UNIVERSITAT DE BARCELONA

Regulation of amino acid catabolism in rats fed diets with different protein content

Silvia Agnelli

UNIVERSITAT DE BARCELONA

Regulation of amino acid catabolism in rats fed diets with different protein content

Silvia Agnelli

Barcelona, 2016

UNIVERSITAT DE BARCELONA

PROGRAMA DE DOCTORAT EN ALIMENTACIÓ I NUTRICIÓ

Regulation of amino acid catabolism in rats fed diets with different protein content

Memòria presentada per Silvia Agnelli per optar al títol de doctor per la Universitat de Barcelona

Els Director/s

Dr. Xavier Remesar

Catedràticde Nutrició i Bromatologia

Dr.Marià Alemany Lamana

Catedràtic de Bioquímica i Biologia Molecularia

La doctoranda

Silvia Agnelli

Barcelona, 2016

Alla mia Famiglia :

per la pazienza e l'amore infinito

"Dubium sapientiae initium"

René Descartes

Meditationes de prima philosophia

Abbreviations

ATF: Activating transcription factor 4
AP-1: Activator protein-1
ARG: Arginase
ASL: Argininosuccinate lyase
ASS: Argininosuccinate synthase
BAT: Brown adipose tissue
BCAA: Branched-chain amino acids
BiP: Immunoglobulin binding protein
BMI: Body mass index
CAFD: Cafeteria diet
CPS: Carbamoyl-phosphate synthase
DAG: Diacylglicerol
eIF2α: Eukaryotic translation initiation factor 2
eNOS: Vascular endothelial constitutive NOS
ER: Endoplasmic reticulum
GADD34: Growth arrest and DNA damage- inducible protein
GLUT-4: Glucose transporter 4
GOT: Glutamic oxaloacetic transaminase
GPT: Glutamate-pyruvate transaminase
HDL: High density lipoprotein
HFD: High fat diet
HPD: High protein diet
IKK: Inhibitor of κ kinase
IL: Interleukin
IMCL: Intramyocellular lipid
iNOS: Inducibile NOS
IR: Insulin resistance
IRE1: Inositol requiring 1
IRS: Insulin receptor substrate
JNK:c-jun N-terminal kinase
LPS: Lipopolisaccharide
MS: Metabolic sindrome
NAFLD: Non-alcoholic fatty liver disease

NF-κB: Nuclear factor κ-light-chain-enhancer of activated β cells
nNOS: Neuronal or brain constitutive NOS
NO·: Nitric oxide
NOS: Nitric oxide synthase
NRF2: Nuclear factor erythroid 2
OTC: Ornithine transcarbamylase
PERK:PKR-like ER kinase
PKC: Protein kinase C
PP1: Protein phosphatase 1
SCFA: Short chain fatty acids
SDH:Serine dehydratase
SFA: Saturated fatty acid
T2DM: Type 2 diabetes mellitus
TAG: Triacylglycerol
TLR: Tool-like receptor
TNFα: Tumor necrosis factor
TRAF2: Tumor necrosis factor receptor-associated factor 2
UC: Urea cycle
UPR: Unfolded protein response
VLDL: Very low-density lipoprotein
WAT: White adipose tissue
WHO: World Health Organization
XBP1:X box-binding protein 1

Abstract

Current lifestyle with high-energy diets and characterized by sedentary is triggering an alarming growth in obesity. Obesity along with metabolic syndrome-related co-morbidities (i.e. insulin resistance, atherosclerosis, sleep apnoea, depression, asthma, hypertension and the alteration of blood lipid transport) are the most apparent consequence of the excess energy.

Under conditions of excess dietary energy, the body cannot easily dispose of the excess amino-N against the evolutively-adapted schemes that prevent its wastage; thus ammonia and glutamine formation and urea excretion are decreased. High lipid and energy availability limit the utilisation of glucose, and high glucose spares the production of ammonium from amino acids, decreasing the synthesis of glutamine and its utilisation by the intestine and kidney. In contrast, high protein diets enhance protein synthesis and growth, and the synthesis of non-protein-N-containing compounds. But these outlets are not enough; consequently, less-conventional mechanisms are activated, such as increased synthesis of NO·followed by higher nitrite (and nitrate) excretion and changes in the microbiota.

In this study we studied how the initial phase of development of metabolic syndrome can affects the function of liver as main site of amino-N metabolism, and to determine whether doubling the protein content in the diet induced significant changes in enzyme of amino acids metabolism along intestine and on liver.

The common result obtained by these studies is that, both in case of hyperlipidic or hyperproteic diets, elimination of excess N is necessary but cannot be easily carried out through the metabolic pathways/tissues we evaluated, although possible alternative pathways have been taken into consideration.

Contents

List of tables and figures

1. INTRODUCTION

1.1 The metabolic syndrome

The *metabolic syndrome* (MS) is a widely recognized clustering of components including insulin resistance (IR)/diabetes, hyperlipidemia, arterial hypertension and obesity, often associated with hyperuricemia, small dense LDL, endothelial inflammation[1,2,3], hypoandrogenism, depression, hepatic steatosis, hyperuricemia[4], and sleep apnoea[5], even if the number of diseases that may fall under the umbrella term of MS grows rapidly[3,6]. This cluster of interrelated biochemical, endocrine and pathological disorders is mainly a consequence of social development[7] and it is probably due, in a large proportion, to the access to rich, abundant and varied food, to sedentary lifestyle, and to the other perks of modern-day life in most human societies[2]. In fact, adult humans are unprepared for this high-load of nutrients and cannot process the excess food ingested because our cortical-derived desire overrides the mechanisms controlling appetite[3]. Indeed, this set of disorders is due not because we lack the biochemical mechanisms to use this excess of energy, but rather, because we are unprepared for excess and adapted to survive scarcity[8]. Furthermore, in condition of high load of nutrients, the ammonium generation and transfer processes[9], another key pathogenic element of MS, don't work properly due to our tendency to preserve amino-N for the food shortage period[10,11].

Beside of environmental and psychosocial, increasing evidence suggests that there is genetic and epigenetic basis for MS[3,12,1].

The concept of the MS has existed for, at least, 100 years. It was first described, independently, in the 1920s by two physicians, as the clustering of hypertension and diabetes mellitus type 2 (T2DM)[13,14]. Then, in the 1960s, several scientists, working independently in different countries, have published their observations on the clustering of various components of the metabolic syndrome, hyperlipidemia, obesity and T2DM, hypertension, and coronary artery disease, giving several names to it[15,16,17] as shown in Table 1. In the early 1980s, Hanefeld and Leonhardt[18] introduced the term metabolic syndrome (*metabolischesSyndrom*) which included T2DM, hyperinsulinaemia, obesity, hypertension, hyperlipidemia, gout, and thrombophilia. In the late 1980s, Gerald M. Reaven[19] hypothesized that IR was the common etiological factor for a group of disorders, consisting of impaired glucose tolerance, hyperinsulinaemia, high levels of very low-density lipoprotein (VLDL), low levels of high-density lipoprotein (HDL) and hypertension. Reaven named the sum of these disorders S*yndrome X* in an attempt to stress its unknown aspects.

Terms	Author
Hypertension–hyperglycaemia–hyperuricaemia syndrome	Kylin, 1923
Metabolic trisyndrome (trisyndromemetabolique)	Camus, 1966
Plurimetabolic syndrome	Avogaro and Crepaldi, 1967
Syndrome of affluence (wohlstandssyndrom)	Mehnert and Kuhlmann, 1968
Metabolic syndrome (metabolischesyndrom)	Hanefeld and Leonhardt, 1981
Syndrome X	Reaven, 1988
Deadly quartet Insulin	Kaplan, 1989
Insulin resistance syndrome	DeFronzo and Ferrannini, 1991 - Haffner, 1992

Table 1. Names given to the clustering of metabolic syndrome disorders. Adapted from Sarafidis et al. [20]

In the same period, Kaplan[21] renamed the syndrome *the deadly quartet* for the combination of upper body obesity, glucose intolerance, hypertension and hyperlipidemia. During the following years, among the most active researchers in the field, the term *insulin resistance syndrom*[22], *dysmetabolic syndrome*[23,24] and *metabolic syndrome*[2,25] was used to describe this entity, considering IR as the key element defining of the syndrome.

As the absence of an international explanation for the syndrome, in 1998, WHO[26] attempted to define the MS, looking for a working definition that would be open to possible future changes according to new evidences. WHO determined a number of criteria corresponding to the major characteristics of the syndrome. In particular, the main proposed component of the syndrome was IR. Apart from that, the diagnosis of the syndrome required the presence of at least two of the variables: hypertension, elevated triacylglycerols (TAG), low HDL-cholesterol, obesity, and microalbuminuria[27]. Since then, there have been several attempts to define the MS[28,29,30], even if they should not be considered as strict classifications but rather as working definitions that help towards the formulation of a complete and globally accepted definition of the syndrome.

1.1.1 The insulin resistance

The most accepted and unifying hypothesis to describe the pathophysiology of the MS is IR[2,31]. IR is characterized by decreased sensitivity to glucose and low peripheral glucose uptake, which often develops into full T2DM[32,35].

Despite periods of feeding and fasting, plasma glucose remains in a narrow range - between 4 and 7 mM in normal individuals[36] - governed by the balance between glucose absorption from the intestine, production by the liver and uptake and metabolism by peripheral tissues. Insulin increases glucose uptake in muscle (up to 75%)[36] and white adipose tissue (WAT)[34,37], and inhibits hepatic glucose production, thus working as the primary regulator of blood glucose concentration.

Insulin promotes glucose uptake by stimulating the translocation of the glucose transporter 4 (GLUT4) from intracellular sites to the cell surface[38]. Animal studies have demonstrated that insulin resistance is associated with a decreased translocation of glucose transporters to the plasma membrane in muscle cells and an attenuate expression of GLUT4 in muscle cells[39]. Insulin also stimulates cell growth and differentiation, and promotes the storage of substrates in WAT, liver and muscle by stimulating lipogenesis, glycogen and protein synthesis, and inhibiting lipolysis, glycogenolysis, gluconeogenesis, and protein breakdown (Figure 1). Figure 1 Furthermore, insulin has an effect in cells not normally considered insulin sensitive, including neurons[40,41], pancreatic islet cell[42,43] and red blood cells[44].

When normal circulating levels of insulin can't regulate properly all these processes in organs and tissues, like liver, skeletal muscle and WAT, IR or deficiency occurs and producing raising in fasting and postprandial glucose and lipid levels[36]. Thus, by definition, IR is a defect in signal transduction[38] and its aetiology is still unclear[32]. In condition of IR, glucose entrance to muscle is minimized and decreased blood flow constrains glucose from adipose tissue uptake, thus most of the remaining glucose could only be used in significant amounts by brown adipose tissue (BAT), by the liver, by the intestine and probably by the microbiota[45]. BAT uses glucose to sustain thermogenesis and for lipid storage, even if this way may represent a significant possibility for rodents, but for humans is limited due to a smaller BAT presence[46]. Liver uses the glucose for lipid synthesis[47] and energy utilization, including thermogenesis. Lipogenesis has to be carried out because of an already large excess of dietary fatty acids and TAGs, temporarily stored in the liver, are waiting for their eventual release as VLDL. But in the same time, the process is limited by the

already excessive availability of acetyl-CoA, because the high levels of glucose fully inhibit the ketone pathway[48]. Other possible pathways for glucose disposal include glucose diffusion across the intestinal wall where it is taken up and metabolizated by microbiota, thus participating in the handling of excess substrate.

In the past decades, researchers tried to define the biological mechanisms that lead to the diseased state. These were recognized in lipid accumulation, systemic inflammation, activation of the unfolded protein response due to the endoplasmic reticulum (ER) stress and change of gastrointestinal microbiota[37,35]. Although other additional mechanisms have been considered, these four represent different aspects of metabolic control that ultimately may converge on common pathways to regulate insulin action.

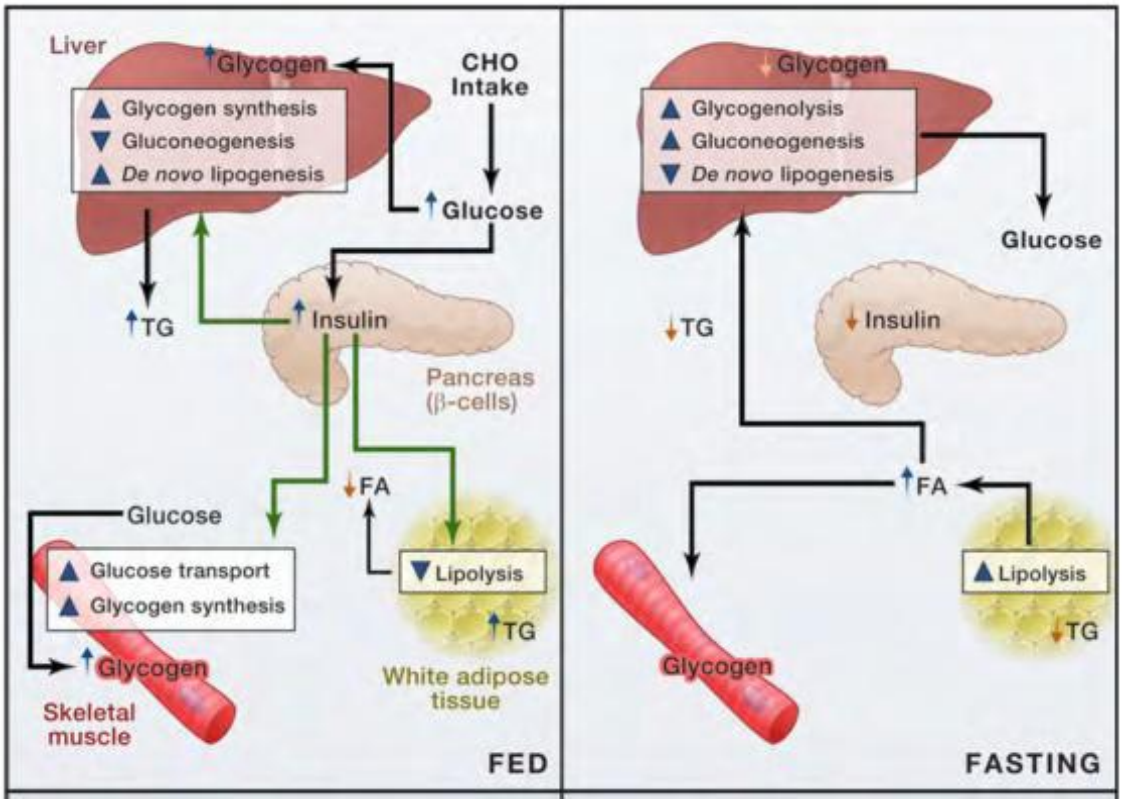


Figure 1. Insulin Action.

In the fed state, dietary carbohydrate (CHO) increases plasma glucose and promotes insulin secretion from the pancreatic β cells. Insulin has numerous actions to promote storage of dietary calories, but only some are illustrated here. In the skeletal muscle, insulin allows glucose entry and glycogen synthesis. In the liver, insulin promotes glycogen synthesis and de novo lipogenesis and inhibits gluconeogenesis. In the adipose tissue, insulin suppresses lipolysis and promotes lipogenesis. (Right) In the fasted state, insulin secretion is decreased. The drop in insulin serves to increase hepatic gluconeogenesis and promote glycogenolysis. Hepatic lipid production diminishes while adipose lipolysis increases. Adapted from Samuel et al.[37]

Lipid accumulation

The metabolic alterations induced by insulin resistance are a direct consequence of the excess availability of dietary lipid responsible of the accumulation of fat in a number of locations of WAT.

Most of the fat is contained in large vacuoles[49] within specialised cells, the adipocytes; the function of this depot is to supply energy to the whole organism under conditions of scarcity of energy[50], even if they are contained in other cells[51], where they are used for their own metabolism. The total lipid content in a rat (i.e. Wistar strain) is in the range of 10-15% of body weight[52], irrespectively of dietary lipid and energy intake; half of these lipids are stored in WAT[53] and the other half are stored, mainly as TAG[52], in other tissues such as skeletal muscle[54], liver[37], heart[49] to carry out other functions: muscle contain adipose tissue, interspersed in its fibres[52], directly involved in supplying energy during exercise, liver contains a sizeable proportion of lipid directly related to the production and export of energy through lipoproteins[55], heart store TAG mainly as an important cell-autonomous energy source[56]. These depots sites in storage-specialized (adipose) or not-specialized (liver, muscle, heart) tissues reacted increasing their size in parallel in front of a hyperlipidic diet[52]. The storage of fat in adipose and in non-adipose tissues may lead to an imbalance in lipid homeostasis and inducing toxicity in the tissue, a phenomenon termed lipotoxicity[49]. The relationship between the lipid accumulation in non-adipose tissues and the development of IR is depicted in Figure 2.

Skeletal muscle is extremely important for the development of systemic IR because it is the tissue in which most of the insulin-dependent disposal of glucose occurs. Mounting evidence indicates that elevated intramyocellular lipid (IMCL) concentrations are associated with diminished insulin sensitivity in skeletal muscle[57]. This lipid accumulation is not only likely due to enhanced fatty acid uptake into the muscle, but also to a diminished mitochondrial lipid oxidation[57]. However, the mechanism by which increased IMCL accumulation induces insulin sensitivity in this tissue is still debated. During obesity, IMCL and bioactive IMCL metabolites (e.g. ceramides, diacylglicerol (DAG) and long-chain fatty acyl-CoAs) accumulate and may cause cellular dysfunction and IR[54].

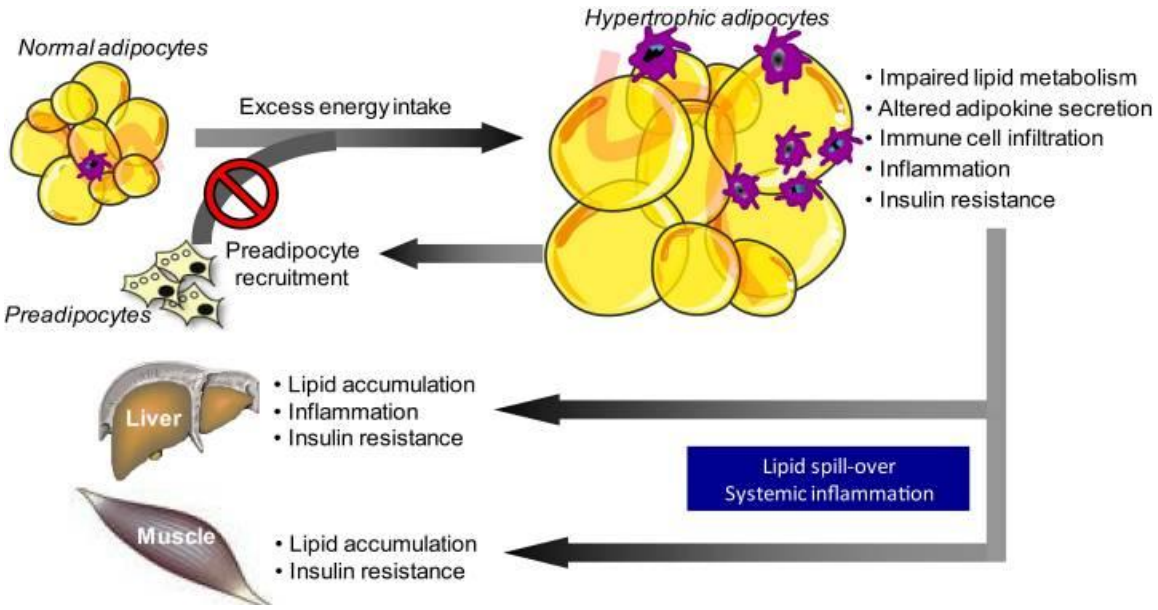


Figure 2. Lipid accumulation in adipose and non-adipose tissues

A rich diet, leading to body weight gain, will increase adipocyte size. Adipocyte hypertrophy in obesity is accompanied by disturbances in lipid metabolism and alterations in adipokine secretion, which a shift toward a pro-inflammatory phenotype. The secretion of pro-inflammatory factors is further boosted by the infiltration of several adaptive and innate immune cells into the adipose tissue in obesity. Together, the impairments in lipid metabolism and the secretory function of adipose tissue not only induce insulin resistance locally within the tissue (via autocrine/paracrine effects) but also have detrimental effects at the whole-body level. The reason for this is that adipose tissue dysfunction in obesity is accompanied by lipid spill over in the circulation and subsequent lipid accumulation in non-adipose tissues. Adapted by Goossens et al.[58]

When DAGs accumulate, activate protein kinase C (PKC) that trigger a serine/threonine kinase cascade, leading to phosphorylation of serine/threonine sites on insulin receptor substrates (IRS-1, IRS-2). This reduces the ability of the IRS to activate phosphatidylinositol 3-kinase, which ultimately results in a reduced GLUT4 translocation to the cell membrane (Figure 3). In this way, increased DAGs concentrations may decrease insulin-stimulated muscle glucose uptake[59,54]. Instead, high levels of ceramides, potent lipid-signalling molecule, can cause IR by inhibiting the ability of insulin to activate the insulin signal transduction pathway and/or via the activation of c-Jun N-terminal kinase (JNK). JNK is responsible of the serine phosphorylation of IRS-1, rather than the normal tyrosine phosphorylation. Serine phosphorylation impairs insulin signaling and causes IR[60].

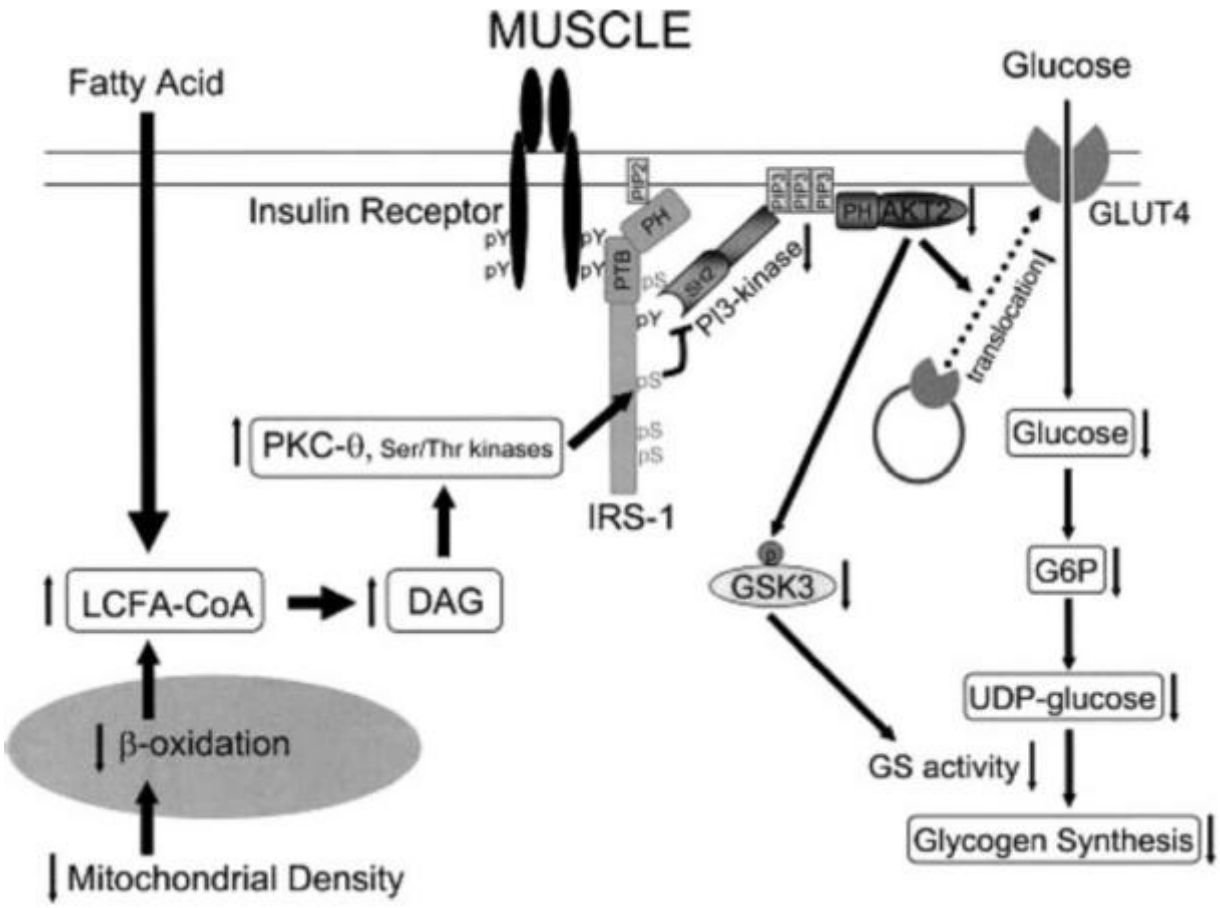


Figure 3. Molecular mechanisms of skeletal muscle insulin resistance

Increases in intramyocellular fatty acyl CoAs and diacylglycerol due to increased delivery from plasma and/or reduced oxidation due to mitochondrial dysfunction trigger a serine/threonine kinases cascade initiated by novel protein kinase C. This ultimately leads to activation of serine residues on IRS-1 and inhibits insulin-induced PI3-kinase activity, resulting in reduced insulin-stimulated muscle glucose transport and reduced muscle glycogen synthesis. DAG, diacylglycerol; GSK3, glycogen synthase kinase-3; PH, pleckstrin homology domain; PI3, phosphatidyl inositol 3-kinase; PTB, phosphotyrosine binding domain. From Jornayvaz et al.[61]

Similar to what just described for skeletal muscle, lipid can accumulate in hepatocytes when IR occurs. Liver receives via portal vein, most of the nutrients extracted from the diet and already shifted out by other splanchnic bed organs, especially the intestine and, probably, mesenteric adipose tissue[62]. In any case, liver controls the flow of glucose into the systemic blood, and detains or processes many amino acids and short-chain fatty acids. In condition of excess of nutrients and of a failure of the insulin system, liver may loss its functionality often provoking non-alcoholic fatty liver disease (NAFLD)[63], associated to insulin resistance. In detail, accumulation of lipid in liver

impairs the ability of insulin to regulate gluconeogenesis and activate glycogen synthesis while lipogenesis remains unaffected[37]. However, it is not completely known how liver steatosis leads to insulin resistance. Likely, very similarly as in skeletal muscle, not the TAG per se, but the lipid intermediates (such as DAGs) are important for the development of IR. Hepatic insulin resistance is also associated with increased insulin plasma concentrations, which in turn stimulates de novo lipogenesis and therefore enhances liver steatosis further. These data indicate that liver steatosis results in a vicious cycle by causing insulin resistance, which again promotes hepatic lipid accumulation.

Inflammation

Inflammation is part of the complex biological response of body tissues, organs or organism to harmful stimuli, such as pathogens, damaged cells, or irritants. The response is mainly performed by immune system agents (cells and proteins), especially against whole organisms, proteins, or large materials susceptible to becoming allergens, that is, parts of invading extraneous materials or organisms. Other locally derived responses are generated from the site of aggression, such as the emission of signalling compounds (histamine[64] and eicosanoids[65] and cytokines[66]) and nervous system signals (pain, itching, heat, and hypersensitivity)[67]. Blood flow and capillary permeability play key roles in the response to localized aggression by causing inflammation[68].

The mechanism of inflammation was described in I century AD by Aulus Cornelius Celsus in his medical work *De Medicina*. In this treatise, he explains the classic signs of acute inflammation (from inflammare, to set on fire) as *rubor* (redness), *turgor* (turgidness, swelling), *calor* (heat), and *dolor* (pain); only one century later, Aelius Galenus identified *functio lesa* (loss of function) as the fifth sign of the acute inflammation. Redness and heat are consequences of increased blood flow, and turgidness is a sign of edema, that is, altered capillary permeability that does not allow for sufficient venous return of fluid, increasing the extracellular (interstitial and plasmatic) space. Pain is largely a consequence of histamine and cytokine release by affected tissues[69]. Inflammation typically ceases within a relatively short time with the elimination of causative agents; but, as well, it may become chronic when elimination is not complete and the defensive activity of the immune system becomes generalized[68].

The classical inflammation characteristic just described are not coincident with that found in the WAT in MS because the massive incorporation of immune cells into tissue, secretion of cytokines,

relative hypoxia, and modification of cell metabolism are not accompanied by an increase in blood flow, by an increased endothelial permeability to water, by the appearance of edema, or by an altered lymph recovery[68]. In fact, the presence of edema is a secondary consequence of altered blood pressure and endothelial damage, but it is not centred in the WAT. The inflammation that characterizes MS is rather a permanent low-level inflammation and it's not derived from a previous acute phase, but is caused by the inability of the body to cope with the continuous influx of excessive energy substrates. Inflamed WAT responds to the 'invasion' of nutrient availability by reducing its blood flow[70,71], thereby decreasing the availability of substrates, but also causing increased generation of oxidative free radicals[72] and generalized hypoxya[73] that results in the glycolytic anaerobic production of lactate and acidosis[74].

Most researches have been recognized the endothelium as the primary tissue where inflammation takes place[75], (mainly) in the liver, WAT, muscle, intestine, and brain. Inflammation is probably the consequence of a breach in homeostasis (i.e. oxygen or nutrient availability and remote or paracrine signalling) that endangers the cells forming the vessel itself.

The initial step of inflammation probably occurs in the WAT as cannot absorb, manage and eliminate an overload of nutrients. This state of suffering and tissue stress is signalized with the production of cytokines such as tumor necrosis factor (TNF-α) and interleukin 6 (IL-6) that elicits the incorporation of immune system cells such as macrophages[76,77]. Macrophage activation enhances the secretion of proinflammatory cytokines and increases IR[78] and TG accumulation[79]. TNF-αsignaling activates intracellular kinases, such as JNK and IkB kinase (IKK), which lead to increased serine phosphorylation of IRS-1, rather than the normal tyrosine phosphorylation causing impairment in insulin signaling. TNF-α and IL-6 also activate the Protein-1 (AP-1) and nuclear factor κ-light-chain-enhancer of activated βcells (NF-κB) which cause increased production of proinflammatory cytokines, thus triggering inflammation[80]. The cytokines can have a paracrine effect, but they can also be secreted into plasma and affect other organs. In Figure 4 are depicted the hallmarks of obesity-induced inflammation in adipocytes.

Another mechanism leading to insulin resistance and thus to inflammation comes from the action of the saturated fatty acids (SFA). SFA activate the tool-like receptor 4 (TLR4), an important receptor of the innate immune system up-regulated in case of inflammation, that induces activation of JNK and IKK. In turn, JNK and IKK up-regulate NF-κB and AP-1[81,82].

Immune system intervention in inflammatory process provokes a glucorticoid response[83] that diminish inflammation caused by immune cells but in the mean time alters not only the response of the insulin system[84] but also the handling of amino acids[85] and liver glucose output[85], enhance overall lipogenesis and fat deposition[85], bone loss/osteoporosis[86], and, in the medium to long terms, interference with estrogens and androgens[87].

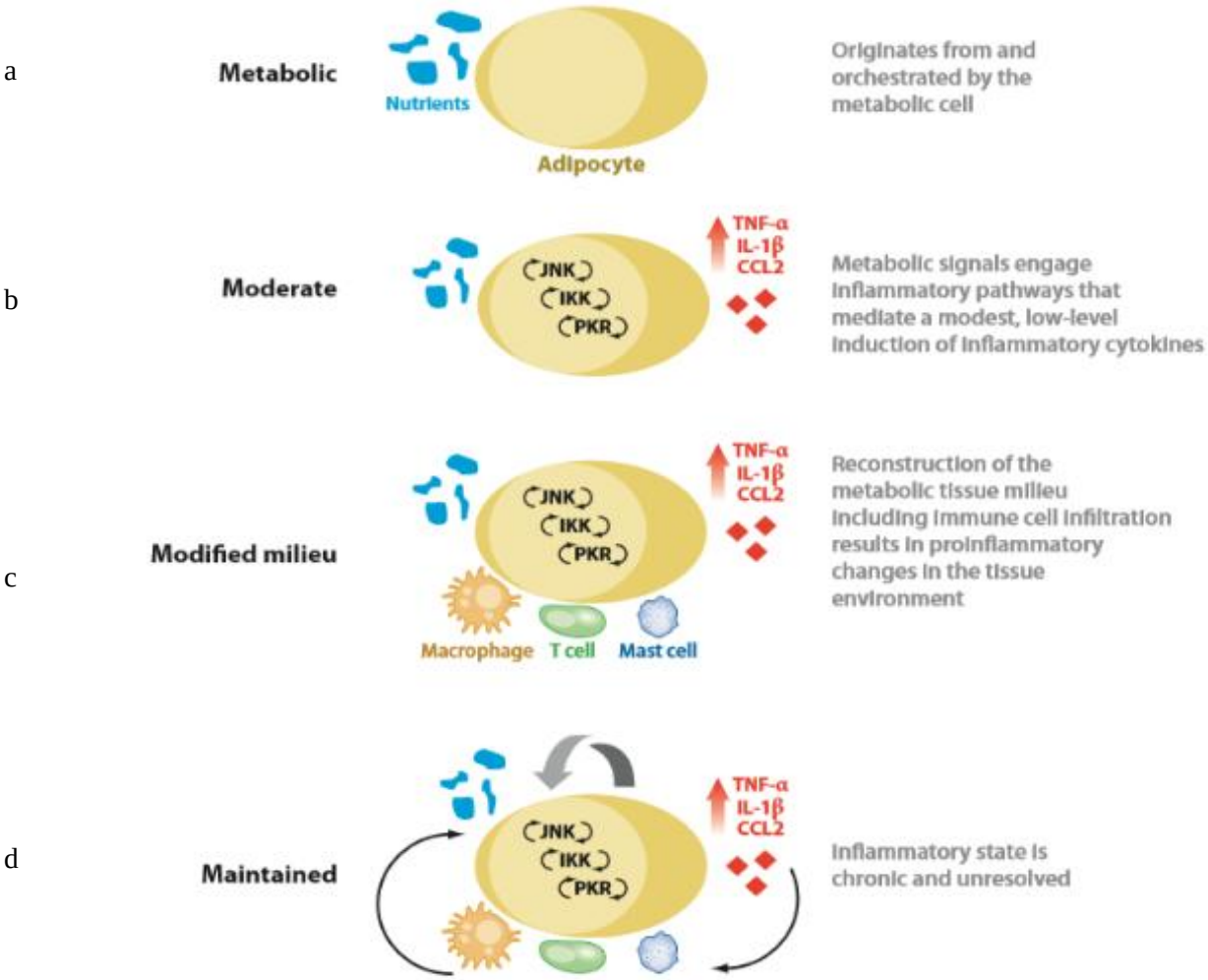


Figure 4. Hallmarks of metabolic inflammation in adipocyte

The hallmarks of obesity-induced inflammation are that it (a) is metabolic—a nutrient-induced inflammatory response orchestrated by metabolic cells; (b) is moderate—low-grade and local expression of inflammatory mediators induced by stress sensors such as IKK and JNK; (c) creates a modified milieu—altered composition of immune cells favouring a proinflammatory tissue environment; and (d) is maintained—chronic maintenance of the inflammatory state without apparent resolution. (Abbreviations: IKK, inhibitor of κkinase; JNK, c-jun N-terminal kinase; PKR, protein kinase R; TNF-α, tumor necrosis factor-α, IL-1β interleukin 1β, CCL2, chemokine (C-C motif) ligand 2). Adapted from Gregor et al.[79]

Another major metabolic organ where inflammatory pathways interfere with normal metabolism and disrupts proper insulin signaling is the liver. In contrast to adipose tissue, the liver does not goes through an infiltration of macrophages during the onset of obesity but instead undergoes an activation of inflammation within cells of the liver[69,81], including the resident macrophage-like Kupffer cells[79] and a substantial gathering of hepatic recruitment of $CCR2^{+}$myeloid cells that promote hepatosteatosis[88]. In animal models of obesity, inflammatory cytokine expression is increased in the liver compared with lean controls[33]. Indeed, obesity is associated with hepatosteatosis that often leads to the more advanced inflammatory state of steatohepatitis. As already described above, inflammatory mediators, especially activation of the Nuclear factor κ-light-chain-enhancer of activated β cells (NF-κB) pathway, have the ability to inhibit insulin signaling. An operative insulin signaling is responsible to inhibit gluconeogenesis but in obese conditions this regulation is lost and hepatic glucose production subsequently contributes to hyperglycaemia. JNK kinase is also activated in the liver during obesity, causing glucose intolerance and increased glucose and lipid production in the liver. More inflammatory mediators and acute-phase reactants activated by inflammation are serum levels of cytokines such as CRP, PAI-1, serum amyloid A, and IL-6[78]. Some of these cytokines have been shown to have adverse metabolic effects on peripheral organs[79], so that the liver inflammatory mediators in obesity may be a strong contributor to the malfunction of peripheral tissues under such nutrient excess. If the inflammation in the liver escalates, cells may die, resulting in the recruitment of immune cells and in steatohepatitis[63]. Thus, the liver remains an important site where metabolic and immune cell signals merge to limit the tissue's response to nutrients.

ER stress and unfolded protein response

During the last decade, ER stress has emerged as a new player in the onset of insulin resistance. Once more time, the excess of substrate availability and deficit of oxygen (hypoxia) provoke a defective protein synthesis that fuels proteasome activity. Moreover, ER stress appears to act directly as a negative modulator of the insulin signaling pathway but also indirectly by promoting lipid accumulation[89].

ER is a multifunctional organelle essential for the synthesis, folding, and all processing of secretory and transmembrane proteins[90]. Environmental (calcium or other ions, excess or lack of

nutrients, infection, changes in the redox state[68]), pathogenic (decreased adipose tissue blood flow responsible of hypoxia[71]), and genetic factors can disrupt ER function causing an accumulation of misfolded and unfolded proteins in the ER lumen, a condition termed ER stress.

ER stress activates a signaling network called the Unfolded Protein Response (UPR) to alleviate this stress and restore ER homeostasis, promoting cell survival and adaptation. However, under irresolvable ER stress conditions, the UPR promotes apoptosis. In higher eukaryotes, the UPR is initiated by three ER transmembrane proteins: Inositol Requiring 1 (IRE1), PKR-like ER kinase (PERK) and Activating Transcription Factor 6 (ATF6). During unstressed conditions, the ER chaperone, immunoglobin binding protein (BiP) binds to the luminal domains of these master regulators keeping them inactive. Upon ER stress, BiP dissociates from these sensors resulting to their activation[91]. Upon ER stress, PERK phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α) causing a reduction of mRNA translation and thus decreasing the load of new proteins in the ER, phosphorylation of eIF2α by PERK allows the translation of activating transcription factor 4 (ATF4) that induces transcription of genes involved in amino acids synthesis and apoptosis. PERK also phosphorylates the nuclear factor erythroid 2 (NRF2), an antioxidant-response transcription factor. Phosphorylation of eIF2α by PERK promotes the expression of the growth arrest and DNA damage-inducible protein (GADD34) that bind the eukaryotic serine/threonine phosphatase protein phosphatase 1 (PP1) to direct eIF-2α dephosphorylation and so reversing the shut-off of protein synthesis initiated by stress-inducible kinases and facilitating recovery of cells from stress[92]. ATF4 also participates in the retro control of PERK signaling[91]. Under stress conditions, IRE1 phosphorylates itself and reveals its endoribonuclease activity that is responsible for the unconventional splicing of the X box-binding protein 1 (XBP1) mRNA and its translation into the transcription factor XBP1s[93]. XBP1s up regulates the transcription of genes encoding ER chaperones components of the ER-associated protein degradation machinery and phospholipids' biosynthesis. Furthermore, IRE1 has also a nonspecific endoribonuclease activity that contributes to the reduction of protein accumulation in the ER. IRE1 also recruits the scaffold protein tumor necrosis factor receptor-associated factor 2 (TRAF2) that regulates activation of NF-κB and JNK and plays a central role in the regulation of cell survival and apoptosis. Finally, accumulation of misfolded proteins in the ER results in the proteolytic cleavage of ATF6. The cytosolic portion of ATF6, an active transcription factor, transactivates genes encoding ER chaperones, ER-associated protein degradation components, and protein foldases. It is important to

underline that several ATF6 homologs have been identified that share the same processing mechanism with ATF6 and that could play tissue-specific roles[94].

Sustained ER stress can lead to ER stress-associated cell death in fact UPR sensors are closely connected to apoptotic pathways.

The pioneer work[95] that linked the interrelationship between ER stress, inflammation and insulin resistance showed an over expression of UPR markers in the liver and adipose tissue of obese rodents.

UPR activation has been proposed to participate in the low-grade inflammation observed in obesity in the liver, WAT and muscle. Indeed, the three arms of the UPR described above, are intimately connected to inflammatory pathways as the activation of NF-kB and JNK responsible of the synthesis of proinflammatory cytokines. These themselves proinflammatory cytokines, could activate ER stress by a feed-forward and non-elucidated mechanism, and thus help to maintain an inflammatory state[96].

ER stress involves insulin-sensitive tissues in different way. In the hepatic tissue, ER stress could contribute to the development of IR in three different ways: modification of transcription of key hepatic enzymes involved in gluconeogenesis or lipogenesis, stimulating stress kinases that interfere with insulin signaling, promoting fat accumulation in hepatocytes[97,98].

In WAT, the contribution of the ER stress to impaired insulin signaling has yet to be confirmed, but it appears that ER stress clearly plays a role in modulating adipose tissue lipolysis[99], in the deregulation of adipokine secretion, frequently observed in obesity and IR, in a decreasing of both leptin and adiponectin secretion and an increase of IL-6 secretion, and in promoting inflammatory pathways through the activation of PERK/ IKK pathway[100].

In muscle, ceramides inhibit insulin signaling. Several studies have addressed the role of ER stress to mediate palmitate-induced IR in muscle cells[101].

In conclusion, although recent literature highlights ER stress an important player in the regulation of insulin signalling WAT, liver, muscle, most of the studies have been performed in vitro, thus, further novel in vivo experimental data will be necessary to confirm the hypothesis that ER stress promotes IR.

Gut microbiota

An increasing body of literature has suggested gut microbiota as contributing to the generation of obesity, obesity-associated inflammation and IR [102,103,104,105] (Figure 5).

The healthy human gut microbiota is comprised of 10^{14} microorganisms, which exceeds the number of human cells in the body by ten-fold[104]. Collectively, the human microbiota encodes 150 times more genes than the human genome (3-4 million). This enables the gut microbiota to perform diverse metabolic activities not encoded in the human genome[106]. The phylum Firmicutes (including *Clostridium, Enterococcus, Lactobacillus and Ruminococcus*) and Bacteroidetes (including *Prevotella* and *Bacteroides* genera) constitute over 90% of known phylogenetic categories and dominate the distal gut microbiota. Factors influencing the microbiota composition may include age, diet, antibiotics and mode of delivery at birth[104] (Figure 5).

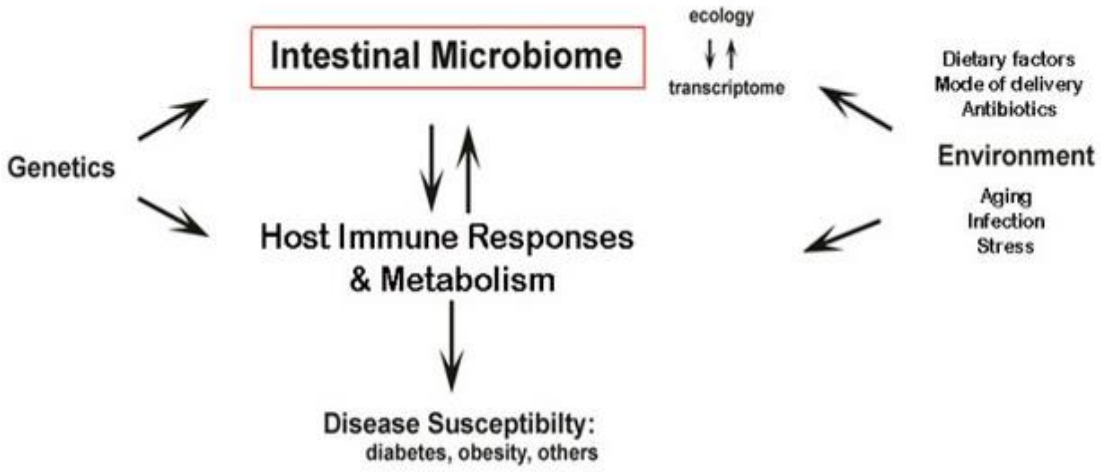


Figure 5. Diet-induced dysbiosis affects disease susceptibility

Diet is implicated as a contributing factor for obesity and T2DM by having direct effects on host metabolism and/or immune responses and influences the composition of the microbiome that, in turn, affects host immunity and metabolism and alters susceptibility to disease**.** Adapted by Brown et al.[107]

In mouse models of obesity, the composition of the microbiota is altered, a phenomenon known as *dysbiosis.* More in detail, obesity is associated with differences in gut microbiota at the phylum level, with a greater proportion of Firmicutes than Bacteroidetes in obese individuals compared with normal weigh individuals[108]. This change is associated with an increased representation of bacterial pathways associated with polysaccharides fermentation and fecal short chain fatty acids (SCFA) production. In fact, germ-free mice are resistant to high-fat diet–induced obesity because of a lack of fermenting bacteria that can process complex polysaccharides and resultant reduced SCFA

production[109]. SCFA (acetate, butyrate and propionate predominantly) are produced by gut microbiota and play an integral role in communication between microbiota and host, in addition to being an energy source for a host.

Furthermore than SCFA, in MS, sizeable levels of lipopolisaccharide (LPS) have been found in serum[4] as obesity is linked with increased intestinal permeability due to a reduced expression of genes encoding for proteins of the tight junctions. LPS is unique glycolipids in the cell wall of gram-negative bacteria, also known as bacterial endotoxins that may trigger acute and chronic inflammation leading to immune cell activation and cytokine release. Modulation of gut microbiota of mice fed high fat diets (HFD) increases intestinal permeability and LPS infusion in mammals leads to the appearance of factors known to be associated with the MS: elevated levels of proinflammatory markers, dyslipidemia, fasting hyperglycaemia, insulin resistance, and obesity[110].

Other than SCFA and LPS, the microbiota, through their activity in the lumen, also produce bioactive metabolites, such as bile acid derivatives that are responsible for influencing systemic insulin sensitivity, inflammation, and energy metabolism[111].

MS is characterized by an altered N metabolism responsible to excrete nitrate and nitrite in the saliva. Salivary nitrate is rapidly reduce to nitrite by nitrate reductase expressed by microorganism in the mouth[112]. Nitrite is a source of nitric oxide (NO·) in the alimentary canal responsible to kill bacteria in the stomach[112] and probably of the bactericidal effect on gut microorganism[9,4].

Despite the growing number of studies linking alterations in the microbiota to obesity and or insulin resistance, it remains to be established whether these are the cause or the consequence. Despite persuasive data in mouse models, the link between the microbiota and MS in humans is less compelling[109].

1.1.2 Obesity and high fat diets

Even if in an early definition of the insulin resistance syndrome Reaven[20] did not include any marker of obesity, at now days, it is clear that in most cases obesity, particularly visceral (or central) obesity, is important for the development of many of the other MS components and downstream manifestations. In fact, in the western countries the MS is driven by the growing obesity 'epidemic'[6].

Obesity is often defined simply as a condition of abnormal or excessive fat accumulation in adipose tissue, to the extent that health may be impaired[113]. The underlying disease is the

undesirable positive energy balance and weight gain. However, obese individuals differ not only in the amount of excess fat that they store, but also in the regional distribution of that fat within the body. The distribution of fat, induced by weight gain, affects the risks associated with obesity and the kinds of disease that result. Indeed, excess abdominal fat is as great a risk factor for diseases as is excess body fat per se.

A useful, albeit crude, population-level measure of obesity is the body mass index (BMI), a simple index of weight-for-height that is commonly used to classify underweight, overweight and obesity in adults. It is defined as the weight in kilograms divided by the square of the height in meters (kg/m^2). The World Health Organization (WHO) considers a BMI of less than 18.5 as underweight and may indicate malnutrition, an eating disorder, or other health problems, while a BMI greater than 25 is regarded overweight and above 30 is considered obese[114]. However, BMI does not account for the wide variation in body fat distribution and may not correspond to the same degree of fatness or associated health risk in different individuals and populations[113], i.e. it does not take in account sex, ages and race difference. In cross-sectional comparisons, therefore, BMI values should be interpreted with caution if estimate of body fat is required. Other methods than the measurement of BMI, are more valuable in identifying individuals at increased risk from obesity-related illness due to abdominal fat accumulation. Over the past decades, it has become accepted that a high waste: hip ratio and increases waist circumference indicates abdominal fat accumulation and associated ill health.

Obesity and overweight are the consequence of the evolutionary inadequacy: the human body is unprepared for a dietary excess of nutrients, especially lipids. In fact, humans have incorporated in their genes the mechanisms needed for survival against scarcity between periods of food availability and have developed mechanisms to store adequate energy reserves, to limit glucose utilization, to preserve protein, and to control energy expenditure in order to maintain thermal homoeostasis at the lowest possible cost[4]. This condition that fit for early human ancestors is no longer adequate at nowadays because food availability is no longer a problem for a large part of Humankind. Excess energy, largely lipids, wreaks havoc on the finely tuned homoeostatic mechanisms controlling energy availability and usage, with the known consequences of difficulties in the elimination of excess available energy. In this condition, a "starvation-like condition"[45] is generated due to the fact that in scarcity there is mobilization of the lipid store to cover body energy needs thus preventing the oxidation of glucose. Furthermore, high glucose availability promotes the protection of dietary

amino acids, in excess too. Finally, excess of dietary glucose could be removed by lipogenesis, lipid oxidation and fat deposition, short term adaptation that cannot be maintained indefinitely, by thermogenesis, turnover and growth that have a limited span of application or by long-term adaptations, affecting tissue structure and function. However, in all cases, these measures represents a forced adaptation of mechanisms not created for these purposes and then, in the medium and long term, they can produce changes we recognize largely as inflammation, the molecular basis of the MS[45].

Obesity is a human disease which is only found in humans and in the animals bred for human use often specifically selected for high fat content[115]. In fact, in the wild, healthy animals tend to maintain a similar body size, and there is no obesity, but neither is there extreme thinness, because of selective predator pressure. According to ponderostat/lipostatic model the body weight and the amount of body fat are fairly well maintained in spite of a wide range of variation in food availability and expenditure at the levels established by the genetic and epigenetic blueprint. In humans, even the obese tend to maintain their anomalously high weight due to the ponderostat system that is probably misadjusted to a higher setting than the optimal[116].

Considering that humanity is facing a new epidemic already nicknamed ‘‘Prosperity’s Plague’’[117], elucidating the causes involved in the pathophysiology of obesity and its related disorders, finding effective therapies and minimizing the enormous costs of the related healthcare are some of the most critical endeavours in modern medical research.

For this purpose, HFD have been used since the 1940s to obtain rodent models of obesity[118] and thus investigating deeper the metabolic derangements typifying this pathologic state. HFD with fat content between 20% and 60% expressed as energy as fat have been used. The main fat component varies between either animal components or vegetable oils[119]. In most of these diets carbohydrate was substituted by fats. Hyperlipidic synthetic diets are alien to rodents, which tend to decrease food intake to maintain the energy intake within tolerable limits. In order to increase energy intake, HFD with high palatability, comparable to those eliciting high intakes in humans, were developed. They were based on a variety of foods sharing their palatability and high energy density. These diets reflect more accurately, the variety of highly palatable, energy dense foods that are prevalent in western society and associated with the current obesity pandemic. They were named collectively “cafeteria diets” (CAFD)[120]. In this model, the experimental animals are allowed free access to standard chow and water while concurrently offered highly palatable, energy dense, human food *ad*

libitum. As CAFD are more energy dense than the standard rat pellet diets and are ingested in larger amounts, they rapidly promote weight gain increasing the mass of body fat[62,121]. As a consequence, a number of metabolic syndrome symptoms appeared, such as glucose and insulin intolerance[122,123], coronary heart disease, stroke, high blood pressure, fatty liver disease, diabetes, and certain cancers[124]. Exposure of young adult rats to a CAFD for one month, induces a number of metabolic changes that are at the limit of normality and corresponds to the initial stages of the MS[125]. Moreover, this diet engages hedonic feeding, consistent with the observed voluntary hyperphagia, alters feeding patterns[126] and may also impact on sensory-specific satiety, a mechanism by which a diet containing different food increases ingestion relative to one lacking variety[127,128]. Although hedonic component, once obesity is well established, hyperphagia decreases and the complete withdrawal from cafeteria-style foods diminishes the impulse to feed[129,126]. However, the accumulated fat remains, and the metabolic consequences of excess energy intake, such as IR and hyperlipidemia, persist.

The direct extrapolation of these findings to humans suggests that the availability and attractiveness of savoury energy dense foods may initially encourage consumption of larger snacks and meals, in terms of both volume, and critically, total energy content[130]. The variety of energy dense food drives over-consumption, and therefore, excessive weight gain. The effects of CAFD are more marked in males[131,132]; this may be because they have less anti-inflammatory[133,134] estrogenic protection than females. More over androgens promote hypertension and cardiovascular disease[135] and low estrogens levels render males more susceptible to be affected by glucocorticoids[136], which in turn decrease the anabolic effects of androgens[137].

Thus, it is generally accepted that CAFD can be used to generate a valid animal model for on late-onset hyperphagic obesity and they are suitable for the study of the effects of high-energy diets, because total protein intake is practically unaffected by the excess dietary energy (mainly from lipid) ingested[121].

1.2 Amino-N disposal in metabolic syndrome

In condition of a high availability of food, as for example during HFD, the glucose oxidation is limited and the amino acids are preserved thus the body cannot easily dispose of the excess amino-N due to the evolutively adapted schemes that prevent its wastage, as mentioned before. In

these conditions, acids are not even used to produce energy because the high availability of energy from others sources limits our body to do so[138] and high amino acids in conjunction with high energy availability can generate a paradoxical scarcity of ammonia leaving a large stock of amino-N.

Amino-N conversion into ammonia is largely done in muscle and in liver by purine nucleotide cycle[139], idle under excess of energy availability. Furthermore, in muscle there is no other major way to produce ammonia[140]. So, muscle cannot use glucose, its standard feed during exercise because blood lipids limit glucose uptake[141] and favour fatty acids oxidation, then, cannot use amino acids as energy source in significant amounts[142] and cannot use their N to produce and release glutamine. Muscle cannot even use branched-chain amino acids[143], oxidised in muscle in the postprandial state to preserve glucose, because excess of nutrients hamper this process and its timing. Glutamine is the main form of blood transport of ammonia toward intestine and kidney[144] where glutaminases release ammonia for its disposal as urea[145] or as ammonium ion[146]. Lower muscle synthesis of glutamine results in lower splanchnic synthesis of carbamoyl-P and so a low production of urea. In condition of high load of nutrients, mainly lipid, urea synthesis is decreased[121] even if there is not a massive accumulation of body-N[147] and, despite amino acid catabolism is restricted, the excess of N is lost, not in the recognized way of urea formation[148]. As a consequence, non conventional mechanisms are activated as there is no body storage of surplus N. A small proportion of N is excreted as N_2 gas[149] by unknown pathways or as urinary nitrate[150] and nitrite, uric acid, creatine, peptides, amino acids, and ammonium ions[151]. Little ammonium amount are also excreted by lungs[152]. The inefficiency of urea cycle under the conditions of excess of energy and amino-N availability indirectly gives advantage to the production of NO·. NO· is synthesizes from arginine by NO synthase[153] (NOS). In addition to NO, NOS yields citrulline which can be used to regenerate arginine[154]. The main function of NO· is relaxing smooth muscle of small vessels and thus increases blood flow and lowers arterial tension[155], creating the obese paradox, that is decreased severity of the consequences of heart failure in the obese[156].

The metabolic effects of the imbalance between amino- and ammonia-N are considerable and should be studied deeply since probably a number of the unexplained phenomena of MS are related with the profound alteration on N homeostasis. The consequence of the excess dietary protein and our inability to eliminate have been not studied in details but the few studies suggest that excess of protein is harmful in the long term for humans.

1.3 High protein diets and their differences with high fat diets

Amino acid metabolism has been only barely studied if compared to carbohydrates and lipids, consequently the knowledge of N metabolism in man is much more limited that the detailed information available about the metabolic effect of carbohydrates and lipids on energy partition. Curiously, the study of proteins has been poorly treated even if they are a key nutritional source of energy. Several reasons could explain this lack of researches: the large number of different molecular species, the multiple catabolic pathways followed by their hydrocarbon skeleton, the methodological difficulties of marking the destiny of N, a multiplicity of functional amino acid pools coupled with an active interorgans metabolism and the superficial knowledge of their catabolic path and regulation in mammals[9]. Despite the lack of information available, the protein has had for almost fifty years a starring role in the development of hyperproteic diets (HPD), very low energy, for treatment of obesity and related diseases[157,158] with mixed success[159,160,161]. Most of the discussion about their effects has been centred on their ketogenic nature[162], even if more basic knowledge are necessary to interpret the result obtained.

One important factor to consider is the definition of a HPD. The protein content of a diet can be determined in different ways: the absolute amount of the protein, the % of total energy as protein and the amount of protein ingested per kg of body weight[163]. In a neutral energy balance and weight stable, dietary protein should account for 10-15% energy equivalent to 0.8-1 g per kg of body weight per day, as recommended by WHO[164]. This level of protein recommended is determined by the minimum daily intake required to maintain nitrogen balance, so preventing the catabolism of own protein stores[165]. Whereas, to be considered high protein and to be effective for weight loss and weight maintenance, a diet should be contained 20-35% of energy derived from proteins[166,167] in energy restricted diet. There are not sufficient studies to determine a tolerable upper limit of proteins, even if, the lack of an upper limit on protein intake does not necessarily mean that there is no potential for danger at very high levels of protein consumption and certain, the difference between a high-protein diet and an excessive protein diet must be taken into account. A very high amount of protein intake might be considered in relationship to osteoporosis[168], kidney stones, renal failure[169], some types of cancer[170,171], changes in microbiota[172] and metabolic changes in liver parenchyma cells[173] due to the large amount of amino acids carried by portal vein that liver should

extensively remove in order to maintain relatively low and constant concentration in the peripheral blood.

Conversely to CAFD, HPD decrease both food intake and growth of rat[174,175] which implies changes in protein metabolism. Furthermore rats fed with an high load of protein (more than 35% of protein-derived energy) had a lower WAT mass if compared with a control isocaloric diet[174], probably because HPD result in a limited availability of energy for metabolic activities in spite that the Energy cost of growth was similar to control group. The depression of food intake is associated with elevated concentrations of essential amino acids in plasma[175]. As the rats adapts to HPD the capacity of the liver to degrade most amino acids increases and the concentration of amino acids other than branch-chain amino acids (BCAAs) in the peripheral blood do not exceed those of animals consuming much less protein[175]. On contrary, in rats fed with CAFD the sums of concentrations of amino acids in plasma increase slightly even if not significantly[121]. The sensitivity of the adaptive mechanism to increasing protein intake is exemplified by the incremental increases observed in liver glutamic oxaloacetic transaminase (GOT)[175], glutamate-pyruvate transaminase (GPT)[176], serine dehydratase (SDH)[175,176] activity, which were almost directly proportional to the degree of dietary amino acid excess. The opposite thing happens in the liver activity of CAFD rats. Serine dehydratase activity in control males is slightly higher than in CAFD rats, while significantly results can be found in SDH gene expression but, being an example of substrate-controlled activity/expression, both are correlated[62]; GPT activity decreases in CAFD rat liver.

The increase in amino acids catabolism during HPD, as stated above, is accompanied by a greeter need for urea likely due to rise of urea cycle enzyme activity[177,178]. In fact, the observed differences in enzyme activities are due to discrepancy in the actual enzyme concentrations rather than to an effect of activators, inhibitors, or an alteration in the kinetics properties of enzyme molecules[178,179].

In conclusion, rats are able to adapt to a wide range of dietary levels of proteins and N partioning is adjusted to protein availability in the diet. Despite this makeable ability, rats accustomed to a HPD when are allowed to select between a high and a low protein diet will reduce their protein intake within few days to obtain a moderate proportion of calories from protein. The proportion of total calories selected as protein under these conditions depends on both the protein content and the amino acids composition of the diets offered and on the amino acid-catabolic state of the animal[175] and it must be taken into account that HPD are not uniform and can vary considerably in relation to

the percentage of energy that represents proteins, the type of protein used and the duration of their dispensation. Thus, in most cases it is difficult to establish comparison between different experiments.

1.4 Outline of nitrogen metabolism

Proteins, ingested with diet or intracellular, undergo to proteolysis responsible of their breakdown into amino acids.

Among more than 300 amino acids in nature, only 20 of them (α-amino acids) serve as building blocks of protein. However, non-protein α-amino acids (e.g. ornithine, citrulline) and non α-amino acids (e.g. taurine and b-alanine) also play important roles in cell metabolism, some amino acids are important regulators of key metabolic pathways that are necessary for maintenance, growth, reproduction[180], and immunity in organisms[180], therefore maximizing efficiency of food utilization, enhancing protein accretion, reducing adiposity, and improving health[181].

In animal, amino acids can undergo to degradative oxidation when not necessary for synthesis of proteins, when amino acids exceeds the metabolic demand for synthesis of proteins i.e. during HPD, when carbohydrates are not available or not used correctly in order to generate energy[182] i.e. during fasting o diabetes. Although each amino acids has its own unique catabolic pathway(s), the catabolism of many amino acids exhibits a number of common characteristics in organisms[182]. Important metabolites of amino acids include NH_4^+[183], CO_2, long-chain and short-chain fatty acids[183], glucose[184], H_2S[183], ketone bodies, NO·[185], polyamines[185], urea[186], uric acid[184], and other nitrogenous substances with enormous biological importance[183] (Figure 6). Complete oxidation of amino acids carbons occurs only if their carbons are ultimately converted to acetyl-CoA, which is oxidized to CO_2 and H_2O via the Krebs cycle and mitochondrial electron transport system (Figure 6).

Whereas part of NH_4^+, generating from amino acids metabolism and during the purine and pyrimidine catabolism, is reused in several biosynthetic pathways (amino acids[62], nucleotide[187], biogenic amines[188]) or in mammals excreted as urea mainly in liver.

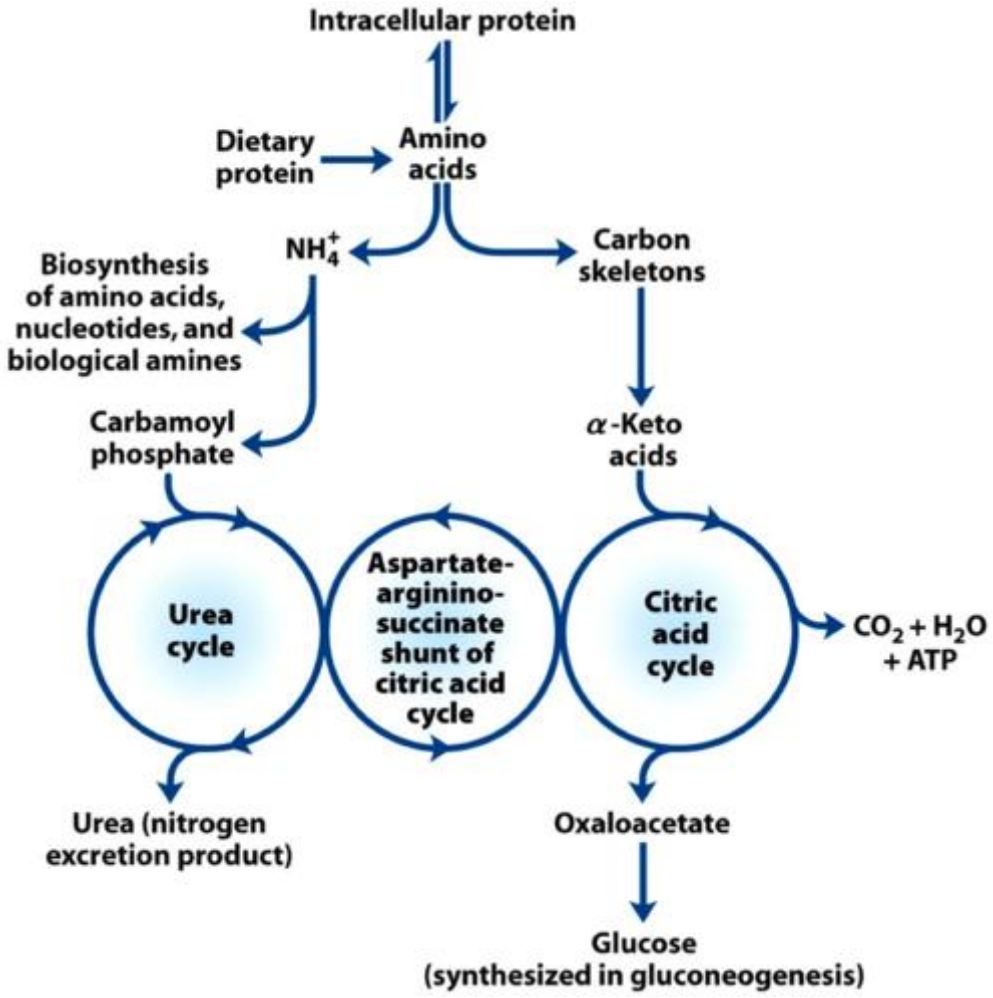


Figure 6. Schematic representation of amino acid catabolism in mammals

The pathways followed by carbons skeletons and by NH_4^+ groups of amino acids are separated but interconnected. From Nelson et al.[182]

NH_4^+ are continually produced and used in mammalian cells. NH_4^+ is a very toxic compound for animal tissues especially for brain where it provokes irreversible damage. The mechanisms leading to these severe brain lesions are still not well understood, but recent studies show that ammonium exposure alters several amino acid pathways and neurotransmitter systems, cerebral energy metabolism, NO· synthesis, oxidative stress and signal transduction pathways[189]. For this reason, NH_4^+ is converted in a non-toxic compound and transported by blood from extra-hepatic tissues to the liver and then to kidney where is excreted by urea. Since urea excretion requires equal proportions of amino and ammonium, any -NH_2:NH_3 ratio above 1·00 represents a relative excess of amino groups. The ratio amino-N:ammonium-N in most dietary proteins is close to 2, which is

responsible of the preservation of N under conditions of starvation, but in the end requires the mineralization of about half of all amino-N to ammonia under normal feeding conditions[9].

Several enzymes/pathways are responsible to convert amino group into ammonium, mainly in the liver[62]: glutamate dehydrogenase[190] (EC 1.4.1.2), glutaminase[191] (EC 3.5.1.2), purine nucleotide cycle, i.e. AMP deaminase[139] (EC 3.5.4.6) with serine dehydratase[192] (EC 4.3.1.17), and the glycine cleavage system protein H[193]. Other sources are amine-oxidases (EC 1.4.3.4) and amino acid oxidases (EC 1.4.3.2). (For all cited enzymes, EC numbers and gene name were taken by Uniprot database http://www.uniprot.org).

Glutamate and glutamine has got an important role in amino acids metabolization and ammonium transport. NH_4^+, removed from amino acids, are transferred to α-ketoglutarate in liver cell cytosol generating glutamate that move to mitochondria where lost NH_4^+. In tissues other than liver, NH_4^+ in excess is incorporated to glutamine and transferred to liver mitochondria. In muscle, NH_4^+ in excess are transferred to pyruvate to form alanine that transfer NH_4^+ from muscle to liver. The separation of the α-aminic group is the first step in catabolism of amino acids and it is promoted by aminotransferase. Aminotransferase, both cytosolic and mithocondrial enzymes are just involved in transferring amino group between amino acid and keto acid pairs without generating or consuming free ammonium ions. An example of aminotransferase is depicted in Figure 7. Several aminotransferase are specific for α-ketoglutarate as NH_4^+ receiving but diverge for the amino acids donor of NH_4^+ for this reason these enzymes take their name for the amino acid donor. Reactions catalyses by aminotransferase are reversible and they have an equilibrium constant equal to 1. Important humans transaminase are aspartate aminotransferase[194] (EC 2.6.1.1), alanine aminotransferase[195] (EC 2.6.1.2) and branched-chain amino-acid aminotransferase[196] (EC 2.6.1.42).

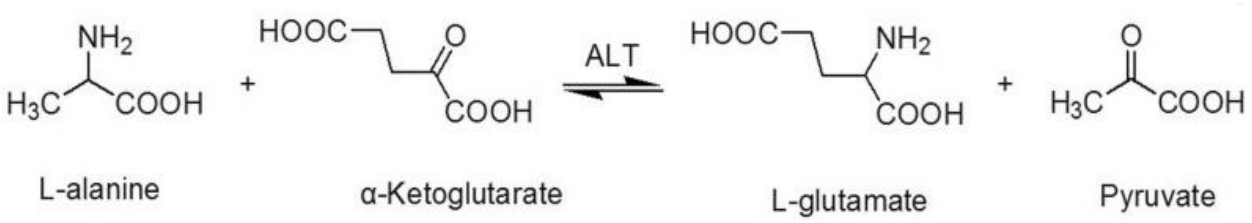


Figure 7. The transamination reaction catalyzed by alanine aminotransferase. From Liu et al.[195]

As mentioned before, NH_4^+ detached from amino acids, is transferred to α-ketoglutarate to yield glutamate. In the mithocondrial matrix, glutamate is deaminated by glutamate dehydrogenase.

Glutamate dehydrogenase[197] is an enzyme localized predominantly in the mitochondrial matrix and catalyzes the reversible oxidative deamination of glutamate to produce α-ketoglutarate and a free ammonium ions (Figure 8). Although in vivo the reaction proceeds predominantly toward the direction of the oxidative deamination of glutamate, probably due to the high concentration of glutamate and low level of free ammonium ions usually present inside the mitochondria under baseline conditions. This enzyme is encoded by two human genes, *GLUD1* and *GLUD2* encoding two glutamate dehydrogenase isoenzymes. *GLUD1* is a housekeeping gene widely expressed in human tissue, including liver, kidney, brain, heart, intestine, skin. *GLUD2* is expressed in retina, brain and testicular tissue. NAD^+ (or $NADP^+$) is a cofactor for the glutamate dehydrogenase reaction. Based on which cofactor is used (NAD^+ or $NADP^+$), glutamate dehydrogenase enzymes are divided into the following two classes: NADH dependent (EC 1.4.1.2) and NADPH dependent (EC 1.4.1.4)[197,190]. The blended action of transaminase and deamination is called transdeamination.

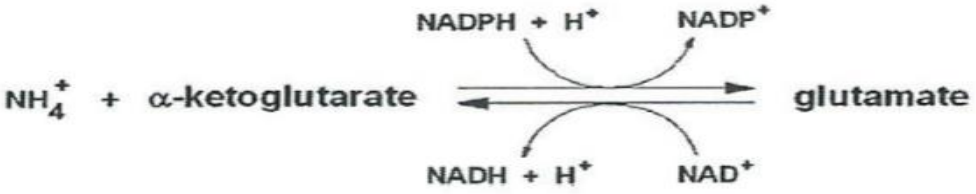


Figure 8. Glutamate dehydrogenase reaction. From Adeva et al.[191]

Glutamate is the pivotal compound involved in NH_4^+ metabolism, while glutamine it is for transport. Glutamine is form by the reaction between NH_4^+ and glutamate catalyzed by glutamine synthetase. Glutamine synthetase (EC 6.3.1.2)[191] is a cytosolic enzyme, encoded by human gene *GLUL*, that catalyzes the synthesis of glutamine from glutamate and free ammonium in an ATP dependent reaction (Figure 9). Its expression and activity have been detected in adult human skin, liver[62], WAT[134], brain and GI tract, especially in the stomach[191]. The reaction required ATP and occurs in two steps through the formation of an intermediate, γ-glutammil phosphate, and ADP.

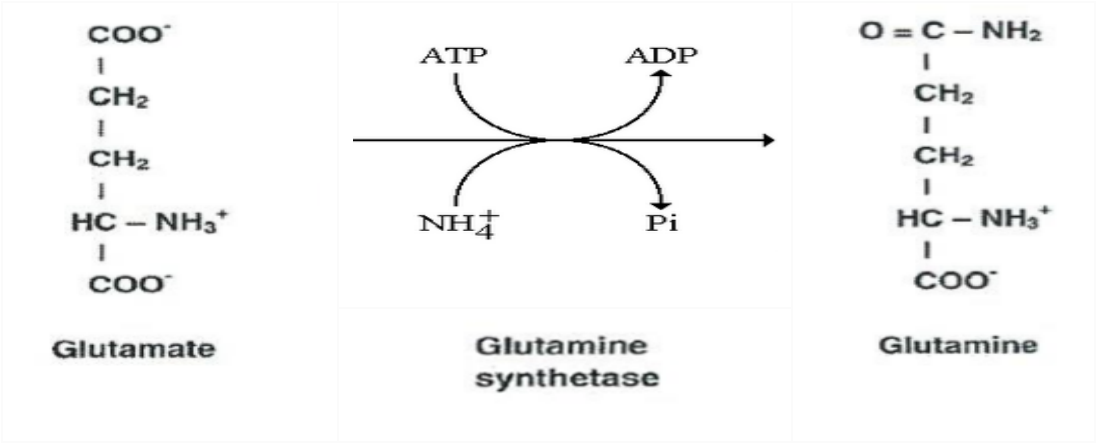


Figure 9. Glutamine synthetase reaction. Adapted from Adeva et al.[191]

As glutamine is the main non-toxic form of NH_4^+ transport, it is present in higher blood concentration than other amino acids[182]. The surplus of glutamine is transferred to liver and kidney where is converted to glutamate and NH_4^+ by glutaminase. Glutaminase (EC 3.5.1.2)[191] is a phosphate-activated mithocondrial matrix enzyme that catalyzes the hydrolysis of the amide group of glutamine to the stoichiometric amount of glutamate and free ammonium ion (Figure 10).

Two human genes, *Gls* and *Gls2*, encode a mRNA transcript that undergoes tissue-specific alternative splicing generating several isoenzymes. *Gls* encodes a mRNA transcript that undergoes tissue-specific alternative splicing generating three variants that are translated into three human glutaminase isoenzymes: Glutaminase-1 or Kidney type, Glutaminase C, and Glutaminase M. Gls2 encodes glutaminase-2 or liver type glutaminase a protein functionally similar to kidney type by smaller insize[191]. Glutaminase-1 or Kidney type expression has been detected prevalently in kidney and brain[198], Glutaminase C is expressed principally in pancreas and cardiac cell but also in kidney, lung and placenta[198]. Glutaminase M is expressed in cardiac and skeletal muscle[198]. Glutaminase-2 or liver type glutaminase distribution is not well defined. While normal human hepatocytes express liver-type glutaminase, hematoma cells express the kidney isoform[191].

Figure 10. Schematic representation of mechanism of action of L- glutaminase. From Elshafei et al.[199]

Serine dehydratase catalyzes the deamination of L-serine and L-threonine to yield pyruvate[192] and requires pyridoxal phosphate as a cofactor (Figure 11). It is encoded by *SDS* gene which expression is controlled only by serine availability[200]. In mammals, the enzyme is found predominantly in the liver. Extensive studies demonstrated that the enzyme has been found to play an important role in gluconeogenesis since its activity is remarkably induced by the consumption of HPD[192], starvation, and CAFD[201].

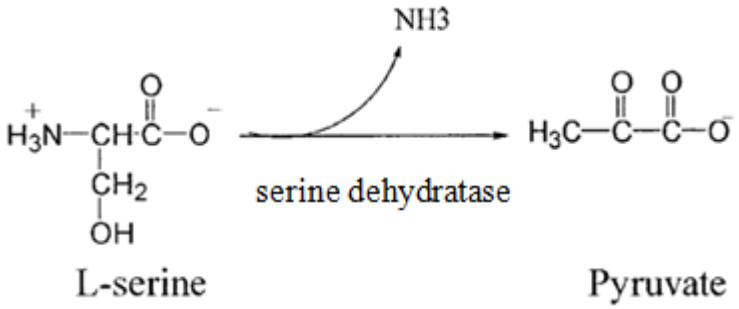


Figure 11. The enzymatic reaction of serine dehydratase. From Sun et al.[192]

AMP deaminase is the first enzyme of purine nucleotide cycle and catalyzes the breaks up the AMP (Figure 12) generated by adenylate kinase under conditions of scarcity of ATP[202] as a way to control glycolysis, often in conjunction with ammonium production[203].

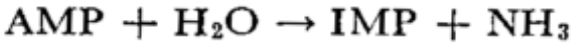


Figure 12. The enzymatic reaction of AMP deaminase. From Lowenstein[204]

The several functions of AMP-deaminase in liver, do not seem to include a significant role in the *in situ* production of ammonium[205], contrary to what happens in muscle where the purine nucleotide cycle as a main mechanism for mineralization of amino-N.

There are three isoforms encoded by the respective genes: *AMPD1* encodes AMPDI[206] that catalyzes the deamination of AMP to IMP in skeletal muscle. *AMPD2* encodes AMPDII[206] expressed in non muscle tissues as liver and *AMPD3* encodes AMPDIII mainly expressed in the erythrocyte.

Glycine cleavage system is the principal pathway by which glycine is catabolized in mammals. This multicomponent system resides exclusively in the mitochondrial compartment and catalyzes the tetrahydrofolate-dependent cleavage of glycine to yield carbon dioxide, ammonia, N^5,N^{10}-Methylene-H_4 folate, and one reducing equivalent as NADH + H[193] (Figure 13).

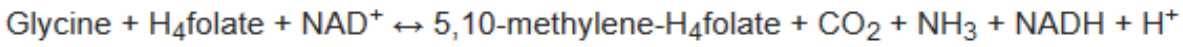


Figure 13. The enzymatic reaction catalyzes by glycine cleavage system. From Kikuchi et al.[207]

The glycine cleavage system is widely distributed in animals, plants and bacteria and consists of three enzymes and a carrier protein: P-protein, (EC 1.4.4.2) a pyridoxal phosphate-containing protein expressed by *GLDC* gene, T-protein (EC 2.1.2.10), a protein required for the tetrahydrofolate-dependent reaction, expressed by *AMT* gene, H-protein, (EC 1.8.1.4) a protein that carries the aminomethyl intermediate and then hydrogen through the prosthetic lipoyl moiety expressed by *GCSH* gene, and L-protein, a common lipoamide dehydrogenase[207]. Glycine cleavage system represents the major pathway of glycine and serine catabolism in vertebrates and its activity is detectable in the liver, kidney and brain[207]. Recently, we found that WAT expresses the H protein of the glycine cleavage system, least so far not described[134].

Monoamine oxidases are a family of mitochondrial-bound flavoproteins located in the mitochondrial outer membrane that catalyze the oxidative deamination of monoamine neurotransmitters, neuromodulators, primary, secondary, and some tertiary xenobiotic amines and hormones to the corresponding aldehydes[208]. This reaction requires flavin adenine dinucleotide (FAD) as a covalently bound redox cofactor **(**Figure 14).

$$RCH_2NH_2+O_2+H_3O^+ \rightarrow RCHO+NH_4^+ +H_2O_2$$

Figure 14. The enzymatic reaction catalyzed by monoamine oxidase. From Bortolato et al.[209]

As the aldehydes produced by monoamine oxidases are toxic species, have to be converted in less harmful metabolites. Thus, this enzyme is functionally coupled with a $NAD(P)^+$-dependent aldehyde dehydrogenase, which oxidizes the aldehyde to the corresponding carboxylic acid; alternatively (depending on the location and the intracellular conditions), aldehydes can be reduced to alcohols or glycols by aldehyde reductase or alcohol dehydrogenase. The role of monoamine oxidases in the homeostasis of monoamine neurotransmitters and xenobiotic amines is essential to modulate the neuroendocrine regulation of the central nervous system and many peripheral organs[209]. In higher vertebrates, the monoamine oxidase family comprises two isoenzymes, termed A and B[209], which, despite a substantial structural overlap, are remarkably different for substrate preference, inhibitor selectivity, anatomical distribution, and functional role in behavioural regulation[195]. These enzymes are encoded by *MAO-A* and *MAO-B* genes. Although both isoenzymes are expressed in most tissues, only MAO-A is characteristically abundant in fibroblasts and placenta; in contrast, MAO-B is the only isoenzyme expressed in platelets and lymphocytes.

L-Amino Acid Oxidases are enzymes found in several organisms, including mammals, insects, fungi, bacteria, snakes and even in plants[210]. L-Amino Acid Oxidases are flavoenzymes belonging to the class of oxidoreductases that catalyze the stereospecific oxidative deamination of L-amino acids (Figure 15). L-Amino Acid Oxidases are considered to be a class of multifunctional enzymes in view of their ability to produce hydrogen peroxide and ammonia, their participation in cell metabolism, and their possible protective effects, including their antiseptic and antimicrobial activities on different organisms[210]. Kinetic studies suggest that LAAO present preferential catalytic specificity for hydrophobic and aromatic L-amino acids, whereas their affinity for polar and basic amino acids is low. LAAO activity was first observed by Krebs in hepatic and renal tissue homogenates. Subsequently, LAAO was isolated from a rat kidney[210]. These enzymes are encoded by *IL4I1*gene.

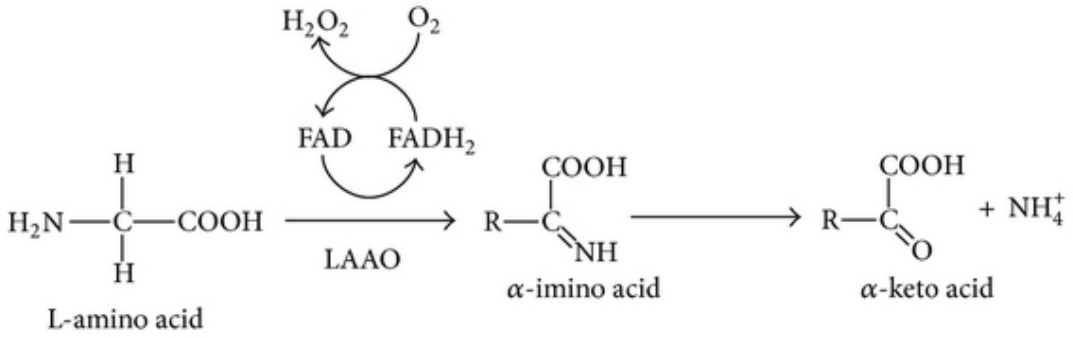


Figure 15. Mechanism of chemical reaction catalyzed by L-amino acid oxidases. From Izidoro et al.[210]

Until the pioneering work of Windmueller on glutamine utilization by rat jejunum[211], it was assumed that all dietary amino acids absorbed by the small intestinal mucosa entered the portal circulation intact and became available to extra intestinal tissues. Studies over the last decades have demonstrated extensive catabolism of non essential amino acids in intestinal mucosa[184,212,213] and that catabolism dominates the first-pass intestinal utilisation of dietary essential amino acids[214]. Intestinal tissue has a high rate of protein metabolism, which is directly linked to the high rates of proliferation, protein secretion, cell death and desquamation of various epithelial and lymphoid cells within the mucosa. The relatively high rates of first-pass metabolism of dietary amino acids may be a phenomenon that is largely confined to the small intestine rather than the stomach or large intestine[212]. In fact, the small intestine, beside to be the primary organ responsible for terminal digestion and absorption of dietary nutrients, is a major site for extensive catabolism of amino acids in humans and animals, therefore modulating the entry of dietary amino acids into the portal circulation and the pattern of them in plasma[145]. Intestinal mucosa amino acids metabolism plays an important role in regulating intestinal functionality through several mechanism: dietary glutamine, aspartate, glutamate and arterial blood glutamine are the main fuel for small intestinal mucosa and are responsible for providing required for intestinal ATP-dependent metabolic processes[213], ornithine, a product of arginine, is the precursor for polyamine synthesis, which is essential to proliferation, differentiation and repair of intestinal epithelial cells[215], arginine is the physiologic precursor of NO·, which plays an important role in regulating intestinal blood flow, integrity secretion and epithelial cell migration[216], glutamate, glycine and cysteine are precursor for the synthesis of glutathione, a tripeptide critical for defending the intestinal mucosa against damage[217]. Arterial glutamine[218], luminal glutamate[218], aspartate[219], and glutamine[218] are extensively

metabolized by rat small intestine indicating that the most of glutamine and almost most of the glutamate and aspartate in the diet do not enter the portal circulation and are not available to extra intestinal tissue. Furthermore, in adult rat small intestine, CO_2 account for the 60% of the total catabolised carbons of these three amino acids, indicating that amino acids, more than glucose, is the mayor fuel for small intestinal mucosa. Serine and glycine may be catabolised by small intestinal mucosa, however, direct evidence for their catabolism via pathways others than glutathione and nucleotide synthesis is lacking. Arginine[219] is degradated by arginase II and to a much lesser extent by NO synthase. Studies indicate that most of the arginine utilisation is accounted for by the small intestinal mucosa, indicating that arginine is not available for extra intestinal tissues. Proline is metabolized by proline oxidase that in intestine has got a high activity and in this tissue start the proline oxidation in the body. BCCA are metabolised by small intestinal mucosa[196] and their nitrogen is used for alanine syntheis[145]. Lysine, methionine, phenylalanine and threonine were traditionally considered not to be catabolised, even if some evidences show that small intestinal mucosa may play an important role in degrading dietary essential amino acids[184].

The urea cycle

As already mentioned before, the amino nitrogen resulting from the utilization of amino acids for energy requirements is excreted largely as urea.

The urea cycle (UC) is an enzyme system that converts ammonia into urea (Figure 16). It was discovered by Krebs and Henseleit in 1932[220].

Urea biosynthesis developed, as a survival mechanism, when primitive animals made the transition from the Devonian seas to a marshy terrestrial existence[221]. While most fish and amphibian larvae excrete ammonia from the gills directly into the surrounding water, terrestrial animals are not able to excrete enough water to dilute ammonia to a non toxic level without becoming dehydrated. In addition to ammonotelic and ureotelic organisms, there are uricotelic ones, such as most insects and terrestrial arthropods, birds and terrestrial reptiles that incorporate ammonia into purines, typically uric acid, which is practically insoluble in water and can be excreted (or stored within the developing egg) in solid form. Other animals excrete products derived from uric acid catabolism as alantoin. Others (i.e. sharks) excrete thrimethylamine oxide, an alternative form of ammonium detoxification to urea. Urea has the advantage of being non-toxic,

highly soluble in water as non-ionic; once synthesized, urea is transported to the kidney and then excreted.

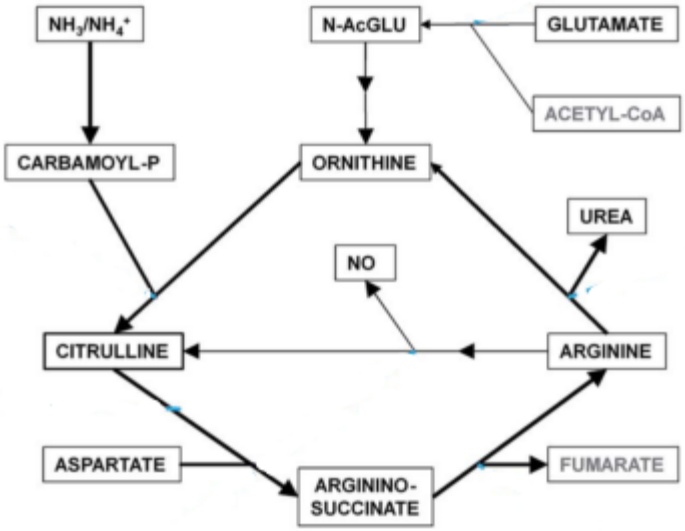


Figure 16. Schematic representation of urea cycle. Adapted from Agnelli et al.[62]

The UC consists of five enzymes: carbamoyl-phosphate synthase (CPS) catalyzes the ATP-dependent synthesis of carbamoyl phosphate from ammonia[201], type I (EC 6.3.4.16) or from glutamine, type II (EC 6.3.5.5)[222] and bicarbonate and may be considered "external" to the cycle proper. CPSI is located in mithocondrial matrix, primarily in the liver and small intestine[223] and requires N-acetyl-L-glutamate (NAG) as allosteric activator[223], but also provides the skeleton for the synthesis of ornithine. It is encoded by *CPS1* gene. CPSII is located in cytoplasm[224] and needs of Zn^{2+} as cofactor[225]. It catalyses the first three steps of the identified pyrimidine biosynthetic pathway, is present ubiquitously in mammalian tissues[226]. This enzyme is a part of a large trifunctional polypeptide, called the CAD complex, which also contains the next two enzymes of the pathway, aspartate transcarbamylase and dihydro-orotase[226]. CPS2 is encoded by *CAD* gene.

Ornithine transcarbamylase (OTC) (EC 2.1.3.3) is located in the mitochondrial matrix and catalyses the formation of citrulline and inorganic phosphate from carbamoyl phosphate and L-ornithine[227]. It is encoded by *OTC* gene and it was detected in liver[62], small intestine[228], lung[228] and WAT[73]. This reaction is therefore a part of urea cycle and also involved in intestinal synthesis of citrulline, which is released into the circulation. The proximal tubules of the kidneys take up most

of the circulating citrulline, which is then converted within the kidney to arginine and again released into the circulation[229].

Argininosuccinate synthetase (ASS) (EC 6.3.4.5) and argininosuccinate lyase (ASL) (EC 4.3.2.1) are cytosolic enzymes responsible for the biosynthesis of arginine from citrulline (and aspartate as a co-substrate). In detail, ASS synthesizes (N(omega)-L-arginino)succinate from L-aspartate and L-citrulline. This enzyme exists in almost all organisms, because it is used by microorganisms to synthesize arginine. ASS is almost ubiquitous, with the higher levels found in the liver and the kidney while lowest levels are found in the intestine (in adults)[230]. Enzyme's regulation essentially acts on the expression of the *ASS* gene, since there is neither post-translational modification nor catalytic kinetic control[153]. Mechanisms of gene regulation differ greatly between tissues[230]. Gene expression in the liver is modulated by hormones and nutritional status: glucocorticoids, glucagon and glutamine increase ASS levels, whereas insulin, growth hormone and oleic acid decrease them, and the underlying mechanisms of this modulation have not been clearly established. Also, the regulating factors in the kidney are not yet known. In endothelial cells, the regulation of ASS expression seems to be closely related to the NOS expression[230].

ASL catalyzes the reversible breakdown of argininosuccinic acid into arginine and fumarate. ASL is expressed constitutively or inducible in many different cell types, but their degree of expression and the efficiency of their catalytic pathways vary between different tissue types[229]. This enzyme is encoded by *ASL* gene.

The last enzyme of the cycle is arginase (ARG) (EC 3.5.3.1) responsible to hydrolyze L-arginine to the products L-ornithine and urea. In mammals, two arginase isoenzymes (arginase I and II) exist. They catalyse the same biochemical reaction but differ in cellular expression, regulation and subcellular localization; furthermore they are encoded by two different genes *ARG1* and *ARG2*. Although arginase I in healthy adult mammals is expressed almost exclusively in the liver, it has recently been found to be highly induced in many tissues and cell types following exposure to a variety of cytokines and other agents[231]. We have found that this isoenzyme is also present in significant amounts in adipose tissue, completing a functional urea cycle in that disperse organ[134]. Arginase II protein is expressed as a mitochondrial protein in a variety of peripheral mammalian tissues, most prominently in kidney, prostate, small intestine and the lactating mammary gland.

Individual UC enzymes are present in many tissues[232,233,229], specifically, significant levels were found in kidney and small intestine[234] while a full operative UC has been described for liver[154].

Inborn errors in any of these enzymes can result in insufficient ammonia detoxification, leading to hyperammonaemia[235].

UC enzymes undergo dietary and hormonal influence. It has been shown that activities of UC enzymes are highest in response to starvation and high protein diet and are reduced to lower protein, free protein[234,154] diet or HFD[236]. Variations in amino acid composition of dietary proteins have no effects on the enzyme activity. Increases in the enzyme activities generally correlate with increases in their corresponding mRNA levels, thus indicating that dietary regulation of enzyme level occurs primarily at a the expression level. Treatment with glucagon or cAMP analogs results in increased activities and mRNA abundance for the UC enzymes in rat liver and hepatoma cell lines[234]. Glucorticoids raise as well activities of UC enzymes in rat hepatoma cell lines, whereas precise correlations between enzyme activity and mRNA levels have not been determined.

Insulin alone has little effect on mRNA urea cycle enzymes in hepatoma cell lines. Effects of growth hormone are unclear[234].

The UC represents one aspect of amino acid metabolism cantered around the amino acid L-arginine and shares some enzymes with other pathways involved in arginine synthesis or catabolism as the citrulline-NO synthesis (Figure 16). In this pathway, arginine is converted into NO· by NOS (EC 1.14.13.39). NO· shows various biological functions such as neurotransmission, macrophage bactericidal and tumoricidal activities, and, as already mentioned, regulation of blood pressure. In addition to NO·, NOS yields citrulline that can be used to regenerate arginine by ASS and ASL. Thus, these three enzymes constitute a shunt or smaller cycle that can be regarded as bypassing the ornithine cycle.

Three isoforms of NOS have been identified so far: neuronal or brain constitutive NOS (nNOS), vascular endothelial constitutive NOS (eNOS), and inducible NOS (iNOS). These enzymes are remarkable for three different reasons: for the rapidity with which they have been characterized, purified and cloned, for the complexity and number of reactions carried out by a medium-sized subunit of 125-155 kDa, albeit probably as a homodimer, and for the number and range of physiological and pathological roles in which they are involved[237].

nNOS was the first enzyme to be purified and cloned in rat. This enzyme, encoded by *NOS1* gene, is highly distributed in brain. Whereas eNOS, encoded by *NOS3* gene, is probably responsible for the NOS activity of vascular endothelium in blood vessels[237]. Moreover, immunohistochemistry has revealed the presence of eNOS immunoreactivity in human lung, liver and skin blood vessels[237].

nNOS and eNOS are clearly Ca^{2+} and calmodulin-dependent[237]. The third type, the iNOS is Ca^{2+} and calmodulin independent and its expression can be induced in many cell types with suitable agents such as bacterial LPS, cytokines, and other compounds[238]. This enzyme is encoded by *NOS2* gene

.

2. OBJECTIVES

This thesis belongs to a wider line of research developed by the Nitrogen and Obesity Research Group which main goal was, and still, is to improve our knowledge of the physiologic, biochemical, and molecular mechanisms responsible of the onset and maintenance of obesity and to find novel ways to fight this disease. Group's main lines of research are:

- investigation of the mechanisms controlling body weight that are responsible of the MS-related diseases (obesity, diabetes, hyperlipidemia, etc); centred mainly in the splanchnic bed and adipose tissue;
- the study of metabolism and endocrine regulation of WAT and BAT;
- general study of amino acids metabolism, using animal models.

My thesis project was conducted in parallel with another one lead by my companion of researches, Dr. Sofía Arriarán. Her research was focused on WAT, while mine was centred on the splanchnic bed. During our laboratory work, we took care, conjointly, of the animals used for our experiments and we developed *ex novo* some of the enzyme assays presented in our works[62,73,134,201,222,239]. We also carried out all experiments, statistical analysis and contributed to the articles' writing and reviewing. The enzymatic essays that I developed are described more extensively in the *Methods* section.

The ability of the organism to metabolize amino acids depends also on the amount, absolute and relative, amount of protein ingested in the diet. The outcome of the catabolic utilization of amino acids is urea production. So, diets with different content of protein might influence the hepatic capacity to produce urea, as much for the availability of amino acids coming from intestine as for its utilization from the liver.

For these reasons, our hypothesis is that the liver ability to produce urea is essentially conditioned by:

- the availability of dietary amino acids, related the intestinal capacity to metabolize them;
- the diet and hormonal environment that influence the activity of enzymes involved in the metabolism of amino acids;
- the plasma levels of amino acids that reflect the interaction of amino acids with tissues.

The main **objective** of this thesis was to investigate the effects of diets with different composition of macronutrients and the relationship between lipid/protein on energy partition and the amino acid metabolism of young adult rats of both sexes. This thesis was centered on the role of liver to produce urea in adult rats and the contribution of small intestine to the metabolization of amino acids. In the first case, the investigation was conducted using an obesogenic hyperlipidic, highly palatable diet, the "cafeteria diet" (CAFD). Its administration for a limited period of time simulates a pre-MS situation in which the immediate effects of the high-energy diet are less complicated by the additional disorders elicited by severe inflammation[240]. The study was done using rats of both sexes. We investigated, as well, the effect of these diets on blood flow distribution. In the second case, the investigation was carried on subjecting male rats to a HPD.

To fulfill the objective, the changes produced on body composition, plasma parameters used as general markers of metabolism, quantitative and qualitative distribution of plasma amino acids, activity and expression of enzymes of splanchnic metabolism of rat were measured, compared and evaluated.

The experiments conducted on liver (and WAT) were performed on Wistar rats, males and females, while the experiments conducted on small intestine and balances were performed on Zucker rats. We used two different rat strains to carry out the experiments because the initial experimental planning requires the comparison of genetic and dietary obesity. The decision to analyze only the dietary obesity on Wistar rat and not only on Zucker lean (Fa/-) was essentially a limitation due to time and the availability of funds. Consequently, the project was not completed in time to be part a completed of this thesis.

3. METHODS

Specific methodological work

3.1 Development of methods for the determination of ornithine carbamoyl transferase and argininosuccinate synthase activities in rat tissues

Development of methods for the determination of ornithine carbamoyl transferase and argininosuccinate synthase activities in rat tissues.

1. Introduction

The urea cycle (Figure 1) transforms toxic ammonia into urea, a nontoxic metabolite, which urinary excretion poses no difficulties. In order to complete the measurement of the activity of all enzymes of urea cycle, we had to adjust/develop a number of viable specific methods not directly available. In particular, along the work described in the accompanying papers, it was necessary to develop assays for the evaluation of ornithine carbamoyl transferase (OTC) and argininosuccinate synthase (ASS) activity. Since the development of an assay for the second enzyme was more complicated than expected, the final steps of this development were done in collaboration with my companion in the Lab, Sofía Arriarán. In parallel to my work on OTC and ASS, she adjusted/developed methods to measure the activities of carbamoyl-P synthases, 1 and 2[1] and argininosuccinate lyase (ASL). The method used for the measurement of arginase activity was already described[2], but was, nevertheless, refined for our purposes.

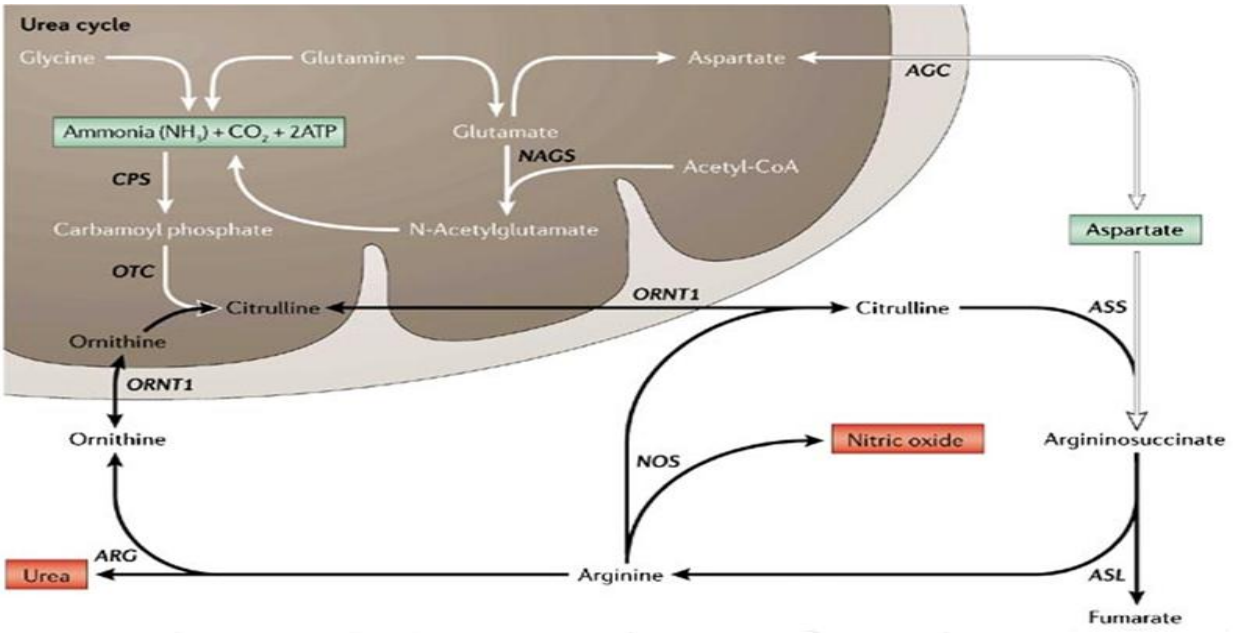
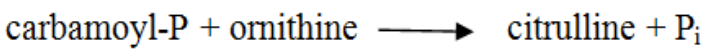


Figure 1: The urea cycle. Abbreviations: NAGS, N-acetylglutamate synthase; CPS, carbamoyl -P synthase; OTC, ornithine carbamoyl transferase; ASS, argininosuccinate synthase; ASL, argininosuccinate lyase; ARG, arginase; ORNT1, the mitochondrial ornithine transporter; AGC, aspartate-glutamate carrier; NOS, nitric-oxide synthase. From Lanpher et al.[3]

2. *Ornithine carbamoyl transferase*

OTC (EC 2.1.3.3) is a condensing enzyme of urea cycle, it is localized to the mitochondrial matrix and catalyzes the synthesis of citrulline from ornithine and carbamoyl-P in an ordered reaction as shown below:



In all eukaryotes examined, the enzyme is a trimer with an approximate molecular weight of 38 kDa; chemical evidence indicates that the subunits are identical in amino acid composition and sequence[4]. Like most mitochondrial enzymes, the enzyme is encoded by a nuclear gene (Otc), synthesized outside the mitochondria and then transported into the organelle. It is expressed in a large variety of organisms from bacteria to mammals, mainly in hepatocytes and in epithelial cells of the intestinal mucosa[5], nevertheless is not an abundant protein, making up only the 0,5-1 % of the total mitochondrial proteome[6,7].

The enzyme is regulated both by nutrients and hormones. In liver, OTC activity is highest in response to starvation and high protein diets and reduced to response to low protein or protein free diets. In small intestine, OTC activity decreases when dietary protein content increases. Variations in amino acid composition between different dietary proteins sources have little or no effects on its activity. Levels of OTC mRNA in liver and small intestine, are modestly or no affected by treatment with glucagon, dexamethasone, glucocorticoids, growth hormones and insulin[8].

The main assay procedures for OTC activity fall into two general categories: colorimetric and radiochemical procedures. The first types measure the amount of citrulline produced through chemical-colorimetric procedures. The second ones rely on the separation of both citrulline and ornithine and on the measure of their radioactivity, as depicted in the *Materials and methods* section. Colorimetric procedures for citrulline rely on the formation of coloured complexes of citrulline, dyacetyl-monoxime and phenazone[9]. We decided not to use this procedure because the colour complex is light-sensitive and diacetyl-monoxime also reacts with carbamido compounds other than citrulline, such as urea. The use of urease may correct this problem but, in doing so, may elicit further complications in the methods because is demonstrated that OTC is present in urease used as reagent in the enzyme activity measurement[10].

2.1. Materials and Methods

A radiochemical method for measuring OTC activity was devised. The idea was to use ^{14}C-carbamoyl-P or ^{14}C-ornithine to generate ^{14}C-citrulline. In first attempt, we tried to generate ^{14}C-carbamoyl-P using ^{14}C-KOCN (Hartmann analytic GmbH, Braunschweig-Germany) according to the reaction:

$$KH_2PO_4 + (KOCN + {}^{14}C\text{-}KOCN) \longrightarrow {}^{14}C\text{- carbamoyl-P}^{2-} + 2K^+$$

Equimolar amounts of KH_2PO_4 and KOCN containing ^{14}C-KOCN were incubated at 37°C for 60 minutes to synthesize of ^{14}C- carbamoyl-P. Then, the medium was neutralized with equimolar amounts of LiOH and $HClO_4$ to promote the precipitation of $Li_3(PO_4)$ and $KClO_4$. The ^{14}C label should remain in the supernatant, with carbamoyl-P. The precipitate was removed by centrifugation at 4°C for 20 minutes at 13,000xg. In order to obtain a more complete precipitation and a good degree of purity, three volumes of chilled absolute ethanol were added to one volume of supernatant and left 4 hours at 4°C; then, the tubes were centrifuged again as described above. The final supernatant was recovered and dried under a gentle flow of nitrogen. The incorporation of ^{14}C-carbamoyl-P to ^{14}C-citrulline was measured as described by Goldstein and others[11] with some modifications. The 14C-citrulline label was measured with a scintillation counter. Unfortunately, under these settings, part of the ^{14}C- carbamoyl-P was unused as carbamidic residue donor. This was checked through paper chromatography: breakup of carbamoyl-P implies the release of phosphate. Thus, samples of the incubated cocktail were run on Whatmann #1 paper, using TRIS 50mM, pH 8 as mobile phase, using sodium ortophosphate as standard. The dried chromatogram was sprayed with 600 g/L perchloric acid: 1N-HCL: 40 g/L ammonium molybdate: water (5:10:25:60 by volume). Yellow spots should indicate the presence of inorganic phosphate[12].

The next attempt implied the analysis of the reaction rate starting with ^{14}C-ornithine to obtain labelled citrulline. Using this approach, we tested thin layer chromatography (TLC) on plastic-backed silicagel sheets (SIL N-HR UV254 Macherey-Nagel GmbH, Co. KG-Germany) to separate a mixture of non-labelled amino acids and and so to find a good separation system; i.e. quite different Rf values for ornithine and citrulline. Sheets of 20x20 cm were used. Vertical lines were etched on the silicagel layer at 2 cm intervals to avoid mixing of samples. The samples were applied in successive depositions, altered with drying the spots with a hand-held hair dryer, on a line 2 cm far from the bottom of the sheet and covering the width of a lane. The eluent used was a 50 mM tris buffer pH 7,5. Amino acids were revealed in the dry chromatograms with a 2.5 g/L ninhidrine in acetone spray[13]; the purple spots were enhanced by heating the sprayed chromatogram with a hair dryer (Figure 2). After this trials, we started to perform the OTC essay. OTC was

measured used the incubation conditions described by McLaren and Ng[14] with some modifications. Frozen tissue samples were weighed and homogenized (1g/10 ml) in chilled 70 mM hepes buffer pH 7.4, containing 1 mM dithiothreitol, 50 mM KCl 1g/L Triton-X-100 and 1 g/L defatted bovine serum albumin using a mechanical tissue disruptor (IKA-T10 basic Ultra-Turrax, IKA, Staufen Germany). The assay mixture consisted of 70 mM hepes, pH 7.4 containing 50 mM KCl, 7 mM $MgCl_2$, 20 mM unlabeled ornithine HCl, 14 mM carbamoyl-P, and 1 KBq/ml of labeled ornithine (Perkin Elmer Waltham, MA,USA).

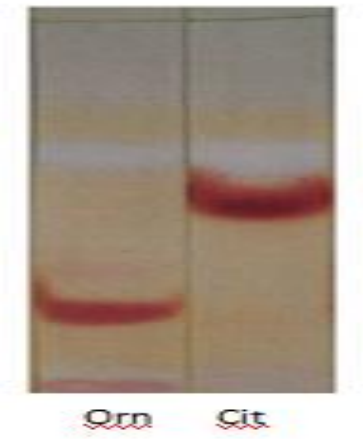


Figure 2 Dry chromatogram of no labeled amino acids revealed with a 2.5 g/L ninhidrine in acetone spray[13]. Abbreviations: Orn, ornithine, Cit, citrulline

The total volume of the complete assay mixture was 75 µl: 50 µl of assay mixture plus 25 µl of homogenate. The final concentrations in the reaction mixture were 47mM hepes, 33 mM KCl, 5mM $MgCL_2$, 13 mM ornithine, 9 mM carbamoyl-P, and 1 KBq/ml of labeled ornithine. The usual procedure was to pipet 50 µl of the assay mixture into 1.5-ml Eppendorf tube for each sample and time of incubation. The tubes were incubated at 37°C in a dry block heater. The reaction was started by the addition of the homogenates. At time intervals, 100 µl of chilled acetone was added to the incubation tubes to stop the reaction. The time 0, was obtained when 100 µl of chilled acetone was added to the incubation tubes before adding the homogenate. After centrifugation at 5,000xg for 3 minutes, the supernatants were transferred to plastic tubes and dried out in a vacuum-centrifuge concentrator (Speedvac, Jouan, Saint-Herblain, France). This way, the product of the reaction was concentrated at the bottom of the tube. This was dissolved in 25 µl of distilled water and the solution was used for TLC. In each TLC sheet a reference standard of labelled ornithine (1 KBq/ml) was included. The chromatogram is represented in figureFigure 3. A mixture of trichloromethane: methanol: acetic acid (1:2:2 by volume) was used as mobile phase to develop the cromatography. After development, the front was marked with pencil and the sheets were dried. Once dried, the

sheets were also marked horizontally and each lane was separated and horizontally cut in 1 cm serial cuts from origin (included) to front.

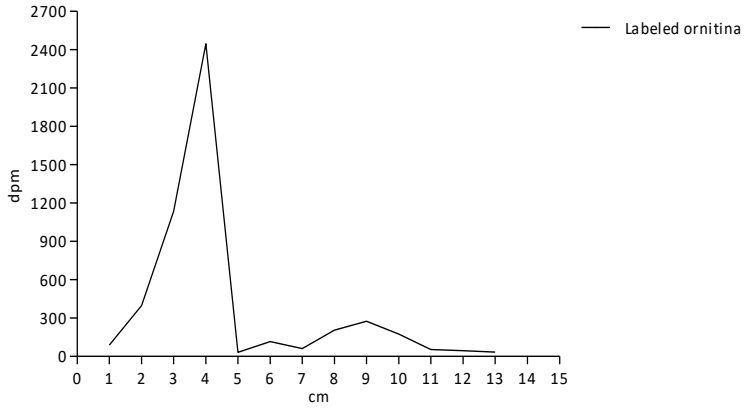


Figure 3 Example of a chromatogram of labelled ornithine used as blank in silica gel sheets with a mixture of trichloromethane: methanol: acetic acid (1:2:2). Cm indicates the distance from the line of the deposition and the dpm indicates the disintegrations per minute encountered in that point**.**

The pieces of sheets were placed into scintillation vials and counted. An example of chromatogram is shown in Figure 4. To limit interferences, the label in the citrulline area was expressed as a percentage of the total label counted in each TLC lane. These data allowed the calculation of newly formed citrulline; the value for each sample was plotted and was considered to be an expression of Vmax under the conditions tested. Protein content in each of the homogenates used for enzyme analysis was estimated with a colorimetric method[15] and the enzyme activity was referred to the homogenate protein content.

If no specified, chemicals and compounds used for this experiment were obtained from Panreac (Castellar del Vallès, Barcelona, Spain) or from Sigma (St Louis, MO, USA).

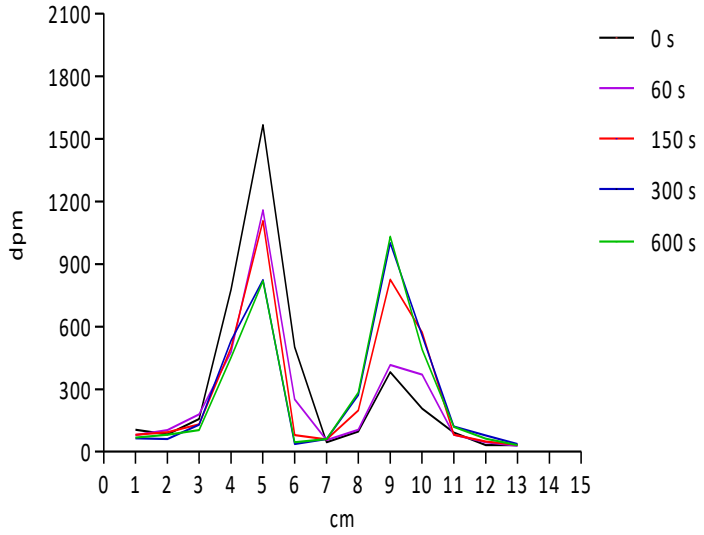


Figure 4 Example of a chromatogram of the conversion of ornithine to citrulline of rat liver homogenate. Each colour represents a different time of incubation. The first peak indicates labelled ornithine as shown in figure 3 and the second one represents the citrulline yeld. Cm indicates the distance from the line of the deposition of the sample and the dpm indicates the disintegrations per minute encountered in that point.

3. Argininosuccinate synthase

Argininosuccinate synthase (ASS) (EC 6.3.4.5) catalyses the reversible ATP-dependent condensation of citrulline with aspartate to form argininosuccinate (AS):

$$MgATP^{2-} + \text{citrulline} + \text{aspartate} \longleftrightarrow \text{argininosuccinate} + MgPPi + AMP \text{ (Figure 1)}$$

The mammalian enzymes have very similar properties and have a homo-tetramer structure, with a subunit size approximately of 46 kDa. The enzyme was first identified in the liver[16] as the limiting enzyme of the urea cycle, but most recently has been recognized as a ubiquitous enzyme in many mammalian tissues. The discovery of the arginine-citrulline nitric oxide synthesis pathway (Figure 65) has increased the interest in this enzyme, which represents also a potential limiting step in the NO synthesis. The highest ASS enzyme activities were found in the liver and kidneys[17] and were lower in the small intestine; these results have been confirmed later with expression data, both at the mRNA and protein levels[18]. The enzyme was initially described as a cytosolic in the liver[17], but posterior studies revealed that the enzyme is linked in part to the outer membrane of mitochondria[19].

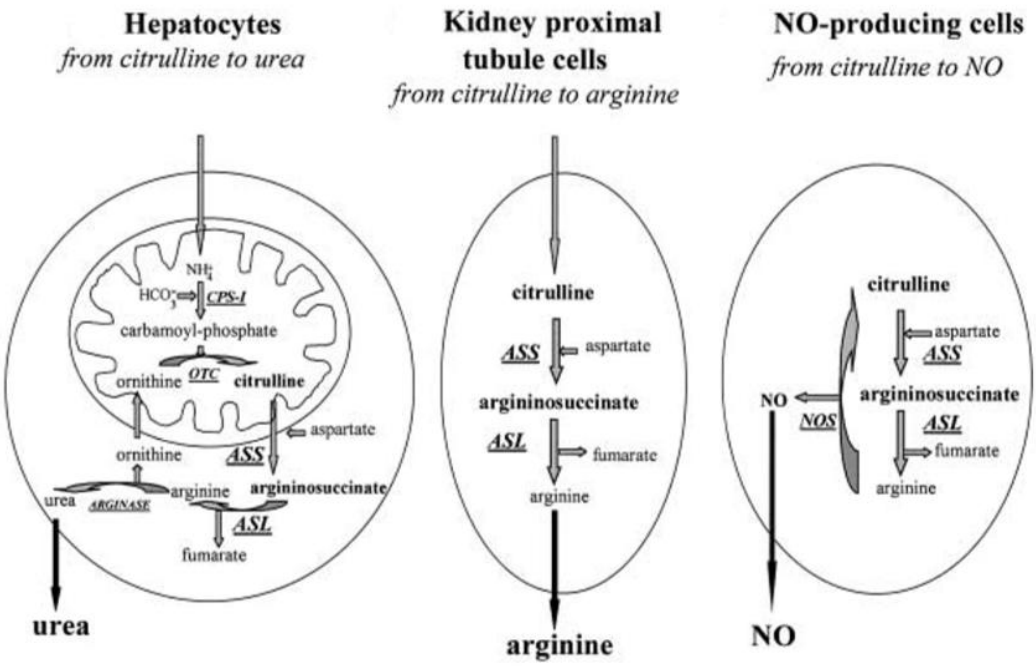


Figure 5 Schematic representation of the three major functions of ASS in the mammalian organism. Abbreviations: CPS-I, carbamoyl-P synthase-I; OTC, ornithine carbamoyl transferase; ASS, argininosuccinate synthase; ASL, argininosuccinate lyase; NOS, nitric oxide synthase.

ASS intracellular location and its regulation are quite different questions, probably related. In the liver, where arginine is largely hydrolysed to form urea and ornithine, the ASS gene (Ass1) is highly expressed and both hormones and nutrients[20] are the major regulating factors[21]. Glucocorticoids, glucagon and insulin, particularly, control the expression of the gene during development and in the adult. Diet, specially protein intake, increase ASS expression, protein levels and activity[22]. In contrast, in cells producing NO, where arginine is the direct substrate in its synthesis, the Ass1 gene is expressed at low levels. Proinflammatory signals seem to be the main factors regulating expression of this gene; it is assumed that regulation of Ass1 gene expression is mainly exerted at the transcriptional level, but the molecular mechanisms controlling the regulation of the enzyme are still poorly understood.

3.1. Material and methods

Most of the few articles published about ASS enzyme activity, measure its activity coupled with ASL, a cytosolic enzyme that breaks, reversibly, argininosuccinate to yield arginine and fumarate. No specific method for the analysis of ASS in animal tissues has been found in the literature, and thus we had to develop a specific individual method that allows differentiating the activities of ASS and ASL. This is an obvious requirement for the study of both the function and regulation of urea

cycle under different physiological conditions, independently of assumptions on which enzyme controls or is controlled when analyses conjointly. Trying to develop a specific method for the determination of ASS activity, we encountered considerable methodological difficulties that largely explain why this task was not accomplished previously by others. We tried a number of different methodological approaches, in part based on the combined methods, but in most cases the tests failed.

Our first try was a radiochemical method. The idea was to use a ^{14}C-labelled amino acid, either aspartate or citrulline in order to generate ^{14}C-AS. Using this general approach, different options were successively tested. In the first instance, we tried the same approach used for OTC that is to say the separation of the labelled amino acids using TLC (silicagel plates) after the incubation of the tissue homogenates with the labelled precursor, taking, as reference, the conditions described in papers [23,24]. Using this approach, we tested TLC to separate a mixture of non-labelled amino acids, to find a good separation system; i.e. quite different Rf values for citrulline, aspartate and AS. The conditions used were the same explicated above for OTC (Figure 6).

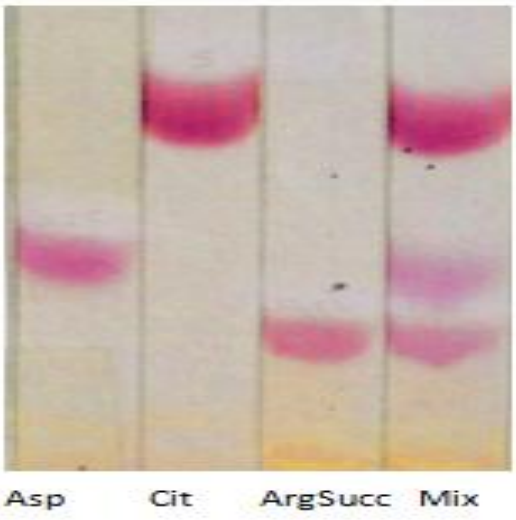


Figure 6 Dry chromatogram of no labelled amino acids revealed with a 2.5 g/L ninhidrine in acetone spray[13]. Abbreviations: Asp, aspartate; Cit , citrulline; ArgSucc,argininosuccinate; Mix, mixtures

Considering that a quite different rf was obtained for citrulline, we decide to use this as labelled amino acid. Frozen tissue samples were weighed and homogenized in the same conditions explained for OTC. The assay mixture consisted of 70 mM hepes, pH=7.4 containing 50 mM KCl, 7 mM $MgCl_2$, 2 mM ATP, 13 mM unlabeled citrulline HCl, 9mM aspartate and about 1 KBq/ml of labelled citrulline (Perkin Elmer Waltham, MA -USA). The total volume of the complete assay mixture was 75 µl: 50 µl of assay mixture plus 25µl of homogenate, so the final concentrations in the reaction mixture were 47 mM hepes, 33 mM KCl, 5mM $MgCl_2$, 1.3 mM ATP, 10 mM citrulline

and 6 mM aspartate . The procedure was exactly the same employed for OTC. In this case, a reference standard of 1 kBq/ml of labelled citrulline was included (Figure 7).

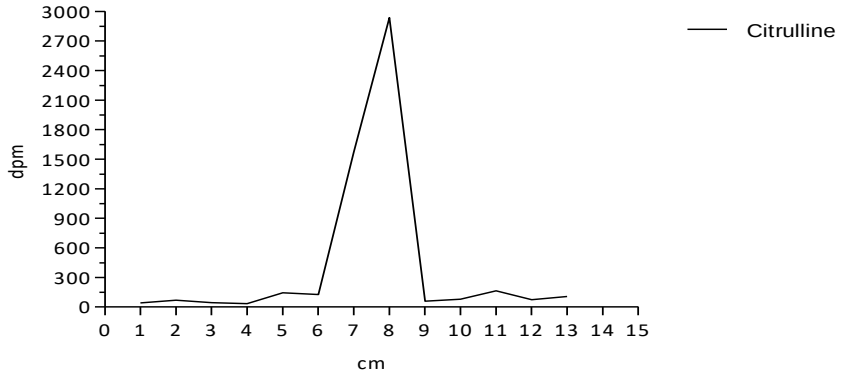


Figure 7 Example of a chromatogram of labelled citrulline used as blank in silica gel sheets developed with a mixture of trichloromethane: methanol: acetic acid (1:2:2). Cm indicates the distance from the line of the deposition and the dpm indicates the disintegrations per minute encountered in that point.

Various solvent mixtures with different polarity were tested to determine the best method for the complete separation of AS and citrulline.

The mixtures tried were:

- trichloromethane: methanol: acetic acid (1:2:2 by volume) (Figure 8 plot A);
- 1-butanol:methanol:ammonium hydroxide: water (33:33:24:10 by volume) (Figure 8 plot B);
- 2-propanol: metanol:ammonium hydroxide: water (33:33:24:10 by volume) (Figure 8 plot C).

After development, the procedure followed was the same for OTC. The pieces of plates were placed into scintillation vials and counted. Unfortunately we didn't find any good mixtures giving us a complete separation of AS and citrulline (Figure 8). The desirable result was obtaining two peaks as achieved for OTC (Figure 4).

As we didn’t get this result, after several tries, we followed another strategy. In an intent to use most of the information available for coupled enzyme analyses, we decided to measure the enzyme activity coupled with an excess of purified ASL to finally yield arginine and fumarate from the argininosuccinate formed in the ASS reaction. The problem was the absence, in the market, of adequate purified ASL enzyme to be used in a coupled reaction. Thus, we tried to purify it from a material rich in this enzyme activity: cotyledons of germinating pea seeds[25].

Seeds of Pisum sativum L. cv. Onyx were bought in a grocery store; they were soaked in tap water and allowed to germinate by transferring them subsequently to moist filter paper in large pots in darkness at 23°C for the appropriate time. We ground about 12 g of cotyledons in a mortar and

pestle with 5 to 8 g of sand and 20 ml of extraction medium containing 400 mM mannitol, 50 mM morpholinopropane sulfonic acid buffer (pH 7.4), and 2.5 g/L defatted bovine serum albumin. After screening in a mesh (width 45 µm), the homogenate was centrifuged at 2,500xg for 5 minutes. The pellet obtained after centrifugation of the 2,500xg supernatant at 40,000xg for 10 minutes, was re-suspended in extraction medium. Five ml of the 40,000xg supernatant were passed through a Sephadex G-25 column (9 x 1.5 cm), equilibrated with 50 mm K-phosphate buffer (pH 7.5). Protein-containing fractions of the eluate were collected. 37 µl of the eluate was mixed with an equal volume of AS. The tubes were incubated at 37°C for 15 minutes in a dry block heater. Then, 95 µl of chilled acetone was added to the tubes to stop the reaction. For time 0, 95 µl of chilled acetone was added to the incubation tubes before adding the AS. After centrifugation at 5,000xg for 10 minutes, the supernatants were transferred to plastic tubes and dried out with a gentle flow of nitrogen. Once dried, 650 µl of distilled water was added and the absorbance was read at 240 nm. If ASL had been presented in the eluate, it would have had to break the AS and to give fumarate and arginine (Figure 1). Reading the absorbance, we would have had to detect the presence of fumarate which absorption wave length is 240 nm. The measurement of ASL activity was even done on concentrated extract before passing it through the column. After considerable effort, several germination cycles, tries at extraction and separation by centrifugation followed by molecular size sieving with the Sephadex column, the idea was abandoned when the measurements of ASL activity in the concentrated extracts was barely higher than that of our samples. Most likely, the problem was that the purification step was not sufficiently precise to yield a pure enzyme (with enough specific activity and devoid of other urea cycle enzymes (ASS itself, AS). In any case, we tested the coupled reaction with liver samples, measuring the fumarate evolved with malate dehydrogenase, as in the measurement of ASL activity, and found no changes in NADH because of inadequate conditions, insufficient enzyme activity or other causes. Thus, we decided not follow this path and try other more promising avenues.

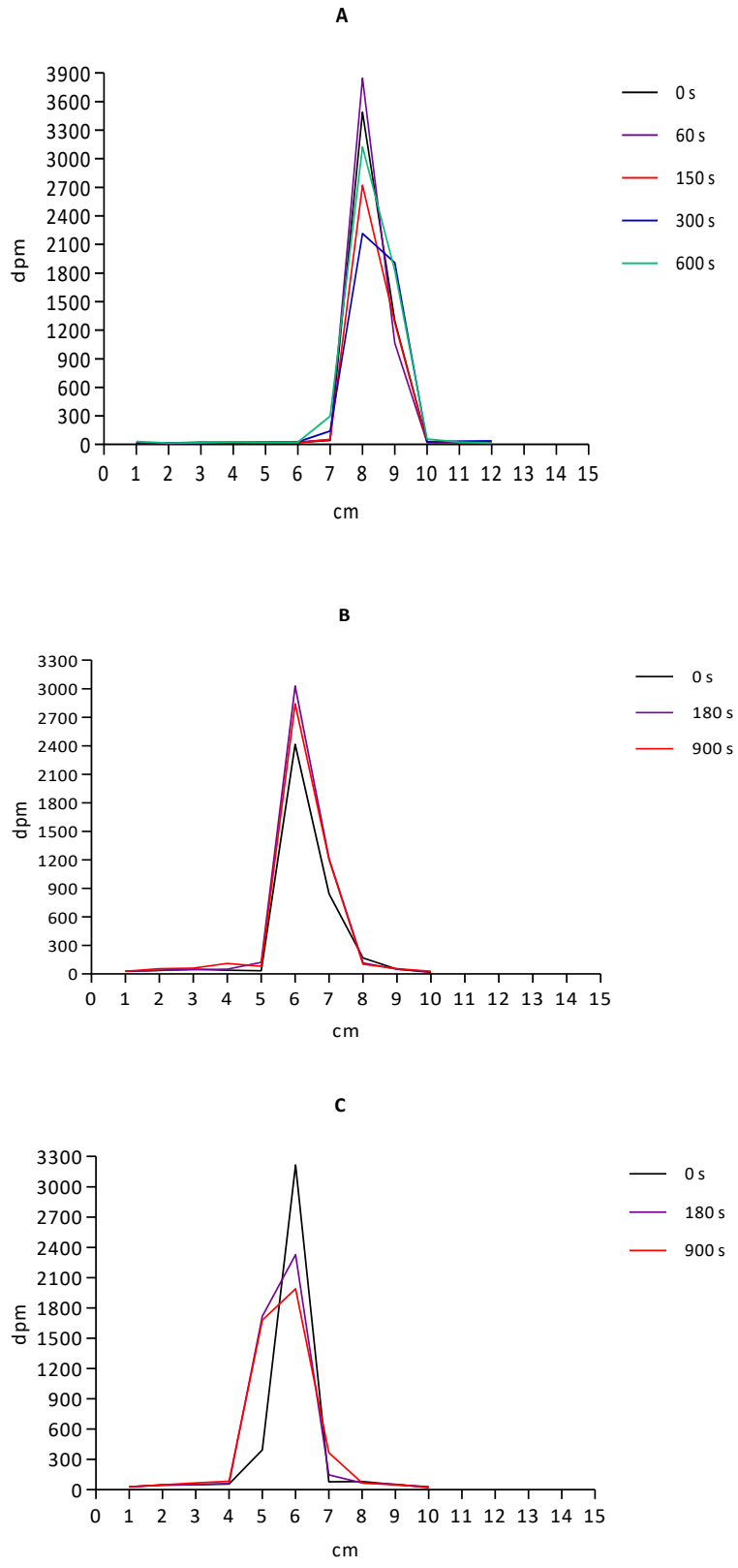


Figure 8 Example of chromatograms of the conversion of citrulline to arginosuccinate of a rat liver homogenate. Each colour represents a different time of incubation. In the plot A, the eluent used was a mixture of trichloromethane: methanol: acetic acid (1:2:2 by volume); in the plot B, the eluent used was a mixture of 1-butanol:methanol:ammonium hydroxide: water (33:33:24:10 by volume); in the plot C, 2-propanol:metanol: ammonium hydroxide: water (33:33:24:10 by volume).Cm indicates the distance from the line of the deposition of the sample and the dpm indicates the disintegrations per minute encountered in that point. The desirable result was obtaining two different peaks as shown in figure 4.

The next procedure tested was an enzymatic/radiochemichal method using ^{14}C-Aspartate (Perkin Elmer Waltham, MA -USA) in presence of α-ketoglutarate in order to generate ^{14}C-oxaloacetic acid by aspartate transaminase. Frozen tissue samples were weighed and homogenized in the same conditions explained above for OTC. The assay mixture consisted of 70 mM hepes, pH=7.4 containing 50 mM KCl, 7 mM $MgCl_2$, 2 mM ATP, 13 mM citrulline HCl, 9mM unlabelled aspartate, 12 mM α- ketoglutarate and about 1 KBq/ml of labelled aspartate. The total volume of the complete assay mixture was 75 µl: 50 µl of assay mixture plus 25µl of homogenate, so the final concentrations in the reaction mixture were 47 mM hepes, 33 mM KCl, 5mM $MgCL_2$, 1.3 mM ATP, 10 mM citrulline and 6 mM aspartate and 9 mM α-ketoglutarate. The usual procedure was pipet 50 µl of the assay mixture into 1.5-ml Eppendorf tube for each sample and time of incubation. The tubes were incubated at 37°C in a dry block heater. The reaction was started by the addition of the homogenates. At time intervals, 0.08 U of aspartate transaminase was added to the incubation tubes to generate ^{14}C-oxaloacetic acid from the remaining labelled aspartate. The reaction was allowed to develop for 20 minutes and stopped with 1 ml of ethanol. After centrifugation at 2,000xg for 5 minutes, the supernatants were transferred to plastic tubes and dried out with a gentle flow of nitrogen. Both pellet and supernatant were dissolved in scintillation liquid and counted. After several tries, the idea was abandoned after encountering all radioactivity in the pellet and not in the supernatant where ^{14}C-oxaloacetic would have had to be.

The next procedure tested was the removal of the amino acids remaining free at the end of the incubation (i.e. not incorporated into AS) by means of their oxidation to nitrogen gas and hydroxy-amino acids using just formed nitrous oxide[26]. Firstly, we tried this procedure without tissue homogenate. The assay mixture consisted of 70 mM hepes, pH=7.4 containing 50 mM KCl, 7 mM $MgCl_2$, 2 mM ATP, 13 mM citrulline HCl, 9mM unlabelled aspartate and about 1KBq/ml of labelled aspartate. We prepared 2M $NaNO_2$ and 2.5 M HCl, we mixed equal volumes of them in order to generate nitrous acid. 5 µl of this mixture, just prepared, was added to 70 µl of the assay buffer in a 1.5 ml eppendorf, shacked and left at room temperature for 1 minute. The reaction was complemented with the selective separation of labelled 2 OH-butanodioic formed from aspartate by precipitation the latter with ethanol. After centrifugation at 2,000xg for 5 minutes, we counted supernatant and pellet. Because of we encountered the radioactivity in the pellet, we supposed that the 2OH-butanodioic was soluble in absolute ethanol. So, we tried the same experiment using unlabelled AS. The assay mixture consisted of 50 mM phosphate buffer, pH=7.5 containing 10 mM AS. 5 µl of the mixture $NaNO_2$/HCl, just prepared, was added to 70 µl of the assay buffer in a 1.5 ml eppendorf, shacked and left at room temperature for 1 minute. The reaction was complemented with the selective separation of the 2 OH-acids formed from AS by precipitation with ethanol.

Looking carefully at the tube and after several tries, it looked like the 2 OH-acids formed from AS wasn't soluble in absolute ethanol because a compound was visible at naked-eye. This way, we thought that we could take advantage of the different solubility in ethanol of hydroxy-amino acids (labeled) produced. As we assumed that the oxidation product of AS (or the amino acid itself) were not soluble, thus, we expected to collect 2OH-butanodioic in the supernatant after centrifuging out all label incorporated into AS. Since we were not sure what was the product(s) formed in the oxidation of AS, we considered more reasonable to measure the label "lost" in the aspartate pool than to measure that incorporated into the AS (assumedly spin-off in the precipitate). We tried this approach with liver homogenates. Frozen tissue samples were weighed and homogenized in the same conditions explained for OTC. The assay conditions and procedure were the same explained above in the experiment without homogenates. As the measurement of radioactivity didn't give the results foreseen, we modified the temperature of exposure of the sample to nitrous acids as suggested by Schmidt [27]. We left the sample reacting with nitrous acids for 1 minute at 37°C. The actual measurements of radioactivity obtained, however, did not stand to our theory, since there was a wide variation in radioactivity levels and most of it was found in the precipitate anyway. Afterwards, it was assumed that probably, under the conditions tested, the newly formed AS was broken up by the homogenate-native ASL and/or solubility in ethanol of the products of oxidation of AS, and both hydroxy and amino acids were largely insoluble in ethanol.

At this point, we thought that the best way to find a good procedure was using a radiochemical method adopted in the first try. So, we repeat the first experiment with some differences. We employed labelled aspartate and not citrulline and we stopped the reaction with 10 µl of 90% trifluoroacetic acid. We decided to test again a TLC to separate a mixture of non-labelled amino acids, to find a good separation system; in this case, we used the three different eluents explained above. The conditions of exposure were the same depicted above. As shown in Figure 10, the mixture that allow achieving the best separation was: trichloromethane: methanol: acetic acid (1:2:2 by volume). In this case, a reference standard of labelled aspartate (1kBq/ml) was included (Figure 10 plot A). Unfortunately, doing the experiment with liver homogenates, we didn't get a good separation (Figure 10 plot B). As we supposed that the presence of ASL in the homogenates could be a limiting factor, we tried to stem-off this problem by adding a large concentration of urea (300 mM) to the incubation buffer, because it can inhibits ASL[28]. Even this last try was not successful because we didn't get a good separation.

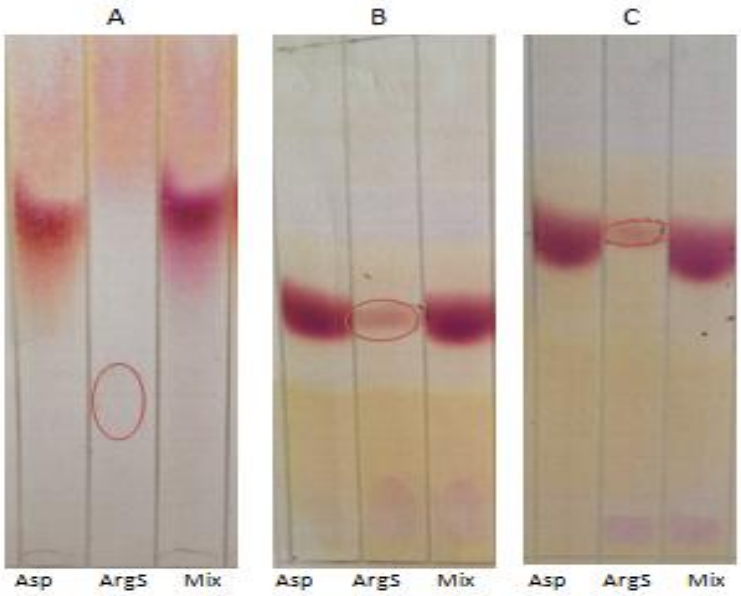


Figure 9 Dry chromatograms of no labelled amino acids revealed with a 0.25% ninhidrine in acetone spray[13]**.** For the chromatogram A the eluate used was trichloromethane: methanol: acetic acid (1:2:2 by volume); for the chromatogram B the eluate used was 1-butanol:methanol: ammonium hydroxide: water (33:33:24:10 by volume); for the chromatogram C the eluate used was 2-propanol:metanol: ammonium hydroxide: water (33:33:24:10 by volume). Abbreviations: Asp, aspartate; Cit, citrulline; ArgS, argininosuccinate; Mix, mixtures.

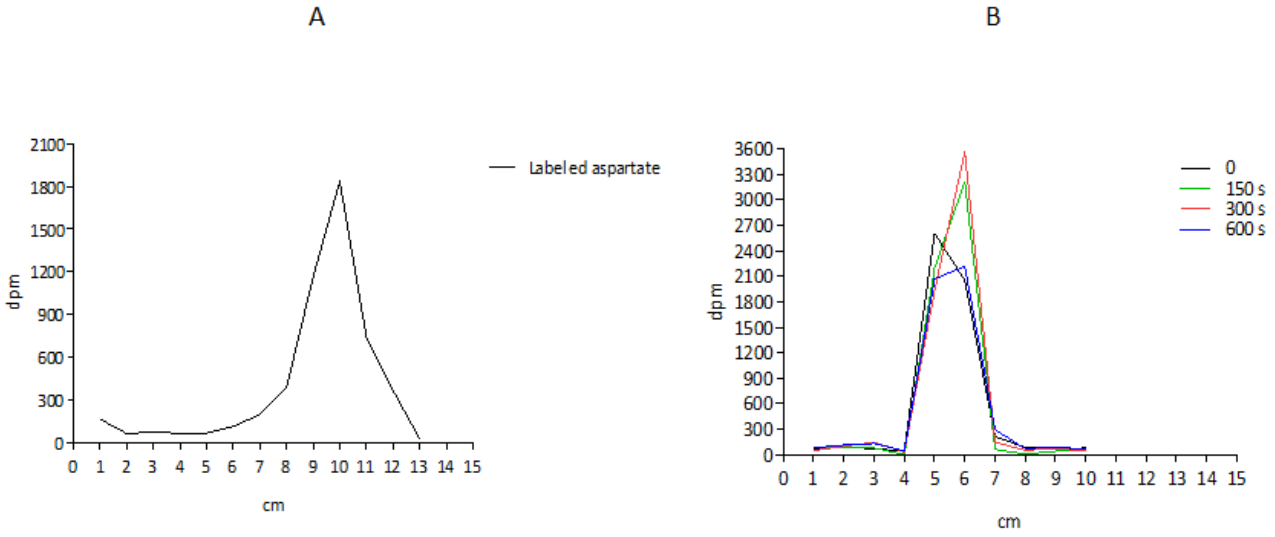
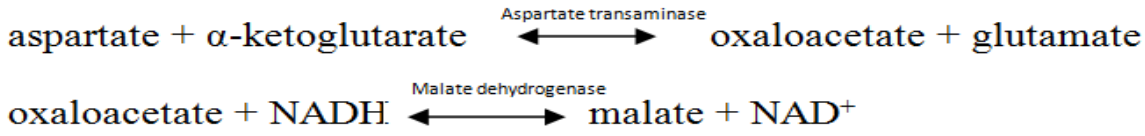


Figure 10 Plot A: example of a chromatogram of labeled aspartate used as blank in silica gel sheets. In the plot A, the eluent used was a mixture of trichloromethane: methanol: acetic acid (1:2:2 by volume).
Plot B: example of chromatograms of the conversion of citrulline to arginosuccinate of a rat liver homogenate. Each colour represents a different time of incubation. Cm indicates the distance from the line of the deposition and the dpm indicates the disintegrations per minute encountered in that point.

Considering the difficulty to isolate labelled AS and because of this strategy of labelling AS didn't give the expected results we decided to follow another strategy: measuring the decreasing of the labelled amino acids used for the synthesis. Thus the focus of the analysis was shifted to the use of a different approach, forfeiting ^{14}C-labelling and reverting to biochemical methods of

measurement of metabolites instead. Reviewing bibliography in order to find a valid method for measuring of ASS, we found an article[29] deals with studies about enzyme sequence mediating arginine synthesis in Saccharomyces cervisiae. One of this is ASS. We decided to use the condition of incubation described in the article with some modifications in order to measure the decreasing of quantity of aspartate in the reaction. Particularly, we measured the remaining amino acid by means of a coupled reaction, transamination to oxaloacetate and reduction of oxaloacetateto malate with NADH as shown below:



To develop this method we needed several attempts, several modification in concentration of reagents and enzymes and several precautions with metabolites interferences and strategies to eliminate the high OD caused by homogenate, but at the end, the procedure worked properly. Considering the complexity of the experiment, we decide to split it in two phases.

Phase I: incubation to form AS

Frozen tissue samples were weighed and homogenized in the same condition of homogenization explained above. The assay mixture consisted of 70 mM hepes buffer at pH 7.4, containing 100 mM citrulline, 168 mM ATP, 84 mM $MgCL_2$, 12.4 mM aspartate. The total volume of the complete assay mixture was 85 µl: 3 µl of citrulline, 5 µl of ATP, 5 µl of $MgCL_2$, 55 µl of homogenate and 17 µl of aspartate. This resulted in final concentrations of: 3 mM ctrulline, 10 mM ATP, 5mM Mg^{2+}, and 2,5 mM aspartate. The usual procedure was to pipet citrulline, ATP, $MgCL_2$ and homogenate into 1.5-ml Eppendorf tube for each sample and multiplied the volume for the number of incubation time tested. The reaction was started with aspartate and was carried out at 37°C in a dry block heater. At defined time intervals, the reaction was stopped with 40 µl of 30 g/L $HCLO_4$. To set the time 0, 40 µl of the acid was added to the incubation tubes before adding the aspartate. The tubes were vortexed and neutralized (pH 7-8) with 10 µl of 100g/L KOH containing 62 g/L $KHCO_3$. This neutralization was necessary because the enzymes in the Phase II work at neutral pH. The tubes were maintained on ice during this process. Then, they were centrifuged at 8,000xg for 15 minutes at 4ºC. The supernatants were transferred to a 1.5-ml Eppendorf tube and kept frozen at -20ºC until processed.

Phase II: measuring of remaining aspartate by means of aspartate transaminase and malate dehydrogenase.

The assay mixture for this phase consisted of 66 mM phosphate buffer at pH 7.4, containing 3 mM NADH, 2.4 mM α-ketoglutarate. The total volume of the complete assay mixture was 225 µl: 25 µl of NADH, 25 µl of α-ketoglutarate, 175 µl of phosphate buffer. This resulted in final concentrations of 0.25 mM of NADH and 0.2 mM of α-ketoglutarate. The usual procedure was to pipet this 225 µl of mixture in each well and read the absorbance at 340 nm in a plate reader (Biotek, Winoosky, VT USA) in order to know the absorbance of NADH. After that, a volume of thawed supernatant of phase I, corresponding to 30 nmol of aspartate, was added and the absorbance was read again in order to know the absorbance of the sample. Then a mix composed by 50 µl of aspartate transaminase (1,2 UI/ml) (Roche Diagnostics, Basel, Switzerland) and 5 µl of malate dehydrogenase (1 UI/ml) (Roche Diagnostics, Basel, Switzerland) was prepared. The reaction starts when the enzymes are added to each well. The drop of absorbance was read during 20 minutes and at intervals of 30 seconds. The fall in NADH was used to determine the levels of aspartate at each incubation time. Its disappearance (versus time zero levels) was used to calculate the aspartate incorporated into arginino-succinate by the enzyme. As for OTC, protein content in each of the homogenates used for enzyme analysis was estimated with a colorimetric method and the enzyme activity was referred to the homogenate protein content.

If no specified, chemicals and compounds used for this experiment were obtained from Panreac (Castellar del Vallès, Barcelona, Spain) or from Sigma (St Louis, MO, USA) .

4. Discussion

The aim of this investigation was to develop sensitive and feasible methods for OTC and ASS.

For OTC activity, a radiochemical, reliable and economic method was developed. This method has got some advantages: it can be used in different tissues, it permits to avoid the employment of a non accurate colorimetric method, it employs the 14C-ornithine that has got a good stability in solution, it is developed by thin layer chromatography that guarantee the accountability of substrate and product, and then, it is characterized by the absence of significant amounts of radioactivity on the chromatograms in region other than those to which ornithine and citrulline migrate.

For ASS activity, a new colorimetric method was developed. The main advantage was to measure the activity of ASS not coupled with the ASL one.

Both methods presented have the advantage of simplicity; several samples can be analysed in parallel, requires a short time of incubation, repetitiveness, and requires minimal manipulation of the incubation medium.

5. Bibliography

1. Arriarán S, Agnelli S, Remesar X, et al. A radiochemical method for carbamoyl-phosphate synthetase-I: Application to rats fed a hyperproteic diet. *J Enz Res* **3,** 29–33 (2012).
2. Schimke RT. Arginase (Rat Liver). *Meth Enzym.* **17,** 313–317 (1970).
3. Lanpher B, Brunetti-Pierri N, Brendan L. Inborn errors of metabolism: the flux from Mendelian to complex diseases. *Nat. Rev Gen* **7,** 449–60 (2006).
4. Lusty CJ, Jilka RL, Nietsch EH. Ornithine Transcarbamylase of Rat Liver. *J Biol Chem* **254,** 100030–10036 (1979).
5. Takiguchi M, Masataka M. Transcriptional regulation of genes for ornithine cycle enzymes. *Biochem J* **312,** 649–659 (1995).
6. Jackson MJ, Beaudet AL, O'Brien WE. Mammalian urea cycle enzymes. *Annu Rev Gen* **20,** 431–464 (1986).
7. Diaz-Muñoz M, Hernandez-Muñoz R. Molecular and biochemical features of the mitochondrial enzyme ornithine transcarbamylase: A possible new role as a signaling factor. *Curr Med Chem* **17,** 2253–2260(8) (2010).
8. Morris SM. Regulation of enzymes of urea and arginine synthesis. *Annu Rev Nutr* (1992).
9. Morizono H, Tuchman M, Rajagopal B, et al. Expression, purification and kinetic characterization of wild-type human ornithine transcarbamylase and a recurrent mutant that produces 'late onset' hyperammonaemia. *Biochem J* **322,** 625–631 (1997).
10. Van der Heiden C, Desplanque J. Ornithine transcarbamilase (oct) in urease used as reagent in the oct activity measurement in biological fluids. *Clin Chim Acta* **73,** 117–120 (1976).
11. Goldstein AS, Hoogenraad NJ, Johnson JD, et al. Metabolic and genetic studies of a family with ornithine transcarbamylase deficiency. *Pediatr Res* **8,** 5–12 (1974).
12. Bandurski RS, Axelrod B. The chromatographic identification of some biologically important phosphate esters. *J Biol Chem* **193,** 405–410 (1951).
13. Yemm EW, Cocking EC, Ricketts RE. The determination of amino-acids with ninhydrin. *Analyst* **80,** 209–214 (1955).
14. McLaren J, Won GNg. Assay of ornithine carbamoyltransferase activity in human liver using carbon-labeled ornithine and thin-layer chromatography. *Clin Chim Acta* **81,** 193–201 (1977).
15. Wang C, Smith RL. Lowry determination of protein in the presence of Triton X-100. *Anal Biochem* **63,** 414–417 (1975).
16. Ratner S, Petrack B. Biosynthesis of urea. III. Further studies on arginine synthesis from citrulline. *J Biol Chem* **191,** 693–705 (1951).
17. Ratner S. Enzymes of arginine and urea synthesis. *Adv Enzym. Rel Areas Mol Biol* **39,** 1–90 (1973).
18. Yu Y, Terada K, Nagasaki A, et al. Preparation of Recombinant Argininosuccinate Synthetase and Argininosuccinate Lyase: Expression of the Enzymes in Rat Tissues. *J Biochem* **117,** 952–957 (1995).
19. Demarquoy J, Fairand A, Gautier C, et al. Demonstration of argininosuccinate synthetase activity associated with mitochondrial membrane: characterization and hormonal regulation. *Mol Cell Biochem* **136,** 145–55 (1994).
20. Ball RO, Urschel KL, Pencharz PB. Nutritional consequences of interspecies differences in

arginine and lysine metabolism. *J Nutr* **137,** 1626S–1641S (2007).

21. Husson A, Brasse-Lagnel C, Fairand A, et al. Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. *Eur J Biochem* **270,** 1887–1899 (2003).
22. Saheki T, Katsunuma T, Sase M. Regulation of Urea Synthesis in Rat Liver: Changes of Ornithine and Acetylglutamate Concentrations in the Livers of Rats Subjected to Dietary Transitions. *J Biochem* **82,** 551–558 (1977).
23. Schimke RT. Adaptive characteristics of urea cycle enzymes in the rat. *J Biol Chem* **237,** 459–468 (1962).
24. O'Brien, W. E. Isolation and characterization of argininosuccinate synthetase from human liver. *Biochemistry* **18,** 5353–5356 (1979).
25. De Ruiter H, Kollöffel C. Arginine catabolism in the cotyledons of developing and germinating pea seeds. *Plant physiol* **73,** 525–528 (1983).
26. Van Slyke DD. A method for quantitative determination of aliphatic amino groups. *J Biol Chem* **12,** 275–284 (1912).
27. Schmidt CLA. The reaction between nitrous acid and certain amino acids and related compounds at 45'. *J Biol Chem* **82,** 587–594 (1929).
28. Menyhárt J, Gróf J. Urea as a selective inhibitor of argininosuccinate lyase. *Eur J Biochem* **75,** 405–409 (1977).
29. Hilger F, Culot M, Minet M, et al. Studies on the kinetics of the enzyme sequence mediating arginine synthesis in Saccharomyces cerevisiae. *J Gen Microbiol* **75,** 33–41 (1973).

4. RESULTS

4.1 Sabater D, Agnelli S, Arriarán S, et al. Altered nitrogen balance and decreased urea excretion in male rats fed cafeteria diet are related to arginine availability. Biomed Res. Int. 2014, 959420 (2014)

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 959420, 9 pages
http://dx.doi.org/10.1155/2014/959420

Research Article

Altered Nitrogen Balance and Decreased Urea Excretion in Male Rats Fed Cafeteria Diet Are Related to Arginine Availability

David Sabater,[1,2] Silvia Agnelli,[1,2] Sofía Arriarán,[1,2] José-Antonio Fernández-López,[1,2,3] María del Mar Romero,[1,2,3] Marià Alemany,[1,2,3] and Xavier Remesar[1,2,3]

[1] *Department of Nutrition and Food Science, Faculty of Biology, University of Barcelona, 08028 Barcelona, Spain*
[2] *Institute of Biomedicine, University of Barcelona, 08028 Barcelona, Spain*
[3] *CIBER Obesity and Nutrition, Institute of Health Carlos III, 28029 Madrid, Spain*

Correspondence should be addressed to Xavier Remesar; xremesar@ub.edu

Received 5 November 2013; Revised 30 December 2013; Accepted 16 January 2014; Published 24 February 2014

Academic Editor: Stelvio M. Bandiera

Hyperlipidic diets limit glucose oxidation and favor amino acid preservation, hampering the elimination of excess dietary nitrogen and the catabolic utilization of amino acids. We analyzed whether reduced urea excretion was a consequence of higher NO_x; (nitrite, nitrate, and other derivatives) availability caused by increased nitric oxide production in metabolic syndrome. Rats fed a cafeteria diet for 30 days had a higher intake and accumulation of amino acid nitrogen and lower urea excretion. There were no differences in plasma nitrate or nitrite. NO_x and creatinine excretion accounted for only a small part of total nitrogen excretion. Rats fed a cafeteria diet had higher plasma levels of glutamine, serine, threonine, glycine, and ornithine when compared with controls, whereas arginine was lower. Liver carbamoyl-phosphate synthetase I activity was higher in cafeteria diet-fed rats, but arginase I was lower. The high carbamoyl-phosphate synthetase activity and ornithine levels suggest activation of the urea cycle in cafeteria diet-fed rats, but low arginine levels point to a block in the urea cycle between ornithine and arginine, thereby preventing the elimination of excess nitrogen as urea. The ultimate consequence of this paradoxical block in the urea cycle seems to be the limitation of arginine production and/or availability.

1. Introduction

Metabolic syndrome is a pathological condition, which develops from localized inflammation and is characterized by the combination of a number of closely related diseases (insulin resistance, obesity, hyperlipidemia, hypertension, etc.) [1]. Administration of "cafeteria" diets [2] to rats has been used as an animal model for the study of late-onset hyperphagic obesity and metabolic syndrome. This model has the advantage of being comparable to some human obesity states induced by the excessive intake of energy-dense food [3, 4]. The effects of the cafeteria diet are more marked in males [5, 6]; this may be because they have less anti-inflammatory [7] estrogen protection than females. Low estrogen levels render males more prone to be affected by glucocorticoids [8, 9], which in turn decrease the anabolic effects of androgens [10, 11].

In rodents, prolonged exposure to a cafeteria diet results in higher energy intake (mainly lipids) [5] and increased body fat (obesity) but also affects lean body mass, favoring growth and protein deposition [12, 13]. Although the hedonic component of the cafeteria diet initially elicits an increase in food consumption [14], once obesity is well established, hyperphagia decreases to almost normal levels of food intake. However, the large mass of accumulated fat remains, and the metabolic consequences of excess energy intake—such as insulin resistance and hyperlipidemia—persist [6, 15].

The study of nitrogen handling under hypercaloric diet conditions has predominantly been limited to measuring plasma amino acid levels [16–18], while less attention has centered on pathways [19, 20] and nitrogen balances [21]. To date, most research on dietary amino acid metabolism has been directed toward the analysis of metabolic adaptation to diets deficient in both energy and amino nitrogen [22–25] or

has focused on specific regulatory pathways. Cafeteria diets are suitable for the study of the effects of high-energy diets, because total protein intake is practically unaffected by the excess dietary energy (mainly lipids) ingested [5].

Dietary or body-protein amino acid nitrogen is spared when other energy sources (such as fat or glucose) abound. Thus, high-energy diet, such as the cafeteria diet, apparently decreases overall amino acid catabolism, inducing a marked decrease in the production of urea [26]. The relative surplus of 2-amino nitrogen can maintain protein turnover and growth [12], but the excess nitrogen must be excreted in some way. The limited operation of the urea cycle suggests that there may be more amino nitrogen available for the operation of the nitric oxide ($NO^{\bullet}$) shunt. Obese humans have been found to excrete more nitrate than their lean counterparts, and loss of $NO^{\bullet}$ in expired air is proportional to body mass index (BMI) [27]. In terms of nitrogen balance, it has been found that cafeteria diet-fed rats show a higher "nitrogen gap," that is, the difference between nitrogen intake and the sum of its accumulation and excretion in the urine and feces [21] than control diet-fed animals.

The objective of the present study was to determine whether cafeteria diet-fed rats show changes in the excretion of nitrate/nitrite in comparison with age-matched animals fed standard rat chow, investigating whether an increase in the excretion of NO_x compensates for the decrease in urea excretion.

2. Materials and Methods

2.1. Animals and Experimental Setup. All animal handling procedures and the experimental setup were carried out in accordance with the animal handling guidelines of the European, Spanish, and Catalan Authorities. The Committee on Animal Experimentation of the University of Barcelona authorized the specific procedures used. This limited keeping the animals in metabolic cages to a maximum of 24 h to prevent unacceptable levels of stress.

Nine-week-old male Wistar rats ($N = 12$) (Harlan Laboratories Models, Sant Feliu de Codines, Spain) were used. The animals were randomly divided into two groups and were fed *ad libitum*, for 30 days on either normal rat chow (Harlan 2014) ($N = 6$) or a simplified cafeteria diet ($N = 6$) [21]. Both groups were housed in solid-bottomed cages with three animals per cage, had free access to water, and were kept in a controlled environment (lights on from 08:00 to 20:00, with a temperature of 21.5–22.5°C; 50–60% humidity). Body weight and food consumption were recorded daily. Calculation of food ingested was performed as previously described by counting the difference between food offered and left, including the recovery of small pieces of food, and compensating for drying [5]. The nitrogen content of the rat chow and the different components used in the cafeteria diet were measured with a semiautomatic Kjeldahl procedure using a Pro-Nitro S semiautomatic system (JP Selecta, Abrera, Spain).

On day 0 (i.e., the day before the experiment began) and day 26, the rats were kept for 24 h in metabolic cages (Tecniplast Gazzada, Buguggiate, Italy), recovering the urine and feces. In the metabolic cages, all rats were fed only standard rat chow and tap water, and their food consumption was measured. Samples of excreta were frozen for later analyses. Urine NO_x was estimated immediately to minimize further oxidation and $NO^{\bullet}$ losses, using a nitric oxide analysis system (ISM-146NOXM system) (Lazar, Los Angeles, CA, USA).

On day 30, rats were anesthetized with isoflurane and then killed by exsanguination through the exposed aorta. Blood plasma and tissue samples were obtained and frozen. Liver samples were rapidly frozen in liquid nitrogen and maintained at −80°C until processed for enzyme analyses. For tissues distributed widely throughout the body (i.e., subcutaneous adipose tissue), all the tissue was carefully dissected and weighed. Hind leg muscle samples were cut from the hind leg, obtaining part of the *quadratus femoris, biceps femoris*, and *semitendinosus* muscle and a smaller proportion of others.

2.2. Diet Composition. In the standard diet (Harlan 2014), 19.9% of energy was derived from protein, 13.8% from lipids, and 65.8% from carbohydrates (10% from sugars).

The cafeteria diet included biscuits spread with liver pâté, bacon, standard chow pellets, water, and milk supplemented with 300 g/L sucrose plus 10 g/L of a mineral and vitamin supplement (Meritene, Nestlé, Esplugues, Spain). All of these compounds were provided fresh daily. From the analysis of the diet components and the ingested items, we calculated that a mean of 33% of energy was derived from lipids, 16% of energy was derived from protein, and 51% of energy was derived from carbohydrates (20% from sugars).

2.3. Body and Metabolite Analyses. Total body muscle mass was estimated, as previously described [28], using the remaining carcass. The method was based on the solubilization of muscle actin and myosin with 1 M LiCl and subsequent precipitation of mainly myosin with distilled water, followed by its estimation with a standard procedure.

Stool nitrogen was measured using the semiautomatic Kjeldahl procedure described above. Nitrogen content and nitrogen accrual were calculated by applying the body composition factors obtained from previous studies [3, 21] to our experimental animals. These data are included as reference values only for comparison. Urine urea was measured with a urease-based test, and creatinine was measured with the Jaffé reaction using commercial kits (BioSystems, Barcelona, Spain).

Plasma was used for the analysis of glucose, total cholesterol, triacylglycerol and urea (using kits from BioSystems, Barcelona, Spain), nonesterified fatty acids (NEFA kit; Wako, Richmond VA, USA), and L-lactate (Spinreact kit, Barcelona, Spain). Nitrite and nitrate were measured with the Arrow-Straight system. Plasma samples were used for amino acid quantification after deproteinization with trifluoroacetic acid; they were measured with ninhydrin, in a Biochrom 30 auto-analyzer (Biochrom, Cambridge, UK), using L-norleucine as internal standard, at the Scientific-Technical Services of the University of Barcelona.

2.4. Enzyme Assays. Frozen liver samples were homogenized in chilled 50 mM Krebs-Ringer phosphate buffer, pH 7.4, containing 0.1% Triton X-100, 2.5 mM mercaptoethanol, 0.1% dextran, 5 mM Na_2-EDTA, and 0.5% bovine serum albumin using a mechanical tissue disruptor (IKA, Staufen, Germany). Arginase I (EC 3.5.3.1) activity was estimated as described elsewhere [29]. The method was based on the colorimetric estimation of urea (Berthelot reaction) released by the action of arginase on arginine. Homogenate protein content was measured with a standard colorimetric method [30] against blanks of the homogenization medium.

Other liver samples were homogenized in 50 mM triethanolamine-HCl buffer, pH 8.0 containing 1 mM dithiothreitol, and 10 mM magnesium acetate. Carbamoyl-P synthetase I (EC 6.3.4.16) activity was measured immediately after homogenization, as previously described [31], by measuring the incorporation of labeled bicarbonate (50 kBq/mmol) into carbamoyl-P in a medium containing 5 mM ATP, 5 mM N-acetyl-glutamate, and 0.05% bovine serum albumin. Enzyme activities were expressed in katals both in reference to the weight of fresh tissue and its protein content.

2.5. Statistical Analysis. Statistical analysis was carried out using one-way ANOVA, with the *post hoc* Bonferroni test, and/or the unpaired Student's *t*-test, using the Statgraphics Centurion XVI software package (StatPoint Technologies, Warrenton, VA, USA).

3. Results

Table 1 shows the body weights and nitrogen balance values for control and cafeteria diet-fed rats at the beginning and end of the study (day 27 for nitrogen data). As expected, the increase in body weight was greater in cafeteria diet-fed rats than in controls. The overall energy and nitrogen intake were also higher in cafeteria diet-fed than in control rats.

In the period that the rats were kept in metabolic cages, significant differences in nitrogen intake were observed between the groups, but not in urine or stool nitrogen excretion. The proportion of urea excreted with respect to the total daily nitrogen budget was lower in cafeteria diet-fed rats than in controls on day 27 (65% versus 75% of nitrogen intake, 82% versus 94% of urea excreted, resp.). This difference was not compensated for by creatinine and NO_x excretion, which was low in comparison to urea, and showed slight changes over time or with dietary treatment. Thus, although the data on nitrogen balance was measured on different days, the estimated "nitrogen gap" showed a wider margin for cafeteria diet-fed rats than for controls.

The effects of diet on organ weight on day 30 are presented in Table 2. The only significant differences between the two groups in organ weights were for muscle, stomach, heart, and adipose tissues. The other organs showed remarkably similar weights.

The plasma nitrate and nitrite concentrations are presented in Table 3. There were no significant differences between control and cafeteria-fed rats for nitrate, nitrite, or their sum. In both groups, however, nitrate was the predominant component (>90%).

Table 4 shows the plasma amino acid concentrations of the control and cafeteria diet-fed groups on day 30. The similarity between the groups was remarkable, with only a few amino acids showing significant differences. Cafeteria diet-fed rats had higher levels of glutamine, threonine, serine, glycine, and ornithine than controls, while the latter showed higher levels of arginine with respect to the cafeteria diet-fed animals. When analyzing the sums of concentrations of groups of related amino acids, no changes were observed for the combined concentrations of glutamate + glutamine, aspartate + asparagine, branched-chain amino acids (leucine + isoleucine + valine), or urea cycle intermediaries (ornithine + citrulline + arginine).

The plasma concentrations of glucose, triacylglycerols, total cholesterol, and urea for both diet groups (Table 5) were similar, and all were within the normal range. These concentrations were similar to data previously published by our group, with no differences between the groups, except for higher glucose and lower urea values in cafeteria diet-fed rats.

Figure 1 presents the measured activities of two key urea synthesis enzymes in the livers of the control and cafeteria diet-fed groups. The activity of carbamoyl-P synthetase I was higher than that of arginase I in the cafeteria diet-fed group; these rats had threefold higher activity rates for carbamoyl-P synthetase I than controls. The results for arginase were the reverse, since the control group had almost twice the activity per unit of tissue weight than the cafeteria diet-fed group, and this result was similar for protein and total tissue.

4. Discussion

The cafeteria diet is essentially hyperlipidic, with identical mean protein and carbohydrate intakes to those of control rats fed a standard diet [5]. As expected, a one-month exposure to a cafeteria diet resulted in overfeeding and increased body weight, leading to a greater increase in the size of adipose tissue deposits and higher muscle mass than in controls. These results are in agreement with previous studies showing that, as with the hyperlipidic diets, a cafeteria diet increases not only fat deposition and growth, but also protein accrual [12, 21] and energy expenditure [32]. A lower excretion of urea, irrespective of the maintained (or increased) amino acid intake, was also observed, again in agreement with previous studies [21, 26].

It has been postulated that a high-energy diet coupled with normal or increased protein intake may hamper 2-amino nitrogen elimination in rats, humans, and other mammals [33]. This problem is largely a consequence of the abundance of energy, mainly in the form of lipids, which is used preferentially by muscle and other peripheral tissues over glucose because of insulin resistance [34]. However, amino acid oxidation is spared due to the availability of energy, that is, in the form of glucose [35]; thus, is to be expected that catabolism of dietary amino acids and, therefore, the production of ammonium from 2-amino nitrogen should also decrease. Consequently, during this buildup,

Table 1: Energy and nitrogen balances of male rats fed a cafeteria diet compared with controls fed a standard rat chow diet.

	Units	Control: initial ($n = 12$)	Control: final ($n = 6$)	Cafeteria diet-fed: final ($n = 6$)	P	Control: (days 1–30)	Cafeteria diet-fed: (days 1–30)
Rat weight	g	291 ± 2^{A}	373 ± 17^{B}	449 ± 8^{C}	<0.001		
Rat weight change	g/30 day					83 ± 7	$158 \pm 7^{*}$
Energy intake	kJ/d	326 ± 6^{A}	365 ± 6^{A}	596 ± 11^{B}	<0.001	350 ± 9	$680 \pm 6^{*}$
Body nitrogen content	g	10.4 ± 0.7^{A}	13.0 ± 0.8^{AB}	15.0 ± 1.2^{B}	0.004		
Body nitrogen accrual	mg/day					87 ± 8	$157 \pm 11^{*}$
Nitrogen intake	mg/day	478 ± 17^{A}	481 ± 14^{A}	652 ± 25^{B}	<0.001	475 ± 11	$813 \pm 13^{*}$
Stool nitrogen[#]	mg/day	23 ± 2	23 ± 3	22 ± 2	NS		
Urea nitrogen excreted[#]	mg/day	374 ± 32	441 ± 33	335 ± 35	NS		
Creatinine nitrogen excreted[#]	μg/day	54 ± 6^{A}	67 ± 10^{B}	$79 + 5^{B}$	0.003		
NO_x nitrogen excreted[#]	μg/day	2.7 ± 0.2^{A}	3.3 ± 0.2^{AB}	3.8 ± 0.4^{B}	0.020		
Nitrogen excreted not accounted (N gap)[#]	mg/day	21 ± 25^{A}	24 ± 14^{A}	99 ± 15^{B}	<0.001		

The values are the mean ± standard error of the mean (SEM) for 6 different animals. Statistical significance of the differences between groups: P (one-way analysis of variance (ANOVA): time); different superscript letters represent statistically significant differences between groups (Bonferroni *posthoc* test) $^{*}P < 0.05$ for Student's t-test (30-day changes). [#]Data between days 0 and 27.

Table 2: Organ and tissue weights of male rats fed a cafeteria diet for one month compared with controls fed a standard rat chow diet.

Tissue/organ	Control (g) ($n = 6$)	Cafeteria diet-fed (g) ($n = 6$)	P
Skeletal muscle	143 ± 4	164 ± 5	0.007
Skin	54.8 ± 1.14	55.5 ± 3.84	NS
Liver	9.86 ± 0.48	11.23 ± 0.38	NS
Small intestine	2.98 ± 0.22	2.90 ± 0.18	NS
Kidneys	2.14 ± 0.08	2.27 ± 0.06	NS
Brain	1.940 ± 0.073	1.884 ± 0.027	NS
Large intestine	1.46 ± 0.05	1.31 ± 0.06	NS
Lungs	1.418 ± 0.164	1.308 ± 0.041	NS
Stomach	1.265 ± 0.039	1.438 ± 0.054	0.023
Heart	0.936 ± 0.048	1.075 ± 0.024	0.027
Interscapular BAT	0.413 ± 0.042	0.442 ± 0.041	NS
Subcutaneous WAT	5.96 ± 0.69	9.11 ± 1.11	0.015
Mesenteric WAT	3.61 ± 0.52	3.86 ± 0.09	NS
Retroperitoneal WAT	2.58 ± 0.36	4.66 ± 0.36	0.002
Epididymal WAT	1.53 ± 0.26	4.43 ± 0.38	<0.001
Pericardial WAT	0.252 ± 0.047	0.311 ± 0.041	NS
Sum of five WAT sites	**13.93 ± 1.89**	**22.23 ± 1.78**	**0.007**

The values are the mean ± SEM for 6 different animals. BAT: brown adipose tissue; WAT: white adipose tissue. Statistical significance of the differences between groups; P was calculated with Student's t test.

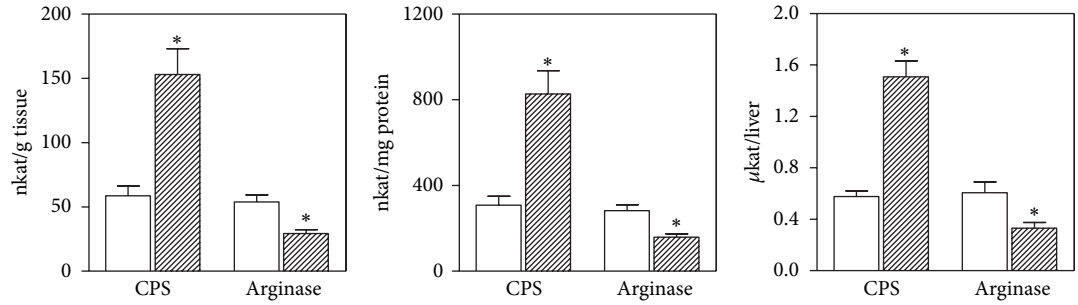


Figure 1: Carbamoyl-P synthetase I and arginase activities in the liver of rats fed a control or cafeteria diet for 30 days. CPS = carbamoyl-P synthetase I. Control: white columns; cafeteria diet-fed: dashed columns. The values are the mean ± SEM for 6 different animals. Comparisons between groups were established with Student's t-test: $^{*}P < 0.05$.

Table 3: Plasma nitrate and nitrite concentrations of male rats fed a cafeteria diet for one month compared with controls fed a standard rat chow diet.

	Units	Control	Cafeteria diet-fed
Nitrite	μM	3.4 ± 1.6	3.3 ± 1.3
Nitrate	μM	48.0 ± 4.7	45.6 + 6.6
Nitrate	% of total	94.5 ± 2.6	92.8 ± 2.8
NO_x total	μM	**51.4 ± 6.0**	**44.8 ± 7.3**

The values are the mean ± SEM for 6 different animals. There were no significant differences between the two groups ($P > 0.05$, Student's t-test) for any parameter. WAT: white adipose tissue, BAT: brown adipose tissue.

the mechanisms of amino nitrogen waste prevention surprisingly create a surplus of available amino acids. The excess of 2-amino nitrogen may be limited, in part, by increased growth (e.g., increased muscle mass) and, to a lesser extent, by increased protein turnover. However, the problem remains that not enough ammonium can be produced from the amino acid pool to maintain the glutamine (or free ammonium) necessary for the splanchnic organs (i.e., the intestines, liver, and kidney) to eliminate the excess of nitrogen as urea [36–38].

The observed plasma levels of amino acids seem to confirm these trends. In cafeteria diet-fed rats, glutamine but not glutamate + glutamine levels were higher than in control rats. High levels of glutamine suggest its decreased splanchnic utilization to provide ammonium for the synthesis of carbamoyl-P. The high circulating levels of ornithine again suggest a diminished production of carbamoyl-P, perhaps because of scarcity of ammonium donors in the liver. The lower arginine levels in the cafeteria diet-fed group versus controls suggest that synthesis of arginine may be insufficient to compensate for the release of urea through arginase activity

Table 4: Plasma amino acid concentrations of male rats fed a cafeteria diet for one month compared with controls fed a standard rat chow diet.

Amino acid	Control (μM)	Cafeteria diet-fed (μM)	P
Alanine	464 ± 5	455 ± 37	NS
Aspartate*	31 ± 3	40 ± 8	NS
Asparagine*	49 ± 5	57 ± 4	NS
Σ Asp + Asn	**81 ± 4**	**87 ± 8**	**NS**
Glutamate*	149 ± 17	160 ± 11	NS
Glutamine*	487 ± 27	582 ± 5	0.005
Σ Glu + Gln	**641 ± 48**	**705 ± 32**	**NS**
Proline	139 ± 12	152 ± 12	NS
Hydroxyproline	22 ± 3	25 ± 4	NS
Threonine	165 ± 10	202 ± 7	0.010
Serine	186 ± 8	245 ± 13	0.003
Glycine	342 ± 9	417 ± 17	0.002
Leucine*	144 ± 7	146 ± 10	NS
Isoleucine*	52 ± 4	61 ± 8	NS
Valine*	157 ± 14	162 ± 15	NS
Σ branched chain	**355 ± 36**	**355 ± 15**	**NS**
Phenylalanine	76 ± 4	87 ± 6	NS
Tyrosine	87 ± 3	93 ± 6	NS
Tryptophan	95 ± 11	101 ± 7	NS
Methionine	51 ± 3	53 ± 4	NS
Cysteine	21 ± 3	22 ± 2	NS
Lysine	376 ± 18	396 ± 14	NS
Histidine	62 ± 4	69 ± 8	NS
Ornithine*	50 ± 3	68 ± 7	0.045
Citrulline*	48 ± 4	51 ± 4	NS
Arginine*	209 ± 10	164 ± 8	0.006
Σ urea cycle	**278 ± 12**	**305 ± 14**	**NS**
Σ total (mM)	**3.98 ± 0.10**	**4.22 ± 0.19**	**NS**

The values are the mean ± SEM for 6 different animals. Statistical significance of the differences between the two groups was determined with Student's t-test. NS: $P > 0.05$. Asterisks "*" indicate the amino acids incorporated in the sums marked in bold below them.

Table 5: Plasma metabolite levels of male rats fed a cafeteria diet for one month compared with controls fed a standard rat chow diet.

Plasma values (mM)	Control	Cafeteria diet-fed	P
Glucose	8.13 ± 0.36	9.20 ± 0.28	0.042
Triacylglycerols	1.91 ± 0.06	1.84 ± 0.08	NS
Cholesterol	1.75 ± 0.17	1.90 ± 0.17	NS
Urea	5.28 ± 0.24	3.94 ± 0.21	0.002

The values are the mean ± SEM for 6 different animals. Statistical significance of the differences between both groups; P was determined with Student's t-test.

or other uses. Arginase I in liver, which is the main site for this enzyme to complete the urea cycle [39], showed lower activity in the cafeteria diet-fed group. This may help maintain circulating arginine, although at levels lower than in control-fed animals.

The higher carbamoyl-P synthetase I activity found in cafeteria diet-fed rats agrees with the clear surplus of 2-amino nitrogen available for excretion, since higher liver ammonium availability increases the activity of this enzyme [40]. The decrease in urea excretion agrees with lower arginase activity, but not with the increased activity of carbamoyl-P synthetase I, which depends on ammonium as its substrate [40]. Thus, the block in urea cycle function (and consequently in "normal" 2-amino nitrogen disposal) should lie between these enzymes in the urea cycle, that is, in the conversion of ornithine to citrulline or the latter to arginine (i.e., argininosuccinate synthetase and argininosuccinate lyase). In addition, the higher ornithine levels in the plasma of cafeteria diet-fed rats suggest that the N-acetyl-glutamate pathway for the exogenous synthesis of ornithine was not sufficiently activated to compensate for the arginine deficit.

Because the circulating levels of citrulline and aspartate were unchanged (or increased) in cafeteria diet-fed versus control rats, it can be assumed that there is probably a key regulatory path, for overall nitrogen disposal, either at the synthesis or breakup of argininosuccinate, which would help explain the lower production of urea. Based on kinetic studies, argininosuccinate synthesis was initially postulated as a key urea cycle control node [41], although normal urea cycle operation is assumed to rely more on other parameters such as pH, ammonium availability, and N-acetyl-glutamate levels [42]. However, the indirect data presented here suggest that argininosuccinate synthesis/breakup may be a significant control point *in vivo* under relatively high nitrogen (and energy) availability.

The involvement of ammonium availability in this context is enhanced by the relatively higher concentrations of threonine, serine, glycine, and glutamine in cafeteria diet-fed rats. These amino acids yield ammonium in their catabolism [43] via threonine/serine dehydratase, glutaminase, or the glycine cleavage system. Serine may also be converted to glycine, which leads to the same fate. These results show an unexpected picture, since, in cafeteria diet-fed rats, there is an excess of 2-amino nitrogen and the higher levels observed correspond to ammoniagenic amino acids. According to the normal catabolic pathways for nitrogen excretion, this excess should activate the production of ammonium, its transport as glutamine, release again as ammonium, and formation of carbamoyl-P, followed by its integration (with more aspartate-derived amino nitrogen) into the guanido group of arginine for its eventual release as urea. However, the amino acids that can yield ammonium directly, in an initial nontransaminative catabolic step, were somehow preserved in cafeteria diet-fed rats. These amino acids were not used in large quantities as was to be expected in a situation in which, theoretically, the lack of 2-amino nitrogen conversion to ammonium could hinder normal nitrogen excretion through the urea cycle. The contrast between preservation of the ammonium donors and high carbamoyl-P synthetase I activity in the cafeteria diet-fed group suggests that the problem does not lie in the availability of ammonium. Higher levels of the main amino acid ammonium donors suggest

instead a constraint on their utilization because elimination via the urea cycle is blocked as indicated above.

The faulty operation of the urea cycle, then, suggests that the main control mechanism sought is not centered on the availability of ammonium-yielding substrates as is usually postulated for normal and starvation conditions [42]. The increased activity of carbamoyl-P synthetase and the low activity of arginase in cafeteria diet-fed rats indicate that the control mechanism lies in the actual synthesis of arginine, which is also essential for the operation of the $NO^{\bullet}$ shunt. Notwithstanding, the $NO^{\bullet}$ shunt does not seem to be significantly altered by the cafeteria diet, as shown by unchanged plasma and urinary NO_x in spite of lower circulating arginine. One possible explanation is that the blockage of arginine production results from the need to prevent an increase in the production of $NO^{\bullet}$ under cafeteria diet conditions, in which blood flow—in part dependent on $NO^{\bullet}$ synthesis—to a number of tissues is markedly altered [44].

The question remains of how the excess nitrogen provided by cafeteria diets is eliminated. The widening of the nitrogen gap under high-energy feeding suggests that nitrogen gas [45] may be involved, since the amount of creatinine, uric acid, and so forth, excreted is only a small fraction of urea nitrogen [46, 47]. The synthesis of $NO^{\bullet}$ results in the excretion, mainly via saliva [48], of nitrite and nitrate. In addition, there is a small direct loss of $NO^{\bullet}$ in the breath [49]. However, the low levels of NO_x measured in the urine and their marked metabolic effects [50] suggest that NO_x as $NO^{\bullet}$ derivatives, could account for at most only a very small part of the "missing" nitrogen. The lack of changes elicited by diet in circulating levels of nitrate and nitrite reinforced this assumption; that is, nitrate excretion is not a significant alternative as a nitrogen-disposal pathway to lower urea synthesis.

The one-month period of exposure to the cafeteria diet proved that this type of diet caused difficulties in the normal mechanisms of amino nitrogen disposal, exemplified by a lower urea production. These problems were not directly related to the potential availability of ammonium as the prime substrate for initiating the urea cycle but instead were probably related to the availability of arginine. No changes were observed in the levels or excretion of NO_x, which were small, but the "nitrogen gap" [21] became significant under cafeteria diet feeding. It is now clear that the decrease in urea excretion is not compensated for by higher NO_x production and elimination. The main pathway for disposal of the excess amino nitrogen generated by energy rich diets remains unsolved, with the additional conundrum of why the urea cycle appears to be disrupted for the only apparent reason of limiting the availability of arginine.

5. Conclusions

The decrease in urea excretion is not compensated for by higher NO_x production and elimination. The defective operation of the urea cycle in rats fed a cafeteria diet seems to be caused by a block in the urea cycle between ornithine and arginine.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

This study was funded by Grants from the Plan Nacional de Investigación en Biomedicina (SAF2012-34895) and the Plan Nacional de Ciencia y Tecnología de los Alimentos (AGL-2011-23635) of the Spanish Government. Ciber-Obn paid for the salary of María del Mar Romero. The funding bodies played no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

[1] M. Alemany, "Metabolic syndrome: a multifaceted disease of affluence," *Journal of Endocrinology and Metabolism*, vol. 2, pp. 155–165, 2012.

[2] A. Sclafani and D. Springer, "Dietary obesity in adult rats: similarities to hypothalamic and human obesity syndromes," *Physiology and Behavior*, vol. 17, no. 3, pp. 461–471, 1976.

[3] M. M. Romero, M. Esteve, and M. Alemany, "Combined effects of oral oleoyl-estrone and limited food intake on body composition of young overweight male rats," *International Journal of Obesity*, vol. 30, no. 7, pp. 1149–1156, 2006.

[4] A. Miesel, H. Müller, M. Thermann, M. Heidbreder, P. Dominiak, and W. Raasch, "Overfeeding-induced obesity in spontaneously hypertensive rats: an animal model of the human metabolic syndrome," *Annals of Nutrition and Metabolism*, vol. 56, no. 2, pp. 127–142, 2010.

[5] E. Prats, M. Monfar, J. Castellà, R. Iglesias, and M. Alemany, "Energy intake of rats fed a cafetaria diet," *Physiology and Behavior*, vol. 45, no. 2, pp. 263–272, 1989.

[6] G. Coatmellec-Taglioni, J.-P. Dausse, Y. Giudicelli, and C. Ribière, "Sexual dimorphism in cafeteria diet-induced hypertension is associated with gender-related difference in renal leptin receptor down-regulation," *Journal of Pharmacology and Experimental Therapeutics*, vol. 305, no. 1, pp. 362–367, 2003.

[7] R. H. Straub, "The complex role of estrogens in inflammation," *Endocrine Reviews*, vol. 28, no. 5, pp. 521–574, 2007.

[8] C. Bouclaous, N. Torbay, C. Nassar, and N. Hwalla, "Modification of glucocorticoid effects on body weight gain, plasma lipids by changes in diet composition," *Nutrition Research*, vol. 23, no. 8, pp. 1105–1115, 2003.

[9] R. V. Carsia, H. Weber, P. J. McIlroy, and C. E. Hock, "Long-term dietary lipid regimen alters adrenocortical function at the cellular level," *Hormone and Metabolic Research*, vol. 40, no. 12, pp. 848–853, 2008.

[10] S. Retana-Márquez, H. Bonilla-Jaime, G. Vázquez-Palacios, R. Martínez-García, and J. Velázquez-Moctezuma, "Changes in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats," *Hormones and Behavior*, vol. 44, no. 4, pp. 327–337, 2003.

[11] W. Zhao, J. Pan, Z. Zhao, Y. Wu, W. A. Bauman, and C. P. Cardozo, "Testosterone protects against dexamethasone-induced muscle atrophy, protein degradation and MAFbx upregulation,"

Journal of Steroid Biochemistry and Molecular Biology, vol. 110, no. 1-2, pp. 125–129, 2008.

[12] R. B. Harris, "The impact of high- or low-fat cafeteria foods on nutrient intake and growth of rats consuming a diet containing 30% energy as fat," *International Journal of Obesity*, vol. 17, no. 6, pp. 307–315, 1993.

[13] I. Lladó, C. Picó, A. Palou, and A. Pons, "Protein and amino acid intake in cafetaria fed obese rats," *Physiology and Behavior*, vol. 58, no. 3, pp. 513–519, 1995.

[14] P. J. Rogers and J. E. Blundell, "Meal patterns and food selection during the development of obesity in rats fed a cafeteria diet," *Neuroscience and Biobehavioral Reviews*, vol. 8, no. 4, pp. 441–453, 1984.

[15] M. B. Davidson and D. Garvey, "Studies on mechanisms of hepatic insulin resistance in cafeteria-fed rats," *American Journal of Physiology: Endocrinology and Metabolism*, vol. 264, no. 1, pp. E18–E23, 1993.

[16] M. Gianotti, P. Roca, and A. Palou, "The effects of cafeteria diet induced obesity on rat blood amino acid compartmentation," *Archives Internationales de Physiologie et de Biochimie*, vol. 98, no. 4, pp. 155–161, 1990.

[17] I. Rafecas, M. Esteve, X. Remesar, and M. Alemany, "Plasma amino acids of lean and obese Zucker rats subjected to a cafeteria diet after weaning," *Biochemistry International*, vol. 25, no. 5, pp. 797–806, 1991.

[18] I. Rafecas, M. Esteve, J. A. Fernandez-Lopez, X. Remesar, and M. Alemany, "Individual amino acid balances in young lean and obese Zucker rats fed a cafeteria diet," *Molecular and Cellular Biochemistry*, vol. 121, no. 1, pp. 45–58, 1993.

[19] F. Serra, L. Bonet, and A. Palou, "Amino-acid-enzyme activities in brown and white adipose tissues and in the liver of cafeteria rats. Effects of 24 hours starving," *Archives Internationales de Physiologie et de Biochimie*, vol. 95, no. 4, pp. 263–268, 1987.

[20] C. V. C. sGhizoni, F. R. Gasparin, A. S. Júnior et al., "Catabolism of amino acids in livers from cafeteria-fed rats," *Molecular and Cellular Biochemistry*, vol. 373, pp. 265–277, 2013.

[21] M. Esteve, I. Rafecas, X. Remesar, and M. Alemany, "Nitrogen balance discrepancy in wistar rats fed a cafeteria diet," *Biochemistry International*, vol. 26, no. 4, pp. 687–694, 1992.

[22] P. Felig, O. E. Owen, J. Wahren, and G. F. Cahill Jr., "Amino acid metabolism during prolonged starvation," *Journal of Clinical Investigation*, vol. 48, no. 3, pp. 584–594, 1969.

[23] R. C. O'Connell, A. P. Morgan, and T. T. Aoki, "Nitrogen conservation in starvation: graded responses to intravenous glucose," *Journal of Clinical Endocrinology and Metabolism*, vol. 39, no. 3, pp. 555–563, 1974.

[24] I. De Blaauw, N. E. P. Deutz, and M. F. Von Meyenfeldt, "In vivo amino acid metabolism of gut and liver during short and prolonged starvation," *American Journal of Physiology: Gastrointestinal and Liver Physiology*, vol. 270, no. 2, pp. G298–G306, 1996.

[25] D. A. Bender, "The metabolism of "surplus" amino acids," *British Journal of Nutrition*, vol. 108, pp. S113–S121, 2012.

[26] T. Barber, J. R. Viña, J. Viña, and J. Cabo, "Decreased urea synthesis in cafeteria-diet-induced obesity in the rat," *Biochemical Journal*, vol. 230, no. 3, pp. 675–681, 1985.

[27] K. W. Tsang, S. K. Ip, R. Leung et al., "Exhaled nitric oxide: the effects of age, gender and body size," *Lung*, vol. 179, no. 2, pp. 83–91, 2001.

[28] L. Arola, E. Herrera, and M. Alemany, "A method for the estimation of striated muscle mass in small laboratory animals," *Revista Espanola de Fisiologia*, vol. 35, no. 2, pp. 215–218, 1979.

[29] X. Remesar, L. Arola, A. Palou, and M. Alemany, "Arginase activity in the organs of fed and 24-hours fasted rats," *Hormone and Metabolic Research*, vol. 12, no. 6, pp. 281–282, 1980.

[30] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein measurement with the Folin phenol reagent," *The Journal of biological chemistry*, vol. 193, no. 1, pp. 265–275, 1951.

[31] S. Arriarán, S. Agnelli, J. A. Fernández-López, X. Remesar, and M. Alemany, "A radiochemical method for carbamoyl-phosphate synthetase-I: Application to rats fed a hyperproteic diet," *Journal of Enzyme Research*, vol. 3, pp. 29–33, 2012.

[32] S. W. Y. Ma and D. O. Foster, "Brown adipose tissue, liver, and diet-induced thermogenesis in cafeteria diet-fed rats," *Canadian Journal of Physiology and Pharmacology*, vol. 67, no. 4, pp. 376–381, 1989.

[33] M. Alemany, "The problem of nitrogen disposal in the obese," *Nutrition Research Reviews*, vol. 25, pp. 18–28, 2012.

[34] M. Roden, M. Krssak, H. Stingl et al., "Rapid impairment of skeletal muscle glucose transport/phosphorylation by free fatty acids in humans," *Diabetes*, vol. 48, no. 2, pp. 358–364, 1999.

[35] D. H. Elwyn, F. E. Gump, and M. Iles, "Protein and energy sparing of glucose added in hypocaloric amounts to peripheral infusions of amino acids," *Metabolism*, vol. 27, no. 3, pp. 325–331, 1978.

[36] H. G. Windmueller and A. E. Spaeth, "Uptake and metabolism of plasma glutamine by the small intestine," *Journal of Biological Chemistry*, vol. 249, no. 16, pp. 5070–5079, 1974.

[37] G. Wu, "Amino acids: metabolism, functions, and nutrition," *Amino Acids*, vol. 37, no. 1, pp. 1–17, 2009.

[38] T. Fujita and K. Yanaga, "Association between glutamine extraction and release of citrulline and glycine by the human small intestine," *Life Sciences*, vol. 80, no. 20, pp. 1846–1850, 2007.

[39] S. M. Morris Jr., "Arginine metabolism: boundaries of our knowledge," *Journal of Nutrition*, vol. 137, no. 6, pp. 1602S–1609S, 2007.

[40] N. S. Cohen, F. S. Kyan, S. S. Kyan, C. W. Cheung, and L. Raijman, "The apparent K(m) of ammonia for carbamoyl phosphate synthetase (ammonia) in situ," *Biochemical Journal*, vol. 229, no. 1, pp. 205–211, 1985.

[41] S. M. Morris Jr., "Regulation of enzymes of the urea cycle and arginine metabolism," *Annual Review of Nutrition*, vol. 22, pp. 87–105, 2002.

[42] S. M. Morris Jr., "Regulation of enzymes of urea and arginine synthesis," *Annual Review of Nutrition*, vol. 12, pp. 81–101, 1992.

[43] G. Wu, A. G. Borbolla, and D. A. Knabe, "The uptake of glutamine and release of arginine, citrulline and proline by the small intestine of developing pigs," *Journal of Nutrition*, vol. 124, no. 12, pp. 2437–2444, 1994.

[44] L. Howitt, S. L. Sandow, T. H. Grayson, Z. E. Ellis, M. J. Morris, and T. V. Murphy, "Differential effects of diet-induced obesity on BKca β1-subunit expression and function in rat skeletal muscle arterioles and small cerebral arteries," *American Journal of Physiology: Heart and Circulatory Physiology*, vol. 301, no. 1, pp. H29–H40, 2011.

[45] G. Costa, L. Ullrich, F. Kantor, and J. F. Holland, "Production of elemental nitrogen by certain mammals including man," *Nature*, vol. 218, no. 5141, pp. 546–551, 1968.

[46] S. A. Bingham and J. H. Cummings, "Urine nitrogen as an independent validatory measure of dietary intake: a study of nitrogen balance in individuals consuming their normal diet," *American Journal of Clinical Nutrition*, vol. 42, no. 6, pp. 1276–1289, 1985.

[47] C. J. K. Henry, J. P. W. Rivers, and P. R. Payne, "Relationship between fasting urinary nitrogen loss and obligatory nitrogen loss in rats," *Nutrition Research*, vol. 5, no. 10, pp. 1131–1141, 1985.

[48] G. Eisenbrand, B. Spiegelhalder, and R. Preussmann, "Nitrate and nitrite in saliva," *Oncology*, vol. 37, no. 4, pp. 227–231, 1980.

[49] C. M. H. H. T. Robroeks, K. D. G. Van De Kant, Q. Jöbsis et al., "Exhaled nitric oxide and biomarkers in exhaled breath condensate indicate the presence, severity and control of childhood asthma," *Clinical and Experimental Allergy*, vol. 37, no. 9, pp. 1303–1311, 2007.

[50] C. E. Carnovale and M. T. Ronco, "Role of nitric oxide in liver regeneration," *Annals of Hepatology*, vol. 11, pp. 636–647, 2012.

4.2 Agnelli S, Arriarán S, Oliva L, et al. Modulation of rat liver urea cycle and related ammonium metabolism by sex and cafeteria diet. RSC Adv 6, 11278–11288 (2016)

RSC Advances

PAPER

Cite this: *RSC Adv.*, 2016, **6**, 11278

Modulation of rat liver urea cycle and related ammonium metabolism by sex and cafeteria diet

Silvia Agnelli,[a] Sofía Arriarán,[a] Laia Oliva,[a] Xavier Remesar,[abc] José-Antonio Fernández-López[abc] and Marià Alemany*[abc]

High-energy (hyperlipidic) cafeteria diets induce insulin resistance limiting glucose oxidation, and lower amino acid catabolism. Despite high amino-N intake, amino acids are preserved, lowering urea excretion. We analysed how energy partition induced by cafeteria diet affects liver ammonium handling and urea cycle. Female and male rats were fed control or cafeteria diets for 30 days. There was a remarkable constancy on enzyme activities and expressions of urea cycle and ammonium metabolism. The key enzymes controlling urea cycle: carbamoyl-P synthase 1, arginino-succinate synthase and arginase expressions were decreased by diet (albeit more markedly in males), and their activities were correlated with the gene expressions. The effects observed, in ammonium handling enzyme activities and expressions behaved in a way similar to that of the urea cycle, showing a generalized downregulation of liver amino acid catabolism. This process was affected by sex. The different strategies of amino-N handling by females and males further modulated the preservation of 2-amino N under sufficient available energy. The effects of sex were more marked than those of diet were, since different metabolism survival strategies changed substrate partition and fate. The data presented suggest a lower than expected N flow to the liver, which overall importance for amino acid metabolism tends to decrease with both cafeteria diet and female sex. Under standard conditions, liver availability of ammonium was low and controlled. The situation was unchanged (or even lowered) in cafeteria-fed rats, ultimately depending on intestinal amino acid catabolism.

Received 26th November 2015
Accepted 12th January 2016

DOI: 10.1039/c5ra25174e

www.rsc.org/advances

Introduction

There is a considerable body of knowledge on the effect of diet on the substrate energy utilization under different physiological conditions. A growing consensus attributes the wide extension of metabolic syndrome (MS) to sustained excess energy (mainly lipid) diets on the ponderostat system.[1] The most apparent consequence of excess energy being the development of obesity[2] and their MS-related co-morbidities, especially insulin resistance[3] and the alteration of blood lipid transport.[4]

High-energy diets show markedly different effects depending on the sex (and age) of the subjects.[5] In general, females are more resistant to the development of MS,[6] in part because of the protective effects of oestrogen,[7] which hampers the obesogenic effects of inflammation and insulin resistance,[8] limiting the full development of obesity.[9] There is a limited antagonism between glucocorticoids and oestrogens,[10] which tend to counteract the increase in fat stores elicited by glucocorticoids.[11] In males, however, the progressive decrease in androgens with age[12] is compounded by the increase in glucocorticoids parallel to the development of MS.[13] Androgen secretion is largely blocked by glucocorticoids, resulting in increased fat storage (obesity, liver steatosis) and a marked alteration of glucose and lipid metabolism, especially in adipose tissue, muscle and liver.[14]

Our knowledge of how sex influences the deleterious effects of excess energy (lipid) intake with respect to energy expenditure, and inflammation is, however, rather sketchy, since sex-related differences have been observed, but most mechanisms remain to be fully clarified.[15,16]

The liver plays a key role in nutrient partition and in the maintenance of body energy homeostasis. It receives, *via* porta vein, most of the nutrients extracted from the diet. However, a large part of the sifting has been done, already, by other splanchnic bed organs, especially the intestine,[17] and, probably, mesenteric/omental adipose tissue.[18] In any case, the liver controls the flow of glucose into the systemic blood, and retains (or metabolizes) many amino acids and short-chain fatty acids. A sustained excess of nutrients and a critical failure of the insulin system may lead to a generalized loss of effectivity of the liver, often provoking hepatic steatosis,[19] associated to insulin resistance.[20] Loss of insulin function resulting also in lower amino acid utilization.[21] The liver condition may even develop

[a]*Department of Nutrition and Food Science, Faculty of Biology, University of Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain. E-mail: malemany@ub.edu*

[b]*Institute of Biomedicine, University of Barcelona, Av. Diagonal, 643, 08028 Barcelona, Spain*

[c]*CIBER-OBN Research Web, Barcelona, Spain*

in a failure to detoxify the portal-carried ammonium, which may result lethal.[22] Of all these critical functions, the liver – in fact the coordinate work of intestine and liver—plays an essential role in the disposal of excess amino N and ammonia. The main pathway for excess N elimination is the urea cycle,[23,24] which has been assumed to be fully operative only in liver.[25] However, both intestine and kidney have functional (albeit complementary) urea cycles.[26,27] We have found, recently, a robust presence of urea cycle in white adipose tissue,[28] which is unaffected by sex and anatomical site.[29]

High-fat diets, such as the cafeteria diets,[30] have been known, to decrease the operation of the urea cycle in liver,[31] with lower overall urinary excretion of N.[32] This decreased excretion, in spite of maintained or increased protein intake, is not paralleled by an increased deposition of protein (or faecal excretion).[32] In fact, nitrogen balances show that a significant portion of the N excreted is not accounted for.[33] It has been speculated that it may be justified (at least partly) by respiratory loss of nitric oxide[34] or, even, release of nitrogen gas.[35]

The control role of the liver on the disposal of ammonium-N and excess amino-N, is a critical process for the maintenance of N homeostasis. Thus, alteration of liver metabolic function induced by diet necessarily influences N homeostasis, albeit in ways so far not known. We assume that this dramatic change may contribute to the pathogenesis of MS. In the present study, we analysed the effects of a relatively short (one-month) exposure of adult rats (female and male) to a cafeteria diet. The objective was to check how the initial phase of development of MS affects, differentially (in adults), both sexes in the critical function of liver as main site for disposal of ammonium through the urea cycle.

Experimental

Ethics statement

All animal handling procedures and the experimental setup were in accordance with the animal handling guidelines of the corresponding European and Catalan Authorities. The Committee on Animal Experimentation of the University of Barcelona approved the present study.

Experimental design and animal handling

Nine week old female and male Wistar rats (Harlan Laboratory Models, Sant Feliu de Codines, Spain) were used. The rats ($N = 6$) were kept in same-sex two-rat cages with wood shards for bedding. The animals were maintained in a controlled animal room (lights on from 08:00 to 20:00; 21.5–22.5 °C; 50–60% humidity). Two groups for each sex were randomly selected and were fed *ad libitum*, for 30 days, with either normal rat chow (Harlan #2014) or a simplified cafeteria diet[36] made of chow pellets, plain cookies, with liver pâté, bacon, whole milk containing 300 g L^{-1} sucrose and a mineral and vitamin supplement. Food/nutrient consumption was measured as previously described.[30] Diet intake composition (expressed as energy content) was: carbohydrate 67%, protein 20%, and lipid 13% for controls; that of rats fed the cafeteria diet (*i.e.* after computing the food ingested) was (mean values, expressed as energy content): carbohydrate 47%, protein 12% and lipid 41%. The simplified cafeteria diet induced a significant increase in body fat, in line with previous studies on metabolic syndrome.[32,36] The rats were killed, under isoflurane anaesthesia, by exsanguination (aortic puncture using a large dry-heparinized syringe) at the beginning of a light cycle. Then, they were rapidly dissected, and two lobes of the liver were excised, blotted, and frozen in liquid nitrogen. These samples were weighed and ground under liquid nitrogen. The coarse powder was aliquoted and stored at −80 °C until processed. Later, the liver remains were dissected to measure its full weight. Blood was centrifuged to obtain plasma, which was frozen and stored as well.

Blood plasma parameters

Plasma samples were used to measure glucose (kit #11504, Biosystems, Barcelona Spain), lactate (kit #1001330, Spinreac, Sant Esteve de Bas, Spain), triacylglycerols and total cholesterol (Biosystems kits #11828, and #11505, respectively). Urea was measured with a chemical method (kit # 11537; Biosystems). Amino acids were analysed individually with an amino acid analyser (LKB-Alpha-plus, Uppsala, Sweden) using plasma samples deproteinized with chilled acetone.[37] Since the method used did not provide reliable data for several amino acids (*i.e.* Gln, Trp, Cys, Asn), we decided to present only the partial sum of the other amino acids as a single indicative value.

Enzyme activity analyses

Homogenate preparation. Frozen liver samples were further homogenized, using a tissue disruptor (Ultraturrax IKA-T10, Ika Werke, Staufen, Germany). Homogenates for arginino-succinate synthase and ornithine carbamoyl-transferase activity measurement were prepared using 10 volumes of chilled 70 mM hepes buffer pH 7.4 containing 1 mM dithiothreitol (Sigma, St Louis MO USA), 50 mM KCl, 1 g L^{-1} Triton X-100 (Sigma), and 1 g L^{-1} lipid-free bovine serum albumin (Sigma). Homogenates for carbamoyl-P synthase analysis were prepared with 10 volumes of chilled 50 mM triethanolamine buffer pH 8.0 containing 1 mM dithiothreitol, 0.5 g L^{-1} Triton X-100, 1 g L^{-1} lipid-free bovine serum albumin and 10 mM magnesium acetate. Homogenates for the analyses of the other enzymes were prepared with 10 volumes of chilled Krebs-Ringer bicarbonate buffer pH 7.4 containing 1 g L^{-1} Triton X-100, 1 mM dithiothreitol and 1 g L^{-1} lipid-free bovine serum albumin. The homogenates were coarsely filtered through nylon-hose to eliminate large debris. They were kept on ice and used for enzyme activity analyses within 2 h. Tissue protein content was estimated with the Lowry method,[38] using the corresponding homogenization buffer (containing albumin) as blank. Enzyme activities were expressed per unit of protein weight. The methods used were largely based in our parallel development of methods for analysis on white adipose tissue, extensively described in a previous publication.[28]

Carbamoyl-P synthase 1. Carbamoyl-P synthase 1 activity was estimated from the incorporation of ^{14}C-bicarbonate (Perkin

Elmer, Bad Neuheim, Germany) into carbamoyl-P using a method previously described by us.[39] Succinctly, we measured the incorporation of label into carbamoyl-P by the activity of the enzyme on ammonium carbonate in the presence of *N*-acetyl-glutamate (Sigma) and flushing out all remaining bicarbonate label with a stream of unlabelled CO_2.

Ornithine carbamoyl transferase. Ornithine carbamoyl transferase activity was measured from the reaction of condensation of carbamoyl-P and ^{14}C-ornithine to yield ^{14}C-citrulline. Aliquots of 25 μL of homogenates were mixed with 50 μL of 70 mM hepes buffer pH 7.4 containing carbamoyl-P, ornithine (all from Sigma), and ^{14}C-ornithine (Perkin-Elmer); final concentrations were 9 mM, 13 mM and 1 kBq mL^{-1}, respectively. The reaction was started with the homogenate, and was carried out at 37 °C during 0, 0.5,1 and 2 min. The reaction was stopped by introducing 75 μL aliquots in tubes, kept on ice, containing 100 μL of chilled acetone. After centrifugation, the clear supernatants were dried in a vacuum-centrifuge (Thermo Scientific, Waltham, MA USA). The residues were dissolved in 25 μL of water; they were run on TLC silica gel plates (200 μm; Macherey-Nagel, Düren, Germany). Standards of ornithine and citrulline were included in one of the lanes of each plate. The plates were developed with trichloromethane: methanol: acetic acid (1 : 2 : 2 by volume). Standards were revealed with a ninhydrin spray. The lanes were cut in 1 cm pieces and counted. The label in the citrulline spot was expressed as a percentage of the total label counted in each TLC lane. These data allowed the calculation of newly formed citrulline at each incubation time. The V_0 value for each sample was plotted, and was considered to represent the value of V_{max} under the conditions tested.

Arginino-succinate synthase. Arginino-succinate synthase activity was measured from the reaction of condensation of aspartate with citrulline in the presence of ATP to yield arginino-succinate. Homogenates (55 μL) were mixed with 30 μL of 70 mM hepes buffer pH 7.4, containing ATP-Na_2, $MgCl_2$, citrulline and aspartate (Sigma); final concentrations were 10 mM, 5 mM, 3 mM, and 2.5 mM, respectively. The reaction was started with aspartate, and was carried out at 37 °C. The reaction was stopped with 40 μL of 30 g L^{-1} perchloric acid. The tubes were vortexed and neutralized (pH 7–8) with 10 μL of 100 g L^{-1} KOH containing 62 g L^{-1} $KHCO_3$. The tubes were vortexed again and centrifuged in the cold 15 min at 8000 × *g*. The aspartate remaining in the supernatants was measured by transamination to oxaloacetate, which was reduced by malate dehydrogenase and NADH. Briefly, 20 μL of the supernatants were brought up to 300 μL in 96-well plates, with 66 mM phosphate buffer pH 7.4 containing NADH, 2-oxoglurarate, aspartate transaminase (pig heart) and malic acid dehydrogenase (pig heart) (all from Sigma); final concentrations were, respectively, 0.25 mM, 0.2 mM, 20 μkat L^{-1} and 17 μkat L^{-1}. The plates were read at 340 nM in a plate reader (Biotek, Winoosky, VT USA) at intervals of 30 s during 20 min. The fall in NADH was used to determine the levels of aspartate at each incubation time. Its disappearance (*versus* time zero levels) was used to calculate the aspartate incorporated into arginino-succinate by the enzyme.

Arginino-succinate lyase. Arginino-succinate lyase activity was measured from the breakup of arginino-succinate to yield fumarate and arginine. This amino acid was analysed in a second reaction, using arginase to form ornithine and urea, which was measured using a sensitive chemical method. Aliquots of 38 μL of homogenates were mixed with 38 μL of 66 mM hepes buffer pH 7.4, containing arginino-succinate (Sigma), at a final concentration 2 mM. Incubations were carried out at 37 °C for 0, 2.5, 5 and 10 min. The reaction was stopped by the addition of 40 μL of 30 g L^{-1} perchloric acid. The tubes were vortexed and brought to pH 8–9 with 10 μL of 100 g L^{-1} KOH, 80 g L^{-1} $HKCO_3$. The tubes were centrifuged for 15 min in the cold at 8000 × *g*. Aliquots of 100 μL of the supernatants were mixed with 50 μL of the reacting mixture, containing 66 mM hepes buffer pH 7.5 (to achieve a final pH 8.5), $MnCl_2$ and arginase (rat liver, Lee Biosolutions, St Louis, MO USA). The final concentrations were 7 mM and 17 μkat L^{-1}, respectively. Arginase already in the buffer containing Mn^{2+}, was previously activated for 5 min at 55 °C. The reaction developed for 30 min at 37 °C, and was stopped by the addition of 35 μL of 160 g L^{-1} perchloric acid. The tubes were centrifuged in the cold for 15 min at 8000 × *g*. The acidic supernatants (175 μL) were used for the estimation of urea. They were mixed with 600 μL of 90 g L^{-1} H_2SO_4 containing 270 g L^{-1} H_3PO_4; then 10 μL of 30 g L^{-1} of 1-phenyl-2-oxime-1,2-propanodione (Sigma) in absolute ethanol were added. The reaction was developed at 100 °C for 30 min in a dry block heater. The absorbance of the tubes (including standards and blanks) was measured at 540 nm with a plate reader. Arginase effectivity (using the method explained above) was tested in all batches. In all cases, conversion of arginine to urea was 100% (*i.e.* there was a full coincidence of the standard curves for both urea and arginine).

Arginase. Arginase activity was measured through the estimation of the urea produced by the activity of the enzyme on arginine in the presence of Mn^{2+} ions.[40,41] Aliquots of 20 μL of homogenates were mixed with 5 μL of $MnCl_2$ in water; final concentration 10 mM. The tubes were heated for 5 min at 55 °C to activate arginase[41,42] After the temperature was brought down to 37 °C, the reaction began with the addition of 75 μL of arginine (Sigma); final concentration 78 mM. Incubations were carried out for 0, 8 and 16 min at 37 °C. The reaction was stopped by the addition of 35 μL 160 g L^{-1} perchloric acid. The tubes were centrifuged 15 min in the cold at 8000 × *g*. Urea was measured as described above.

Glutamine synthetase. Glutamine synthetase, activity was estimated using a method we had used previously,[43] based on the reaction of glutamine and hydroxylamine in the presence of ADP, Mn^{2+} and arsenate to yield γ-glutamyl-hydroxamate. The addition of $Fe(NO_3)_3$ in trichloroacetic acid results in the development of colour, read at 500 nm using a plate reader.

Serine dehydratase. Serine dehydratase activity was analysed, by measuring the pyruvate freed by the enzyme in the presence of pyridoxal-P.[44] This reaction was coupled with the reduction of pyruvate to lactate with lactate dehydrogenase, measuring the decrease in NADH,[45] by UV spectrometry using a plate reader.

AMP deaminase. AMP deaminase activity was estimated by the determination of the ammonium released by the action of the enzyme on AMP, in the presence of KCl, yielding IMP.[46] The ammonium evolved was estimated with the classical Berthelot indophenol reaction,[47] in which indophenol was formed by reaction of ammonium with phenol in the presence of an oxidative agent (hypochlorite) and nitroprusside as catalyser.

Gene expression analysis

Total tissue RNA was extracted from frozen samples (about 30 mg) using the GenEluteTM (Sigma-Aldrich, St Louis MO USA) procedure, and was quantified in a ND-100 spectrophotometer (Nanodrop Technologies, Wilmington DE USA). RNA samples were reverse transcribed using the MMLV reverse transcriptase (Promega, Madison, WI USA) system and oligo-dT primers. The data were also used to determine the total RNA content of the tissue in order to establish quantitative comparisons between different gene expressions.

Real-time PCR (RT-PCR) amplification was carried out using 10 μL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), 10 ng of reverse-transcribed RNA and primers (300 nM). Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set to 0.15 for all runs.

A semi-quantitative approach for the estimation of the concentration of specific gene mRNAs per unit of tissue or protein weight was used.[48] Cyclophyllin A (*Ppia*) was used as charge control gene.[49] The data were expressed as the number of transcript copies per gram of protein in order to obtain comparable data between the groups. The genes analysed, and a list of primers used, are presented in Table 1.

The possible contamination of RNA with DNA was checked, before PCR cycling, by charging a number of samples of each batch with known internal standards of RNA. No spurious signals were observed. All the primers used for measurement of the enzyme gene expressions were checked, with Northern blots of the PCR-synthesized cDNAs. In all cases, the cDNAs obtained had the expected molecular weights.

Total DNA was estimated with a fluorimetric method.[50] Approximate cellularity was calculated, assuming that the mean mammalian cell DNA content was 6 pg.[51] Mean cell volume was estimated from liver weight, liver density: 1.1 g mL^{-1}, and the estimated number of cells.

Statistics

Two-way ANOVA comparisons between groups, correlations and curve fitting (including V_i estimations) were carried out with the Prism 5 program (GraphPad Software, San Diego CA USA).

Results

General parameters

Table 2 presents the rat weights, and liver size and composition of the four groups of rats. As expected, body and liver weight

Table 1 Primer sequences used in the analysis of liver gene expressions

Protein	Gene	EC number		Primer sequence	bp
Carbamoyl-phosphate synthase [ammonia], mitochondrial type 1	*Cps1*	6.3.4.16	5′ > 3′	ACCCATCATCCCCTCTGACT	118
			3′ > 5	ACACGCCACCTCTCCAGTAG	
Ornithine carbamoyl-transferase	*Otc*	2.1.3.3	5′ > 3′	CTTGGGCGTGAATGAAAGTC	126
			3′ > 5	ATTGGGATGGTTGCTTCCT	
Arginino-succinate synthase 1	*Ass1*	6.3.4.5	5′ > 3′	CAAAGATGGCACTACCCACA	100
			3′ > 5	GTTCTCCACGATGTCAATGC	
Arginino-succinate lyase	*Asl*	4.3.2.1	5′ > 3′	CCGACCTTGCCTACTACCTG	104
			3′ > 5	GAGAGCCACCCCTTTCATCT	
Arginase, liver (type 1)	*Arg1*	3.5.3.1	5′ > 3′	GCAGAGACCCAGAAGAATGG	126
			3′ > 5	GTGAGCATCCACCCAAATG	
N-Acetyl-glutamate synthase	*Nags*	2.3.1.1	5′ > 3′	GCAGCCCACCAAAATCAT	82
			3′ > 5	CAGGTTCACATTGCTCAGGA	
Nitric oxide synthase 3, endothelial cell type	*Nos3*	1.14.13.39	5′ > 3′	CAAGTCCTCACCGCCTTTT	138
			3′ > 5	GACATCACCGCAGACAAACA	
Glutamate-ammonia ligase [glutamine synthetase]	*Glul*	6.3.1.2	5′ > 3′	AACCCTCACGCCAGCATA	148
			3′ > 5	CTGCGATGTTTTCCTCTCG	
Glutaminase kidney isoform, mitochondrial	*Gls*	3.5.1.2	5′ > 3′	CCGAAGGTTTGCTCTGTCA	63
			3′ > 5	AGGGCTGTTCTGGAGTCGTA	
Glutamate dehydrogenase 1, mitochondrial	*Glud1*	1.4.1.3	5′ > 3′	GGACAGAATATCGGGTGCAT	122
			3′ > 5	TCAGGTCCAATCCCAGGTTA	
Glycine cleavage system H protein, mitochondrial	*Gcsh*	—	5′ > 3′	AAGCACGAATGGGTAACAGC	146
			3′ > 5	TCCAAAGCACCAAACTCCTC	
Adenosine monophosphate deaminase 2	*Ampd2*	3.5.4.6	5′ > 3′	CGGCTTCTCTCACAAGGTG	78
			3′ > 5	CGGATGTCGTTACCCTCAG	
Peptidyl-prolyl-*cis-trans* isomerase A[a]	*Ppia*	—	5′ > 3′	CTGAGCACTGGGGAGAAAGGA	87
			3′ > 5	GAAGTCACCACCCTGGACA	

[a] Housekeeping gene.

were affected by sex and diet. However, liver weight was maintained at about 3.1–3.3% of body weight in all groups. Whole-liver cellularity was (mean values for groups, in 10^9 cells): 4.0 and 4.9 for control and cafeteria males, as well as 2.4 and 2.7 for control and cafeteria females. Estimated cell size was higher in females: 3.3 and 3.2 ng per cell in control and cafeteria rats, respectively, *versus* 2.9 and 2.8 ng per cell in males ($P < 0.05$ for both diets). However, no overall significant effects of diet and sex were observed for DNA content in mg g^{-1} tissue.

Concentrations of protein and RNA in liver were affected by sex; males had higher protein and females had higher RNA concentrations; but no significant effects of diet were observed. RNA/DNA ratios were, again, not affected by diet, but sex resulted in higher values ($P < 0.05$) for females (3.2 controls and 3.0 cafeteria) than males (2.4 for both dietary groups).

Table 3 shows the main plasma energy parameters of the rats. Glucose levels were increased, and those of lactate decreased, significantly by feeding the cafeteria diet. Cholesterol was unaffected by either sex or diet. Triacylglycerols, however, were affected by sex (but not by diet), with female values being higher than those of males, especially in control rats. The sum of plasma amino acids was also affected by sex (with females showing higher combined levels). Finally, urea concentrations were decreased by cafeteria diet, but were overall higher in female rats.

Urea cycle

Fig. 1 depicts a scheme of the urea cycle in liver, showing the enzyme activities (and their corresponding gene expressions) for ornithine carbamoyl-transferase, arginino-succinate synthase, arginino-succinate lyase and arginase 1. The figure shows also the expressions of the genes coding for endothelial nitric oxide synthase and *N*-acetyl-glutamate synthase (acetyl-transferase). Arginase activity was about three orders of magnitude higher than those of the arginino-succinate enzymes; ornithine carbamoyl-transferase activity was also high, but only one order of magnitude higher than those of the arginino-succinate enzymes. These extreme differences in activity were less marked when comparing the expressions of the four enzymes, since all were in the same range except arginino-succinate synthase, one order of magnitude higher. These differences resulted in disparate activity/expression ratios.

The patterns of activity were similar for all four enzymes, but there were significant effects of sex only in arginino-succinate synthase and arginase, and of diet in these same enzymes plus arginino-succinate lyase. The patterns for gene expression of the urea cycle enzymes were also similar and followed the same profile than their corresponding enzyme activities. Sex affected only (*i.e.* significantly) the expression of arginino-succinate synthase. The expression of their corresponding genes was affected by diet in the same enzyme plus arginino-succinate lyase and arginine. In all enzymes of Fig. 1, except ornithine carbamoyl-transferase, both activities and expressions were decreased in cafeteria diet-fed rats *vs.* controls.

The possible direct relationship between gene expression and enzyme activity (unaffected by post-translational modification) was checked analysing the correlation between the data

Table 2 Body and liver weight and composition of male and female rats fed control or cafeteria diets for 30 days[a]

		Male		Female		*P*	
Parameter	Units	Control	Cafeteria	Control	Cafeteria	Sex	Diet
Body weight	g	373 ± 6	420 ± 20	232 ± 8	267 ± 16	<0.0001	0.0074
Liver weight	g	11.6 ± 0.5	13.8 ± 1.1	7.72 ± 0.31	8.36 ± 0.50	<0.0001	0.0473
DNA	mg g^{-1}	2.07 ± 0.17	2.13 ± 0.32	1.83 ± 0.22	1.91 ± 0.22	NS	NS
RNA	mg g^{-1}	4.96 ± 0.31	5.21 ± 0.18	5.95 ± 0.30	5.75 ± 0.15	0.0065	NS
Protein	mg g^{-1}	191 ± 7	185 ± 5	160 ± 5	157 ± 9	0.0003	NS

[a] The data correspond to the mean ± sem of 6 different animals. Statistical significance of the differences between groups was established with a 2-way anova program.

Table 3 Main plasma metabolites of male and female rats fed control or cafeteria diets for 30 days[a]

	Male		Female		*P*	
Plasma parameters (mM)	Control	Cafeteria	Control	Cafeteria	Sex	Diet
Glucose[b]	10.2 ± 0.4	10.8 ± 0.4	8.64 ± 0.34	11.5 ± 0.3	NS	0.0001
Lactate	3.10 ± 0.29	2.64 ± 0.21	3.78 ± 0.24	2.57 ± 0.21	NS	0.0023
Cholesterol	1.97 ± 0.07	2.28 ± 0.21	1.98 ± 0.16	2.07 ± 0.19	NS	NS
Triacylglycerols	1.50 ± 0.06	1.50 ± 0.01	1.69 ± 0.06	1.51 ± 0.03	0.0390	NS
Urea	3.90 ± 0.17	3.82 ± 0.20	5.13 ± 0.25	3.78 ± 0.20	0.0094	0.0025
Amino acids[c]	3.34 ± 0.08	3.68 ± 0.10	3.96 ± 0.18	4.07 ± 0.12	0.0007	NS

[a] The data correspond to the mean ± sem of 6 different animals. Statistical significance of the differences between groups was established with a two-way anova program. [b] The glucose values were higher than expected because of the necessary exposure of the animals to isoflurane anaesthesia during the process of killing and sampling. [c] These values do not include Gln, Asn, Trp and Cys.

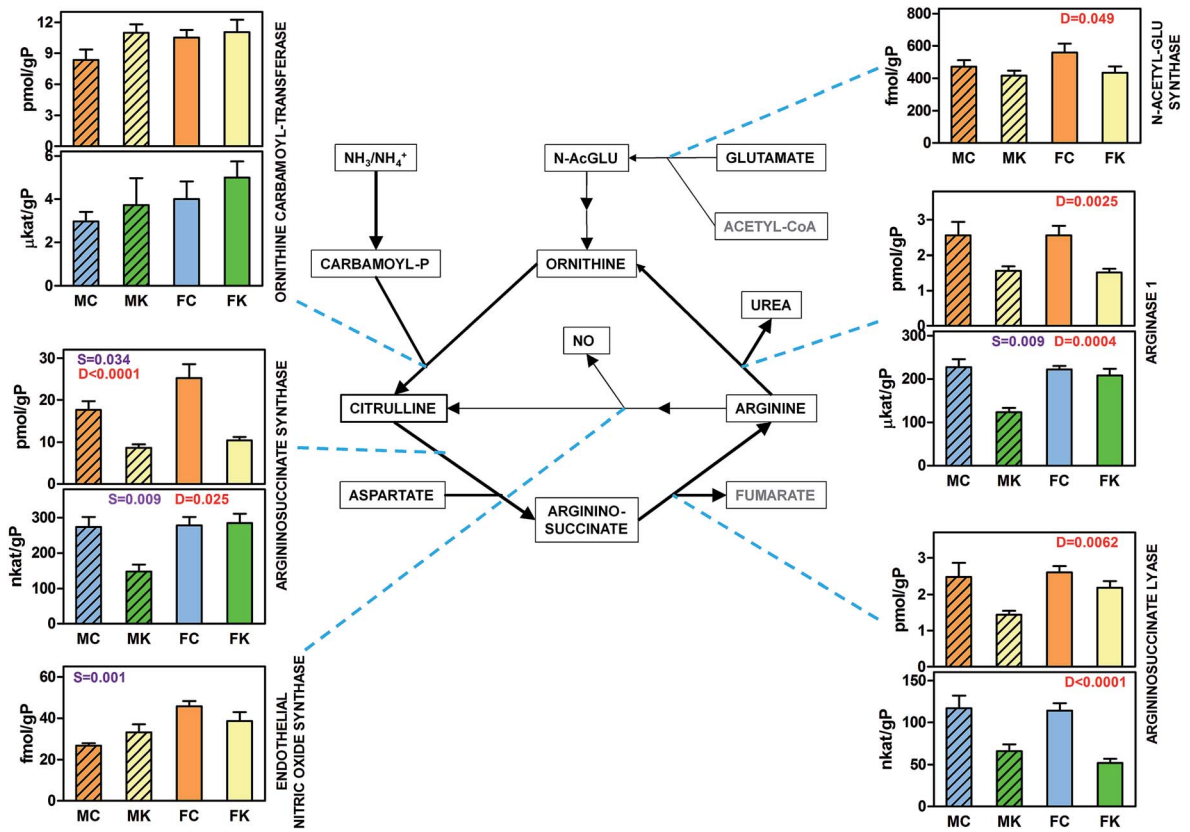


Fig. 1 Gene expressions and enzyme activities of the urea cycle in the liver of male and female rats fed control or cafeteria diet for 30 days. The data correspond to the mean ± sem of 6 different animals, and are all expressed per gram of tissue protein (gP). Statistical analysis of the differences between groups was done using a two-way anova program and the variables "sex" and "diet". Only significant ($P < 0.05$) values have been represented. Purple data, marked with a "S" correspond to the overall effect of "sex", and red data marked with a "D" correspond to the overall effect of "diet". MC = male fed the control diet; MK = male fed the cafeteria diet; FC = female fed the control diet; FK = female fed the cafeteria diet. Males: dashed columns; females: no-pattern columns. Enzyme gene expressions: orange: control diet; yellow: cafeteria diet enzyme activities: blue: control diet; green: cafeteria diet.

for both parameters of all animals studied, irrespective of sex and diet (*i.e.* $N = 24$). There were significant correlations between enzyme activity and expression for arginino-succinate synthase ($R^2 = 0.283$; $P = 0.023$), arginase 1 ($R^2 = 0.184$; $P = 0.037$), carbamoyl-P synthase ($R^2 = 0.440$; $P = 0.0008$) and serine dehydratase ($R^2 = 0.635$; $P = 0.0002$). No significant correlations were found for any of the other enzymes studied.

Acetylation of glutamate was also affected by diet, following the same pattern described above. The expression of endothelial nitric oxide synthase showed a clear effect of sex, with higher values in females but the effects of diet were not significant.

Ammonium metabolism

Fig. 2 shows a general outline of liver ammonium metabolism, including the activities of carbamoyl-P synthase 1, serine dehydratase, AMP deaminase and glutamine synthetase, as well as the expressions of their corresponding genes. The figure includes, also the expressions of glutaminase, the cytoplasmic (NADPH-dependent) glutamate dehydrogenase and a component of the glycine cleavage system (H protein).

Carbamoyl-P synthetase 1 showed higher enzyme activities in females; these effects were not observed in its gene expression, which presented considerable variability. AMP deaminase showed no significant effects of sex or diet on activity or gene expression. The activity of serine dehydratase was markedly affected by sex, with lower female values, an effect that was parallel to the changes in gene expression. Diet also affected the gene expression of serine dehydratase, with values even lower for cafeteria-fed rats.

Glutamine synthetase activity in females was higher than in males, a difference also observed in the expression of its gene. Glutaminase expression did not show effects of sex, but cafeteria diet decreased the expression of the enzyme. This pattern was paralleled by glutamate dehydrogenase, which also showed an effect of sex (higher values in females). The expression of the glycine cleavage system (in fact that of representative H protein) was strongly influenced by sex, with—again—higher values in female rats.

Discussion

We have shown evidence that both sex and diet, rather independently, affected the activities and gene expressions of the urea cycle enzymes in rat liver. However, this apparent

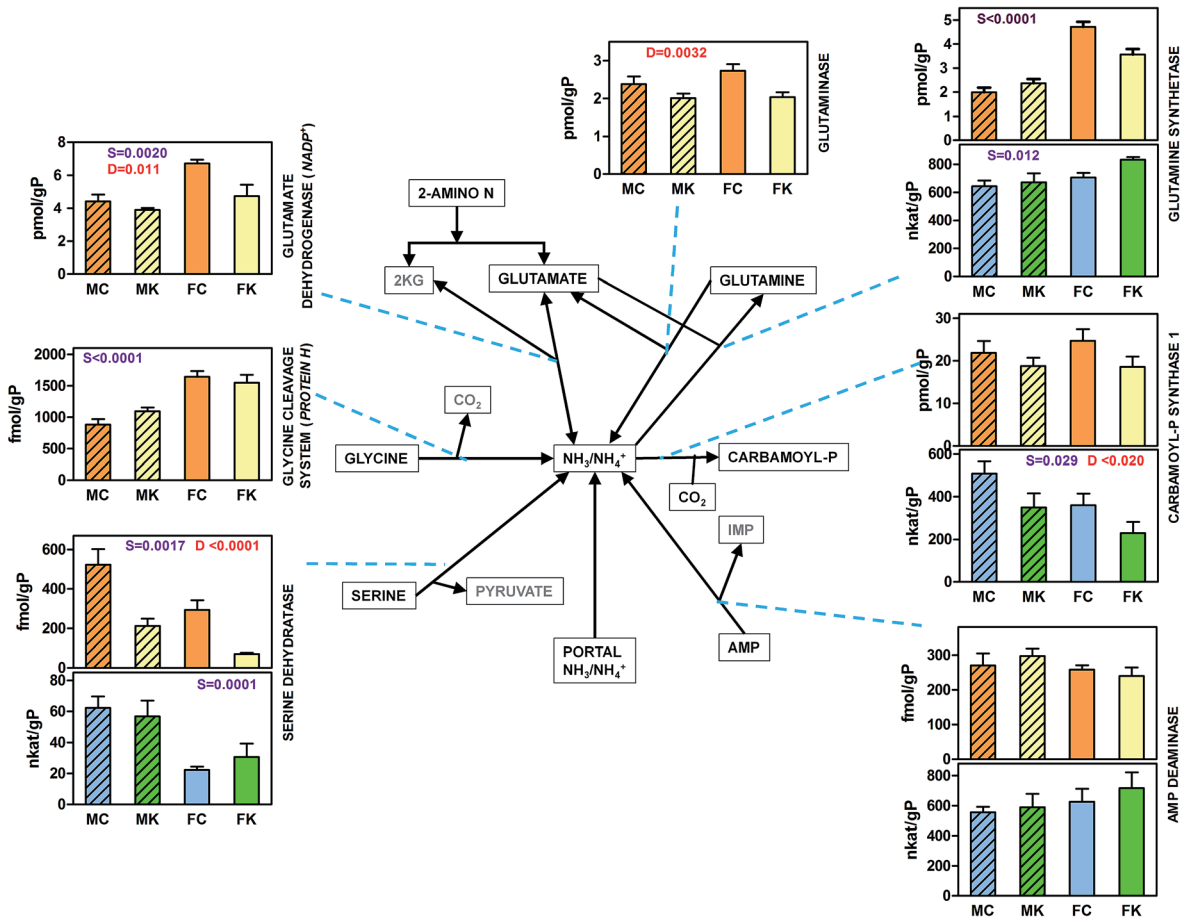


Fig. 2 Gene expressions and enzyme activities of enzymes related to ammonia/ammonium metabolism in the liver of female and male rats fed control or cafeteria diet for 30 days. The data correspond to the mean ± sem of 6 different animals, and are expressed per gram of tissue protein (gP). Statistical analysis, abbreviations and colour conventions are the same described for Fig. 1.

similarity of effect shifts towards more extensive effects of sex on the management of ammonium in the liver, thus affecting, albeit indirectly, the overall operation of the urea cycle and the final excretion of N as urea. The model used behaved as expected both in increased WAT fat deposition[52,53] and relative normalcy of plasma parameters, including insulin[54] as repeatedly found under these same conditions in previous studies.[32,36]

We used a cafeteria diet model well studied by us previously, which shows a discrete increase in body weight due to the accumulation of fat, but the metabolic alterations induced by inflammation in the context of MS are essentially incipient.[52] The effects are strongly influenced by sex;[55] a question that we also found applies to amino acid metabolism.[29] The obesogenic effects of cafeteria diets are maximal during early postnatal development,[56] preventing the weaning shift from a high fat to a high carbohydrate diet.[57] The timing and extent of exposure are critical to enhance the ability of this type of diets to induce MS.[55,58] We used young adult rats, and subjected them to a moderate exposure time to the hyperlipidic diet in order to obtain not a frankly pathological state but a pre-MS situation in which the immediate effects of the high-energy diet are not confounded by the additional disorders elicited by a severe inflammation.[59,60]

The so far scarcely studied effect of sex on amino acid catabolism may have deeper roots than usually assumed. The lack of direct studies on the mechanisms has driven our attention to the overall picture of effects of sex on amino-N economy. In males, the main trend is accumulation of body protein, largely muscle, an effect facilitated by insulin,[61] GH and androgens,[62] and hampered by glucocorticoids.[63] In females, however, the main drive seems to be somewhat different: to enhance N sparing probably to fulfil the burden of reproduction, first the foetuses, and then the energy economy ordeal of lactation. In both sexes, in addition, the overall trend to preserve amino-N is a primeval drive that prevents its wasting[64,65] even under (rare in Nature) conditions of dietary excess of protein.[66] We can speculate that androgen predominance (*i.e.* in males), acting as counterbalance to glucocorticoids, may diminish the hepatic conversion of amino acid N to urea; oestrogen (*i.e.* in females) showing a less marked influence on this aspect.

Enzyme activities are not direct estimations of the enzyme function within the cell, but are a widely accepted correlate of the overall enzyme ability to carry out its function. Thus, the V_i values presented are a correlate of V_{max} and of functional protein enzyme levels. These values, consequently; reflect

potential ability of the tissue to catalyse the reaction, albeit being estimated under result-maximizing non-physiological conditions. The closeness of gene expression patterns and enzyme activities mutually support the data presented. However, the large differences in activity observed between enzymes (*i.e.* arginase *vs.* arginino-succinate synthase) but also between expression and activity may be a consequence of different turnover number or enzyme (as protein) turnover,[67] but place the control of the cycle, precisely on these key enzymes. In arginino-succinate synthase as rate limiting step;[68] and arginase as final factor in the release of urea,[69] but also as the main site for arginine break-up and maintenance of body arginine-citrulline equilibrium.[70] In the urea cycle, N disposal and guanido-amino acid maintenance for their multiple regulatory tasks intermix to a considerable extent, as can be deduced from the model presented here. The different needs for arginine possibly modulate the sex differences in expression, but the enzyme activities follow more closely the regulation mechanisms for N handling.

It has been known for long, that urea production is decreased by diets rich both in energy and in protein,[31] assumedly because of lower urea cycle enzyme activities in the liver. Our results are consistent with this observation, but, at the same time, we provide evidence that these differences, largely down regulation of enzyme activities, were described, essentially, in males. No sufficient data for comparison is available for females. This is best observed in the halving of three key enzyme activities in males by feeding a cafeteria diet: arginino-succinate synthase, arginino-succinate lyase and arginase. Of these, female rats maintained only the effect on arginino-succinate lyase; thus, males' downregulation may be traced to more control points than females, which may help explain the sex-related differences in regulation described above. The consequences on overall function of the cycle are consistent with the lower urea production observed in rats fed a cafeteria diet, in studies using mainly males.[31,32,71]

The relatively low and largely unchanged expression of endothelial nitric oxide synthase suggests a relatively low activity, compared with arginase, in their competence for arginine, an effect best seen in peripheral tissues.[72] In the present study, the expression of the enzyme did not change significantly at all, which seems to disconnect this enzyme from the main hepatic degradative pathway represented by the urea cycle. The fate of the "unaccounted for" dietary nitrogen *i.e.* that portion of dietary N not excreted in urine (mainly urea) or faeces, and neither accumulated in body protein does not seem, thus, to be related to changes in the capacity of liver for higher nitric oxide synthesis. Limiting its contribution to the excess nitric oxide production caused by metabolic syndrome and/or cafeteria diet feeding.[33,73]

Liver ability to synthesize citrulline was not decreased by diet, which suggests that liver may also contribute to the overall production of citrulline,[74] as that observed in adipose tissue.[29] The relative inability of the liver to retain and process citrulline[74] hints at this amino acid not being, in the liver, a critical factor in the regulation of the cycle, in addition to its overall importance for arginine metabolism regulation.[75]

It has been generally assumed that ammonia arriving to the liver (and that produced in its own catabolism of amino acids) is a main factor for the control of its disposal through the urea cycle.[76,77] It is obvious that the liver is a formidable barrier that prevents ammonium from entering the systemic circulation and thus possibly damaging the nervous system.[78] The liver counts not only with the urea cycle (essentially carbamoyl-P synthase 1) to incorporate it into urea with amino-N taken from aspartate, but also with two additional and powerful ammonium-handling systems (and nitrogen salvage[79]): glutamine synthetase,[80] and glutamate dehydrogenases[81] within the mitochondrion and in the cytoplasm. Compartmentation of ammonium/ammonia in the cell is also an important[82] aspect that has not been sufficiently studied.

Carbamoyl-P synthases convert ammonium (or glutamine amido N) into carbamoyl-P in liver, were practically only the isozyme 1 has significant activity.[83] The reaction provides carbon and nitrogen to start the formation of the guanido group on ornithine, *via* ornithine carbamoyl-transferase. Following the trend described for the urea cycle enzymes, no differences were observed in gene expressions but cafeteria diet slightly decreased the enzyme activity in both sexes. These differences support the overall function of the cycle described above, since this enzyme incorporation of ammonia is a key control point in the synthesis of urea.[77]

The existence of significant correlations between activity and expression for carbamoyl-P synthase and the key regulatory urea cycle enzymes arginino-succinate synthase and arginase, attest to a direct translational control of the urea cycle in liver. As indicated above, these three enzymes have been postulated as main control points for urea cycle operation. It is worth noting that only these enzymes, and serine dehydratase, which gene expression is controlled only by serine availability,[84] showed a direct (statistically significant) relationship between expression and measured enzyme activity. Since the analyses have been done under different dietary and sex conditions, the maintenance of this basic process shows that regulation of gene translation is a key mechanism of control of the cycle.

The liver is a main site for amino acid partition and N disposal. This is a consequence of its peculiar placement, at the end of the portal system, which carries the N debris of intestinal and microbiota catabolism, modulated by intestinal function.[85] In addition, liver has the advantage of using the ammonia evolved from catabolic reactions directly in its cells. Glutamine synthetase is placed essentially in the peri-venous cells,[86] acting as last defence barrier against release of ammonium into the systemic circulation; this enzyme shows a marked sex difference. $NADP^+$-glutamate dehydrogenase showed a similar pattern with respect to sex, but cafeteria diet tended to decrease its expression. Since glutamate dehydrogenase is assumed to act (in the liver) mainly in the direction of glutamate synthesis,[81,87] its increase in females agrees with the hypothesis of their enhanced focussing on amino-N sparing.

The significant (from a quantitative point of view) functional urea cycle in white adipose tissue introduces a critical question on the primacy of liver in overall 2-amino N disposal.[28,29] Probably, the main role of adipose tissue urea cycle is

complementary to that of liver, providing arginine and citrulline to the rest of the body[28] and, perhaps acting as backup system for the liver amino-N elimination. The limited effect of diet on the urea cycle of adipose tissue[28,29] contrasts with the marked effects observed here in liver, and help support the hypothesis that the function of the cycle is not subjected to the same parameters of control, nor, probably, shares the same metabolic function in both organs.

The main liver ammonium producing mechanisms are the purine nucleotide cycle,[88] *i.e.* AMP deaminase,[89] glutaminase,[90] serine (and threonine) dehydratase[91] and the glycine cleavage system.[92] There are other sources, such as amine-oxidases, amino acid oxidases and a number of enzymes acting on the catabolism of essential amino acids, but the nature of the N donors suggest a conjointly limited contribution to the liver ammonium pool. However, a main source is the ammonia/ammonium carried from the intestine (and microbiota) by the portal blood.[93]

The role of AMP deaminase in liver is more complex than its simple participation in the purine nucleotide cycle,[94] since it is part of the purine salvage pathway.[95] In addition, the enzyme breaks up the AMP generated by adenylate kinase under conditions of scarcity of ATP or nutrients,[96] as a way to control glycolysis, often in conjunction with ammonium production.[97] Breakup of AMP to IMP also affects AMP-kinases and their control of energy partition.[98] The varied functions of AMP-deaminase in liver, do not seem to include a significant role in the *in situ* production of ammonium,[99] a condition largely different from that of the muscle enzyme, which places the purine nucleotide cycle as a main mechanism for mineralization of amino-N.

The analysis of diet/sex effects on serine dehydratase are consistent with a sex-related preservation of amino-N in females, enhanced by the additional protective effects of cafeteria diet. Serine dehydratase is a classic example of substrate-controlled expression/activity,[84] and thus, both are correlated. The expression of protein H of the glycine cleavage system showed, again an effect of sex. Glycine is also a by-product of serine, thus the probable increase in its cleavage may not only represent a way of amino-N disposal, but a much needed source of 1C fragments for synthesis,[100] especially under conditions of excess energy and amino acids.

It may be assumed that, under conditions of sufficient glucose and energy availability (controls) or in their excess (compounded by the presence of large amounts of lipid in cafeteria diet), amino acid metabolism must be hampered in intestine (and liver) by the ultimate need to preserve amino-N.[101] In consequence, it is probable that the porta vein ammonia would not be increased by cafeteria diet. The final picture, then, could be summarized in a controlled, relatively low, availability of ammonium in the liver under standard conditions, which may be unchanged or even lowered in cafeteria-fed rats, depending on intestinal amino acid catabolism.[93]

Conclusions

The effects observed, both in enzyme activities and expressions contributing to remove ammonium and those producing it tend to run in a way similar to that described for the urea cycle: a generalized down regulation of amino acid metabolism.[71] This conclusion is in agreement with a decreased urea production, and markedly contrasts with the actually higher availability of 2-amino N in the rats fed a cafeteria diet.[102]

This complex intertwining of mechanisms is affected by sex, in a way that the different strategies of amino-N handling by females and males further modulate the preservation of 2-amino-N when sufficient energy is available. The sex-related differences are important both in direction and in extension, and open new avenues for understanding how amino acids are used for energy, but also how survival and/or sex-related metabolism strategies modify substrate partition and fate.

The confrontation between amino-N preservation and the need to dispose of its excess seem to show a winning hand for preservation as all the data presented above suggest. In any case, elimination of excess N is necessary and cannot be easily carried out through the metabolic pathways we know. In any case, the experimental data show that excess N is removed. The problem is that we have not yet identified which (necessarily major) pathway is used for that elimination, so far, we can only add that it is not urea, and also that the main agent does not seem to be the liver. In any case, the critical question of the fate of the 2-amino N ingested but not excreted (faeces, urine) or accrued in the body of rats fed a high-energy protein-rich self-selected (cafeteria) diet, remains open.

Conflict of interest

The authors declare that they have no conflict of interests.

Acknowledgements

This study was financed in part, by grants of the Plan Nacional de Investigación en Biomedicina (SAF2012-34895) and the Plan Nacional de Ciencia y Tecnología de los Alimentos (AGL-2011-23635) of the Government of Spain. It also counted with the collaboration of the CIBER-OBN Research Web. Silvia Agnelli obtained a Leonardo da Vinci fellowship, and Sofía Arriarán was the recipient of a predoctoral fellowship of the Catalan Government, in both cases covering part of the time invested in this study.

References

1 M. Alemany, *Nutr. Res.*, 2013, **33**, 1–11.
2 A. Sclafani and D. Springer, *Physiol. Behav.*, 1976, **17**, 461–471.
3 J. P. Felber, E. Haesler and E. Jéquier, *Diabetologia*, 1993, **36**, 1221–1229.
4 J. P. Després, *Clin. Endocrinol. Metab.*, 1994, **8**, 629–660.
5 X. Wang, J. W. Choi, T. S. Oh, D. K. Choi, R. Mukherjee, H. Liu and J. W. Yun, *Proteomics*, 2012, **12**, 284–299.
6 M. R. Meyer, D. J. Clegg, E. R. Prossnitz and M. Barton, *Acta Physiol.*, 2011, **203**, 259–269.

7 T. M. d'Eon, S. C. Souza, M. Aronovitz, M. S. Obin, S. K. Fried and A. S. Greenberg, *J. Biol. Chem.*, 2005, **280**, 35983–35991.
8 R. E. Stubbins, K. Najjar, V. B. Holcomb, J. Hong and N. P. Núñez, *Diabetes, Obes. Metab.*, 2012, **14**, 58–66.
9 R. E. Stubbins, V. B. Holcomb, J. Hong and N. P. Núñez, *Eur. J. Nutr.*, 2012, **51**, 861–870.
10 H. Gong, M. J. Jarzynka, T. J. Cole, J. H. Lee, T. Wada, B. Zhang, J. Gao, W. C. Song, D. B. DeFranco, S. Y. Cheng and W. Xie, *Cancer Res.*, 2008, **68**, 7386–7393.
11 C. Asensio, P. Muzzin and F. Rohner-Jeanrenaud, *Int. J. Obes.*, 2004, **28**, S45–S52.
12 L. Antonio, F. C. W. Wu, T. W. O'Neill, S. R. Pye, E. L. Carter, J. D. Finn, M. K. Rutter, M. R. Laurent, I. T. Huhtaniemi, T. S. Han, M. E. J. Lean, B. G. Keevil, N. Pendleton, G. Rastrelli, G. Forti, G. Bartfai, F. F. Casanueva, K. Kula, M. Punab, A. Giwercman, F. Claessens, B. Decallonne and D. Vanderschueren, *J. Clin. Endocrinol. Metab.*, 2015, **100**, 1396–1404.
13 M. Wang, *Nutr. Metab.*, 2005, **2**, 3.
14 M. Alemany, *Front. Endocrinol.*, 2012, **3**, 27.
15 M. E. Estrany, A. M. Proenza, M. Gianotti and I. Lladó, *Cell Biochem. Funct.*, 2013, **31**, 504–510.
16 H. Yokomizo, T. Inoguchi, N. Sonoda, Y. Sasaki, Y. Maeda, T. Inoue, E. Hirata, R. Takei, N. Ikeda, M. Fujii, K. Fukuda, H. Sasaki and R. Takayanagi, *Am. J. Physiol.*, 2014, **306**, E1163–E1175.
17 T. Aikawa, H. Matsutaka, H. Yamamoto, T. Okuda, E. Ishikawa, T. Kawano and E. Matsumura, *J. Biochem.*, 1973, **74**, 1003–1017.
18 Y. Eguchi, T. Eguchi, T. Mizuta, Y. Ide, T. Yasutake, R. Iwakiri, A. Hisatomi, I. Ozaki, K. Yamamoto, Y. Kitajima, Y. Kawaguchi, S. Kuroki and N. Ono, *J. Gastroenterol.*, 2006, **41**, 462–469.
19 M. Carmiel-Haggai, A. I. Cederbaum and N. Nieto, *FASEB J.*, 2005, **19**, 136–138.
20 G. Marchesini, M. Brizi, A. M. Morselli-Labate, G. Bianchi, E. Bugianesi, A. J. McCullough, G. Forlani and N. Melchionda, *Am. J. Med.*, 1999, **107**, 450–455.
21 G. Bianchi, G. Marchesini, N. Brunetti, E. Manicardi, F. Montuschi, R. Chianese and M. Zoli, *Dig. Liver Dis.*, 2003, **35**, 722–727.
22 A. H. Lockwood, J. M. McDonald, R. E. Reiman, A. S. Gelbard, J. S. Laughlin, T. E. Duffy and F. Plum, *J. Clin. Invest.*, 1979, **63**, 449–460.
23 H. A. Krebs, *Biochem. J.*, 1942, **36**, 758–767.
24 H. A. Krebs, R. Hems, P. Lund, D. Halliday and W. W. C. Read, *Biochem. J.*, 1978, **176**, 733–737.
25 M. Takiguchi, T. Matsubasa, Y. Amaya and M. Mori, *BioEssays*, 1989, **10**, 163–166.
26 A. Aperia, O. Broberger, A. Larsson and K. Snellman, *Scand. J. Clin. Lab. Invest.*, 1979, **39**, 329–336.
27 P. K. Davis and G. Y. Wu, *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.*, 1988, **119**, 527–537.
28 S. Arriarán, S. Agnelli, X. Remesar, J.-A. Fernández-López and M. Alemany, *RSC Adv.*, 2015, **5**, 93403–93414.
29 S. Arriarán, S. Agnelli, X. Remesar, J. A. Fernández-López and M. Alemany, *PeerJ*, 2015, **3**, e1399.
30 E. Prats, M. Monfar, R. Iglesias, J. Castellà and M. Alemany, *Physiol. Behav.*, 1989, **45**, 263–272.
31 T. Barber, J. R. Viña, J. Viña and J. Cabo, *Biochem. J.*, 1985, **230**, 675–681.
32 D. Sabater, S. Agnelli, S. Arriarán, J.-A. Fernández-López, M. D. M. Romero, M. Alemany and X. Remesar, *BioMed Res. Int.*, 2014, **2014**, 959420.
33 M. Esteve, I. Rafecas, X. Remesar and M. Alemany, *Biochem. Int.*, 1992, **26**, 687–694.
34 C. Bucca, A. Cicolin, G. Guida, E. Heffler, L. Brussino and G. Rolla, *J. Breath Res.*, 2012, **6**, 027104.
35 G. Costa, L. Ullrich, F. Kantor and J. F. Holland, *Nature*, 1968, **218**, 546–551.
36 R. Ferrer-Lorente, C. Cabot, J. A. Fernández-López and M. Alemany, *Life Sci.*, 2005, **77**, 2051–2058.
37 L. Arola, E. Herrera and M. Alemany, *Anal. Biochem.*, 1977, **82**, 236–239.
38 O. H. Lowry, R. W. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265–275.
39 S. Arriarán, S. Agnelli, J. A. Fernández-López, X. Remesar and M. Alemany, *J. Enzyme Res.*, 2012, **3**, 29–33.
40 R. T. Schimke, *Methods Enzymol.*, 1970, **17**, 313–317.
41 E. L. Oginsky, *Methods Enzymol.*, 1957, **3**, 639–643.
42 D. M. Greenberg, *Enzymes*, 1960, **4**, 257–267.
43 L. Arola, A. Palou, X. Remesar and M. Alemany, *Horm. Metab. Res.*, 1981, **13**, 199–202.
44 A. Palou, M. Alemany, L. Arola and X. Remesar, *Rev. Esp. Fisiol.*, 1980, **36**, 151–154.
45 E. Ishikawa, T. Ninagawa and M. Suda, *J. Biochem.*, 1965, **57**, 506–513.
46 N. Ogasawara, H. Goto and T. Watanabe, *FEBS Lett.*, 1974, **44**, 63–66.
47 J. K. Fawcett and J. E. Scott, *J. Clin. Pathol.*, 1960, **13**, 156–159.
48 M. M. Romero, M. M. Grasa, M. Esteve, J. A. Fernández-López and M. Alemany, *Nutr. Metab.*, 2007, **4**, 26.
49 J. Q. He, A. J. Sandford, I. M. Wang, S. Stepaniants, D. A. Knight, A. Kicic, S. M. Stick and P. D. Paré, *Eur. Respir. J.*, 2008, **32**, 755–762.
50 R. Vytašek, *Anal. Biochem.*, 1982, **120**, 243–248.
51 GSP Consortium, *Nature*, 2004, **428**, 493–521.
52 S. Arriarán, S. Agnelli, D. Sabater, X. Remesar, J. A. Fernández-López and M. Alemany, *PLoS One*, 2015, **10**, e0119572.
53 M. D. M. Romero, S. Roy, K. Pouillot, M. Feito, M. Esteve, M. D. M. Grasa, J.-A. Fernández-López, M. Alemany and X. Remesar, *PLoS One*, 2014, **9**, e90995.
54 R. Ferrer-Lorente, C. Cabot, J. A. Fernández-López and M. Alemany, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 2010, **381**, 339–348.
55 R. Díaz-Rúa, E. García-Ruiz, A. Caimari, A. Palou and P. Oliver, *Food Funct.*, 2014, **5**, 3117–3131.
56 H. Castro, C. A. Pomar, C. Picó, J. Sánchez and A. Palou, *Int. J. Obes.*, 2015, **39**, 430–437.
57 M. Alemany, *Reprod. Biol. Endocrinol.*, 2011, **9**, 101.

58 D. A. Correa Pinto and P. Monteiro Seraphim, *Rev. Nutr.*, 2012, **25**, 313–319.
59 J. F. Lalanza, A. Caimari, J. M. del Bas, D. Torregrosa, I. Cigarroa, M. Pallàs, L. Capdevila, L. Arola and R. M. Escorihuela, *PLoS One*, 2014, **9**, e85049.
60 C. J. Bailey, P. R. Flatt and N. S. Radley, *Nutr. Res.*, 1985, **5**, 1003–1010.
61 D. R. Bolster, L. S. Jefferson and S. R. Kimball, *Proc. Nutr. Soc.*, 2004, **63**, 351–356.
62 J. Grigsby, W. Bergen and R. Merkel, *Growth*, 1976, **40**, 303–316.
63 R. C. May, J. L. Bailey, W. E. Mitch, T. Masud and B. K. England, *Kidney Int.*, 1996, **49**, 679–683.
64 D. H. Elwyn, F. E. Gump, M. Iles, C. L. Long and J. M. Kinney, *Metabolism*, 1978, **27**, 325–331.
65 J. Van Raaij, C. M. Schonk, S. H. Vermaat-Miedema, M. Peek and J. Hautvast, *Am. J. Clin. Nutr.*, 1991, **53**, 612–619.
66 C. Moundras, C. Remesy and C. Demigne, *Am. J. Physiol.*, 1993, **264**, G1057–G1065.
67 B. K. Speake, R. Dils and R. J. Mayer, *Biochem. J.*, 1976, **154**, 359–370.
68 A. Husson, C. Brasse-Lagnel, A. Fairland, S. Renouf and A. Lavoinne, *Eur. J. Biochem.*, 2003, **270**, 1887–1899.
69 S. M. Morris, *Annu. Rev. Nutr.*, 1992, **12**, 81–101.
70 J. C. Marini, *J. Nutr.*, 2012, **142**, 572–580.
71 C. Vizioli de Castro Ghizoni, F. Rodrigues Silva Gasparin, A. Sueiti Maeda, F. Olinto Carreño, R. Polimeni Constantin, A. Bracht, E. L. Ishii Iwamoto and J. Constantin, *Mol. Cell. Biochem.*, 2013, **373**, 265–277.
72 W. Durante, F. K. Johnson and R. A. Johnson, *Clin. Exp. Pharmacol. Physiol.*, 2007, **34**, 906–911.
73 A. Ghasemi, S. Zahediasl and F. Azizi, *Scand. J. Clin. Lab. Invest.*, 2012, **72**, 523–530.
74 H. G. Windmueller and A. E. Spaeth, *Am. J. Physiol.*, 1981, **241**, E473–E480.
75 C. Breuillard, L. Cynober and C. Moinard, *Amino Acids*, 2015, **47**, 685–691.
76 S. Grisolía, M. D. Miñana, E. Grau and V. Felipo, *Adv. Exp. Med. Biol.*, 1994, **341**, 1–12.
77 A. J. Meijer, *Trends Biochem. Sci.*, 1979, **4**, 83–86.
78 C. H. C. Dejong, M. T. Kampman, N. E. P. Deutz and P. B. Soeters, *J. Neurochem.*, 1992, **59**, 1071–1079.
79 C. Rémésy, C. Moundras, C. Morand and C. Demigné, *Am. J. Physiol.*, 1997, **272**, G257–G264.
80 E. Cersosimo, P. Williams, R. Geer, T. Lairmore, F. Ghishan and N. N. Abumrad, *Am. J. Physiol.*, 1989, **257**, E514–E519.
81 L. Boon, W. J. C. Geerts, A. Jonker, W. H. Lamers and C. J. F. van Noorden, *Histochem. Cell Biol.*, 1999, **111**, 445–452.
82 L. R. Soria, J. Marrone, G. Calamita and R. A. Marinelli, *Hepatology*, 2013, **57**, 2061–2071.
83 V. Rubio, G. Ramponi and S. Grisolía, *Biochim. Biophys. Acta*, 1981, **659**, 150–160.
84 H. Nakagawa, S. Miura, H. Kimura and T. Kanatsuna, *J. Biochem.*, 1969, **66**, 549–564.
85 O. M. Wrong and A. Vince, *Proc. Nutr. Soc.*, 1984, **43**, 77–86.
86 R. da Silva, O. Levillain, J. T. Brosnan, S. Araneda and M. E. Brosnan, *Can. J. Physiol. Pharmacol.*, 2013, **91**, 362–368.
87 J. D. McGivan and J. B. Chappell, *FEBS Lett.*, 1975, **52**, 1–7.
88 K. K. Moss and J. D. McGivan, *Biochem. J.*, 1975, **150**, 275–283.
89 M. Szydlowska, G. Nagel-Starczynowska, I. Rybakowska, A. Swieca and K. Kaletha, *Mol. Cell. Biochem.*, 2002, **241**, 81–82.
90 A. J. Verhoeven, J. F. van Iwaarden, S. K. Joseph and A. J. Meijer, *Eur. J. Biochem.*, 1983, **133**, 241–244.
91 T. Yamada, J. Komoto, Y. Takata, H. Ogawa, H. C. Pitot and F. Takusagawa, *Biochemistry*, 2003, **42**, 12854–12865.
92 R. K. Hampson, M. K. Taylor and M. S. Olson, *J. Biol. Chem.*, 1984, **259**, 1180–1185.
93 N. M. Anderson, F. I. Bennett and G. A. O. Alleyne, *Biochim. Biophys. Acta*, 1976, **437**, 238–243.
94 J. M. Lowenstein, *Int. J. Sports Med.*, 1990, **11**, S37–S46.
95 Y. A. Kim, M. T. King, W. E. Teague, G. A. Rufo, R. L. Veech and J. V. Passoneau, *Am. J. Physiol.*, 1992, **262**, E344–E352.
96 P. Dzeja and A. Terzic, *Int. J. Mol. Sci.*, 2009, **10**, 1729–1772.
97 K. Tornheim, *J. Theor. Biol.*, 1979, **79**, 491–541.
98 G. Lutoslawska and M. Niedbalska, *Med. Sportiva*, 2004, **8**, 85–104.
99 K. Tornheim and J. M. Lowenstein, *J. Biol. Chem.*, 1972, **247**, 162–169.
100 T. Yoshida and G. Kikuchi, *Arch. Biochem. Biophys.*, 1970, **139**, 380–392.
101 M. Alemany, *Nutr. Res. Rev.*, 2012, **25**, 18–28.
102 M. Esteve, I. Rafecas, J. A. Fernández-López, X. Remesar and M. Alemany, *Biochem. Mol. Biol. Int.*, 1993, **29**, 1069–1081.

4.3 Sabater D, Agnelli S, Arriarán S, et al. Cafeteria diet induce changes in blood flow that are more related with heat dissipation than energy accretio. PeerJ 4, e2302 (2016)

Cafeteria diet induce changes in blood flow that are more related with heat dissipation than energy accretion

David Sabater[1], Silvia Agnelli[1], Sofía Arriarán[1], María del Mar Romero[1,2], José Antonio Fernández-López[1,2,3], Marià Alemany[1,2,3] and Xavier Remesar[1,2,3]

[1] Department of Biochemistry an Molecular Biomedicine, Faculty of Biology, Universitat de Barcelona, Barcelona, Spain
[2] CIBER OBN Research Network, Barcelona, Spain
[3] Institute of Biomedicine, Universitat de Barcelona, Barcelona, Spain

ABSTRACT

Background. A "cafeteria" diet is a self-selected high-fat diet, providing an excess of energy, which can induce obesity. Excess of lipids in the diet hampers glucose utilization eliciting insulin resistance, which, further limits amino acid oxidation for energy.
Methods. Male Wistar rats were exposed for a month to "cafeteria" diet. Rats were cannulated and fluorescent microspheres were used to determine blood flow.
Results. Exposure to the cafeteria diet did not change cardiac output, but there was a marked shift in organ irrigation. Skin blood flow decreased to compensate increases in lungs and heart. Blood flow through adipose tissue tended to increase in relation to controls, but was considerably increased in brown adipose tissue (on a weight basis).
Discussion. The results suggest that the cafeteria diet-induced changes were related to heat transfer and disposal.

Subjects Anatomy and Physiology, Diabetes and Endocrinology, Hematology, Nutrition, Metabolic Sciences
Keywords Flux, Cafeteria diet, Rat, Tissue blood flow

Submitted 8 April 2016
Accepted 7 July 2016
Published 3 August 2016

Corresponding author
Xavier Remesar, xremesar@ub.edu

Academic editor
Cheorl-Ho Kim

Additional Information and Declarations can be found on page 8

DOI **10.7717/peerj.2302**

INTRODUCTION

The cafeteria diets (CD) were devised as a dietary model in which palatability could overcome the intrinsic control of energy intake of experimental animals and induce hyperphagia, consequently, its basis is taste and variety (*Sclafani & Springer, 1976*). There is a wide variety of formulations of cafeteria diets, all based on these principles, and have been widely used for the study of late-onset hyperphagic obesity and metabolic syndrome in animal models (*Sampey et al., 2011*), since it induces the increase of fat storage (*Rothwell & Stock, 1976*). These models have the advantage of being comparable, at least in its basic characteristics (self-selection, excess energy intake), to human obesity induced by energy-dense diets (*Romero, Esteve & Alemany, 2006*). In general, the lipid intake with CD is grossly increased, while other nutrients are consumed in proportions barely different from controls: most CD are, thus, hyperlipidic (*Prats et al., 1989*), and their effects are more marked in males (*Prats et al., 1989*; *Coatmellec-Taglioni et al., 2003*), not protected by estrogen, and are thus more prone to be affected by glucocorticoids (*Bouclaous et al., 2003*; *Carsia et al., 2008*), which in turn compound the obesogenic effect

by decreasing the anabolic and protective effects of androgens (*MacAdams, White & Chipps, 1986*; *Retana-Márquez et al., 2003*).

In rats, CD increase the accumulation of body fat, but also affect their lean body mass by favoring growth and protein deposition (*Harris, 1993*; *Lladó et al., 1995*). The hedonic component of CD initially elicits marked increase in food consumption (*Rogers & Blundell, 1984*); however, when obesity has been already developed, hyperphagia decreases, often down to normal food intake. Nevertheless, the accumulated excess persists even after normalization of energy intake (*Davidson & Garvey, 1993*; *Coatmellec-Taglioni et al., 2003*). The variable presence of sucrose in a number of CD diets can elicit binge eating in humans (*Latner, 2008*) and in rodents induce similar situations to metabolic syndrome (*Santuré et al., 2002*), probably because of the additional obesogenic effects of fructose availability (*Bocarsly et al., 2010*).

All the changes elicited by CD-induced excess energy intake, necessarily influence the interorgan metabolic relationships, changing their ability to control and metabolize substrates (*Macedo et al., 2015*), the storage of excess energy as reserves (mostly fat), affecting the whole body energy economy to the new pathologic situation. Since dietary-restricted (*Nutter et al., 1979*) or malnourished rats (*Sakanashi, Brigham & Rasmussen, 1987*) show decreased heart blood output, in connection with their decreased energy consumption mode, we expected that overfed animals, with excess energy available could show a reverse blood output pattern. The hypothesis was to assume that a higher blood flow in the rat tissues would favor the metabolic interchange and thus help increase energy expenditure and substrate disposal (including storage). Thus, in the present study we have intended to determine whether a common hyperlipidic CD could affect significantly the overall and individual organ blood flow.

MATERIALS & METHODS

Animal handling conditions

The experimental setup and all the animal handling procedures were carried out in accordance with the guidelines established by the European, Spanish and Catalan Authorities. The specific procedures used in this study were authorized by the Committee on Animal Experimentation and Ethics of the University of Barcelona (Procedure DAAM 6911).

Animals and diets

Nine week old male Wistar rats (Harlan Laboratories Models, Sant Feliu de Codines, Spain) (initial weight 288 ±2 g) were used. Two groups of 12 animals were randomly selected and were fed *ad libitum* for 30 days with either normal rat chow (#2014 Harlan) or a simplified hyperlipidic cafeteria diet (*Esteve et al., 1992b*); the groups were named "control" and "cafeteria," respectively. The rats were housed in 2-rat cages, and were kept in a controlled environment (lights on from 08:00 to 20:00; 21.5–22.5 °C; 50–60% humidity). They had free access to water. The rats in the Cafeteria group were fed with fresh offerings of excess chow pellet, liver pâté spread on cookies, bacon and milk enriched with 300 g/L sucrose and 10 g/L of a protein, vitamin and mineral supplement (Meritene, Nestlé Nutrition, Barcelona, Spain) (*Esteve et al., 1992a*; *Esteve et al., 1992b*). Consumption of every item in

the CD was recorded daily and converted into nutrient/energy equivalents to establish the actual intake of nutrients in their food selected by the cafeteria group. Body weight was recorded along with food consumption. In the standard diet, 13% of total energy was derived from lipids, 20% from protein and 67% from carbohydrates. In the cafeteria diet, those percentages (mean values) were 43%, 14% and 43%, respectively for the whole period analyzed.

Permanent cannulations

On day 27, all rats were implanted with two cannulas through the left carotid artery using Intramedic PE-10 polyethylene tubing (Becton Dickinson, Parsipanny, NJ, USA), (0.28 mm id/0.61 mm od) under isoflurane anesthesia. The first carotid cannula was used to draw blood from the descending aorta and the other to inject microspheres directly into the heart outflow as previously described (*Ardévol et al., 1998*). At 12 h intervals, the viability of the cannulas was checked (without disturbing the animals) by drawing blood up a few mm into the capillary tube, followed by refilling with heparinized saline. On day 30, the rats were injected through the left ventricle cannula with 10^5 red latex beads (Molecular Probes, Carlsbad, CA, USA) suspended in 0.1 ml of 9 g/L NaCl. At the same time, about 0.2 ml of blood were slowly drawn through the other cannula for exactly one minute. Then, the rats were anesthetized with isoflurane, and larger blood samples were drawn by aortic puncture, euthanizing them. The blood was used to obtain plasma, which was frozen and kept at −80 °C. After sacrifice, the position of the cannulas was checked; no placement errors nor cannula clotting were found. Tissue samples were obtained, frozen, and then maintained at −80 °C. The weights of all organs sampled were also recorded.

Blood flow analysis

Blood and tissue samples of known volume/weight were digested with 4 M KOH at 25 °C for 24 h with occasional stirring. The samples were filtered through glass-fiber filters (GF/D, 2.5 μm; Whatman, Maidstone, Kent, UK), which retained the colored latex microspheres. The filters were washed with 20 g/L Tween-20 followed by distilled water. Then, the fluorospheres were extracted from the filter with 2.5 ml of etoxyethyl acetate. A fluorimeter (RF1501 Shimadzu, Kyoto, Japan) was used to measure the fluorescence (at 598 nm emission wavelength) under a red excitation light (565 nm), using adequately diluted samples compared against tissue blanks (i.e., pieces of the same tissue of rats from other experiments which had not received fluorescent beads). At least two samples for each tissue and rat were analyzed. The whole procedure was performed in accordance with the instructions provided by the supplied of the fluorescent beads. The number of beads in the tissue samples were estimated from the differential bead extract fluorescence, which was, then, extrapolated to the whole tissue or organ mass. Percent distribution of bead numbers between the different organs was used to determine the distribution of blood flow between the different organs (as percentage), since the total amount of beads injected was known. A sample of the injected material was also analyzed to correct for possible errors in the evaluation of injected bead numbers (*Bassingthwaighte et al., 1990*).

The calculation of cardiac output was performed by measuring the amount of beads in the blood drawn from the artery for exactly one minute. The estimated bead concentration

and the known amount of beads injected allowed the calculation of cardiac output (*Closa et al., 1993*). In any case, as additional checking, an indirect value of heart output was calculated from body mass (*Delp, Evans & Duan, 1998*) at rest; the data obtained using both approaches were similar, and thus we used the experimental value for the ensuing calculations.

Absolute blood flows were calculated from the number of beads leaving the heart per unit of time (i.e., absolute heart output), blood volume and the percentage of beads distributed between the different organs of each rat.

Other analyses

Total body muscle mass was estimated from selective myosin precipitation from the minced and high-concentration lithium chloride-dissolved rat carcass, as previously described (*Arola, Herrera & Alemany, 1979*). Glucose in plasma with a glucose oxidase kit #11504 (Biosystems, Barcelona, Spain) supplemented with mutarotase (490 nkat/mL of reagent) (Calzyme, San Luis Obispo CA, USA). Mutaroratase was added to speed up epimerization equilibrium of α- and β-D-glucose and thus facilitate the oxidation of β-D-glucose by glucose oxidase (*Miwa et al., 1972*).

Statistical analysis

The data were expressed as mean $\pm$ standard error. The unpaired Student's t test was used for comparisons between groups ($n = 12$). Statistical analyses were performed using the Prism 5 program (Graph Pad Software, La Jolla, CA USA). Significant differences were stablished at $p < 0.05$.

RESULTS

After one moth of treatment, control animals weighted 370 $\pm$ 15 g and those in the cafeteria-fed group 450 $\pm$ 11 g ($p < 0.05$ vs. controls), these values and those of plasma metabolites were similar to other previously described obtained the same dietary model and handling setup (*Herrero et al., 1997*). Mean daily metabolizable energy intake of control rats was 7.34 $\pm$ 0.36 kJ, rats in the cafeteria group ingested 18.4 $\pm$ 0.76 kJ/per day ($p < 0.05$ vs. controls). Plama glucose values were similar in both groups (9.97 $\pm$ 0.18 mM for controls and 9.68 $\pm$ 0.33 mM for cafeteria group).

Table 1 presents the effects 30-day exposure to the cafeteria or standard chow diet on relative organ weight, blood flow and cardiac output. The only significant individual differences in relative organ weight between both groups corresponded to brain, large intestine, lungs and a couple of white adipose tissue sites. The rest of organs showed remarkably similar weights in spite of a slight trend of cafeteria diet-fed rats to increase their size in, in accordance with their higher body size.

The cardiac output data were similar in both dietary groups. However, in absolute terms, blood flows were higher in most organs and tissues of cafeteria group compared to those in the control group but their weight/size were also higher. The ratios of blood flow for each organ (irrespective of size) between the cafeteria and control groups were higher than 1 for all tissues, except for skin, with IBAT (x4), heart (x3) and lungs (x2.5)

Table 1 Organ and tissue weight and blood flow of rats fed a cafeteria diet for one month compared with controls on a standard rat chow diet.

Tissue/organ	Control diet			Cafeteria diet			Absolute blood flow ratios: cafeteria/control	*P* values		
	Organ/tissue weight (%bw)	Relative blood flow (mL/min g)	Absolute blood flow (mL/min)	Organ/tissue weight (%bw)	Relative blood flow (ml/min g)	Absolute blood flow (mL/min)		Weight	Relative flow	Absolute flow
Skeletal muscle	39.51 ± 2.41	0.29 ± 0.029	37.7 ± 6.3	39.23 ± 3.11	0.31 ± 0.042	56.0 ± 5.1	1.49	NS	NS	0.049
Skin	15.14 ± 0.63	0.48 ± 0.19	26.3 ± 6.3	13.27 ± 0.64	0.12 ± 0.03	6.8 ± 1.7	0.26	NS	NS	0.014
Liver (arterial)	2.72 ± 0.11	0.24 ± 0.067	2.27 ± 0.64	2.67 ± 0.18	0.56 ± 0.11	5.34 ± 0.60	2.35	NS	0.032	0.006
Liver (total)			14.6 ± 3.98			17.2 ± 3.07	1.18			NS
Small intestine	0.82 ± 0.02	1.77 ± 0.75	8.47 ± 3.57	0.69 ± 0.06	2.34 ± 0.2	8.65 ± 1.90	1.02	NS	NS	NS
Kidneys	0.59 ± 0.02	4.56 ± 1.23	9.43 ± 2.46	0.54 ± 0.02	4.77 ± 0.69	10.8 ± 1.7	1.15	NS	NS	NS
Brain	0.53 ± 0.01	0.65 ± 0.24	1.27 ± 0.47	0.45 ± 0.02	1.09 ± 0.31	2.09 ± 0.61	1.65	0.005	NS	NS
Large intestine	0.41 ± 0.02	1.46 ± 0.37	2.17 ± 0.59	0.31 ± 0.01	2.29 ± 0.38	2.88 ± 0.46	1.33	0.001	NS	NS
Lungs	0.39 ± 0.01	0.47 ± 0.11	0.60 ± 0.10	0.31 ± 0.01	1.08 ± 0.41	1.49 ± 0.24	2.48	<0.001	NS	0.006
Stomach	0.34 ± 0.02	0.91 ± 0.3	1.14 ± 0.38	0.34 ± 0.02	1.1 ± 0.15	1.60 ± 0.26	1.40	NS	NS	NS
Heart	0.26 ± 0.01	0.34 ± 0.18	0.31 ± 0.17	0.26 ± 0.01	0.81 ± 0.38	0.94 ± 0.22	3.03	NS	NS	0.043
Pancreas	0.14 ± 0.003	1.68 ± 0.46	0.62 ± 0.16	0.12 ± 0.003	1.62 ± 0.41	0.80 ± 0.18	1.29	NS	NS	NS
Adrenal glands	0.020 ± 0.005	3.12 ± 0.67	0.22 ± 0.04	0.017 ± 0.004	5.11 ± 0.77	0.35 ± 0.04	1.59	NS	NS	NS
Interscapular BAT	0.11 ± 0.002	0.21 ± 0.074	0.073 ± 0.021	0.11 ± 0.001	0.59 ± 0.22	0.30 ± 0.09	4.11	NS	NS	0.034
Subcutaneous WAT	1.65 ± 0.03	0.12 ± 0.049	0.70 ± 0.30	2.17 ± 0.02	0.084 ± 0.036	0.77 ± 0.33	1.10	<0.001	NS	NS
Mesenteric WAT	0.92 ± 0.02	0.27 ± 0.038	0.49 ± 0.16	0.92 ± 0.02	0.18 ± 0.06	0.71 ± 0.25	1.46	NS	NS	NS
Retroperitoneal WAT	0.72 ± 0.02	0.062 ± 0.02	0.18 ± 0.05	1.11 ± 0.01	0.039 ± 0.008	0.35 ± 0.18	1.94	<0.001	NS	NS
Epididymal WAT	0.42 ± 0.01	0.093 ± 0.04	0.12 ± 0.06	1.06 ± 0.02	0.041 ± 0.008	0.30 ± 0.13	2.50	<0.001	NS	NS
Pericardial WAT	0.07 ± 0.007	0.40 ± 0.125	0.088 ± 0.029	0.07 ± 0.004	1.79 ± 0.51	0.45 ± 0.11	1.16	NS	0.024	0.009
Sum of 5 WAT sites	3.84 ± 0.02		1.34 ± 0.25	5.31 ± 0.04		2.29 ± 0.34	1.71	<0.001		0.045
Cardiac output			**111 ± 10**			**116 ± 14**				**NS**

Notes.

Values are presented as means ± sem.

Liver (total) = Σ liver (arterial) + small intestine + pancreas + stomach + large intestine; BAT = brown adipose tissue; WAT = white adipose tissue.

Statistical significance of the differences between groups was estimated with Student's *t* test.

showing the maximal differences between both dietary groups. The maximum differences observed were in the interscapular brown adipose tissue mass, which irrigation was more than four-fold higher in the cafeteria than in controls. This contrasts with the case of skin, which, in cafeteria group, only received about one fourth of the blood than controls. In white adipose tissue, when compared on a tissue weight basis, the only significant increase in irrigation corresponded to the small but highly active pericardial WAT mass, followed (in decreasing order) by epididymal, retroperitoneal and mesenteric sites, which did not show significant increases in cafeteria vs. control groups in spite of their larger size. However, the combined total adipose mass showed a significant increase in blood flow.

In the liver, experimental microsphere data represent only the arterial inflow, which was higher in cafeteria than in the control group. Total liver blood flow, which can be calculated adding the arterial inflow to the plus portal flow data (i.e., approximately the sum of pancreas, stomach, intestines and mesenteric WAT) did not show, either, differences between both groups.

When the absolute blood flows were corrected by weight, most of the differences described above disappeared, leaving only those for pericardial adipose tissue and liver arterial, but not total, blood inflow. These data suggest that the cafeteria diet altered only partialy, but significantly, blood flow distribution; the differences observed being largely a consequence of different tissue or organ relative mass, ultimately a consequence of excess energy availability and deposition.

DISCUSSION

The cafeteria diets are essentially hyper-energetic, tipically hyperlipidic, with mean protein and total carbohydrate intakes proportionally not different from controls fed standard rat chow (*Prats et al., 1989*; *Ferrer-Lorente et al., 2007*). As expected, a one-month exposure to the cafeteria diet caused overfeeding and resulted in higher body weights, which translated into a marked increase in adipose tissue depot size and higher muscle mass than those of controls. This is in agreement with previous studies showing that cafeteria (and other high-energy, self-selected tasty hyperlipidic diets) increased fat deposition (*Sclafani & Springer, 1976*), growth, protein accrual (*Harris, 1993*; *Esteve et al., 1992a*) and, also, increased energy output (*Ma & Foster, 1989*). Glucose plasma levels did not show differences caused by dietary treatment, as a direct consequence of increase in plasma glucose caused by anaesthesia (*Zuurbier et al., 2008*).

The comparison of the patterns of distribution of blood flow between the control and cafeteria groups agrees with the increase in the importance of brown adipose tissue-related thermogenesis (*Tulp, Frink & Danforth, 1982*; *Ma & Foster, 1989*) since this tissue blood flow in was almost four-fold higer in cafeteria group then in controls. Thermogenesis was essentially based on the uncoupling of the mitochondrial proton inflow (used to drive the synthesis of ATP via the directional ATPase system) generated in the oxidative part of the respiratory chain because of the adrenergic activation of a specific uncoupling protein in the inner mitochodrial membrane (*Ricquier et al., 1984*; *Rial & González-Barroso, 2001*). This mechanism allows dissipating energy as a heat, and its presence is magnified in small mammals such as rodents, with a high surface to volume ratio.

The blood flow of heart and lungs were also higher in the cafeteria group, which contrasts with the similar heart output of both groups. Consequently, more oxygen was available to maintain the activity of the heart in the rats of cafeteria group, despite showing a similar pumping effectiveness than in controls. The same can be said of lungs. However, the skin blood flow in cafeteria rats was greatly reduced compared with controls. We know that cafeteria diets increase the body energy expenditure and heat output (*Ma & Foster, 1989*), and, in rats, most of this heat is eliminated through evaporation, largely throughout the respiratory tract rather than from the loss through the skin surface observed in larger mammals as man, by means of higher blood circulation (conduction, radiation, convection). This lowered skin blood flow has been previously observed in other metabolic conditions in which energy expenditure was increased, such as active exercise in obese rats, in which skin circulation was maintained in lean but decreased in obese rats (*Ardévol et al., 1998*). This suggests that heat loss through skin radiation in rats may be inversely related to body size (including changes in fat stores), thus favoring lung evaporation as main heat-loss control mechanism. Probably, this form of control is more flexible and has less thermic inertia and effectiveness than the loss of heat though a skin evolutively prepared (at least in small, furry mammals) to prevent heat loss. The data on blood flow presented here, under standard, i.e., non-exercise, conditions, supports the postulated heat loss control mechanism shift.

The similarity of blood flows to the liver suggests that the intestine is already efficient organ for the extraction of nutrients, nutrients does not need additional blood (essentially oxygen input and substrates output) supply compared with controls. This may be, in part, a consequence of the global easier digestibility overall of the non-lipid components of the fiber-laden chow compared with the heavy disaccharide content of the CD. The CD energy density is larger than that of the control diet, but the volume of solids actually ingested was not much different between both groups.

The trend to increase blood flow in white adipose tissue suggests a harmonic growth pattern affecting all of them, which is probably a consequence of being part of a single disperse organ (*Cinti, 2005*), but also by the uniform metabolic response to excess energy. This includes fairly uniform distribution of fat stores in adipose and non-adipose tissues (*Romero et al., 2014*).

The higher blood flow to striated muscle observed in the cafeteria group is in agreement with a steadier (and increased) supply of substrates to sustain muscle growth and maintenance, coincident with the described increase of amino acid availability (*Herrero et al., 1997*) and body protein accrual of cafeteria diet-fed rats.

The ingestion of a self-selected hyperlipidic cafeteria diet induces the modification of energy partition (*Sclafani & Springer, 1976*), insulin resistance (*Davidson & Garvey, 1993*), accumulation of fat (*Rogers & Blundell, 1984*; *Harris, 1993*), increased energy expenditure (*Tulp, Frink & Danforth, 1982*; *Ma & Foster, 1989*) and decreased production of urea (*Barber et al., 1985*). To this list we should add that after a relatively short-time (one month) of exposure to the diet, thermogenesis is most likely kept high, but blood flow distribution is markedly changed in a way most likely related (at least in part) to the disposal

of excess heat (i.e., lungs' evaporation vs. skin radiation and conduction) and to support normal function and body groth and accrual of protein and fat.

Abbreviations

BAT	Brown Adipose Tissue
WAT	White Adipose Tissue

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This study was done with the partial support of grants of the Plan Nacional de Ciencia y Tecnología de los Alimentos (AGL-2011-23635) and the Plan Nacional de Investigación en Biomedicina (SAF2012-34895) of the Government of Spain. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Plan Nacional de Ciencia y Tecnología de los Alimentos: AGL-2011-23635.
Plan Nacional de Investigación en Biomedicina: SAF2012-34895.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- David Sabater, Silvia Agnelli, Sofía Arriarán, María del Mar Romero and José Antonio Fernández-López performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Marià Alemany conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.
- Xavier Remesar conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Committee on Animal Experimentation and Ethics of the University of Barcelona. Procedure DAAM 6911.

Data Availability

The following information was supplied regarding data availability:

Dipòsit Digital de la Universitat de Barcelona: http://hdl.handle.net/2445/96861.

REFERENCES

Ardévol A, Adán C, Remesar X, Fernández-López JA, Alemany M. 1998. Hind leg balance in obese Zucker rats during exercise. *Pflugers Archives* **435**:454–464.

Arola L, Herrera E, Alemany M. 1979. A method for the estimation of striated muscle mass in small laboratory animals. *Revista Española de Fisiología* **35**:215–218 DOI 10.1042/bj2300675.

Barber T, Viña JR, Viña J, Cabo J. 1985. Decreased urea synthesis in cafeteria-diet-induced obesity in the rat. *Biochemical Journal* **230**:675–681 DOI 10.1161/01.RES.66.5.1328.

Bassingthwaighte JB, Malone MA, Moffett TC, King RB, Chan IS, Link JM, Krohn KA. 1990. Molecular and particulate depositions for regional myocardial flows in sheep. *Circulation Research* **66**:1328–1344 DOI 10.1016/j.pbb.2010.02.012.

Bocarsly ME, Powell ES, Avena NM, Hoebel BG. 2010. High-fructose corn syrup causes characteristics of obesity in rats: increased body weight, body fat and triglyceride levels. *Pharmacology, Biochemistry and Behavior* **97**:101–105 DOI 10.1016/S0271-5317(03)00104-0.

Bouclaous C, Torbay N, Nassar C, Hwalla N. 2003. Modification of glucocorticoid effects on body weight gain, plasma lipids by changes in diet composition. *Nutrition Research* **23**:1105–1115 DOI 10.1055/s-0028-1086025.

Carsia RV, Weber H, McIlroy PJ, Hock CE. 2008. Long-term dietary lipid regimen alters adrenocortical function at the cellular level. *Hormone and Metabolic Research* **40**:848–853 DOI 10.1016/j.plefa.2005.04.010.

Cinti S. 2005. The adipose organ. *Prostaglandins Leukotrienes & Essential Fatty Acids* **73**:9–15 DOI 10.1113/expphysiol.1993.sp003684.

Closa D, Gómez-Sierra JM, Latres E, Alemany M, Remesar X. 1993. Short-term oscillations of aortic core temperature and thermogenic organ blood flow in the rat. *Experimental Physiology* **78**:243–253 DOI 10.1124/jpet.102.045682.

Coatmellec-Taglioni G, Dausse J-P, Giudicelli Y, Ribière C. 2003. Sexual dimorphism in cafeteria diet-induced hypertension is associated with gender-related difference in renal leptin receptor down-regulation. *Journal of Pharmacology Experimental and Therapeutics* **305**:362–367.

Davidson MB, Garvey D. 1993. Studies on mechanisms of hepatic insulin resistance in cafeteria- fed rats. *American Journal of Physiology* **264**:E18–E23.

Delp MD, Evans MV, Duan C. 1998. Effects of aging on cardiac output, regional blood flow, and body composition in Fischer-344 rats. *Journal of Applied Physiology* **85**:1813–1822.

Esteve M, Rafecas I, Remesar X, Alemany M. 1992a. Nitrogen balance discrepancy in Wistar rats fed a cafeteria diet. *Biochemistry International* **26**:687–694.

Esteve M, Rafecas I, Remesar X, Alemany M. 1992b. Nitrogen balances of lean and obese Zucker rats subjected to a cafeteria diet. *International Journal of Obesity* **16**:237–244 DOI 10.1254/jphs.FP0061441.

Ferrer-Lorente R, Cabot C, Fernández-López JA, Alemany M. 2007. Effects of combined Oleoyl-estrone and Rimonabant on overweight rats. *Journal of Pharmacological Sciences* **104**:176–182.

Harris RBS. 1993. The impact of high-fat or low-fat cafeteria foods on nutrient intake and growth of rats consuming a diet containing 30 percent energy as fat. *International Journal of Obesity* **17**:307–315 DOI 10.1038/sj.ijo.0800464.

Herrero MC, Remesar X, Bladé L, Arola L. 1997. Muscle amino acid pattern in obese rats. *International Journal of Obesity* **21**:698–703.

Latner JD. 2008. Macronutrient effects on satiety and binge eating in bulimia nervosa and binge eating disorders. *Appetite* **40**:309–311 DOI 10.1016/0031-9384(95)00081-S.

Lladó I, Picó C, Palou A, Pons A. 1995. Protein and amino acid intake in cafeteria fed obese rats. *Physiology & Behavior* **58**:513–519 DOI 10.1139/y89-061.

Ma SWY, Foster DO. 1989. Brown adipose tissue, liver, and diet-induced thermogenesis in cafeteria diet-fed rats. *Canadian Journal of Physiology and Pharmacology* **67**:376–381 DOI 10.7326/0003-4819-104-5-648.

MacAdams MR, White RH, Chipps BE. 1986. Reduction of serum testosterone levels during chronic glucocorticoid therapy. *Annals of Internal Medicine* **104**:648–651 DOI 10.1016/j.npep.2015.01.002.

Macedo IC, Rozisky JR, Oliveira C, Oliveira CM, Laste G, Nonose Y, Santos VS, Marques PR, Ribeiro MFM, Caumo W, Torres ILS. 2015. Chronic stress associated with hypercaloric diet changes the hippocampal BDNF levels in male Wistar rats. *Neuropeptides* **51**:75–81 DOI 10.1016/0009-8981(72)90483-4.

Miwa J, Okudo J, Maeda K, Okuda G. 1972. Mutarotase effect on colorimetric determination of blood-glucose with b-D-glucose oxidase. *Clinica Chimica Acta* **37**:538–540 DOI 10.1007/s004240050539.

Nutter DO, Murray TG, Heymsfield SB, Fuller EO. 1979. The effect of chronic protein-calorie undernutrition in the rat on myocardial function and cardiac function. *Circulation Research* **45**:144–152 DOI 10.1161/01.RES.45.1.144.

Prats E, Monfar M, Iglesias R, Castellà J, Alemany M. 1989. Energy intake of rats fed a cafeteria diet. *Physiology & Behavior* **45**:263–272 DOI 10.1016/0031-9384(89)90128-5.

Retana-Márquez S, Bonilla-Jaime H, Vázquez-Palacios G, Martínez-García R, Velázquez-Moctezuma J. 2003. Changes in masculine sexual behavior, corticosterone and testosterone in response to acute and chronic stress in male rats. *Hormones and Behavior* **44**:327–337 DOI 10.1016/j.yhbeh.2003.04.001.

Rial E, González-Barroso MM. 2001. Physiological regulation of the transport activity in the uncoupling proteins UCP1 and UCP2. *Biochimica et Biophysica Acta* **1504**:70–81 DOI 10.1016/S0005-2728(00)00240-1.

Ricquier D, Mory G, Bouillaud F, Thibault J, Weissenbach J. 1984. Rapid increase of mitochondrial uncoupling protein and its mRNA in stimulated brown adipose tissue. *FEBS Letters* **178**:240–244 DOI 10.1016/0014-5793(84)80608-0.

Rogers PJ, Blundell JE. 1984. Meal patterns and food selection during the development of obesity in rats fed a cafeteria diet. *Neuroscience & Biobehavioral Reviews* **8**:441–453 DOI 10.1016/0149-7634(84)90003-4.

Romero MM, Esteve M, Alemany M. 2006. Combined effects of oral oleoyl-estrone and limited food intake on body composition of young overweight male rats. *International Journal of Obesity* **30**:1149–1156 DOI 10.1038/sj.ijo.0803224.

Romero MM, Roy S, Pouillot K, Feito M, Esteve M, Grasa M, Fernández-López JA, Alemany M, Remesar X. 2014. Treatment of rats with self-selected hyperlipidic diet increases the lipid content of the main adipose tissue sites in a proportion similar to that of the rest of body lipid stores. *PLoS ONE* **9**:e90995 DOI 10.1371/journal.pone.0090995.

Rothwell NJ, Stock MJ. 1976. Mechanism of weight gain and loss in reversible obesity in the rat. *Journal of Physiology* **276**:60P–61P.

Sakanashi TM, Brigham HE, Rasmussen KM. 1987. Effect of dietary restriction during lactation on cardiac output, organ blood flow and organ weights of rats. *Journal of Nutrition* **117**:1469–1474.

Sampey BP, Vanhoose AM, Winfield HM, Freemerman AJ, Muehlbauer MJ, Fueger PT, Newgard CB, Makowski L. 2011. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet. *Obesity* **19**:1109–1117 DOI 10.1038/oby.2011.18.

Santuré M, Pitre M, Marette A, Deshaies Y, Lemieux C, Larivière R, Nadeau A, Bachelard H. 2002. Induction of insulin resistance by high-sucrose feeding does not raise mean arterial blood pressure but impairs haemodynamic responses to insulin in rats. *British Journal of Pharmacology* **137**:185–196 DOI 10.1038/sj.bjp.0704864.

Sclafani A, Springer D. 1976. Dietary obesity in adult rats: similarities to hypothalamic and human obesity syndromes. *Physiology and Behavior* **17**:461–471 DOI 10.1016/0031-9384(76)90109-8.

Tulp OL, Frink R, Danforth E. 1982. Effect of cafeteria feeding on brown and white adipose tissue cellularity, thermogenesis, and body composition in rats. *Journal of Nutrition* **112**:2250–2260.

Zuurbier CJ, Keijzers PJ, Koeman A, Van Wezel HB, Hollmann MW. 2008. Anesthesia's effects on plasma glucose and insulin and cardiac hexokinase at similar hemodynamicsand without major surgical stress in fed rats. *Anesthesia and Analgesia* **106**:135–142 DOI 10.1213/01.ane.0000297299.91527.74.

4.4 Agnelli S, Arriarán S, Fernández-López JA, et al. The activity of amino acid metabolism enzymes along the small intestine of male rats is slightly affected by high-protein diet. Under evaluation (European Journal of Nutrition)

The activity of amino acid metabolism enzymes along the small intestine of male rats is slightly affected by high-protein diet.

Silvia Agnelli[1], Sofía Arriarán[1], José-Antonio Fernández-López[1,2,3], Xavier Remesar[1,2,3], Marià Alemany[1,2,3]

[1]Department of Nutrition & Food Science, Faculty of Biology, University of Barcelona, Barcelona, Spain

[2]Institute of Biomedicine, University of Barcelona, Barcelona, Spain

[3]CIBER Obesity and Nutrition, Institute of Health Carlos III, Spain

Author for correspondence:

Dr. Marià Alemany. Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, University of Barcelona. Av. Diagonal, 643; 08028 Barcelona, Spain

E-mail: malemany@ub.edu TEL: +34 94 403 4606; FAX: +34 93 4021559

Abstract

Objective. To determine whether doubling the protein content in the diet induced significant changes in enzymes of amino acid metabolism along the intestine.

Design. Six adult male rats were fed a standard (control diet), and six more were given a hyperproteic diet (HPD) for 30 days. Then they were killed and the small intestine divided in three equal-length segments. These were used for the measurement of amino acid (AA) metabolism enzyme activities and enzyme gene expressions.

Results Most AA enzyme genes were expressed along a marked gradient: higher near the stomach. AMP-deaminase, and carbamoyl-P synthetase activities followed this pattern, but there were no changes in Orn-transcarbamylase. There was a fair coincidence between gene expression and enzyme activities. There were no significant differences between HPD and control diets. However, the longitudinal patterns of expression and activity were less marked in HPD than in control rats.

Conclusion In conclusion, we observed an anisotropic distribution of the main amino acid metabolism enzymes along the small intestine, with generally highest expressions (and activities) in the upper segments and lowest near the gross intestine. Feeding for one-month HPD induced little change (not significant) in the distribution of enzyme activities and expressions per unit of protein weight. It is speculated that this lack of changes may be part of a mechanism preventing amino acid wasting.

Key words:

Intestine; AMP-deaminase; urea cycle; amino acid metabolism; protein digestion; ammonium

Summary box

What is already known about this subject:

- Hyperproteic diets result in increased nitrogen excretion
- Excess amino-nitrogen in the intestine elicits the activation of urea cycle in the intestine, exporting its intermediary metabolites and ammonia to the liver for completion of the cycle
- Hyperproteic diets have been used (with mixed success) for the treatment of obesity, but the mechanisms are unknown

What are the new findings

- Doubling the protein content in the diet does not affect the expression of genes or activities of the main amino acid metabolism enzymes in the intestine
- There is a marked gradient in gene expression and activities of the enzymes from (maximal) duodenum to the end of ilion
- Maximal amino acid enzyme activities and gene expressions do not coincide with the expected intestinal zones of amino acid absorption resulting from dietary protein digestion

How might it impact on clinical practice in the foreseeable future?

- Hyperproteic diets should not be used to treat obesity, since the small intestine amino acid metabolism is not altered by dietary protein, i.e. not acting on the problems posed by the handling of excess amino nitrogen

Introduction

In spite of the wide use of hyperproteic diets for the treatment of obesity and related diseases (1;2), our knowledge of their actual metabolic effects on energy partition and on general regulation of metabolism are largely unknown (3;4). There are several reasons explaining the limited interest for this important field, which reached its zenith in the late seventies of last century (5), namely, the considerable difficulties posed by the fast interconversion of amino acids, with the N moiety being processed by a complex network of inter-organ paths, and the hydrocarbon moieties being incorporated, again in different organs and multiple paths, in the intermediate metabolism (such as glycolysis / gluconeogenesis, Krebs cycle, One-carbon pathways). These functions add to the role of amino acids as building bricks for proteins and as donor of N for the synthesis of purine, pyrimidine, porphyrins and a number of signalling molecules (i.e. nitric oxide, catecholamines) of critical importance in regulation.

In addition, the whole of body protein may be considered a reserve of energy and potential energy and gluconeogenic substrates (6), with intricately complex mechanisms set in place to preserve body N, for the salvage and turnover of amino N under conditions of starvation (5;7;8). However, under excess substrate availability, these mechanisms may pose a serious problem for energy and N homoeostasis (9). Unfortunately, most of the studies available refer to conditions of scarcity, malnutrition and starvation (7;8), and thus we keep using high-energy high-protein diets for a number of assumed physiological effects, but too often without analyzing the fate of the excess of dietary protein (or amino acid) intake (10;11).

Since protein digestion is slow, and requires the sequential denaturation and breakup of peptide bonds in dietary proteins, set at different places along the gut, we hypothesized that a high protein diet may alter this process, in concordance with changes in the microbiota, already known (12), and a modulation of the N balance, favouring a higher N excretion (13;14), largely via increased urea production (15). However, the use of high-energy, high-protein diets (i.e. cafeteria diets) in experimental animals results in a lower production of urea (10), excess N being eliminated through so far not fully identified pathways (16). The alteration of N balance under conditions of excess energy availability results in a deeply altered excretion of N (11;16). In the present study we have studied the expression of some of the most critical enzymes in overall amino acid metabolism in the splanchnic bed along the small intestine. In this case we selected the small intestine because in the stomach, dietary proteins are cut down by endopeptidases to produce peptones, with no significant amino acid uptake and metabolism. Similarly, in the large intestine contents, most of the dietary protein amino acids have been already released and absorbed, with most of the remaining catabolic activity being carried out by the microbiota. It is in the small intestine where most of the protein digestion takes place, where amino acids are assimilated, and in a significant part modified (7). It is in the small intestine (in parallel and in coordination with the kidney and other splanchnic organs) where most of the urea cycle is initiated under conditions of excess amino-N, and urea cycle precursors are conducted to the liver via porta vein for the cycle to continue there (17). Thus, we analyzed a number

of genes controlling the synthesis of amino acid metabolism enzymes along the small intestine, complementing the study with the measurement of the specific activities of key enzymes of the intestinal leg of the urea cycle, under conditions of high-protein feeding. We expected the pattern of enzyme gene expression (and activities) to change in parallel to the increased supply of amino acids.

Materials and Methods

Ethics statement

All animal handling procedures and the experimental setup were carried out in accordance with the animal handling guidelines of the European, Spanish and Catalan Authorities. The Committee on Animal Experimentation of the University of Barcelona authorized the specific procedures used.

Diet

Common rat chow (Teklad global 14% protein rodent maintenance diet, Harlan Laboratories, Madison WI) was used after weaning and for the duration of the experiment in control animals, and up to 14 weeks in animals later receiving hyperproteic-diet (HPD). The HPD was prepared by crushing to a coarse powder the common rat chow described above. To produce about 1 kg of HPD pellets, a mixture 87 g of powdered alimentation-grade pure gelatin, 100 g of purified powdered casein, 702 g of crushed standard chow, and 9.5 g of sunflower oil were suspended in 725 mL of water, and stirred, forming a paste which was thoroughly mixed and then extruded through cut-off syringes to form pellets; they were dried in an oven at 45ºC for 24 h.

The final composition of the pellets, compared with control chow is shown in Table 1. The consistency of the dried HPD pellets was comparable to that of the standard one. Their composition was the expected with respect to N content (about twice that of control pellets), and its calculated (from its composition) and crude energy (adiabatic bomb calorimeter, IKA C7000; IKA, Staufen, Germany) per g were comparable (Table 1). The main difference between control and HPD diets were limited to lower carbohydrate versus protein and a relative decrease in mineral and specific micronutrient content in the HPD.

Animals

Fourteen-week-old male Zucker lean rats (Harlan Laboratories Models, Sant Feliu de Codines, Spain) were used. Half of the rats (N=6 for each group, randomly selected) were shifted to the HPD for 30 days, while the controls continued ingesting the standard chow. The animals were kept in 3-rat solid-bottom cages (with wood shards as bedding material, changed every 2 days) in a controlled environment: free access to their diet and tap water, lights on from 08:00 to 20:00, humidity 60-70 %, temperature 20.5-21.5ºC.

Food intake was measured, as well as the weights of the animals (in fact consumption per cage) on alternate days. On day 30, the rats were killed by exsanguination under isoflurane anesthesia.

Dissection and intestine sampling

The rats were dissected immediately after death, their small intestine cut out of the visceral cavity, cleaned of mesentery and fat cordons, their contents carefully extruded out and then internally washed with a gentle stream of ice-cold KCl (9 g/L). The excess water was extruded and the intestine blotted and weighed; then it was extended unstretched, and cut in three equal length parts: I, II and III, I being that closer to the stomach and III to the gross intestine. I contained the duodenum and part of jejunum, II jejunum and part of ilion, and III ilion alone. The parts were frozen in liquid nitrogen, crushed to a fine powder, and distributed in several containers, before storage at -80ºC until processed.

Gene expression analysis

Tissue total RNA was extracted from frozen tissue samples (about 30 mg) using the GenEluteTM procedure. Total RNA was quantified using a ND-100 spectrophotometer (Nanodrop Technologies, Wilmington DE USA). RNA samples were reverse transcribed using the MMLV reverse transcriptase (Promega, Madison, WI USA) and oligo-dT primers.

Real-time PCR amplification was carried out using 10 µL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), equivalent to 4 ng of reverse-transcribed RNA and 300 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set to OD 0.150 for all runs.

A semiquantitative approach for the estimation of the concentration of specific gene mRNAs per cell or unit of tissue weight was used (18). *Rplp0* was used as charge control gene. The data were presented as the number of transcript copies per gram of protein. The genes analyzed and a list of primers used is presented in Table 2.

Measurement of enzyme activities

Frozen intestine samples were homogenized using a tissue disruptor (IKA-T10 basic Ultra-Turrax, IKA, Stauffen Germany) in 10 volumes of chilled Krebs-Ringer bicarbonate solution, pH 7.8 containing 5 mM dithiothreitol, 0.5% bovine serum albumin, 1% dextran (MW 200,000), 0.1% Triton X-100, and 1 mM EDTA (19). All incubations were carried out at 37ºC using freshly homogenized homogenates. Adenylate deaminase (EC 3.4.5.6) was estimated with a previously described method (20), based essentially on incubation of the crude homogenate with AMP, stopping the reaction from 0 up to 16 minutes (3-4 time-points) with cold trichoroacetic acid and then measuring the ammonia released with the Berthelot reaction. Initial (V_0) activities were determined from the course of the reaction at different times. V_0 was assumed to correspond to V_{max} under the conditions of analysis. Activity was expressed as nkat/g of protein.

Carbamoyl-P synthetase 1 (EC 6.3.4.16) was measured with a method developed by us and previously published (21), based essentially on the utilization of ^{14}C bicarbonate and ammonium (in the presence of N-acetyl-glutamate) to synthesize carbamoyl-P. Excess $^{14}CO_2$ was flushed with a current of cold CO_2 and the remaining label (i.e. ^{14}C-carbamoyl-P) was counted. The same multiple measured and analyses of V0 described above were also used for this enzyme.

Ornithine carbamoyl-transferase (EC 2.1.3.3) was measured using the incubation conditions described by McLaren and Ng (22), in a setting of multiple measurements along time as described above for other enzymes. The homogenization medium was a pH 7.4 HEPES 75 mM buffer, containing 1 mM dithiothreitol, 50 mM KCl and 10 g/L defatted bovine serum albumin (Sigma-Aldrich, St.Louis, MO USA). The method was based on the incorporation of carbamoyl-P to ^{14}C-ornithine to yield ^{14}C-citrulline. The amino acids were separated through thin-layer chromatography (silicagel sheets SIL G25 Macherey-Nagel, Düren, Germany) and counted. After correction for blanks and specific activity of labelled amino acids, production of citrulline was estimated.

Protein content in each of the homogenates used for enzyme analysis was estimated with a colorimetric method (23). These data were also used to establish the tissue protein content.

Statistical analyses

Comparisons between groups were done using two-way ANOVA analyses and the Bonferroni post-hoc test with the Prism 5 (GraphPad Software, San Diego CA USA) graphics/statistics package. Analyses of correlations and curve fitting (i.e. for V_0 estimation) were carried out using the same program set.

Results

Table 3 shows the comparison of rat and intestine weights, and intake at the end of the 30-day dietary treatment. There were no differences in body weight (neither in body composition, data not shown). Intestine weight was slightly (albeit significantly) smaller in HPD-treated rats than in controls (mean 1.4% of body weight versus 1.6%).

There were no differences in mean gross energy intake between both groups, but N intake was about twice (as expected) in HPD than in controls; the proportion of energy derived from diet protein versus total diet energy was twice in HPD than in controls.

Tissue protein content was uniform for the three segments and there were no differences between control and HPD groups (range: 17-18 g/kg fresh weight; maximal difference between segments about 6%).

Figure 1 presents the patterns of gene expression for the amino acid metabolism enzymes studied. There were no differences between expressions for any gene between HPD and control intestines. However, in most cases, there were significant differences in expressions along the three intestine segments (alanine transaminase 1, AMP deaminase, both carbamoyl-P synthetases, arginino-succinate synthetase, arginase 2, glutamine synthetase, N-acetyl-glutamate synthetase and neural nitric oxide synthetase). No detectable expression of the gene for arginase 1 was found in intestine.

In general, most patterns showed the highest levels of expression in segment I and the lower in III (i.e. the pattern showed by AMP deaminase), this general pattern showed slight modifications, with the II fraction expression levels being closer either to those of I or III, but in general, the loss of expression along the intestine was the norm for most genes analyzed, the differences being significant for the

gene-enzymes listed above. In a number of cases there were no appreciable differences in gene expression between the three segments (controls of ornithine transcarbamylase, glutamine synthetase, and both groups of glutaminase and arginino-succinate lyase).

Figure 2 shows the results for enzyme activites in the intestine fractions of rats fed control of HPD protein. The pattern for AMP deaminase activity closely followed that of its gene expression (Figure 1), with a pattern of rapidly decreasing activity in controls, and a more contained decrease (observed only in fraction III) for HPD-fed rats. The differences were significant for diet and segment. The decrease in activity of carbamoyl-P-synthetase 1 in controls was similar (decreased along the length of the intestine) than its expression, with little, if any, change in HPD-fed rats; no significant differences were observed between groups. Ornithine transcarbamylase activity showed also some differences with the data for gene expression, especially in controls, where the maximal activity was found in segment II, making the differences between segments significant.

Discussion

The most remarkable finding of this study is the lack of changes elicited on the expression (and largely on activity) of enzymes of amino acid metabolism in the intestine of rats subjected to a hyperproteic diet. We expected an adaptive modulation of the intestinal capacity to begin the process of disposal of excess amino-N (24), but the changes observed were minimal, with no reflection on statistical significance, which contrasts with the adaptive uptake of amino acids and adjustment of the rate of digestion to amino acid availability (25-27).

It is known that excess protein intake favours the utilization of intestinal amino acids for the synthesis of urea cycle intermediates (15), thus initiating an urea cycle that is later completed in the liver via formation of arginine (28), largely from portal vein-carried urea cycle intermediaries (29;30). Their origin of this citrulline can be traced to the production of ammonia, or utilization of glutamine (31) followed by the combined actuation of carbamoyl-P synthetases and ornithine transcarbamylase (28;32): This process is in part modulated by enterocyte NADPH, which is dependent on glucose availability (29). Thus, protein amino acids are partially spared under conditions of high dietary glucose content (or release through digestion), in a way parallel to N sparing under starvation (7;8).

The results obtained for enzyme activities and their gene expression show similar patterns, which helps reinforce the assumption that the lack of changes elicited by diet is only of limited extent. The rather uniform energy intake, lack of changes in body weight and composition, in spite of doubling the intake of 2-amino N, agree with this “passive” role of intestine in the control of amino acid availability.

We have found a significant presence of all urea cycle enzymes in intestine, including arginase 2 but not arginase 1 (*unpublished results*). Not all genes for amino acid enzymes behaved in the same way: maximal differences between control and HPD were found for neural-type nitric oxide synthase, and the limited change observed in glutamine metabolism enzymes, in spite of glutamine being a critical nutrient for intestine (30) and this splanchnic organ one of the main sites for glutamine conversion to ammonia (33;34).

In addition to the lack of effect of a hyperproteic diet, we also observed a clear distribution of amino acid metabolism enzymes (through the differential expression of the genes for these enzymes) that tends to accumulate most activities in the upper (duodenum, jejunum) segments in detriment of the lower small intestine (ilion). This is, again, counterintuitive, since protein digestion is a slow process (25), needing the sequential action of endopeptidases, exopeptidades and, finally dipeptidases and transport systems. Logically, the maximal availability of amino acids released from dietary protein would be found in the farthest parts of the intestine, where the digestion took more time. Precisely, the short extent of duodenum, and the perennial absence of contents in the jejunum (etymologically "*I fast*") loads the brunt of protein digestion in the ilion. We have found, consistently, that both enzyme activities and gene expression were lowest for the distal small intestine, where most of amino acid absorption is supposed to take place (35;36) in spite of the highest transporting capacity of amino acids in small intestine having been identified --too-- in its initial segments (37). However, absorption is regulated by dietary protein load as a way to regulate the flow of 2-amino N to the liver (26) .

Then, why most amino acid degrading enzymes, including the starters of urea cycle (and even transporters) are concentrated in the fractions I or I+II?. We can speculate that this disposition prevents a massive oxidation of dietary amino acids, since most of them would be released in the III or II+III segments, where they have more probabilities of being absorbed and transferred to the portal vein intact. However, when the diet is frankly rich in amino acids, the secretion of peptidases increases (24;27), and probably a significant part of amino acids may be released in the upper small intestine within reach of catabolic pathways. We can further speculate that the imperviousness of intestine amino acid enzyme distribution to excess protein intake (and expected parallel increase in 2-amino N availability) is in itself a mechanism to limit amino acid oxidation, leaving to the automatic release of peptidases and transport (26;27) the brunt of control of digestion (and that of absorption and oxidation).

A particular case is that of ornithine transcarbamylase activity, which pattern resembles that of of its gene expression, but the peak activity is (in both groups) placed in fraction II, which suggests that a key enzyme in the intestine side of the urea cycle operation (38) works along the whole length of the intestine, with a maximum at the center of the organ length.

Another peculiar finding is the marked differences observed in AMP deaminase, a critical enzyme of the purine nucleotide cycle (39), which produces ammonia from aspartate (in fact from almost any amino acid (40)) for local use by carbamoyl-P synthetase or exportation to the liver (41). In controls, the fall of gene expression and enzyme activity is clear and linear, but in HPD-fed rats, the fall only is observed in fraction III. This "delay" may be part of a mechanism in which the only sensitive part of the intestine to a protein load may reside in its median part. AMP deaminase is a critical regulatory enzyme controlling the levels of AMP (42), and thus AMP-kinases (43) and the control of glycolysis (44). AMP deaminase is inhibited by metformin (43), a fundamental drug for the treatment of hyperglycemia. But most of AMP deaminase is present in the upper part of the intestine, precisely where free glucose is absorbed and not in the rest of the intestine, which absorbs the glucose, released from polysaccharide digestion. The placing of AMP deaminase largely in the duodenum

suggests that its main role is related to control glucose uptake/metabolism through AMP-kinase (45) rather than its participation in ammoniagenesis of the purine nucleotide cycle.

In any case, the data from rats fed HPD show a less marked precipitous fall of expression/activity from fractions I to III, suggesting a possible localized extension of highly catabolic upper intestine environmental conditions as a response to HPD, but this is only an observation apparent in Figures 1 and 2 but not clearly backed by hard statistical data.

In conclusion, we observed an anisotropic distribution of the main amino acid metabolism enzymes along the small intestine, with generally highest expressions (and activities) in the upper segments and lowest near the gross intestine. Feeding for one-month a hyperproteic diet induced little change (not significant) in the distribution of enzyme activities and expressions per unit of protein weight. It is speculated that this lack of changes may be part of a mechanism preventing amino acid wasting.

Acknowledgements

This study was done with the partial support of grants of the Plan Nacional de Investigación en Biomedicina (SAF2012-34895) and the Plan Nacional de Ciencia y Tecnología de los Alimentos (AGL-2011-23635) of the Government of Spain, as well as of CIBER-OBN (Institute of Health Carlos III). S.Agnelli was the recipient of a Leonardo da Vinci fellowship, and S. Arriarán a predoctoral fellowship of the Catalan Government, in both cases covering part of the time invested in this study. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

References

(1) dalle Grave R, Calugi S, Gavasso I *et al*. A randomized trial of energy-restricted high-protein versus high-carbohydrate, low-fat diet in morbid obesity. *Obesity* 2013;**21**(9):1774-81.

(2) Dong JY, Zhang ZL, Wang PY *et al*. Effects of high-protein diets on body weight, glycaemic control, blood lipids and blood pressure in type 2 diabetes: meta-analysis of randomised controlled trials. *Br J Nutr* 2013;**110**:781-9.

(3) Kitabchi AE, McDaniel KA, Wan JY *et al*. Effects of high-protein versus high-carbohydrate diets on markers of β-cell function, oxidative stress, lipid peroxidation, proinflammatory cytokines, and adipokines in obese, premenopausal women without diabetes. A randomized controlled trial. *Diabetes Care* 2013;**36**:1919-25.

(4) Tang MH, Armstrong CLH, Leidy HJ *et al*. Normal vs. high-protein weight loss diets in men: effects on body composition and indices of metabolic syndrome. *Obesity* 2013;**21**:E204-E210.

(5) Alemany M. Adjustment to dietary energy availability: from starvation to overnutrition. *RSC Advances* 2013;**3**:1636-51.

(6) Cahill GF. Protein and amino acid metabolism in man. *Circulation Res Suppl* 1976;**1**:I109-

I114.

(7) de Blaauw I, Deutz NEP, von Meyenfeldt MF. In vivo amino acid metabolism of gut and liver during short and prolonged starvation. *Am J Physiol* 1996;**270**:G298-G306.

(8) Felig P, Owen OE, Wahren J *et al*. Amino acid metabolism during prolonged starvation. *J Clin Invest* 1969;**48**:584-94.

(9) Alemany M. The problem of nitrogen disposal in the obese. *Nutr Res Rev* 2012;**25**(1):18-28.

(10) Barber T, Viña JR, Viña J *et al*. Decreased urea synthesis in cafeteria-diet-induced obesity in the rat. *Biochem J* 1985;**230**:675-81.

(11) Sabater D, Agnelli S, Arriarán S *et al*. Altered nitrogen balance and decreased urea excretion in male rats fed cafeteria diet are related to arginine availability. *BioMed Res Int* 2014;**2014:**959420.

(12) Scott KP, Gratz SW, Sheridan PO *et al*. The influence of diet on the gut microbiota. *Pharmacol Res* 2013;**69**:52-60.

(13) Schemmel RA, Stoddart KA, Stone M *et al*. Nitrogen and protein losses in rats during weigh reduction with a high protein, very low-energy diet or fasting. *J Nutr* 1983;**113**:727-34.

(14) Bingham SA. Urine nitrogen as a biomarker for the validation of dietary protein intake. *J Nutr* 2003;**133**:921S-4S.

(15) Petersen KF, Vilstrup H, Tygstrup N. Effect of dietary protein on the capacity of urea synthesis in rats. *Horm Metabol Res* 1990;**22**:512-615.

(16) Esteve M, Rafecas I, Remesar X *et al*. Nitrogen balance discrepancy in Wistar rats fed a cafeteria diet. *Biochem Int* 1992;**26**:687-94.

(17) Matsuzawa T, Kobayashi T, Tashiro K *et al*. Changes in ornithine metabolic enzymes induced by dietary protein in small intestine and liver - Intestine liver relationship in ornithine supply to liver. *J Biochem* 1994;**116**:721-7.

(18) Romero MM, Grasa MM, Esteve M *et al*. Semiquantitative RT -PCR measurement of gene expression in rat tissues including a correction for varying cell size and number. *Nutr Metabol* 2007;**4:**6.

(19) Remesar X, Arola L, Palou A *et al*. Arginase activity in the organs of fed and 24-hours fasted rats. *Horm Metabol Res* 1980;**12**:281-2.

(20) Arola L, Remesar X, Palou A *et al*. Adenylate deaminase activity in the rat: effect of 24 hours of fasting. *Horm Metabol Res* 1981;**13**:264-6.

(21) Arriarán S, Agnelli S, Fernández-López JA *et al*. A radiochemical method for carbamoyl-phosphate synthetase-I: Application to rats fed a hyperproteic diet. *J Enzyme Res* 2012;**3**:29-33.

(22) McLaren J, Ng WG. Assay of ornithine carbamoyl transferase activity in human liver using carbon-labeled ornithine and thin-layer chromatography. *Clin Chim Acta* 1977;**81**:193-201.

(23) Lowry OH, Rosebrough RW, Farr AL *et al*. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;**193**:265-75.

(24) Jean C, Rome S, Mathé V *et al*. Metabolic evidence for adaptation to a high protein diet in rats. *J Nutr* 2001;**131**:91-8.

(25) Masanés RM, Rafecas I, Remesar X. Absorption of a protein gavage in Zucker lean rats. Influence of protein content in the diet. *Arch Physiol Biochem* 2001;**109**(2):168-74.

(26) Nolles JA, van Straten EME, Bremer BI *et al*. Dietary amino acids fed in free form and as protein components do not differently affect postprandial plasma insulin, glucagon, growth hormone and corticosterone responses in rats. *J Anim Physiol Anim Nutr* 2006;**90**:289-99.

(27) Raul F, Goda T, Gossé F *et al*. Short-term effect of a high-protein/low-carbohydrate diet on aminopeptidase in adult rat jejunoileum. Site of aminopeptidase response. *Biochem J* 1987;**247**:401-5.

(28) Hartman WJ, Prior RL. Dietary arginine deficiency alters flux of glutamine and urea cycle intermediates across the portal-drained viscera and liver of rats. *J Nutr* 1992;**122**:1472-82.

(29) Wu GY. An important role for pentose cycle in the synthesis of citrulline and proline from glutamine in porcine enterocytes. *Arch Biochem Biophys* 1996;**336**:224-30.

(30) van de Poll MCG, Lighart-Melis GC, Boelens PG *et al*. Intestinal and hepatic metabolism of glutamine and citrulline in humans. *J Physiol* 2007;**581**:819-27.

(31) Weber FL, Veach G, Friedman DW. Stimulation of ammonia production from glutamine by intraluminal glucose in small intestine of dogs. *Am J Physiol* 1982;**242**:G552-G557.

(32) Wrong OM, Vince A. Urea and ammonia metabolism in the human large intestine. *Proc Nutr Soc* 1984;**43**:77-86.

(33) Gate JJ, Parker DS, Lobley GE. The metabolic fate of the amido-N group of glutamine in the tissues of the gastrointestinal tract in 24 h-fasted sheep. *Br J Nutr* 1999;**81**:297-306.

(34) Windmueller HG, Spaeth AE. Uptake and metabolism of plasma glutamine by the small intestine. *J Biol Chem* 1974;**249**:5070-9.

(35) Munck LK, Munck BG. Variation in amino acid transport along the rabbit small intestine. Mutual jejunal carriers of leucine and lysine. *Biochim Biophys Acta* 1992;**1116**:83-90.

(36) Munck LK. Chloride-dependent amino acid transport in the small intestine: occurrence and significance. *Biochim Biophys Acta* 1995;**1241**:195-214.

(37) Silk DB, Grimble GK, Rees RG. Protein digestion and amino acid and peptide absorption. *Proc Nutr Soc* 1985;**44**:63-72.

(38) Raijman L. Citrulline synthesis in rat tissues and liver content of carbamoyl phosphate and ornithine. *Biochem J* 1974;**138**:225-32.

(39) Tornheim K, Lowenstein JM. The purine nucleotide cycle. The production of ammonia from aspartate by extracts of rat skeletal muscle. *J Biol Chem* 1972;**247**:162-9.

(40) Turner LV, Fern EB. Changes in the activity of rat muscle AMP deaminase in relation to the proportion of dietary protein. *Br J Nutr* 1974;**32**:539-48.

(41) Bogusky RT, Steele KA, Lowenstein LM. The purine nucleotide cycle in the regulation of ammoniagenesis during induction and cessation of chronic acidosis in the rat kidney. *Biochem J* 1981;**196**:323-6.

(42) Flanagan WF, Holmes EW, Sabina RL *et al*. Importance of purine nucleotide cycle to energy production in skeletal muscle. *Am J Physiol* 1986;**251**:C795-C802.

(43) Ouyang JY, Parakhia RA, Ochs RS. Metformin activates AMP kinase through inhibition of AMP deaminase. *J Biol Chem* 2011;**287**:1-11.

(44) Yoshino M, Murakami K. AMP deaminase reaction as a control system of glycolysis in yeast. Activation of phosphofructokinase and pyruvate kinase by the AMP deaminase ammonia system. *J Biol Chem* 1982;**257**:2822-8.

(45) Fujii N, Jessen N, Goodyear LJ. AMP-activated protein kinase and the regulation of glucose transport. *Am J Physiol* 2006;**291**:E867-E877.

Table 1 Composition of control and hyperproteic diet fed to the experimental animals for the duration of the experiment

diet composition	units	control diet	HPD
protein	g/kg	143	286
	% of energy	20	41
plant-derived protein	% of protein	100 [1]	35
gelatin	% of protein	0	30
casein	% of protein	0	35
fibre	g/kg	180	125
carbohydrates	g/kg	480	334
	% of energy	67	47
lipid	g/kg	40	38
	% of energy	13	12
water	g/kg	95	123
mineral and vitamin mix	g/kg	47	33
total metabolizable energy (calculated)	kJ/g	11.9	11.7
total energy (bomb calorimeter)	kJ/g	16.5	17.6

[1] Essential amino acids (lysine and methionine) were added to supplement the this protein source (natural cereal protein content and gluten concentrate)(manufacturer's specifications).

Dietary protein was measured by its N content (Kjeldahl), and total energy was either calculated from the composition of metabolizable nutrients in the diet (Atwater factors), or measured as crude energy with a bomb calorimeter. Water was estimated by differential weighing after 24 h at 105ºC. Fibre was estimated with a neutral detergent method. Control diet was used without changes, and the data correspond to the manufacturer's specifications (except for total energy and water estimations).

Table 2 Primers used for the analysis of gene expression

enzyme	gene	5'➔3'	3'➔5'	bp
alanine transaminase 1	*Gpt*	GTATTCCACGCAGCAGGAG	CACATAGCCACCACGAAACC	85
alanine transaminase 2	*Gpt2*	CATTCCCTCGGATTCTCATC	GCCTTCTCGCTGTCCAAA	146
AMP deaminase	*Ampd2*	CGGCTTCTCTCACAAGGTG	CGGATGTCGTTACCCTCAG	78
carbamoyl-P synthetase 1	*Cps1*	ACCCATCATCCCCTCTGACT	ACACGCCACCTCTCCAGTAG	118
carbamoyl-P synthetase 2	*Cad*	AGTTGGAGGAGGAGGCTGAG	ATTGATGGACAGGTGCTGGT	90
ornithine transcarbamylase	*Otc*	CTTGGGCGTGAATGAAAGTC	ATTGGGATGGTTGCTTCCT	126
arginino-succinate synthetase	*Ass1*	CAAAGATGGCACTACCCACA	GTTCTCCACGATGTCAATGC	100
arginino-succinate lyase	*Asl*	CCGACCTTGCCTACTACCTG	GAGAGCCACCCCTTTCATCT	104
arginase 1	*Arg1*	GCAGAGACCCAGAAGAATGG	GTGAGCATCCACCCAAATG	126
arginase 2	*Arg2*	GCAGCCTCTTTCCTTTCTCA	CCACATCTCGTAAGCCAATG	122
glutamine synthetase	*Glul*	AACCCTCACGCCAGCATA	CTGCGATGTTTTCCTCTCG	148
glutaminase	*Gls*	CCGAAGGTTTGCTCTGTCA	AGGGCTGTTCTGGAGTCGTA	63
N-acetyl glutamate synthetase	*Nags*	GCAGCCCACCAAAATCAT	CAGGTTCACATTGCTCAGGA	82
endothelial nitric oxide synthetase	*Nos3*	CAAGTCCTCACCGCCTTTT	GACATCACCGCAGACAAACA	138
neural nitric oxide synthetase	*Nos1*	CCTGAGAACGGGGAGAAAT	GCTGTTGAATCGGACCTTGT	99
housekeeping gene	*Rplp0*	GAGCCAGCGAAGCCACACT	GATCAGCCCGAAGGAGAAGG	62

Table 3 Body weight, food intake and diet composition for male rats subjected to 30 days of a hyperproteic diet.

parameter	units	control diet	HPD	P
rat final weight	g	389±9	371±14	NS
intestine weight	g	6.24±0.07	5.24±0.23	0.0021
intestine protein	g/kg	17.4±0.45	17.8±0.68	NS
energy intake	kJ/day	272±19	263±23	NS
amino-N intake	mgN/day	514±18	1019±65	0.0008
	% of energy	20.0	40.6	-

The values are the mean ± sem of 6 rats for each group; food consumption was measured in 2 cages per group and are the composite mean of 15 different measurement days.

Legends to Figures

Figure 1 Amino acid metabolism enzyme gene expression in the small intestine of male rats fed control or hyperproteic diet for 30 days

The values correspond to the mean ± sem of 6 different animals per group. White = control diet; gray = HPD; I, II and III are the three intestine segments (I was closer to the stomach). Statistical comparison between groups (2-way ANOVA): the P values for diet and site (i.e. intestine fraction) are presented in each graph. All expressions are related to tissue protein (gP or g of protein).

Figure 2 Amino acid enzyme activities in the small intestine of male rats fed control or hyperproteic diet for 30 days

The values correspond to the mean ± sem of 6 different animals per group. dashed = control diet; black = HPD; I, II and III are the three intestine segments (I was closer to the stomach). Statistical comparison between groups (2-way ANOVA): the P values for diet and site (i.e. intestine fraction) are presented in each graph. All activities are related to tissue protein (gP or g of protein).

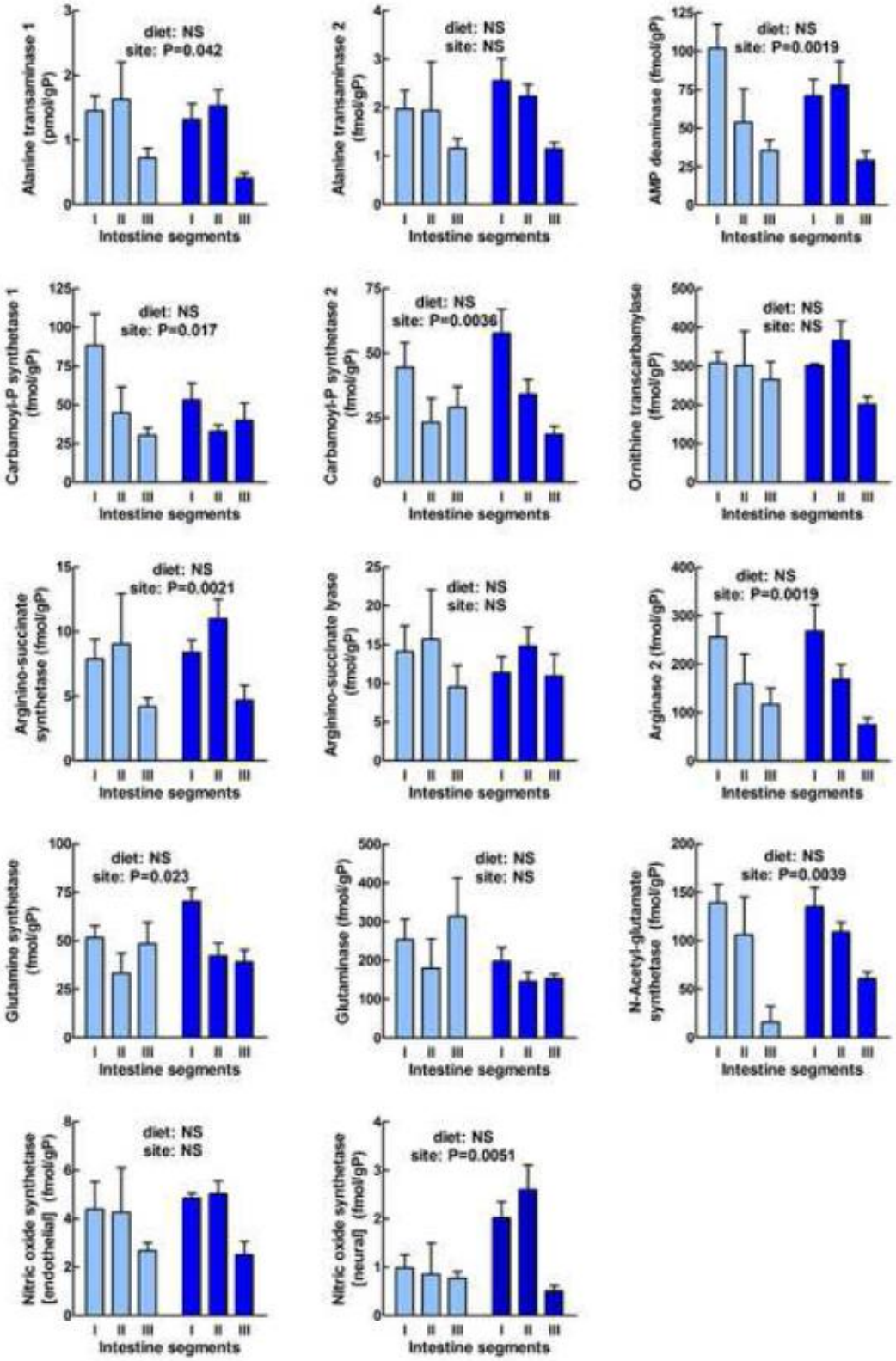
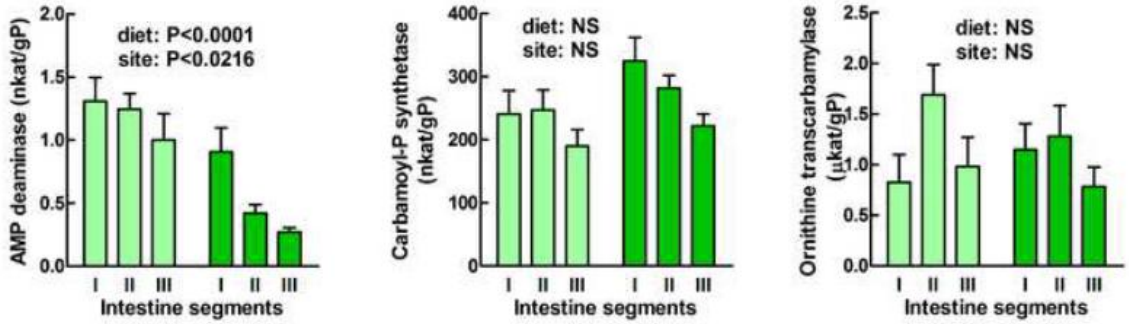

Alanine transaminase 1 (pmol/gP)
diet: NS
site: P=0.042
Alanine transaminase 2 (fmol/gP)
diet: NS
site: NS
AMP deaminase (fmol/gP)
diet: NS
site: P=0.0019
Carbamoyl-P synthetase 1 (fmol/gP)
diet: NS
site: P=0.017
Carbamoyl-P synthetase 2 (fmol/gP)
diet: NS
site: P=0.0036
Ornithine transcarbamylase (fmol/gP)
diet: NS
site: NS
Arginino-succinate synthetase (fmol/gP)
diet: NS
site: P=0.0021
Arginino-succinate lyase (fmol/gP)
diet: NS
site: NS
Arginase 2 (fmol/gP)
diet: NS
site: P=0.0019
Glutamine synthetase (fmol/gP)
diet: NS
site: P=0.023
Glutaminase (fmol/gP)
diet: NS
site: NS
N-Acetyl-glutamate synthetase (fmol/gP)
diet: NS
site: P=0.0039
Nitric oxide synthetase [endothelial] (fmol/gP)
diet: NS
site: NS
Nitric oxide synthetase [neural] (fmol/gP)
diet: NS
site: P=0.0051
I II III I II III
Intestine segments

FIGURE 1

FIGURE
2


AMP deaminase (nkat/gP)
diet: P<0.0001
site: P<0.0216
Intestine segments
Carbamoyl-P synthetase (nkat/gP)
diet: NS
site: NS
Intestine segments
Ornithine transcarbamylase (µkat/gP)
diet: NS
site: NS
Intestine segments

4.5 Agnelli S, Arriarán S, Fernández-López JA, et al. The effects of high-protein diet on amino acid metabolism of lean and obese Zucker rats. Under evaluation (Food&Function)

The effects of high-protein diet on amino acid metabolism of lean and obese Zucker rats.

Silvia Agnelli[1], Sofía Arriarán[1], José-Antonio Fernández-López[1,2,3], Marià Alemany[1,2,3], Xavier Remesar[1,2,3]

[1]Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, University of Barcelona, Barcelona, Spain

[2]Institute of Biomedicine, University of Barcelona, Barcelona, Spain

[3]CIBER Obesity and Nutrition, Spain

Author for correspondence:

Prof. Xavier Remesar. Department of Biochemistry and Molecular Biomedicine, Faculty of Biology, University of Barcelona. Av. Diagonal, 643; 08028 Barcelona, Spain

E-mail: xremesar@ub.edu TEL: +34 94 4021521; FAX: +34 93 4021559

Abstract

Objective. To determine whether doubling diet protein content induced significant changes in amino acid metabolism of lean and obese rats.

Design. Adult male rats (fourteen weeks old) of the Zucker strain (lean and obese) were fed either a standard (control diet), or a high-protein diet (HPD) for 30 days. Then they were killed and the liver and small intestine were used for the measurement of enzyme activities. The whole carcass was used to measure protein and lipid, and thus calculate their balances. Plasma was used for metabolite quantification, including individual amino acids.

Results.

Rats fed a high-protein diet ingested less energy than lean animals; both in those fed standard or high-protein diet. Obese rats showed a higher lipid, and unchanged protein balances that were not affected by diet. Obesity altered more markedly plasma amino acids than diet. Glucose and lipid-related metabolites were increased in obese animals. However, the increased levels of plasma urea showed different patterns for lean and obese animals, since the latter did not reflect higher levels with a high-protein diet. The lower ornithine transcarbamylase activity in high-protein diet-fed hints at this change being critical for the differences in urea production.

Conclusion.

The administration of a moderate high-protein diet alters amino acid metabolism, increasing, selectively, the levels of a number of them; however, the effects of obesity were more marked, also affecting lipid metabolism, and especially the lipid balance. Nevertheless, the most significant change induced by a high-protein diet on the obese was the decrease in urea synthesis, largely attributable to a decrease of ornithine transcarbamylase activity.

Key words:

High-protein diet, urea cycle enzymes; amino acid metabolism; balances

Introduction

There is a growing disenchantment with the effectiveness of most hypocaloric diets in the treatment of obesity,[1] This includes their usage alone or combined with drugs and exercise. Ketogenic, dissociate, and high-protein diets have been also widely used (not always tested, however) with limited success, if any.[2,3,4] High-protein diets, are currently used by sportsmen and body builders, in the assumption that excess dietary protein increases body muscle mass and helps eliminate fat tissue.[5] Most of the subjects consuming these diets also limit the intake of lipid and carbohydrate; and often combine this dietary manipulation with strenuous exercise training and the consumption of anabolic hormones and dietary supplements.[6] The problem is, however, that our knowledge of body amino acid metabolism, and the fate of dietary amino acids, and even which are their pathways, has been severely neglected in the last decades.[7] High-protein diets have been used for the treatment of obesity without actually knowing how the dietary modification may alter the body energy homeostasis, and, especially, ignoring the elaborated mechanisms that prevent the loss of "precious" 2-amino N, especially that of essential amino acids.[8] This well-known evolutionary trend helps maintain alive, the most disfavoured half of the World's population, but we do not know yet (in their full extent) the mechanisms and regulative paths that determine this fundamental biological trait.[9]

The results obtained so far using high-protein diets for obesity are a widely discordant mixed bag, since there is no uniform pattern of results obtained from different experimental designs, and more often than not the effects observed fall well within the wide range of variation of "normal" diets.[10] In many experiments done on rodents, the additional dietary N load often consists of purified, high quality protein. This fact makes even more complicated the evaluation of the results, since they combine the alteration of the proportion of N in the diet with the often higher (and unnatural) essential amino acid availability; in addition, this practice modifies the energy density and the proportions of other nutrients provoking havoc in the regulation of energy partition and disposal.[11,12] The case of cafeteria diets is paradigmatic: they are hyperlipidic,[13] but their protein content is usually in the higher range of normalcy.[14] Notwithstanding, urea production, i.e. amino acid oxidation, decreases, and the altered balance is not compensated by a parallel body protein accrual.[15]

Obesity affects the management of dietary nitrogen in different ways depending on the origin of the obesity. Hyperphagia-induced models, such as that induced by the palatability of the cafeteria diet components, often result in the development of amino nitrogen sparing mechanisms, such as decreased urea cycle enzyme activities,[16] that may conduce to higher carcass retention of N[17] and increased amino acid availability.[18,19] On the other hand, the genetically obese Zucker *fa/fa* rats do not show this altered urea production/nitrogen excretion despite being also obese.[20] This situation coexists with increased protein accrual, in part related to extreme fat storage.[21]The effects of cafeteria diet-induced hyperphagia on genetically obese rat result in additive effects with respect to N management, since the amino acid sparing effect of the excess energy available, counteracts, in some way, the

nitrogen-wasting effect associated to genetic obesity energy handling.[22] Most studies on nitrogen retention and efficiency of deposition have been focussed on the effects of diet protein on growth, energy balances or the fight against malnutrition.[23]

The relative scarcity of some individual (essential) amino acids in the diet is partly counteracted by an increase in their intestinal absorption [24]and tissue reutilization, with decreased oxidation and urea excretion rates. [25]

When protein constitutes a large proportion of the diet energy, especially under condition so limited energy availability, as in starvation or dieting, [26] much of this dietary proteins necessarily used for energy, which results in negative N balance,[27] lowered protein synthesis and very often net loss of body protein[28].The dietary excess of amino nitrogen when their energy substrate availability is limited requires the oxidation of part of the s e amino acids far energy**.** We assume that doubling the diet amino acid content will affect the amino acid metabolism in the whole animal, affecting especially the liver, but we also expect that these effects will induce different effects in genetically obese animals since their more efficient energy metabolism will be severely stretched.

Materials and Methods

Ethics statement

All animal handling procedures and the experimental setup were carried out in accordance with the animal handling guidelines established by the corresponding Catalan, Spanish and European, Authorities. The Committee on Animal Experimentation of the University of Barcelona authorized the specific procedures used in this study.

Diet

Common rat chow pellets (C) (Teklad global 14% protein rodent maintenance diet, Harlan Laboratories, Madison, WI USA) was used after weaning and for the duration of the experiment in control animals, and up to 14 weeks in animals later receiving a hyperproteic diet (HPD). The HPD was prepared by crushing to a coarse powder the common rat chow described above. To produce about 1 kg of HPD pellets, a mixture 87 g of powdered alimentation-grade pure gelatin, 100 g of purified powdered casein, 702 g of crushed standard chow, and 9.5 g of sunflower oil were suspended in 725 mL of water, and stirred, forming a paste which was thoroughly mixed and then extruded through cut-off syringes to form pellets; they were dried in an oven at 45ºC for 24 h.

The final composition of the pellets, compared with control chow is shown in Table 1. The consistency of the dried HPD pellets was comparable to that of the standard one. Their analysed composition was as expected with respect to N content (about twice that of control pellets). In addition, the calculated energy content estimated from its composition) and the measured crude energy (using and adiabatic bomb calorimeter, IKA C7000; IKA, Staufen, Germany) per g were the same (Table 1). The main difference between control and HPD were a lower carbohydrate versus protein ratio and a relative decrease in mineral and specific micronutrient content in the HPD.

Animals

Fourteen-week-old male Zucker lean and obese rats (Harlan Laboratories Models, Sant Feliu de Codines, Spain) were used. After 30 d of acclimation using the standard

diet, half of the rats (N=6 for each group, randomly selected) were shifted to the HPD for 30 days, while the controls continued under the standard chow. All animals were kept in 2-rat solid-bottom cages (with wood shards as bedding material, changed every 2 days) in a controlled environment: free access to diet and water, lights on from 08:00 to 20:00, humidity 60-70 %, temperature 20.5-21.5ºC.

Food intake (in fact consumption per cage) was measured daily, as well as the weights of the animals. On day 30, the rats were killed by exsanguination under isoflurane anaesthesia, using a dried-heparinized syringe. Blood was maintained in an ice bath until centrifugation to obtain plasma.

Additional groups of six lean and obese control diet-fed animals (30 days acclimation only), were used to obtain the basal values of protein and lipid content in order to calculate the nutrient balances.

Dissection and sampling

The small intestine of the rats was dissected immediately after death. The organs were blotted, cleaned of mesentery and fat cordons. The intestine contents were carefully extruded out with a wide glass rod, and then internally washed with a gentle stream of ice-cold KCl (9 g/L). The excess water was extruded and the intestine blotted and weighed. Samples of the tissues were frozen in liquid nitrogen, crushed to a fine powder, and distributed in several containers, before storage at -80ºC until processed.

Metabolite analysis

Urine and plasma parameters were measured using commercial kits (BioSystems, Barcelona, Spain): urea (#11537), glucose (#11504), total cholesterol (#11505) and triacylglycerols (#11528). Plasma samples were deproteinized with acetone [29], and the supernatants were used for amino acid analysis as previously described.[30]

Measurement of enzyme activities

Frozen tissue samples were homogenized using a tissue disruptor (IKA-T10 basic Ultra-Turrax, IKA, Stauffen Germany) in 10 volumes of chilled Krebs-Ringer bicarbonate solution, pH 7.8 containing 5 mM dithiothreitol, 5 g/L bovine serum albumin, 10 g/L dextran, º10g/L Triton X-100 , and 1 mM EDTA (all from Sigma-Aldrich, St Louis MO USA). Incubations were carried out at 37ºC using freshly homogenized homogenates.

Adenylate deaminase (AMD) (EC3.4.5.6) was estimated using a previously described method,[31] based essentially on incubation of the crude homogenate with AMP, stopping the reaction from 0 up to 16 minutes (3-4 time-points) with cold trichloroacetic acid, and then measuring the ammonia produced with the Berthelot reaction. Initial (V_0) activities were determined from the course of the reaction at different times. V_0 was assumed to correspond to V_{max} under the conditions of analysis. Activity was expressed as nkat/g of protein.

Ornithine carbamoyl-transferase (OTC) (EC 2.1.3.3) was measured using the incubation conditions described previously, [32] in a setting of multiple measurements along time as described above for other enzymes. The homogenization medium was a pH 7.4 HEPES75 mM buffer, containing 1 mM dithiothreitol, 50 mM KCl and 10 g/L defatted bovine serum albumin (Sigma-Aldrich). The method was based on the incorporation of carbamoyl-P to ^{14}C-ornithine to yield ^{14}C-citrulline. The amino acids were separated

through thin-layer chromatography (Silicagel sheets SIL G25 Macherey-Nagel, Düren, Germany), identified, cut and counted. After correction for blanks and specific activity of labelled amino acids, production of citrulline was estimated.

Arginase (ARG) (EC 3.5.3.1) activity was measured through the estimation of the urea produced by the activity of the enzyme on arginine in the presence of Mn^{2+} ions. Aliquots of 20 mL of homogenates were mixed with 5 mL of $MnCl_2$ in water; final concentration 10 mM. The tubes were heated for 5 min at 55ºC to activate arginase.[33] The reaction began with the addition of 75 mL of arginine (Sigma); final concentration 78 mM. Incubations were carried out for 0, 8 and 16 min at 37°C. The reaction was stopped by the addition of 35 mL 160 g/L perchloric acid. The tubes were centrifuged 15 min in the cold at 8000 x g. Urea was measured as described above.

Glutamine synthetase (GST) (EC 6.3.1.2)activity was estimated using a method we had used previously, [34] based on the reaction of glutamine and hydroxylamine in the presence of ADP, Mn^{2+} and arsenate to yield 4-glutamyl-hydroxamate. The addition of $Fe(NO_3)_3$ in trichloroacetic acid results in the development of colour, read at 500 nm using a plate reader.

Protein content in each of the homogenates used for enzyme analysis was estimated with a colorimetric method.[35]

Analytical procedures

Diet components and animal's total homogenates were used for nitrogen, lipid an energy analysis. Nitrogen content was measured with a semi-automatic Kjeldah lprocedure using a ProNitro S system (JP Selecta, Abrera, Spain), whereas lipid content was measured with a classical trichloromethane : methanol extraction method. [36]Total energy content was determined using a calorimetric pump as described above.

Statistical comparisons Groupswere compared with two-way ANOVA analyses and the Bonferroni post-hoc test using the Prism 5 (GraphPad Software, San Diego CA USA) graphics/ statistics package.

Results

Figure 1 shows the weights of the rats during the experiment. Obese rats weighed more than the other groups, but the increase was smaller in the rats fed the HPD compared with its controls. Energy and lipid content were higher in obese animals, but their protein content was lower than that of controls. No differences were observed between controls and HPD groups.

The animals fed with HPD ingested a significantly lower amount of energy than controls (Figure2), in spite that obese rats ingested more energy than the lean ones. There were no differences in protein balance. Since obese animals showed an increased lipid balance, their cost of accrual was significantly lower than that of controls.

Figure 3 shows that glucose, cholesterol and triacylglycerol values of obese animals were significantly higher than the corresponding values for lean rats. However, urea concentrations did not follow this pattern, although the levels of lean HPD group were markedly higher than those of controls.

Table 1 depicts the plasma individual amino acid levels. Obesity caused increases in Arg, Thr, Ser, Asn, Asp, Gly, Met and Lys, in contrast with decreased values for Ala, Val and Ile. The effect of HP diet resulted in

increased levels of Ser, Gly, Orn, Val, Leu, Ile, Cys and Lys with decreases in Thr, Tyr and His values.

Figure 4 shows the changes observed in liver enzyme activities with the diets tested. Obesity increased the activities of glutamine synthetase, ornithine transcarbamylase and arginase, with no significant changes in AMP-deaminase activity. Diet caused a decrease in GST activity. The activity of OTC was clearly decreased in obese HPD group.

Figure 5 shows the activities of enzymes in intestine that indicate points out a clear increase caused by obesity except in AMD. However, the activity of this enzyme, decreased under the effect of the HPD diet.

Discussion

As expected, obese rats showed a higher rate of increase in body weight than their lean counterparts, both when fed standard or high-protein diet, in agreement with previous results. [37] Consequently, the total energy (and lipid) content of the obese animals we also higher than in the lean; and, as a consequence, the accrual cost was lower in obese animals, independently of diet. The described decrease in energy intake caused by high-protein diets in rats[38]was also observed in the obese. These data suggest that the mechanisms inducing satiation are not limited by obesity. Obviously, the increased lipid balance is a direct consequence of the higher energy intake and lower accretion rate of the obese. The maintained protein balance in adult obese animals is in agreement with the data obtained in young Zucker rats. [39]

The fact that HPD did not induce patent changes in essential metabolic parameters such as glucose, cholesterol and triacylglycerols, in contrast with the marked increases caused by obesity, coincides with the pattern observed in younger animals. [37] However, in this case, the absence of an increase in urea levels did not match with the usual pattern. .The maintenance of normal urea levels in the obese fed HPD hints to a differential regulation of amino acid metabolism in the liver, resulting in changes in the regulation of the urea cycle activity.

The effects on liver amino acid metabolism depend on the maintenance of equilibrium in the amino acid flux from intestine and the systemic circulation. From the plasma amino acid levels, we can assume that obesity affects more deeply the amino acid profile than HPD did. The ornithine levels were higher in HPD-fed animals, whereas arginine and citrulline maintained levels similar to those of controls. Thus, the arterial supply to the liver did not limit the supply of precursors to sustain the urea cycle. Other potential ureogenic amino acids, such as glycine, were increased levels in HPD-treated animals. Furthermore, increased activity of the amino acid transport systems has been described in the liver of obese animals, [37]that bring us to conclude that liver, under a HPD maintains an unaltered substrate amino acid supply.

Since HPD necessarily induce a higher portal vein flow of absorbed 2-amino nitrogen from the diet towards liver, [40] and considering that in obese rats there is a lower fractional extraction of ammonia by the liver, [41] we can assume that the ability of liver to dispose of N for urea synthesis should be affected, because the portal flow represents almost 2/3rds of the blood supply to the liver.[42]

On the other hand, the maintenance of ornithine transcarbamilase and arginase activities in small intestine may help guarantee

a sustained supply to the liver, via portal vein, of ornithine, citrulline and arginine. This is in agreement with the well-known fact that liver overall metabolic status plays a critical role in the control of ureagenesis.

Obese rats fed HPD showed a marked decrease in the ornithine transcarbamilase activity, a fact which may affect the normal function of the urea cycle, despite transcarbamylase being considered to be the main point of control of the urea cycle .[43]

Since the protein balance in HPD-fed obese rats remained unaltered in comparison with controls, the animals can maintain their lean mass, assumedly maintaining the same protein synthesis rates. The maintenance, in the obese, of activities as in HPD fed rat liver should be interpreted as a proof that amino acid catabolism was not increased in spite of the higher supply of dietary amino acids. The logical consequence to this apparent conundrum lies on the fate of the excess amino-N that simply disappears. As of today, this question remains unanswered although the possible alternative pathways for amino N to form nitrogen gas should be taken in consideration.[44]

Acknowledgements

This study was done with the partial support of grants of the Plan Nacional de Investigación en Biomedicina (SAF2012-34895) and the Plan Nacional de Ciencia y Tecnología de los Alimentos (AGL-2011-23635) of the Government of Spain, as well as of CIBER-OBN. S.Agnelli was the recipient of a Leonardo da Vinci fellowship, and S. Arriarán a predoctoral fellowship of the Catalan Government, in both cases covering part of the time invested in this study.

References

1 R.L. Williams, L.G. Wood, C.E. Collins and R. Callister, *Obesity Rev*., 2015, **16**, 171–186.

2 A.M. Johnstone, *Proc. Nutr. Soc.,* 2012, **71**, 339–349.

3 A. Paoli, A. Rubini, J.S. Volek and K.A. Grimaldi,*Eur. J. Clin. Nutr.,* 2013, **67**, 789–796.

4 K.J. Petzke, A. Freudenberg and S. Klaus, *Int. J. Mol. Sci.,* 2014, **15**, 1374-1391.

5 D.H. Pesta and V.T. Samuel, *Nutr. Metabol.,* 2014, **11**, 53.

6 C.P. Lambert and L.L. Frank, Sports.Med. 2004,**34**, 317-327.

7 M. Alemany M, *Nutr. Res. Rev.*, 2012, 25, 18-28.

8 G. Wu, Amino Acids, 2009, **37**, 1-17.

9 C.D. Morrison and T. Laeger, *Trends Endocrinol. Metab.*, 2015, 26, 256-262.

10 F.M. Sacks, G.A. Bray, V.J. Carey *N. Engl. J. Med.*, 2009, 360, 859–873.

11 J.A.Gilbert, A.Bendsen, A.Tremblay, and A.Astrup, *Nutr. Metabol. Cardiovasc. Dis.*, 2011, **21**, suppl. 2, B16-B31.

12 M. Cuenca-Sánchez, D. Navas-Carrillo and E. Orenes-Piñero, *Adv. Nutr.*, 2015, **15**, 260-266.

13 N.J. Rothwell and M.J. Stock, *J. Nutr.,* 1988, **118**, 925-928.

14 I. Rafecas, M. Esteve, J.A. Fernández-López, X. Remesar and M. Alemany, *Mol. Cell. Biochem.,* 1993, **121**, 45-58.

15 M. Esteve, I. Rafecas, X. Remesar and M. Alemany, *Int. J. Obesity,* 1992, **16**, 237-244.

16 T.Barber, J.R.Viña,J.Viña and J.Cabo, *Biochem.J., 1985,***230**:675--681.

17 H.G.Barr and K.J.McCracken ,*Br.J.Nutr.*, 1984,**51**: 379-387.

18 J. Calles-Escandon, J.Cunningham and P.Felig, *Metabolism*, 1984, **33**:364-368.

19 I.Rafecas, M.Esteve, X.Remesar and M.Alemany, *Biochem. Internat*., 1991, **25**: 797-806.

20 M.Esteve, I.Rafecas, X.Remesar and M.Alemany, *Int. J,Obesity*, 1992, **160**: 237-244.

21 G.A.Bray and D.A.York, *Physiol. Rev*., 1979, **590** : 719-809.

22 J.Kielanowski, in: K.L. Blaxter (ed.) Energy Metabolism. Academic Press, London, 1985. pp 13-19

23 R.A. Wapnir and F.Lifshitz, *J. Nutr*., 1974, **104**: 843-849.

24 I.M.Brookes, F.N.Owens and U.S.Garrigus, *J. Nutr.*, 1972, **102**: 27-36.

25 D.O.Sapir and M.Walser,*Metabolism*, 1977, **26**: 301-308.

26 L.Scalfi, F.Conlaldo, R.Borrelli, M.De Caterina, G.Spagnuolo, R.Alficri and M.Mancini, *Ann. Nutr. Metab.*, 1987, **31**: 154-159.

27 J.S.Fisler, E.J.Drenick, D.E.Blumficld and M.E.Swendseid, *Am. J. Clin.Nutr*.,1982, **35**: 471-486.

28 S.Tsukahara, M.Ohno and Y.lkeda, in; G.A.Bray. J.LeBlanc, S.lnoue and M.Suzuki (eds.) Diet and Obesity. Japan Scientific Societies Press/Karger. Tokyo/Basel. 1988. pp 205-217

29 L. Arola, E. Herrera and M. Alemany, *Anal. Biochem.,* 1977, **82**, 236-239.

30 I. Rafecas, M. Esteve, J.A. Fernández-López, X. Remesar and M. Alemany, *Mol. Cell. Biochem.,* 1993, **121**, 45-58

31 L.Arola, X.Remesar, A.Palou and M.Alemany, *Horm.Metabol.Res.,* 1981;**13**:264-266.

32 J.McLaren and W.G.Ng, *Clin.Chim.Acta,* 1977;**81**:193-201.

33 D. M. Greenberg, *Enzymes*, 1960, **4**: 257–267.

34 L. Arola, A. Palou, X. Remesar and M. Alemany, *Horm.Metab. Res.*, 1981, **13**: 199–202.

35 O.H.Lowry, R.W.Rosebrough, A.L.Farr and J.P.Randall, *J. Biol. Chem.,* 1951;**193**:265-75.

36 J.Folch, M.Lees and G.H.Sloane-Stanley, *J. Biol.Chem.,* 1957, **226**:497-509.

37 R.M.Masanés, I.Rafecas and X.Remesar *J. Nutr. Biochem*., 1999, **10**:716 –722.

38 F.Rotondo, T.Sanz, J.A.Fernández-López, M.Alemany and X.Remesar, *RSC Advances*, 2016, **6**: 69177-69184

39 I.Rafecas, M.Esteve, J.A.Fernández-López, X.Remesar and M.Alemany, Mol. Cell. Biochem, 1993, **121**: 45-58.

40 R.M.Masanés, I.Rafecas and X.Remesar, *Int. J. Food Sci. Nutr.*, 2001, **52**:183-192.

41 M.C.Herrero, N.Anglès, X.Remesar, L.Arola, C.Bladé, 1994, *Int. J.Biochem*, **18**:255-261.

42 D.Closa, J.M.Gómez-Sierra, E.Latres, M.Alemany, X.Remesar, *Exp. Physiol.*, **78**: 243-253.

43 S.Agnelli, S.Arriarán, L.Oliva, X.Remesar, J.A.Fernández-López and M.Alemany, *RSC Advances,*2016, **6**: 11278-11288.

44 G.Costa, L.Ullrich, F.Kantor and
J.F.Holland, *Nature*, **218**: 546-551.

Table 1 Composition of control and hyperproteic diet fed to the experimental animals for the duration of the experiment

diet composition	units	control diet	HPD
protein	g/kg	143	286
	% of energy	20	41
plant-derived protein	% of protein	100 [1]	35
gelatine	% of protein	0	30
casein	% of protein	0	35
fibre	g/kg	180	125
metabolizable carbohydrates	g/kg	480	334
	% of energy	67	47
lipid	g/kg	40	38
	% of energy	13	12
water	g/kg	95	123
mineral and vitamin mix	g/kg	47	33
total metabolizable energy (calculated)	kJ/g	11.9	11.7
total energy (bomb calorimeter)	kJ/g	16.5	17.6

[1] Essential amino acids (Lys and Met) were added to supplement the protein source (natural cereal protein content and gluten concentrate according to the manufacturer's specifications).

Dietary protein was measured by its N content (Kjeldahl), and total energy was either calculated from the composition of metabolizable nutrients in the diet (Atwater factors), or measured as crude energy with a bomb calorimeter. Water was estimated by differential weighing after 24 h at 105ºC. Fibre was estimated with a neutral detergent method. Control diet was used without changes, and the data correspond to the manufacturer's specifications (except for totalenergy and water estimations).

LEGENDS TO FIGURES 1

Figure 1. Weight, weight increase and protein, lipid and energy content of animals fed standard or high-protein diet. White columns represents animals fed with standard diet, and brown columns represent animals fed high-protein diet. Statistical comparisons: Two-way ANOVA: O (Obesity), D(Diet). O and D = $P<0.05$. Post-hoc Bonferroni test: * = $P<0.05$.

Figure 2. Energy intake, accrual cost and lipid and protein balances of animals fed standard or high-protein diet. White columns represents animals fed with standard diet, and brown columns represent animals fed high-protein diet. Statistical comparisons: Two-way ANOVA: O (Obesity), D(Diet). O and D = $P<0.05$. Post-hoc Bonferroni test: * = $P<0.05$.

Figure 3. Plasma glucose, cholesterol, triacylglycerols and urea of animals fed standard or high-protein diet. White columns represents animals fed with standard diet, and brown columns represent animals fed high-protein diet. Statistical comparisons: Two-way ANOVA: O (Obesity), D(Diet). O and D = $P<0.05$. Post-hoc Bonferroni test: * = $P<0.05$.

Figure 4. Activities of AMD, OTC, ARG and GST in liver of of animals fed standard or high-protein diet. White columns represents animals fed with standard diet, and brown columns represent animals fed high-protein diet. Statistical comparisons: Two-way ANOVA: O (Obesity), D(Diet). O and D = $P<0.05$. Post-hoc Bonferroni test: * = $P<0.05$.

Figure 5. Activities of AMD, OTC, ARG and GST in small intestine of animals fed standard or high-protein diet. White columns represents animals fed with standard diet, and brown columns represent animals fed high-protein diet. Statistical comparisons: Two-way ANOVA: O (Obesity), D(Diet). O and D = $P<0.05$. Post-hoc Bonferroni test: * = $P<0.05$.

Table 2. Plasma amino acid levels of animals fed standard or high-protein diet.

Statistical differences: two-way ANOVA: O (obesity) and D (diet). Bonferroni post-hoc test: * P<0.05 vs. Control. NS: not significant

Amino acid	**Control Lean µM**	**HPD Lean µM**	**Control ObeseµM**	**HPD Obese µM**	**ANOVA**
Arginine	101 ± 13	137 ± 19	62.0 ± 9.25	12.3 ± 5.85 *	Obesity P<0.0001
Taurine	177 ± 2.1	121 ± 5.50	152 ± 13.0	193 ± 17.0	NS
Threonine	247 ± 7.84	235 ± 9.28	237 ± 17.0	194 ± 12.1 *	Diet p=0.030 Obesity p=0.042
Serine	231 ± 6.54	285 ± 12.1 *	179 ± 14.1	198 ± 18.3	Diet p= 0.0171 Obesity P<0.0001
Aspartate	26.2 ± 4.87	19.0 ± 1.47	34.4 ± 3.71	31.2 ± 3.77	Obesity P=0.0079
Asparagine	29.0 ± 1.53	28.2 ± 1.22	20.0 ± 1.44	20.9 ± 1.88	Obesity P<0.0001
Glu+Gln	282 ± 28.0	285 ± 32.9	369 ± 22.8	275 ± 21.7	NS
Proline	66.1 ± 4.80	92.1 ± 7.11	77.6 ± 6.44	62.4 ± 10.2	NS
Glycine	210 ± 16.8	415 ± 16.1 *	84.2 ± 6.85	176 ± 20.7 *	Obesity and Diet p>0.0001
Alanine	335 ± 9.95	339 ± 10.8	492 ± 31.0	487 ± 47.3	Obesity p>0.0001
Citrulline	37.1 ± 4.19	37.2 ± 1.93	48.2 ± 4.08	37.1 ± 3.61	NS
Ornithine	231 ± 17.8	354 ± 37.9 *	183 ± 34.0	354 ± 37.5 *	Diet p=0.0004
Arginine	105 ± 13.7	152 ± 14.1 *	61.1 ± 9.26	42.6 ± 18.4	Obesity p>0.0001
Valine	133 ± 5.43	179 ± 9.90	167 ± 15.2	266 ±19.9 *	Diet p>0.0001 Obesity p=0.0004
Isoleucine	58.0 ± 4.44	69.2 ± 3.72	78.4 ± 6.86	105 ± 5.27 *	Diet P=0.0011 Obesity P>0.0001
Leucine	160 ± 5.42	169 ± 8.79	157 ± 11.8	217 ± 14.0 *	Diet p=0.0049
Cysteine	30.4 ± 3.41	30.9 ± 4.60	4.69 ± 0.50	47.8 ± 6.62 *	Diet p=0.0002
Methionine	75.1 ± 2.27	64.0 ± 3.45	55.5 ± 5.60	47.6 ± 6.62	Obesity p=0.0018
Tyrosine	166 ± 5.81	136 ± 9.50	181 ± 22.1	144 ± 15.7	Diet p=0.0293
Phenylalanine	69.1 ± 0.92	71.1 ± 3.46	82.4 ± 5.28	73.2 ± 2.54	Obesity 0.0380
Lysine	583 ± 22.9	656 ± 35.5	472 ± 33.9	547 ± 27.7	Diet P=0.0277 Obesity p=0.0019
Histidine	84.1 ± 2.95	75.1 ± 3.22	98.5 ± 6.90	79.2 ± 7.05 *	Diet p=0.0154
Tryptophan	22.4 ± 1.17	52.6 ± 8.03 *	37.5 ± 7.03	34.2 ± 8.51	NS
Total AA	3358 ± 80.3	3744 ± 126	3280 ± 212	3615 ± 225	Diet 0.0049

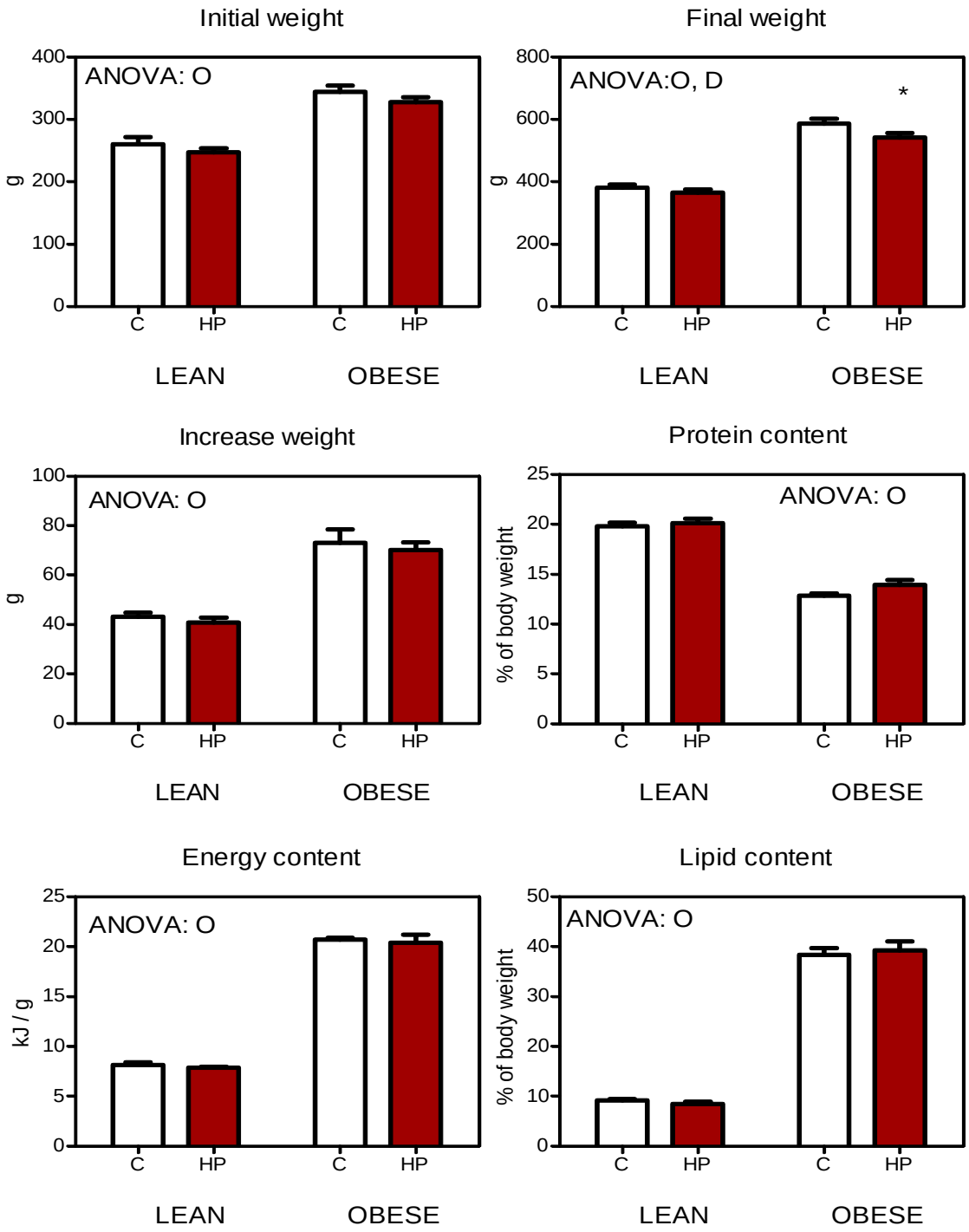

Initial weight
ANOVA: O
g
400
300
200
100
0
C
HP
C
HP
LEAN
OBESE
Final weight
ANOVA:O, D
*
g
800
600
400
200
0
C
HP
C
HP
LEAN
OBESE
Increase weight
ANOVA: O
g
100
80
60
40
20
0
C
HP
C
HP
LEAN
OBESE
Protein content
ANOVA: O
% of body weight
25
20
15
10
5
0
C
HP
C
HP
LEAN
OBESE
Energy content
ANOVA: O
kJ / g
25
20
15
10
5
0
C
HP
C
HP
LEAN
OBESE
Lipid content
ANOVA: O
% of body weight
50
40
30
20
10
0
C
HP
C
HP
LEAN
OBESE

FIGURE1

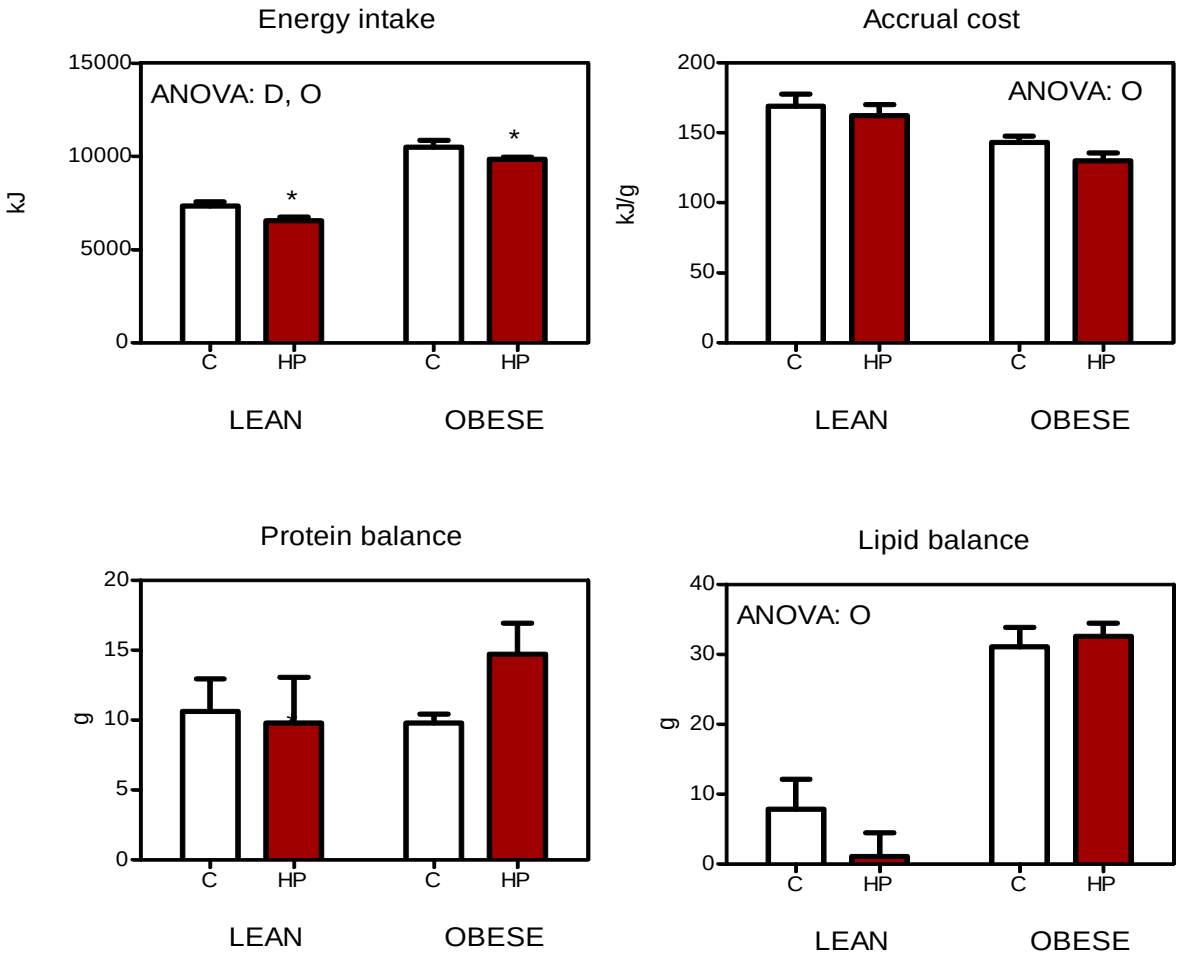

Energy intake
ANOVA: D, O
kJ
15000
10000
5000
0
*
*
C
HP
C
HP
LEAN
OBESE
Accrual cost
ANOVA: O
kJ/g
200
150
100
50
0
C
HP
C
HP
LEAN
OBESE
Protein balance
g
20
15
10
5
0
C
HP
C
HP
LEAN
OBESE
Lipid balance
ANOVA: O
g
40
30
20
10
0
C
HP
C
HP
LEAN
OBESE

FIGURE 2

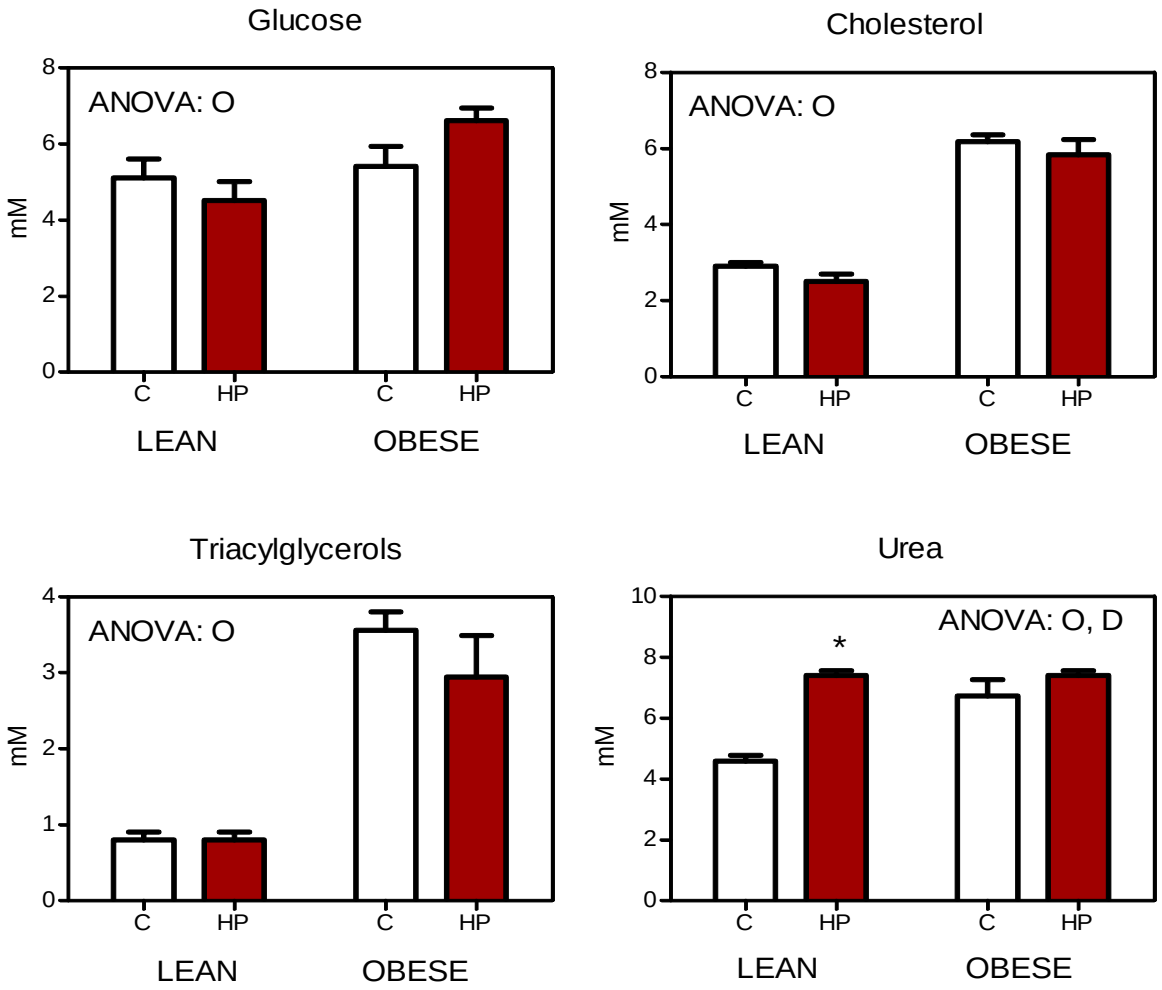

Glucose
ANOVA: O
mM
8
6
4
2
0
C
HP
C
HP
LEAN
OBESE
Cholesterol
ANOVA: O
mM
8
6
4
2
0
C
HP
C
HP
LEAN
OBESE
Triacylglycerols
ANOVA: O
mM
4
3
2
1
0
C
HP
C
HP
LEAN
OBESE
Urea
ANOVA: O, D
*
mM
10
8
6
4
2
0
C
HP
C
HP
LEAN
OBESE

FIGURE 3

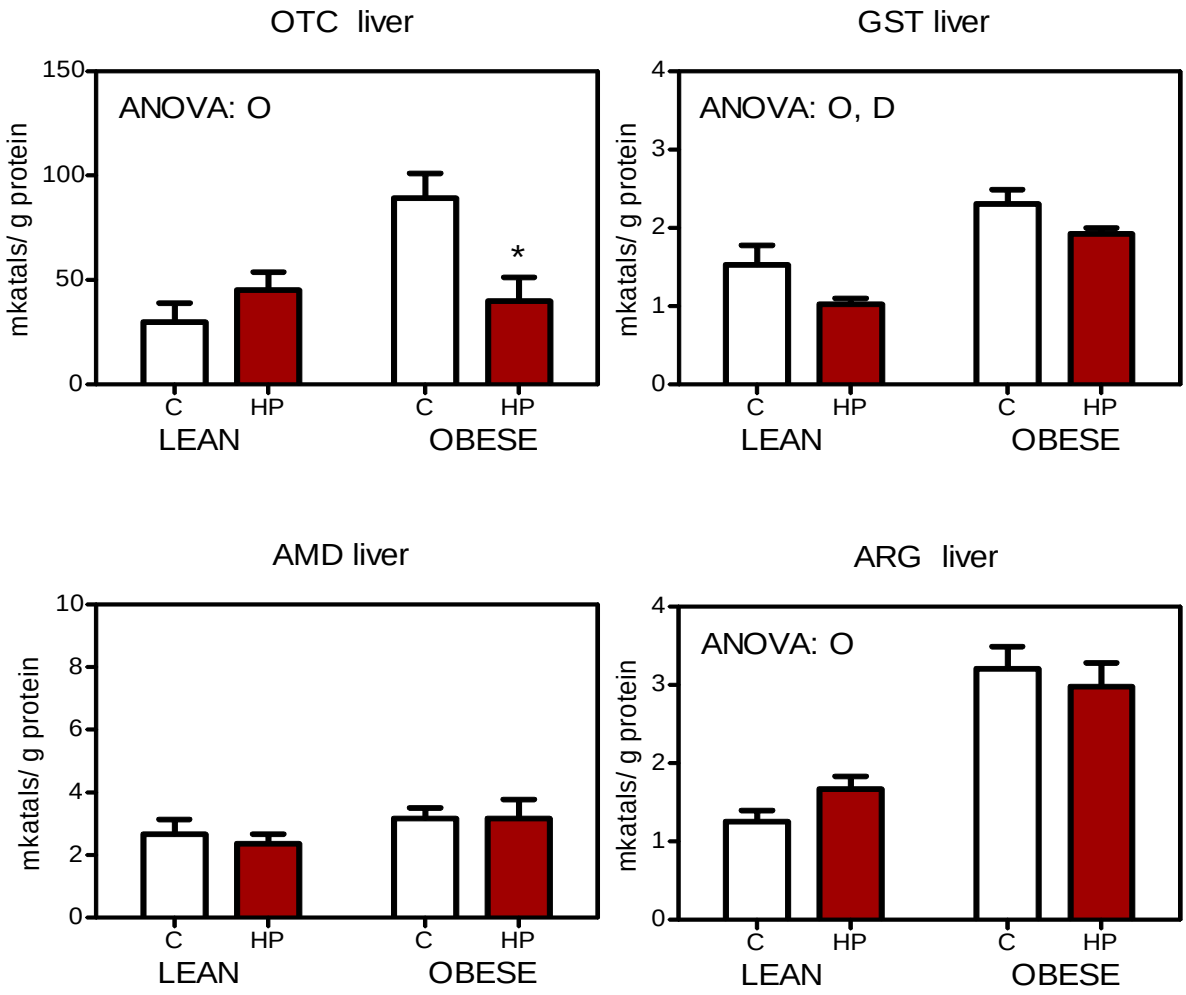

OTC liver
ANOVA: O
mkatals/ g protein
150
100
50
0
*
C
HP
C
HP
LEAN
OBESE
GST liver
ANOVA: O, D
mkatals/ g protein
4
3
2
1
0
C
HP
C
HP
LEAN
OBESE
AMD liver
mkatals/ g protein
10
8
6
4
2
0
C
HP
C
HP
LEAN
OBESE
ARG liver
ANOVA: O
mkatals/ g protein
4
3
2
1
0
C
HP
C
HP
LEAN
OBESE

FIGURE 4

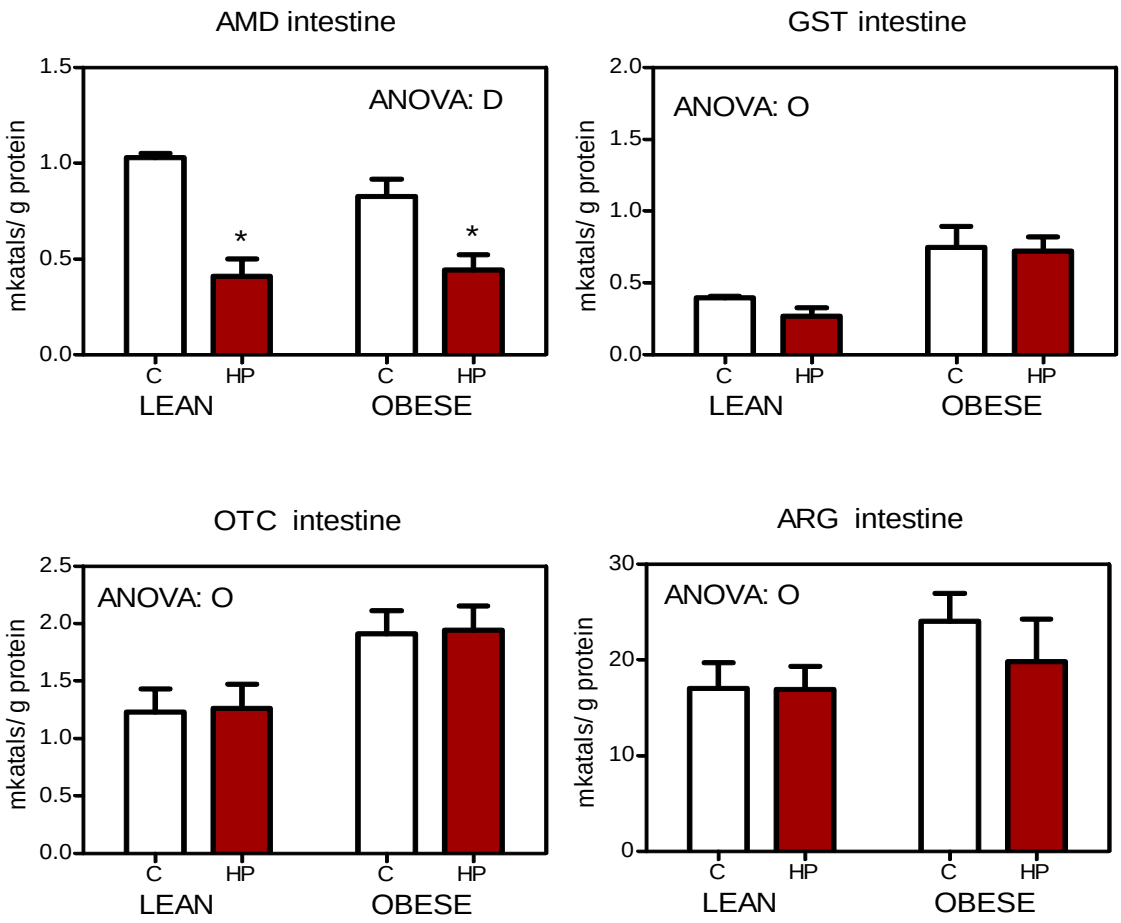

AMD intestine
ANOVA: D
mkatals/ g protein
1.5
1.0
0.5
0.0
*
*
C
HP
C
HP
LEAN
OBESE
GST intestine
ANOVA: O
mkatals/ g protein
2.0
1.5
1.0
0.5
0.0
C
HP
C
HP
LEAN
OBESE
OTC intestine
ANOVA: O
mkatals/ g protein
2.5
2.0
1.5
1.0
0.5
0.0
C
HP
C
HP
LEAN
OBESE
ARG intestine
ANOVA: O
mkatals/ g protein
30
20
10
0
C
HP
C
HP
LEAN
OBESE

FIGURE 5

5. GENERAL DISCUSSION

Effect of an obesogenic diet on N metabolism in liver and blood flow in Wistar rats

As expected, one month of CAFD caused overfeeding of adult rats resulted in higher body weight, increasing visceral adipose tissue, muscle and liver weight in when compared to controls. A lower urea excretion, largely due to lower enzyme activities in liver, was observed besides the identical (or higher protein) intake of CAFD-fed rats and their controls. These results are in agreement with previous studies that showed not only a higher body weight but also increased energy expenditure[241] and protein accrual[242]. In fact, it was assumed that high-energy diets, coupled with normal or high protein intake, interferes with 2-amino N elimination in mammals, and are related to an imbalance in N excretion, through pathways are which are still unknown.

Amino-N retention is a primeval drive that prevents or limits its wasting even under condition of dietary excess of protein. Thus, in presence of high load of nutrients, lipids are preferred over glucose by muscle and peripheral tissues because of insulin resistance; furthermore the high presence of glucose too hinders amino acid oxidation. As a consequence, the production of ammonia from 2-amino-N decreased. This is confirmed by the high levels of glutamine in plasma of CAFD-fed rats suggesting its decreased splanchnic utilisation to provide ammonium for the synthesis of carbamoyl-P, as well as the high levels of ornithine in CAFD-fed rats confirms a diminished production of carbamoyl-P. Instead, the unchanged levels of citrulline and aspartate in CAFD versus control rats seem to indicate that there is a key regulatory point either at synthesis or breakup of argininosuccinate for the overall nitrogen disposal that justifies the lower urea production. The control of urea cycle may be recognized in ASS, as the rate limiting step but also in ARG as final factor in the release of urea and as main site for arginine break up.

The lower production of urea in CAFD occurred in spite of high circulating levels of ammoniagenic amino acids (threonine, serine, glycine, and glutamine) which should trigger the production of ammonium, its transport as glutamine, its release again, and the formation of carbamoyl-P, powering the urea cycle. Thus, high levels of the most common ammonia-generating amino acids can be explained because the urea cycle is blocked and unable to absorb the N load, not responding to the stimulus of excess substrate. This is in agreement with a possible blockage in the control of the synthesis of arginine which is also essential for the operation of the NO· shunt. Since both plasma and urinary NOx levels did not change significantly, a possible explanation to this

situation may lie on the lower activity of arginase to prevent an excessive production of NO· during CAFD.

The effects of CAFD as well as the amino acid metabolism in liver were strongly influenced by sex. In general, females were more resistant to the development of MS[131,132] mainly because of the protective effects of estrogen[243] which prevents the obesogenic effects of inflammation and insulin resistance, limiting the full development of obesity. In fact, estrogens tend to counteract the increase in fat stores elicited by glucorticoids[244]. In males, the progressive decrease of androgens with advancing age is blended with an increase of glucocorticoids that run parallel to the development of MS. Furthermore, N sparing is managed in a different way according to sex. In males, the main trend is the accumulation of body protein, an effect facilitated by insulin[245], GH and androgens, and hampered by glucocorticoids[85]. In females, the N-sparing seems to be more related to save energy (and N) in preparation for pregnancy. Both in males and in females the urea cycle was down-regulated, but, apparently, the control points were not the same: ASS, ASL and ARG were halved in CAFD-fed male rats while in females only ASL follows this tendency. The low and unchanged expression of eNOS in both sexes suggests a relatively low activity, compared with arginase in their competence for arginine. The lack of changes in the expression of eNOS seems to disconnect this enzyme from the main degradative pathway represented by the urea cycle.

The liver is a formidable barrier that prevents ammonium from entering the systemic circulation and thus damaging the nervous system[189], and is also the main site for amino acids partition and N disposal. The liver counts not only with the urea cycle to incorporate ammonium into urea but also with other powerful ammonium-handling systems influenced by sex and diet as well. Glutamine synthetase represents the last line of defence to avoid the release of ammonium into systemic circulation; this enzyme did not change with diet but showed a marked sex-related difference; furthermore, $NADP^+$-glutamate dehydrogenase (cytoplasmic) was affected by both sex and diet; in particular it is down regulated by CAFD and shows increased values in females backing up the hypothesis of their focus on amino-N sparing.

The main liver ammonium producing mechanisms are purine nucleotide cycle, i.e. AMP deaminase[139], glutaminase[191–193], serine/threonine dehydratase and the glycine-cleavage system. Other sources, such as amine-oxidases and amino acid oxidases, may contribute in a limited manner to the liver ammonium pool. AMP deaminase participates in the purine nucleotide cycle which is also part of purine catabolism and the purine salvage pathway. Furthermore, AMPD is responsible

to breakup of the AMP generated by adenylate kinase under conditions of scarcity of ATP or nutients[202] as a way to control glycolysis, often together with ammonium production[246]. The breakup of AMP to IMP obviously modifies the substrate for AMP-kinase, consequently modulating the glycolytic pathway and overall energy partition[247]. The several functions of AMP-deaminase in liver, do not seem to include a significant role in the *in situ* production of ammonium[205], contrary to what happens in muscle where the purine nucleotide cycle is a main mechanism for mineralization of amino-N.

Serine dehydratase was also affected by sex and diet; favouring, in both cases the preservation of N. The expression/activity of this enzyme is substrate-controlled; we observed these correlations, as expected[200]. The protein H of glycine-cleavage system-expression was, again, influenced by sex: females showed higher expression values, suggesting a higher activity of the complex. However that might not represent only an increased amino-N disposal but, more probably, a key source of 1C fragments for synthesis[248], of purines and other critical building blocks.

CAFD also affects the blood flow distribution. In particular in CAFD-fed male rats, BAT shows a four-fold higher absolute blood flow than that of the control group. BAT is involved in thermogenesis and this higher blood flow towards BAT agrees with an increased heat dissipation of excess energy as heat.

In CAFD-fed rats, the heart showed a higher absolute blood flow than controls, despite heart output was not influenced by diet. This suggests that sufficient oxygen was available to maintain the activity of the heart of CAFD-fed rats despite showing similar pumping capacity than that of control rats.

Skin is the only organ that shows a decreased absolute blood flow in CAFD rats. It is known that in rats most of the excess heat is eliminated by evaporation throughout the respiratory tract, rather than from the skin surface, and that CAFD increase the body energy expenditure and heat output[241]. The lower absolute blood flow to the skin and the higher blood flow to the lungs suggest that lung evaporation was enhanced with this diet, becoming the main heat-loss mechanism (at the expense of increased water loss).

The lack of differences elicited by diet on liver blood flow suggests an already well-kept and adjusted balance of portal and arterial blood flows that cover sufficiently the different load of substrates and intestinal products to the liver, in a way that no changes are needed to adapt the blood supply to the widely different metabolic situation.

The trend to increase the blood flow in parallel in all sub-location of WAT of CAFD-fed rats compared to controls suggest their belonging to a single dispense organ[50] and support their uniform metabolic response to excess energy. The higher blood flow to striated muscle in CAFD-fed rats suggests a steadier or increased supply of substrates to sustain muscle growth and maintenance.

Effect of high protein ingestion on N metabolism in obese and lean male Zucker rats

At the end of the dietary treatment, the HPD groups increased their weight less than their controls. As expected, obese rats ingested more energy than the lean ones, showed a higher rate of increase in body weight than their lean counterparts and consequently their total energy and lipid content was higher than in the lean. Obviously, the obese showed a marked increase in metabolic parameters such as glucose, cholesterol and triacylglycerols in contrast with the lack of changes induced by HPD. Urea increased significantly in lean HPD-fed rats while in the obese urea levels were maintained, suggesting a differential regulation of amino acid metabolism in liver, resulting in changes in the regulation of the urea cycle activity. The effects on liver amino acid metabolism depend on the maintenance of equilibrium in the amino acid flux from intestine and the systemic circulation. Observing plasma amino acid concentrations we can postulate that obesity affected amino acid concentrations more intensely than exposure to a HPD did. In short: circulating glycine and ornithine increased in HPD-fed rats whereas arginine and citrulline maintained similar levels; and there was a higher amino acid transport systems activity in the liver of obese animals[174]; we can assume that, during HPD, liver maintained an unaltered substrate amino acid supply.

Since liver overall metabolic status plays a critical role in the control of ureagenesis, the unaltered activity of ornithine transcarbamylase and arginase activities in small intestine may help to guarantee a sustained supply to the liver, via portal vein, of ornithine, citrulline and arginine. The significant decrease in OTC activity observed in liver of HPD-fed obese rats may affect the normal function of urea cycle; the lack of changes in the liver enzyme activity of HPD-fed rats suggests that amino acids catabolism was not increased in spite of the higher supply of dietary amino acids. Furthermore, it has been recently found that amino-N disposal depends essentially on amino acids availability[249], in fact HPD rats excreted higher amounts of amino-N in the urine than controls, but the quantitative importance of the alternative (unknown) pathway of N disposal has not yet evaluated in this model.

One month of HPD was used as well to study the effects of a dietary protein load on the activity of amino acid metabolism enzymes along the small intestine of male lean Zucker rats. The treatment did not result in significant changes of activity and expression of the enzymes involved in N metabolism in the small intestine. This was an unexpected result, since the intestine is known to adapt its metabolism to the composition of the diet, especially when there are amino acids in excess for disposal[250]. An excess of protein intake favours the utilization of intestinal amino acids for the synthesis of urea cycle intermediates[251], carried to the liver by the portal vein, where the urea cycle is completed[252].

Another unexpected finding was the limited changes observed in glutamine metabolism enzymes, although glutamine is a pivotal amino acid for nitrogen homeostasis and intestinal energy supply[252]. In addition, we found a clear polarization of the activities of the enzymes analysed, with high activity in the proximal intestine (duodenum and jejunum segments) than in the distal, ileum, part. In fact, protein digestion is a slow process that foresees sequential actions of different proteases and peptidases that seem to logically point to the distal part of the small intestine as the place where more free amino acids could be found, absorbed and eventually metabolised. Higher presence of amino acid metabolism enzymes in the proximal segment can be tentatively explained as a way to prevent the massive oxidation of dietary amino acids in the distal part of intestine. The paradoxical longitudinal distribution of amino acid catabolism enzymes along the small intestine perhaps, in addition to be a mechanism to limit amino acids oxidation, leaves most of the control of digestion to the automatic release of peptidases and amino acid or peptide transport[253].

Curiously OTC did not follow this pattern suggesting that this enzyme works along the whole length of small intestine with a peak of activity in the central segment following a need for its activity unrelated to the direct events of digestion occurring along the gut.

Another finding of this study is the marked differences in activity and expression of AMP deaminase, an enzyme that produces ammonium for local use by carbamoyl-P or exportation to the liver. Activity and expression show the same trend: in control rats, the enzyme decrease its activity/expression along the length of the small intestine, while in HPD-fed rats the fall can be observed only in distal segment. AMP deaminase controls AMP levels[142] and thus AMP-kinase[254], which regulates glycolysis[255], and it is inhibited by metformin, the first-line medication for hyperglycaemia. The presence of AMP deaminase mainly in the duodenum, where free glucose is

absorbed suggests that its main role may lie on the control glucose uptake/metabolism through AMP-kinase rather than (or in addition to) its role in the purine nucleotide cycle.

6. SUMMARY

One month of CAFD overfeeding increased body and liver weight, largely because of increased WAT and muscle mass compared to controls. The excess substrate and energy ingested increased thermogenesis and promoted protein and fat accrual. The excess energy was largely lost as heat, with a significant deviation in the overall blood flow, favouring heat dissipation by lung (evapo-transpiration) rather than through the skin (conduction-radiation).

CAFD induced a marked decrease in urea synthesis in comparison with controls and despite higher protein intake. In CAFD, the operation of urea cycle was decreased. This effect was extended to the main liver enzymes providing ammonia as substrate for the urea cycle, which were down-regulated. Both urea cycle and these other enzymes were not only influenced by diet but also by sex. In fact females and males implemented different strategies to prevent amino-N wasting under excess of energy. By the way, the elimination of amino-N is mandatory but it was not performed by already discovered pathways.

One month of HPD decreased the energy ingested both in lean and obese male Zucker rats, suggesting that obesity didn't interfere with mechanisms responsible of inducing satiation, didn't alter amino acids metabolism and didn't increased some plasmatic amino acids. However, deeper changes were caused by obesity that affected also lipid balance and lipid metabolism. A significant change was observed in OTC activity that decreased significantly in the liver of HPD-fed obese rats, thus decreasing the ability to synthesize urea. The lack of changes in enzyme activity in the liver of HPD-fed rats suggests that amino acid catabolism was not increased in spite of the higher supply of dietary amino acids.

Along the small intestine of lean male Zucker rats, a not polarized distribution of the main amino N handling enzymes was observed, with highest activity and expression in the proximal segments. However, exposure for one month to HPD did not induce significant changes in this pattern, which recesses the probable availability of free amino acids liberated by digestion. It is postulated that this enzyme distribution helps protect the integrity of amino acids for eventual distribution, and to prevent amino-N wasting.

Both CAFD and HPD-fed rats showed nitrogen balances incomplete, with a large portion (higher than that of controls) of N ingested but not accrued, excreted in the urine or stool. The lower the urea excretion, the higher the size of this fraction which catabolic pathway is so far unknown, but which importance seems magnified in the case of HPD, coexisting with a normal synthesis and excretion of urea.

7. CONCLUSIONS

In the CAFD-fed rats, operation of urea cycle was decreased. This effect was extended to the main liver enzymes that provide ammonia as substrate for the urea cycle.

The changes in urea cycle and other related enzymes were influenced not only by diet but also by sex.

The excess energy ingested during a CAFD was largely lost as heat, with a significant deviation in the overall blood flow, favouring heat dissipation by lung (evapo-transpiration) rather than through the skin (conduction-radiation).

In HPD-fed rats, genetic obesity didn't interfere with mechanisms responsible of inducing satiation.

The low OTC activity justified the decreased ability to synthesize urea in high protein diet-fed obese rats.

A not polarized distribution of the main amino-N handling enzymes was observed in the small intestine of rats. Exposure for one month to high protein diet did not provoke marked changes in male Zucker rats.

8. BIBLIOGRAPHY

1. Cornier MA, Dabelea D, Hernandez TL, et al. The metabolic syndrome. *Endocr Rev* **29,** 777–822 (2008).

2. Kaur J. A comprehensive review on metabolic syndrome. *Cardio Res Pr.* **2014,** 943162–943183 (2014).

3. Alemany M. Metabolic syndrome: a multifaceted disease of affluence. *J Endocrinol Metab* **2,** 155–165 (2012).

4. Alemany M. Adjustment to dietary energy availability: From starvation to overnutrition. *RSC Adv* **3,** 1636–1651 (2013).

5. Coughlin SR, Mawdsley L, Mugarza JA, et al. Obstructive sleep apnoea is independently associated with an increased prevalence of metabolic syndrome. *Eur Hear. J* **25,** 735–741 (2004).

6. Bruce KD, Byrne CD. The metabolic syndrome: common origins of a multifactorial disorder. *Postgr. Med J* **85,** 614–21 (2009).

7. Bhurosy T, Jeewon R. Overweight and Obesity Epidemic in Developing Countries: A Problem with Diet, Physical Activity, or Socioeconomic Status? *Sci World J* **2014,** 964236 (2014).

8. Alemany M. Different effects of hyperlipidic diets in human lactation and adulthood: growth versus the development of obesity. *Reprod Biol Endocrin* **9,** 101 (2011).

9. Alemany M. The problem of nitrogen disposal in the obese. *Nutr Res Rev* **25,** 18–28 (2012).

10. O'Connell RC, Morgan AP, Aoki TT, et al. Nitrogen Conservation in starvation: graded responses to intravenous glucose. *J. Clin. Enidocrinol. Metab* **39,** 555–563. (1974).

11. Palou A, Remesar X, Arola LI, et al. Metabolic effect of short term food deprivation in the rat. *Horm Metab Res* **13,** 326–330 (1981).

12. Eckel RH, Grundy SM, Z. P. The metabolic syndrome. *Lancet* **365,** 1415–28 (2005).

13. Kylin E. Hypertonie und Zuckerkrankheit. *Z Inn Med* **45,** 873–877 (1921).

14. Marañon G. Uber Hypertonie und Zuckerkrankheit. *Z Inn Med* **43,** 169–176 (1922).

15. Camus JP. Goutte, diabete, hyperlipemie: un trisyndrome metabolique. *Rev Rhum* **33,** 10–15 (1966).

16. Avogaro P, Crepaldi G. Associazione di iperlipidemia, diabete mellito e obesità di medio grado. *Acta Diabetol Lat* 572–590 (1967).

17. Mehnert H, Kuhlmann H. Hypertonie und Diabetes Mellitus. *Dtsch Med J* **19,** 567–571 (1968).

18. Hanefeld M, Leonhardt W. Das metabolische Syndrom. *Dt Gesundh Wes.* **36,** 545–551 (1981).

19. Reaven GM. Role of insulin resistance in human disease. *Diabetes* **37,** 1595–1607 (1988).

20. Sarafidis PA, Nilsson PM. The metabolic syndrome: a glance at its history. *J Hypertens.* **24,** 621–626 (2006).

21. Kaplan N. The deadly quartet. *Arch Intern Med* **149,** 1514–1520 (1989).

22. DeFronzo RA, Ferrarini E. Insulin resistance: a multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia and atherosclerotic cardiovascular disease. *Diabet Care* **14,** 173–194 (1991).

23. Zimmet P, Shaw J, Alberti KGMM. Preventing Type 2 diabetes and the dysmetabolic syndrome in the real world: a realistic view. *Diabet Med* **20,** 693–702 (2003).

24. Eschwège E. The dysmetabolic syndrome, insulin resistance and increased cardiovascular morbidity and mortality in type 2 diabetes: aetiological factors in the development of cardiovascular complications. *Diabet Metab* **29,** 6S19-6S27 (2003).

25. Córdova-Pluma VH; Castro-Martínez G; Rubio-Guerra et al. Breve crónica de la definición del síndrome metabólico Definition. *Med Int Méx* 312–328 (2014).

26. World Health Organisation. Definition diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus. *Rep. a WHO Consult.* (1999).

27. Yudkin JS. Microalbuminuria: a genetic link between diabetes and cardiovascular disease? *Ann Med* **24,** 517–22 (1992).

28. 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 report. *Circulation* **106,** 3143–3421 (2002).

29. Einhorn D, Reaven GM, Cobin RH, et al. American College of Endocrinology Position Statement on the Insulin Resistance Syndrome. *Endocrin Pr.* **9,** 236–252 (2003).

30. Balkau B, Charles MA. Comment on the provisional report from the WHO consultation. *Diabet Med* **16,** 442–443 (1999).

31. Moller DE, K. K. Metabolic syndrome: a clinical and molecular perspective. *Annu Rev Med* **56,** 45–62 (2005).

32. Murrow BA, Hoehn KL. Mitochondrial regulation of insulin action. *Int J Biochem Cell Biol* **42,** 1936–9 (2010).

33. Guo S. Insulin signaling, resistance, and the metabolic syndrome: insights from mouse models into disease mechanisms. *J Endocrinol* **220,** T1–T23 (2014).

34. Wilcox, G. Insulin and insulin resistance. *Clin Biochem Rev* **26,** 19–39 (2005).

35. Johnson AMF, Olefsky JM. The Origins and Drivers of Insulin Resistance. *Cell* **152,** 673–684 (2013).

36. Saltiel AR, Kahn CR. Glucose and Lipid Metabolism. *Nature* **414,** 799–806 (2001).

37. Samuel VT, Shulman GI. Mechanisms for insulin resistance: Common threads and missing links. *Cell* **148,** 852–871 (2012).

38. Pessin JE, Saltiel AR. Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest* **106,** 165–169 (2000).

39. Weiss M, Steiner DF, Philipson LH. *Insulin Biosynthesis, Secretion, Structure, and Structure-Activity Relationships*. (MDText.com, Inc., 2014).

40. Plum L, Schubert M, Brüning JC. The role of insulin receptor signaling in the brain. *Tr Endo Met* **16,** 59–65 (2005).

41. Schulingkamp RJ, Pagano TC, Hung D, Raffa RB. Insulin receptors and insulin action in the brain: review and clinical implications. *Neurosci. Biobehav. Rev.* **24,** 855–872 (2000).

42. Zhang B, Roth RA. The insulin receptor-related receptor. *J Biol Chem267* **267,** 18320–183284w4 (1992).

43. Burks DJ, White MF. IRS Proteins and betaCell Function. *Diabetes* **50,** S140–S145 (2001).

44. Gambhir KK, Agarwal VR. Red blood cell insulin receptors in health and disease. *Biochem. Med. Metab. Biol.* **45,** 133–153 (1991).

45. Alemany M. Utilization of dietary glucose in the metabolic syndrome. *Nutr Metab* **8,** 74 (2011).

46. Grimpo K, Völker MN, Heppe EN, et al. Brown adipose tissue dynamics in wild-type and UCP1-knockout mice: in vivo insights with magnetic resonance. *J. Lipid Res.* **55,** 398–409 (2014).

47. McArdle MA, Finucane OM, Connaughton RM, et al. Mechanisms of obesity-induced inflammation and insulin resistance: Insights into the emerging role of nutritional strategies. *Front. Endocrinol. (Lausanne).* **4,** 1–23 (2013).

48. Sunny NE, Satapati S, Fu X, et al. Progressive adaptation of hepatic ketogenesis in mice fed a high-fat diet. *Am. J. Physiol. Endocrinol. Metab.* **298,** E1226–E1235 (2010).

49. Borén J, Taskinen MR, Olofsson SO, et al. Ectopic lipid storage and insulin resistance: a harmful relationship. *J Inter Med* **274,** 25–40 (2013).

50. Cinti S. The adipose organ at a glance. *Dis. Model. Mech.* **5,** 588–94 (2012).

51. Walther TC, Farese RV. Lipid droplets and cellular lipid metabolism. *Annu. Rev. Biochem.* **81,** 687–714 (2012).

52. Romero MdM, Roy S, Pouillot K, et al. Treatment of rats with a self-selected hyperlipidic diet, increases the lipid content of the main adipose tissue sites in a proportion similar to that of the lipids in the rest of organs and tissues. *PLoS One* **9,** e90995 (2014).

53. Remesar X, Fernández-López JA, Blay MT, et al. Effect of oral oleoyl-estrone on adipose tissue composition in male rats. *Int. J. Obes.* **26,** 1092–1102 (2002).

54. Lara-Castro C, Garvey WY. Intracellular Lipid accumulation in liver and muscle and the insulin resistance syndrome. *Endocr Metab Clin NAm* **37,** 841–856 (2008).

55. Adiels M, Taskinen MR, Packard C, et al. Overproduction of large VLDL particles is driven by increased liver fat content in man. 755–765 (2006).

56. Heier C, Haemmerle G. Fat in the heart: The enzymatic machinery regulating cardiac triacylglycerol metabolism. *Biochim. Biophys. Acta* (2016).

57. Corcoran MP, Lamon-fava S, Fielding R. Skeletal muscle lipid deposition and insulin resistance : effect of dietary fatty acids and exercise. *Am J Clin Nutr* **85,** 662–677 (2007).

58. Goossens GH, Blaak EE. Adipose tissue dysfunction and impaired metabolic health in human obesity: a matter of oxygen? *Front Endocrinol* **6,** 55 (2015).

59. Van Herpen NA, Schrauwen-Hinderling VB. Lipid accumulation in non-adipose tissue and lipotoxicity. *Physiol Behav* **94,** 231–241 (2008).

60. Febbraio MA. Role of interleukins in obesity: implications for metabolic disease. *Tr Endo Met* **25,** 312–319 (2014).

61. Jornayvaz FR, Samuel VT, Shulman GI. The role of muscle insulin resistance in the pathogenesis of atherogenic dyslipidemia and nonalcoholic fatty liver disease associated with the metabolic syndrome. *Annu Rev Nutr* **30,** 273–290 (2010).

62. Agnelli S, Arriarán S, Oliva L, et al. Modulation of rat liver urea cycle and related ammonium metabolism by sex and cafeteria diet. *RSC Adv* **6,** 11278–11288 (2016).

63. Papandreou D, Andreou E. Role of diet on non-alcoholic fatty liver disease: An updated narrative review. *Wrld J Hepat* **7,** 575–82 (2015).

64. MacGlashan D. Histamine: A mediator of inflammation. *J. Allergy Clin. Immunol.* **112,** (2003).

65. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arter. Thromb Vasc Biol* **31,** 986–1000 (2011).

66. Jun-Ming Z, Jianxiong A. Cytokines, inflammation and pain. *Int Anesth. Clin.* **69,** 482–489 (2009).

67. Eming S, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. *J. Invest. Dermatol.* **127,** 514–525 (2007).

68. Alemany M. Relationship between energy dense diets and white adipose tissue inflammation in metabolic syndrome. *Nutr Res* **33,** 1–11 (2013).

69. Loeser JD, Melzack R. Pain : an overview. *Lancet* **353,** 1607–1609 (1999).

70. Pasarica M, Sereda OR, Redman LM, et al. Reduced adipose tissue oxygenation in human obesity. *Diabetes* **58,** 718–725 (2009).

71. Alemany M. Regulation of adipose tissue energy availability through blood flow control in the metabolic syndrome. *Free Rd Biol Med* **52,** 2108–2119 (2012).

72. Netzer N, Gatterer H, Faulhaber M, et al. Hypoxia, Oxidative Stress and Fat. *Biomolecules* **5,** 1143–1150 (2015).

73. Arriarán S, Agnelli S, Remesar X, et al. White adipose tissue urea cycle activity is not affected by one-month treatment with a hyperlipidic diet in female rats. *Food Funct.* **7,** 1554–1563 (2016).

74. Sabater D, Arriarán S, Romero MdM, et al. Cultured 3T3L1 adipocytes dispose of excess medium glucose as lactate under abundant oxygen availability. *Sci. Rep.* **4,** 3663 (2014).

75. Marchesi C, Ebrahimian T, Angulo O, et al. Endothelial nitric oxide synthase uncoupling and perivascular adipose oxidative stress and inflammation contribute to vascular dysfunction in a rodent model of metabolic syndrome. *Hypertension* **54,** 1384–1392 (2009).

76. Lumeng CN, DeYoung SM, Bodzin JL, et al. Increased Inflammatory Properties of Adipose Tissue Macrophages Recruited During Diet-Induced Obesity. *Diabetes* **56,** 16–23 (2007).

77. Olefsky JM, Glass CK. Macrophages, Inflammation, and Insulin Resistance. *Annu Rev Physiol* **72,** 219–246 (2010).

78. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. *J Clin Invest* **116,** 1793–801 (2006).

79. Gregor MF, H. G. Inflammatory Mechanisms in Obesity. *Annu Rev Imm* **29,** 415–445 (2011).

80. Osborn O, Olefsky JM. The cellular and signaling networks linking the immune system and metabolism in disease. *Nat. Med* **18,** 363–74 (2012).

81. Welty FK, Alfaddagh A, Elajami TK. Targeting Inflammation in Metabolic Syndrome. *Transl. Res* **167,** 257–280 (2015).

82. Lumeng, C. N., Saltiel, A. R. Inflammatory links between obesity and metabolic disease. *J Clin Invest* **121,** 2111–7 (2011).

83. Hermoso MA, Cidlowski JA. Putting the Brake on Inflammatory Responses: the Role of Glucocorticoids. *IUBMB Life* **55,** 497–504 (2003).

84. Bailey CJ. Treating insulin resistance: future prospects. *Diabet Vasc Dis Res* **4,** 20–31 (2007).

85. Romero, MdM, Holmgren-Holm F, Grasa MdM, et al. Modulation in Wistar Rats of Blood Corticosterone Compartmentation by Sex and a Cafeteria Diet. *PLoS One* **8,** (2013).

86. Henneicke H, Gasparini SJ, Brennan-Speranza TC, et al. Glucocorticoids and bone: Local effects and systemic implications. *Tr Endo Met* **25,** 197–211 (2014).

87. Nicolaides NC, Kyratzi E, Lamprokostopoulou A, et al. Stress, the stress system and the role of glucocorticoids. *Neuroimmunomodulation* **22,** 6–19 (2015).

88. Obstfeld AE, Sugaru E, Thearle M, et al. C-C chemokine receptor 2 regulates the hepatic recruitment of myeloid cells that promote obesity-induced hepatic steatosis. *Diabetes* **59,** 916–25 (2010).

89. Flamment M, Hajduch E, Ferré P, et al. New insights into ER stress-induced insulin resistance. *Tr Endo Met* **23,** 381–390 (2012).

90. Oslowski CF, Urano F. Measuring endoplasmatic reticulum stress and the unfolded protein response using mammalian tissue culture system. *Meth Enzym.* 71–92 (2011).

91. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev Mol Cell Biol* **8,** 519–529 (2007).

92. Brush MH, Weiser DC, Shenolikar S. Growth arrest and DNA damage-inducible protein GADD34 targets protein phosphatase 1 alpha to the endoplasmic reticulum and promotes dephosphorylation of the alpha subunit of eukaryotic translation initiation factor 2. *Mol cell Biol* **23,** 1292–1303 (2003).

93. Yoshida H, Matsui T, Yamamoto A, et al. XBP1 mRNA Is Induced by ATF6 and Spliced by IRE1 in Response to ER Stress to Produce a Highly Active Transcription Factor. *Cell* **107,** 881–891 (2001).

94. Chen X, Shen J, Prywes R. The Luminal Domain of ATF6 Senses Endoplasmic Reticulum (ER) Stress and Causes Translocation of ATF6 from the ER to the Golgi. *J Biol Chem* **277,** 13045–13052 (2002).

95. Ozcan U, Cao Q, Yilmaz E, et al. Endoplasmic Reticulum Stress Links Obesity, Insulin Action, and Type 2 Diabetes. *Science (80-.).* **304,** 1469–1476 (2004).

96. Cnop M, Foufelle F, Velloso LA. Endoplasmic reticulum stress, obesity and diabetes. *Tr Mol Med* **18,** 59–68 (2012).

97. Dufour JF, Clavien PA. Endoplasmic reticulum stress in nonalcoholic fatty liver disease. *Hepatology* (2015).

98. Hudson WM, Pagliassotti MJ. in *Chapter 5 - Studies on Hepatic Disorders* 113–122 (2015).

99. Bogdanovic E, Kraus N, Patsouris D, et al. Endoplasmic reticulum stress in adipose tissue augments lipolysis. *J Cell Mol Med* **19,** 82–91 (2015).

100. Salvadó L, Palomer X, Barroso E, et al. Targeting endoplasmic reticulum stress in insulin resistance. *Tr Endo Met* **26,** 438–448 (2015).

101. Rodriguez J, Gilson H, Jamart C, et al. Pomegranate and green tea extracts protect against ER stress induced by a high-fat diet in skeletal muscle of mice. *Eur J Nutr* **54,** 377–89 (2015).

102. Hannelore D, Gholami AM, Berry D, et al. High-fat diet alters gut microbiota physiology in mice. *ISME J.* **8,** 295–308 (2014).

103. Velloso LA. TLR4 at the Crossroads of Nutrients, Gut Microbiota, and Metabolic Inflammation. *Endocr Rev* **36,** (2015).

104. Moran CP, Shanahan F. Gut microbiota and obesity: Role in aetiology and potential therapeutic target. *Best Pr Res Clin Gastr* **28,** 585–597 (2014).

105. Shen J, Obin M, Zhao L. The gut microbiota, obesity and insulin resistance. *Mol. Aspects Med.* **34,** 39–58 (2013).

106. Qin J, Li R, Raes J, et al. A human gut microbial gene catalogue established by metagenomic sequencing : Article : Nature. *Nature* **464,** 59–65 (2010).

107. Brown K, DeCoffe D, Molcan E, et al. Diet-Induced Dysbiosis of the Intestinal Microbiota and the Effects on Immunity and Disease. *Nutrients* **4,** 1095–1119 (2012).

108. Turnbaugh PJ, Hamady M, Yatsunenko T, et al. A core gut microbiome in obese and lean twins. *Nature* **457,** 480–484 (2009).

109. Blumberg R, Powrie F. Microbiota, Disease, and Back to Health: A Metastable Journey. *Sci Transl. Med* **4,** 137rv7-137rv7 (2012).

110. Lassenius MI, Pietiläinen KH, Kaartinen K, et al. Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation. *Diabet Care* **34,** 1809–1815 (2011).

111. Ridaura VK, Faith JJ, Reay FE, et al. Gut Microbiota from Twins Discordant for Obesity Modulate Metabolism in Mice. *Science (80-.).* **341,** 1241214-1-10 (2013).

112. Dykhuizen R S, Frazer R, Duncan C, et al. Antimicrobial effect of acidified nitrite on gut pathogens : importance of dietary nitrate in host defense. Antimicrobial Effect of Acidified Nitrite on Gut Pathogens : Importance of Dietary Nitrate in Host Defense. *Antimicrob Ag Chemother* **40,** 1422–1425 (1996).

113. *Obesity: preventing and managing the global epidemic. Report of a WHO consultation*. *World Health Organization technical report series* **894,** (2000).

114. Ahima RS. Digging deeper into obesity. *J Clin Invest* **121,** 2076–2079 (2011).

115. Abrams H.L. in *In Food and evolution: Toward a theory of human food habits.* 207–223 (1987). doi:10.1017/CBO9781107415324.004

116. M, A. Steroid hormones interrelationships in the metabolic syndrome: An introduction to the ponderostat hypothesis. *Hormones* **11,** 272–289 (2012).

117. Taubes G. Insulin resistance Prosperity's plague. *Science (80-.).* **325,** 256–60 (2009).

118. Oakes ND, Cooney GJ, Camilleri S, et al. Mechanisms of Liver and Muscle Insulin Resistance Induced by Chronic High-Fat Feeding. *Diabetes* **46,** 1768–1774 (1997).

119. Buettner R, Schölmerich J, Bollheimer LC. High-fat diets: modeling the metabolic disorders of human obesity in rodents. *Obesity* **15,** 798–808 (2007).

120. Sampey BP, Vanhoose AM, Winfield HW, et al. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet. *Obesity* **19,** 1109–17 (2011).

121. Sabater D, Agnelli S, Arriarán S, et al. Altered nitrogen balance and decreased urea excretion in male rats fed cafeteria diet are related to arginine availability. *Biomed Res. Int.* **2014,** 959420 (2014).

122. Oliva L, Baron C, Fernández-López JA, et al. Marked increase in rat red blood cell

membrane protein glycosylation by one-month treatment with a cafeteria diet. *PeerJ* **3,** e1101 (2015).

123. Goularte JF, Ferreira MBC, Sanvitto GL. Effects of food pattern change and physical exercise on cafeteria diet-induced obesity in female rats. *Br J Nutr* **108,** 1511–8 (2012).

124. Tan X, Xie G, Sun X, et al. High Fat Diet Feeding Exaggerates Perfluorooctanoic Acid-Induced Liver Injury in Mice via Modulating Multiple Metabolic Pathways. *PLoS One* **8,** (2013).

125. Romero MdM, Fernandez-Lopez JA, Alemany M, et al. Gene expression modulation of liver energy metabolism by oleoyl-oestrone in overweight rats. *Biosci Rep* **30,** 81–89 (2010).

126. South T, H. N., Martire SI, et al. Rats eat a cafeteria-style diet to excess but eat smaller amounts and less frequently when tested with chow. *PLoS One* **9,** e93506 (2014).

127. Reichelt AC, M. M., Westbrook RF. Cafeteria diet impairs expression of sensory-specific satiety and stimulus-outcome learning. *Front Psych* **5,** 852 (2014).

128. Esteve M, Rafecas I, Fernández-López JA, et al. Effect of a cafeteria diet on energy intake and balance in Wistar rats. *Physiol Behav* **56,** 65–71 (1994).

129. South T, Westbrook F, Morris MJ. Neurological and stress related effects of shifting obese rats from a palatable diet to chow and lean rats from chow to a palatable diet. *Physiol Behav* **105,** 1052–7 (2012).

130. Martire SI, Holmes N, Westbrook RF, et al. Altered feeding patterns in rats exposed to a palatable cafeteria diet: increased snacking and its implications for development of obesity. *PLoS One* **8,** e60407 (2013).

131. Romero MdM, Fernández-López JA, Remesar X, et al. Effect of sex and prior exposure to a cafeteria diet on the distribution of sex hormones between plasma and blood cells. *PLoS One* **7,** e34381 (2012).

132. Oliveira RB, Maschio DA, Carvalho CPF, et al. Influence of gender and time diet exposure on endocrine pancreas remodeling in response to high fat diet-induced metabolic disturbances in mice. *Ann Anat* **200,** 88–97 (2015).

133. Christensen A, Pike CJ. Menopause, obesity and inflammation: interactive risk factors for Alzheimer's disease. *Front Aging Neurosci* **7,** 1–14 (2015).

134. Arriarán S, Agnelli S, Remesar X, et al. Effects of sex and site on amino acid metabolism enzyme gene expression and activity in rat white adipose tissue. *PeerJ* **3,** e1399 (2015).

135. Maranon R, Reckelhoff JF. Sex and gender differences in control of blood pressure. *Clin Sci* **125,** 311–318 (2013).

136. Alemany M. Do the interactions between glucocorticoids and sex hormones regulate the development of the metabolic syndrome? *Front Endocrinol* **3,** 1–13 (2012).

137. Urban RJ, Dillon EL, Choudhary S, et al. Translational studies in older men using testosterone to treat sarcopenia. *Trans Am Clin Clim. Assoc* **125,** 27–44 (2014).

138. Esteve M, Rafecas I, Fernandez-Lopez JA, et al. Dietary amino acids balances in young Wistar rats fed a cafeteria diet. *Biochem Mol Biol Int* **29,** 1069–1081 (1993).

139. Goodman MN, Lowenstein JM. The purine nucleotide cycle – studies of ammonia production by skeletal muscle in situ and in perfused preparations. *J Biol Chem* **252,** 5054–5060 (1977).

140. Lowenstein JM. The purine nucleotide cycle revisited. *Int J Sport Med* **11 Suppl 2,** :S37-46 (1990).

141. Yu J, Zheng J, Liu XF, et al. Exercise improved lipid metabolism and insulin sensitivity in rats fed a high-fat diet by regulating glucose transporter 4 (GLUT4) and musclin expression. *Braz J Med Bio Res* **49,** 1–6 (2016).

142. Flanagan WF, Holmes EW, Sabina RL, et al. Importance of purine nucleotide cycle to energy production in skeletal muscle. *Am J Physiol* **251,** C795-802 (1986).

143. Brestenský M, Nitrayov S, Patráš P, et al. Branched chain amino acids and their importance in nutrition. *J Micro Biotech Food Sci* **5.2,** 197–202 (2015).

144. Marliss EB, Aoki TT, Pozefsky T, et al. Muscle and splanchnic glutamine and glutamate. Metabolism in postabsorptive and starved man. *J Clin Invest* **50,** 814–817 (1971).

145. Windmueller HG, Spaeth AE. Intestinal metabolism of glutamine and glutamate from the lumen as compared to glutamine from blood. *Arch Biochem Biophys* **171,** 662–672 (1975).

146. Good DW, Burg MB. Ammonia production by individual segments of the rat nephron. *J. Clin. Invest.* **73,** 602–610 (1984).

147. Esteve M, Rafecas I, Remesar X, et al. Nitrogen balances of lean and obese Zucker rats subjected to a cafeteria diet. *Int J Obes.* **16,** 237–244 (1991).

148. Costa G, Ullrich L, Kantor L, Al., E. Production of elemental nitrogen by certain mammals including man. *Nature* **218,** 546–551 (1988).

149. Cissik JH, Johnson RE, Rokosch DK. Production of Gaseous Nitrogen in Human Steady-state Conditions. *J. Appl. Physiol.* **32,** 155–159 (1972).

150. Green LC, Ruiz de Luzuriaga K, Wagner DA, Al., E. Nitrate biosynthesis in man. *Proc Natl Acad Sci U S A* **78,** 7764–8 (1981).

151. Desir G, Bratusch-Marrain P, DeFronzo RA. Effect of hyperketonemia on renal ammonia excretion in man. Metabolism. **35,** 736–743 (1986).

152. Robin ED, Travis DM, Bromberg PA, et al. Ammonia excretion by mammalian lung. *Science (80-.).* **129,** 270–271 (1959).

153. Curis, E. *et al.* Almost all about citrulline in mammals. *Amino Acids* **29,** 177–205 (2005).

154. Takiguchi M, Masataka M. Transcriptional regulation of genes for ornithine cycle enzymes. *Biochem J* **312,** 649–659 (1995).

155. Tousoulis D, Kampoli AM, Tentolouris C, et al. The role of NO on endothelial function. *Curt Vasc Pharmacol* **10,** 4–18 (2012).

156. Badheka AO, Rathod A, MA, K., et al. Influence of Obesity on Outcomes in Atrial Fibrillation: Yet Another Obesity Paradox. *Am. J. Med.* **123,** 646–651 (2010).

157. Veldhorst MAB, Westerterp KR, Westerterp-Plantenga MS. Gluconeogenesis and protein-induced satiety. *Br J Nutr* **107,** 595–600 (2012).

158. Dalle Grave R, Calugi S, Gavasso I, et al. A randomized trial of energy-restricted high-protein versus high-carbohydrate, low-fat diet in morbid obesity. *Obesity* **21,** 1774–1781 (2013).

159. Sargrad KR, Homko C, Mozzoli M, Al, E. Effect of high protein vs high carbohydrate intake on insulin sensitivity, body weight, hemoglobin A1c, and blood pressure in patients with type 2 diabetes mellitus. *J Acad. Nutr Diet* **105,** 573–580 (2005).

160. Lobley GE, Bremner DM, Holtrop G, et al. Impact of high-protein diets with either moderate or low carbohydrate on weight loss, body composition, blood pressure and glucose tolerance in rats - download.php. *Bri J Nutr* **97,** 1099–1108 (2007).

161. Tang M, Armstrong CLH, Leidy HJ, et al. Normal vs. high-protein weight loss diets in men: Effects on body composition and indices of metabolic syndrome. *Obesity* **21,** 204–210 (2013).

162. Yancy WS, Olsen MK, Guyton JR, et al. Annals of Internal Medicine Article A Low-Carbohydrate, Ketogenic Diet versus a Low-Fat Diet To Treat Obesity and Hyperlipidemia. *Ann Inter Med* **140,** 769–779 (2004).

163. Tipton KD. Efficacy and consequences of very-high-protein diets for athletes and exercisers. *Proc Nutr Soc* **70,** 205–214 (2011).

164. Westerterp-Plantenga, M. S. How are normal, high- or low-protein diets defined? *Bri J Nutr* **97,** 217 (2007).

165. Astrup A, Raben A, Geiker N. The role of higher protein diets in weight control and obesity-related comorbidities. *Int J Obes.* **39,** 721–726 (2015).

166. Westerterp-Plantenga MS, Lemmens SG, Westerterp KR. Dietary protein – its role in satiety, energetics, weight loss and health. *Bri J Nutr* **108,** S105–S112 (2012).

167. Westerterp-Plantenga, M. S. *et al.* Dietary protein, metabolism, and body-weight regulation: dose–response effects. *Int J Obes.* **30,** S16–S23 (2006).

168. Heaney RP, Layman DK. Amount and type of protein influences bone health. *Am. J. Clin. Nutr.* **87,** 1567–1570 (2008).

169. Martin WF, Armstrong LE, Rodriguez NR. Dietary protein intake and renal function. *Nutr Metab* **2,** (2005).

170. Institute of Medicine of the national academies. *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids.* (2002).

171. Metges CC, Barth CA. Issues and Opinions Metabolic Consequences of a High Dietary-Protein Intake in Adulthood : Assessment of the Available Evidence. *Am S Nutr Sc* 886–889 (2000).

172. Scott KP, Gratz SW, Sheridan PO, et al. The influence of diet on the gut microbiota. *Pharmacol. Res.* **69,** 52–60 (2013).

173. Didier R, Remesy C, Demigne C, et al. Hepatic proliferation of mitochondria in response to a high protein diet. *Nutr Res* **5,** 1093–1102 (1985).

174. Masanes R, Fernandez-Lopez JA, Alemany M, et al. Effect of dietary protein content on tissue protein synthesis rates in Zucker lean rats. *Nutr Res* **19,** 1017–1026 (1999).

175. Peters JC, Harper AE. Adaptation of rats to diets containing different levels of protein: effects on food intake, plasma and brain amino acid concentrations and brain neurotransmitter metabolism. *J Nutr* **115,** 382–398 (1985).

176. Jean, C. *et al.* Metabolic evidence for adaptation to a high protein diet in rats. *J. Nutr.* **131,** 91–8 (2001).

177. Melorose J, Perroy R, Careas S. Effect of dietary protein level on urea synthesis in isolatd rat hepatocytes. *J Nutr* 561–566 (1977).

178. Schimke RT. Adaptive characteristics of urea cycle enzymes in the rat. *J Biol Chem* **237,** 459–468 (1962).

179. Payne E, Morris JG. The effect of protein content of the diet on the rate of urea formation in sheep liver. *Biochem. J.* **113,** 659–62 (1969).

180. Wu G, Bazer FW, Davis TA, et al. Important roles for the arginine family of amino acids in swine nutrition and production. *Livest Sci* **112,** 8–22 (2007).

181. Wu G, Bazer FW, Cudd TA, et al. Pharmacokinetics and safety of arginine supplementation in animals. *J Nutr* **137,** 1673S–1680S (2007).

182. Nelson DL, Cox MM. *Lehninger Principles of Biochemistry*. (2002).

183. Blachier F, Mariotti F, Huneau JF, et al. Effects of amino acid-derived luminal metabolites on the colonic epithelium and physiopathological consequences. *Amino Acids* **33,** 547–562 (2007).

184. Wu G. Amino acids: Metabolism, functions, and nutrition. *Amino Acids* **37,** 1–17 (2009).

185. Montañez R, Rodríguez-Caso R, Sánchez-Jiménez F, et al. In silico analysis of arginine catabolism as a source of nitric oxide or polyamines in endothelial cells. *Amino Acids* **34,** 223–229 (2008).

186. Morris SM. Arginine metabolism: boundaries of our knowledge. *J Nutr* **137,** 1602S–1609S (2007).

187. JM, B., Tymoczko JL, Stryer L. in *Biochemistry 5th edition* Chapter 25 (2002).

188. Purves D, Augustine GJ, Fitzpatrick D, et al. in *Neuroscience 2nd edition Sunderland MA editors* (2001).

189. Braissant O, McLin VA, Cudalbu C. Ammonia toxicity to the brain. *J Inherit Met Dis.* **36,** 595–612 (2012).

190. Grisolia S, Quijada CL, Fernandez M. Glutamate dehydrogenase from yeast and from animal tissues. *Biochim Biophys Acta* - **81,** 61–70 (1964).

191. Adeva M, Souto G, N, B., et al. Ammonium metabolism in humans. *Metab. Clin Exp* **61,** 1495–511 (2012).

192. Sun L, Bartlam M, Liu Y, et al. Crystal structure of the pyridoxal-5'-phosphate-dependent serine dehydratase from human liver. *Protein Sci.* **14,** 791–798 (2005).

193. Hampson RK, Melanie KT, Olson MS. Regulation of the glycine cleavage system in the isolated prefused rat liver. *J Biol Chem* **259,** 1180–1185 (1984).

194. Ford GC, Eichele G, Jansonius JN. Three-dimensional structure of a pyridoxal-phosphate-dependent enzyme, mitochondrial aspartate aminotransferase. *Proc. Natl. Acad. Sci. USA* **77,** 2559–2563 (1980).

195. Liu Z, Que S, Xu J, et al. Alanine aminotransferase-old biomarker and new concept: A

review. *Int J Med Sci* **11,** 925–935 (2014).

196. Harper AE, Miller RH, Block KP. Branched-chain amino acid metabolism. *Annu Rev Nutr* **4,** 409–454 (1984).

197. Spanaki C, Kotzamani D, Petraki Z, et al. Heterogeneous Cellular Distribution of Glutamate Dehydrogenase in Brain and in Non-neural Tissues. *Neuro Res* **39,** 500–515 (2014).

198. Elgadi KM, Meguid RA, Qian M, et al. Cloning and analysis of unique human glutaminase isoforms generated by tissue-specific alternative splicing. *Physiol Genom* **1,** 51–62 (1999).

199. Elshafei AM, Hassan MM, Ali NH, et al. Purification, kinetic properties and antitumor activity of L-glutaminase from Penicillium brevicompactum NRC 829. *Br. Microbiol. Res. J.* **4,** 97–115 (2014).

200. Nakagawa H, Miura S, Kimura H. Studies on Substrate Induction of Serine Dehydratase of Rat Liver. *J Biochem* **66,** 549–564 (1969).

201. Arriarán S, Agnelli S, Remesar X, et al. A radiochemical method for carbamoyl-phosphate synthetase-I: Application to rats fed a hyperproteic diet. *J Enz Res* **3,** 29–33 (2012).

202. P, D., Terzic A. Adenylate kinase and AMP signaling networks: metabolic monitoring, signal communication and body energy sensing. *Int J Mol SciSci* **10,** 1729–1772 (2009).

203. Kim YA, King MT, Teague WE, et al. Regulation of the purine salvage pathway in rat liver. *Am J Physiol* **262,** E344–E352 (1992).

204. Lowenstein JM. Ammonia production in muscle and other tissues: the purine nucleotide cycle. *Physiol Rev* **52,** 382–414 (1972).

205. Tornher K, Lowenstein JM. The Purine. *J Biol Chem* 162–169 (1972).

206. Morisaki T, Sabinat R, Holmes EW. Adenylate deaminase. *Biochemistry* **265,** 11482–11486 (1989).

207. Kikuchi G, Motokawa Y, Yoshida T, et al. Glycine cleavage system : reaction mechanism, physiological significance, and hyperglycinemia. *Proc Jap Acad Ser* **84,** 246–263 (2008).

208. Vilar S, Ferino G, Quezada E, et al. Predicting Monoamine Oxidase Inhibitory Activity through Ligand-Based Models. *Curr Top Med Chem* **12,** 2258–2274 (2012).

209. Bortolato M, Shih JC. Behavioral outcomes of monoamine oxidase deficiency: preclinical and clinical evidence. *Int Rev Neurobiol* **100,** 13–42 (2012).

210. Izidoro LF, Sobrinho JC, Mendes MM, et al. Snake venom L-amino acid oxidases: Trends in pharmacology and biochemistry. *Biomed Res. Int.* **2014,** (2014).

211. Windmueller HG. Glutamine utilization by the small intestine. *Adv Enzym. Rel Areas Mol Biol* **53,** 201–237 (1982).

212. Burrin DGG. in *Biology of the Intestine in Growing Animals R. Zabielski, P.C. Gregory and B. Westrom (Eds.)* **1,** 695–725 (2002).

213. Burrin DG, Reeds PJ. Alternative fuels in the gastrointestinal tract. *Curr Op Gastroent* **13,** 165–170 (1998).

214. Stoll B, Henry J, Reeds PJ, et al. Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk protein-fed piglets. *J Nutr* **128,** 606–614 (1998).

215. Lux GD, Marton LJ, Baylin SB. Ornithine decarboxylase is important in intestinal mucosal maturation and recovery from injury in rats. *Science (80-.).* **210,** 195–198 (1980).

216. Alican I, Kubes P. A critical role for nitric oxide in intestinal barrier function and dysfunction. *Am J Physiol* **270,** G225–G237 (1996).

217. Reeds PJ, Burrin DG, Stoll B, et al. Enteral glutamate is the preferential source for mucosal glutathione synthesis in fed piglets. *Am J Physiol* **272,** E408–E415 (1997).

218. Windmueller HG, Spaeth AE. Intestinal metabolism of glutamine and glutamate from the lumen as compared to glutamine from blood. *Arch Biochem Biophys* **171,** 662–672 (1975).

219. Windmueller HG, Spaeth AE. Metabolism of absorbed aspartate, asparagine, and arginine by rat small intestine in vivo. *Arch Biochem Biophys* **175,** 670–676 (1976).

220. Krebs HA, Henseleit K. Urea formation in the animal body. *Z Physiol Chem* **210,** 33–66 (1932).

221. Shambaugh GE. Urea biosynthesis I. The urea cycle and relationships acid cycle. *Nutrition* 2083–2087 (1977).

222. Arriarán S, Agnelli S, Remesar X, et al. The urea cycle of rat white adipose tissue. *RSC Adv* **5,** 93403–93414 (2015).

223. Heibel, S. K. *et al.* Transcriptional regulation of N-acetylglutamate synthase. *PLoS One* **7,** e29527 (2012).

224. Makoff AJ, Radford A. Genetics and biochemistry of carbamoyl phosphate biosynthesis and its utilization in the pyrimidine biosynthetic pathway. *Microbiol Rev* **42,** 307–328 (1978).

225. Function of Conserved Histidine Residues in Mammalian Dihydroorotase. *Biochemistry* **34,** 7038–7046 (1995).

226. Morris CM, Knox WE. Glutamine-dependent carbamyl phosphate synthetase. *J Biol Chem*

245, 2199–2204 (1970).

227. Shi D, Morizono H, Yu X, et al. Crystallization and preliminary X-ray crystallographic studies of wild-type human ornithine transcarbamylase and two naturally occurring mutants at position 277. *Acta Crystall* **57,** 719–721 (2001).

228. Neill, M. A., Aschner, J., Barr, F., Summar, M. L. Quantitative RT-PCR comparison of the urea and nitric oxide cycle gene transcripts in adult human tissues. *Mol Genet Met* **97,** 121–127 (2009).

229. Rath M, Muller I, Kropf P, et al. Metabolism via Arginase or Nitric Oxide Synthase: Two Competing Arginine Pathways in Macrophages. *Front Immunol* **5,** 1–10 (2014).

230. Husson A, Brasse-Lagnel C, Fairand A, et al. Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. *Eur J Biochem* **270,** 1887–1899 (2003).

231. Morris SM. Regulation of enzymes of the urea cycle and arginine metabolism. *Annu Rev Nutr* **22,** 87–105 (2002).

232. Emmanuel B. Urea cycle enzymes in tissues (liver, rumen epithelium, heart, kidney, lung and spleen) of sheep (Ovis aries). *Comp Biochem. Physiol* **65,** 693–697 (1980).

233. Nishibe H. Urea cycle enzymes in human erythrocytes. *Clin Chim Acta* **50,** 305–310 (1974).

234. Morris SM. Regulation of enzymes of urea and arginine synthesis. *Annu Rev Nutr* (1992).

235. Haeberle J, Boddaert N, Burlina A, et al. Suggested Guidelines for the Diagnosis and Management of Urea Cycle Disorders. *Orphanet J Rare Dis* **7,** 32 (2012).

236. Barber T, Viña JR, Viña J, et al. Decreased urea synthesis in cafeteria-diet-induced obesity in the rat. *Biochem J* **230,** 675–681 (1985).

237. Knowles RG, Salvador M. Nitric oxide synthases in mammals. *Biochem. J.* **298,** 249–58 (1994).

238. Kleinert H, Swharz PM, Förstermann U. Regulation of the expression of inducible nitric oxide synthase. *Biol Chem* **384,** 1363–1364 (2003).

239. Arriarán S, Agnelli S, Sabater D, et al. Evidences of Basal Lactate Production in the Main White Adipose Tissue Sites of Rats. Effects of Sex and a Cafeteria Diet. *PLoS One* **10,** e0119572 (2015).

240. Lalanza JF, A, C., Del Bas JM, et al. Effects of a post-weaning cafeteria diet in young rats: Metabolic syndrome, reduced activity and low anxiety-like behaviour. *PLoS One* **9,** 1–9 (2014).

241. Ma SWY, Foster DO. Brown adipose tissue, liver and diet-induced thermogenesis in cafeteria diet-fed rats. *Can J Physiol Pharmacol* **67,** 376–381 (1989).

242. Esteve M, Rafecas I, Remesar X, et al. Nitrogen balance discrepancy in wistar rats fed a cafeteria diet. *Biochem Int* **26,** 687–694 (1992).

243. D'Eon TM, Souza SC, Aronovitz M, et al. Estrogen regulation of adiposity and fuel partitioning: Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. *J Biol Chem* **280,** 35983–35991 (2005).

244. Asensio C, Muzzin P, Rohner-Jeanrenaud F. Role of glucocorticoids in the physiopathology of excessive fat deposition and insulin resistance. *Int J Obes.* **28 Suppl 4,** S45–S52 (2004).

245. Bolster DR, Jefferson LS, Kimball SR. Regulation of protein synthesis associated with skeletal muscle hypertrophy by insulin-, amino acid- and exercise-induced signalling. *Proc Nutr Soc* **63,** 351–356 (2004).

246. Tornheim K. Oscillations of the glycolytic pathway and the purine nucleotide cycle. *J Theor Biol* **79,** 491–541 (1979).

247. Lutoslawska G, Niedbalska M. Adenilate kinase and 5'-AMP-activated protein kinase contribution to the regulation of skeletal muscle energetics. *Med Sport.* **8,** 85–104 (2004).

248. Yoshida T, Kikuchi G. Mayor pathways of glycine and serine catabolism in rat liver. *Arch Biochem* **139,** 30–392 (1970).

249. Rotondo F, Sanz T, Fernández-López JA, et al. Stable isotope analysis of dietary arginine accrual and disposal efficiency in male rats fed diets with different protein content. *RSC Adv* **6,** 69177–69184 (2016).

250. Jean C, Rome S, Aattouri N, et al. Nutrient Metabolism Metabolic Evidence for Adaptation to a High Protein Diet in Rats. 91–98 (2001).

251. Petersen KF, Vilstrup H, Tygstrup N. Effect of Dietary Protein on the Capacity of Urea Synthesis in Rats. *Horm Metab. Res* **22,** 512–615 (1990).

252. Van de Poll MCG, Ligthart-Melis GC, Boelens PG, et al. Intestinal and hepatic metabolism of glutamine and citrulline in humans. *J Physiol* **581,** 819–27 (2007).

253. Nolles JA, Van Straten EME, Bremer BI, et al. Dietary amino acids fed in free form and as protein components do not differently affect postprandial plasma insulin, glucagon, growth hormone and corticosterone responses in rats. *J. Anim. Physiol. Anim. Nutr. (Berl).* **90,** 289–299 (2006).

254. Ouyang J, Parakhia RA, Ochs RS. Metformin activates AMP kinase through inhibition of AMP deaminase. *J Biol Chem* **286,** 1–11 (2011).

255. Yoshino M, Murakami K. AMP deaminase reaction as a control system of glycolysis in yeast. *J Biol Chem* **257,** 2822–2828 (1982).

A chi mi ha svegliato al mattino… A chi mi ha incoraggiato nei miei cammini… A chi si è commosso e mi ha fatto commuovere… A chi mi ha regalato continue risate… A chi mi ha insegnato a vivere… A chi mi ha lasciato sbagliare… A chi mi ha regalato i propri ricordi… A chi mi ha visto nascere… A chi mi ha guardato crescere… A chi, nonostante tutto, è orgoglioso di me… A chi mi ha supportato… A chi mi ha sopportato… A chi mi ha ascoltato… A chi mi ha dato consigli… A chi mi è stato vicino… A chi ha creduto in me… A chi mi ha reso il cammino più facile… A chi mi ha permesso di arrivare fin qui …

Grazie

Gracias

Gràcies

"Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e conoscenza"

Dante Alighieri
Divina Commedia - Inferno - Canto XXVI (vv. 118-120)