in *vastus lateralis*. Protein carbonylation assessment has been demonstrated to be a marker of oxidative stress occurrence (Greilberger et al. 2008). In fact, protein carbonylation is a consequence of ROS action on protein carbon groups, determining alterations in enzyme structure and activity (Stadtman 2001). An increase in this index has been previously associated to disease progression in muscle wasting patients with leukemia (Ahmad 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; Grune et al. 2003). Carbonylated proteins are selectively degraded by the 20S core proteasome without ubiquitination (Grune et al. 2003; Grune and Davies 2003) and there is evidence that carbonylated proteins are more efficiently and rapidly scavenged by proteolytic degradation than their non-oxidized counterparts (Dukan et al. 2000; Grune et al. 2003; Bota and Davies 2002). This suggests that carbonylation can trigger or sustain muscle atrophy.

The inverse relationship between bed rest mediated changes in protein carbonylation and in *vastus lateralis* thickness further suggests that oxidative damage by carbonylation in human muscle proteins is one of pathways leading to muscle atrophy during physical inactivity. Other mechanisms triggering protein catabolism and then muscle atrophy involve caspases, calpain (Du et al. 2004; Goll et al. 2003) and ubiquitin proteasome system regulation (Ikemoto et al. 2001; Purintrapiban, Wang, and Forsberg 2003). Enhanced ROS production, in fact, modifying calcium availability, affects caspases and calpain activities (Siems et al. 2003). Moreover, oxidative stress, can up-regulate E3 ubiquitin ligases as muscle atrophy F-box/atrogin1 and muscle ring finger-1 in myotubes (Li et al. 2003): these alterations could enhance skeletal muscle proteolysis and atrophy (Bodine et al. 2001). 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 up-regulated homocysteine availability: even though this alteration failed to be related to worsened muscle atrophy, a possible contribution can not be rouled out.
The relationship between physical activity level and oxidative stress is complex; strenuous exercise in fact induces fiber skeletal muscle damage (Close et al. 2004) by enhanced ROS production. On the contrary, long term moderate training up-regulated antioxidant systems leading to improved redox balance (Gomez-Cabrera, Domenech, and Vina 2008). However, further studies are required to understand pathways linking unloading and muscle architecture changes. In fact, carbonylation changes failed to be related to changes in fiber orientation, suggesting that other oxidative processes, as e.g. reactive nitrogen species (Bar-Shai and Reznick 2006), could be involved in regulation of this parameter. Moreover, different mechanisms affecting muscle protein turnover, as changes in insulin-like growth factor expression, could contribute to alterations of muscle morphology (Clemmons 2009).
When excessive ROS production occurs, antioxidant system activation is stimulated (Pastore et al. 2003) in order to limit the biological damage on substrates. There are several enzymatic and non-enzymatic mechanisms, activated to reduce oxidative damage; among them, in muscle, the glutathione system is quantitatively the most important antioxidant (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 may lead to increased glutathione availability (Ji, Fu, and Mitchell 1992; Biolo et al. 2008). Thus, kinetic assessment of glutathione pools effectively monitors oxidative stress onset. By the novel and validated one-sample and double isotopic tracer infusion approach the impact of bed rest on muscle glutathione synthesis rate was assessed. After one month of bed rest, muscle glutathione synthesis is significantly increased. This underlines that in humans, physical inactivity enhances glutathione antioxidant activity in muscle: this 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. 2002) and activities of key enzymes in glutathione system as glutathione reductase and glutathione peroxidase were demonstrated to be increased (Sen et al. 1992), unaltered or down-regulated (Tauler et al. 2006). Reasons leading to this wide range of results can be ascribed to model and experimental design differences. The presence of erythrocytes on explanted fibres could, theoretically, biases glutathione assays in muscle biopsies. This is an intrinsic limitation for muscle studies and its impact was strongly minimized by accurate cleaning of each biopsy. Glutathione synthesis was also assessed in erythrocytes and, in agreement with previous publication, erythrocyte glutathione synthesis failed to be affected by bed rest. Glutathione

FSR assessed in red blood cells and in muscles was strongly different. This is in accordance with previous studies demonstrating that even if glutathione turnover rates could be slightly different in erythrocytes and in muscles (Flaring et al. 2009), glutathione turnover rate in muscle can be three time higher than in erythrocytes (Malmezat et al. 2000). Moreover, additional publications showed that glutathione synthesis rate is markedly higher in muscles when compared to other tissues (Griffith and Meister 1979) and that glutathione turnover is particularly low in erythrocytes (Mortensen , HALEY, and ELDER 1956). Thus, present glutathione FSR measurements performed in muscle and in red blood cells are aligned with previous publications. Moreover, published values of glutathione concentrations in muscle are distributed over a quite wide range, starting from 0.5 $mmol\times(kg\ wet\ tissue)^{-1}$ until 1.7-1.8 $mmol\times(kg\ wet\ tissue)^{-1}$ (Rutten et al. 2008; Medved et al. 2004; Griffith and Meister 1979; Luo et al. 1998). In the Valdoltra Bed Rest 2007, muscle glutathione concentration ranged around 2 $mmol\times(kg\ wet\ tissue)^{-1}$: considering the range of published data, concentrations measured in these healthy young subjects can be considered as reliable.

Up-regulation of glutathione synthesis in muscle could be considered a consequence of previously occurred oxidative protein carbonylation in atrophying muscle after bed rest. However, significant correlation between bed rest mediated changes of muscle protein oxidation and glutathione availability failed to be evidenced in this work. The reduced sample size could provide an explanation for the lacking direct correlation; however, the involvement of other antioxidant systems, e.g. heme oxygenase (Dalla Libera et al. 2009), in inactivity-induced muscle oxidative stress could also be proposed.

Moreover, in the present work, also a reliable correlation between glutathione synthesis rate and muscle atrophy markers failed to be identified. Nevertheless, previous studies suggest that altered availability of glutathione could affect clinical conditions and muscle wasting in patients.

In sarcopenic critically ill patients, glutathione levels are decreased, suggesting an impaired antioxidant response to oxidative stress (Biolo, Antonione, and De Cicco 2007). Moreover, glutathione depletion was demonstrated to influence the degree of symptoms and severity of selected pathologies (Najim, Sharquie, and Abu-Raghif 2007) as well as clinical outcome (Crimi et al. 2006). An impairment of muscle antioxidant systems has been associated to cachexia (Laviano et al. 2007). On the contrary, the increase in glutathione precursors, such as cysteine or N-acetyl-cysteine, ameliorates glutathione availability and antioxidant activity (Badaloo et al. 2002) and reduces muscle protein ubiquitination in animals (Ikemoto et al. 2002).

In addition to dietary glutathione precursor supplementation, other metabolic factors can affect body composition during immobility (Clemmons 2009). Particularly, energy intake level is a

critical factor for muscle mass maintenance control in bed resting patients. As previously demonstrated, both negative (Biolo et al. 2007) and positive (Biolo et al. 2008) energy balance worsen muscle atrophy during bed rest respectively affecting whole body protein turnover and systemic inflammation. Moreover, fat mass gain was shown to enhance in bed resting subjects erythrocyte glutathione synthesis while fat maintenance prevented this response (Biolo et al. 2008). In the present study, diet was strictly controlled to maintain a near neutral energy balance.

THE ROLE OF PHYSICAL INACTIVITY ON SYSTEMIC INFLAMMATORY STATUS: ERYTHROCYTE MEMBRANE FATTY ACID COMPOSITION

Sedentary lifestyle is known to induce chronic low grade inflammation, characterised by a slight increase in pro-inflammatory cytokines, and oxidative stress, both in animals and humans (Ischander et al. 2007; Fischer et al. 2007). Inflammation and altered redox balance are recognised pathogenic mediators of cardiometabolic diseases (Hopps et al. 2010; Alberti, Zimmet, and Shaw 2006). During experimental bed rest in healthy young subjects, the plasma ratio between IL-6 and IL-10 increased as well as C reactive protein and pentraxin-3 levels (Bosutti et al. 2008). Fatty acid composition in erythrocyte membranes represents a reliable marker of whole body inflammatory status and reflects cell membrane composition in different tissues (Harris and Von 2004). The n-6 and n-3 fatty acids series in fact are involved in up-regulation and down-regulation of the inflammatory response, respectively.

The assessment of erythrocyte membrane fatty acid pattern, performed in the Valdoltra Bed Rest Studies, evidenced that one month of bed rest promotes the development of a pro-inflammatory status at whole body level. Bed rest, in fact, led to a significant increase in pro-inflammatory n-6 fatty acid, including arachidonic acid. Interestingly, n-6 precursor, i.e., linoleic acid, was significantly decreased, suggesting an active role of bed rest in enhancing n-6 fatty acid metabolism. These data are in agreement with previous evidences demonstrating that exercise reduces arachidonic acid content (Helge et al. 2001). Bed rest mediated increases in arachidonic acid can lead, in turn, to increased pro-inflammatory eicosanoid production.

On the contrary, experimental bed rest reduced eicosapentaenoic acid, a member of n-3 fatty acid series, displaying anti-inflammatory properties (Babcock, Helton, and Espat 2000). Moreover, physical inactivity reduced the amount of the n-3 fatty acid precursor, the α-linolenic acid. Interestingly, docosahexaenoic acid levels were significantly up-regulated at the end of the experimental period.

Interestingly, n-6 and n-3 fatty acids are competitively metabolized by the same enzymes (Arterburn, Hall, and Oken 2006); it is then possible hypotheses that the enhanced n-6 fatty acid availability could have reduced the metabolism of n-3 fatty acids, with consequent decrease in α-linolenic and eicosapentaenoic fatty acids in cell membranes. Explanations for bed rest-induced increase in docosahexaenoic acid levels are lacking. However, the increase in docosahexaenoic acid could, at least in part, derives from eicosapentaenoic acid retrotransformation, a metabolic peculiarity of this n-3 fatty acid (Conquer and Holub 1997).

The arachidonic-to-eicosapentaenoic acid ratio, that is calculated to monitor the competitive roles of arachidonic acid and eicosapentaenoic acid on inflammatory processes, significantly increased after

33 days of physical inactivity. As previously demonstrated, the balance between pro-inflammatory eicosanoids, mainly derived arachidonic acid metabolism, and anti-inflammatory molecules, mainly deriving from the metabolism of arachidonic and eicosapentaenoic acid respectively, is a critical point for the maintenance of cell membrane functions (Serhan, Haeggstrom, and Leslie 1996; Kelley 2001).

The present data are in agreement with previous reports in exercised subjects. Muscle skeletal membrane fatty acids after regular exercise training display the opposite pattern as compared to that assessed after 33 days of unloading. Exercise training in fact leads to a reduction in saturated and n-6 fatty acids whereas determines an increase in monounsaturated and n-3 fatty acids (Helge et al. 2001; Andersson et al. 2000).

The pro-inflammatory effect of bed rest is further confirmed by changes in the estimated activities of enzymes involved in n-3 and n-6 metabolism.

Prolonged inactivity decreased Δ-9 desaturase activity, as estimated from product-to-precursor ratio. Δ-9 desaturase catalyses the conversion of stearic and palmitic acids to monounsaturated oleic and palmitoleic acid, respectively (Ntambi and Miyazaki 2004). The inactivity-mediated reduction in Δ-9 desaturase activity led to a significant reduction in monounsaturated fatty acids and a tendency towards increases in the saturated fatty acids. The reduction in monounsaturated fatty acid, not balanced by a reduction in unsaturated fatty acids, could contribute to a pro-inflammatory condition mediated by decreased clearance of pro-oxidant metabolites of saturated fatty acids, such as diacylglycerol and ceramides (Peter et al. 2009).

Finally, alterations in membrane fatty acid composition have been associated to changes in insulin sensitivity. The activities of Δ-5, Δ-6 and Δ-9 desaturases are affected by insulin action (Brenner 2003). Moreover, the reduction in Δ-5 desaturase activity, leading to a decrease in long-chain polyunsaturated fatty acid content could affect cell membrane physical properties, potentially leading to altered receptor binding capacities and further impairment of insulin sensitivity (Borkman et al. 1993). On the contrary, Δ-5 desaturase activity is improved by regular exercise training, in parallel with insulin sensitivity (Andersson et al. 2000; Helge et al. 1999). As reported below, bed rest significantly impairs insulin sensitivity and such metabolic alteration is paralleled, in erythrocyte membranes, by a decrease in Δ-5 desaturase activity.

In conclusion, these abnormalities observed after 33 days of bed rest are consistent with results obtained in other insulin resistance conditions, as type 2 diabetes and metabolic syndrome (Borkman et al. 1993) and could contribute to the activation of a pro-inflammatory condition. Moreover, these alterations could, in turn, contribute to metabolic and cardiovascular alterations observed after exposure to unloading.

THE ROLE OF PHYSICAL INACTIVITY ON HYPERHOMOCYSTEINEMIA

Homocysteine is a non-proteinogenic amino acid, involved in methionine metabolism.
High plasma homocysteine levels have been associated to high cardiovascular risk and coronary diseases (Graham et al. 1997; Boushey et al. 1995). Hyperhomocysteinemia, in fact, induces endothelial damage and promotes the development of pro-atherogenic and pro-trombotic profiles (van den Berg et al. 1995). In diagnosed coronary heart disease patients, homocysteinemia is directly related to flogosis and oxidative stress (Jonasson et al. 2005). Oxidative stress could represent the major mechanism of homocysteine-induced cardiovascular alteration. The conversion of homocysteine in homocystine increases reactive oxygen species production (Welch and Loscalzo 1998) and homocysteine *per se* inhibits glutathione peroxidase activity (Nishio and Watanabe 1997) and expression (Upchurch, Jr. et al. 1997). Nevertheless, the increase in homocysteine observed in inflammatory condition (Dudman 1999), associated to homocysteine-induced stimulation of pro-inflammatory and procoagulant thromboxane A2 synthesis (Di Minno et al. 1993), leads to hypothesise that both oxidative stress and inflammation are the mediators of homocysteine deleterious effects on cardiovascular system.
In the present thesis, the net impact of physical inactivity on homocysteine kinetics has been assessed in the frame of the WISE Study, through the approach of isotopic tracers infusions. Two month of bed rest significantly increased plasma homocysteine concentrations in women. Interestingly, in the same subjects, cardiac atrophy was evidenced at the end of the experimental bed rest (Dorfman et al. 2007), further supporting the role of hyperhomocysteinemia as marker of cardiovascular risk. Reported data are in agreement with literature. As previously reported, in fact, sedentary lifestyle is characterised by hyperhomocysteinemia, independently from genetic factors, age, gender or from dietary habits (Dankner et al. 2007); (Nygard et al. 1995). Moreover, regular physical training down-regulates homocysteine concentrations, reducing also cardiovascular risk (Duncan et al. 2004); the same results are also reported in both lean and obese adult subjects after 6 months of regular resistance exercise (Vincent, Bourguignon, and Vincent 2006).
The isotope tracer approach permitted to analyse homocysteine kinetics. Hyperhomocysteinemia, during bed rest, was induced by a decrease in remethylation paralleled by an increase in the transulfuration rate, whereas no significant changes in the transmethylation were detected. To complete understood metabolic abnormalities underlying homocysteine accumulation after prolonged physical inactivity, clearance changes by each disposal pathway were determined. Results evidenced that inactivity-induced hyperhomocysteinemia was the consequence of the reduction of homocysteine catabolism, coupled with unaltered homocysteine synthesis. In fact, homocysteine clearance by remethylation was significantly reduced whereas homocysteine

clearance by transulfuration was unchanged after 2 months of inactivity. Molecular mechanisms leading to hyperhomocysteinemia during inactivity are presently unknown. However, it is possible to hypothesise that bed rest down-regulates the activity or the availability of MTHFR (methylentetrahydrofolate reductase), a key enzyme in remethylation pathway of homocysteine, that is considered the preferential process of homocysteine catabolism (Ueland and Refsum 1989). Moreover, oxidative stress develops in parallel to hyperhomocysteinemia and is considered the mediator of negative effects of homocysteine accumulation on cardiovascular system, as evidenced in endothelial function (van den Berg et al. 1995), vasodilation (Tawakol et al. 1997) and atheromatose processes (Huang et al. 2001). The increase in oxidative stress could also explain the enhanced cardiovascular risk associated to hyperhomocysteinemia. Moreover, evidences in coronary heart disease patients demonstrate that homocysteine accumulation is directly related also to the activation of systemic inflammation (Jonasson et al. 2005). The activation of a low-grade systemic inflammation during bed rest was already demonstrated (Bosutti et al. 2008; Biolo et al. 2008) and confirmed by results reported above, on inactivity-induced changes in membrane fatty acid composition.

As previously reported, homocysteine availability could be influenced by gender and dietary habits. In males, homocysteine concentrations average 5-15 $\mu mol \times L^{-1}$ whereas this range is almost 20% lower in females (Fukagawa et al. 2000). The risk of coronary diseases is usually associated to plasma homocysteine levels greater than 10 $\mu mol \times L^{-1}$ (Aguilar, Rojas, and Collados 2004), stressing the relevance of homocysteine abnormalities observed in females after 2 months of inactivity.

Another important factor influencing homocysteine kinetics is dietary intake of folate and protein (Lee and Frenkel 2003). In order to assess the net impact of physical inactivity on homocysteine, nutrition was strictly controlled during the experimental period. Particularly, levels of protein and folic acid were tailored and monitored to be maintained constant through 60-day bed rest, avoiding contribution of dietary folate and protein availability changes on observed altered homocysteine metabolism following prolonged physical inactivity.

THE ROLE OF PHYSICAL INACTIVITY ON DYSLIPIDAEMIA AND IN CETP AVAILABILITY

Effects of bed rest on dyslipidaemia and CETP concentration were assessed in the Valdoltra Bed Rest Studies.

Dyslipidaemia is known to develop in sedentary condition. As expected, 33 days of bed rest led to increase in triglycerides and decrease in HDL levels. Availability of CETP, a plasma protein transferring cholesteryl esters and triglycerides from HDL to VLDL and LDL, significantly increased following bed rest. Inactivity decreased the ratio between HDL and non-HDL cholesterol. Changes of this ratio inversely correlated with inactivity-mediated changes in CETP concentrations, suggesting that modifications in CETP availability contribute to inactivity-mediated alterations of plasma lipid pattern.

It is well known that obesity and overfeeding are associated with increased CETP levels (Arai et al. 1994) and that body fat mass directly correlates with plasma CETP levels (Arai et al. 1994). On the contrary, energy restriction and fat loss decrease CETP availability (Laimer et al. 2009). To avoid potential interferences of changes in fat mass as well as of over- or under-feeding on CETP synthesis, energy balance was maintained throughout the experimental bed rest and subjects who failed to adapt to decreased energy requirement and increased in fat mass, were excluded by the analysis (6 over 30 analyzed subjects).

Moreover, as reported below, in parallel to altered lipid pattern and CETP availability, insulin sensitivity was impaired, independently from variations of fat mass, as consequence of increased plasma insulin levels. Hyperinsulinemia is known to have direct inhibitory action on CETP activity (Maclean et al. 2001; Arii et al. 1997) and synthesis (Berti et al. 2003). In transgenic mice, insulin availability directly down-regulates hepatic CETP expression (Berti et al. 2003). Moreover, hyperinsulinemia, achieved during hyperinsulinemic-euglycemic clamp studies, significantly suppresses plasma CETP activity in type 2 diabetic patients, in insulin-resistant obese subjects and in lean healthy subjects (Maclean et al. 2001; Arii et al. 1997). In agreement with these previous observations, results obtained in the Valdoltra Bed Rest campaigns evidence that inactivity-mediated changes in insulin concentrations are inversely correlated with changes in CETP concentrations. The stratification of subjects according to the median value of bed rest mediated changes in fasting insulin levels evidences that the group with lower changes in plasma insulin exhibits the greatest increases in CETP concentrations. On the contrary, in the group displaying higher insulin changes, CETP levels did not change significantly following bed rest.

All together, these data lead to conclude that insulin resistance was not responsible for the inactivity-mediated increases of CETP availability reported in our study, whereas, in those subjects

developing insulin resistance following bed rest, compensatory hyperinsulinemia prevented CETP levels to increase. These results may indicate dissociation between insulin actions on glucose and lipid metabolism following inactivity. Moreover, the present results indicate that changes in CETP mass, leading to decreased HDL levels, may proceed independently from insulin resistance development during unloading. This is further supported by the lack of statistical significance in the bed rest × insulin change interactions for the effects of inactivity on triglycerides.

Even though a conclusive mechanistic insights into the effects of physical inactivity on CETP mass can not be provide, it is possible to speculate that muscle unloading have increased CETP expression in skeletal muscle (Tall 1993) through down-regulation of PPAR-α (Allen et al. 2009) and acceleration of intracellular cholesteryl ester flux (Izem and Morton 2001; Chinetti et al. 2003). Previous evidences in fact indicate that exercise training is associated with decreased CETP mass and activity (Maclean et al. 2001; Serrat-Serrat et al. 1993) as well as with increased PPAR-α expression in skeletal muscle (Horowitz et al. 2000).

THE ROLE OF PHYSICAL INACTIVITY AND INACTIVITY-INDUCED INSULIN RESISTANCE ON AUTONOMIC DYSREGULATION

Altered insulin sensitivity after 33 days of bed rest.

Insulin resistance is a metabolic alteration typically observed during physical inactivity as well as in sedentary condition (Alibegovic et al. 2009; Alibegovic et al. 2010; Brower 2009; Hamburg et al. 2007; Biolo et al. 2005; Biolo, Antonione, and De Cicco 2007). Data obtained by reported bed rest studies confirm the development of insulin resistance in healthy young subjects after exposure to prolonged inactivity. Moreover, in the Valdoltra Bed Rest Study 2008, a time-course of insulin resistance development has been performed, using the oral glucose tolerance test procedures. Interestingly, obtained results demonstrated that insulin resistance rapidly and completely developed after 5 days of inactivity and that this metabolic abnormality is than maintained at long term. These results underline an early impairment in insulin activity that could reflect a direct role of insulin resistance on other metabolic alterations observed during unloading.

Autonomic nervous system dysregulation following one month of bed rest: effects of fasting condition and glucose load.

The autonomic nervous system regulation is impaired in subjects affected by the metabolic syndrome, as evidenced by an increase in the sympathetic tone associated to a reduction in the parasympathetic activity (Licht et al. 2010). Interestingly, the degree of autonomic dysregulation is directly related to the number of metabolic syndrome components (Stein et al. 2007; (Lindgren et al. 2006) and sympathetic over-activity *per se* is known to be directly involved in the pathogenesis of the syndrome (Brunner et al. 2002) as well as of several distinct metabolic alterations, including altered blood pressure and plasma lipid pattern (Anagnostis et al. 2009; Tentolouris, Argyrakopoulou, and Katsilambros 2008) as well as impaired insulin sensitivity (Berntson et al. 2008).

Moreover, the level of physical activity modulates autonomic nervous system response, inducing a reduction in sympathetic and an increase in parasympathetic activities (Furlan 1993) (Iellamo et al. 2002), in healthy young (Achten and Jeukendrup 2003) and old subjects (Stein et al. 1999) as well as in patients suffering type 2 diabetes and hypertension (Sridhar et al. 2010). A physiological adaptation to enhanced cardiac work induced by physical exercise has been suggested as linking mechanism between improved autonomic regulation and increased level of physical activity. Regular physical activity reduces systolic and diastolic blood pressures (Sridhar et al. 2010) as well as heart rate whereas exercise improves heart rate variability (Stein et al. 1999; Sridhar et al. 2010)

as well as the high and low-frequency components of heart rate variability (Achten and Jeukendrup 2003).

Systolic and diastolic blood pressures

In agreement with previous reports (Stein et al. 1999; Sridhar et al. 2010), data obtained in the Valdoltra Bed Rest Study 2008 evidenced that prolonged exposure to inactivity determines an increase in the systolic and diastolic blood pressures, as measured by sphygmomanometer, and enhance in heart rate. Alterations in blood pressure can be induced by impaired insulin sensitivity. The vasodilator response to insulin is impaired in insulin resistant states such as obesity and type 2 diabetes, possibly contributing to the development of hypertension in these subjects (Laakso et al. 1990). The strong correlations we observed between bed rest induced changes in indexes of insulin resistance (as insulin AUC index) and systolic blood pressure strongly support this hypothesis.

Heart rate and heart rate variability

One month of unloading leads to increase in heart rate. Such alteration occurs only at the end of the experimental period in the fasting state whereas is evidenced and completely developed after only 5 days of inactivity, after glucose load. The increase in heart rate has been previously demonstrated to be the consequence of the increase in sympathetic and the decrease in parasympathetic tones (Aubert, Seps, and Beckers 2003; Rajendra et al. 2006).

Heart rate variability is not affected by prolonged exposure to unloading. On the contrary, heart rate variability is affected by the association between glucose load and bed rest also after few days of unloading. A decreased in heart rate variability has been previously observed in patients with heart failure and myocardial ischemia (Dekker et al. 2000) as well as in young, middle-age and elderly subjects affected by the metabolic syndrome (Liao et al. 1998; Stein et al. 2007; Koskinen et al. 2009). The stimulatory effect of glucose load in insulin release and action could evidence precocious or small alterations induced by inactivity-induced insulin resistance on autonomic regulations. The reduction of heart rate variability, in fact, has been demonstrated in hyperglycaemic condition (Singh et al. 2000) and during a glucose load, the impairment in insulin sensitivity is stressed.

Finally both the components of heart rate variability are affected following 33 days of bed rest. The HF component is significantly decreased, evidencing a decrease in the vagal tone. On the contrary, the LF component is enhanced, indicating an over-activation of the sympathetic nervous system as well as an unbalance between sympathetic and parasympathetic systems activity on heart rate. In accordance, the LF-to-HF ratio, index of sympathovagal balance influence on heart rate control (Pagani et al. 1986) is more than duplicate at the end of the experimental period, indicating an higher sympathetic activation (Malliani 1999; Heart rate variability. Standards of measurement,

physiological interpretation, and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology1996). As previously demonstrated, insulin resistance is directly associated to the LF-to-HF ratio independently from body mass and, in particular, body fat (Emdin et al. 2001).

Baroreflex sensitivity

Baroreceptors activity is strictly related to autonomic adaptation to everyday living environmental perturbations (Eckberg et al. 1992). Baroreflex sensitivity reflects reflex vagal activity that can be evaluated through the use of two main indexes, the baroreflex sensitivity index, as assess by the sequence technique (Di Rienzo, Mancia, and Pedotti 1985; Bertinieri et al. 1988), and the α-index, defined considering fluctuations in RR interval and blood pressure. Both indexes provide similar results. A decrease in baroreflex sensitivity reflects a depression of reflex vagal activity and impairment in body response to external input changes. Moreover, a reduction in baroreflex sensitivity has been reported in subjects suffering for the metabolic syndrome. Moreover, the degree of reduction in baroreflex sensitivity directly correlated to the number of metabolic syndrome components (Stein et al. 2007; (Lindgren et al. 2006). A decrease in baroreflex sensitivity has been previously reported in resting condition, as evidenced after 5 days of spaceflight (Fritsch et al. 1992) as well as in insulin resistant states (Kuusisto et al. 1994) (Smith 1599-601;Pikkujamsa et al. 523-31). Moreover, a decrease in baroreflex sensitivity has been previously related to enhanced cardiovascular risk in healthy population (Tsuji et al. 1994) and to increased adverse event risk in subjects affected by several cardiovascular diseases (La Rovere et al. 1998; Airaksinen et al. 1998). In accordance with these observations, in our study, baroreflex sensitivity was significantly reduced following inactivity. Such alteration, evidenced by decrease in both baroreflex sensitivity indexes, rapidly developed after only 5 days of bed rest and then was maintained at long term. A rapid impairment in baroreflex sensitivity in inactivity condition, has been already reported (Fritsch et al. 1992). Baroreflex in fact plays a key role in cardiovascular response to exercise (O'Leary 1996; Iellamo 2001) and regular physical activity is known to ameliorate baroreflex sensitivity in type 2 diabetes (Loimaala et al. 2003). Even though a direct cause-effect relationship between physical activity level and baroreflex response has not been clarified, few hypothesis has been suggested. First, increase in muscle mass, following a period of regular training program, ameliorates glycaemic control glucose tolerance and is directly correlated with baroreflex sensitivity in diabetic subjects (Loimaala et al. 2003). These evidences suggest that exercise-induced improvement of autonomic function could be related to improved glucose tolerance (Loimaala et al. 2003). Our results indicate that increased plasma glycaemia decreases baroreflex sensitivity, as evidenced by the decrease in baroreflex sensitivity after glucose load. However, we observed the same effect also

in ambulatory condition, when subjects were still physically active. Moreover, prolonged bed rest determines an impairment in insulin sensitivity whereas does not have effects on plasma glucose levels. Another possible hypothesis explaining the activity-induced increase in baroreflex sensitivity is that the repeated activation of the baroreflex and cardiovascular end-organ responses induced by regular training could improve baroreflex sensitivity. This is supported also by the observation that the decrease in baroreflex sensitivity induced by microgravity is restored few days after the end of the spaceflight (Cooke et al. 2000). The absence of repeated baroreflex activation and exercise-related cardiovascular end-organ responses could explain the reduction in baroreflex response we observed in healthy young subjects after only 5 days of inactivity.

Autonomic nervous system dysregulation and insulin resistance after one month of physical inactivity.

Our results indicate that exposure to physical inactivity induces an impairment in autonomic nervous system regulation, characterised by an increase in sympathetic and a reduction in the parasympathetic tones. These alterations are the same observed in subjects suffering the metabolic syndrome (Licht et al. 2010). In our study, altered insulin sensitivity induced by physical inactivity could be strictly related to changes in autonomic regulation we observed. In fact, strong correlations have been evidenced between indexes of autonomic regulation and of insulin resistance. Nevertheless, we demonstrated that insulin resistance completely develops in the early phase of unloading and that such alterations are maintained at long term. Analogously, some autonomic regulation indexes are affected by inactivity following the same trend that insulin sensitivity. Sympathetic nervous system activation could play an important role in insulin resistance development (Mancia et al. 2007); in fact, sympathetic activity inhibits insulin release, reducing thus glucose uptake in peripheral tissues and stimulating hepatic gluconeogenesis (Nonogaki 2000; Fehm, Kern, and Peters 2006). Otherwise, insulin centrally stimulates the sympathetic nervous system (Muntzel et al. 1994), as evidenced during physiological and pharmacological hyperinsulinemia (Scherrer et al. 1993; Vollenweider et al. 1993), both in obese (Reaven, Lithell, and Landsberg 1996) and lean healthy subjects (Hausberg et al. 1997; Paolisso et al. 2000). Additionally, when elevated but physiological plasma insulin levels are maintained at long time (36 hours), a decrease in parasympathetic nervous system activity occurs. Nevertheless, mechanisms responsible for the interaction between insulin sensitivity and autonomic nervous system regulation are not elucidated. Moreover, further analyses are required to define a cause-effect relationship between inactivity-induced autonomic dysregulation and inactivity-induced insulin resistance.

CONCLUSION

In the present thesis the net impact of physical inactivity on the development of different component of the metabolic syndrome has been evaluated.

To reach this aim, the experimental model of bed rest in young healthy volunteers has been employed, allowing to exclude the potential interferences of confounding factors such as aging and diseases. Moreover energy balance has been strictly monitored and subjects were maintained in a near neutral balance to eliminate the impact of altered nutritional status.

Presented results evidenced that physical inactivity *per se* directly interfere with human physiology and metabolism, leading to muscle atrophy as well as to a pattern of metabolic alterations similar to that observed in subjects affected by the metabolic syndrome.

Our data indicate that a drastic reduction in physical activity is *per se* sufficient for the development of (a,b) insulin resistance and altered autonomic nervous system regulation, occurring in the first phases of exposure to unloading; (c) oxidative stress at muscle level; (d) systemic inflammation; (e) dyslipidaemia and (f) hyperhomocysteinemia. Furthermore, oxidative stress and inflammation as well as dyslipidaemia and hyperhomocysteinemia are known to increase the risk of cardiovascular diseases.

These results further stress the importance of regular physical activity in the population, independently from age and pathological condition. Moreover, these data suggest that exercise training is an optimal choice in care strategy in sedentary or hospitalized patients as well as in the prevention of several metabolic alterations.

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