Efecto de la proporción de los componentes de la dieta en distintos aspectos del metabolismo de la rata en un contexto de obesidad

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FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ

Tesis doctoral en Alimentación y Nutrición

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Memoria presentada por Laia Oliva Lorenzo para optar al título de doctor por la Universitat de Barcelona

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Hay tiempo para todo y todo lleva su tiempo

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RESUMEN

El sobrepeso y la obesidad son condiciones patológicas, con una prevalencia elevada, que están consideradas como una de las principales causas de mortalidad. Los factores genéticos, y especialmente, los factores ambientales y los estilos de vida son determinantes para su desarrollo. Existen varias patologías asociadas a la obesidad como la resistencia a la acción de la insulina, la hipertensión, la hiperlipidemia y las enfermedades cardiovasculares, que son las principales comorbilidades del síndrome metabólico. A lo largo de las décadas se han desarrollado infinidad de estudios enfocados en la prevención y el tratamiento de la obesidad y sus complicaciones, la mayoría de ellos relacionados con el control del peso a través de la intervención nutricional, que se basa principalmente en la modificación de la proporción de los macronutrientes de la dieta. Sin embargo, dichas modificaciones conllevan adaptaciones metabólicas que, con la tentativa de mantener la homeostasis en el organismo, pueden comprometer algunas funciones biológicas.

En la presente Tesis, se han estudiado algunas de las adaptaciones metabólicas que se producen tras aplicar una intervención nutricional con cuatro dietas distintas durante un mes. La intervención se realizó en ratas Wistar hembra y macho de 10 semanas de edad. Las dietas utilizadas diferían en la proporción y/o la composición de macronutrientes, y a la vez, fueron comparables entre sí al estar ajustadas al resto de componentes.

Los resultados obtenidos en relación a la medida de la glucemia indican que las lecturas de glucosa estarían sujetas a una infraestimación sistemática, pudiendo comprometer los criterios diagnósticos. Las medidas de glucemia y glucosilación obtenidas apuntan a una influencia de la dieta sobre la proporción anomérica de la glucosa, y a diferencias en la compartimentación de la glucosa sanguínea; por ello, se sugiere la medida de la glucosilación de las proteínas de membrana de los eritrocitos (debido a su contacto continuado con la glucosa del plasma) como mejor indicador del desarrollo de la hiperglucemia.

En relación a los efectos obesogénicos de la dieta, parece que las consecuencias metabólicas no vienen dadas únicamente por un exceso de nutrientes de la dieta sino también por un desequilibrio en su proporción. Se ha observado que la ingesta de componentes sabrosos (dulce/salado) junto con la textura que aporta el lípido, son los principales responsables de la inducción de hiperfagia y el desarrollo de obesidad. La relación lípido / proteína de la dieta influye positivamente en la acumulación de lípido ectópico, siendo esta también diferente entre sexos. Por otro lado, la composición lipídica de la dieta generó efectos importantes en el metabolismo de carbohidratos y de lípidos, que parecen estar mediados por el estradiol. Respecto al metabolismo nitrogenado, la relación energía / proteína de la dieta parece tener un papel clave en la regulación del ciclo de la urea hepático. Ante un exceso energético y proteico simultáneo proveniente de la dieta, el funcionamiento del ciclo y la producción de urea se ven comprometidos, dificultando la excreción de nitrógeno amínico. Por otro lado, la capacidad del tejido adiposo marrón para contribuir a la eliminación de nitrógeno amínico está limitada debido a la presencia parcial del ciclo de la urea, que parece estar relacionado con la síntesis de arginina. Estos hallazgos refuerzan las hipótesis relacionadas con la existencia de mecanismos alternativos para eliminar un superávit de nitrógeno amínico.

ABSTRACT

Overweight and obesity are pathologic conditions with a high prevalence, and are considered as one of the leading risks for global deaths. Genetic factors, and especially, environmental factors and lifestyle habits are crucial for their development. There are several obesity-associated pathologies, as insulin resistance, hypertension, cardiovascular disease and hiperlipidemia, which are the main comorbidities of Metabolic Syndrome. Over the decades several approaches have been carried out for the prevention and treatment of obesity and its complications. Most of them are related to weight control through nutritional intervention, which is mainly based on macronutrient's proportion modifications. However, these modifications involve metabolic adaptations for homeostasis maintenance that can compromise some biological functions.

Some of those metabolic adaptations have been studied by carrying out a one-month nutritional intervention with four different diets. It has been performed in 10-week old female and male Wistar rats. The diets used differed in macronutrients proportion or composition, and were comparable to each other since the rest of the components were adjusted.

The results obtained in relation to glycaemia measurement suggest the existence of a systematic underestimation of glucose readings, which could be compromising the diagnostic criteria. The obtained glycaemia and glycosylation measurements point to an influence of the diet on glucose anomer proportion and to a different blood glucose compartmentalization, suggesting the measurement of red blood cell membrane protein glycosylation (due to its sustained exposure to glucose) as better marker of hyperglycaemia development.

Regarding diet obesogenic effects, it seems that the metabolic consequences are not only given by an excess of nutrients from the diet but also by an imbalance in their proportion. It has been observed that the intake of tasty components (sweet / salty) together with lipid texture, are the main responsible factors for hyperphagia induction and obesity development. The lipid / protein ratio of the diets positively influences the accumulation of ectopic lipid, which also exhibited sex-related differences. On the other hand, the lipid composition of the diet promoted important effects on carbohydrate and lipid metabolism, which seemed to be mediated by oestradiol. In relation to nitrogen metabolism, the energy / protein ratio of the diet seems to play a key role in liver urea cycle regulation. When high amounts of energy and protein simultaneously come from the diet, the operation of urea cycle, and so the excretion of nitrogen as urea, is compromised. Furthermore, the capacity of brown adipose tissue to contribute to the nitrogen excess elimination is limited due to its partial urea cycle presence, which may be more related to arginine synthesis. These findings reinforce the hypotheses related to the existence of alternative mechanisms to eliminate the nitrogen surplus.

Introducción

1. SÍNDROME METABÓLICO

El síndrome metabólico es el término médico que se usa para englobar varias condiciones fisiopatológicas, todas ellas relacionadas con una disfunción metabólica que afecta al organismo de manera global [1]. Las cuatro patologías consideradas principales en el síndrome metabólico son: obesidad, hiperglucemia e hiperinsulinemia, hiperlipidemia e hipertensión arterial, aunque frecuentemente van asociadas a otras patologías o complicaciones [1] como la hiperuricemia, artritis y gota [2], la inflamación endotelial, el hipoandrogenismo [3], la depresión [4], la esteatosis hepática [5], el síndrome de ovario poliquístico [6], la apnea del sueño [7], las enfermedades autoinmunes [8] e incluso algunos tipos de cáncer [9, 10].

El concepto de síndrome metabólico, aunque bajo distintos nombres, existe desde ya hace varias décadas. De hecho, fue descrito por primera vez en los años 80 por Gerald M. Reaven como el síndrome X [11, 12], el cual se caracterizaba por la presencia de 4 condiciones fisiopatológicas (obesidad, hiperglucemia, hipertensión, hipertrigliceridemia) a las que Kaplan nombró "el cuarteto de la muerte" [13]. Es importante establecer unos criterios de diagnóstico válidos para la comunidad médica y científica, puesto que los individuos con síndrome metabólico tienen mayor riesgo de sufrir complicaciones como la diabetes tipo 2 y la enfermedad cardiovascular. Aunque estos criterios han ido evolucionando a lo largo de los años, actualmente establecen que el paciente debe presentar al menos 3 de los siguientes 5 factores de riesgo: obesidad abdominal, hipertrigliceridemia, niveles de colesterol-HDL bajos, hipertensión e hiperglucemia [14].

La relación entre el síndrome metabólico y sus patologías asociadas no está totalmente aclarada, por lo que existe una importante controversia al respecto; sin embargo el nexo común parece ser la incapacidad del organismo para gestionar un exceso de nutrientes y energía provenientes de la dieta [15]. El modo y el grado en el que el síndrome metabólico se manifieste se verán condicionados por la interacción entre los factores genéticos, ambientales y de estilo de vida [1, 16].

El síndrome metabólico implica varias afecciones que, al fin y al cabo, están relacionadas entre sí, siendo causa-consecuencia unas de otras. Aunque en el desarrollo de esta tesis se hace referencia a algunos de estos factores fisiopatológicos que definen el síndrome metabólico, se toma la obesidad como base por su etiología principalmente nutricional.

2. OBESIDAD

2.1. Epidemiologia y etiología

La obesidad está considerada como una enfermedad desde 1948, año en el que la Organización Mundial de la Salud publicó la Clasificación Internacional de Enfermedades. Puesto que entre los años 60 y 80 la prevalencia de la obesidad era relativamente baja y estable [17], gran parte de la comunidad médica internacional consideró irrelevantes las advertencias que la resaltaban como un problema potencial de salud pública [18]. No obstante, en las últimas décadas, su incidencia a nivel global ha incrementado, doblándose en más de 70 países desde 1980, incluyendo Norteamérica, Europa, Asia y Australia [19, 20]. En 2015, se estimó que aproximadamente 107 millones de niños y 603 millones de adultos eran obesos, datos que establecieron unos valores de prevalencia global estimada del 5% en niños y del 12% en adultos [19].

Actualmente, la obesidad y el sobrepeso son una de las mayores preocupaciones de salud pública, dado que están altamente asociadas a un mayor riesgo de morbilidad y mortalidad [17, 21]. Además, contribuyen al desarrollo de enfermedades crónicas como, por ejemplo, enfermedades de tipo cardiovascular, diabetes de tipo 2, hígado graso, complicaciones respiratorias e incluso algunos tipos de cáncer [22, 23].

La obesidad y el sobrepeso son una condición heterogénea de origen multifactorial [24] que deriva de un desequilibrio entre la energía ingerida y la energía gastada. Dicho desequilibrio conlleva una acumulación anormal y excesiva de reservas energéticas en forma de grasa corporal [20]. En el desarrollo de la obesidad están implicados tanto factores ambientales como genéticos, dándose entre ellos una interacción compleja [20] (Figura 1).

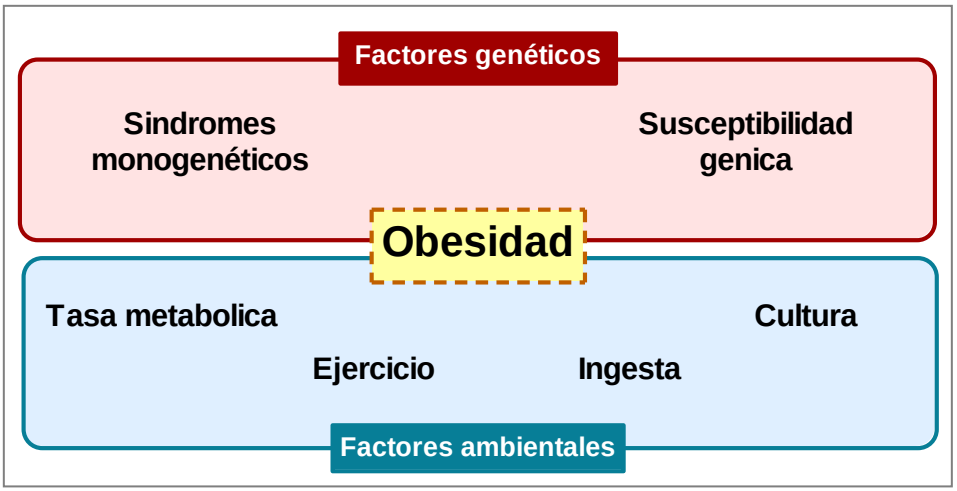

Figura 1. Factores que influyen en el desarrollo de la obesidad. Adaptado de Kopelman *et al.* 2000 [22].

Aunque la predisposición genética es indudable y está bien establecida [25, 26], en las últimas décadas se ha dado un incremento en la prevalencia de la obesidad, difícilmente justificable teniendo en cuenta únicamente los cambios genéticos ocurridos en un periodo de tiempo tan corto. En las últimas décadas, se han generado cambios sociales importantes, que de algún modo han influenciado en los estilos de vida [27, 28]. Dichos cambios han podido propiciar el desarrollo de obesidad en aquellos individuos con genotipos susceptibles (p. ej. el genotipo ahorrador) como resultado de la interacción entre la genética y el ambiente. De hecho, varios estudios apuntan a una asociación directa entre el incremento en la incidencia de la obesidad y los cambios sociales producidos [20, 22, 29].

2.1.1. Factores genéticos:

El componente genético de la obesidad se sugirió tras algunos estudios con gemelos adoptados que presentaron un fenotipo, en cuanto a peso corporal, más parecido a sus progenitores que al de los padres adoptivos [30-32]. Los genes candidatos a considerar como genes de susceptibilidad para el desarrollo de obesidad probablemente serían aquellos relacionados con la composición y contenido de grasa corporal, su distribución anatómica, la ingesta y el gasto energético [22].

Dicha contribución genética se reafirmó tras conocerse la existencia de formas monogénicas de obesidad severa, relacionadas con el desarrollo temprano de la misma y causadas por mutaciones específicas en genes concretos, como por ejemplo las mutaciones en genes codificantes para proteínas reguladoras del apetito como la leptina, su propio receptor [33-35], el receptor 4 de melanocortina [36] y la propiomelanocortina [37].

Por otro lado, la presencia de variantes genéticas o polimorfismos de un único nucleótido (SNPs, por sus siglas en inglés) mostraron tener un papel moderador sobre la susceptibilidad individual para desarrollar obesidad [20]. En la última década y mediante estudios de asociación del genoma completo, se han identificado más de 50 *loci* relacionados con el desarrollo de obesidad o diabetes de tipo 2 [38]. El polimorfismo del gen *FTO* (*fat mass and obesity associated gen*) fue el primero en ser descrito como SNPs de susceptibilidad a la obesidad [39, 40]. Posteriormente, también se describieron polimorfismos en el gen del receptor 4 de melanocortina asociados con el riesgo de desarrollar obesidad [41]. Actualmente, las bibliotecas de marcadores que cubren el genoma humano entero permiten tener la oportunidad de hacer búsquedas aleatorias de genes candidatos que contribuyen en el desarrollo de la obesidad. De hecho, se especula que cientos o miles de genes podrían estar implicados, cada uno de ellos aportando un pequeño efecto [42].

2.1.2. Factores ambientales

La predisposición genética suele estar enmascarada por los factores ambientales, entre los que se incluyen los hábitos de vida, las costumbres culturales y los cambios socio-económicos [22].

El desequilibrio en el balance energético, es decir, la descompensación entre la ingesta energética (dieta) y el gasto energético (actividad física, metabolismo basal y termogénesis adaptativa) es el factor directamente responsable del desarrollo de la obesidad [43, 44]. En este sentido, la tendencia actual a ingerir alimentos de alta densidad energética conjuntamente con unos hábitos de vida cada vez más sedentarios, se relacionan directamente con el incremento de peso [45].

Además, los cambios sociodemográficos experimentados en las últimas décadas, propiciados en mayor medida por la innovación tecnológica (automatización de los procesos) y los cambios en los sistemas de comidas (mayor disponibilidad y accesibilidad a alimentos de alto contenido energético y campañas de marketing persuasivas) son probablemente los factores de mayor impacto en el desarrollo de obesidad [45, 46].

2.1.3. Otros factores

Existen otros factores que también pueden tener un papel importante en el desarrollo de la obesidad. Varios estudios evidencian la influencia de deleciones cromosómicas (Síndrome de Prader Willi) [47, 48], la presencia previa de enfermedades endocrinas [49], los cambios hormonales durante la menopausia en las mujeres [50], la nutrición fetal [51, 52], los mecanismos neurofisiológicos [44, 53] e incluso la microbiota intestinal [54].

2.2. Clasificación

En la práctica clínica, el grado de obesidad se clasifica principalmente mediante un índice antropométrico, el Índice de Masa Corporal (IMC) (Tabla 1), calculado como el peso corporal en quilogramos dividido por la altura en metros al cuadrado [55]. El IMC proporciona información aproximada en relación al peso y la grasa corporal y, a la vez, permite realizar una predicción en la evaluación del riesgo de complicaciones asociadas, morbilidad y mortalidad [56].

El IMC debería usarse únicamente como una herramienta para la clasificación general, la comparación de poblaciones y los estudios epidemiológicos [17, 20, 23], habiéndose establecido actualmente una relación entre un valor de IMC elevado (sin llegar necesariamente a la obesidad) y el 39% de las muertes[19]. Sin embargo, la aplicación de este índice presenta limitaciones serias al utilizarlo en individuos concretos, dado que no tiene en cuenta la composición corporal y asume que la variación de peso de individuos de la misma estatura se debe únicamente al incremento de grasa corporal [57].

Clasificación de la obesidad según el Índice de Masa Corporal (IMC)		
Peso corporal	**IMC (kg/m²)**	**Descripción popular**
Bajo	<18,5	Delgado
Normal o aceptable	18,5-24,9	Normal/Sano
Sobrepeso	25,0-29,9	Sobrepeso
Obesidad	≥30,0	
Grado 1	30,0-34,9	Obesidad
Grado 2	35,0-39,9	Obesidad moderada
Grado 3	≥40	Obesidad mórbida
Grado 4	≥50	Obesidad mórbida
Grado 5	≥60	Obesidad mórbida

Tabla 1. Clasificación de la obesidad según el índice de masa corporal. Adaptado de Kopelman *et al.* 2000 [22] y Poirer *et al.* 2009 [56].

Por otro lado, el IMC no tiene en cuenta la distribución de la grasa corporal ni la funcionalidad del tejido adiposo, aspectos que influyen notablemente en el desarrollo de complicaciones metabólicas o de tipo cardiovascular, y que dependen en mayor medida de la distribución de los depósitos de grasa [23, 58], siendo este otro factor de clasificación de la obesidad (Figura 2). El acumulo de tejido adiposo en la zona abdominal está altamente relacionado con el desarrollo de dichas complicaciones, mientras que el acumulo en la zona gluteofemoral parece tener una acción protectora [59]. Así pues, en función de la distribución de la grasa corporal se puede discernir entre un tipo de obesidad metabólicamente sana y otra asociada a anormalidades metabólicas, principalmente la resistencia a la acción de la insulina [57]. La obesidad metabólicamente sana se caracteriza por una mayor acumulación de tejido adiposo subcutáneo abdominal, menor masa grasa visceral, menor acumulación de grasa en hígado y músculo esquelético y menor tamaño de los adipocitos [60, 61].

Aunque los factores que determinan donde se deposita el exceso de calorías son complejos y permanecen poco establecidos, varios estudios apuntan a la influencia de las hormonas sexuales, los glucocorticoides y los mecanismos epigenéticos [58].

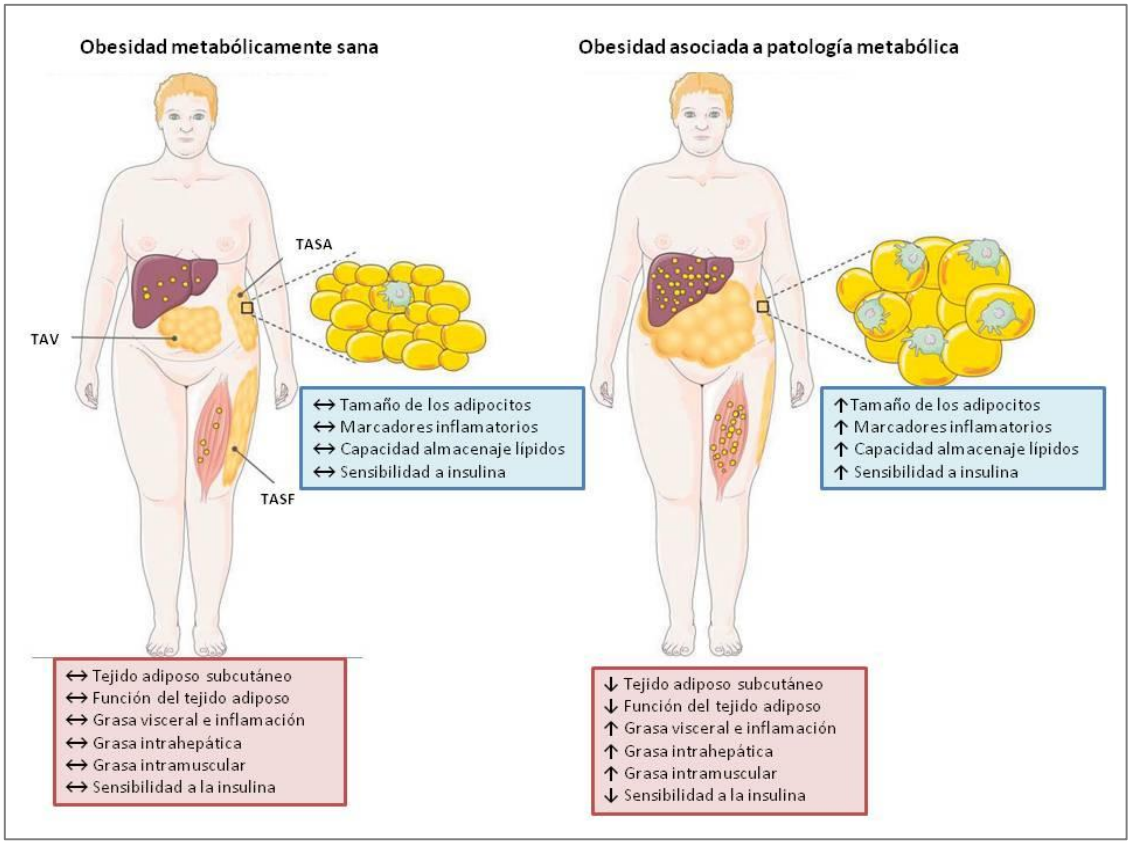

Figura 2. **Diferencias entre fenotipos obesos en función de la distribución de los depósitos de grasa y la función del tejido adiposo.** La expansión del tejido adiposo no implica necesariamente el desarrollo de patología metabólica y complicaciones asociadas. TAV: tejido adiposo visceral; TASA: tejido adiposo subcutáneo abdominal; TASF: tejido adiposo subcutáneo femoral. Adaptado de Goosens *et al*. 2017 [58].

3. HOMEOSTASIS ENERGÉTICA Y PESO CORPORAL

Durante la evolución, y según la teoría del genotipo ahorrador [62, 63], se han ido seleccionando aquellos mecanismos fisiológicos que han permitido optimizar el uso de la energía ingerida en épocas de abundancia nutricional, propiciando el almacenaje eficiente de energía en forma de reservas de grasa como adaptación para afrontar periodos de escasez de recursos y hambruna [64]. Por ello, ante la tendencia actual en la que la ingesta de nutrientes es abundante y continuada, el organismo se enfrenta a una situación de exceso simultáneo de glucosa y ácidos grasos para la que no está fisiológicamente preparado, hecho que conlleva una desregulación del mantenimiento homeostático del balance energético [64].

Como se ha apuntado anteriormente, y desde un punto de vista simplificado, el sistema de balance energético depende básicamente del equilibrio entre la ingesta y el gasto de energía [44]. Así, ante una situación de desequilibrio energético por exceso de ingesta, el organismo pone en marcha diferentes mecanismos para afrontar la situación. Sin embargo, la regulación del balance energético y, por ende, del peso corporal es compleja y está influenciada por diferentes mecanismos que, a su vez, están interrelacionados: 1) hábitos alimentarios y actividad física; 2) actividad del sistema nervioso autónomo (regulación del gasto energético,

apetito, termogénesis y otros aspectos metabólicos); 3) efectos en el sistema neuroendocrino (secreción de hormonas, como por ejemplo: hormona del crecimiento, insulina, hormonas tiroideas, hormonas esteroideas…) [44, 65, 66].

El gasto energético está parcialmente regulado por el sistema nervioso cognitivo (actividad física) y también por mecanismos autónomos que involucran procesos como la termogénesis [67]. Sin embargo, el incremento del gasto energético mediante la práctica voluntaria de ejercicio promueve respuestas compensatorias [68] que conllevan mayor apetito y como consecuencia mayor ingesta energética [65]. En cuanto a la termogénesis, es un proceso controlado fisiológicamente por el sistema nervioso simpático [69, 70], en el que el exceso de energía proveniente de la dieta se disipa en forma de calor por acción del sistema mitocondrial de la UCP (por sus siglas en inglés, UnCoupling Protein), específico del tejido adiposo marrón [71, 72].

La percepción sensorial de los alimentos, el efecto saciante de los mismos, la liberación de hormonas desde tejidos periféricos y de neuropéptidos son, todos ellos, consecuencia de una interacción entre los circuitos cerebrales autonómicos de ejecución, de recompensa y de las señales homeostáticas circulantes. Todos ellos juegan un papel relevante en el control del apetito y de la homeostasis energética [67].

3.1. Control del apetito

El control del apetito y la homeostasis energética se gestiona mediante la liberación de hormonas o péptidos que se generan en órganos periféricos (a nivel gastrointestinal, pancreático y de tejido adiposo) y que actúan sobre el sistema nervioso central (principalmente el hipotálamo), con el objetivo de regular la ingesta según los requerimientos energéticos. La producción de dichas señales, así como su concentración circulante, está asociada con determinadas situaciones fisiológicas (prandial, posprandial, ayunas), permitiendo regular la homeostasis en función del estado nutricional y energético del organismo [73].

El mantenimiento energético mediante el control de la ingesta se basa en la interacción entre señales que actúan a largo plazo, provenientes en mayor medida del tejido adiposo (liberación de adipoquinas y leptina) [74] y señales periféricas mediante la liberación circunstancial de péptidos gastrointestinales (ghrelina, colecistoquinina, insulina, péptido-1 similar al glucagón (GLP-1), péptido tirosina-tirosina (PYY), amilina y oxintomodulina principalmente) [75] (Figura 3).

La señalización de origen adiposo refleja el estado de las reservas adiposas. Por otro lado, las señales de origen gastrointestinal muestran una sincronización rítmica según los episodios de ingesta de alimentos. La integración de ambas señales permite el control del balance energético a través del reconocimiento del estado de las reservas energéticas y la oscilación del flujo de nutrientes a nivel de sistema nervioso central [76].

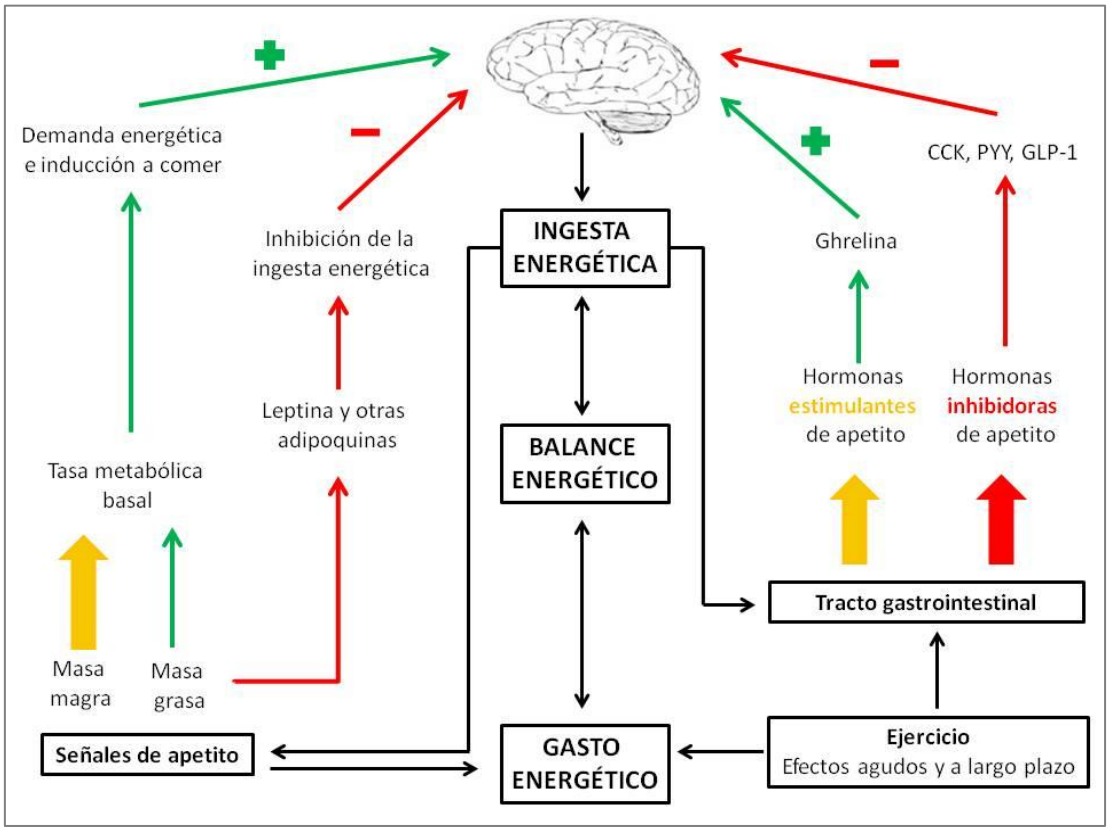

Figura 3. Factores que influyen en el control del apetito y la regulación del balance energético. Se pueden distinguir los procesos tónicos (relativamente estables a medio plazo y que surgen de los tejidos y metabolismo) y los episódicos (variables a lo largo del día como consecuencia del consumo alimentario). El efecto de la masa grasa sobre la ingesta energética refleja un control de origen lipostático; la leptina es el mediador principal para la inhibición de la ingesta. La demanda energética surge de los requerimientos energéticos de aquellos órganos con mayor gasto (corazón, hígado, cerebro, tracto gastrointestinal, músculo esquelético). El equilibrio entre los procesos tónicos inhibitorios y estimuladores modulará la ingesta energética. CCK: colecistoquinina; PYY: péptido tirosina-tirosina; GLP-1: péptido-1 similar al glucagón. Adaptado de Blundell *et al.* 2015 [65].

La producción de leptina y de otras adipoquinas segregadas por el propio tejido adiposo depende de la masa grasa y está regulada por el tamaño de sus adipocitos [77]. La leptina es una hormona con efectos homeostáticos a largo plazo, que actúa a nivel hipotalámico limitando la deposición de energía mediante la reducción de la ingesta energética y la estimulación del gasto energético [78, 77]. La deficiencia en la producción de leptina o en su receptor propicia el desarrollo de obesidad [33, 79].

La saciedad, que se define como el proceso de cese o retraso de la ingesta por sensación de llenado, es una de las respuestas desencadenadas tras la ingesta. La saciedad se puede evaluar mediante la percepción de sensaciones (hambre, llenado, deseo de ingerir), respuesta a hormonas gastrointestinales e inicio de la ingesta [65].

La liberación de hormonas desde el tracto gastrointestinal fluctúa en función del patrón de ingesta, reflejando cambios homeostáticos agudos [76, 80]. Por ejemplo, la colecistoquinina y GLP-1 se liberan en respuesta a la ingesta, propiciando la interrupción de la misma, influyendo en el tamaño de la comida e inhibiendo el apetito durante el periodo posprandial, considerándose, por lo tanto, hormonas con efecto saciante. En cambio la ghrelina es una hormona inductora del apetito, cuyos niveles aumentan en ayunas, siendo su objetivo incrementar la ingesta energética en situaciones de escasez [73]. Además, la insulina producida por las células β-pancreáticas, cuya acción se relaciona principalmente con la regulación del metabolismo de la glucosa, también tiene un efecto anorexigénico en el sistema nervioso central [73, 81].

Aunque la ingesta de alimentos desencadena multitud de señales neuronales y hormonales, todavía no está claro el papel que tiene la composición en macronutrientes de la dieta sobre la ingesta. Existen estudios, tanto en animales como humanos, que indican que los macrocomponentes dietéticos tienen efectos jerárquicos en la saciedad y la inhibición de la ingesta a corto plazo [82-84]. Sin embargo, otros autores sustentan la inexistencia de dicha jerarquía y apoyan la contribución de todos los macronutrientes, de forma individual o en combinación, en la secreción de hormonas con efecto saciante [85].

Otro factor que influye en el control del apetito y en la homeostasis energética es el placer sensitivo ante los alimentos. Así, la pérdida de la eficiencia en el control del apetito podría relacionarse no sólo con un mal funcionamiento de las vías homeostáticas a partir de señales neuroquímicas sino también con una sensibilización inapropiada del sistema hedónico [86] (Figura 4). Este sistema involucra las vías del glutamato, opioides, benzodiacepinas, ácido gamma-aminobutírico, endocanabinoides y dopamina, cuyo centro de integración de señales es el *nucleus accumbens* [76]. Los agonistas opioides inducen el apetito mientras que sus antagonistas producen el efecto contrario. El hecho que este efecto se vea incrementado ante alimentos "palatables" o sabrosos, sugiere que la regulación del apetito y de la ingesta se ajustan a una respuesta hedónica ante los alimentos [87, 88]. Por otro lado, el consumo en exceso de alimentos sabrosos genera una respuesta neuroadaptativa en los circuitos de recompensa dopaminérgicos, hecho que puede conllevar el desarrollo de un patrón de ingesta compulsiva [89, 90].

Una respuesta exagerada a las características sensoriales de los alimentos constituye un factor de riesgo que puede comprometer la eficacia en el control del apetito mediante el control homeostático. En cualquier caso, el control del apetito es complejo y se ve afectado por secuencias variables en el comportamiento, que frecuentemente están influenciadas por estados fisiológicos oscilantes (hambre, saciedad). En el contexto de la obesidad, la integración neuronal favorece el consumo de alimentos en exceso propiciando un aumento de peso. El incremento de peso, principalmente en el tejido adiposo, desregula el control del apetito, mediado por señales anorexigénicas, sobreponiendo los mecanismos hedónicos ante los homeostáticos [76].

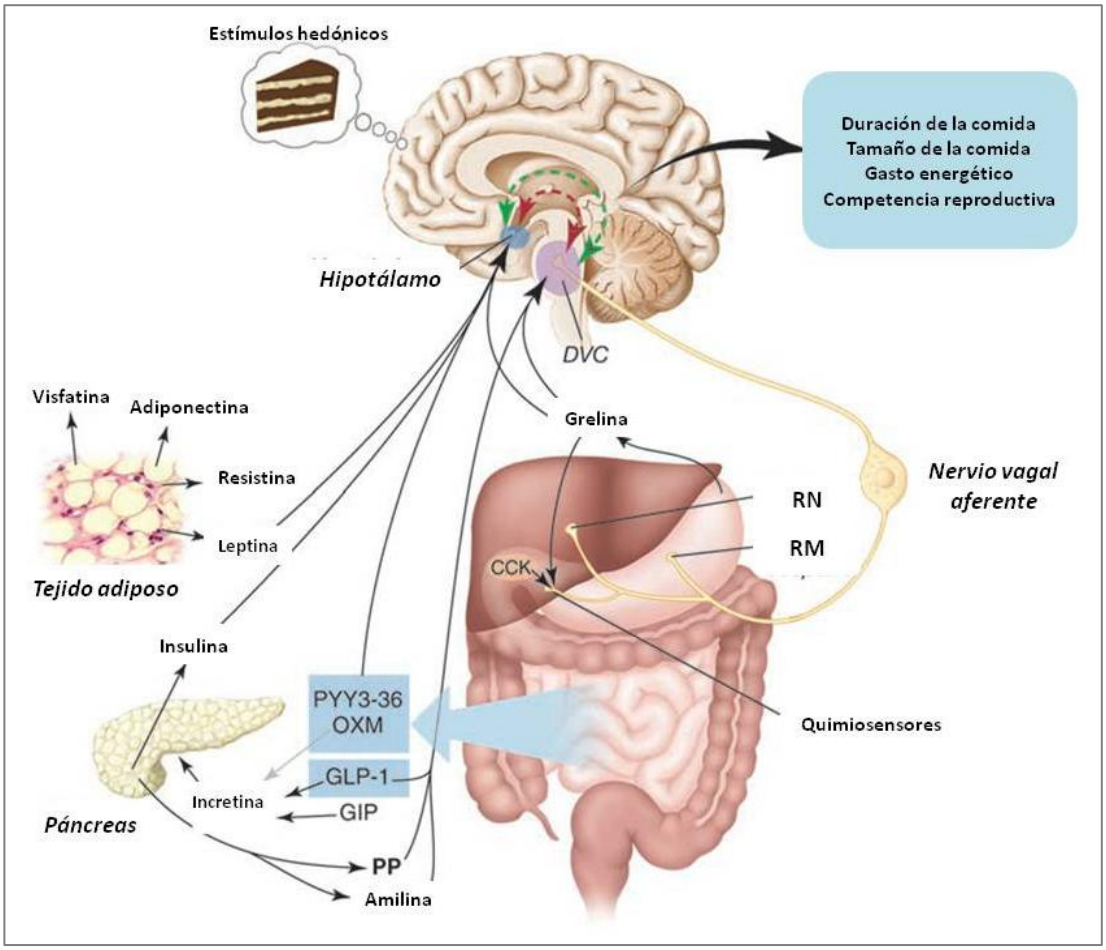

Figura 4. Integración del balance energético. Las señales periféricas relacionadas con la reserva energética sostenida se producen en el tejido adiposo (leptina) y en el páncreas (insulina) La información relacionada con el estado nutricional reciente viene dada por los nutrientes absorbidos, las señales neuronales y los péptidos intestinales. Las vías de señalización neuronales, principalmente a través del nervio vago, aportan información al núcleo *tractus solitarius* del complejo vagal dorsal sobre la distensión del estómago y el estado químico y hormonal del intestino delgado superior. Las hormonas liberadas por el intestino (incretinas) tienen un efecto estimulante de la sensación de saciedad. Las incretinas GLP-1, GIP y OXM mejoran la respuesta endocrina del páncreas ante la absorción de nutrientes. GLP-1 y OXM también reducen la ingesta. La ghrelina se libera desde el estómago y estimula el apetito. Las hormonas intestinales estimuladoras de la saciedad también incluyen la CCK, que se libera a nivel de duodeno y yeyuno. La OXM y el PPY se liberan desde el tracto gastrointestinal inferior y el PP desde los islotes de Langerhans. CCK: colecistoquinina; DCV: complejo vagal dorsal; GIP: polipéptido insulinotrópico dependiente de glucosa; GLP-1: péptido-1 similar al glucagón; OXM: oxintomodulina; PP: polipéptido pancreático; PYY: péptido tirosina-tirosina; RM: receptores mecánicos; RN: receptores de nutrientes. Adaptado de Badman *et al*. 2005 [75].

4. RESPUESTA FISIOLÓGICA EN LA OBESIDAD

Ante la llegada de nutrientes en exceso, el organismo pone en marcha varios mecanismos con el objetivo de mantener la homeostasis y afrontar la nueva situación. La respuesta fisiológica que determina una situación de sobrepeso u obesidad se caracteriza por la acumulación de lípidos, un estado de inflamación crónico, hipoxia tisular, estrés del retículo endoplasmático y desarrollo de resistencia a la insulina. Todas las respuestas mencionadas están conectadas entre sí y se retroalimentan, siendo así tanto causa como consecuencia de la situación.

4.1. Acumulación de lípidos

El exceso de energía proveniente de la alimentación se almacena en forma de reservas lipídicas principalmente en el tejido adiposo, un tejido disperso, cuyas células mayoritarias (adipocitos) tienen como función principal almacenar lípidos y/o liberarlos cuando hay demanda energética. El número y tamaño de adipocitos varían en función de la localización anatómica del tejido adiposo y están fuertemente influenciados por el sexo [91].

Ante un exceso energético, el tejido adiposo blanco se expande de forma hipertrófica, provocando un aumento del tamaño celular por acumulación de lípidos en la vacuola de los adipocitos ya existentes [92, 93]. En una situación fisiológica normal, cuando los adipocitos alcanzan un volumen celular crítico lanzan señales para reclutar y diferenciar nuevos preadipocitos y así aumentar la cantidad de adipocitos del tejido (hiperplasia) [94, 95]. No obstante, un excesivo aporte energético de forma crónica, provocará la incapacidad de reclutar nuevos adipocitos, por lo que, para almacenar el exceso de lípidos, se producirá una excesiva expansión hipertrófica del tejido adiposo, promoviendo el desarrollo de las complicaciones propias de la obesidad [91, 96] (Figura 5).

Cuando el tejido adiposo es incapaz de seguir acumulando lípidos pero el exceso calórico no cesa, el organismo afronta la sobrecarga acumulando también grasa ectópica en otros tejidos, como por ejemplo el hígado, corazón y músculo esquelético [97, 91]. El incremento de grasa visceral e intraabdominal es un marcador de acumulación de grasa ectópica en otros órganos y tejidos [98]. El incremento de grasa corporal en exceso supone cambios y adaptaciones en las funciones fisiológicas del organismo, que generalmente derivan en la aparición de complicaciones o enfermedades asociadas [60].

La acumulación de grasa ectópica induce la secreción de citoquinas que propician el desarrollo de resistencia a la acción de la insulina, inflamación y progresión de enfermedad cardiovascular [99]. La acumulación de grasa ectópica en el páncreas puede contribuir a la disfunción de las células β [100], y en el riñón, a la pérdida de la función nefrítica favoreciendo el desarrollo de hipertensión [101].

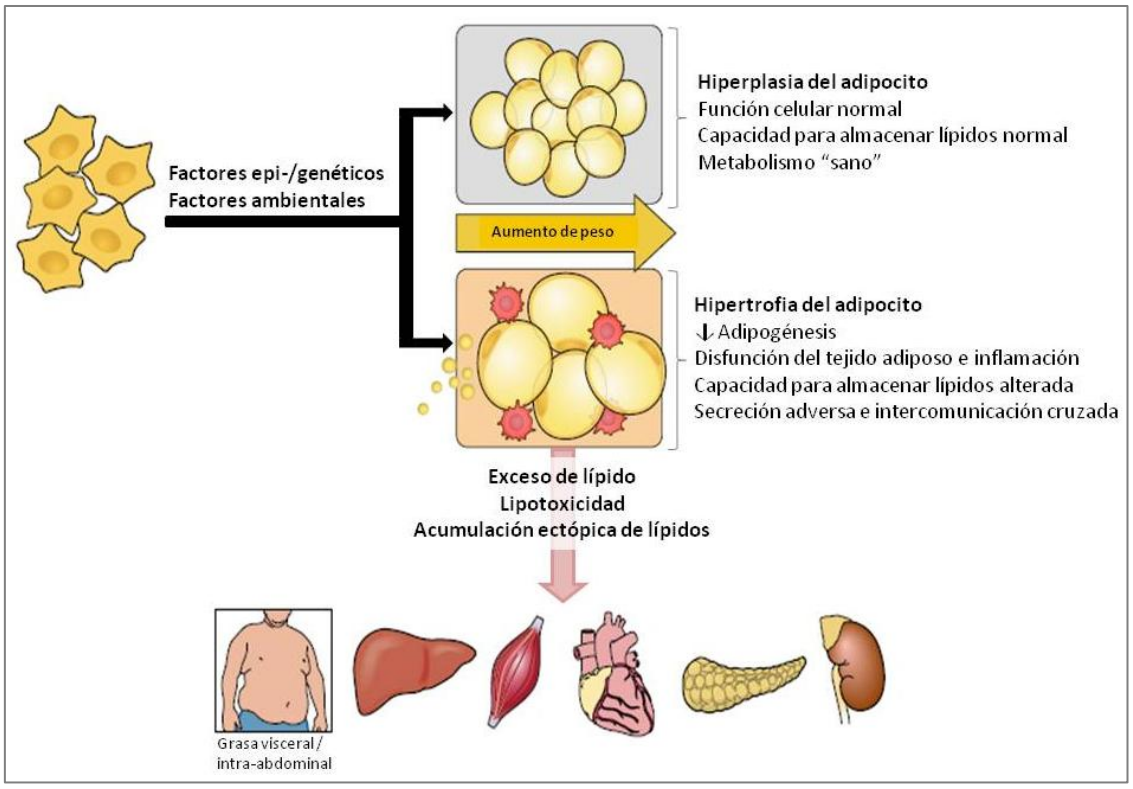

Figura 5. Expansión hiperplásica o hipertrófica del tejido adiposo y sus efectos en la función celular y metabólica. La expansión del tejido ocurre mediante la combinación de la hiperplasia y la hipertrofia del adipocito en respuesta a factores genéticos, epigenéticos y ambientales. La expansión hiperplásica mantiene la función normal del adipocito y la capacidad suficiente de almacenamiento de lípidos en el interior del tejido. Cuando la capacidad de almacenaje del tejido adiposo es limitada y no puede hacer frente a la sobrecarga nutricional, se promueve la acumulación de lípidos ectópica, es decir, en otros órganos o tejidos; se da un incremento de la captación lipídica en estos órganos pero sin un incremento de la utilización de lípidos, de modo que se promueve la acumulación de intermediarios lipídicos tóxicos que conllevan una disfunción celular. Adaptado de Hammarsted *et al.* 2018 [91].

La acumulación ectópica de lípidos en el hígado se conoce como esteatosis hepática. Dicha acumulación puede conllevar el desarrollo de cirrosis hepática no alcohólica, frecuentemente asociada a la obesidad [102]. El exceso de lípidos provenientes de la dieta, los ácidos grasos liberados por la lipólisis del tejido adiposo y la lipogénesis *de novo* son aspectos que generan un aumento en la disponibilidad de ácidos grasos en el hígado, contribuyendo a la formación y acumulación de triacilgliceroles [103]. Aunque los triacilgliceroles son usados principalmente como fuentes de energía celular, ante un exceso se produce un incremento de los intermediarios de su catabolismo, como los diacilgliceroles, las ceramidas y los ácidos grasos de cadena larga, que pueden tener un papel señalizador proinflamatorio e incluso efectos adversos sobre la función celular [104].

4.2. Inflamación

La inflamación es una respuesta biológica de elevada complejidad que se genera en el organismo ante la presencia de un cuerpo extraño. La respuesta fisiológica inflamatoria clásica se caracteriza principalmente por la presencia de señales desencadenadas por el sistema nervioso como el calor, rubor, turgor, dolor y pérdida de función. Dichos síntomas son consecuencia, en parte, de la presencia de compuestos circulantes como la histamina, eicosanoides y citoquinas [105, 106].

La obesidad y el síndrome metabólico se caracterizan por presentar un estado de inflamación crónica de bajo nivel, en la que se da una activación del sistema inmunológico en respuesta a la condición de estrés tisular. En este caso, el agente dañino o agresor causante de la respuesta inmunológica es principalmente la disponibilidad excesiva de nutrientes y energía [107, 108], La respuesta inflamatoria en el síndrome metabólico es generalizada y se da en varios tejidos como el hígado, músculo, intestino y cerebro, aunque el tejido adiposo es el principal y mayor tejido afectado [78]. Los daños en la vía de señalización de la insulina, característicos de la obesidad, limitan la capacidad del tejido adiposo para hacer frente a la sobrecarga de nutrientes, afectando su capacidad para absorberlos, gestionarlos o eliminarlos [109, 110].

El incremento del tamaño de los adipocitos supone una condición de estrés para las células, por lo que se desencadena la liberación de citoquinas [110] y proteínas quimioatrayentes de macrófagos como la MCP1 (por sus siglas en inglés: Macrophague chemoattractant protein), hecho que resulta en la infiltración de macrófagos en el tejido adiposo [111]. El porcentaje de macrófagos infiltrados en el tejido adiposo correlaciona positivamente con la adiposidad y el tamaño de los adipocitos. Además, los macrófagos infiltrados expresan factores proinflamatorios como el factor de necrosis tumoral α (TNF-α) y la interleuquina 6 (IL-6), y activan la óxido nítrico sintasa inducible [112]. Todo ello favorece una mayor infiltración de macrófagos y una mayor respuesta inflamatoria en el tejido, con el objetivo fallido de vencer al "agente agresor".

Por otro lado, el exceso de ácidos grasos y la acumulación de triacilgliceroles promueven la formación de intermediarios lipídicos tóxicos (ceramidas, diacilgliceroles y acil-CoAs) cuya presencia desencadena una situación de estrés y, en consecuencia, una activación de las vías de señalización proinflamatoria. Dicha situación genera un incremento en la secreción de citoquinas como el TNF-α, IL-1β e IL-6, y a la vez, disfunción mitocondrial. Además, la presencia de dichos intermediarios lipídicos está asociada con daños en la vía de señalización de la insulina [113, 91].

A diferencia del tejido adiposo, el hígado no presenta infiltración de macrófagos sino que son sus propios macrófagos, las células de Kupffer, los que sufren el proceso inflamatorio [78]. Las células de Kupffer, de manera similar a los macrófagos del tejido adiposo, se activan al detectar las señales moleculares endógenas propias de un estado homeostático alterado, mediadas a través de los receptores TLR-4 (toll-like receptor 4, por sus siglas en inglés) [114]. Una vez activas y en coordinación con los hepatocitos y otras células del hígado, inician la secreción

de citoquinas, eicosanoides, enzimas proteolíticas, especies reactivas de oxígeno y óxido nítrico, y reclutan y retienen células del sistema inmunitario mediante la expresión de moléculas de adhesión, entre otros mecanismos relacionados con el sistema inmunológico [114].

La obesidad no induce una respuesta inflamatoria específica del músculo ni infiltración de macrófagos en las fibras musculares [78]. Sin embargo, los mediadores inflamatorios secretados desde el hígado o el tejido adiposo influyen en el metabolismo propio del tejido muscular y, en consecuencia, disminuyen la sensibilidad a la insulina [78, 115].

4.3. Hipoxia y estrés del retículo endoplasmático

El mayor tamaño del tejido adiposo influye directamente en el flujo sanguíneo y la irrigación del tejido, por lo que en la obesidad cabría esperar un incremento de la irrigación [116]. Sin embargo, un incremento o mantenimiento del flujo sanguíneo sobrecargaría al tejido adiposo de nutrientes forzándolo a su crecimiento hipertrófico e hiperplásico. Ante esta situación, que es considerada como una agresión, se ponen en marcha los mecanismos proinflamatorios comentados anteriormente, favoreciendo la disminución del flujo sanguíneo y, por lo tanto, limitando la llegada de nutrientes y el crecimiento descontrolado del tejido [117, 118].

La disminución del flujo sanguíneo disminuye la carga nutricional que llega a los adipocitos, pero también la oxigenación del tejido, por lo que como consecuencia de la activación de los mecanismos de defensa, se genera otro problema, la hipoxia [119]. El principal agente vasodilatador del tejido adiposo es el óxido nítrico, cuya producción se ve aumentada ante una situación de hipoxia [120]. Sin embargo, en la obesidad la respuesta vasodilatadora está deteriorada por lo que una mayor producción de óxido nítrico no se traduce directamente en vasodilatación [121]. Como consecuencia, la situación de hipoxia en el tejido se mantiene y los niveles de oxígeno disminuyen significativamente, desencadenando una respuesta glucolítica anaerobia que permita obtener energía en forma de ATP, que a la vez altera el estado redox de la célula e induce una producción masiva de lactato y acidificación del tejido [122]. Esta situación provoca la disfunción de algunos orgánulos celulares, especialmente de la mitocondria y del retículo endoplasmático [123], daña la vía de señalización de la insulina en el tejido [124] y contribuye a la secreción de citoquinas proinflamatorias [125, 126], empeorando la situación. Sin embargo, algunos estudios muestran que, a diferencia de lo que ocurre en roedores, la disfunción del tejido adiposo de humanos obesos no se correlaciona con una situación de hipoxia tisular [127, 128]. Además, estudios recientes sugieren que el tejido adiposo mantiene la vía glucolítica anaerobia activa independientemente de la presencia de oxígeno con el objetivo de limitar la lipogénesis y derivar la glucosa a fragmentos de 2 y 3 carbonos, contribuyendo así a la regulación de la glucemia [129-132].

La disfunción del retículo endoplasmático, también conocida como estrés del retículo endoplasmático, se da cuando hay un malfuncionamiento del complejo de producción de proteínas, ya sea por una síntesis excesiva de proteínas o por una acumulación de proteínas sin plegar o mal plegadas en el lumen del retículo endoplasmático [126, 133]. Esta condición genera

una respuesta celular llamada respuesta a proteínas desplegadas (UPR, por sus siglas en inglés) cuyo objetivo es restablecer la homeostasis aumentando la capacidad de plegar proteínas y disminuyendo la carga de proteínas sin plegar [134]. Cualquier condición fisiológica o patológica (modificaciones en la homeostasis del calcio, niveles insuficientes de chaperonas, cambios del estado redox celular, depleción de fosfolípidos o acumulación de colesterol) que interfiera en el proceso de plegado de proteínas del retículo endoplasmático activará dicha respuesta [133, 135]. Cuando el mecanismo UPR no funciona apropiadamente, la célula inicia el proceso de apoptosis [136]. La activación de la UPR está directamente relacionada con la inflamación y la resistencia a la insulina observada en la obesidad [133, 135]. Gran parte de las proteínas involucradas en el desarrollo de la UPR también tienen capacidad de activar el factor de necrosis κB (NFκB) y las quinasas c-jun N-terminal (JNK), cuya señalización propicia la síntesis de citoquinas proinflamatorias. Al mismo tiempo la presencia de citoquinas inflamatorias empeorará el estrés del retículo endoplasmático induciendo y facilitando el mantenimiento de la inflamación [137].

En el tejido adiposo, el estrés del retículo endoplasmático modula la capacidad lipolítica del tejido, al disminuir la síntesis de perilipina A. Además, participa en la desregulación de la secreción de adipoquinas, disminuyendo la secreción de leptina y adiponectina y a la vez, aumentando la producción de IL-6 [138].

En el hígado, el estrés de retículo endoplasmático juega un papel importante en el desarrollo de la resistencia a la insulina mediante distintos mecanismos: 1) la transcripción de enzimas relacionadas con las vías gluconeogénicas y lipogénicas se ve directamente alterada por la activación de la UPR; 2) las quinasas involucradas en la activación de la UPR interfieren en la señalización de la insulina a través de la activación de JNK e IKK (IκB kinase, por sus siglas en inglés); 3) la acumulación de lípidos en los hepatocitos se ve inducida por la UPR mediante la activación de SREBP-1c (proteína de unión al elemento regulador de esteroles-1c, por sus siglas en inglés), contribuyendo indirectamente a la resistencia a la acción de la insulina [133, 138, 139].

4.4. Resistencia a la acción de la insulina

La insulina es una hormona anabólica cuya función principal es la de regular y mantener la glucemia, cuyo rango de normalidad, en ayunas, se establece entre 4 y 7 mM [140]. Tras la ingesta de carbohidratos la glucosa circulante incrementa y, en respuesta, las células β de los islotes pancreáticos de Langerhans secretan insulina. La presencia de insulina promueve la translocación de transportadores de glucosa sensibles a insulina (GLUT4) desde el espacio intracelular hasta la membrana plasmática [141], facilitando así la captación de la glucosa circulante por tejidos periféricos como el músculo y el tejido adiposo [140, 142]. En presencia de insulina circulante, la captación de glucosa por estos tejidos incrementa. Además, se activa la síntesis de glucógeno en el hígado y músculo, proceso favorecido por la desfosforilación y consiguiente activación de la glucógeno sintasa [143, 144]. La insulina bloquea la actividad de la glucógeno sintasa quinasa 3, que fosforilaría la glucógeno quinasa y activa la proteína fosfatasa

1, encargada de la desfosforilación de la glucógeno quinasa [145]. Además la insulina regula directamente, inhibiendo las enzimas hepáticas involucradas en la gluconeogénesis y glucogenólisis, también por procesos de fosforilación/desfosforilación, así como su expresión génica [146]. En relación al metabolismo de lípidos, la insulina ejerce un efecto lipogénico, promoviendo su síntesis (lipogénesis) e inhibiendo su degradación (lipólisis) mediante la activación de SREBP-1c [147].

La falta de respuesta fisiológica ante la insulina está altamente asociada a una situación de exceso energético crónico, con una sobrecarga simultánea de nutrientes circulantes en sangre (glucosa, ácidos grasos libres, lípidos) [148].

La resistencia a la insulina es un estado fisiopatológico en el que, ante la presencia de niveles normales de insulina circulante, la respuesta celular es insuficiente, produciéndose una afectación en su acción reguladora de la glucemia. En otras palabras, la resistencia a la insulina se da cuando hay un defecto en la señalización de la insulina. En muchos casos, la alteración de la vía de señalización de la insulina involucra a su propio receptor, que puede verse alterado a nivel de expresión, de unión al sustrato, del estado de fosforilación y de su actividad quinasa [141]. En condiciones fisiológicas normales, la insulina desencadena cambios metabólicos mediante la activación de una cascada de señalización intracelular basada en la fosforilación de proteínas [141]. Cuando la insulina se une a su receptor este se activa y una tirosina quinasa fosforila los sustratos del receptor de la insulina (IRS, por sus siglas en inglés). Dicha fosforilación recluta proteínas que contienen dominios SH2 (del inglés, Src Homology 2) como la fosfatidil inositol 3,4,5 quinasa, las cuales pueden activar las dos vías principales de señalización de las proteínas quinasa: la vía mediada por la serina-treonina quinasa Akt que coordina y regula el tráfico vesicular, la síntesis de proteínas y la recaptación de glucosa; y la vía de la MAPK/ERK, que tiene un papel en el control del crecimiento celular y la expresión génica. Además, la insulina también puede unirse al receptor de IGF-1 y viceversa, aunque la afinidad de la insulina es entre 10 y 100 veces menor [149] (Figura 6).

El músculo tiene un papel de gran importancia en la regulación del metabolismo de la glucosa y la utilización de lípidos, puesto que el 40% de la masa corporal corresponde al músculo esquelético [150]. La acumulación excesiva de lípidos en los miocitos está directamente relacionada con el desarrollo de resistencia a la acción de la insulina y diabetes de tipo 2 [151]. La acumulación de triacilgliceroles y la elevada presencia de ácidos grasos saturados promueven la formación de intermediarios lipídicos tóxicos como los diacilgliceroles y las ceramidas, cuya presencia influye negativamente en la señalización de la insulina [152, 153]. Los diacilgliceroles activan la proteína quinasa C, que a su vez, activa la fosforilación de IRS-1 [154], mientras que las ceramidas inhiben la actividad Akt resultando en la activación de FoxO1 (del inglés, Forkhead box protein O1) e hiperglucemia [155].

En el tejido adiposo la señalización de la insulina se ve afectada por el estado de inflamación crónica; la secreción de TNF-α por los macrófagos infiltrados incrementa, hecho que desencadena la activación de quinasas de señalización intracelular: JNK e IKK, promoviendo la

fosforilación en residuos de serina de IRS-1 [156, 157], inhibiéndose la cascada de fosforilaciones que permite la traslocación a la membrana de GLUT4, generando así resistencia a la acción de la insulina en el tejido adiposo, músculo y otros tejidos [157, 158].

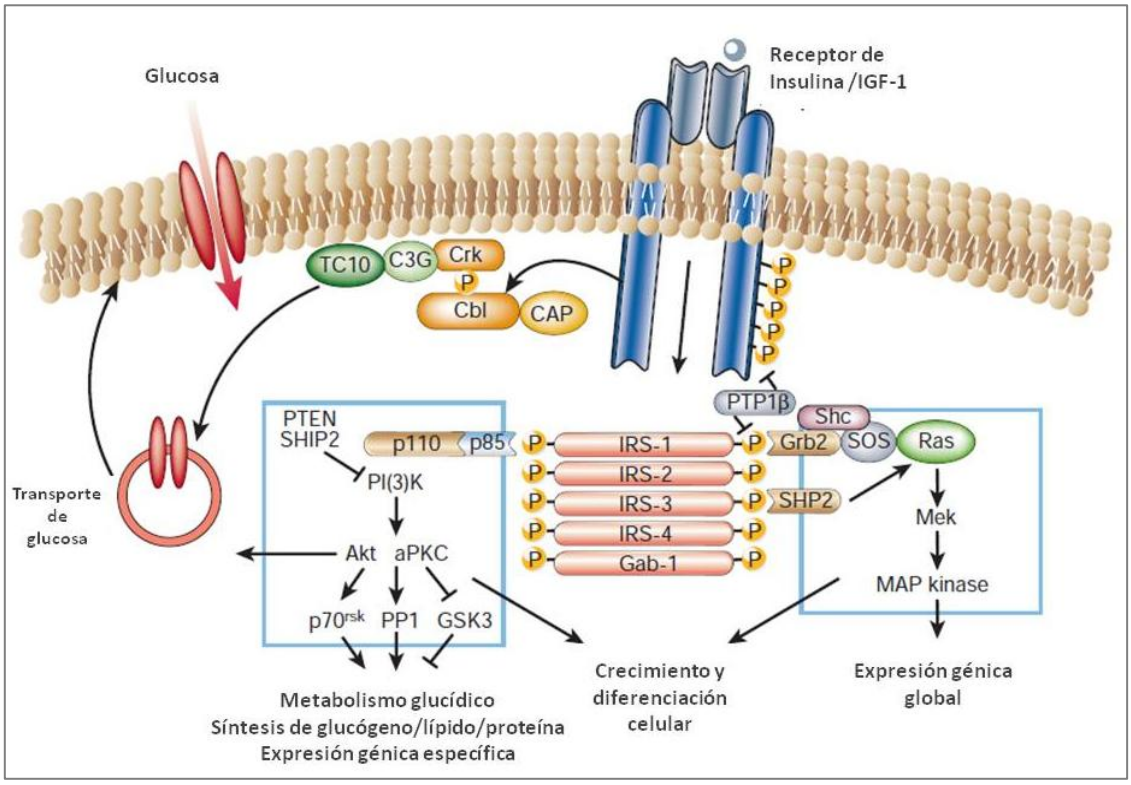

Figura 6. Señalización de la insulina y sus efectos. El receptor de la insulina es una tirosina quinasa que se autofosforila y cataliza la fosforilación de otras proteínas celulares como las IRS. La fosforilación de los residuos de tirosina de estas proteínas permite iniciar una interacción mediante sus dominios SH2 con otras moléculas señalizadoras, resultando en la activación de diversas cascadas de señalización y en definitiva regulando el metabolismo glucídico, lipídico y proteico. Adaptado de Saltiel *et al.* 2001 [140].

Como se ha comentado anteriormente en el apartado 4.3, el estrés del retículo endoplasmático también es un factor contribuyente a la resistencia a la insulina, mediante distintos mecanismos conectados con la activación de la UPR.

La señalización de la insulina también parece estar afectada por varios cambios a nivel intracelular que contribuyen al desarrollo de la resistencia a la acción de la insulina: cambios en el perfil lipídico celular (incremento de ceramidas [155, 159] y diacilgliceroles [160]), estrés del retículo endoplasmático [135], alteración de la función mitocondrial [161], y el incremento de daños mediados por la generación de especies reactivas de oxígeno [162, 163]. Dichos cambios podrían ser propiciados directamente como consecuencia de la dieta o indirectamente por cambios en el microambiente tisular (por ejemplo, hipoxia [124] e inflamación [164]). Por ello, algunos autores sugieren que la resistencia a la acción de la insulina es un desorden altamente heterogéneo.

5. COMPOSICIÓN DE LAS DIETAS EN FUNCIÓN DE SU CONTENIDO EN MACRONUTRIENTES Y SU USO COMO MODELOS DE ESTUDIO

La intervención dietética para el control de peso o de patologías de origen metabólico se lleva aplicando desde el siglo pasado. En el ámbito clínico, la intervención dietética es comúnmente usada como tratamiento de primera elección (previa a la intervención farmacológica y/o quirúrgica) para la prevención y manejo de condiciones fisiopatológicas como la obesidad, hipertensión, enfermedad cardiovascular, insuficiencias e intolerancias. En el ámbito académico y de investigación, el uso de dietas específicas está cada vez más extendido, siendo el impacto de la composición nutricional en diferentes aspectos (metabólicos, farmacológicos, neurológicos, etc.) su objeto de estudio principal.

La presencia de diferencias significativas en la composición de macronutrientes (lípidos, carbohidratos y proteínas) puede generar una gran variedad de efectos; variaciones en el peso corporal, influencia en la liberación de neuropéptidos gastrointestinales que modulan la saciedad y la ingesta, variaciones a nivel metabólico y también neurocognitivo. Por ello, la comunidad científica centrada en la investigación básica emplea de manera usual modelos animales expuestos a distintas dietas para evaluar sus efectos moduladores en una gran variedad de aspectos fisiológicos y patológicos.

La dieta es uno de los factores más relevantes asociados con el desarrollo de obesidad y del síndrome metabólico. El contenido nutricional y energético de la dieta humana ha sufrido cambios considerables a lo largo de la historia. Así, la dieta correspondiente a la época del Paleolítico era claramente hipocalórica, basada en plantas silvestres y animales de tamaño relativamente pequeño, con bajo contenido lipídico y sin sal [165]. Sin embargo, en los llamados países desarrollados la dieta actual se compone de una gran variedad de alimentos a nuestra elección, cuyo contenido en proteína de alta calidad, azúcares, lípidos y sal es, generalmente, elevado. Este tipo de dieta favorece el incremento de peso y el desarrollo de obesidad junto con las complicaciones metabólicas asociadas.

Por otro lado, las propiedades organolépticas como el sabor, olor, textura y aspecto juegan un papel importante en la elección de los alimentos. En la actualidad encontramos mucha variedad de alimentos, concentrados en establecimientos específicos, de obtención rápida y mayormente sabrosos, hecho que favorece la acción de comer [166]. Además, el estilo de vida actual incluye una gran variedad de situaciones estresantes, las cuales favorecen los comportamientos motivados por la recompensa, que promueven la obesidad a la vez que debilitan el autocontrol. De hecho, los individuos tienden a buscar alimentos sabrosos y densos en energía para combatir el estrés [167, 168], acto llamado alimentación emocional.

El uso de dietas como modelo de estudio tiende a adaptarse y a mimetizar la composición dietética actual de la dieta humana, con el objetivo de encontrar paralelismos y oportunidades para una aplicación translacional. En cuanto al control de peso y al tratamiento de la obesidad, la introducción de cambios en la composición de la dieta es generalmente la opción más

empleada. No obstante, el éxito de la intervención dietética como tratamiento único o en conjunto con el ejercicio está todavía en duda.

5.1. Lípidos y dietas hiperlipídicas

Los lípidos (aceites y grasas) son los macrocomponentes de la dieta que mayor energía aportan (39,4 kJ/g). Incluyen triacilgliceroles, fosfolípidos y esteroles, siendo los triacilgliceroles el representante lipídico más abundante en los alimentos y en el organismo. Los ácidos grasos que componen los triacilgliceroles se clasifican en función de la longitud de la cadena (corta, media o larga), el grado de insaturación (saturados, monoinsaturados o poliinsaturados) y la forma (cis o trans).

La ingesta de grasa está directamente relacionada con el incremento de peso y de las reservas de grasa corporal debido a su alto contenido energético [169]. Con el objetivo de mimetizar el exceso de ingesta de grasas y la consecuente aparición de obesidad o complicaciones cardiovasculares que se da actualmente en humanos, las dietas hiperlipídicas han sido ampliamente utilizadas en modelos animales desde la década de los 40 [170], para generar un modelo de obesidad y así profundizar en el conocimiento de las condiciones que la generan, así como del impacto metabólico de dicho estado patológico [171, 172].

Existe gran variedad de dietas hiperlipídicas, teniendo todas ellas en común un contenido lipídico y energético elevado, aunque la proporción de la energía de la dieta proveniente de lípidos pueda ser variable (entre el 20 y 70%) y pueda contener grasas de distinta naturaleza (origen vegetal o animal), afectando a su composición [173-175]. En cualquier caso, el porcentaje de energía derivada de lípidos de estas dietas es superior al estándar requerido, por lo que su ingesta generará un desequilibrio del balance energético favoreciendo el incremento de peso y del contenido de grasa corporal.

Los efectos metabólicos de las dietas hiperlipídicas pueden ser variables en función de varios factores: la composición lipídica, el periodo de exposición, la edad, el género, la especie y cepa del modelo animal [172, 176, 177]. Sin embargo, está bien descrito que una dieta cuyo contenido lipídico sea superior al 30% (equivalente a aproximadamente el 50% del aporte calórico) es obesogénica, eleva la glucemia basal y posprandial [178], genera hipertrigliceridemia posprandial, aumenta el número y tamaño de los adipocitos [179], induce resistencia a la acción de la insulina [180], eleva los niveles de leptina [181], disminuye los niveles de adiponectina [182], inhibe la lipólisis [183] y genera esteatosis hepática [184] e inflamación [185].

Uno de los factores más importantes a tener en cuenta a la hora de crear un modelo de estudio o de generar unos efectos fisiopatológicos concretos, es el tipo de lípido que tendrá mayor presencia en la dieta. Los ácidos grasos saturados, como el ácido palmítico y el ácido láurico inducen la activación de la vía IKK/NF-kB a través del receptor TLR4 [186, 187], propiciando la liberación de citoquinas proinflamatorias por el tejido adiposo y contribuyendo al riesgo del desarrollo de enfermedades inflamatorias relacionadas con el síndrome metabólico [188]. Los

ácidos grasos monoinsaturados, en cambio, están asociados con un perfil antiinflamatorio [108, 189] y los poliinsaturados, como el ácido linoleico [187] y el ácido docosahexaenoico [186], parecen ejercer un papel protector ante el desarrollo de la obesidad y sus complicaciones asociadas [190, 191]. Por ello, en modelos de inducción de obesidad, resistencia a la acción de la insulina o síndrome metabólico es muy común el uso de dietas ricas en ácidos grasos saturados [139, 192].

La ingesta de grasa también tiene un papel modulador sobre el control de la ingesta mediante la liberación de péptidos saciantes como la colecistoquinina, el GLP-1, o el PYY a nivel gastrointestinal, así como enlenteciendo el vaciado gástrico y la absorción [193], propiciando de esta manera la inhibición de la ingesta a corto plazo. Los efectos son diferentes en función del tipo de ácido graso; los ácidos grasos de cadena larga estimulan la liberación de colecistoquinina y PYY, se absorben y entran en circulación en los *quilomicra*, evitando la oxidación hepática y aumentando la probabilidad de acumulación periférica. Los ácidos grasos de cadena media se absorben y llegan al hígado directamente por la vena porta siendo rápidamente oxidados. Entre los ácidos grasos de cadena media encontramos los ácidos grasos saturados, que tienen un mayor efecto saciante [85].

Además, las dietas ricas en grasas se han aplicado en el estudio de otros aspectos, como por ejemplo la capacidad reproductora, el desarrollo cognitivo y la carcinogénesis. La dieta hiperlipídica parece tener efectos negativos destacables a nivel neurocognitivo; en ratas, induce la apoptosis en neuronas hipotalámicas mediante la transducción de la señal inflamatoria [194], y se correlaciona con el deterioro del aprendizaje y memoria [195]. Además, en roedores un alto consumo de grasa afecta la capacidad reproductora y el mantenimiento durante el periodo perinatal [196], así como favorece la aparición de tumores en las mamas [197].

El uso de dietas hiperlipídicas en modelos animales de investigación está muy extendido; sin embargo, la mayoría de las dietas no son estrictamente hiperlipídicas y en muchas ocasiones también contienen un excedente de mono y disacáridos. Por esta razón, la comparación de los efectos desencadenados por la grasa dietética en dietas bien definidas puede confundirse con los efectos generados por otros componentes de la dieta, que en ocasiones no están definidos o contemplados en el diseño experimental [174].

5.2. Carbohidratos y dietas hiperglucídicas

Los carbohidratos son el sustrato energético principal para el organismo, con un aporte energético de 15,6 kJ/g. Entre los carbohidratos se distinguen tres grandes categorías: fibra, azúcares simples y almidones, los cuales generan distintos efectos fisiológicos debido a su digestibilidad.

La fibra (soluble e insoluble) es un conjunto de carbohidratos complejos indigeribles por los humanos; sin embargo, tiene efectos beneficiosos en cuanto al control de la glucemia y perfil lipídico, la inducción de saciedad y el mantenimiento del equilibrio de la microbiota intestinal [198]. La fibra soluble forma un gel en el tracto intestinal que provoca un retraso en la digestión y

absorción de otros macronutrientes favoreciendo la distensión gástrica, la cual genera una respuesta aferente del nervio vago y contribuye a la disminución de la ingesta [199]. La ingesta de fibra también tiene efectos en la saciedad mediante la secreción de incretinas y la producción de ácidos grasos de cadena corta como consecuencia de la fermentación bacteriana del intestino grueso [200].

Entre los azúcares distinguimos los monosacáridos como la glucosa, fructosa y galactosa, y los disacáridos como la sacarosa, maltosa y lactosa. Todos ellos son de fácil digestibilidad y absorción, por lo que favorecen el rápido incremento de la glucemia y, en consecuencia, la liberación de insulina, la cual favorece la activación de las vías lipogénicas y la acumulación de lípidos en el tejido adiposo, a la vez que contribuye a la activación de la saciedad [201].

Las dietas hiperglucídicas pueden ser muy variables puesto que dependen del tipo de glúcido que contengan en exceso. Las dietas con un elevado contenido de sacarosa son las utilizadas por excelencia como modelo de desarrollo de obesidad y diabetes tipo 2 [202]. Sin embargo, su capacidad para generar obesidad o resistencia a la acción de la insulina no solo depende de las especies o cepas animales sino que en gran medida depende de la forma en la que el azúcar es aportado (bebida o alimento sólido).

En roedores, un elevado contenido de sacarosa en la dieta genera daños en la vía de señalización de la insulina a través de sus efectos en el hígado [203], de manera independiente a los cambios en la composición corporal. De hecho, aunque el exceso de azúcar no altera la cantidad de IRS-1 o IRS-2 o de PI3K en los hepatocitos, sí que reduce la fosforilación de IRS-1 e IRS-2, hecho que indica un daño a nivel de señalización tras la unión de la insulina [204]. A nivel de músculo, el contenido elevado de sacarosa reduce la cantidad de receptores de insulina y la consiguiente fosforilación de IRS-1 [205]. Además, el uso de dietas ricas en fructosa (provenientes del jarabe de maíz) es muy común para inducir resistencia a la acción de la insulina, debido a su efecto reductor de la sensibilidad a la insulina en un periodo de tiempo corto [206]. En roedores, este tipo de dietas generan múltiples síntomas del síndrome metabólico (resistencia a la acción de la insulina, hiperinsulinemia, hipertensión e hipertrigliceridemia) [207]. Además, el consumo crónico propicia un incremento de peso, probablemente debido a un incremento de la palatabilidad que en definitiva genera hiperfagia [208].

Generalmente las dietas hiperglucídicas no se usan como tales, sino que comúnmente se usan en combinación con un elevado contenido lipídico, generando así una dieta hiperenergética. La problemática para definir cada uno de estos casos viene dada por la gran variedad de composiciones y proporciones que se encuentran en la literatura, por lo que los efectos descritos podrían ser no comparables.

5.3. Proteínas y dietas hiperproteicas

Las proteínas son un sustrato cuyo aporte energético teórico es de 22,1kJ/g, aproximadamente. Su oxidación para la obtención de energía solo se da cuando está en exceso de forma continua (como en el caso de los animales carnívoros) o bien cuando no hay otro sustrato que oxidar (por ejemplo en ayunas o en dietas hipocalóricas) [209, 210]. Por otro lado, en una dieta no solo es necesario considerar la cantidad de proteínas que aporta dicha dieta, sino también la calidad de la proteína, siendo aquellas de mala calidad las que presentan un bajo grado de digestibilidad y absorción a nivel de intestino [211] y/o una composición inadecuada de aminoácidos, en especial de aminoácidos esenciales.

Las dietas hipocalóricas, es decir, las dietas cuyo aporte energético es bajo, suelen emplearse como tratamiento dietético para la pérdida de peso [212, 213]. Sin embargo, también propician la pérdida de masa magra, pues las proteínas que contienen se usan como sustrato principal para mantener la glucemia [214]. Con el objetivo de evitar la pérdida de masa muscular, dichas dietas tienden a incorporar un exceso proteico que, al fin y al cabo, propician el uso de aminoácidos como sustrato energético principal y el incremento de la oxidación de las reservas lipídicas [215], aunque la preservación de la masa magra puede verse comprometida.

Se considera que una dieta es hiperproteica cuando el contenido en proteína es superior a los requerimientos estándar (10-15% de la energía), cuando excede el 20% del total de energía ingerida, o en términos absolutos, es superior a 60–75 gramos de proteína al día (en el caso de los humanos) [215, 216]. El exceso de proteína procedente de la dieta genera un exceso de nitrógeno en el organismo que es fácilmente eliminable por excreción urinaria en forma de urea, por lo que un incremento de la ingesta proteica no conlleva necesariamente un problema cuando el aporte energético total es adecuado. Sin embargo, las dietas hiperproteicas pueden conllevar complicaciones a nivel renal; algunos estudios muestran alteraciones en la hemodinámica y la excreción renal a corto plazo [215, 217].

Una mayor ingesta de proteína, en comparación con la ingesta de carbohidratos o lípidos, incrementa en mayor medida el gasto energético en el periodo posprandial mediante la inducción de la termogénesis [218, 219]. La proteína tiene un poder saciante elevado [82], que favorece la regulación de la ingesta mediante la secreción de péptidos anorexigénicos como la colecistoquinina, GLP-1 y PYY [220]. Adicionalmente, las dietas hiperproteicas generan un incremento de la oxidación de grasa y, en consecuencia, de los valores de los cuerpos cetónicos, especialmente del β-hidroxibutirato circulante que contribuye al efecto supresor del apetito [221]. Por otro lado, el tipo y la calidad de proteína podrían tener un papel importante en el efecto saciante, directamente relacionado con la concentración y proporción de aminoácidos circulantes en sangre [218]. Ante la sobrecarga de aminoácidos circulantes, algunos estudios sugieren que las dietas hiperproteicas estimulan la síntesis de glucosa a partir de sustratos con potencial glucogenogénico para hacer frente al exceso de aminoácidos y ajustar la homeostasis de aminoácidos y glucosa [214].

Además de la reducción de peso y masa grasa, el uso de las dietas hiperproteicas propician mejoras a nivel metabólico y del riesgo cardiovascular asociado a la obesidad: mejora de la función endotelial, la inflamación sistémica y la sensibilidad a la insulina [212, 213, 222, 223]. No obstante, algunos estudios discrepan y sugieren que la ingesta elevada de proteína no tiene efectos en relación a los marcadores de inflamación [224] y que también puede conllevar el desarrollo de resistencia a la acción de la insulina [225, 226].

En definitiva y a pesar de las controversias, las dietas hiperproteicas siguen siendo la intervención dietética de primera opción para la reducción de peso corporal y tratamiento de la obesidad a corto y medio plazo [212, 227], aunque algunos autores ponen en entredicho su uso a largo plazo, especialmente si tienen un bajo contenido en carbohidratos, debido a su naturaleza cetogénica [228].

5.4. Dieta de cafetería

Las dietas hiperenergéticas son aquellas que conllevan una elevada ingesta de energía en la dieta, que puede llegar a ser superior al gasto energético, por lo que pueden provocar un desequilibrio energético positivo y propiciar el incremento de reservas de grasa y de peso corporal [229]. Cualquier dieta hiperlipídica o hiperglucídica podría considerarse hiperenergética dado su elevado aporte energético, por lo que las dietas que combinan ambos aspectos son ampliamente utilizadas en modelos animales para el estudio de la obesidad o complicaciones asociadas.

La dieta de cafetería se caracteriza por ser una dieta apetitosa y compuesta por una gran variedad de ingredientes; su consumo altera el control de la ingesta induciendo hiperfagia [230, 231] y contribuyendo al incremento de peso y de grasa corporal [232, 233]. El almacenamiento de las reservas lipídicas se da de manera similar en tejidos especializados (tejidos adiposos) y no especializados (hígado y músculo) [97]. Como consecuencia de la elevada ingesta de glúcidos y lípidos, y en definitiva de la energía ingerida [234], genera los estados fisiopatológicos característicos del síndrome metabólico: obesidad, hiperglucemia, resistencia a la acción de la insulina, inflamación y dislipidemia [235-237]. Por este motivo, junto con la similitud que presenta con los patrones alimentarios de los humanos de los países desarrollados [238], es ampliamente usada en modelos animales para el estudio del síndrome metabólico y la obesidad [176, 239].

La hiperfagia inducida por la dieta de cafetería implica la ingesta de elevadas cantidades de glúcidos, lípidos y también proteínas, por lo que podría considerarse también como una dieta hiperproteica en comparación con las dietas estándar. Sin embargo, el exceso de proteína en combinación con el exceso energético compromete la excreción de amonio [240, 241] el intercambio de glutamina y la funcionalidad del ciclo de la urea [242, 243]. El destino del exceso amínico en condiciones de superávit energético todavía permanece en duda, por lo que el término “Nitrogen Gap” descrito décadas atrás [244, 245] permanece vigente (ver apartado 6.3).

Así pues, la dieta cafetería resulta ser una dieta muy efectiva para crear un modelo de rata con síndrome metabólico e inflamación, en un periodo de tiempo relativamente corto. Este modelo permite, además, determinar el potencial de la reversibilidad de patologías metabólicas y cardiovasculares a lo largo de la vida [237].

6. METABOLISMO NITROGENADO EN EL CONTEXTO DEL SÍNDROME METABÓLICO

El metabolismo de los aminoácidos, en comparación con el metabolismo de lípidos y carbohidratos, es el aspecto del metabolismo menos estudiado hasta la fecha. Por ello, el conocimiento de ciertos aspectos sigue siendo limitado a pesar de que las proteínas son esenciales para el crecimiento, mantenimiento y recambio de estructuras, incluso, tras aplicar intervenciones nutricionales para el tratamiento de la obesidad. Algunos de los factores contribuyentes al desconocimiento del metabolismo nitrogenado y su regulación residen en la gran variedad de especies moleculares implicadas, su fácil interconversión, las múltiples vías catabólicas y las dificultades metodológicas para su análisis [246].

Las proteínas se forman por una combinación de veinte aminoácidos, aunque en el organismo también encontramos otros aminoácidos como producto de diversas reacciones; cada uno de estos aminoácidos tiene su propia vía de síntesis y de degradación, a excepción de los aminoácidos esenciales, los cuales no pueden ser sintetizados por el organismo humano, por lo que deben obtenerse a través de la dieta. Por otro lado, algunos aminoácidos se consideran condicionalmente esenciales, pues en determinadas situaciones o patologías su síntesis puede verse limitada y no alcanzar los requerimientos del organismo. Del mismo modo que los carbohidratos y los lípidos, los aminoácidos también pueden ser oxidados para la obtención de energía; no obstante, en condiciones normales su finalidad principal no es la producción de energía y por ello la proteína corporal está muy preservada [247].

Los aminoácidos pueden formar parte de las proteínas, o bien, encontrarse libres, siendo captados de la circulación por los tejidos a través de mecanismos de transporte específicos [248]. El músculo esquelético es el órgano que más carga proteica presenta, pues representa aproximadamente un 40% del peso corporal, del cual un 15% son proteínas. El hígado desempeña un papel importante en su metabolismo dado que es el órgano que recibe, a través de la vena porta, los aminoácidos procedentes de la dieta, absorbidos en el intestino; también es el órgano en el que se dan gran parte de las interrelaciones metabólicas con el metabolismo de carbohidratos y la síntesis de urea.

6.1. Biosíntesis de urea. Ciclo de la urea

El nitrógeno amínico proveniente de la utilización de aminoácidos con fines energéticos, así como el que se encuentra en exceso, puede resultar tóxico para el organismo, por lo que debe ser excretado. Dicho nitrógeno se excreta en forma de urea, un compuesto polar, altamente soluble en agua y no tóxico. Aproximadamente el 90 % de la urea se elimina a través de la

orina tras la filtración glomerular del riñón, aunque en pequeña proporción se excreta a través del sudor, saliva y heces. En humanos, la presencia de urea en plasma es relativamente baja (aproximadamente 5 mM), mientras que la concentración en orina puede ser hasta más de 60 veces mayor [249].

La biosíntesis de urea se da mediante un sistema enzimático conocido como el ciclo de la urea, que se expresa principalmente en el hígado, aunque también se ha detectado en el intestino [250]. Recientemente, se ha descrito en el tejido adiposo blanco la presencia de un ciclo de la urea completo, plenamente funcional [251]. En algunos tejidos como el riñón, cerebro, glándula mamaria y endotelio, entre otros, sólo se expresan parte de las enzimas del ciclo (principalmente de las enzimas que se encuentran en el citoplasma celular), siendo su sentido fisiológico la síntesis de poliaminas, óxido nítrico y la conversión de arginina a ornitina [249].

La biosíntesis de urea consiste en 5 reacciones en las que participan 5 enzimas con distinta localización subcelular (Figura 7 y Tabla 2): carbamoil fosfato sintetasa, ornitina transcarbamilasa, argininosuccinato sintetasa, argininosuccinato liasa, y arginasa.

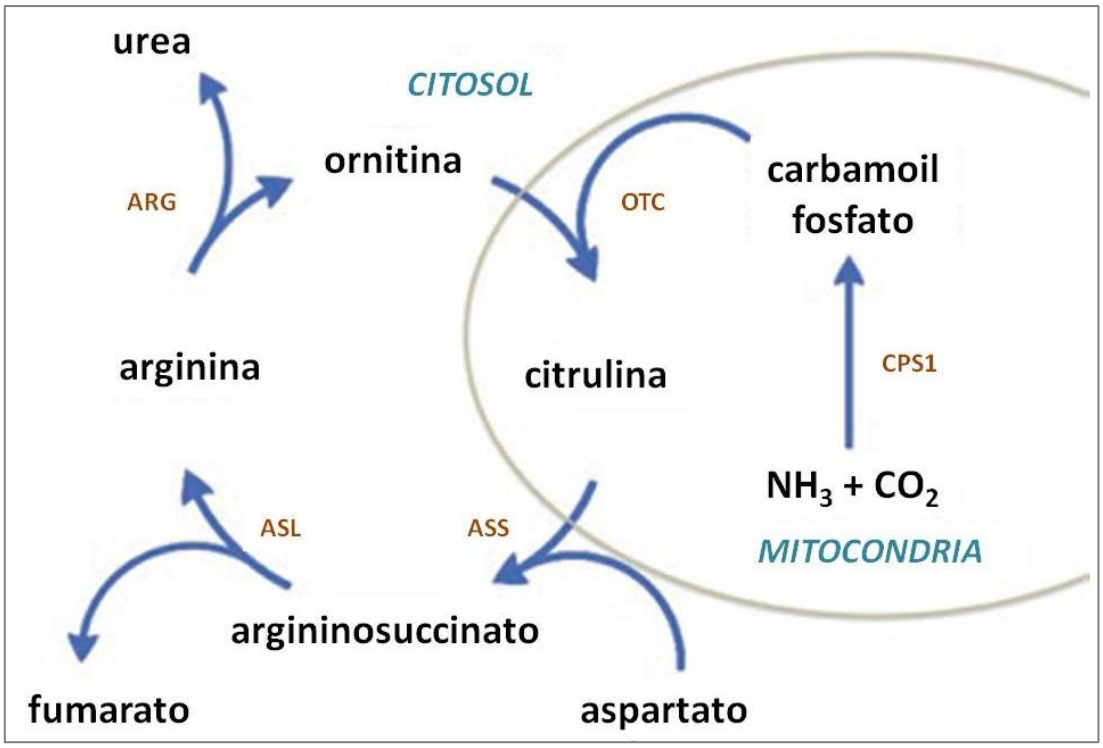

Figura 7. Ciclo de la urea. CPS1: carbamoil fosfato sintetasa de tipo 1; OTC: ornitina transcarbamilasa; ASS: argininosuccinato sintetasa; ASL: argininosuccinato liasa; ARG: arginasa. Adaptado de Wang, H *et al.* 2014 [249].

La carbamoil fosfato sintetasa cataliza la síntesis del carbamoil fosfato mediante una reacción dependiente de ATP. Existen dos isoformas diferentes en función de su localización celular y de la fuente de nitrógeno que usan como sustrato; la carbamoil fosfato sintetasa de tipo 1 (EC 6.3.4.16) que utiliza amonio como sustrato [252], se localiza en la matriz mitocondrial y requiere la presencia de N-acetil-L-glutamato, que actúa como activador alostérico. La carbamoil

fosfato sintetasa de tipo 1 aporta uno de los dos nitrógenos que se incorporarán para dar urea, por lo que es una enzima crítica en el control de la eliminación de nitrógeno amínico a través del ciclo de la urea [253]. Esta isoforma está presente en el hígado e intestino [254]. En cambio, la carbamoil fosfato sintetasa de tipo 2 (EC 6.3.5.5), que utiliza glutamina como sustrato, se localiza en el citoplasma y requiere de Zn^{2+} como cofactor. Esta isoforma se encuentra en todos los tejidos de mamíferos de forma ubicua y cataliza las tres primeras reacciones de la vía de síntesis de pirimidinas, por lo que se considera una enzima externa al ciclo de la urea [255].

La ornitina transcarbamilasa (EC 2.1.3.3) cataliza la formación de citrulina a partir de carbamoil fosfato y L-ornitina. Esta enzima es activa en tejidos como el hígado [242], intestino delgado, pulmón [256] y tejido adiposo blanco [251].

En cuanto a la argininosuccinato liasa (EC6.3.4.5) y la argininosuccinato sintetasa (EC4.3.2.1), son las responsables de la biosíntesis de arginina a partir de citrulina y aspartato. En el caso de la argininosuccinato sintetasa, está presente en la mayoría de tejidos aunque su mayor expresión se da en el hígado y el riñón [257]. La argininosuccinato liasa es un homotetrámero que se expresa en diversos tejidos y que cataliza la descomposición reversible del L-argininosuccinato, producido por la acción de la argininosuccinato sintetasa, en arginina y fumarato. Aunque ambas enzimas tienen un importante papel regulador del funcionamiento del ciclo de la urea, pues son las responsables de aportar el segundo átomo de nitrógeno para la síntesis de urea, la reacción limitante del ciclo recae en la acción de la argininosuccinato sintetasa, cuya expresión es modulada por factores hormonales y nutricionales [257].

Enzimas e isoformas del ciclo de la urea y localización

Enzima	Gen	Localización celular	Sustrato(s)	Producto(s)	Presencia en tejidos
CPS1	*Cps1*	Matriz mitocondrial	Amonio	Carbamoil fosfato	Hígado Intestino
CPS2	*Cad*	Citoplasma	Glutamina Bicarbonato	Carbamoil fosfato	Ubícua
OTC	*Otc*	Matriz mitocondrial	Carbamoil-fosfato L-ornitina	L-citrulina Fosfato inorgánico	Hígado Intestino delgado Pulmón Adiposo blanco
ASS	*Ass*	Citoplasma	L-aspartato L-citrulina	L-arginino succinato	Ubicua
ASL	*Asl*	Citoplasma	L-Arginino succinato	L-arginina Fumarato	Ubicua
ARG1	*Arg1*	Citoplasma	L-arginina	L-ornitina Urea	Hígado
ARG2	*Arg2*	Matriz mitocondrial	L-arginina	L-ornitina Urea	Riñón

Tabla 2. Características de las enzimas del ciclo de la urea.

La enzima encargada del último paso del ciclo es la arginasa (EC 3.5.3.1.), responsable de hidrolizar la L-arginina a L-ornitina y urea. En mamíferos podemos distinguir 2 isoformas, la tipo 1 y la tipo 2, que aunque catalizan la misma reacción difieren en su localización celular, distribución tisular, regulación, expresión y función fisiológica [258]. La isoforma mayoritaria es la arginasa 1, cuya expresión se da principalmente en el hígado, como componente del ciclo de la urea, aunque también se ha detectado en piel, médula ósea y, recientemente, en el tejido adiposo [251, 259]. Por el contrario, la arginasa 2 se expresa en tejidos extrahepáticos y parece tener un papel en la regulación de los niveles intracelulares de arginina y ornitina.

La arginina es un aminoácido con múltiples destinos metabólicos posibles. No sólo es metabólicamente interconvertible con los aminoácidos prolina y glutamato, sino que también sirve como precursor para la síntesis de proteínas, óxido nítrico, creatina, poliaminas, agmatina y urea. La mayoría de estas actividades (síntesis de prolina, glutamato, poliaminas y urea) requieren la acción de la arginasa [259].

La arginina puede ser oxidada a través de la acción de la familia de enzimas de la óxido nítrico sintasa [260, 261]. El óxido nítrico es un regulador crítico de la función de las células y los tejidos, con múltiples acciones biológicas [262], siendo la regulación del flujo sanguíneo la función más conocida. Otras funciones pleiotrópicas incluyen la regulación de la transmisión sináptica a largo plazo, el aprendizaje, la memoria, la agregación plaquetaria, las interacciones leucocitarias y endoteliales, la función inmunitaria, la angiogénesis y la arteriogénesis. La biodisponibilidad del óxido nítrico depende del equilibrio entre su generación y su degradación. Además, aunque la afinidad de las óxido nítrico sintasas por la arginina es aproximadamente 1000 veces mayor que la afinidad de la arginasa, la Vmax de la arginasa es aproximadamente 1000 veces mayor que la de las óxido nítrico sintasas [263]. Así, en principio, la arginasa debería poder competir eficazmente con las óxido nítrico sintasas por el sustrato, limitando la producción de óxido nítrico. Esto se ha confirmado experimentalmente ya que la inhibición de la actividad de la arginasa da lugar a un aumento de la producción de óxido nítrico. [264, 265].

Por otro lado, cabe enfatizar que la ornitina producida por la arginasa dentro del ciclo de la urea está esencialmente restringida al reciclaje dentro del propio ciclo, probablemente no siendo una fuente importante de sustrato para la ornitina decarboxilasa (vía de la síntesis de poliaminas) o de la ornitina aminotransferasa (síntesis de prolina y glutamato) [259].

Además de las 5 enzimas descritas anteriormente, varias proteínas participan en el proceso para el correcto funcionamiento del ciclo *in vivo*: glutaminasa, glutamato deshidrogenasa, N-acetilglutamato sintetasa, transportador mitocondrial de aspartato/glutamato y transportador mitocondrial de ornitina/citrulina [266-269].

6.2. Regulación del ciclo de la urea

En mamíferos, el significado biológico del ciclo de la urea es convertir el amonio citotóxico en un compuesto menos tóxico (urea) [270], que no puede ser metabolizado en ningún tejido, por lo que ha de ser excretado. Puesto que la presencia de urea inhibe la ureogénesis por retroalimentación negativa [271], el balance de nitrógeno corporal estará controlado a partir de la regulación de la generación de urea [272], aunque la operatividad del ciclo también puede estar modulada por aspectos dietéticos y hormonales.

El hígado tiene una capacidad ureogénica superior a las tasas habituales de producción de urea, por lo que es capaz de responder rápidamente ante aumentos agudos de la carga de nitrógeno residual, siendo la disponibilidad del sustrato el factor que determina los cambios en dichas tasas a corto plazo [259]. Un exceso de proteína proveniente de la dieta puede generar un incremento de la producción de urea, aumentando la expresión de las enzimas del ciclo en un 300% respecto al nivel de expresión que hay en ayunas [273]. En cambio, alteraciones en la calidad de la proteína ingerida y, por lo tanto en la presencia de diferentes proporciones de aminoácidos libres en el hígado, limitan la producción de urea sin afectar a la actividad enzimática de las enzimas del ciclo [274]. Por otro lado, las actividades enzimáticas del ciclo de la urea disminuyen con la ingesta de dietas hiperlipídicas [240, 242]. Generalmente, las variaciones en las actividades enzimáticas correlacionan con sus correspondientes ARN mensajeros, hecho que indica que la regulación del ciclo por influencia dietética se da a nivel de expresión génica [242].

Los cambios a largo plazo en las actividades enzimáticas del ciclo de la urea ocurren en respuesta a alteraciones del flujo de nitrógeno amínico, generalmente provocados por cambios en la ingesta de proteína o bien por el catabolismo proteico endógeno. En cualquiera de estas situaciones, el glucagón, la insulina y los glucocorticoides juegan un papel importante en la regulación transcripcional de las enzimas y en la funcionalidad del ciclo [275]. La insulina disminuye la capacidad de síntesis de urea [276] al disminuir la actividad de la carbamoil fosfato sintetasa 1, la ornitina transcarbamilasa, la argininosuccinato sintetasa y la arginasa 1 [277], mientras que el glucagón la acelera [278, 279] mediante la estimulación de la carbamoil fosfato sintetasa 1, la argininosuccinato liasa y la arginasa 1 [280]. Los agonistas adrenérgicos y los glucocorticoides incrementan la síntesis de urea [281, 282], mientras que la hormona del crecimiento e IGF-1 la disminuyen al reducir la actividad de todas las enzimas del ciclo [283, 284].

Además, la capacidad de síntesis de urea puede verse afectada por otras condiciones como la sobrecarga de hierro, la cirrosis hepática, la presencia de hepatocarcinoma, la artritis reumatoide, el estrés severo, la deficiencia de vitamina A, el síndrome del intestino irritable, la esteatosis hepática y la obesidad, entre otras [249].

6.3. Adaptación del metabolismo nitrogenado en la obesidad

Ante una situación de elevada ingesta proteica y de exceso de nutrientes, el metabolismo de aminoácidos también se ve afectado. Como se ha comentado anteriormente en el apartado 5.3, una mayor ingesta de proteína implica un incremento de su oxidación, en el recambio proteico de estructuras y en la eliminación del nitrógeno excedente. Ante dicho incremento, en el hígado se da una mayor actividad catabólica, de modo que, y de manera proporcional al exceso proteico, hay un aumento de la actividad de las enzimas alanina transaminasa, glutamato transaminasa y serina deshidratasa [285]. El incremento del catabolismo proteico, inicialmente se acompaña de una mayor excreción de nitrógeno amínico mediante el incremento del funcionamiento de las enzimas del ciclo de la urea y, por lo tanto, de una mayor producción de urea. Sin embargo, cuando el exceso proteico va acompañado de un exceso de sustratos energéticos (lípidos y carbohidratos), aparecen dificultades para procesar y oxidar el exceso proteico, pues el organismo esta metabólicamente adaptado y condicionado para preservar la proteína [245, 286], aunque como en este caso, la conservación de nitrógeno amínico y de aminoácidos esenciales no sea necesaria.

En situación de exceso energético y proteico se da una desregulación del equilibrio entre la cantidad de nitrógeno amínico y de amonio presentes para la producción de urea. Una parte de la conversión de nitrógeno amínico a amonio se produce en el hígado y en el músculo a través del ciclo de los nucleótidos de purina [287], cuya funcionalidad va estrechamente asociada a la activación de la glucólisis [288] y a la baja disponibilidad energética, por lo que ante una situación de exceso energético el ciclo es inoperativo. Además, en el músculo se da una disminución de la síntesis y exportación de glutamina hacia el intestino y riñón, donde la acción de las glutaminasas que contribuyen a la liberación de amonio para la síntesis de urea y/o excreción de amonio iónico a través de la orina se ve comprometida [289]. Adicionalmente, la disminución en la producción de glutamina se traduce en una limitación en la síntesis de carbamoil fosfato, resultando en una insuficiencia en el funcionamiento del ciclo de la urea [246]. Otra enzima importante en la síntesis de amonio es la glutamato deshidrogenasa, con una considerable actividad en el hígado y el riñón pero más limitada en el músculo [290]. No obstante, su función es principalmente la de resintetizar glutamato a partir de 2-cetoglutarato y del exceso de amonio [291, 292].

En el caso de la obesidad y de la ingesta hiperenergética crónica, como ocurre en los estudios que usan modelos animales expuestos a dietas como la de cafetería, la funcionalidad de las enzimas del ciclo de la urea, y en definitiva la producción de urea, disminuyen [240, 293, 242], consecuencia principalmente de que los aminoácidos tienden a ser preservados a pesar del exceso de sustratos energéticos [294]. Sin embargo, en dicha situación no se observa un aumento masivo de masa magra en forma de proteína estructural, por lo que se asume que el exceso amínico debe ser eliminado por otra vía, no convencional [245, 295]. En los individuos con obesidad se ha observado una mayor producción de óxido nítrico [296], así como un incremento en la exhalación de óxido nítrico [297], y de su conversión a nitratos y nitritos [298]. También se ha descrito que la mayor parte de este exceso de nitrógeno, eliminado por vías desconocidas y en

mayor proporción en situación de obesidad, podría ser eliminado a través de los pulmones en forma de N_2 gas [299, 300]. Aunque el destino del exceso amínico en condiciones de elevada ingesta energética y proteica permanece sin resolver, los estudios realizados en modelos animales sugieren que las consecuencias metabólicas en humanos de dicho desequilibrio pueden ser dañinas a largo plazo [246].

7. EL TEJIDO ADIPOSO

El tejido adiposo es un órgano disperso, al que se le atribuyen diversas funciones, entre ellas la de amortiguador mecánico o de aislante térmico [301]. Sin embargo, su función principal, desde un punto de vista metabólico, es la de almacenamiento de energía en forma de triacilgliceroles (cuando hay abundancia energética, por ejemplo, en estado absortivo) o bien, la de movilización de ácidos grasos no esterificados (cuando hay escasez energética, por ejemplo, en ayunas o en ejercicio). Por ello, el tejido adiposo tiene un papel clave en la regulación de la homeóstasis energética.

Se pueden diferenciar dos tipos de tejido adiposo en función de las características del tejido, la apariencia microscópica, la función metabólica, la distribución corporal, las características bioquímicas y los patrones de expresión génica: el tejido adiposo blanco y el tejido adiposo marrón [302] (Figura 8).

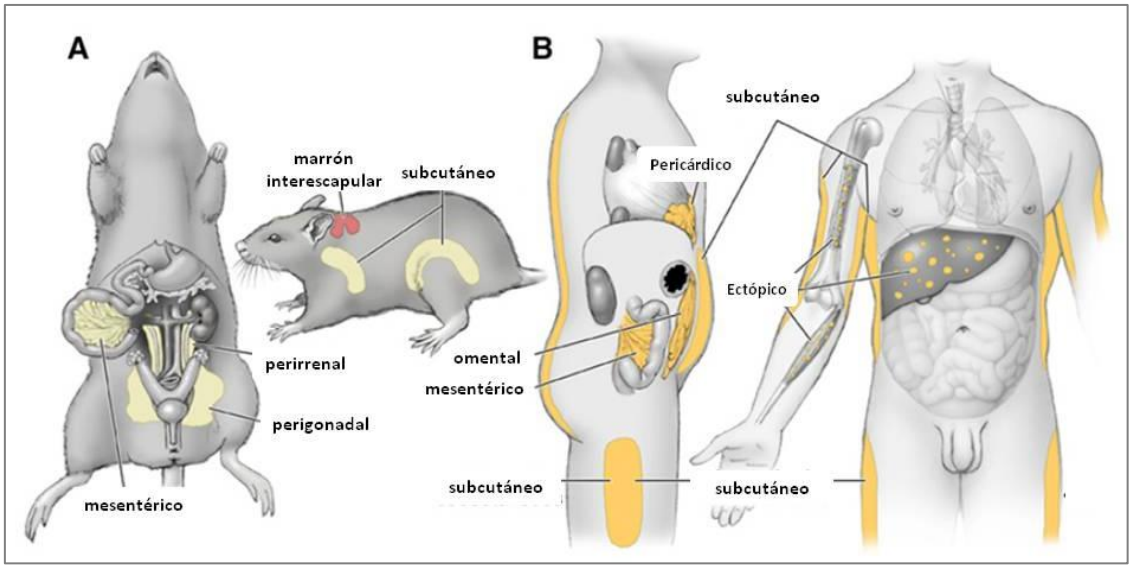

Figura 8. Tipos y localizaciones de tejido adiposo. (A: rata; B: humano) Existen dos tipos de tejido adiposo, el blanco y el marrón. Este último, en la rata y otros roedores, se encuentra principalmente en la zona interescapular, mientras que su presencia en humano es limitada y se da de forma dispersa. El tejido adiposo blanco es mayoritario y se distribuye por distintas localizaciones del organismo, las cuales se pueden subdividir en dos tipos representativos: el tejido adiposo de tipo visceral, que se distribuye alrededor de los órganos proporcionando una protección de acolchado, o bien, el tejido adiposo subcutáneo, el cual se localiza debajo de la piel y proporciona un efecto aislante. Adaptado de Tchkonia *et al.* 2013 [104].

El principal tipo celular que define el tejido adiposo son los adipocitos, aunque también hay presencia de otros tipos celulares (conocidos como células mesenquimales o estromales) que forman parte del estroma. En cuanto a los adipocitos, se pueden distinguir 3 tipos celulares (blanco, marrón y beige), que se diferencian según su origen, función metabólica y distribución en función del tipo de tejido adiposo (Tabla 3).

Clasificación y características de los adipocitos.

	Blanco	Beige	Marrón
Localización	Tejido adiposo blanco visceral y subcutáneo	Tejido adiposo blanco subcutáneo	Tejido adiposo marrón interescapular y otros depósitos
Origen	Mesenquimal Linaje adipogénico (Myf5-)	Mesenquimal Linaje adipogénico (Myf5-)	Mesenquimal Linaje miogénico (Myf5+)
Presencia de mitocondrias	Baja	Incrementada en respuesta a estímulos (frío)	Elevada
Presencia del complejo UCP1	No	Si (en respuesta a estímulos)	Si
Función	Almacenamiento de energía química en forma de lípidos	Contribuye a la producción de calor ante estímulos (frío y ejercicio)	Disipa energía mediante la producción de calor en respuesta al frío
Vascularización	Baja	Incrementada en respuesta a estímulos (frío)	Elevada
Contribución a la obesidad y S. metabólico	Contribuye	Contrarresta	Contrarresta

Tabla 3. Diferencias entre los tres tipos de adipocitos. Adaptado de Bartelt *et al*. 2014 [303] y Park *et al*. 2014 [302].

7.1. Tejido adiposo blanco

El tejido adiposo blanco es el tejido adiposo más abundante. En general, se caracteriza por tener unos adipocitos de gran tamaño, con un número de mitocondrias limitado y una única vacuola, formada por una gran gota de grasa que ocupa gran parte del volumen celular, dejando el citoplasma, el núcleo y el resto de orgánulos apilados junto a la membrana celular [304].

A nivel metabólico es un tejido principalmente glucolítico, con bajo consumo de oxígeno y con una elevada capacidad de almacenar triacilgliceroles principalmente a partir de la captación de ácidos grasos de las lipoproteínas del plasma pero también, mediante la lipogénesis *de novo* en el propio tejido a partir de glucosa [305]. El tejido adiposo blanco también es capaz de producir lactato [306] y alanina [307], o de usar aminoácidos como sustrato energético o para la lipogénesis [308]. En el tejido adiposo blanco la captación de la glucosa se da principalmente en respuesta a

la acción de la insulina mediante GLUT4, aunque los adipocitos también pueden captar glucosa en ausencia de insulina a través de GLUT1 [309]. Estudios recientes ponen en evidencia que, ante un exceso de glucosa, parte de esta glucosa captada en el tejido se libera en forma de lactato y glicerol (sustratos de 3 carbonos) con el objetivo de reciclar y derivar los sustratos energéticos hacia otros tejidos y, así, reducir la glucemia y limitar la sobreproducción de ácidos grasos y su acumulación patológica en el tejido [131, 132, 309].

En relación al metabolismo proteico, se ha descrito la expresión y funcionalidad del ciclo de la urea al completo en diferentes localizaciones anatómicas de tejido adiposo [251]. Sin embargo, el funcionamiento del ciclo parece estar enfocado principalmente a proporcionar intermediarios como la citrulina, para ser utilizados por otros tejidos periféricos [251]. La operatividad del ciclo de la urea en el tejido adiposo se considera, por lo tanto, complementaria a la función ureogénica del hígado, del mismo modo que ocurre con el intestino [310].

Tradicionalmente, el tejido adiposo blanco se ha considerado únicamente como un tejido de almacenaje energético. No obstante, hoy día también se considera como un tejido con importante actividad endocrina y paracrina. Como se ha tratado en apartados anteriores, el tejido adiposo libera señales en forma de citoquinas, hormonas y factores de crecimiento, que influyen en los procesos fisiológicos y patológicos y, cuyo efecto va dirigido a sus células vecinas y a tejidos involucrados en el metabolismo energético [311].

7.2. Tejido adiposo marrón

El tejido adiposo marrón (también llamado pardo) se distribuye principalmente en la zona interescapular, aunque también pueden encontrarse pequeños depósitos en la zona perirrenal (roedores y otros mamíferos) y en pequeñas áreas del tórax (humanos). Tradicionalmente, en humanos y otros mamíferos de gran tamaño, la presencia del tejido adiposo marrón está restringida a periodos perinatales y de la niñez [312]. Los adipocitos del tejido se caracterizan por tener un gran número de mitocondrias en el citoplasma, las cuales le dan cierta coloración marrón, y de ahí su nombre. El almacenamiento de grasa celular, a diferencia del tejido adiposo blanco, es multivacuolar y en forma de microgotas.

El tejido adiposo marrón, presenta una vascularización e inervación elevada y es altamente oxidativo. Como el tejido adiposo blanco es capaz de almacenar triacilgliceroles, oxidar sustratos y liberar ácidos grasos, pero además tiene la capacidad metabólica única de disipar energía química en forma de calor mediante la acción de una proteína mitocondrial desacopladora, la UCP1 (por sus siglas en inglés) [313], contribuyendo a la regulación de la homeostasis energética [72]. La UCP1 es una proteína específica de las mitocondrias de los adipocitos marrones, que se encarga de desacoplar el funcionamiento de la cadena de transporte de electrones de la síntesis de ATP, promoviendo, al oxidarse los sustratos, la producción de calor (termogénesis) y no la síntesis de ATP [314]. La termogénesis se produce al activarse la UCP1, la cual está controlada mediante el sistema nervioso simpático,

generalmente asociada a estímulos como la exposición al frío o la sobrecarga de nutrientes procedentes de la dieta [70, 315].

En relación al metabolismo proteico el tejido adiposo marrón presenta elevadas actividades de enzimas como la glutamina sintetasa, glutamato deshidrogenasa, aspartato transaminasa, alanina transaminasa, adenilato deaminasa y transaminasas de aminoácidos de cadena ramificada; sus expresiones y actividades son de un rango similar a las del hígado y músculo. En cambio, la arginasa y otras enzimas propias del ciclo de la urea no presentan actividad, por lo que no tiene capacidad para producir urea [316]. Cuando el tejido está estimulado por exposición al frío, ya sea de forma aguda o crónica, y hay suficiente disponibilidad de sustratos gluconeogénicos, el uso de aminoácidos como sustrato energético no se ve priorizado, hecho que sustenta la adaptación en cuanto a la preservación de proteína corporal. Sin embargo, ante la escasez de nutrientes y la exposición al frío, el tejido adiposo marrón usa aminoácidos como sustrato termogénico (principalmente los de cadena ramificada) y exporta alanina y prolina para equilibrar el balance de nitrógeno [317].

7.3. Adipocitos beige

Recientemente, las investigaciones relacionadas con el tejido adiposo y su papel en el abordaje terapéutico de la obesidad y la enfermedad metabólica se han centrado en la capacidad que tienen los adipocitos del tejido adiposo blanco para convertirse en adipocitos con características similares a los del tejido adiposo marrón, los llamados adipocitos beige, en un proceso denominado de pardeamiento [318]. Los adipocitos beige tienen un perfil fenotípico distinto a los blancos y a los marrones, pero son considerados como adipocitos marrones inducibles ya que tienen la capacidad de adaptarse a la demanda termogénica. El proceso de pardeamiento se da en respuesta a cambios fisiológicos y ambientales (exposición al frío, ejercicio, exceso de ingesta energética), y están regulados por una interacción compleja entre hormonas liberadas desde el propio tejido adiposo y desde otros órganos metabólicamente activos [303]. En relación al origen de los adipocitos beige recientemente se han propuesto dos teorías; por diferenciación *de novo* de células progenitoras residentes o por transdiferenciación de los adipocitos blancos maduros (Figura 9).

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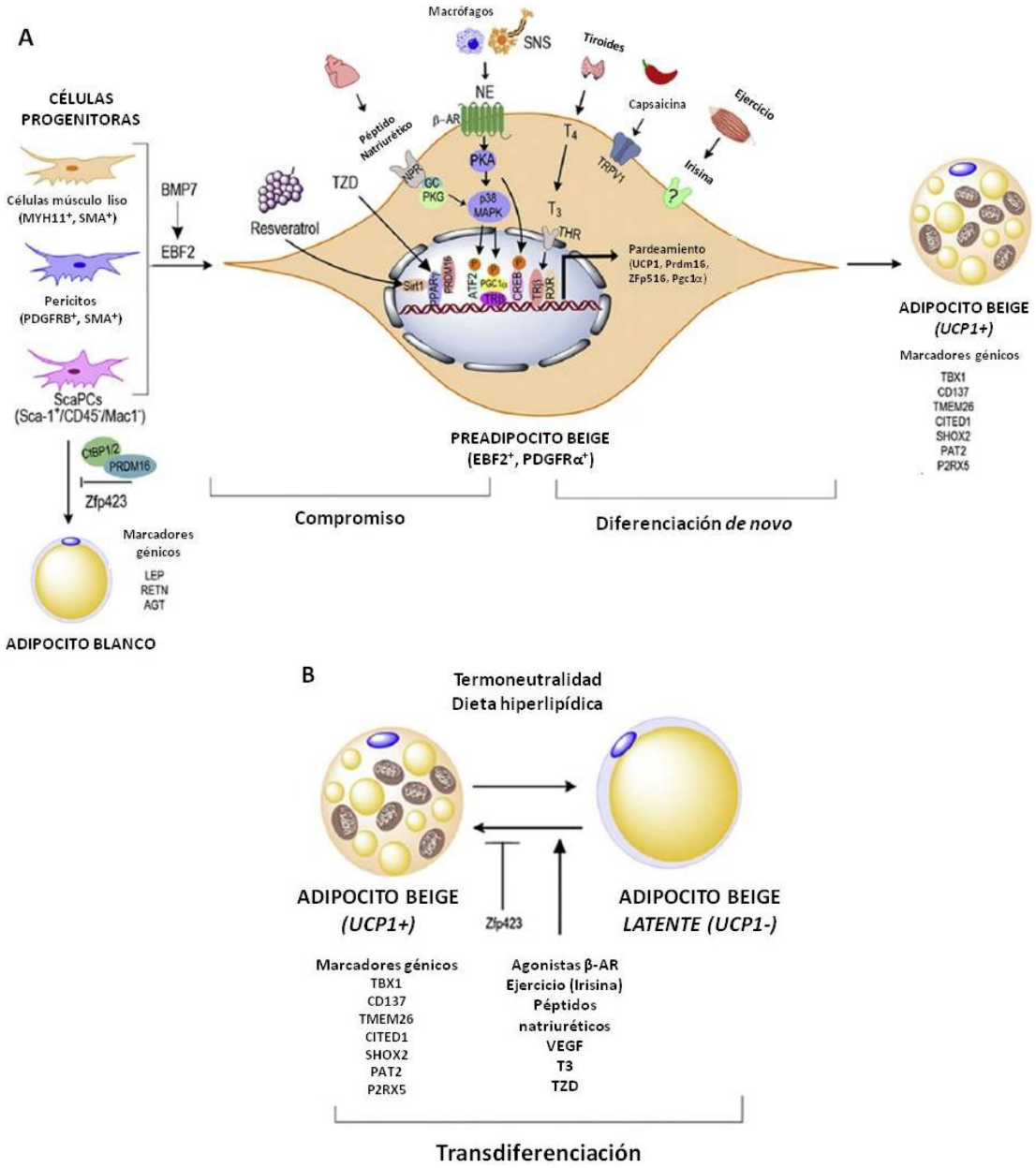

Figura 9. Procesos de pardeamiento. A) El origen por diferenciación *de novo* consiste en la diferenciación a partir de células progenitoras que, ante estímulos hormonales, ambientales y compuestos nutricionales determinados, se diferencian a preadipocitos beige como consecuencia de la activación de factores de transcripción específicos, culminando el proceso de diferenciación en un adipocito que expresa el fenotipo beige. **B)** En cambio, la transdiferenciación a partir de adipocitos blancos maduros, sustenta que el adipocito blanco maduro solo expresaría el fenotipo beige ante la presencia de los estímulos termogénicos mientras que dicho fenotipo quedaría "adormecido" ante la falta de estímulos. MYH11^{+}: Cadena pesada 11 de la miosina; SMA^{+}: Actina del músculo liso; PDGFRβ^{+}: Receptor β del factor de crecimiento derivado de plaquetas; Sca-1^{+}: antígeno 1 de la célula germinal; Mac1^{-}: antígeno del macrófago-1; LEP: Leptina; RETN: Resistina; AGT: Angiotensinógeno; GC: Guanilato ciclasa; T4: Tiroxina; THR: Receptor de hormonas tiroideas; TRPV1: Receptor de potencial transitorio V1; TRβ: Receptor β de las hormonas tiroideas; PDGFRα^{+}: Receptor α del factor de crecimiento derivado de plaquetas; VEGF: Factor de crecimiento endotelial vascular. Adaptado de Vargas-Castillo *et al.* 2017 [318].

8. LA DETERMINACIÓN DE LA GLUCEMIA Y SUS IMPLICACIONES EN EL DIAGNÓSTICO

La hiperglucemia es una de las comorbilidades características del síndrome metabólico y de la obesidad, y está relacionada con la existencia de daños en la señalización de la insulina y el desarrollo de diabetes de tipo 2 [14]. Como se ha apuntado anteriormente en el apartado 4.4, la glucemia incrementa en estado absortivo, produciéndose, en consecuencia, un incremento de la insulina circulante que contribuye a la regulación y el mantenimiento de dicha glucemia. La deficiencia en la respuesta celular ante la acción de la insulina implica que la captación de la glucosa por parte de los tejidos periféricos no se dé correctamente, por lo que la concentración de glucosa circulante incrementa favoreciendo el desarrollo de diabetes de tipo 2 [140, 142].

La medida de la glucemia en ayunas es una prueba bioquímica rutinaria y sencilla que permite el diagnóstico y control de patologías metabólicas como la diabetes [319]. En las últimas décadas se han desarrollado multitud de métodos y dispositivos que permiten medir la glucemia de forma rápida y sencilla, incluso por el propio paciente [320, 321]. Sin embargo, para el diagnóstico fisiopatológico generalmente se requiere de pruebas adicionales y específicas como el test de la tolerancia a la glucosa. Dicha prueba consiste en el análisis de la glucemia durante un periodo de tiempo corto tras una sobrecarga de glucosa administrada por vía oral, permitiendo evaluar la funcionalidad y sensibilidad a la insulina [322].

Una elevada concentración de glucosa circulante contribuye a la formación de productos finales de glucosilación avanzada [323] mediante procesos de glucosilación no enzimática o glicación entre la glucosa y las proteínas, en un proceso tradicionalmente conocido como reacción de Maillard [324]. La glicación tiene un impacto negativo en las proteínas, ya que implica la generación de cambios estructurales en la propia proteína y, en definitiva, cambios en su función biológica [325] que, a la vez, puede contribuir a la aparición de complicaciones asociadas [326].

Los individuos que presentan hiperglucemia también muestran niveles elevados de productos de glucosilación avanzada [327, 328]. Así pues, en pacientes con hiperglucemia, una de las medidas más empleadas para el manejo clínico y el control de la glucemia es la valoración de dichos productos de glicación. La hemoglobina glucosilada y la albumina glucosilada, cuyas vidas medias son de 3 meses y 21 días respectivamente, permiten hacer un seguimiento a medio y largo plazo del control de la glucemia del paciente [329, 330].

8.1. La glucosa

La glucosa es un azúcar sencillo que desde el punto de vista químico, es un polialcohol y, a la vez un aldehído (aldosa) de seis carbonos. La molécula de glucosa tiene quiralidad, hecho que permite la existencia de 2 configuraciones moleculares distintas; la configuración dextrógira o D que se da de forma natural (D-glucosa), y la configuración levógira o L que se obtiene únicamente por procesos sintéticos (L-glucosa).

Los grupos carbonilo de la D-glucosa son reactivos, por lo que suelen conllevar la formación de un enlace hemiacetálico con un grupo hidroxilo y, por consiguiente, la reorganización espacial de la molécula formando una estructura cíclica. Cuando el grupo aldehído forma un hemiacetal con el grupo hidroxilo del C5 obtenemos una *piranosa,* mientras que si el carbono implicado es el C4 obtenemos una *furanosa,* siendo la primera la estructura más estable. La ciclación de la glucosa genera un nuevo carbono quiral (C1), por lo que se dan 2 configuraciones anoméricas en función de la posición que adopte el grupo hidroxilo del C1 respecto al plano (debajo del plano: α; encima del plano β) (Figura 10).

La D-glucosa puede encontrarse tanto en forma sólida (dextrosa hidrato) como en solución acuosa debido a su naturaleza hidrofílica, coexistiendo en este caso tanto la forma de cadena abierta como ambas formas cíclicas (piranosa y furanosa) en sus 2 posibles configuraciones anoméricas del C1 (α y β), aunque en diferente proporción.

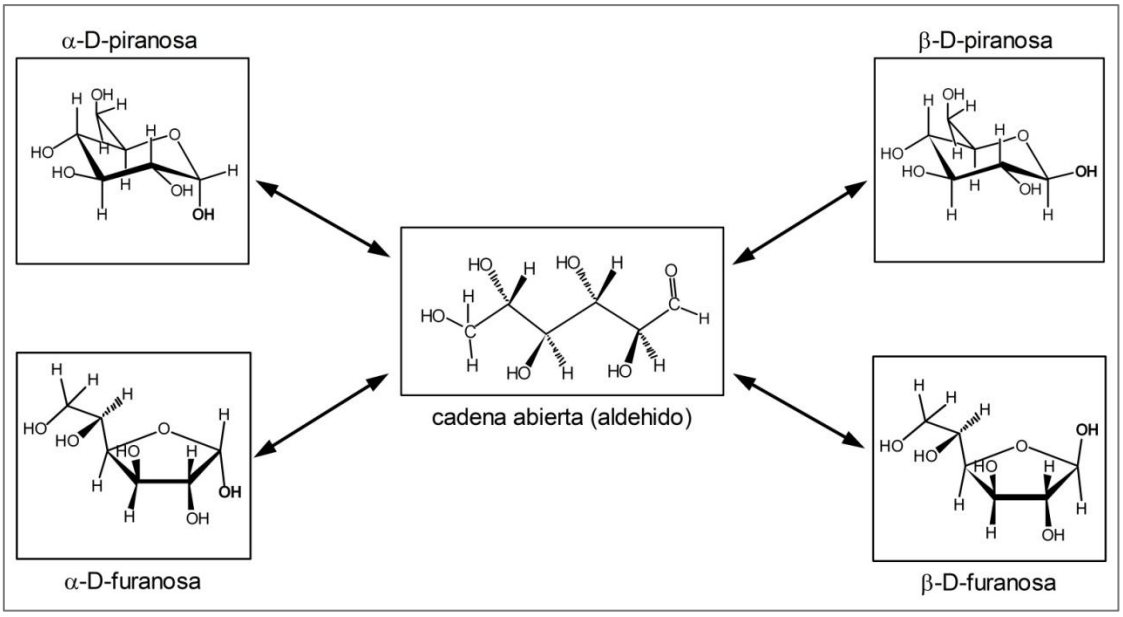

Figura 10. Configuraciones anoméricas de la D-glucosa.

Desde el punto de vista del metabolismo energético, la glucosa es la fuente de energía principal para la gran mayoría de organismos vivos, a partir de la cual las células sintetizan energía mediante su oxidación, o bien la acumulan en forma de polímeros como el almidón (en vegetales) y el glucógeno (en animales) a modo de almacenamiento energético.

La concentración de glucosa circulante en sangre, cuyo rango de normalidad en ayunas se establece entre 4 y 7 mM [140], está altamente regulada y tiende a ser constante, por lo que cualquier variación "crónica" en su concentración suele ser indicativo de patología [319]. La determinación de la glucemia es uno de los parámetros bioquímicos más analizados debido a su importancia en el diagnóstico y control de patologías como la diabetes. De ahí, la necesidad de desarrollar y obtener métodos fiables, precisos y sencillos para su análisis.

8.2. Métodos para la determinación de la glucosa

En la década de los 40, se desarrollaron varios métodos químicos basados en la capacidad reductora de la glucosa, es decir, en la susceptibilidad del grupo aldehído del C1 a ser oxidado a carboxilo. Los primeros métodos para la detección cuantitativa de la glucosa fueron de tipo colorimétrico, basados en la capacidad de oxidación del grupo aldehído por reactivos como el cobre (reacciones de Fehling y de Benedict) o el arsenomolibdato (reacción de Nelson-Somogy) [331, 332]. Sin embargo, la existencia de algunos inconvenientes como la interferencia con otros compuestos, la falta de especificidad y la baja sensibilidad del análisis propiciaron el desarrollo de otros métodos, cuya base fuese la especificidad del sustrato. Entonces surgieron otro tipo de métodos colorimétricos, en este caso basados en reacciones de tipo enzimático. Dichos métodos resultaron ser específicos, precisos y económicos en general, aunque los más efectivos y los más usados en la actualidad son aquellos que incluyen 2 o más reacciones enzimáticas acopladas como la hexoquinasa / glucosa 6-P deshidrogenasa [333] y la glucosa oxidasa / peroxidasa [334]. Sin embargo, algunos problemas de interferencia analítica e inespecificidad anomérica permanecen sin resolver.

En las últimas décadas, se han desarrollado métodos basados en técnicas separativas de alta eficiencia [335] y detección mediante espectrofotometría de masas [336], aunque su aplicación se limita esencialmente a la investigación. Además, se han desarrollado métodos más manejables como los biosensores, basados en diferentes tipos de transducción de señales (electroquímica, óptica, piezoeléctrica, termométrica y magnética) [320], que se utilizan en el ámbito clínico para el control y monitorización de la glucemia. Los más comunes son los biosensores enzimáticos amperométricos, que detectan corrientes generadas por el intercambio de electrones entre el sistema biológico y un electrodo [337]. Los avances recientes en nanotecnología han permitido desarrollar dispositivos para la medida o monitorización de la glucemia de manera continua y no invasiva [320], e incluso métodos intracelulares basados en métodos enzimáticos [338]. Sin embargo, todavía no hay métodos no invasivos o basados en la medida del diferencial de potenciales lo suficientemente fiables y precisos [321].

A pesar de que la determinación de la concentración de glucosa es un procedimiento común y aparentemente bien establecido, los procesos para alcanzar medidas fiables y precisas han involucrado varias cuestiones a lo largo de las décadas. Algunos problemas destacables son el tipo de muestra biológica (sangre total, plasma, suero) [339], la estabilidad y conservación de la muestra [340], la interferencia debida a diferentes condiciones fisiológicas o a la presencia de sustancias exógenas [319, 341, 342], y la variabilidad inter- e intraindividual [343]. Sin embargo, la mayoría de ellos son bien conocidos y están controlados o parcialmente resueltos [344].

8.3. Reactividad de la glucosa: glucosilaciones no enzimáticas

Los azúcares reductores como la glucosa, tienen la capacidad de reaccionar de manera espontánea con los grupos amino libres de las proteínas [326] o de otros componentes biológicos. Este proceso se conoce como reacción de Maillard y conlleva la formación de una glucosilación no enzimática o glicación [345].

El grupo aldehído de la glucosa se condensa con los residuos amino libres generando una base de Schiff, que se caracteriza por ser inestable termodinámicamente, pudiendo darse una reorganización espontánea e isomerización irreversible para formar los denominados productos de Amadori [323, 346] (Figura 11). El proceso de glicación puede complicarse mediante otras reorganizaciones y reacciones irreversibles para generar compuestos más estables como los productos finales de glucosilación avanzada, que implican modificaciones en la estructura y función de las proteínas [346, 347] y que pueden jugar un papel importante en cuanto al control de utilización de sustratos [348], función celular [349] e inflamación [350].

La glicación es un proceso frecuente, pues gran parte de las proteínas que entran en contacto directo con la glucosa, como las proteínas plasmáticas [351], las proteínas de la membrana de los eritrocitos [352], las células endoteliales y la hemoglobina [353], están glucosiladas. La proporción de proteínas glucosiladas incrementa cuando los niveles de glucosa circulante son elevados y sostenidos en el tiempo, como sucede en la diabetes. De hecho, actualmente se usan métodos que correlacionan los valores promedio estimados de glucemia y los niveles de hemoglobina glucosilada (Hb_{1AC}) para hacer el seguimiento y control de glucemia rutinaria [354].

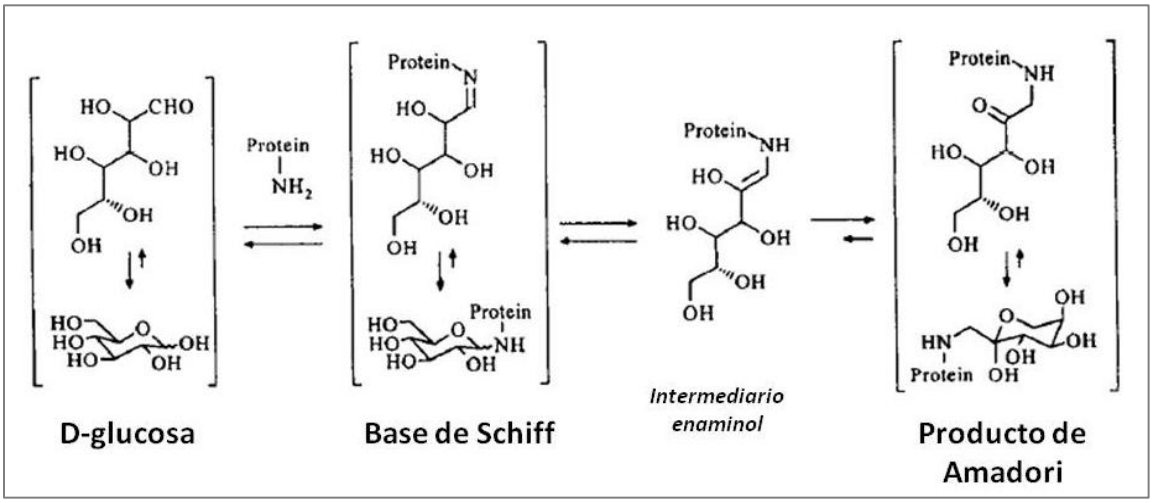

Figura 11. Formación de la base de Schiff a partir de la interacción entre la glucosa y el residuo amino libre de una proteína, y reorganización hasta la formación de un producto de Amadori. Adaptado de Ulrich *et al.* 2001 [323].

Objetivos

OBJETIVOS

El desarrollo de la obesidad y el sobrepeso depende esencialmente del exceso del aporte energético (mayormente en forma de lípido) procedente de la dieta, que conlleva un desequilibrio del balance energético que favorece la acumulación de reservas energéticas en el tejido adiposo. El abordaje nutricional con dietas hipocalóricas y con un porcentaje elevado de proteína es una de las opciones de primera elección para el control de peso y el tratamiento de la obesidad. La intervención nutricional con dietas de composición específica para estudiar los efectos sobre el metabolismo en modelos animales, ya sea en condiciones fisiológicas o patológicas, es una práctica habitual en el ámbito de la investigación básica.

El objetivo principal de esta tesis doctoral es profundizar en el conocimiento del efecto que genera la variación de la proporción y la composición de macronutrientes de la dieta sobre el metabolismo de ratas adultas de ambos sexos. Actualmente, se dispone de múltiples combinaciones en relación a la proporción de los macronutrientes y a su composición específica. Como consecuencia de ello, el conocimiento y los datos disponibles en la literatura aportan mucha variabilidad y, en la mayoría de ocasiones, dificultad para compararlos entre ellos. En este sentido, en la presente tesis se ha tratado de facilitar la comparación de los efectos generados por la dieta mediante el desarrollo de un modelo de dieta que permita generar un cambio en la proporción de un macronutriente específico sin implicar grandes variaciones respecto a la dieta estándar de referencia. Además, este modelo permitiría ajustar las proporciones para que se asemejen a las de otras dietas experimentales, como por ejemplo a la dieta de cafetería. Ante diferentes situaciones nutricionales, el organismo pone en marcha toda una serie de mecanismos encaminados a mantener la homeostasis y el balance energético. En un esfuerzo por comprender las adaptaciones metabólicas que ocurren, se ha tratado de abordar e integrar diferentes aspectos del metabolismo. Por ello, se plantearon los objetivos específicos descritos a continuación:

- Determinar la reactividad de la glucosa y evaluar la influencia de la dieta en la proporción de sus formas anoméricas mediante el efecto modulador de la mutarrotasa en el análisis de la glucemia por el método de la glucosa oxidasa/peroxidasa.

- Determinar el grado de compartimentación de la glucosa en sangre y los cambios en el grado de glucosilación de la hemoglobina, las proteínas plasmáticas y las proteínas de membrana de los eritrocitos en condiciones fisiológicas y obesogénicas.

- Establecer la influencia de la proporción lípido/energía y de la composición lipídica de de la dieta, utilizando dos dietas hiperlipídicas distintas, en relación al incremento del peso y la adiposidad corporal, la acumulación ectópica de lípidos y la alteración del metabolismo hepático de carbohidratos y lípidos.

- Evaluar el efecto del contenido lipídico y de la relación proteína/energía de la dieta en la regulación del ciclo de la urea hepático y caracterizar la capacidad ureogénica del tejido adiposo marrón y su contribución en la eliminación de nitrógeno amínico.

Informes

1. INFORME DE PARTICIPACIÓN

Esta tesis ha sido realizada en el grupo de investigación Nitrógeno-Obesidad. El método de trabajo del grupo implica la participación de diversas personas, en período de formación, que abordan los experimentos de manera coordinada. Por esta razón, en la autoría de los trabajos publicados o enviados para publicar se incluyen las personas implicadas en el desarrollo de los experimentos y los responsables del grupo. En cualquier caso, ninguna publicación ha sido utilizada en ninguna otra Tesis doctoral.

La participación de Laia Oliva en los trabajos presentados ha sido la siguiente:

1. *L. Oliva, J.A. Fernández-López, X. Remesar, M. Alemany. The anomeric nature of glucose: Implications on its analyses and the influence of diet. Are routine glycaemia measurements reliable enough? Enviado para publicar.*

Este es un trabajo en el que todo el trabajo experimental, el tratamiento estadístico de los datos y el redactado del borrador del trabajo final fueron realizados por Laia Oliva.

2. *L. Oliva, C. Barón, J.A. Fernández-López, X. Remesar, M. Alemany. Marked increase in rat red blood cell membrane protein glycosylation by one-month treatment with a cafeteria diet. Peer J (2015) 3:e1101.*

En este trabajo, Cristian Barón realizó los trabajos preliminares (Trabajo Final de Grado). Laia completó los experimentos, hizo el tratamiento estadístico de los datos y participó en la discusión de los resultados.

3. *L. Oliva, T. Aranda, G. Caviola, A. Fernández-Bernal, M. Alemany, J.A. Fernández-López, X. Remesar. In rats fed high-energy diets, taste, rather than fat content, is the key factor increasing food intake: a comparison of a cafeteria and a lipid-supplemented standard diet. PeerJ (2017) 5: e3697.*

Este trabajo es el primero de la serie que se comenta a continuación y que ha representado una ingente cantidad de trabajo. Para ello, participaron otros alumnos en formación: Giada Caviola (estudiante Erasmus), Tània Aranda y Ana Fernández Bernal (Trabajo de Final de Grado), que participaron en la fase inicial de elaboración de dietas y tratamiento de los animales, así como en algunas de las determinaciones realizadas. Sin embargo, la mayor parte de las determinaciones enzimáticas, expresiones génicas y todo el tratamiento de los datos fue realizada por Laia Oliva, que también participó en la discusión de los resultados.

4. *L. Oliva, T. Aranda, M. Alemany, J.A. Fernández-López, X. Remesar. Dietary fatty acid composition influence on liver cholesterol and triacylglycerols accumulation. Effect of oestradiol. Enviado para publicar.*

En este trabajo, en parte del tratamiento inicial de los animales y de las valoraciones participó Tània Aranda, pero las determinaciones finales de lípidos en tejidos, de expresiones génicas, actividades enzimáticas y balances, así como su tratamiento estadístico, lo realizó exclusivamente Laia Oliva, que también participó en la discusión de los resultados.

5. *L. Oliva, M. Alemany, X. Remesar, J.A. Fernández-López. The food energy/protein ratio regulates the rat urea cycle but not total nitrogen losses. Nutrients (2019) 11, 316; doi: 10.3390/nu11020316*

Esta parte del trabajo global comentado anteriormente, la realizó por entero Laia Oliva.

6. *L. Oliva, S. Arriarán, M. Alemany, X. Remesar, J.A. Fernández-López. Urea cycle enzyme activities are partially present in brown adipose tissue, contrarily to white adipose tissue. Enviado para publicar.*

Laia Oliva ha medido las actividades enzimáticas y determinado las expresiones génicas en los tejidos implicados con la colaboración de Sofía Arriarán. También ha participado en los cálculos estadísticos y en la discusión de los resultados.

Codirector Codirector

José Antonio Fernández-López Xavier Remesar Betlloch

2. INFORME SOBRE EL FACTOR DE IMPACTO DE LAS PUBLICACIONES

Los artículos que forman parte de esta tesis doctoral presentada por Laia Oliva Lorenzo han sido publicados o sometidos para su publicación en revistas internacionales indexadas tal como se detalla a continuación:

L. Oliva, C. Barón, J.A. Fernández-López, X. Remesar, M. Alemany. Marked increase in rat red blood cell membrane protein glycosylation by one-month treatment with a cafeteria diet. Peer J (2015) 3:e1101.

En el momento de su publicación, la revista PeerJ tenía un Factor de Impacto de 2,183 y estaba clasificada dentro del primer cuartil (Q1) del ámbito de *Multidisplinary Sciences* del WOS (*Web of the Science*).

L. Oliva, T. Aranda, G. Caviola, A. Fernández-Bernal, M. Alemany, J.A. Fernández-López, X. Remesar. In rats fed high-energy diets, taste, rather than fat content, is the key factor increasing food intake: a comparison of a cafeteria and a lipid-supplemented standard diet. PeerJ (2017) 5: e3697.

En el momento de su publicación, la revista PeerJ tenía un Factor de Impacto de 2,118 y estaba clasificada dentro del segundo cuartil (Q2) del ámbito de *Multidisplinary Sciences* del WOS (*Web of the Science*).

L. Oliva, M. Alemany, X. Remesar and J.A. Fernández-López. The food energy/protein ratio regulates the rat urea cycle but not total nitrogen losses. Nutrients (2019) 11, 316; doi:10.3390/nu11020316

En el momento de su publicación, la revista Nutrients tenía un Factor de Impacto de 4,196 y estaba clasificada dentro del primer cuartil (Q1) del ámbito de *Nutrition & Dietetics* del WOS (*Web of the Science*).

De este modo, dos artículos se han publicado en revistas del Primer Cuartil (Q1) y una en el Segundo Cuartil (Q2), a la espera de los que resulten de los tres artículos enviados para publicar.

Codirector | Codirector

José Antonio Fernández-López | Xavier Remesar Betlloch

Publicaciones

1. LA GLUCEMIA Y LA GLUCOSILACIÓN NO ENZIMÁTICA

La hiperglucemia es una condición patológica muy frecuente en la obesidad, característica del síndrome metabólico y de otras patologías metabólicas como la diabetes. La hiperglucemia sostenida puede conllevar el desarrollo de complicaciones importantes. La presencia de valores de glucemia elevados y sostenidos favorece los procesos de glucosilación no enzimática (o glicación) de proteínas, principalmente en aquellas que están directamente expuestas a la glucosa circulante. La glucosilación de proteínas puede interferir en la estructura y funcionalidad de dichas proteínas, contribuyendo a la aparición de complicaciones asociadas.

En la actualidad, existe una gran cantidad de métodos y dispositivos que permiten medir la glucemia de forma rápida y sencilla, aunque en algunos casos, dicha rapidez podría estar influyendo en la validez de las lecturas. Por ello, se ha realizado un estudio en el que se evalúa la influencia de las características químicas de la glucosa y del tiempo de reacción en la medición de la misma en condiciones fisiológicas y patológicas, empleando uno de los métodos más comunes, el método de la glucosa oxidasa / peroxidasa. Además, se ha llevado a cabo un análisis del grado de glucosilación de las proteínas de la membrana del eritrocito, que están mayormente expuestas a la glucosa y, en consecuencia, son susceptibles de sufrir glucosilación especialmente en condiciones de hiperglucemia inducida por la dieta.

1.1. The anomeric nature of glucose: Implications on its analyses and the influence of diet. Are routine glycaemia measurements reliable enough?

Laia Oliva, José Antonio Fernández-López, Xavier Remesar and Marià Alemany.

La naturaleza química de la glucosa implica que esté presente en sus distintas formas anoméricas, en concreto, los anómeros α y β. Aunque dichos anómeros están en equilibrio, su presencia podría estar interfiriendo en los métodos empleados para su propio análisis. Para estudiar la interferencia de dichos anómeros en la medida de la glucemia se ha utilizado el método de la glucosa oxidasa / peroxidasa (método muy común que utiliza la β-glucosa como sustrato) en presencia o ausencia de mutarrotasa (catalizador de la interconversión anomérica α/β). Las medidas se realizaron en patrones de glucosa pura a diferentes concentraciones y en muestras de plasma de ratas sujetas a una intervención nutricional estándar o hiperglucémica (dieta de cafetería). La adición de mutarrotasa al sistema enzimático proporcionó, en todos los casos, un incremento en las lecturas de glucosa, independientemente de las concentraciones de glucosa. Las valoraciones realizadas en las muestras de plasma de ambos grupos de intervención dietética mostraron diferencias en la glucemia en ausencia de mutarrotasa, no obstante, al añadir mutarrotasa al sistema enzimático dichas diferencias por el efecto de la dieta desaparecieron, hecho que sugiere una posible alteración en la proporción anomérica propiciada por la dieta. Todo ello apunta a que en ausencia de mutarrotasa y, tras el tiempo de reacción recomendado, la oxidación de la glucosa presente en la muestra es incompleta, proporcionando lecturas subestimadas de la concentración de glucosa.

1.2. Marked increase in rat red blood cell membrane protein glycosilation by one-month treatment with a cafeteria diet

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La glucosa reacciona de manera espontánea con las proteínas generando proteínas glucosiladas. Ante concentraciones de glucosa elevadas y sostenidas en el tiempo, las tasas de glucosilación incrementan. Actualmente, la determinación analítica de la albúmina plasmática y de la hemoglobina se utiliza como un marcador para el control de la glucemia a largo plazo en estados patológicos. En este estudio se han evaluado los cambios generados en la glucosilación de proteínas en las fases iniciales de la patología. Para ello se han alimentado ratas de ambos sexos con una dieta estándar (grupo control) o bien con una dieta de cafetería (grupo potencialmente obeso) durante 30 días, tiempo suficiente para un desarrollo temprano del síndrome metabólico. Tras el periodo de intervención nutricional se recogieron muestras de sangre en las que se analizó la glucemia, el contenido de glucosa intracelular y el grado de glucosilación tanto de la hemoglobina, como de las proteínas plasmáticas y de la membrana de los eritrocitos, estas últimas en contacto directo con la glucosa plasmática. Los resultados obtenidos mostraron una ausencia de cambios significativos, por el efecto de la dieta o del sexo, en la glucosilación de la albúmina plasmática o de la hemoglobina, aunque si se detectaron cambios inducidos por la intervención nutricional en el grado de glucosilación de las membranas de los eritrocitos. La concentración de glucosa intracelular fue significativamente inferior a la del plasma, y prácticamente insignificante en las muestras de los animales alimentados con dieta de cafetería. Todo ello, sugiere la existencia de un mecanismo de compartimentación de la glucosa sanguínea para la regulación del transporte/transferencia de esta, y que la glucosilación de las membranas de los eritrocitos valdría como un posible indicador del desarrollo de hiperglucemia asociada al síndrome metabólico.

The anomeric nature of glucose: Implications on its analyses and the influence of diet. Are routine glycaemia measurements reliable enough?

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Running title:

Anomeric form of glucose affects its analysis

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ABSTRACT

Background & Aims: Glucose is the main inter-organ energy supplying metabolite in humans and other vertebrates. In clinical analyses, its measurement is probably the most performed and used for diagnostic, monitoring and control of the physiological status. However, glucose chemical structure, specially its anomeric forms (α/β), may deeply interfere in their own analyses, often resulting in misleading results. *Methods:* These effects on glucose estimation were studied by using a common glucose oxidase / peroxide based method, in the presence or absence of added mutarotase, which speeds up the α/β conversion rate. Glucose concentrations were measured in pure standards and plasma samples from control and cafeteria diet-fed rats. *Results:* The addition of mutarotase resulted in higher glucose readings, independently of glucose concentration and its initial anomeric proportions in the sample. In the absence of mutarotase, cafeteria-fed rats had higher glucose levels than controls, but the differences disappeared in its presence, because under experimental conditions, a proportion of the α-anomer was not isomerized and thus was not measured. *Conclusions*: Diet altered the proportion of anomers, suggesting that glucose usage by physiological processes affects the anomers' ratio and may have an important metabolic meaning, which deserves a detailed study in addition to the need to correct the methods in use to obtain real 'total glucose' readings.

INTRODUCTION

In humans, and in most vertebrates, glucose is considered the main inter-organ energy substrate. Consequently, its levels are highly regulated and monitored. We often take for granted the precision and validity of the methods used for its measurement. However, most methods are not fully reliable [1], and thus, most experimental glucose measures may not actually reflect the true glucose levels. Given its relevance, we question whether routine measures suffice to build upon critical decisions on diagnostic, control and treatment of malnutrition, diabetes, metabolic syndrome and so on.

In the (near) past, glucose was measured chemically, using its reducing power to develop colorimetric procedures (e.g. Fehling, Benedict, Nelson-Somogyi, etc.) [2,3]. The main problems were the need for large samples, low sensitivity, chemical interference, and, especially, lack of specificity. The use of enzymatic methods solved the specificity question and often required smaller samples [4]. The methods most effective were those based on the use of paired enzymes, such as hexokinase / glucose-6P dehydrogenase [5] or glucose oxidase / peroxidase [6]. The former, is quite specific, with good linearity, sensitive and precise for glucose determination [7], although it may be affected by other sugars, limiting somehow its full applicability [5,8]. The other main method, glucose oxidase / peroxidase (GOxP) is, at present, the most commonly used method for glucose because of its easy use, resilience, precision and specificity [7]; and more importantly, its enzymes do not react (theoretically) with other monosaccharides [9]. However, reducing agents such as ascorbate, bilirubin or

urate may interfere [10]. A powerful factor modifying the measurements (often ignored) is the almost omnipresent catalase. Nevertheless, the most critical problem of the method is that β-D-glucose: oxygen-1-oxidoreductase (EC 1.1.3.4) acts only on the β–D-pyranose anomer of glucose [11]. The widespread use of these methods, however, relies on its cheapness and robustness. The coupled enzymes are easy to produce and are more resistant to extreme pH, ion strength and temperature range variations than other possible alternatives [9,12]. Its extension is largely based on the widespread availability of enzymes and adaptation to systems, platforms and other technical developments [12]. They have lowered the cost, time and need of specialized laboratory equipment, allowing their portability, instant response and miniaturization.

In recent decades, powerful separation methods (i.e. those based on mass spectrometry) have been developed [13,14] for investigation. But slowly extending metabolomic studies showed that α- and β-D-glucose anomers are both routinely found in biological samples [15]. The absence of reliable quantitative data usually hampers the interpretation of these results.

Free or saccharide-incorporated glucose is regularly found in the more stable cyclic form. Glucose anomeric carbon (C1) forms a hemiacetalic bond with C5 (pyranose cycle) or C4 (furanose cycle) hydroxyl groups [16]. For hexoses, pyranose forms predominate at room temperature since they are thermodynamically more stable followed by the less stable furanoses; open chain, found only in a small proportion.

The spontaneous closing of the ring via hemiacetalic bond results in an additional source of isomery on the anomeric carbon (C1), (α or β configurations). Most of solid crystalline glucose (i.e. obtained from starch) is in the α-D-glucopyranose form. However, when dissolved in water yields a mixture of the open-chain form (aldehyde) and its four cyclic isomers (Figure 1)**.** In plasma, D-glucose is found essentially as a mixture of two anomeric forms (roughly one third of α-D-glucopyranose and about two thirds as β-D glucopyranose), with practically no furanose forms [16,17]. The interconversion between open-chain aldehyde and ring forms is fast and is affected by the medium conditions. Thus, any aldose may, in practice, react within a short time frame as if it were fully in the open aldehyde form [17].

Hexokinase (EC 2.7.1.1) transfers a phosphate group from ATP to aldo- or ketohexoses, forming the corresponding phosphate ester in C6. This carbon is accessible in both glucose anomeric forms; thus hexokinase may phosphorylate either α or β-D-glucose [8,18,19], producing the corresponding anomeric forms of glucopyranose-6P. However, yeast glucose-6-P dehydrogenase (the most common enzyme used in glucose analysis kits) acts specifically on β-D-glucose-6-P only. The spontaneous anomerization rate of α-D-glucopyranose-6-P at physiological pH is two-fold faster than that of free glucose [7,19]. Thus, the hexokinase / glucose 6-P dehydrogenase enzyme pair has often been used successfully for quantitative glucose determination, under adequate medium conditions [5,19]. The high cost and the conditions for analysis have limited the use of this paired-enzyme approach (Figure 2).

When solid (α-D-glucose) is dissolved to prepare standards, it tends to achieve an equilibrium with somewhat more β than α-isomer [20]. When both, standards and samples, are exposed to glucose oxidase, the enzyme oxidizes only β-D-glucopyranose, whilst the α-anomer needs to be previously isomerized before oxidation. This interconversion is rather slow at physiological pH [21], although the widespread presence in biological systems of a specific enzyme, mutarotase (aldose 1-epimerase: EC 5.1.3.3) [22], speeds up the α-β interconversion. Absent, insufficient (or variable) natural mutarotase, as is –obviously-- the case of deproteinized samples, may result in a potentially underestimation of 'total' (for 'true') glucose. This is due to an incomplete conversion of α-D-glucopyranose to the β-D isomer in time for its oxidation during the analytical process [23]. The addition of mutarotase to the medium [23,24], accelerates the interconversion, and increases the proportion of measured glucose. In the practice, however, most current methods “measure” only a large, albeit unspecified, portion of the actual glucose present in the sample. This problem is often overlooked too because the glucose in biological samples is compared with standards (often just prepared), which anomer proportion (equilibrium) is also affected by time pH, temperature, etc.

The existence of these problems is well-known (textbook material) [25,26] but, surprisingly, it is seldom, discussed or corrected. This widespread ellipsis results in the scarcity of reliable quantitative data on glucose levels, which may seriously compromise serial or comparative studies. In order to get some insight as to the range of changes expectable from the sources of variability indicated in the previous sections, we designed a short series of experiments to show the modulatory effects of mutarotase on glucose analysis and the eventual advantages of its regular use.

METHODS

Samples

Standard glucose solutions were just prepared D-glucose (Sigma-Aldrich, St Louis, MO USA) standard solution (0, 2.5, 5.0, 10 mM). No differences were observed with the standards provided with the kits after storing our standards overnight. The effect of different concentrations of mutarotase (#136A5000; Calzyme, San Luis Obispo, CA, USA; specific activity 75 nkat/g protein) was tested with standard solutions.

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 (#DAAM 6911).

Samples of ten-week-old female and male Wistar rats (Janvier, le Genest Saint-Isle France) from a study carried out by our group [27] were used for the analysis of plasma glucose levels. The animals were randomly divided in two groups (n=6 for each sex) and were

fed *ad libitum* either a standard diet or a hyperglycemia-inducing simplified cafeteria diet [28,29] for 30 days. All animals had free access to water. The rats were housed (in same-sex pairs) under standard conditions. On day 30, at the beginning of light cycle, the rats were anesthetized with isoflurane and then killed by exsanguination through the exposed aorta using a dry-heparinized syringe. Plasma was obtained by centrifugation and kept at -20°C until processed.

Analytical procedure

Mutarotase solutions were prepared just before each experiment (0.3, 0.5, and 0.7 nkat/L). Glucose standards were also prepared fresh a couple of hours before measurement of glucose. Standards and samples were analyzed using a glucose oxidase/peroxidase kit (#11504; Biosystems, Barcelona, Spain). In all cases, measurements were duplicated, one series receiving 250 µL of reagent and the other the same volume with added mutarotase. Samples, blanks and standards were incubated at 30ºC in 96-well plates. The changes in absorbance at 490 nm were measured at 30 s intervals for 15 min (or up to stabilization) using a plate reader spectrophotometer (ELx808 Ultra Microplate Reader, Biotek, Winooski, VT USA).

Statistics

Statistical comparison between groups was carried out using 1- or 2-way ANOVA analyses, and the Bonferroni post-hoc test for further differences between specific groups, using the Prism 5 program (GraphPad Software, La Jolla, CA, USA).

RESULTS

The effects of different mutarotase concentrations on the oxidized glucose percentage using a pure D-glucose standard (10mM) are shown in Figure 3**.** The presence (in excess) of mutarotase was found to speed-up the reaction, providing a complete oxidation of glucose (the curve became asymptotic before 10 minutes of incubation, the method-established incubation time). The amount of mutarotase used changed little the form of the curves. However, in the absence of added mutarotase, the time required to achieve a similar reading of glucose levels was dilated up to 15 minutes. The absence of mutarotase implies that not all the glucose was oxidized (measured), and consequently the overall concentration was underestimated. Mutarotase addition did not affect the readings of buffer blanks. Further analyses were performed using a fixed mutarotase concentration of 0.5 nkat/L, derived from this experiment.

The effects of mutarotase were assessed using different glucose concentration standards (Figure 4). Eventually, all D-glucose seemed to be completely converted to β-D-glucose (and then oxidized) in the presence of mutarotase. At 10 minutes of incubation in the absence of added mutarotase, a significant part (25±1%, p<0.0037) of the initial D-glucose remained unmeasured due to the slowness of the process of spontaneous equilibrium under the (common) experimental conditions used. The profiles obtained using three different

glucose concentrations were almost identical. These results showed, as expected, that the isomeric ratio of glucose was independent of its concentration. Thus, the actual magnitude of the differences obtained from relative data may result in marked differences when translated to absolute concentrations.

Figure 5 shows the comparative values of plasma glucose concentrations of adult female and male rats fed with either standard or cafeteria diet, measured in the presence / absence of added mutarotase. In all cases, higher absorbances, translated also into higher plasma glucose concentrations, were found in the presence of mutarotase. On this small sample, no significant differences were found for sex. Nevertheless, in the absence of mutarotase, diet elicited borderline significant differences. This is a common occurrence in the literature, since cafeteria-fed rats are considered hyperglycemic when compared to controls fed chow diet.

The higher glucose concentrations of cafeteria fed rats using the non-mutarotase data, compared with standard diet-fed rats, disappeared when mutarotase was present. The estimated α-D-glucose proportion (i.e non-oxidized glucose remnants at the end of the analysis) of standard-fed rat plasma (10 minutes incubation with the coupled enzymes) was similar to that found in standard glucose solutions, being in both situations 1.5-fold higher than that estimated for cafeteria fed rats. The fact that the method of measurement of glucose was apparently "more efficient" in cafeteria rats than in controls, suggests the existence of a different initial proportion of α/β glucose anomers. This fact elicits the question of the probable differential disposal rates of glucose anomers depending on diet (or pathologies) related to diet modulation of substrate energy utilization.

Another series of plasma samples were deproteinized using the classic Somogyi method [30], in order to eliminate any possible effect of pre-existing plasma mutarotase [21]. This procedure, widely used in the past for deproteinization, was used because the exposure to extreme pH, heat or residual chemicals was minimal before analysis of glucose levels. No differences in the levels of glucose were observed between direct plasma and deproteinized samples (data not shown), suggesting that the assumption of a 'sufficient' presence of mutarotase in natural plasma was not enough to minimize (not even fractionally decrease) the analytical problem of slow interconversion between glucose anomers.

DISCUSSION

The presence of two main glucose isoforms in plasma and tissues causes differences in reactivity, metabolic use and disposal. Anomeric proportions of glucose in plasma and tissues tend to an α/β equilibrium, although β-D-glucose proportion is usually higher [16]. The predominance of the β-anomer may initially suggest a higher utilization of the α-anomer, although, the actual (preferred or exclusive) utilization of glucose anomers in metabolic processes has been sparsely studied. Initially, α-D-glucose was proposed as a signal for D-glucose sensing cells (e.g. α- and β- Langherhans cells and taste buds cells) while β-D-glucose

has been proposed as main substrate for energy production in a number of cells and tissues [21]. Later on, several authors found that different tissues showed specific preferences for transport or metabolization of the α- or β-anomers [31]. Thus, liver [32], pancreas β-cells [33], skeletal muscle or adipose tissue [34] seem to prefer α-D-glucose, while brain [35,36], retina [37], erythrocytes and lens cells [31,38] were found to preferably take up β-D-glucose.

These fragmentary findings emphasized the existence of a tissue-specific physiology of anomer glucose transport, hinting that cells expressing GLUT1 or GLUT3 may prefer the β-anomer and those expressing GLUT2 or GLUT4 may prefer the α-form [31]. However, we have not found studies assessing the anomer preferences for the highly regulated GLUT4 transporter. Recent analyses, however, suggest that in some situations glucose anomerization may not be necessary for structure recognition by GLUTs [39,40], since in red blood cells, glucose transporter GLUT1 has been found to transport α- and β-glucose with similar efficiency [41]. Nevertheless, this question may be largely rhetorical, since red blood cells have mutarotase bound to membranes and hemoglobin [41] thus facilitating a faster interconversion between anomers, speeding glucose disposal for the cell's own metabolic use. Notwithstanding, glucose needs of mammal red cells are low compared with 'standard' nucleated cells, thus enhancing the relative importance of mutarotase.

Plasma mutarotase is assumed to speed up the interconversion between hexose anomers [22], but we found no effects at all, i.e. no differences using fresh or deproteinized plasma. Previous studies confirmed that the addition of mutarotase to glucose oxidase-based methods increased the glucose readings of varied experimental samples [23,24]. We can assume that plasma mutarotase [21] (if any functional activity is present) can hardly help speed up significantly the reactions to allow for 'true' glucose results. In the present study, we have found that the addition of enough mutarotase to the coupled glucose oxidase/peroxidase enzymes for the measurement of glucose allowed a sufficiently rapid anomeric interconversion to get a complete oxidation of glucose, resulting in 'higher' (in fact closer to reality) measurements of glucose, independently of its concentration within the method range.

Physiological glucose concentration is (assumedly) a well-known entity, around 5-6 mM for rats. In our study, the animals were (necessarily) anesthetized with isoflurane, a hyperglycemic agent [42], which may explain the relatively high values found, specially, in standard diet fed rats. In fact, they were even higher when mutarotase allowed a more accurate estimation. The values obtained were higher than those assumed as physiological, even when anesthesia do not interfere [29], and strongly hint at a generalized and systematic underestimation of glycaemia.

Cafeteria diets are widely used as a non-stressing method to induce overfeeding and excessive energy intake, inducing obesity and chronic hyperglycemia as part of the development of metabolic syndrome [43]. The significant differences found in glucose anomer proportion between standard (assumedly physiological glycaemia) and that of cafeteria fed rats (assumedly altered) suggest an alteration of the α/β anomer equilibrium in plasma. These

changed anomer proportions can affect (in an undetermined way and extent) glucose measurements when performed in absence of mutarotase. This unexpected finding may further compound the series of unknown factors when compared to the data of 'standards' of pure glucose. The consequences may be deeply misleading with respect to the evaluation of physiological status, and diagnosis.

The presence of different anomeric forms of glucose, in biological fluids may be a consequence of different rates of metabolization, and the unknown entity of how much mutarotation takes effect in biological samples, either by mutarotase, or spontaneously depending on time, storage, etc. These factors may provide additional insight on how glucose is used. The bioavailability of anomeric forms of an aldose may deeply affect the ability of enzymes (and complete biological systems) to use them as substrates. Glucose is a key inter-organ substrate, in practical terms; the effectiveness of the systems acting on glucose (transporters, kinases) will be influenced by the total glucose levels but also by the proportions of its anomeric forms (i.e. glucose available for the cell or system). Unfortunately, this factor is almost systematically overlooked, ignored or dismissed.

We have routinely added mutarotase to our analyses of glucose in the last years [44-47]. The wide differences observed in our studies using different diets must be now contemplated from a different point of view, since we now know that most studies of glycaemia are (probably) underestimations of the true values, the problem is that we do not know which ones and in which proportion. The finding that diet may change the anomeric proportion is in fact much more disturbing, since an old line of work (metabolic specificity of glucose anomer utilization) may be asked to return from practical oblivion. First, transporter and enzyme preferences or specificities should be checked, followed by an analysis in depth of the effects of diet, since our data show clearly that underestimation of glucose levels may depend ultimately on diet (or inflammation).

In conclusion, the addition of sufficient mutarotase allows the common glucose oxidase/peroxidase methods to account for practically all glucose present in the samples irrespective of their initial anomeric form. This procedure should be made extensive to standards. The results shown here hint at a variable underestimation of glucose levels using (at least) one of the most common enzymatic methods. It is imperative to revise these methods in order to obtain accurate values; and thus allow the analysis of possible metabolic differences of anomer specificity handling under physiological and pathological situations. The need for easy to perform quantitative anomeric glucose analyses becomes another obviously necessary step to clarify most of the present day available data, which reliability may be suspect because of the considerable extent of result variations and uncontrolled factors described in the present study.

DECLARATIONS

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Conflict of Interest

The Authors declare no conflict of interests.

Informed Consent

Not applicable

Author Contributions

Concept and overall design, analysis and redaction (MA). Ideas and experimental design details (all Authors). Experimental work and data analysis (LO). Bibliography (LO, MA). Provision of samples and materials, administration and budget (XR). Revision and Statistics (JAFL). General discussion and revision of drafts (all Authors). XR and JAFL are the directors of LO PhD thesis, from which this study is a methodological part.

Institutional Review Board Approval

Not required at the University of Barcelona

Ethical Compliance with Animal Study

Already inserted in the main text as recommended

FIGURE 1

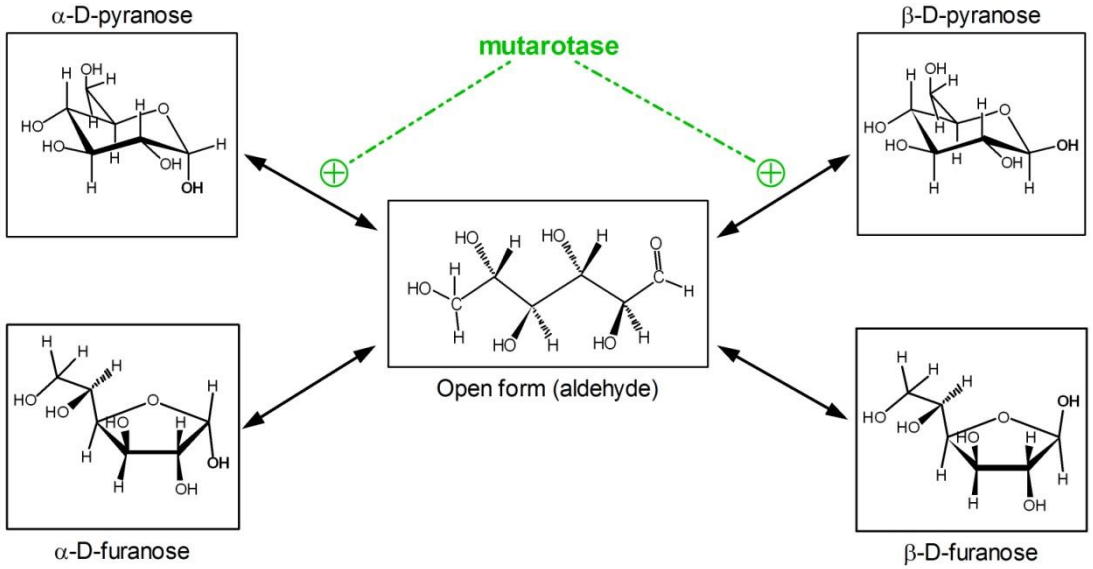

Figure 1. **Anomeric forms of glucose in solution and its spontaneous or facilitated interconversions.**

FIGURE 2

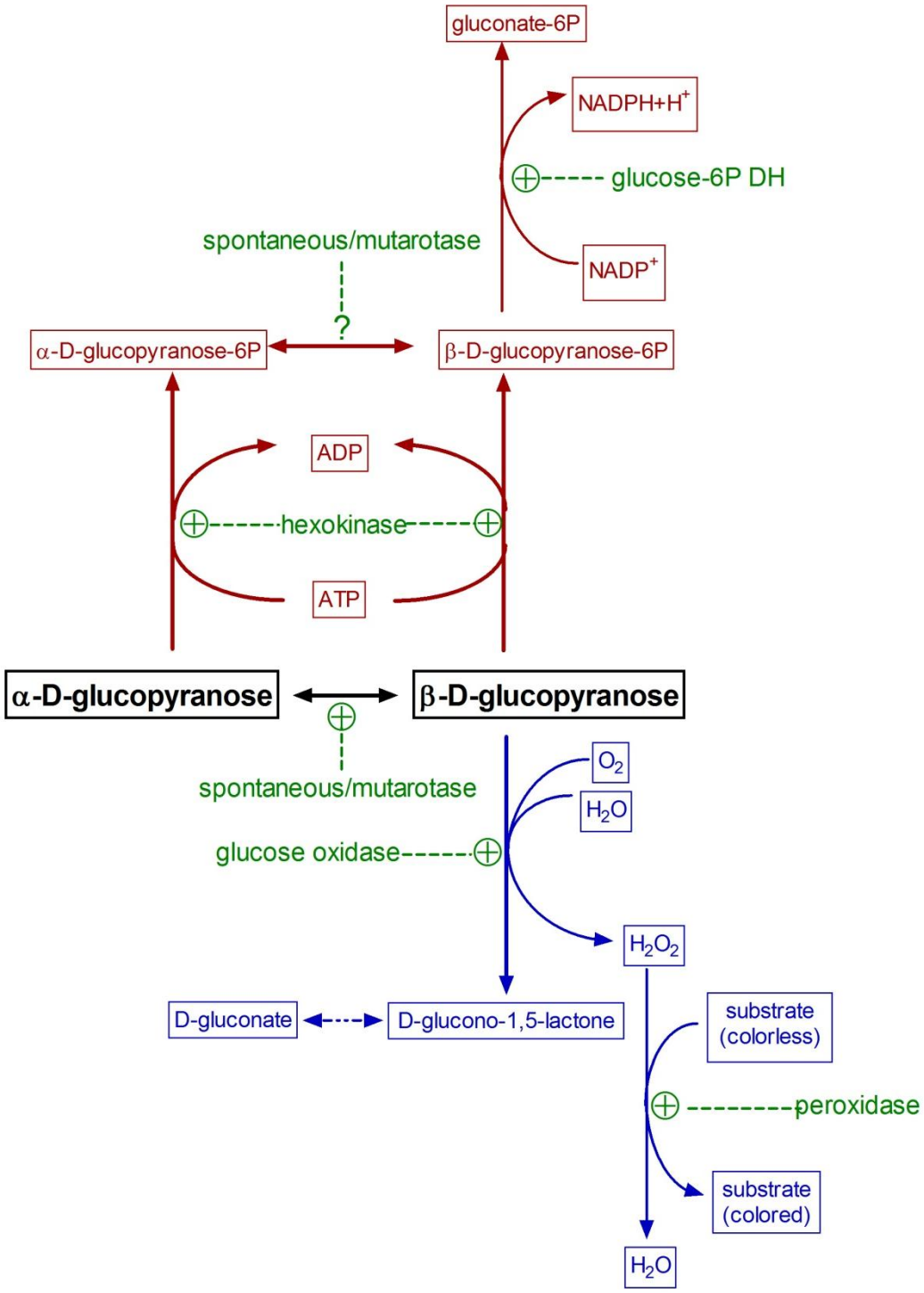

Figure 2. **Common paired enzymatic reactions used in the specific measurement of glucose.** Hexokinase/glucose 6-P dehydrogenase reaction is represented in dark red, glucose oxidase/peroxidase in blue, and all enzymes involved in green.

FIGURE 3

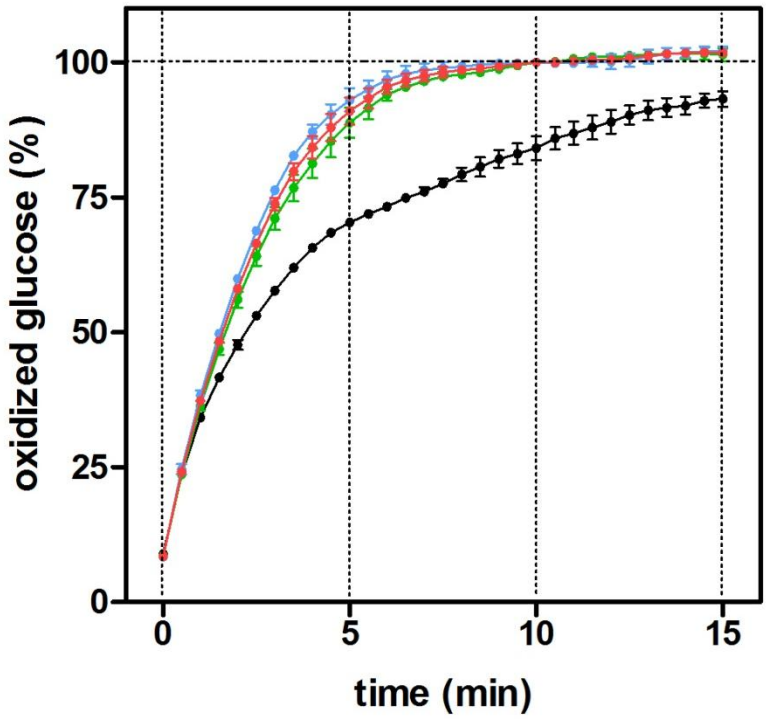

Figure 3. **Percentage of oxidized glucose with incubation time in the presence of varying mutarotase concentrations, using a glucose oxidase/peroxidase method.**

The data (mean ± SEM) are expressed as of an estimated percentage of oxidized glucose of 10 replicates obtained from a 10 mM α-D-glucose standard. Black circles: absence of mutarotase; green squares: 0.3 nkat/L of mutarotase; red inverted triangle: 0.5 nkat/L and blue triangle: 0.7 nkat/L.

FIGURE 4

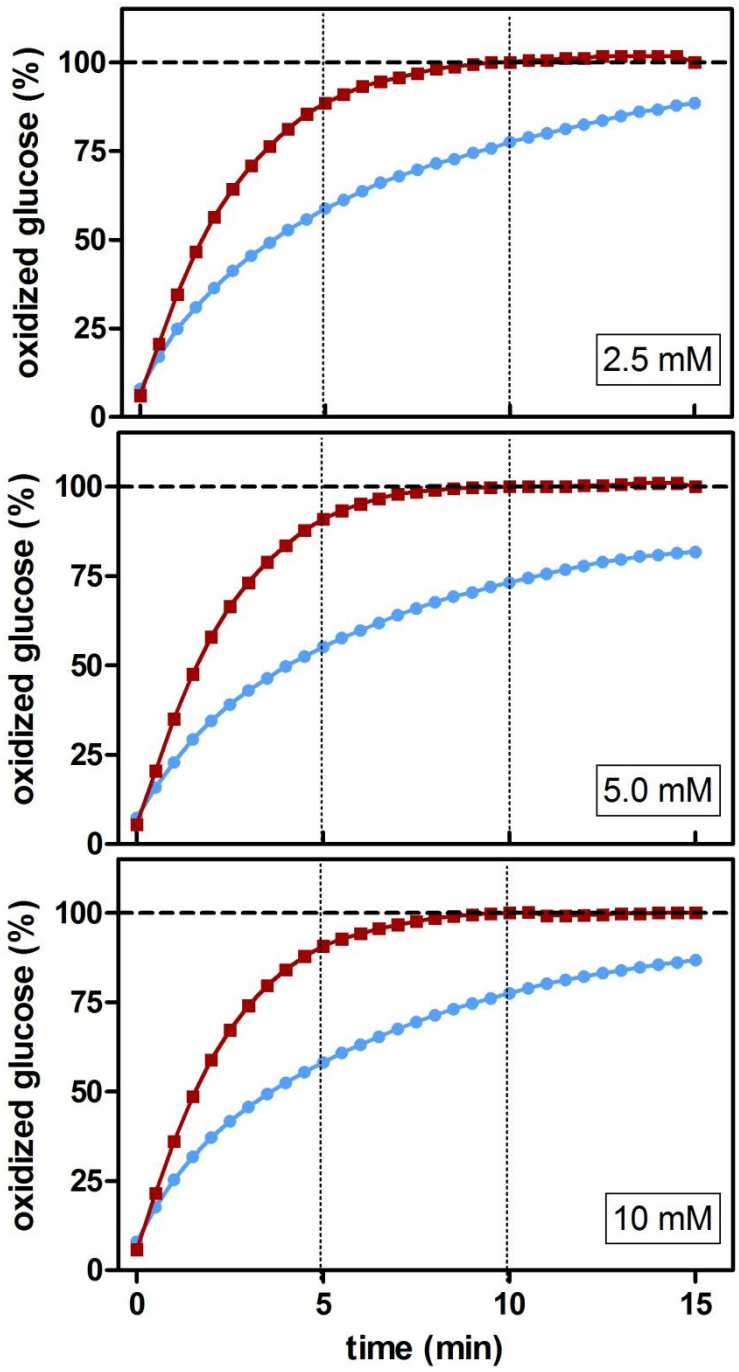

Figure 4. **Estimated percentage of glucose oxidized during incubation at different glucose concentrations using a glucose/oxidase assay in the presence / absence of mutarotase.**

The data are expressed as an estimated percentage of oxidized glucose (calculated from the absorbance of the combined reaction). Data related to absence of mutarotase are represented in blue (line and circles); the data for mutarotase (0.5 nkat/L) are shown in red (line and squares).

FIGURE 5

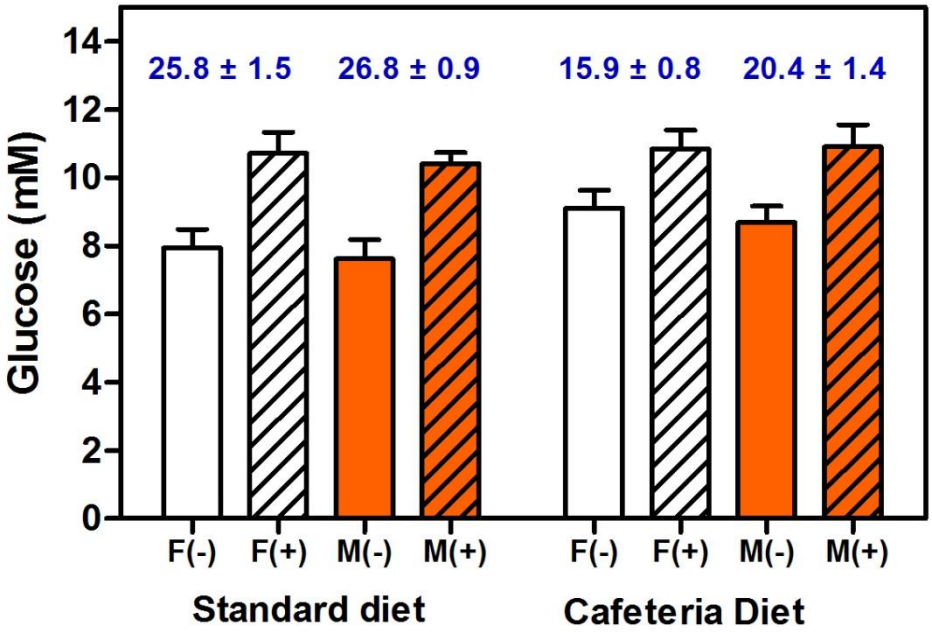

Figure 5. **Plasma glucose concentrations of female and male rats fed with either standard chow or cafeteria diet. Glucose was measured with a glucose oxidase/peroxidase method in the absence or presence of added mutarotase**.

The data are expressed as mean ± SEM of 6 animals per group. White bars: females (F), orange bars: males (M).Results obtained in the presence of mutarotase (+) are shown as dashed columns; open columns have been used for the groups measurements' in absence (-) of mutarotase..The numbers in blue at the top of each pair of same-sex and-diet columns show the estimated percentage of non-measured α-glucose anomer, as a consequence of the absence of (enough) mutarotase.

Statistical analysis of the differences between groups was done using two-way ANOVA. Significant differences were found between absence / presence of mutarotase: (p<0.0001). Diet elicited significant statistical differences only in the absence of mutarotase (p=0.0477). Significant differences of unmeasured α-anomer were found between control and cafeteria groups (p<0.0001). No differences were found for sex.

REFERENCES

1. Vashist SK. Non-invasive glucose monitoring technology in diabetes management: A review. Analytica Chimica Acta. 2012;750:16-27.
2. Benedict SR. The analysis of whole blood. 2. The determination of sugar and of saccharoids (non-fermentable copper-reducing substances). Journal of Biological Chemistry. 1931;92:141-159.
3. Nelson N. A photometric adaptation of the Somogyi method for the determination of glucose. Journal of Biological Chemistry. 1944;153(2):375-380.
4. Neeley WE. Simple automated determination of serum or plasma glucose by a hexokinase/glucose-6-phosphate dehydrogenase method. Clinical Chemistry. 1972;18(6):509-515.
5. Carroll JJ, Smith N, Babson AL. A colorimetric serum glucose determination using hexokinase and glucose-6-phosphate dehydrogenase. Biochemical Medicine. 1970;4(2):171-180.
6. Trinder P. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Annals of Clinical Biochemistry. 1969;6(1):24-27.
7. Ambade VN, Sharma Y, Somani B. Methods for estimation of blood glucose: A comparative evaluation. Medical Journal of the Armed Forces India. 1998;54(2):131-133.
8. Lange CF, Kohn P. Substrate specificity of hexokinases. Journal of Biological Chemistry. 1961;236(1):1-5.
9. Bankar SB, Bule MV, Singhal RS, Ananthanarayan L. Glucose oxidase—an overview. Biotechnology Advances. 2009;27(4):489-501.
10. Meites S, Saniel-Banrey K. Modified glucose oxidase method for determination of glucose in whole blood. Clinical Chemistry. 1973;19(3):308-311.
11. Wong CM, Wong KH, Chen XD. Glucose oxidase: natural occurrence, function, properties and industrial applications. Applied Microbiology and Biotechnology. 2008;78(6):927-938.
12. Yoo E-H, Lee S-Y. Glucose biosensors: an overview of use in clinical practice. Sensors. 2010:10(5):4558-4576.
13. Wilson CM. Quantitative determination of sugars on paper chromatograms. Analytical Chemistry. 1959;31(7):1199-1201.
14. Lopes JF, Gaspar EM. Simultaneous chromatographic separation of enantiomers, anomers and structural isomers of some biologically relevant monosaccharides. Journal of Chromatography A. 2008;1188(1):34-42.
15. Breier M, Wahl S, Prehn C, Fugmann M, Ferrari U, Weise M, et al. Targeted metabolomics identifies reliable and stable metabolites in human serum and plasma samples. PloS One. 2014;9(2):e89728.
16. Miwa I, Maeda K, Okuda J. Anomeric compositions of D-glucose in tissues and blood of rat. Cellular and Molecular Life Sciences. 1978;34(2):167-169.

17. BeMiller JN. Carbohydrate chemistry for food scientists: Saint Paul, American Association of Cereal Chemists (AACC International); 2007.

18. Sols A, Crane RK. Substrate specificity of brain hexokinase. Journal of Biological Chemistry. 1954;210(2):581-595.

19. Salas M, Viñuela E, Sols A. Spontaneous and enzymatically catalyzed anomerization of glucose 6-phosphate and anomeric specificity of related enzymes. Journal of Biological Chemistry. 1965;240(2):561-568.

20. Nelson J, Beegle FM. Mutarotation of glucose and fructose. Journal of the American Chemical Society. 1919;41(4):559-575.

21. Okuda J, Taguchi T, Tomimura A. Mutarotation of D-glucose in body fluids and perfused rat liver. Chemical and Pharmaceutical Bulletin. 1987;35(10):4332-4337.

22. Okuda J, Miwa I. Mutarotase effect on micro determinations of D-glucose and its anomers with β-D-glucose oxidase. Analytical Biochemistry. 1971;43(1):312-315.

23. Miwa I, Okuda J, Maeua K, Okuda G. Mutarotase effect on colorimetric determination of blood glucose with β-D-glucose oxidase. Clinica Chimica Acta. 1972;37:538-540.

24. Okuda J, Miwa I, Maeda K, Tokui K. Rapid and sensitive, colorimetric determination of the anomers of D-glucose with D-glucose oxidase, peroxidase, and mutarotase. Carbohydrate Research. 1977;58(2):267-270.

25 Glick D (Ed). Methods of Biochemical Annalysis, volume 118. New York & London, John Wiley & Sons, 2009.

26 Preedy VR. Dietary Sugars. Chemistry, Analysis, Funtion and Effects. Food and Nutritional Components in Foicus. London, Royal Society of Chemistry, 2012.

27. Oliva L, Baron C, Fernández-López J-A, Remesar X, Alemany M. Marked increase in rat red blood cell membrane protein glycosylation by one-month treatment with a cafeteria diet. PeerJ. 2015;3:e1101.

28. Prats E, Monfar M, Castellà J, Iglesias R, Alemany M. Energy intake of rats fed a cafeteria diet. Physiology and Behavior. 1989;45(2):263-272.

29. Esteve M, Rafecas I, Fernández-López J, Remesar X, Alemany M. Fatty acid utilization by young Wistar rats fed a cafeteria diet. Molecular and Cellular Biochemistry. 1992;118(1):67-74.

30. Somogyi M. Determination of blood sugar. Journal of Biological Chemistry. 1945;160:69-73.

31. Leitch JM, Carruthers A. ATP-dependent sugar transport complexity in human erythrocytes. American Journal of Physiology-Cell Physiology. 2007;292(2):C974-C986.

32. Malaisse W, Willem R. Anomeric specificity of D-glucose production by rat hepatocytes. Molecular and Cellular Biochemistry. 2004;266(1-2):145-150.

33. Malaisse WJ, Sener A. Mathematical modelling of alpha-and beta-D-glucose metabolism in pancreatic islets exposed to equilibrated D-glucose. International Journal of Molecular Medicine. 2004;14(4):677-759.

34. Zhang Y, Courtois P, Sener A, Malaisse WJ. Anomeric specificity of D-[U-^{14}C] glucose incorporation into glycogen in rat hemidiaphragms. Biochimie. 2004;86(12):913-918.

35. Malaisse WJ, Malaisse-Lagae F. Anomeric specificity of D-glucose metabolism in rat brain cells. Brain Research. 1987;419(1-2):147-155.

36. Nagata Y, Nanba T, Ando M, Miwa I, Okuda J. Anomeric preferences of D-glucose uptake and utilization by cerebral cortex slices of rats. Neurochemical Research. 1979;4(4):505-516.

37. Okuda J, Miwa I, Sato M, Murata T. Uptake of D-glucose anomers by rat retina. Experientia. 1977;33(1):19-20.

38. Miwa I, Fukatsu H, Toyoda Y, Okuda J. Anomeric preference of glucose utilization in human erythrocytes loaded with glucokinase. Biochemical and Biophysical Research communications. 1990;173(1):201-217.

39. Leitch JM, Carruthers A. α-And β-Monosaccharide transport in human erythrocytes. American Journal of Physiology-Cell Physiology. 2009;296(1):C151-C61.

40. Deng D, Sun PC, Yan CY, Ke M, Jiang X, Xiong L, Ren WL, et al. Molecular basis of ligand recognition and transport by glucose transporters. Nature. 2015;526(7573):391-396.

41. Carruthers A, Melchior D. Transport of. alpha.-and. beta.-D-glucose by the intact human red cell. Biochemistry. 1985;24(15):4244-4250.

42. Zuurbier CJ, Keijzers PJ, Koeman A, van Wezel HB, Hollmann MW. Anesthesia's effects on plasma glucose and insulin and cardiac hexokinase at similar hemodynamics and without major surgical stress in fed rats. Anesthesia and Analgesia. 2008;106(1):135-142.

43. Mašek J, Fabry P. High-fat diet and the development of obesity in albino rats. Experientia. 1959;15(11):444-445.

44. Oliva L, Aranda T, Caviola G, Fernández-Bernal A, Alemany M, Fernández-López JA, Remesar X. In rats fed high-energy diets, taste, rather than fat content, is the key factor increasing food intake: a comparison of a cafeteria and a lipid-supplemented standard diet. PeerJ. 2017;5:e3697.

45. Rotondo F, Ho-Palma AC, Remesar X, Fernández-López JA, Romero MM, Alemany M. Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycerogenesis and increased acyl-glycerol turnover. Scientific Reports. 2017;7(1):8983.

46. Ho-Palma AC, Rotondo F, Romero MM, Fernández-López JA, Remesar X, Alemany M. Use of ^{14}C-glucose by primary cultures of mature rat epididymal adipocytes. Marked release of lactate and glycerol, but limited lipogenesis in the absence of external stimuli. Adipocyte. 2018:7(3):204-217.

47. Rotondo F, Ho-Palma AC, Remesar X, Fernández-López JA, Romero MM, Alemany M. Effect of sex on glucose handling by adipocytes isolated from rat subcutaneous, mesenteric and perigonadal adipose tissue. PeerJ. 2018;6:e5440.

Marked increase in rat red blood cell membrane protein glycosylation by one-month treatment with a cafeteria diet

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ABSTRACT

Background and Objectives. Glucose, an aldose, spontaneously reacts with protein amino acids yielding glycosylated proteins. The compounds may reorganize to produce advanced glycosylation products, which regulatory importance is increasingly being recognized. Protein glycosylation is produced without the direct intervention of enzymes and results in the loss of function. Glycosylated plasma albumin, and glycosylated haemoglobin are currently used as index of mean plasma glucose levels, since higher glucose availability results in higher glycosylation rates. In this study we intended to detect the early changes in blood protein glycosylation elicited by an obesogenic diet.

Experimental Design. Since albumin is in constant direct contact with plasma glucose, as are the red blood cell (RBC) membranes, we analyzed their degree or glycosylation in female and male rats, either fed a standard diet or subjected to a hyper-energetic self-selected cafeteria diet for 30 days. This model produces a small increase in basal glycaemia and a significant increase in body fat, leaving the animals in the initial stages of development of metabolic syndrome. We also measured the degree of glycosylation of hemoglobin, and the concentration of glucose in contact with this protein, that within the RBC. Glycosylation was measured by colorimetric estimation of the hydroxymethylfurfural liberated from glycosyl residues by incubation with oxalate.

Results. Plasma glucose was higher in cafeteria diet and in male rats, both independent effects. However, there were no significant differences induced by sex or diet in either hemoglobin or plasma proteins. Purified RBC membranes showed a marked effect of diet: higher glycosylation in cafeteria rats, which was more marked in females (not in controls). In any case, the number of glycosyl residues per molecule were higher in hemoglobin than in plasma proteins (after correction for molecular weight). The detected levels of glucose in RBC were lower than those of plasma, even when expressed in molal units, and were practically nil in cafeteria-diet fed rats compared with controls; there was no effect of sex.

Conclusions. RBC membrane glycosylation is a sensitive indicator of developing metabolic syndrome-related hyperglycemia, more sensitive than the general

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measurement of plasma or RBC protein glycosylation. The extensive glycosylation of blood proteins does not seem to be markedly affected by sex; and could be hardly justified from an assumedly sustained plasma hyperglycemia. The low levels of glucose found within RBC, especially in rats under the cafeteria diet, could hardly justify the extensive glycosylation of hemoglobin and the lack of differences with controls, which contained sizeable levels of intracellular glucose. Additional studies are needed to study the dynamics of glucose *in vivo* in the RBC to understand how such extensive protein glycosylation could take place.

Subjects Cell Biology, Diabetes and Endocrinology, Hematology, Metabolic Sciences
Keywords Erythrocyte, Protein glycosylation, Glycemia, Glycosylated hemoglobin, Blood cell membrane, Cafeteria diet, Glycosylation

INTRODUCTION

Glucose, in addition to being the main intercellular energy staple, is a reducing aldose. Thus, it may easily react with a number of chemical groups in proteins and other biological compounds. The direct condensation with protein free amino groups (Maillard reactions) (*John & Lamb, 1993*) is fairly common, to the degree that a significant proportion of circulating plasma proteins are glycosylated (*Gragnoli et al., 1982*), as well as proteins in the red blood cell (RBC) membrane (*Miller, Gravallese & Bunn, 1980*) and the hemoglobin they contain (*Bunn, Gabbay & Gallop, 1978*). The proportion of hemoglobin glycosylated in the terminal valine of chain B (Hb_{1AC}) is currently used as an index of overall exposure to free plasma glucose over time (*Siu & Yuen, 2014*). Glycosylation products may undergo Amadori reorganizations, producing a number of complex compounds known as advanced glycosylation products (AGP) (*Henning et al., 2011*), which play a significant role in the control of substrate utilization (*Wu et al., 2011*), cell function (*Guo et al., 2012*) and inflammation (*Poulsen et al., 2014*).

The chemical reactivity of glucose is often overlooked because of its overwhelming function in energy supply and rapid turnover, but direct non-enzymatic glycosylation remains a common mechanism of alteration of protein function and interference in signaling pathways (*Asahi et al., 2000*; *Itkonen & Mills, 2013*). It is commonly accepted that higher sustained circulating levels of glucose, as in diabetes, result in increased proportions of glycosylated proteins in plasma, RBCs and endothelial cells, Hb_{1AC} being a case in point (*Carson et al., 2010*). In fact, equations based on the correlation between mean estimated plasma glucose concentration and Hb_{1AC} proportion are currently in use (*Borg & Kuenen, 2009*; *Nathan et al., 2008*).

The self-selected cafeteria diets (*Sclafani & Springer, 1976*) are essentially hyperlipidic (*Prats et al., 1989*), and its consumption by rats causes hyperphagia, insulin resistance and obesity (*Correa Pinto & Monteiro Seraphim, 2012*; *Prats et al., 1989*). Exposure for one month of young adult rats to a cafeteria diet induces a number of metabolic changes that are in the limit of normalcy and correspond to the initial stages of the metabolic syndrome

(*Romero et al., 2010*). The effects are more marked in male than in female rats (*Romero et al., 2012*), probably because of the anti-inflammatory effects of estrogen (*Thomas et al., 2003*); but, in any case, the obesity is already patent. Short-term treatment with cafeteria diets induce a mild hyperglycemia and hyperinsulinemia (*Romero et al., 2010*), but not frank diabetes, which is more developed after prolonged exposure (*Castell-Auví et al., 2012*; *Correa Pinto & Monteiro Seraphim, 2012*).

In the present study, we have analyzed whether the glycosylation degree of total plasma or RBC proteins, as well as those of RBC membranes, are a direct correlate of their prolonged contact with plasma glucose in an early stage of development of hyperglycemia. We wanted, also, to check whether sex exerts any influence on the glycosylation response to comparable glucose concentrations.

MATERIALS AND METHODS

Animals and animal handling

All animal handling procedures were carried out in accordance with the norms of the European, Spanish and Catalan Governments. The study was specifically approved (DMAH-5483) by the Animal Ethics Committee of the University of Barcelona.

Wistar adult male and female (9 week-old) rats were used (Harlan Laboratories Models, Sant Feliu de Codines, Spain). The rats were adapted to the Animal House environment for at least one week prior to the beginning of the experiment, and were fed a standard (Harlan, type 2014) chow. The rats were kept in solid-bottomed adjoining collective 2-rat cages, with wood shards as bedding material. Half of the rats in each group were subjected to an energy-rich limited-item cafeteria diet (*Ferrer-Lorente et al., 2005*) for a month. The items of cafeteria diet (plain cookies spread with liver pâté, bacon, standard chow, water and whole cow's milk containing 300 g/L sucrose and a mineral and vitamin supplement) were renewed daily. Food consumption per cage and rat weights were recorded every day.

The four experimental groups ($N = 6$ for each) were: female-control (FC), female-cafeteria, (FCAF) male-control (MC) and male-cafeteria (MCAF). On day 29, a small sample of blood was taken from a cut in the rat tail's tip, centrifuged in capillary tubes, and the plasma was frozen for later measurement of glucose levels.

At the end of the experiment (day 30), the rats were anaesthetized with isoflurane and killed by exsanguination (blood drawn from the aorta using a dry-heparinized syringe). Part of the blood was centrifuged immediately (at 1,300×*g* for 25 min and 2–4 °C). Plasma and packed cells were frozen and kept at −20 °C. A sample of fresh blood was deproteinized with 0.5 volumes of 6,7 M perchloric acid, mixed, neutralized with 4.5 M KOH containing 1.55 M potassium bicarbonate, centrifuged again at the same speed (at 4 °C), and the supernatants used for the measurement of total blood glucose.

Packed cell volume was estimated from the weight of blood before centrifugation, that of plasma obtained after that centrifugation and the (redundant) weight of packed cells sedimented. Since the densities of cells and packed cells were known, and the proportion of packed cell volume was a direct correlate of time and acceleration generated during centrifugation, we used the previously described graphs, obtained under the same

conditions (*Romero et al., 2012*) to estimate the actual proportion of plasma trapped between the cells, and thus determine the real packed cell volume.

A known weight of frozen packed cells was suspended in 10 volumes of chilled pure water. After gentle shaking for 20 min at 4 °C, the suspension was centrifuged 10 min at 2,000× *g* and 2–4 °C. The clear supernatant (hemoglobin and cytosolic RBC proteins) was used for the analyses of total and glycosylated protein.

RBC membrane separation

About 0.5 g samples of frozen packed cells were weighed and suspended in 3.5 mL of chilled tris–HCl buffer 10 mM pH 7.4, the cells were gently stirred until a uniform solution was obtained. Then, 4 mL of chilled 250 mM glucose were added and gently mixed. After standing 15 min (*Tomoda et al., 1984*), the suspension was coarse-filtered through a small wad of glass fiber to remove debris, and then was centrifuged for 3 min at 8,000× *g* in the cold (2–4 °C). The fluffy precipitate was suspended in medium, and centrifuged again. A small translucent sediment of RBC membranes was obtained; it was weighed, and used for the analysis of protein, total phosphate and glycosylation.

Chemical analyses

Glucose in plasma and deproteinized fresh blood was measured with a glucose oxidase kit (Biosystems, Barcelona, Spain), supplemented with mutarotase (490 nkat/mL of reagent) (Calzyme, San Luis Obispo, CA, USA). Mutarotase 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*). The enzyme addition was complemented with a precise control of the time (15 min) and temperature (30 °C) conditions of development of the reaction, in order to make sure all glucose in the sample was oxidized to gluconate. Protein content was estimated with a variant of the Lowry method (*Lowry et al., 1951*) using fatty acid-free bovine serum albumin (Sigma, St Louis MO, USA) as standard.

RBC membranes were mineralized with perchloric acid (700 g/L) in 15 mL Teflon-stoppered glass tubes, in a dry block heater, at 150 °C for 24 h (*Stein & Smith, 1982*). Aliquots of the clear mineralized samples were used, after centrifugation, for the estimation of phosphate using the phosphomolybdate reaction using sodium mono-phosphate as standard (*Gomori, 1942*; *Stein & Smith, 1982*). A standard of phosphatidyl-choline (Sigma) was processed along with the samples. The measurements of phosphate from the phosphatidyl-choline standards proved that mineralization was complete (98–101%). Each batch of samples was corrected using their own standards, ran in parallel.

The degree of glycosylation was estimated by direct measurement of the 5-hydroxymethylfurfural (HMF) liberated by treatment of the samples with 1 N oxalic acid at 100 °C for 24 h (*Gabbay et al., 1979*) in 15 mL Teflon-stoppered tubes set in a dry heating block. After cooling, trichloroacetic acid was added (final concentration 100 g/L), and the tubes were shaken and centrifuged for 15 min at 5,000× *g*. The precipitate was discarded. The amount of HMF released was measured through the condensation of HMF with 50 mM thiobarbituric acid (Sigma) (*Gabbay et al., 1979*). After 20 min at 37 °C for development of color, the OD was measured at 443 nm, using blanks and pure HMF

Table 1 Body weight changes, energy intake and plasma glucose of Wistar rats fed control or cafeteria diet for 30 days. The data are the mean ± sem of 6 animals per group. Plasma glucose was measured on day 29. Statistical significance of the differences between means were determined using a 2-way ANOVA program.

Parameter	Units	Male		Female		*P* values		
		Control	Cafeteria	Control	Cafeteria	Sex	Diet	Interaction
Initial weight	g	394 ± 9	379 ± 3	238 ± 5	217 ± 4	<0.0001	NS	NS
Final weight	g	474 ± 10	511 ± 3.5	275 ± 11	290 ± 8	0.0068	<0.0001	NS
Weight increase	g/30 d	82 ± 10	137 ± 4	41 ± 5	74 ± 7	<0.0001	<0.0001	NS
Eenergy intake	MJ/30 d	8.62 ± 0.04	21.4 ± 1.5	6.32 ± 0.39	18.0 ± 1.0	0.0055	<0.0001	NS
	W	3.33 ± 0.01	8.26 ± 0.59	2.44 ± 0.15	6.93 ± 0.38			
Plasma glucose	mM	7.58 ± 0.32	9.13 ± 0.15	6.83 ± 0.26	8.53 ± 0.12	0.0082	<0.0001	NS

(Sigma) standards, and was used to determine the HMF (i.e., unaltered glycosyl residues in proteins) in each sample.

Blood cell glucose estimation

Blood glucose is the composite of the glucose carried by the cells and that in plasma using the common (*Higgins, Garlick & Bunn, 1982*) formula:

$$blood\ glucose = plasma\ glucose \times (1 - \mathrm{PCV}) + cell\ glucose \times \mathrm{PCV}$$

where PCV (Packed Cell Volume) is the net cell volume fraction (i.e., discounting trapped plasma volume) of total blood volume (in this case = 1). In that equation, we had, for each rat, the PCV value as well as plasma and blood glucose. Crude cell-transported glucose was derived from these data. Since it was assumed that trapped plasma glucose concentration was the same than in plasma obtained by centrifugation, the glucose present in that plasma fraction was discounted from the total packed cell glucose (and added to the final data for "plasma glucose"). These calculations were carried out for each individual rat, thus all data used for the calculations were homologous.

Statistics

Statistic comparison between groups was carried out using 2- and 3-way ANOVA analyses, and the Bonferroni *post-hoc* test for further differences between specific groups (Prism 5 program; GraphPad Software, La Jolla, CA, USA).

RESULTS

Table 1 presents the changes in body weight experienced by the rats during one month of exposure to a cafeteria diet. The initial weight difference between female and male rats widened with time, since control males increased about 20% of their weight, compared with 15% of females; cafeteria diet increased body weight 35% in males and 34% in females. Males ate more energy than females: 36% (control diet) or 19% (cafeteria diet). Males' food (expressed as energy content) intake was 2.5-fold higher in cafeteria than in control diet; the value for females was 2.8×.

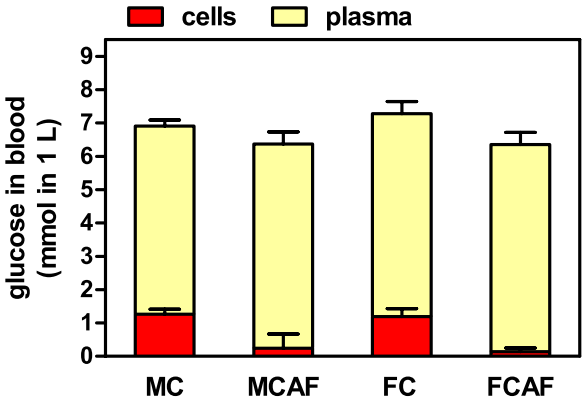

Figure 1 Distribution of blood glucose in plasma and cell compartments of Wistar rats fed control or cafeteria diet during 30 days. Data are the mean ± sem of 6 animals per group (killed under isoflurane anesthesia), and were calculated from the data presented in Table 2. Statistical significance (two-way ANOVA) of the differences between groups: No differences were found for "sex", but "diet" showed $P < 0.0001$ for cells and was not significant for plasma. Blood cell data for cafeteria diet were not statistically different from zero.

Standard plasma glucose (measured on day 29) showed both an effect of sex (female levels being lower) and diet (cafeteria diet data being higher).

Table 2 shows the data obtained from the analysis of blood extracted under isoflurane anesthesia. In this case, all plasma glucose data were higher than those obtained on day 29 under basal conditions, and there were no statistical differences between the groups attributable to sex or diet. Total blood glucose values were lower than those of plasma, and showed neither differences by sex or diet. However, the estimated data for cell glucose showed a clear effect of diet (Fig. 1). In both groups of cafeteria rats, the levels were minimal, not statistically different from zero, while those of rats under the control diet were lower than in plasma but clearly positive, the differences being not significant for "sex" but significant for "diet". In control rats, when water content of plasma (about 92%) and packed cells (about 70%) was included in the calculations, the molal concentrations of cell glucose were in the range of 1/3rd of those of plasma; female rats presented similar values. Cafeteria diet-fed rats showed values in the range of only 4–7%.

The proportions of glycosylated protein, both in RBC and in plasma, are presented in Fig. 2. No significant differences were observed between the groups for "sex" and "diet". However, cell protein was more heavily glycosylated than plasma proteins. In the case of cells, since most of the protein (>95%) is hemoglobin, it can be assumed that most glycosyl residues were bound to this protein; since its molecular weight (tetramer) is about 64,000, the molar ratio of HMF to hemoglobin was about 320, i.e., about 80 glycosyl residues per hemoglobin subunit. This value is about six-fold higher than the number of sites representing 7% Hb_{1AC}, which is limited to the terminal chains of hemoglobin. In the case of plasma, since albumin makes about 55% of plasma proteins and its molecular weight is close to 66,500, we obtain about 90 glycosyl residues per molecule. Evidently, this is only an imprecise approximation but shows that under the particular conditions of this

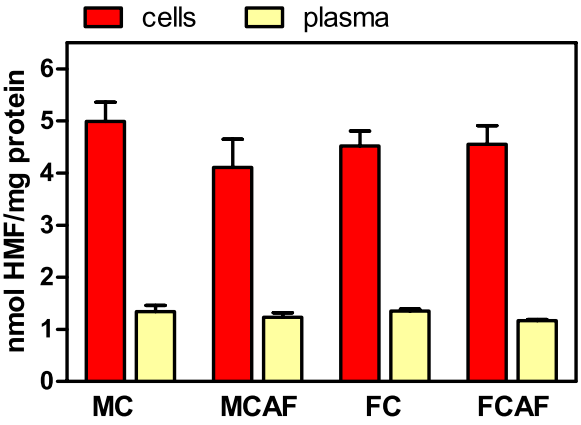

Figure 2 Degree of glycosylation expressed in nmol HMF per mg total protein in the cells and plasma of Wistar rats fed control or cafeteria diet during 30 days. Data are the mean ± sem of 6 animals per group. Statistical significance (three-way ANOVA) of the differences between groups: No differences were found for "sex" and "diet", but the differences between "compartments" (i.e., blood cells vs. plasma) was $P < 0.0001$.

Table 2 Blood glucose and packed cell volume of Wistar rats fed control or cafeteria diet for one month, after exsanguination under isoflurane anesthesia on day 30. The data are the mean ± sem of 6 animals per group. Packed cell volume data were corrected for trapped plasma as explained in the text. Statistical significance of the differences between means were determined using a 2-way ANOVA program.

Parameter	Units	Male		Female		*P* values		
		Control	Cafeteria	Control	Cafeteria	Sex	Diet	Interaction
Blood glucose	mM	6.83 ± 0.13	5.93 ± 0.37	6.43 ± 0.14	6.31 ± 0.31	NS	NS	NS
Plasma glucose	mM	10.41 ± 0.33	10.90 ± 0.64	10.71 ± 0.63	10.84 ± 0.64	NS	NS	NS
Packed cell volume	% blood volume	45.7 ± 0.9	43.8 ± 0.7	43.1 ± 1.1	42.7 ± 1.8	NS	NS	NS
Blood cell glucose[a]	µmol/g	2.75 ± 0.32	0.53 ± 0.96[*]	2.76 ± 0.56	0.33 ± 0.27[*]	NS	<0.0001	NS

Notes.

[a] Blood cells' glucose concentration was calculated for each animal from glucose data (whole blood and plasma) and the net packed cell volume.

[*] Statistically not different from zero.

experiment, protein glycosylation was significant and about 3.5 times more intensive in cells (on a molar ratio) than in plasma proteins as a whole.

Figure 3 depicts the rate of glycosylation observed in purified membranes of blood cells. Since purification of membranes is not even close to quantitative, we could not determine in which proportion RBC membranes were glycosylated. In fact, we were not able to ascertain the degree of the purity of samples. Thus, membrane proteins could be contaminated by hemoglobin (in spite of the appearance of total elimination at the expense of dwindling recovery of membranes), spectrin or other molecules. Thus, we decided to also relate the degree of glycosylation to phospholipid, an exclusive membrane component in RBC. The molar ratio of released HMF to phospholipid phosphate (Fig. 3) showed an image quite different from that of Fig. 2. There were no statistical differences between groups attributable to "sex". This was clear for control diet, but the post-hoc test showed a significant ($P < 0.05$) sex-related difference in cafeteria-fed rats. The effect of "diet" was significant, with several-fold higher values in cafeteria- than in control-fed rats.

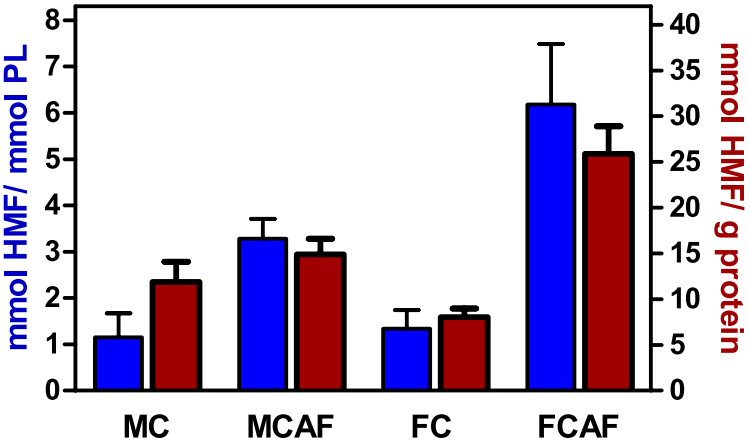

Figure 3 Degree of glycosylation of blood cell membranes, expressed as mmoles of HMF per mmol of phospholipid *P* or unit of membrane protein weight, of Wistar rats fed control or cafeteria diet during 30 days. Data are the mean ± sem of 6 animals per group. Statistical significance (two-way ANOVA) of the differences between groups: When analyzed for HMF *vs*. protein, no statistical differences were found. When analyzed for HMF *vs*. phospholipid *P*. No differences were found for "sex" but the difference for "diet" was $P < 0.0001$. There was a significant interaction between sex and diet. Glycosylation was higher ($P < 0.05$, Bonferroni post-hoc test) in the female cafeteria rats, compared with males.

Presentation of the data of HMF per mg of membrane preparation protein shown in Fig. 3, yields almost the same pattern, but statistical significance was lower because the individual variation of data was higher.

DISCUSSION

In the development of this apparently simple study, we tried to maintain a close control of methodology, since the problems of glycosylation of blood components have generated a sizeable number of studies, but their integrated analysis is scarce, in a way that only limited comparisons have been studied. We intended to present homologous data for plasma and RBC proteins, including also samples of RBC membranes, and using a model in which the metabolic syndrome pathologies, especially insulin resistance and hyperglycemia, were not fully set in.

The problem of anesthesia as hyperglycemic agent (*Arola et al., 1981*; *Zuurbier et al., 2008*) has not been solved; we opted by using this avenue to obtain sufficient blood to carry out all the compartmentation and membrane experiments in the same samples. Consequently we had to obtain separate plasma samples to compare the basal results with previous studies (*Palou et al., 1980*). The changes elicited by cafeteria diet agree with previously published studies (*Ferrer-Lorente et al., 2005*). We assumed that the brief isoflurane anesthesia-induced hyperglycemia (*Zuurbier et al., 2008*) (less than 5 min from start to exsanguination) changes plasma glucose levels, but its effects on RBC glucose (if any) would be at least partly buffered. In any case, it is highly improbable that these changes would affect differentially the rats depending on their diet. The uniformity of the data

obtained seem to support this weak point of our experimental setup. We have not been able to circumvent the problem within the ethical standards of our Laboratory.

The lower blood *vs.* plasma glucose levels, more marked in cafeteria diet-fed rats, attest directly to a lower cell compartment glucose content (there were minimal differences in packed cell volume). The accuracy of the calculations used to quantify the cell glucose content notwithstanding, do not change the fact that cafeteria rats had higher glucose content in the blood plasma fraction compared with that of cells; precisely the glucose in direct contact with hemoglobin.

Metabolic syndrome, diabetes and in general, high exposure to inflammation and hyperglycemia increase the glycosylation of plasma proteins (*Matsuura, Hughes & Khamashta, 2008*; *Roohk & Zaidi, 2008*). In fact, glycosylated albumin has been proposed as an indicator of maintained hyperglycemia (i.e., exposure of plasma proteins to higher aldose levels for long periods) (*Abe & Matsumoto, 2008*). However, the most used indicator of long-time maintained hyperglycemia is the measurement of glycosylated hemoglobin (*Siu & Yuen, 2014*), which initially was applied to whole RBC hemoglobin (*Carson et al., 2010*), but soon was focused on the terminal amino groups of hemoglobin (Hb_{1AC}) alone, giving rise to a much more sensitive (and extended) assay methodology (*Little et al., 2008*; *Weykamp et al., 2008*). The study of glycosylated hemoglobin (Hb_{1AC}) has become one of the standard elements for the evaluation of diabetes (metabolic syndrome) and, in general, sustained hyperglycemia (*Ong et al., 2010*). The critical point, however, is that all hemoglobin is contained within the RBC, and is not in direct contact with plasma glucose. This obvious circumstance would make *a priori* glycosylated albumin a more acceptable indicator of hyperglycemia. The conundrum of a marker of glycosylation not in direct contact with the parameter it measures has not been sufficiently explained so far. Nevertheless, its widespread use and clinical reliability are powerful reasons in favor of its continued use despite the largely unexplained nature of its origins.

In mammals, the direct permeability of the RBC membrane to glucose is low, if any (*Britton, 1964*; *Rich et al., 1967*), however, interchange of plasma and RBC glucose is active thanks to a facilitated-diffusion transport system (*Levine, Oxender & Stein, 1965*). The transport has been attributed, mainly to GLUT1 (*Graybill et al., 2006*), which function may be regulated by insulin, glucocorticoids and other factors (*Kahn & Flyer, 1990*). However, no differences in glucose transport through erythrocyte membranes were found between diabetic and euglycemic children (*Mortensen & Brahm, 1985*). There is, also, a high variability in the permeability of RBC membranes to glucose, due to species differences, individual factors and transporter modulation/saturation (*Khera et al., 2008*).

Compartmentation of blood glucose between plasma and cells may be an important regulatory factor by itself (*Palou et al., 1980*), since glucose carried by blood cells is rapidly interchanged with tissues (*Jacquez, 1984*). This is in overt contradiction with the slow velocity of glucose interchange of RBC when measured *in vitro* (*Sen & Widdas, 1962*). In addition, given the glycolytic nature of mammalian RBC, it can be expected that a sizeable part of the glucose entering the cell is rapidly glycolyzed to lactate, a process that is the only significant source of ATP for the cell. This inefficient mechanism converts blood in a

sizeable source of lactate, which implies that a variable part of glucose will be converted to hexoses-P on entering the cell, and thus (at least in part, when isomerized to ketose-P) lose its aldose-related glycosylating capacity.

The high proportions of Hb_{1AC} found under conditions of assumed sustained hyperglycemia (*Giuffrida et al., 2010*) contrast with the physical existence of barriers between hyperglycemic plasma and hemoglobin. We could not explain why Hb_{1AC} is so highly correlated with hyperglycemia, since total hemoglobin glycosylation does not reflect only hyperglycemia (*Adams et al., 2009*; *Chan et al., 2014*), which is in agreement with our data. In addition, diabetogenic conditions, such as those presented here and those found in the literature (*Koga et al., 2007*; *Miyashita et al., 2007*; *Wakabayashi, 2012*) do not show the expected changes in hemoglobin glycosylation. Our data on the lack of significant changes elicited by diet on plasma protein glycosylation do not agree with the common occurrence of increased glycosylated proteins in plasma of humans and rodents alike under already settled metabolic syndrome or its associated pathologies (*Gornik & Lauc, 2008*). The probable differences lie in the fact that in our model of initial stages of metabolic syndrome, the pathologic markers have not been yet developed fully, as we have previously found (*Ferrer-Lorente et al., 2005*). It must be also taken into account that metabolic syndrome-induced modifications on plasma proteins (*Marliss et al., 2006*; *Welle et al., 1992*) and RBC (*Cohen, Franco & Joiner, 2004*; *Manodori & Kuypers, 2002*) increase their cell turnover rates which compounds the problem and makes more difficult the comparisons unless the data maintain their homology.

The elevated degree of glycosylation found in RBC membranes, however, shows that even the small differences in basal glycemia found in our model are enough to already induce several-fold changes in the glycosylative activity of plasma glucose. Probably, other factors so far not identified, may help explain the increased glycosylation observed even at early stages of the development of metabolic syndrome. The relationship with high-energy (lipid) diet is clear, but the common assumption that these changes are a correlate of hyperglycemia remain unproven, and largely based only on indirect evidence.

In our experiment, the degree of glycosylation of hemoglobin was high, even under conditions in which practically no free glucose was found within the RBC. We only measured glycosyl residues, not those recombined by Amadori rearrangements, i.e., those able to liberate hydroxymethylfurfural. However, the ratio of up to 90 glycosyl residues per subunit of hemoglobin is close to the level of saturation of glycosylable sites. This was necessarily achieved in at most one month, a time shorter than the mean rat RBC half-life, 45–50 days (*Burwell, Brickley & Finch, 1953*), a value considerably decreased in rats with metabolic syndrome (*Kung, Tseng & Wang, 2009*). However, compared with albumin which median life span is close to 2 days (*Reed et al., 1988*), the differences can be better explained, since exposure of hemoglobin was 15-fold higher (30 days out of 45–50) than that of plasma proteins (2 days, assuming a behavior comparable to that of albumin). The shorter exposure was predictably more intense (as that of RBC membrane protein) because plasma proteins were in constant contact with plasma glucose.

The levels of glycosylation of plasma proteins and hemoglobin observed do not reflect the (limited) changes in plasma glucose, however, RBC membranes do. The results we obtained are puzzling; they agree with the known fact that exposure to hyperglycemia results in increased protein glycosylation, as shown by membrane proteins' differences, but not observed in plasma proteins; this may be due to their shorter half-lives and limited span of glucose level change.

On the other side, the low free glucose levels observed in cafeteria diet-fed rat RBC, agree with a slower rate of uptake (*Prats et al., 1987*) compared with plasma, but cannot directly explain how the overall glycosylation of hemoglobin was unaffected by one month of consumption of a hyper-energetic obesogenic diet.

CONCLUSIONS

We conclude that blood glucose compartmentation, as previously indicated, may play a role, in the regulation of plasma/blood versus tissue glucose transport/transfer, more important than usually assumed, but also, that glycosylation of blood proteins widely affects non-diabetic young experimental animals, both under standard or hyper-energetic diet conditions. This extensive glycosylation does not seem to be markedly affected by sex; and could be hardly justified from an assumedly sustained plasma hyperglycemia. More detailed—and comprehensive—analyses should be carried out to study the dynamics of glucose *in vivo* in the RBC to understand how so extensive protein glycosylation as that found here could take place, including an special emphasis on the hormonal regulation of RBC glucose transporters.

We have also found that RBC membrane glycosylation is a sensitive indicator of developing metabolic syndrome-related hyperglycemia, more sensitive than the general measurement of plasma or RBC protein glycosylation.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Laia Oliva performed the experiments, prepared figures and/or tables, reviewed drafts of the paper.
- Cristian Baron performed the experiments.
- José-Antonio Fernández-López analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, reviewed drafts of the paper.
- Xavier Remesar 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, prepared figures and/or tables.

Animal Ethics

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

All animal handling procedures were carried out in accordance with the norms of European, Spanish and Catalan Governments. The study was specifically approved (DMAH-5483) by the Animal Ethics Committee of the University of Barcelona.

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REFERENCES

Abe M, Matsumoto K. 2008. Glycated hemoglobin or glycated albumin for assessment of glycemic control in hemodialysis patients with diabetes? *Nature Clinical Practice Nephrology* **4**:482–483 DOI 10.1038/ncpneph0881.

Adams RJ, Appleton SL, Hill CL, Wilson DH, Taylor AW, Chittleborough CR, Gill TK, Ruffin RE. 2009. Independent association of HbA_{1c} and incident cardiovascular disease in people without diabetes. *Obesity* **17**:559–563 DOI 10.1038/oby.2008.592.

Arola L, Alemany M, Herrera E, Palou A, Remesar X. 1981. Effect of ether, sodium pentobarbital and chloral hydrate anesthesia on rat plasma metabolite concentrations. *Revista Española de Fisiología* **37**:379–385.

Asahi K, Ichimori K, Nakazawa H, Izuhara Y, Inagi R, Watanabe T, Miyata T, Kurokawa K. 2000. Nitric oxide inhibits the formation of advanced glycation end products. *Kidney International* **58**:1780–1787.

Borg R, Kuenen J. 2009. HbA_{1c} levels can be expressed as an estimated Average glucose (eAG). *Clinical Laboratory International* **33**:14–16.

Britton HG. 1964. Permeability of the human red cell to labelled glucose. *Journal of Physiology* **170**:1–20 DOI 10.1113/jphysiol.1964.sp007310.

Bunn HF, Gabbay KH, Gallop PM. 1978. The glycosylation of hemoglobin: relevance to diabetes mellitus. *Science* **200**:21–27 DOI 10.1126/science.635569.

Burwell EL, Brickley BA, Finch CA. 1953. Erythrocyte life span in small animals. Comparison of two methods employing radioiron. *American Journal of Physiology* **172**:718–724.

Carson AP, Reynolds K, Fonseca VA, Muntner P. 2010. Comparison of A1C and fasting glucose criteria to diagnose diabetes among US adults. *Diabetes Care* **33**:95–97 DOI 10.2337/dc09-1227.

Castell-Auví A, Cedó L, Pallarès V, Blay M, Ardévol A, Pinent M. 2012. The effects of a cafeteria diet on insulin production and clearance in rats. *British Journal of Nutrition* **108**:1155–1162 DOI 10.1017/S0007114511006623.

Chan CL, McFann K, Newnes L, Nadeau KJ, Zeitler PS, Kelsey M. 2014. Hemoglobin A_{1c} assay variations and implications for diabetes screening in obese youth. *Pediatric Diabetes* **15**:557–563 DOI 10.1111/pedi.12132.

Cohen RM, Franco RS, Joiner CH. 2004. Is poor glycemic control associated with reduced red blood cell lifespan? *Diabetes Care* **27**:1013–1014 DOI 10.2337/diacare.27.4.1013.

Correa Pinto DA, Monteiro Seraphim P. 2012. Cafeteria diet intake for fourteen weeks can cause obesity and insulin resistance in Wistar rats. *Revista de Nutriçao* **25**:313–319 DOI 10.1590/S1415-52732012000300001.

Ferrer-Lorente R, Cabot C, Fernández-López JA, Alemany M. 2005. Combined effects of oleoyl-estrone and a β_3-adrenergic agonist (CL316,243) on lipid stores of diet-induced overweight male Wistar rats. *Life Sciences* **77**:2051–2058 DOI 10.1016/j.lfs.2005.04.008.

Gabbay KH, Sosenko JM, Banuchi GA, Mininsohn MJ, Flückiger R. 1979. Glycosylated hemoglobins: increased glycosylation of Hemoglobin A in diabetic patients. *Diabetes* **28**:337–340 DOI 10.2337/diab.28.4.337.

Giuffrida FMA, Sallum CFC, Gabbay MAL, Gomes MB, Pires AC, Dib SA. 2010. Relationship between glycated hemoglobin and metabolic syndrome of type 1 and type 2 diabetes. A factor analysis study. *Diabetes Care* **33**:e80–e80 DOI 10.2337/dc09-2280.

Gomori G. 1942. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. *Journal of Laboratory and Clinical Medicine* **27**:955–960.

Gornik O, Lauc G. 2008. Glycosylation of serum proteins in inflammatory diseases. *Disease Markers* **25**:267–278 DOI 10.1155/2008/493289.

Gragnoli G, Tanganelli I, Signorini AM, Tarli P, Paoli C. 1982. Non-enzymatic glycosylation of serum proteins as an indicator of diabetic control. *Acta Diabetologica Latina* **19**:161–166 DOI 10.1007/BF02581152.

Graybill C, Van Hoek AN, Desai D, Carruthers AM, Carruthers A. 2006. Ultrastructure of human erythrocyte GLUT1. *Biochemistry* **45**:8096–8107 DOI 10.1021/bi060398x.

Guo CX, Zeng XJ, Song JJ, Zhang M, Wang HX, Xu XW, Du FH, Chen BX. 2012. A soluble receptor for advanced glycation end-products inhibits hypoxia/reoxygenation-induced apoptosis in rat cardiomyocytes via the mitochondrial pathway. *International Journal of Molecular Sciences* **13**:11923–11940 DOI 10.3390/ijms130911923.

Henning C, Smuda M, Girndt M, Ulrich C, Glomb MA. 2011. Molecular basis of Maillard amide-advanced glycation end product (AGE) formation *in vivo*. *Journal of Biological Chemistry* **286**:44350–44356 DOI 10.1074/jbc.M111.282442.

Higgins PJ, Garlick RL, Bunn HF. 1982. Glycosylated hemoglobin in human and animal red cells: role of glucose permeability. *Diabetes* **31**:743–748 DOI 10.2337/diab.31.9.743.

Itkonen HM, Mills IG. 2013. N-Linked glycosylation supports cross-talk between receptor tyrosine kinases and androgen receptor. *PLoS ONE* **8**:e65016 DOI 10.1371/journal.pone.0065016.

Jacquez JA. 1984. Red blood cell as glucose carrier: significance for placental and cerebral glucose transfer. *American Journal of Physiology* **246**:R289–R298.

John WG, Lamb EJ. 1993. The Maillard or browning reaction in diabetes. *Eye* **7**:230–237 DOI 10.1038/eye.1993.55.

Kahn BB, Flyer JS. 1990. Regulation of glucose-transporter gene expression *in vitro* and *in vivo*. *Diabetes Care* **13**:548–564 DOI 10.2337/diacare.13.6.548.

Khera PK, Joiner CH, Carruthers A, Lindsell CJ, Smith EP, Franco RS, Holmes YR, Cohen RM. 2008. Evidence for interindividual heterogeneity in the glucose gradient across the human red blood cell membrane and its relationship to hemoglobin glycation. *Diabetes* **57**:2445–2452 DOI 10.2337/db07-1820.

Koga M, Otsuki M, Matsumoto S, Saito H, Mukai M, Kasayama S. 2007. Negative association of obesity and its related chronic inflammation with serum glycated albumin but not glycated hemoglobin levels. *Clinica Chimica Acta* **378**:48–52 DOI 10.1016/j.cca.2006.10.013.

Kung CM, Tseng ZL, Wang HL. 2009. Erythrocyte fragility increases with level of glycosylated hemoglobin in type 2 diabetic patients. *Clinical Hemorheology and Microcirculation* **43**:345–351.

Levine M, Oxender DL, Stein W. 1965. The substrate-facilitated transport of the glucose carrier across the human erythrocyte membrane. *Biochimica et Biophysica Acta* **109**:151–163 DOI 10.1016/0926-6585(65)90099-3.

Little RR, Rohlfing CL, Hanson S, Connolly S, Higgins T, Weykamp CW, D'Costa M, Luzzi V, Owen WE, Roberts WL. 2008. Effects of hemoglobin (Hb) E and HbD traits on measurements of glycated Hb (HbA_{1C}) by 23 methods. *Clinical Chemistry* **54**:1277–1282 DOI 10.1373/clinchem.2008.103580.

Lowry OH, Rosebrough RW, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**:265–275.

Manodori AB, Kuypers FA. 2002. Altered red cell turnover in diabetic mice. *Journal of Laboratory and Clinical Medicine* **140**:161–165 DOI 10.1067/mlc.2002.126504.

Marliss E, Chevalier S, Gougeon R, Morais J, Lamarche M, Adegoke O, Wu G. 2006. Elevations of plasma methylarginines in obesity and ageing are related to insulin sensitivity and rates of protein turnover. *Diabetologia* **49**:351–359 DOI 10.1007/s00125-005-0066-6.

Matsuura E, Hughes GR, Khamashta MA. 2008. Oxidation of LDL and its clinical implication. *Autoimmunity Reviews* **7**:558–566 DOI 10.1016/j.autrev.2008.04.018.

Miller JA, Gravallese E, Bunn HF. 1980. Nonenzymatic glycosylation of erythrocyte membrane proteins. Relevance to diabetes. *Journal of Clinical Investigation* **65**:896–901 DOI 10.1172/JCI109743.

Miwa I, Maeda K, Okuda J, Okuda G. 1972. Mutarotase effect on colorimetric determination of blood-glucose with β-D-glucose oxidase. *Clinica Chimica Acta* **37**:538–340 DOI 10.1016/0009-8981(72)90483-4.

Miyashita Y, Nishimura R, Morimoto A, Matsudaira T, Sano H, Tajima N. 2007. Glycated albumin is low in obese, type 2 diabetic patients. *Diabetes Research and Clinical Practice* **78**:51–55 DOI 10.1016/j.diabres.2007.02.021.

Mortensen HB, Brahm J. 1985. Glucose transport in erythrocytes of diabetic and healthy children as related to hemoglobin A_{1c}. *Clinical Chemistry* **31**:1387–1389.

Nathan DM, Kuenen J, Borg R, Zheng H, Schoenfeld D, Heine RJ. 2008. Translating the A1C Assay into estimated average glucose values. *Diabetes Care* **31**:1473–1478 DOI 10.2337/dc08-0545.

Ong KL, Tso AWK, Lam KSL, Cherny SS, Sham PC, Cheung BMY. 2010. Using glycosylated hemoglobin to define the metabolic syndrome in United States adults. *Diabetes Care* **33**:1856–1858 DOI 10.2337/dc10-0190.

Palou A, Remesar X, Arola L, Alemany M. 1980. Blood and plasma glucose relationships during pregnancy, the breeding cycle and development in the rat. *Diabete et Metabolisme* **6**:271–275.

Poulsen MW, Bak MJ, Andersen JM, Monosik R, Giraudi-Futin AC, Holst JJ, Nielsen J, Lauritzen L, Larsen LH, Bugel S, Dragsted LO. 2014. Effect of dietary advanced glycation end products on postprandial appetite, inflammation, and endothelial activation in healthy overweight individuals. *European Journal of Nutrition* **53**:661–672 DOI 10.1007/s00394-013-0574-y.

Prats E, Monfar M, Argilés JM, Alemany M. 1987. Effects of starvation and a high-energy diet on rat blood compartmentation of injected radioactive alanine and glucose. *Biochemistry International* **14**:95–101.

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

Reed RG, Davidson L, Burrington C, Peters T. 1988. Non-resolving jaundice: bilirubin covalently attached to serum albumin circulates with the same metabolic half-life as albumin. *Clinical Chemistry* **34**:1992–1994.

Rich G, Sha'afi R, Barton T, Solomon A. 1967. Permeability studies on red cell membranes of dog, cat, and beef. *The Journal of General Physiology* **50**:2391–2405 DOI 10.1085/jgp.50.10.2391.

Romero MM, Fernández-López JA, Alemany M, Esteve M. 2010. Gene expression modulation of liver energy metabolism by oleoyl-estrone in overweight rats. *Bioscience Reports* **20**:81–89.

Romero MM, Fernández-López JA, Remesar X, Alemany M. 2012. Effect of sex and prior exposure to a cafeteria diet on the distribution of sex hormones between plasma and blood cells. *PLoS ONE* **7(3)**:e34381 DOI 10.1371/journal.pone.0034381.

Roohk HV, Zaidi AR. 2008. A review of glycated albumin as an intermediate glycation index for controlling diabetes. *Journal of Diabetes Science and Technology* **2**:1114–1121 DOI 10.1177/193229680800200620.

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.

Sen A, Widdas W. 1962. Determination of the temperature and pH dependence of glucose transfer across the human erythrocyte membrane measured by glucose exit. *The Journal of physiology* **160**:392–403 DOI 10.1113/jphysiol.1962.sp006854.

Siu PM, Yuen QS. 2014. Supplementary use of HbA1c as hyperglycemic criterion to detect metabolic syndrome. *Diabetology and Metabolic Syndrome* **6**:Article 119 DOI 10.1186/1758-5996-6-119.

Stein J, Smith G. 1982. Phospholipids. In: *Techniques in lipid and membrane biochemistry*. County Clare: Elsevier/North Holland, 1–15.

Thomas TN, Rhodin JA, Clark L, Garces A, Bryant M. 2003. A comparison of the anti-inflammatory activities of conjugated estrogens and 17-β estradiol. *Inflammation Research* **52**:452–460 DOI 10.1007/s00011-003-1198-0.

Tomoda A, Kodaira K, Taketo A, Tanimoto K, Yoneyama Y. 1984. Isolation of human erythrocyte membranes in glucose solution. *Analytical Biochemistry* **140**:386–390 DOI 10.1016/0003-2697(84)90182-9.

Wakabayashi I. 2012. Obesity-independent inverse association between regular alcohol consumption and hemoglobin A_{1C}. *Obesity Facts* **5**:60–67 DOI 10.1159/000336067.

Welle S, Barnard R, Statt M, Amatruda J. 1992. Increased protein turnover in obese women. *Metabolism* **41**:1028–1034 DOI 10.1016/0026-0495(92)90133-U.

Weykamp C, John WG, Mosca A, Hoshino T, Little R, Jeppsson J-O, Goodall I, Miedema K, Myers G, Reinauer H. 2008. The IFCC reference measurement system for HbA1c: a 6-year progress report. *Clinical Chemistry* **54**:240–248 DOI 10.1373/clinchem.2007.097402.

Wu CH, Huang HW, Huang SM, Lin JA, Yeh CT, Yen GC. 2011. AGE-induced interference of glucose uptake and transport as a possible cause of insulin resistance in adipocytes. *Journal of Agricultural and Food Chemistry* **59**:7978–7984 DOI 10.1021/jf201271y.

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 hemodynamics and without major surgical stress in fed rats. *Anesthesia and Analgesia* **106**:135–142 DOI 10.1213/01.ane.0000297299.91527.74.

2. VARIACIONES EN LA COMPOSICIÓN DE LA DIETA: EFECTOS EN LA INGESTA Y EL PESO

El uso de dietas hiperenergéticas en modelos animales es extenso, y tiene como objetivo determinar las causas del desarrollo de la obesidad y de otras complicaciones metabólicas. La textura que aportan los lípidos suele ser un componente atractivo que induce la ingesta en humanos y roedores, y que puede ser un factor determinante para la desregulación de los mecanismos que controlan el apetito. Sin embargo, los efectos de una dieta hiperlipídica pueden ser muy variables en función de su composición lipídica y de la presencia de otros componentes, como disacáridos o monosacáridos, hecho que resulta en la obtención de datos heterogéneos dificultando la comparación de los mismos. En los estudios presentados a continuación se evaluaron los efectos obesogénicos exhibidos en ratas de ambos sexos tras la ingesta, durante un mes, de dos tipos distintos de dieta hiperlipídica perfectamente comparables, pues aunque difieren en la composición lipídica, ambas tienen un contenido de energía procedente de lípidos de aproximadamente el 40%. Por un lado, una dieta de cafetería, apetitosa y con una cierta variedad de alimentos a elegir y por otro lado, una dieta hiperlipídica creada a partir de pienso estándar enriquecido con aceite de coco rico en ácidos grasos saturados.

2.1. In rats fed high-energy diets, taste rather than fat content, is the key factor increasing food intake: a comparison of a cafeteria and a lipid-supplemented standard diet

Laia Oliva, Tània Aranda, Giada Caviola, Anna Fernández-Bernal, Marià Alemany, José Antonio Fernández-López and Xavier Remesar.

En este estudio se evaluaron los patrones de ingesta y los efectos en el peso corporal asociados a cada una de las dietas. Tras la intervención nutricional durante un mes se pudo observar que los animales alimentados con la dieta de cafetería incrementaron el peso corporal más rápidamente que los animales alimentados con la dieta estándar o con la dieta estándar enriquecida con aceite de coco. El incremento de peso de los animales sujetos a la dieta de cafetería se correspondió con un patrón de ingesta hiperfágico, que provocó un mayor índice de adiposidad, aunque el gasto energético estimado también fue más elevado; dichos efectos fueron más evidentes en machos. El análisis de la ingesta de los componentes nutricionales mostró que los animales sujetos a la dieta de cafetería ingirieron más cantidad de todos los componentes, aunque la mayor diferencia vino marcada por la ingesta de grandes cantidades de disacáridos y sal, ambos relacionados con el sabor. Los resultados obtenidos sugieren que, en un periodo de tiempo corto, la ingesta de únicamente grandes cantidades de lípido no ejerce efectos obesogénicos, siendo el sabor dulce y/o salado el componente clave para la inducción de hiperfagia y, en consecuencia, de una mayor ingesta energética y un incremento de las reservas energéticas en forma de tejido adiposo.

2.2. Dietary fatty acid composition influences on liver triacylglycerol and cholesterol accumulation. Effect of oestradiol

Laia Oliva, Tània Aranda, Marià Alemany, José Antonio Fernández-López and Xavier Remesar.

En este estudio se analizaron los efectos de la composición de ácidos grasos de ambas dietas hiperlipídicas (dieta estándar enriquecida con aceite de coco y dieta de cafetería) sobre la deposición de lípido corporal y la acumulación de lípido ectópico en el hígado, así como el efecto del estradiol en ambos procesos. Como ya se observa en el estudio anterior, los animales con dieta de cafetería presentaron mayor peso corporal, siendo la relación lípido/proteína de la dieta un factor que influye positivamente en la acumulación de lípido en el tejido adiposo y/o en localizaciones ectópicas. El grado de acumulación de lípido ectópico mostró diferencias entre sexos, de forma que solo en los machos alimentados con ambas dietas hiperlipídicas se observó una excesiva acumulación de triacilgliceroles en el hígado, mientras que el contenido de colesterol hepático incrementó solo en los machos alimentados con la dieta de cafetería. Los animales alimentados con la dieta enriquecida exhibieron una menor concentración de colesterol y mayores concentraciones de glicerol y lactato en plasma. Esta última se correspondió con una mayor expresión y actividad de la lactato deshidrogenasa del músculo, contrariamente a lo que ocurrió con la isoforma del hígado. En cuanto a las concentraciones de estradiol circulante, se vieron incrementadas en los grupos alimentados con la dieta hiperlipídica, siendo sus valores equivalentes entre machos y hembras. Además, se hallaron correlaciones significativas entre los valores de estradiol y las concentraciones de colesterol y de lactato, así como con la expresión y la actividad de la lactato deshidrogenasa del músculo. Todo ello sugiere que la relación lípido / proteína y la composición de ácidos grasos de la dieta tienen efectos significativos sobre el metabolismo de lípidos y de carbohidratos a través de cambios mediados por el estradiol.

In rats fed high-energy diets, taste, rather than fat content, is the key factor increasing food intake: a comparison of a cafeteria and a lipid-supplemented standard diet

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ABSTRACT

Background. Food selection and ingestion both in humans and rodents, often is a critical factor in determining excess energy intake and its related disorders.
Methods. Two different concepts of high-fat diets were tested for their obesogenic effects in rats; in both cases, lipids constituted about 40% of their energy intake. The main difference with controls fed standard lab chow, was, precisely, the lipid content. Cafeteria diets (K) were self-selected diets devised to be desirable to the rats, mainly because of its diverse mix of tastes, particularly salty and sweet. This diet was compared with another, more classical high-fat (HF) diet, devised not to be as tasty as K, and prepared by supplementing standard chow pellets with fat. We also analysed the influence of sex on the effects of the diets.
Results. K rats grew faster because of a high lipid, sugar and protein intake, especially the males, while females showed lower weight but higher proportion of body lipid. In contrast, the weight of HF groups were not different from controls. Individual nutrient's intake were analysed, and we found that K rats ingested large amounts of both disaccharides and salt, with scant differences of other nutrients' proportion between the three groups. The results suggest that the key differential factor of the diet eliciting excess energy intake was the massive presence of sweet and salty tasting food.
Conclusions. The significant presence of sugar and salt appears as a powerful inducer of excess food intake, more effective than a simple (albeit large) increase in the diet's lipid content. These effects appeared already after a relatively short treatment. The differential effects of sex agree with their different hedonic and obesogenic response to diet.

Subjects Biochemistry, Nutrition, Metabolic Sciences
Keywords High-fat diet, Cafeteria diet, Taste, Food intake, Rat

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INTRODUCTION

Fat intake is correlated with weight gain and increased body fat content (*Rothwell & Stock, 1984*). The use of different diets with high energy content has been widely used to determine the conditions eliciting overweight or obesity (*Hariri & Thibault, 2010*). Obesogenic diets have been used to provoke important changes in rodents, especially those related to adipose tissue growth and, as a consequence, their increased involvement in carbohydrate and lipid metabolism (*Peckham, Entenman & Carroll, 1977*; *Archer et al., 2007*). A wide variety of high-energy diets have been used, in which the high lipid content is the common link, thus indicating that dietary fat is a critical factor for fat accumulation (*Buettner, Schölmerich & Bollheimer, 2007*). However, there is considerable variability in the composition of the high-fat diets (HF) used in different obesity models, since the proportion of lipids and their fatty acid composition make these diets highly heterogeneous (*Oakes et al., 1997*; *Briaud et al., 2002*), by far, different from controls on standard chow. In addition, most HF diets contain high fructose or sucrose to enhance their obesogenic effects. They are often simplified (standardized), using a single fat and/or protein source (*Sato et al., 2010*). The metabolic effects of these diets are variable depending on several factors, such as the age of the animals (*Sclafani & Gorman, 1977*), the duration course of the intervention (*Schemmel, Mickelsen & Tolgay, 1969*), the energy density of the diet and, especially, sex (*Agnelli et al., 2016*).

The cafeteria diet is a palatable food diet model in which the range (and variety of tastes and texture) of the foods offered induce a marked hedonic-driven increase in food (and thus energy) consumption (*Sclafani & Springer, 1976*; *Prats et al., 1989*). This consequent excess of energy intake results in the excessive accrual of fat, despite the homoeostatic response to lower food intake and increased thermogenesis (*Rothwell, Saville & Stock, 1982*). Cafeteria diets have been widely used to fatten rats, but a number of Authors tend to consider that the variability attributed to self-selection by taste may be a serious handicap of this model (*Moore, 1987*). Cafeteria diets are very effective creating a model of metabolic syndrome (*Gomez-Smith et al., 2016*), which can cause oxidative damage in adipose tissues (*Johnson et al., 2016*), although it also lowers the anxiety of rats (*Pini et al., 2016*) attenuating their response to stress (*Zeeni et al., 2015*) because of the "comfort food effect" (*Ortolani et al., 2011*). On the other hand, the analysis of what food items were selected by the rats is laborious, but the results obtained are precise, and may allow us to measure the change with exposure time during different phases of development (*Prats et al., 1989*; *Lladó et al., 1995*). The fact remains that cafeteria diets are more obesogenic than standard high-lipid diets with equivalent energy content; despite the variability associated to selection, the actual precise and statistically invariable nutrient intake (*Esteve et al., 1992a*) overcomes the rat strict energy intake control. The consequence is a higher lipid deposition, metabolic change and inflammation (*Rafecas et al., 1992*; *Romero et al., 2014*).

A critical difference between cafeteria diet and "fixed composition" HF diets, in spite of their equivalence in lipid-derived energy, is the (constant) abundance of at least two key tasty components, salt and sugar, which enhance the appetite for food, and consequently increase energy intake (*Tordoff & Reed, 1991*; *Breslin, Spector & Grill, 1995*). A number of

HF diets are also additionally sugar-laden, being very effective in eliciting fat deposition (*Sato et al., 2010*).

In this study, we used a model of HF diet matched in composition (except fat) to the standard rat chow. We used coconut oil (rich in saturated fat), that has a moderate obesogenic capacity (*Buettner et al., 2006*; *Hariri & Thibault, 2010*) when not supplemented with sucrose. This fat content was selected to coincide with the known "usual" percentage of fat self-selected by rats using our simplified cafeteria diet model (ca. 40%) (*Esteve et al., 1992a*; *Rafecas et al., 1993*). The proportion of essential lipids in control diet and our HF diet was the same (i.e., PUFA), being the difference essentially C12–C16 (saturated and monounsaturated) fatty acids. The uniformity in the energy derived from lipids between HF diet *vs.* cafeteria, and the equivalence in everything else except lipid between control diet and HF diet allowed us to establish comparisons based on comparable facts, a point that, as far as we know, has not been previously attempted.

We tried to analyse the influence of tasty food (and consequent activation of the satisfaction circuits) on body energy balance and the known metabolic alterations induced by hyperlipidic diets. Our aim was to determine whether a relatively short treatment is sufficient to show the hedonic response to diet on increased food (and energy) consumption and lipid deposition, taking into account the influence of sex.

MATERIALS & METHODS

Diets

Standard diet (C) (Teklad 2014, Teklad diets, Madison WI, USA) contained 20% of digestible energy derived from protein, 13% from lipids, and 67% from carbohydrates (including 0.10% oligosaccharides). This diet essentially contained plant-derived foods.

The high-fat diet (HF) was prepared by the addition of coconut oil (Escuder, Rubí, Spain) to coarsely ground standard chow. The mix, containing 33 parts (by weight) of standard chow, 4 of coconut oil, and 16 parts of water, was thoroughly kneaded, to form a rough paste which was extruded using cut-end syringes to form 1 × 6 cm cylindrical pellets which were dried at 40 °C for 24 h. This diet contained 14.5% of digestible energy derived from protein, 37.0% from lipids, and 48.5% from carbohydrates. Aversion tests to this diet gave negative results, i.e., not different from control diet.

The simplified cafeteria diet (K) was formed by excess offering of the standard chow pellets, plain cookies spread with liver pâté, bacon, water and milk, which was supplemented with 300 g/L sucrose and 30 g/L of a mineral and vitamin supplement (Meritene, Nestlé, Esplugues, Spain) (*Esteve et al., 1992a*; *Rafecas et al., 1993*). All components were kept fresh (i.e., renewed daily). From the analysis (*a posteriori*) of the ingested items and diet composition, we calculated that approximately 41% of ingested energy was derived from lipids, 12% from protein, and 47% of energy was derived from carbohydrates (23% oligosaccharides and 24% starches), with fair uniformity between sexes ($p > 0.05$).

Table 1 presents the composition of the diets used. For K rats we used the actual food consumption data. Both, crude and digestible energy content per g were higher in the HF diet, since it contains more energy per g than the C and K diets. Cafeteria diet had the

Table 1 Diet composition.

	Standard diet (C)	High-fat diet (HF)	Cafeteria diet* (K)
g/kg			
Protein	143	116	91.6 ± 2.1
Fat	40	134	139 ± 3.5
Carbohydrate	480	390	361 ± 2.9
of which sugars	<1	<1	153 ± 7.4
Fibre	181	146	25.7 ± 4.8
Minerals	47	38	11.4 ± 0.6
Crude energy content (kJ/g)	16.5	18.8	12.4 ± 0.15
Digestible energy content (kJ/g)	12.1	14.6	12.0 ± 0.14

Notes.

*The data for K were the mean ± SEM of six pairs of rats; no significant differences in the proportions of food eaten were observed between sexes.

lowest crude energy value because of its low content of fibre, although its digestible energy was similar to that of control chow. The diet fat content was essentially the same for K and HF diets, i.e., 3-fold higher than that of C diet.

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 (# DAAM 6911).

Ten-week-old male and female Wistar rats (Janvier, Le-Genest-Saint-Isle, France) were used ($N = 39$). The animals were randomly divided in three groups ($n = 6$–8 for each sex) and were fed *ad libitum* for 30 days, either standard rat chow, oil-enriched rat chow (HF) or a simplified cafeteria diet (K). All animals had free access to water. They were housed (in same-sex pairs) in solid-bottom cages with wood shards as bedding material and were kept in a controlled environment (lights on from 08:00 to 20:00, temperature 21.5–22.5 °C, and 50–60% humidity). Body weight and food consumption were recorded daily. Calculation of ingested food in cafeteria diet fed rats was done as previously described by weighing the differences in food offered and debris left (*Prats et al., 1989*), correcting for dehydration.

On day 30, at the beginning of light cycle, the rats were anesthetized with isoflurane and then killed by exsanguination through the exposed aorta using a dry-heparinized syringe. Plasma was obtained by centrifugation and kept at −20 °C until processed. The carcass (and remaining blood and debris) were sealed in polyethylene bags, which were subsequently autoclaved at 120 °C for 2 h (*Esteve et al., 1992b*); the bag contents were weighed and then minced to a smooth paste with a blender (thus obtaining a total rat homogenate).

Analytical procedures

Diet components were used for nitrogen, lipid and energy analyses. Nitrogen content was measured with a semi-automatic Kjeldahl procedure using a ProNitro S system (JP Selecta, Abrera, Spain), whereas lipid content was measured with a solvent extraction method (trichloromethane/methanol 2:1 v/v) (*Folch, Lees & Sloane-Stanley, 1957*). These

procedures were also used for the determination of carcass lipid and protein content determination. The energy content of diet components and rat carcasses were determined using a bomb calorimeter (C7000, Ika, Staufen, Germany).

Glucose in plasma was measured under controlled conditions (15 min, 30 °C) with a glucose oxidase kit #11504 (Biosystems, Barcelona, Spain) supplemented with mutarotase (490 nkat/mL of reagent) (Calzyme, San Luis Obispo, CA, USA). Mutarotase 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*; *Oliva et al., 2015*). Other plasma parameters were measured with commercial kits; thus urea was measured with kit #11537, total cholesterol with kit #11505, creatinine with kit #11802 and triacylglycerols with kit # 11528 (all from BioSystems, Barcelona, Spain). Lactate was measured with kit #1001330 (Spinreact, Sant Esteve d'en Bas, Spain) and non-esterified fatty acids with kit NEFA-HR (Wako, Neuss, Germany); 3-hydroxybutyrate and acetoacetate were estimated with a ketone bodies kit (Biosentec, Toulouse, France) based on 3-hydroxybutyrate dehydrogenase. Total plasma protein was measured using the Folin-phenol reagent (*Lowry et al., 1951*).

Calculations and statistical procedures

Energy intake was calculated from daily food consumption converted with the energy equivalence of the different foods and components measured with the bomb calorimeter. Energy expenditure was calculated as previously described (*Rothwell, Saville & Stock, 1982*) from the difference between the ingested energy and the increase in body energy content of the animals. Energy content increase was estimated using reference data from our previous studies using rats of the same stock, age and sex (*Romero et al., 2013*; *Romero et al., 2014*). Sodium (salt) intake was calculated from food intake and the sodium content of the different food components used (*Fernández-López et al., 1994*).

Statistical comparisons were done with two-way ANOVA analyses (diet and time for weight changes, and sex and diet for the other data) and the *post hoc* Bonferroni test, using the Prism 5.0 program (GraphPad Software Inc, La Jolla CA, USA). Differences were considered significant when p value was <0.05.

RESULTS

Figure 1 presents the changes on rat body weight after one-month of exposure to the diets. The males fed the cafeteria diet showed a significant weight gain (35%) with 1 month treatment; C and HF groups showed a similar, albeit lower, weight gain (18% and 22% respectively). The female K group showed the same pattern as males did (increase of 36%), but differences between K and C (16%) or HF (15%) groups were more marked than in males. There were no differences between C and HF groups. Nevertheless, K and C male weights were different from day 25 onwards. In females, the K group differed from HF from day 12 onwards, and the control group from day 19 onwards. Cafeteria-fed groups showed higher *in vivo* weight increases (males: 126 ± 3 g; females: 74 ± 7 g) than C (males: 79 ± 8 g; females: 40 ± 4 g) and HF (males: 83 ± 6 g; females: 28 ± 2 g) groups (Two-way ANOVA: Sex = $p < 0.0001$; Diet = $p < 0.0001$).

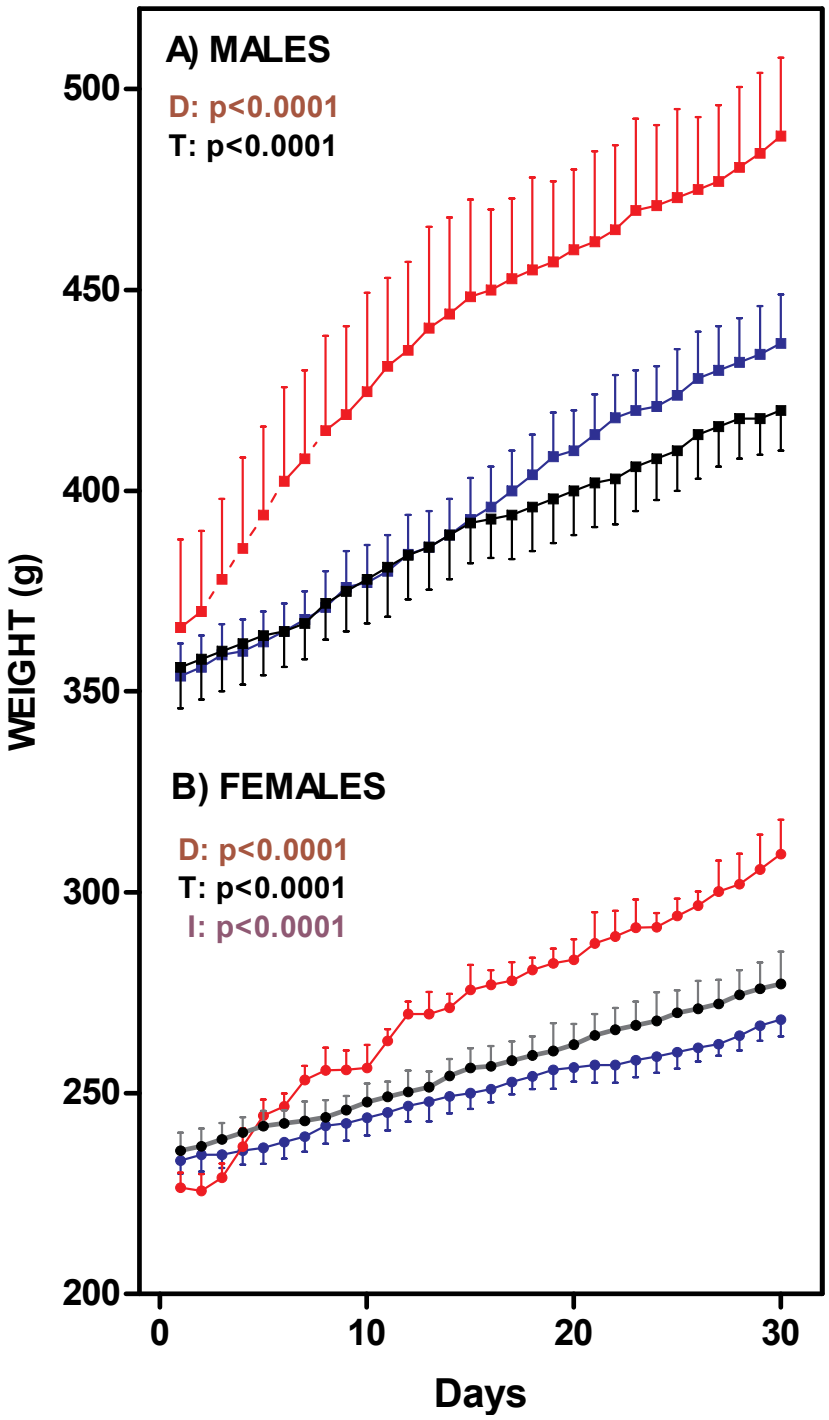

Figure 1 Rat weight changes through 30-days of dietary treatment. (A) represent males (squares) and (B) represent females (circles). Data are expressed as mean ± SEM of six to eight animals per group. Black: rats fed standard diet (C); Blue: rats treated with standard diet supplemented with fat (HF); Red: rats fed cafeteria diet (K). Statistical comparison were established by two-way ANOVA (T, time; D, diet; I, their interaction) and the Bonferroni *post-hoc* test ($p < 0.05$).

Table 2 Plasma parameters of rats fed Standard diet (C), High-fat diet (HF) or Cafeteria diet (K).

	Males			Females			*p*-value	
	C ($n=8$)	HF ($n=6$)	K ($n=7$)	C ($n=6$)	HF ($n=6$)	K ($n=6$)	D	S
Glucose (mM)	10.4 ± 0.33^{A}	9.53 ± 0.47^{A}	10.9 ± 0.64^{A}	10.7 ± 0.63^{a}	8.58 ± 0.21^{b}	10.8 ± 0.56^{a}	0.0029	ns
Lactate (mM)	2.17 ± 0.07^{A}	4.67 ± 0.39^{B}	2.71 ± 0.20^{A}	2.11 ± 0.21^{a}	3.91 ± 0.20^{b}	2.55 ± 0.19^{a}	<0.0001	ns
Cholesterol (mM)	2.57 ± 0.18^{A}	1.82 ± 0.02^{B}	2.38 ± 0.12^{A}	2.57 ± 0.09^{a}	1.76 ± 0.19^{b}	2.69 ± 0.12^{a}	<0.0001	ns
Triacylglycerols (mM)	1.27 ± 0.07^{A}	1.87 ± 0.12^{B}	1.82 ± 0.21^{B}	0.95 ± 0.10^{a}	0.98 ± 0.16^{a}	0.99 ± 0.09^{a}	ns	<0.0001
Non-esterified fatty acids (mM)	0.32 ± 0.05^{A}	0.44 ± 0.03^{AB}	0.51 ± 0.06^{B}	0.34 ± 0.07^{a}	0.36 ± 0.02^{a}	0.40 ± 0.06^{a}	ns	ns
Total protein (g/L)	67.7 ± 0.68^{A}	69.1 ± 0.64^{A}	70.5 ± 1.72^{A}	63.4 ± 1.92^{a}	64.8 ± 1.78^{a}	66.9 ± 1.27^{a}	ns	0.0032
Urea (mM)	2.67 ± 0.21^{A}	3.41 ± 0.05^{A}	1.93 ± 0.18^{B}	2.05 ± 0.13^{a}	3.20 ± 0.37^{b}	1.84 ± 0.21^{a}	0.0001	ns
3-Hydroxybutyrate (μM)	30.2 ± 4.91^{A}	50.5 ± 4.42^{A}	30.8 ± 5.80^{A}	45.3 ± 6.92^{ab}	61.9 ± 10.9^{a}	30.3 ± 6.83^{b}	0.0028	ns
Acetoacetate (μM)	188 ± 43.1^{A}	157 ± 31.1^{A}	126 ± 16.1^{A}	143 ± 61.0^{a}	177 ± 29.8^{a}	202 ± 51.8^{a}	ns	ns

Notes.

Data expressed as mean ± SEM. Statistical analysis: two-way ANOVA, *p*-values for diet (D) or sex (S); *ns* = $p > 0.05$. No significant differences were found for the interaction between diet and sex. Bonferroni's *post-hoc* test statistical significance, established at $p < 0.05$, is represented by different superscript letters.

Table 2 shows the concentration of plasma metabolites. Female HF rats had lower glycaemia than C. When compared with controls, HF elicited a significantly higher lactate levels in both males and females. This HF diet also lowered cholesterol levels *vs.* controls irrespective of sex, but only males showed high triacylglycerols similar to those found in K. Compared with controls, the K males (but not females) showed higher free fatty acids. Urea levels were lower in K males *vs.* C, in contrast with females, which HF group also showed higher urea levels than C. Ketone bodies, especially 3-hydroxybutyrate levels, were affected by diet tending to show higher levels in the HF groups.

Figure 2 shows that the percentage of body lipid was increased in both male and female cafeteria-fed rats, whereas there were no differences between the C and HF groups. The same pattern was observed when body lipid content was expressed in absolute values. Thus, body lipid was a main determinant of absolute body weight gain.

Figure 3 shows the daily energy intake and estimated energy expenditure of rats fed the three experimental diets. Cafeteria fed groups showed the highest values for both daily energy intake and energy expenditure. No differences were found between C and HF, in spite of the significantly lower polysaccharide and protein intake and higher lipid ingestion of the HF groups. The energy values for the different components were balanced, and thus the total energy intake was similar for C and HF groups. Cafeteria-fed rats showed significant increases in the energy intake derived from all diet components, especially for oligosaccharides, which represented 47 ± 2% of carbohydrate energy intake for males and 53 ± 2% for females (ns). Protein, lipid and polysaccharide intake showed different values ($p < 0.0001$) for diet and sex. Lipid and polysaccharide intake also showed statistically significant interaction between diet and sex ($p = 0.0030$).

Figure 4 shows the mean daily rat intake of sugar and salt. The differences in sugar (either lactose or sucrose) intake were considerable, since C and HF intake (only sucrose) was very low compared with that ingested by the K groups. There were no differences between sexes. The daily salt intake was also higher in cafeteria groups (higher in males than in females), and a significant interaction with sex was observed. However, when expressed in mg/g of

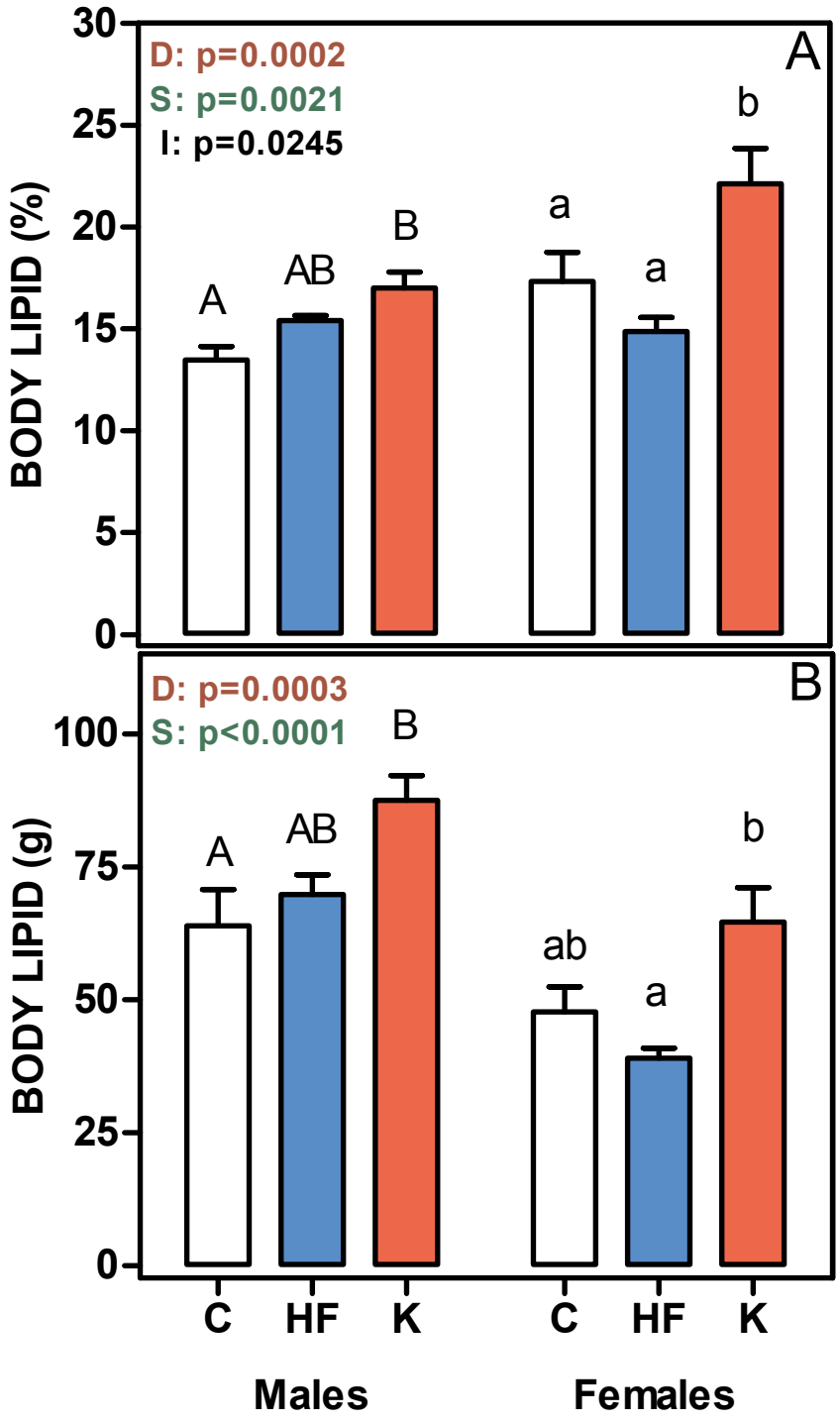

Figure 2 Body lipid content, expressed as a percentage of body weight, and in absolute values. (A) body lipid content as a percentage of body weight. (B) represent the total body lipid content (g). Data are the mean ± SEM of six to eight animals per group. White bars: standard diet (C); blue: high-fat diet (HF) and red: cafeteria diet (K). Statistical differences between groups: two-way ANOVA (D, diet; S, sex; I, their interaction). Bonferroni *post-hoc* test: different letters represent statistically significant ($p < 0.05$) differences between groups of the same sex.

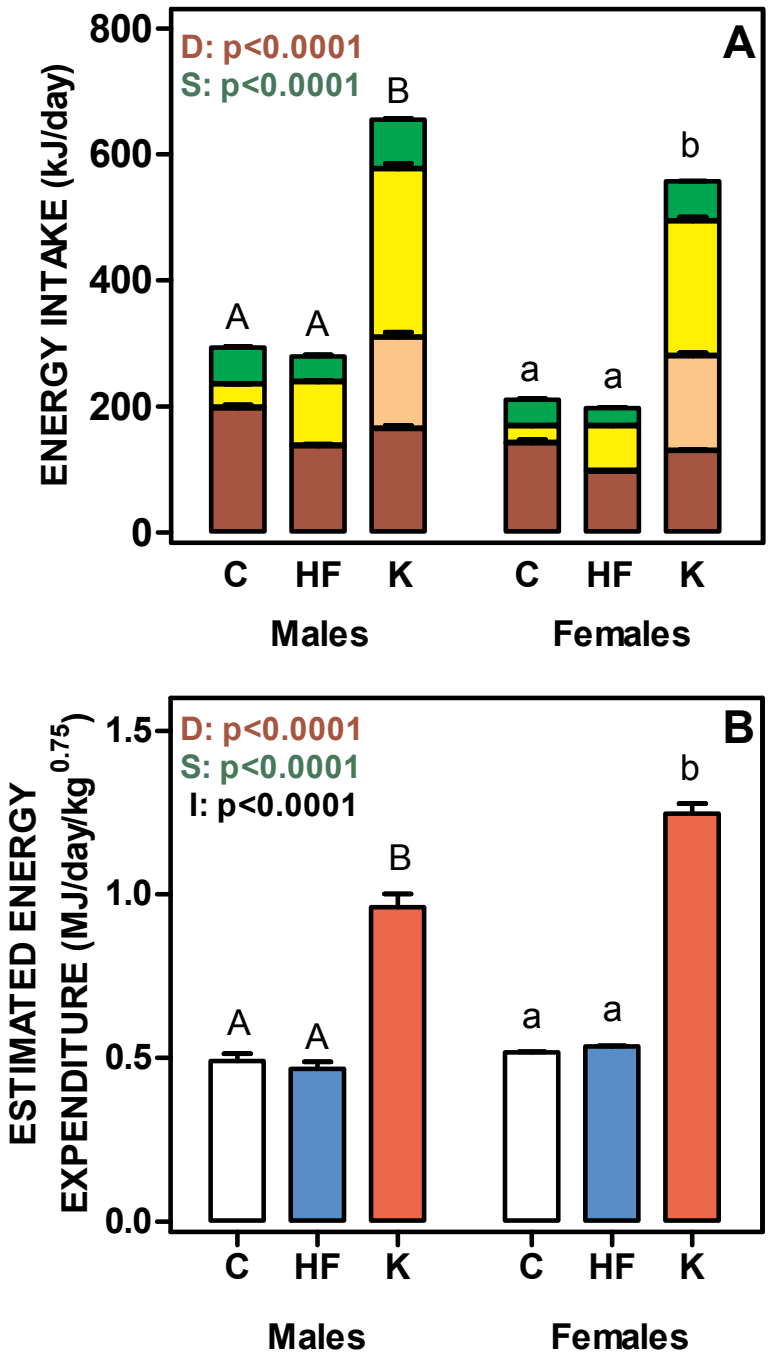

Figure 3 Total daily nutrient intake and estimated daily energy expenditure of rats treated for 30-days with standard, high-fat or cafeteria diets. (A) daily intake of protein, lipids, oligosaccharides and polysaccharides. Energy intake is expressed as kJ/day for each nutrient as stacked columns: brown bars represents polysaccharides; light brown bars oligosaccharides; yellow bars lipid and green bars protein. (B) estimated total daily energy expenditure expressed as MJ/day/weight$^{0.75}$. White bars: standard diet (C); blue: high-fat diet (HF) and red: cafeteria diet (K). Data are the mean ± SEM of six to eight animals per group. Statistical significance of the differences were estimated for each nutrient group using two-way ANOVA (D, diet; S, sex; I, their interaction) and the Bonferroni *post-hoc* test showed differences between groups. Different letters represent statistically significant ($p < 0.05$) total energy intake/expenditure differences between groups of the same sex.

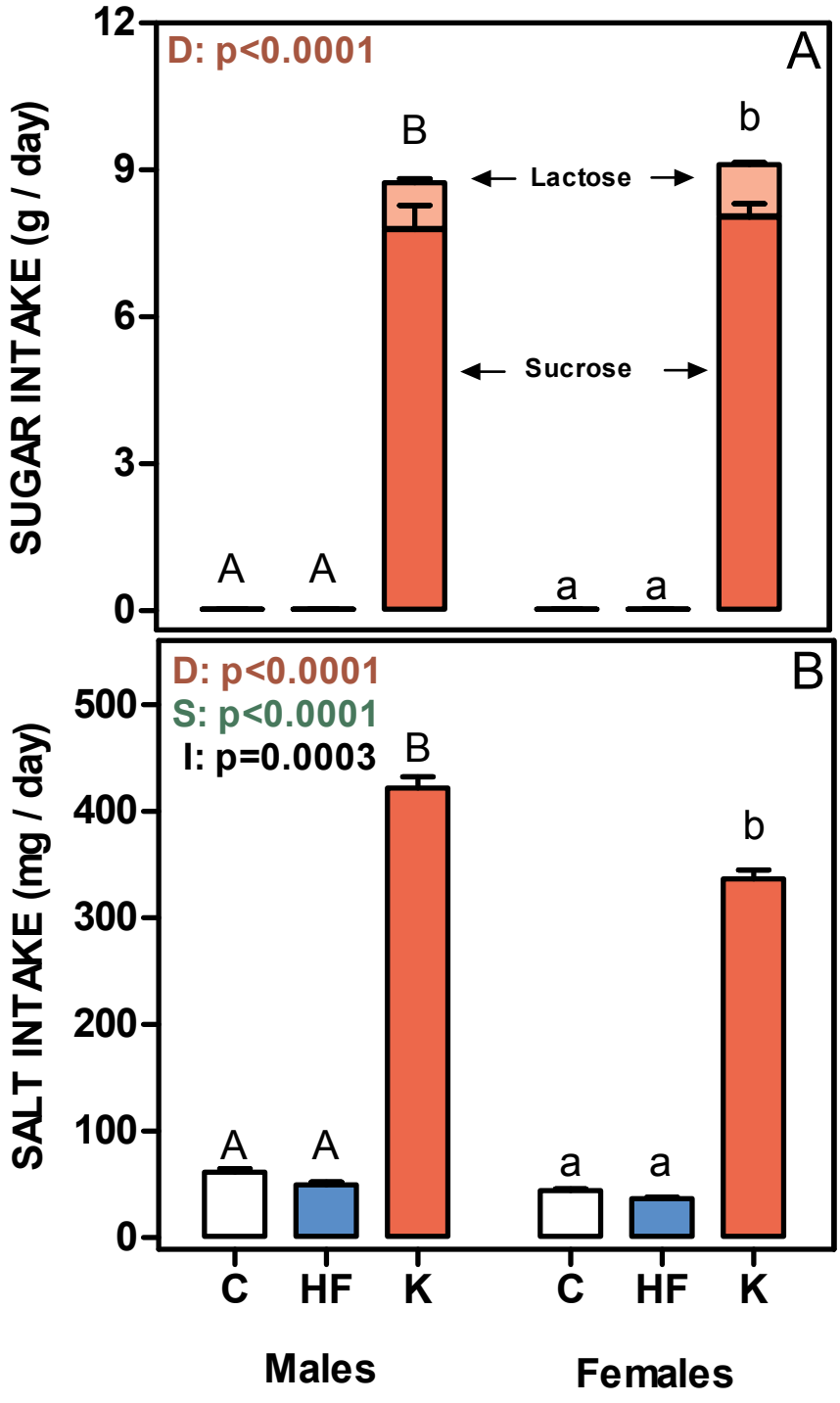

Figure 4 Sugar and salt intakes of rats treated for 30-days with standard, high-fat or cafeteria diets. (A) sugar intake. Values are given as g/day of lactose (light red) + sucrose (red). (B) Salt intake. Values are presented as mg of NaCl per day. Data are the mean ± SEM of six to eight animals per group. White bars: standard diet (C); blue: high-fat diet (HF) and red: cafeteria diet (K). Statistical differences between groups: two-way ANOVA (D, diet; S, sex; I, their interaction); Bonferroni *post-hoc* test: different letters represent statistically significant ($p < 0.05$) differences between groups of the same sex.

accrued weight, female rats ingested more salt than males (39 ± 0.7 in males and 56 ± 1.2 in females; $p = 0.0061$).

DISCUSSION

The main finding of this study is that, paradoxically, 30-day exposure to two types of high fat diet, with similar fat content but markedly different taste, texture and food variety, elicited widely different effects in body weight. The gain in weight shown by the HF-diet fed animals was similar to that of controls on fed standard food pellets, and agrees with data previously described for rats of the same-age kept on a standard diet (*Buettner, Schölmerich & Bollheimer, 2007*; *Martire et al., 2013*), although the results were also influenced by sex. The known obesogenic effects of cafeteria diets resulted in a significant increase in body weight in a relatively short-term (*Romero et al., 2014*). This increase was largely caused by the accumulation of fat, mainly in adipose tissue, although the increase in fat content is generalized to all tissues (*Esteve et al., 1992a*). Body lipid accrual was more marked in males. The absence of significant water retention again confirms that the main cause of weight increase was a consequence of the massive lipid accretion. Both high-lipid diets contained the same proportion of fat and had a similar proportion of the other macronutrients, but HF did not elicit an increase in body weight as K did. The difference was in the higher overall amount of energy ingested by the rats in the K group.

The differences in energy intake between HF and K groups were not caused by the dissimilar fibre content, since energy intake is a function of energy density irrespective of the presence of fibre content (*Ramirez & Friedman, 1990*). High fibre content induces a drastic reduction of food intake (and body weight) in rats previously fattened with high fat diet (*Adam et al., 2016*), probably as a consequence of lower diet energy density. The scant differences in digestible energy content between C and K, for instance, is an additional argument to assume that fibre has a minimal effect on food consumption in our model.

Tasty components of the diet have been considered as the main agents responsible of cafeteria diets overcoming the rat strict control on energy intake of cafeteria diets (*Radcliffe & Webster, 1976*; *Mrosovsky & Powley, 1977*), and also of decreasing their satiety threshold (*Reichelt, Morris & Westbrook, 2014*), even with relatively short periods of exposure. These effects may help explain the hyperphagia (causing the increased energy intake) observed in the rats fed a cafeteria diet, since its effects on appetite are mediated by a short-term increase in sympathetic activity (*Muntzel et al., 2012*). The effect of the high energy density of the diet, tending to decrease the overall food intake (*Ramirez & Friedman, 1990*), seems not to be effective in the K groups. Thus, the acknowledged taste components of cafeteria diet (essentially sugar and salt, i.e., sweet and salty) seem to be more effective agents acting on the control of appetite than the possible palatability of fats (and fatty acids) also present in the HF diet in amounts similar to those of cafeteria diet. This factor should be considered with the context of both the induction of food intake caused by variety (and novelty) of foods and tastes (*Moore et al., 2013*), which in part exploits the "explorative" drive shared by rats and humans. In addition, the intake of pleasing food (such as sweets), lowers the levels of anxiety (*Faturi et al., 2010*), and is used (by humans and experimental animals

alike) as "comfort food" (*Ortolani et al., 2011*) to escape of conflict situations, or simply for pleasure (*Pini et al., 2016*).

The estimated values for energy expenditure and the percentage of body lipid content indicate that HF rats closely paralleled the energy balance of the control diet groups, and differ markedly from those fed K diet. The lower lipid storage in the HF rats, despite their high intake of lipids (largely consisting in saturated fatty acids and the PUFA from the standard diet from which HF diet was fabricated) suggests that in HF rats, dietary lipids were oxidized almost quantitatively. Their energy simply compensated the decreased carbohydrate utilization due to the compounded effect of its lower presence in the diet and lower food intake.

It must be taken into account that the lipids ingested were almost exclusively as acylglycerols, not free fatty acids, and thus it is unlikely that the actions on lingual fatty acid receptors (*Mizushige, Inoue & Fushiki, 2007*) could play a significant role in the taste of this diet.

Nevertheless, the greasy texture that lipid confer to high-fat diets seems to be attractive for rats (*Hamilton, 1964*) (as in humans (*Kant et al., 2008*)). Notwithstanding, our data showed that rats fed HF diet did not show a higher food intake than controls, which seems to just eliminate the "lipid taste" as a critical factor for hyperphagia. This conclusion may be an unexpected consequence of the HF diet formulation we used, being essentially the standard diet with added fat, and not a wholly different diet, formed by a few simple components (protein, starches, sugars and fats), as those commonly used for studies on obesity (*Crescenzo et al., 2015*).

Our data help clarify the situation, since they prove that fat (alone) could not be the key factor eliciting a higher food (energy) intake. The case in point being the sucrose-oil HF diets commonly used to induce obesity in rodents (*Kanarek & Marks-Kaufman, 1979*) even when coconut oil was used (*Portillo et al., 1998*; *Ellis, Lake & Hoover-Plow, 2002*). Probably, in these diets, the sugar plays a deeper effect on the obesogenic properties of diet than usually assumed (*Sclafani, 1987*). The significant increase in 3-hydroxybutyrate levels caused by diet (indicating active fatty acid disposal), especially marked in HF rats, may act also as a satiety signal (*Scharrer, 1999*), thus helping maintain food intake in an already relatively low setting. This was compounded mainly in females, by an efficient catabolic use of lipids.

The results obtained using this model, proved that fat alone was not the main inducer of hyperphagia. Consequently, we should determine what other dietary factors could justify the marked differences in food (and energy) intake between HF and K diets (sharing a similar proportion of dietary fat content). We postulate that this difference should be attributed to the massive intake of sugar and salt in addition to other psychological variables such as variety and comfort. These nutrients are present in relatively large proportions in all cafeteria diet formulations, and are often absent or in low proportion in most standard rodent diets, much closer to natural life conditions. Up to now, these components have received only scant attention as inducers of cafeteria diet-driven hyperphagia. Sugar (sweet taste) causes pleasurable sensations in rodents due to their oral sensory properties (*Peciña, Smith & Berridge, 2006*) that seek and stimulate the consumption of sweet foods,

an intake that can be modulated with exposure (*Sclafani, 2006*) associated to the energy that the sugars provide (*McCaughey, 2008*). The increase in sucrose (energy) ingestion may contribute to increase fat deposition, since fructose has been recognized as highly obesogenic (*Bocarsly et al., 2010*). Fructose (largely as sucrose) is widely present in many Western diets and can induce obesity, including prenatal obesity (*Szostaczuk et al., 2017*). In rodents, a sucrose-rich diet can rapidly induce a pathologic condition comparable to human metabolic syndrome (*Santuré et al., 2002*). We assume that the effect of sweet taste may complement the *flavour* of fat texture, in K, despite fatty acids with more powerful "fat taste" not being directly available (*Strik et al., 2010*).

Rats, like humans, prefer to drink sweet or salty solutions rather than plain water (*Khavari, 1970*). We can add that salt is known for its taste-enhancing properties, thus increasing the taste effects of all diet components, as well as a reward response, since preferences for both, sweet and salty tastes are mediated by endogenous opioids (*Nascimento et al., 2012*). In fact, the contrast sweet/salty is one of the key factors establishing the powerful drive to eat, elicited (in humans, at least) by varied food offerings (*Low, Lacy & Keast, 2014*), thus the factor "variety" could largely be correlated with the presence of these main ancestral sought-for tastes (*Naim et al., 1985*). Sweets are the most classical "comfort food" (*Rho et al., 2014*). In humans this slot is covered largely by sweet chocolate, but previous experiments showed that rats do not like the bitter taste of chocolate (*Prats et al., 1989*), thus sugared milk may be a very good substitute.

Sodium is an essential element actively sought and massively consumed by animals (and evidently including humans) when found (*Dahl, 1958*), hence our evolutionary drive to consume salt in excess (*Morris, Na & Johnson, 2008*). The maintenance of normal plasma protein levels suggest limited, if any, effects of high salt intake on the rat water balance, as previously found (*Fernández-López et al., 1994*). Despite these antecedents, salt intake has not been described as an essential factor eliciting hyperphagia of cafeteria diets. In the case of humans, it is almost impossible to avoid even minimal amounts of salt in present-day diets, whereas its presence in foods akin to cafeteria diets points to a relevant role in the hyperphagia. Furthermore, the effects of salt intake on the renin-angiotensin system (*Drenjancevic-Peric et al., 2011*), and their effect on corticosteroid secretion along the corticosterone-aldosterone axis have seldom been taken into account in this context. We can speculate that the increased secretion of corticoids as a response to salt (*Lewicka, Nowicki & Vecsei, 1998*) may help elicit metabolic changes that favour the development of the conditions driving to metabolic syndrome (*Alemany, 2012*), and the consequent increased lipid deposition (*Moosavian et al., 2017*).

There were distinctive differences between sexes in taste preferences when the rats were allowed to select foods, as is the case of cafeteria diets. Female rats ingested almost 40% more salt than males when the intake was expressed with respect to body weight increase. These data confirm that female rats show a higher preference for salt than males (*Flynn, Schulkin & Havens, 1993*). Furthermore, female rats also ingested more sugar, either in absolute or in relative values (i.e., g ingested per g of body weight increase) than males. The preferences of female rats for these nutrients, however, did not result in increased weight, in part because of their higher energy expenditure (*Rodríguez-Cuenca et al., 2002*) even after correction of

size by an allometric factor (*Nair & Jacob, 2016*). These sex differences could be traced to sex-specific factors of architecture and maturation of the reward system (*Gugusheff, Ong & Muhlhausler, 2015*). In this context, we have no data about the implication of lactose in taste, although it is well known that the intake of milk (for its taste) also implies the consumption of other milk components, such as active peptides and oestrone (*García-Peláez et al., 2004*) responsible for higher efficiency in energy deposition during lactation. Furthermore, female rats showed lower increases in circulating triacylglycerols, and lower urea levels than males, in agreement with previous reports (*Agnelli et al., 2016*), being largely "protected" from excess fat deposition by oestrogens (*Zhu et al., 2013*).

In this study we assumed that the contribution of protein taste (umami) to food consumption increase can be considered minimal, since the presence of protein (and its quality) was similar (and more than enough in quantities) in all diets; but essentially because dietary protein limits food intake (*Anderson & Moore, 2004*) in part due to its high satiating effect (*Bensaid et al., 2002*). The possible effect of protein on food intake in HF groups was, probably, of limited extent, since it was the same (albeit partially diluted) protein of the control diet, and the lack of differences C *vs.* HF prove that they did not act as a differential inductor of satiety as in other models (*Bensaid et al., 2002*). Conversely, the higher intake of protein in cafeteria groups should elicit a higher satiating effect of proteins; opposing, in fact, the combined actions of sugar and salt (and fat taste) inducing higher food intake. The balance of these opposing effects did not support a significant role of protein in the control of food intake in this model, being superseded by the hedonic influence of more intense tastes (sweet-salty) of food. To our knowledge, no effects of salt enhancing the properties on amino acid and umami taste has been described, so far, in rats (*Kurihara, 2015*).

CONCLUSIONS

The data presented confirm the higher taste-induced appetite of rats for cafeteria diets, which we can also describe as multichoice high-fat, high-sugar and high-salt compared with most high-fat diets. The higher overall energy intake, in part a consequence of the attenuated satiation mechanisms, the increased variety of food items, and the comfort-food effect (the latter—probably largely as a consequence of the admixture and abundance of sweet-salty taste of food items) enhance the effect of the cafeteria diet to rapidly increase body energy stores. These combined actions favour the development of metabolic syndrome. The perils linked to cafeteria diets are not, thus, limited to high dietary fat content and energy density, but largely to a powerful hedonic component (taste) which can effectively override the normal mechanisms, controlling food (energy) intake.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Laia Oliva performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Tània Aranda, Giada Caviola and Anna Fernández-Bernal performed the experiments, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Marià Alemany analyzed the data, wrote the paper, reviewed drafts of the paper.
- José Antonio Fernández-López analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Xavier Remesar conceived and designed the experiments, analyzed the data, 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):

The Committee on Animal Experimentation of the University of Barcelona authorized the specific procedures used: Procedure DAAM 6911.

Data Availability

The following information was supplied regarding data availability:

University of Barcelona institutional repository:
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REFERENCES

Adam CL, Gratz SW, Peinado DI, Thompson LM, Garden KE, Williams PA, Richardson AJ, Ross AW. 2016. Effects of dietary fibre (pectin) and/or increased protein (casein or pea) on satiety, body weight, adiposity and caecal fermentation in high fat diet-induced obese rats. *PLOS ONE* **11**:e0155871 DOI 10.1371/journal.pone.0155871.

Agnelli S, Arriarán S, Oliva L, Remesar X, Fernández-López J-A, Alemany M. 2016. Modulation of rat liver urea cycle and related ammonium metabolism by sex and cafeteria diet. *RSC Advances* **6**:11278–11288 DOI 10.1039/C5RA25174E.

Alemany M. 2012. Do the interactions between glucocorticoids and sex hormones regulate the development of the metabolic syndrome? *Frontiers in Endocrinology* **3**:Article 27 DOI 10.3389/fendo.2012.00027.

Anderson GH, Moore SE. 2004. Dietary proteins in the regulation of food intake and body weight in humans. *Journal of Nutrition* **134**:974S–979S.

Archer ZA, Corneloup J, Rayner DV, Barrett P, Moar KM, Mercer JG. 2007. Solid and liquid obesogenic diets induce obesity and counter-regulatory changes in hypothalamic gene expression in juvenile Sprague-Dawley rats. *Journal of Nutrition* **137**:1483–1490.

Bensaid A, Tome D, Gietzen D, Even P, Morens C, Gausseres N, Fromentin G. 2002. Protein is more potent than carbohydrate for reducing appetite in rats. *Physiology & Behaviour* **75**:577–582 DOI 10.1016/S0031-9384(02)00646-7.

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 Behaviour* **97**:101–106 DOI 10.1016/j.pbb.2010.02.012.

Breslin PA, Spector AC, Grill HJ. 1995. Sodium specificity of salt appetite in Fischer-344 and Wistar rats is impaired by chorda tympani nerve transection. *American Journal of Physiology* **269**:R350–R356.

Briaud I, Kelpe CL, Johnson LM, Tran PO, Poitut V. 2002. Differential effects of hyperlipidemia on insulin secretion in islets of Langerhans from hyperglycemic versus normoglycemic rats. *Diabetes* **51**:662–668 DOI 10.2337/diabetes.51.3.662.

Buettner R, Parhofer KG, Woenckhaus M, Wrede CE, Kunz-Schugart LA, Schölmerich, Bollheimer LC. 2006. Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. *Journal of Molecular Endocrinology* **36**:485–501 DOI 10.1677/jme.1.01909.

Buettner R, Schölmerich J, Bollheimer LC. 2007. High-fat diets: modeling the metabolic disorders of human obesity in rodents. *Obesity* **15**:798–808 DOI 10.1038/oby.2007.608.

Crescenzo R, Bianco F, Mazzoli A, Giacco A, Cancelliere R, Di Fabio G, Zarrelli A, Liverini G, Iossa S. 2015. Fat quality influences the obesogenic effect of high fat diets. *Nutrients* **7**:9475–9491 DOI 10.3390/nu7115480.

Dahl LK. 1958. Salt intake and salt need. *New England Journal of Medicine* **258**:1205–1208 DOI 10.1056/NEJM195806122582406.

Drenjancevic-Peric I, Jelakovic B, Lombard JH, Kunert MP, Kibel A, Gros M. 2011. High-salt diet and hypertension: focus on the renin-angiotensin system. *Kidney Blood Pressure Research* **34**:1–11 DOI 10.1159/000320387.

Ellis J, Lake A, Hoover-Plow J. 2002. Monounsaturated canola oil reduces fat deposition in growing female rats fed a high or low fat diet. *Nutrition Research* **22**:609–621 DOI 10.1016/S0271-5317(02)00370-6.

Esteve M, Rafecas I, Fernández-López JA, Remesar X, Alemany M. 1992a. Fatty acid utilization by young Wistar rats fed a cafeteria diet. *Molecular and Cellular Biochemistry* **118**:67–74 DOI 10.1007/BF00249696.

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

Faturi CB, Leite JR, Alves PB, Canton AC, Teixeira-Silva F. 2010. Anxiolytic-like effect of sweet orange aroma in Wistar rats. *Progress in Neuropsychopharmacol & Biological Psychiatry* **34**:605–609 DOI 10.1016/j.pnpbp.2010.02.020.

Fernández-López J, Rafecas I, Esteve M, Remesar X, Alemany M. 1994. Effect of genetic and dietary obesity on sodium, potassium, calcium and magnesium handling by the rat. *International Journal of Food Science and Nutrition* **45**:191–201 DOI 10.3109/09637489409166158.

Flynn FW, Schulkin J, Havens M. 1993. Sex differences in salt preference and taste reactivity in rats. *Brain Research Bulletin* **32**:91–95 DOI 10.1016/0361-9230(93)90061-F.

Folch J, Lees M, Sloane-Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* **226**:497–509.

García-Peláez B, Ferrer-Lorente R, Gómez-Ollés S, Fernández-López JA, Remesar X, Alemany M. 2004. A method for the measurement of estrone content in foods. Application to dairy products. *Journal of Dairy Science* **87**:2331–2336 DOI 10.3168/jds.S0022-0302(04)73354-8.

Gomez-Smith M, Karthikeyan S, Jeffers MS, Janik R, Thomason LA, Stefanovic B, Corbett D. 2016. A physiological characterization of the Cafeteria diet model of metabolic syndrome in the rat. *Physiology & Behavior* **167**:382–391 DOI 10.1016/j.physbeh.2016.09.029.

Gugusheff JR, Ong ZY, Muhlhausler BS. 2015. The early origins of food preferences: targeting the critical windows of development. *FASEB Journal* **29**:365–373 DOI 10.1096/fj.14-255976.

Hamilton CL. 1964. Rat's preference for high fat diets. *Journal of Comparative Physiological Psychology* **58**:459–460 DOI 10.1037/h0047142.

Hariri N, Thibault L. 2010. High-fat diet-induced obesity in animal models. *Nutrition Research Reviews* **23**:270–299 DOI 10.1017/S0954422410000168.

Johnson AR, Wilkerson MD, Sampey BP, Troester MA, Hayes DN, Makowski L. 2016. Cafeteria diet-induced obesity causes oxidative damage in white adipose. *Biochemical and Biophysical Research Communications* **473**:545–550 DOI 10.1016/j.bbrc.2016.03.113.

Kanarek RB, Marks-Kaufman R. 1979. Developmental aspects of sucrose-induced obesity in rats. *Physiology & Behaviour* **23**:881–885 DOI 10.1016/0031-9384(79)90195-1.

Kant AK, Andon MB, Angelopoulus TJ, Rippe JM. 2008. Association of breakfast energy density with diet quality and body mass index in American adults: national health and nutrition examination surveys, 1999–2004. *American Journal of Clinical Nutrition* **88**:1396–1404 DOI 10.3945/ajcn.2008.26171.

Khavari KA. 1970. Some parameters of sucrose and saline ingestion. *Physiology & Behaviour* **5**:663–666 DOI 10.1016/0031-9384(70)90227-1.

Kurihara K. 2015. Umami the fifth basic taste: history of studies on receptor mechanisms and role as a food flavor. *BioMed Research International* **2015**:189402 DOI 10.1155/2015/189402.

Lewicka S, Nowicki M, Vecsei P. 1998. Effect of sodium on urinary excretion of cortisol and its metabolites in humans. *Steroids* **63**:401–405 DOI 10.1016/S0039-128X(98)00015-4.

Lladó I, Picó C, Palou A, Pons A. 1995. Protein and amino acid intake in cafeteria fed obese rats. *Physiology & Behaviour* **58**:513–519 DOI 10.1016/0031-9384(95)00081-S.

Low YQ, Lacy K, Keast R. 2014. The role of sweet taste in satiation and satiety. *Nutrients* **2**:3431–3450 DOI 10.3390/nu6093431.

Lowry OH, Rosebrough RW, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**:265–275.

Martire SI, Holmes N, Westbrook RF, Morris MJ. 2013. Altered feeding patterns in rats exposed to a palatable cafeteria diet: increased snacking and its implications for development of obesity. *PLOS ONE* **8**:e60407 DOI 10.1371/journal.pone.0060407.

McCaughey SA. 2008. The taste of sugars. *Neuroscience & Biobehavioral Reviews* **32**:1024–1043 DOI 10.1016/j.neubiorev.2008.04.002.

Miwa I, Maeda K, Okuda J, Okuda G. 1972. Mutarotase effect on colorimetric determination of blood-glucose with b-D-glucose oxidase. *Clinica Chimica Acta* **37**:538–540 DOI 10.1016/0009-8981(72)90483-4.

Mizushige T, Inoue K, Fushiki T. 2007. Why is fat so tasty? Chemical reception of fatty acid on the tongue. *Journal of Nutritional Science and Vitaminology* **53**:1–4 DOI 10.3177/jnsv.53.1.

Moore BJ. 1987. The cafeteria diet. An inappropriate tool for studies of thermogenesis. *Journal of Nutrition* **117**:227–231.

Moore CJ, Michopoulos V, Johnson ZP, Toufexis D, Wilson ME. 2013. Dietary variety is associated with larger meals in female rhesus monkeys. *Physiology & Behavior* **2**:190–194 DOI 10.1016/j.physbeh.2013.06.014.

Moosavian SP, Haghighatdoost F, Surkan PJ, Azadbakht L. 2017. Salt and obesity: a systematic review and meta-analysis of observational studies. *International Journal of Food Sciences and Nutrition* **68**:265–277 DOI 10.1080/09637486.2016.1239700.

Morris MJ, Na ES, Johnson AK. 2008. Salt craving: the psychobiology of pathogenic sodium intake. *Physiology & Behaviour* **94**:709–721 DOI 10.1016/j.physbeh.2008.04.008.

Mrosovsky N, Powley TL. 1977. Set points for body weight and fat. *Behaviour Biologica* **20**:205–223.

Muntzel MS, Al-Naimi OAS, Barclay A, Ajasin D. 2012. Cafeteria diet increases fat mass and chronically elevates lumbar sympathetic nerve activity in rats. *Hypertension* **60**:1498–1502 DOI 10.1161/HYPERTENSIONAHA.112.194886.

Naim M, Brand JG, Kare MR, Carpenter RG. 1985. Energy intake, weight gain and fat deposition in rats fed flavored, nutritionally controlled diets in a multichoice (''cafeteria'') design. *Journal of Nutrition* **115**:1447–1448.

Nair AB, Jacob S. 2016. A simple practice guide for dose conversion between animals and human. *Journal of Basic Clinical Pharmacy* **7**:27–21 DOI 10.4103/0976-0105.177703.

Nascimento AIR, Ferreira HS, Saraiva RM, Almeida TS, Fregoneze JB. 2012. Central kappa opioid receptors modulate salt appetite in rats. *Physiology & Behaviour* **106**:506–514 DOI 10.1016/j.physbeh.2012.03.028.

Oakes ND, Cooney GJ, Camilleri S, Chisholm DJ, Kraegen EW. 1997. Mechanisms of liver and muscle insulin resistance induced by chronic high-fat feeding. *Diabetes* **36**:1768–1774 DOI 10.2337/diab.46.11.1768.

Oliva L, Baron C, Fernández-López J-A, Remesar X, Alemany M. 2015. Marked increase in rat red blood cell membrane protein glycosylation by one-month treatment with a cafeteria diet. *PeerJ* **3**:e1101 DOI 10.7717/peerj.1101.

Ortolani D, Oyama LM, Ferrari EM, Melo LL, Spadari-Bratfisch RC. 2011. Effects of comfort food on food intake, anxiety-like behavior and the stress response in rats. *Physiology & Behavior* **103**:487–492 DOI 10.1016/j.physbeh.2011.03.028.

Peciña S, Smith KS, Berridge KC. 2006. Hedonic hot spots in the brain. *The Neuroscientist* **12**:500–511 DOI 10.1177/1073858406293154.

Peckham SC, Entenman C, Carroll HW. 1977. The influence of a hypercaloric diet on gross body and adipose tissue composition in the rat. *Journal of Nutrition* **62**:187–197.

Pini RTB, Do Vales LDMF, Braga Costa TM, Almeida SS. 2016. Effects of cafeteria diet and high fat diet intake on anxiety, learning and memory in adult male rats. *Nutritional Neuroscience* **1**:1–13 DOI 10.1080/1028415X.2016.1149294.

Portillo MP, Serra F, Simon E, Del Barrio AS, Palou A. 1998. Energy restriction with high-fat diet enriched with coconut oil gives higher UCP1 and lower white fat in rats. *International Journal of Obesity* **22**:974–978 DOI 10.1038/sj.ijo.0800706.

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.

Radcliffe J, Webster A. 1976. Regulation of food intake during growth in fatty and lean female Zucker rats given diets of different protein content. *British Journal of Nutrition* **36**:457–469 DOI 10.1079/BJN19760100.

Rafecas I, Esteve M, Fernández-López JA, Remesar X, Alemany M. 1992. Deposition of dietary fatty acids in young Zucker rats fed a cafeteria diet. *International Journal of Obesity* **16**:775–787.

Rafecas I, Esteve M, Fernández-López JA, Remesar X, Alemany M. 1993. Individual amino acid balances in young lean and obese Zucker rats fed a cafeteria diet. *Molecular and Cellular Biochemistry* **121**:45–58 DOI 10.1007/BF00928699.

Ramirez I, Friedman MI. 1990. Dietary hyperphagia in rats—role of fat, carbohydrate, and energy content. *Physiology & Behaviour* **47**:1157–1163 DOI 10.1016/0031-9384(90)90367-D.

Reichelt AC, Morris MJ, Westbrook R. 2014. Cafeteria diet impairs expression of sensory-specific satiety and stimulus-outcome learning. *Frontiers in Psychology* **5**:Article 852 DOI 10.3389/fpsyg.2014.00852.

Rho SG, Kim YS, Choi SC, Lee MY. 2014. Sweet food improves chronic stress-induced irritable bowel syndrome-like symptoms in rats. *World Journal of Gastroenterology* **20**:2365–2373 DOI 10.3748/wjg.v20.i9.2365.

Rodríguez-Cuenca S, Pujol E, Justo R, Fronteras M, Oliver J, Gianotti M, Roca P. 2002. Sex-dependent thermogenesis, differences in mitochondrial morphology and function, and adrenergic response in brown adipose tissue. *Journal of Biological Chemistry* **277**:42958–42963 DOI 10.1074/jbc.M207229200.

Romero MM, Holmgren F, Grasa MM, Esteve M, Remesar X, Fernández-López JA, Alemany M. 2013. Modulation in Wistar rats of blood corticosterone compartmentation by sex and prior exposure to a cafeteria diet. *PLOS ONE* **8**:e57342 DOI 10.1371/journal.pone.0057342.

Romero MM, Roy S, Pouillot K, Feito M, Esteve M, Grasa MM, Fernández-López JA, Remesar X, Alemany M. 2014. 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 rest of body lipid stores. *PLOS ONE* **9**:e90995 DOI 10.1371/journal.pone.0090995.

Rothwell NJ, Saville ME, Stock MJ. 1982. Effects of feeding a "cafeteria" diet on energy balance and diet-induced thermogenesis in four strains of rat. *Journal of Nutrition* **112**:1515–1524.

Rothwell NJ, Stock MJ. 1984. The development of obesity in animals: the role of dietary factors. *Clinical Endocrinology and Metabolism* **13**:437–449 DOI 10.1016/S0300-595X(84)80032-8.

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.

Sato A, Kawano H, Notsu T, Ohta M, Nakakuki M, Mizuguchi K, Itoh M, Suganami T, Ogawa Y. 2010. Antiobesity effect of eicosapentaenoic acid in high-fat/high-sucrose diet—induced obesity. Importance of hepatic lipogenesis. *Diabetes* **59**:2495–2504 DOI 10.2337/db09-1554.

Scharrer E. 1999. Control of food intake by fatty acid oxidation and ketogenesis. *Nutrition* **15**:704–714 DOI 10.1016/S0899-9007(99)00125-2.

Schemmel R, Mickelsen O, Tolgay Z. 1969. Dietary obesity in rats: influence of diet, weight, age, and sex on body composition. *American Journal of Physiology* **216**:373–379.

Sclafani A. 1987. Carbohydrate-induced hyperphagia and obesity in the rat: effects of saccharide type, form, and taste. *Neuroscience & Biobehavioral Reviews* **11**:155–162 DOI 10.1016/S0149-7634(87)80020-9.

Sclafani A. 2006. Enhanced sucrose and Polycose preference in sweet "sensitive"(C57BL/6J) and "subsensitive"(129P3/J) mice after experience with these saccharides. *Physiology & Behaviour* **87**:745–756 DOI 10.1016/j.physbeh.2006.01.016.

Sclafani A, Gorman AN. 1977. Effects of age, sex, and prior body weight on the development of dietary obesity in adult rats. *Physiology & Behavior* **18**:1021–1026 DOI 10.1016/0031-9384(77)90006-3.

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

Strik CM, Lithander FE, McGill AT, MacGibbon AK, McArdle BH, Poppitt SD. 2010. No evidence of differential effects of SFA, MUFA or PUFA on post-ingestive satiety and energy intake: a randomised trial of fatty acid saturation. *Nutrition Journal* **9**:Article 24 DOI 10.1186/1475-2891-9-24.

Szostaczuk N, Priego T, Palou M, Palou A, Picó C. 2017. Oral leptin supplementation throughout lactation in rats prevents later metabolic alterations caused by gestational calorie restriction. *International Journal of Obesity* **41**:360–371 DOI 10.1038/ijo.2016.241.

Tordoff MG, Reed DR. 1991. Sham-feeding sucrose or corn oil stimulates food intake in rats. *Appetite* **17**:97–103 DOI 10.1016/0195-6663(91)90065-Z.

Zeeni N, Bassil M, Fromentin G, Chaumontet C, Darcel N, Tome D, Daher CF. 2015. Environmental enrichment and cafeteria diet attenuate the response to chronic variable stress in rats. *Physiology & Behavior* **139**:41–49 DOI 10.1016/j.physbeh.2014.11.003.

Zhu L, Brown WC, Cai Q, Krust A, Chambon P, McGuiness OP, Stafford JM. 2013. Estrogen treatment after ovariectomy protects against fatty liver and may improve pathway-selective insulin resistance. *Diabetes* **62**:424–434 DOI 10.2337/db11-1718.

Dietary fatty acid composition influences on liver triacylglycerol and cholesterol accumulation. Effect of oestradiol.

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ABSTRACT

Large amounts of fat intake are associated with an increase in body weight, adiposity and ectopic fat accumulation in the liver. However, specific lipid composition of the diet may elicit different metabolic and obesogenic effects. Ten-week-old male and female rats were fed with either standard rat chow (SD), standard chow enriched with coconut oil (high-fat diet, HF) or a cafeteria diet (CAF) during one month. Both HF and CAF contained the same lipid derived percentage of energy (40%), but different lipid composition. After the treatment there were no differences in energy intake, energy expenditure, and total body weight and lipid content between SD and HF fed rats, contrarily to CAF groups that showed significant increases, being those higher in males. Hepatic lipid accumulation showed sex-related differences; triacylglycerol accumulation was significant in both HF and CAF fed males, although it was higher in CAF group, while cholesterol accumulation was only found in CAF fed males. Cholesterol circulating levels were lower in HF fed animals, whereas lactate and oestradiol were increased, being that of oestradiol similar in both males and females. Moreover, plasma oestradiol positively correlated with muscle lactate dehydrogenase expression and activity. Significant differences were also found in some enzymatic liver expressions related to lipid metabolism. Fatty acid composition of diet may elicit essential changes in lipid and glucose metabolism through oestradiol mediated changes.

INTRODUCTION

The accumulation of body fat is well correlated with fat intake (Rothwell and Stock, 1984). Thus, diets with high energy content have been used for long time to achieve overweight or obese animal subjects (Hariri and Thibault, 2010). However, the lack of uniformity of these diets generate so far wide-ranging effects, depending on the age of animals (Sclafani and Corman, 1977), the time course of the intervention (Schemmel *et al.*, 1969), the energy density of diet's components, the gender (Agnelli *et al.,* 2016) and finally, the palatability of the diet (Ghibaudi *et al.*, 2002). As a consequence, the growth patterns obtained are heterogeneous, including the variability in the ratio of weight gain and lipid deposition (Woods *et al.*, 2003). In humans, a high-energy diet, together with enough amounts of carbohydrates and proteins, help fat deposition (Lissner *et al.*, 1987). Dietary fat improves diet's palatability, but other flavours may increase the hedonic response in humans (Davis and Loxton, 2014) or rats (Oliva *et al.*, 2017).

Protein content in the diet is an essential factor for the growth and maintenance of mammals, due to their essential role in the plastic body structures and in their turnover. Their energy content represents ca. 20% of total energy in standard diets. In normal metabolic states, the increase of this proportion induces protein catabolism and lipid mobilization, as well as lower food intake due to the satiating effect of proteins (Bensaïd *et al.,* 2002). When protein constitutes a large proportion of the diet, especially under conditions of low energy

availability, like dieting (Scalfi *et al.*, 1987), much of this dietary protein is used as energy, which results in negative N balance (Fisler *et al.,* 1982), lowering protein synthesis and -very often– inducing the net loss of body protein (Tsukuhara *et al.*, 1988). Thus, high-protein diets have been widely used for body weight reduction. However, the data related to dietary protein/lipid relationship and its role in energy balance are scarce. In animal models, when protein availability is sufficient, the higher is the lipid content the higher is the growth rate of adipose tissue. However, changes in the protein/lipid ratio may condition the rate of lipid and protein accretion.

The case of cafeteria diets is paradigmatic since they are hyperlipidic (Rothwell and Stock, 1988), but their protein content is usually in the higher range of normalcy (Rafecas *et al.*, 1993). This model extremely contrasts with the described models where only protein or lipid component are modified. As cafeteria diets are diverse, their content in sucrose and salt may be essential for their palatability (Oliva *et al.,* 2017). Furthermore, most of data provided with modified diets are based in highly excess of some component, but there are scarce data about the effects on moderate modifications in the relative proportion of macronutrients.

As the composition of high-fat diets is varied, their effects on body weight and metabolism are diverse (Buettner *et al*., 2006). Furthermore, fatty acids exhibit an important role on oestrogen synthesis (Williams, 2012) and its signalling on metabolic pathways related to lipid metabolism; the lack of oestrogen signalling promotes liver triacylglycerol accumulation (Palmisano *et al.,* 2017) or induces non-genomic effects (Watson *et al.,* 2007). Increases in oestradiol levels stimulate its own extraglandular synthesis through aromatase upregulation (Dieudonne *et al.,* 2006). All these evidences point to a possible differential effect of the diet, depending on its fatty acid composition.

We have tried to compare an isocaloric high-fat supplemented diet with a high-fat/high-protein diet (cafeteria) to determine the relationship between their lipid/protein content and body lipid accrual, independently of diet's palatability, and the effect of diet's lipid composition on hepatic lipid accumulation through oestrogen action.

MATERIALS AND METHODS

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 (# DAAM 6911).

Ten-week-old male (initial weight 264±12 g) and female (initial weight 233±8 g) Wistar rats (Janvier, Le-Genest-Saint-Isle, France) were used (N=40). The animals were randomly divided in three groups (n= 6-8 each) and were fed *ad libitum* for 30 days, either a standard rat chow (SD) diet, rat chow enriched with coconut oil (high-fat (HF) diet) or a simplified cafeteria diet (CAF). All animals had free access to water. They were housed (in same-sex pairs) in solid-

bottom cages with wood shards as bedding material and were kept in a controlled environment (lights on from 08:00 to 20:00, temperature 21.5-22.5ºC, and 50-60% humidity). Body weight and food consumption were recorded daily. Calculation of ingested food in cafeteria diet fed rats was done as previously described by weighing the differences in food offered and debris left (Prats *et al*., 1989) and correcting for dehydration.

Diets

Standard diet (Teklad 2014, Teklad diets, Madison WI, USA) contained 20% of digestible energy derived from protein, 13% from lipids, and 67% from carbohydrates (including 10% oligosaccharides). This diet essentially contained plant-derived foods.

The supplemented high-fat diet was prepared by the addition of coconut oil to coarsely ground standard chow. The mix, containing 33 parts (by weight) of standard chow, 4 of coconut oil and 16 parts of water, was thoroughly kneaded, to form a rough paste, which was extruded using cut end syringes to form 1x6 cm cylindrical pellets which were dried at 40ºC for 24 hours. This diet contained 15% of digestible energy derived from protein, 37% from lipids, and 49% from carbohydrates. Aversion tests to this diet gave negative results, not different from control diet.

The simplified cafeteria diet was formed by the standard chow pellets, plain cookies spread with liver pâté, bacon, water and milk, which was supplemented with 300 g/L sucrose containing 30 g/L of a mineral and vitamin supplement (Meritene, Nestlé, Esplugues, Spain) (Esteve *et al*., 1992a). All components were kept fresh (i.e. renewed daily). From the analysis of diet components and the ingested items, we calculated that approximately 40% of energy was derived from lipids, 12% from protein, and 49% of energy from carbohydrates (20% from oligosaccharides).

Experimental procedure

After 30 days of treatment, at the beginning of light cycle, the rats were anesthetized with isoflurane and blood was withdrawn, with a dry-heparinized syringe, through the exposed aorta. Plasma was obtained by centrifugation and kept at -20ºC until processed. Adipose tissue from different locations, liver, hind leg muscle and interscapular brown adipose tissue were dissected and immediately frozen in liquid nitrogen. The gastrointestinal content was cleaned and the carcass, the remaining blood and debris were sealed in polyethylene bags, which were subsequently autoclaved at 120°C for 2 h. The bag contents were weighed and then minced to a smooth paste with a blender (obtaining a total rat homogenate) as previously described (Esteve *et al*., 1992b).

Analytical procedures

Nitrogen, lipid and energy content of diet components were analysed. Nitrogen content was measured with a semi-automatic Kjeldahl procedure using a ProNitro S system (JP Selecta, Abrera, Spain), whereas lipid content was measured with a solvent extraction method

(trichloromethane/methanol 2:1 v/v) (Folch *et al*., 1957). These procedures were also used for carcass lipid and protein content determination. The energy content of diet components and rat carcasses were determined using a calorimetric bomb (C7000, Ika, Staufen, Germany).

Fatty acid analyses

Lipids from grounded food samples were extracted overnight with chloroform/methanol (2:1 v/v) and processed for fatty acid analysis as previously described (Remesar *et al.,* 2015). Briefly, samples were suspended in 10 % boron trifluoride (Fluka, Buchs, Switzerland) dissolved in methanol and maintained in the dark at 4ºC for 12 h. After hexane and pure water addition, the residues were dissolved in HPLC-quality hexane (Panreac, Castellar del Vallès, Spain). Samples were analysed with a CG-MS system (QP2010 Shimadzu, Kyoto Japan) using a SP-2560 Supelco (Supelco, Bellefonte, PA USA) column. The samples were run using, as standards, an extended methylated fatty acid mixture (Supelco FAME mix C4-C24). Calculations were done using the Shimadzu FASST for GC-MS program (version 2). The rates of recovery of lipids (and those of fatty acids) were analysed with internal standards of bis-C17:0 diacylglycerol (Sigma-Aldrich, Darmstadt, Germany) randomly added to several duplicate samples.

Plasma determinations

Plasma parameters were measured with commercial kits; thus, urea was measured with kit #11537, total cholesterol with kit #11505 and triacylglycerols with kit # 11528 (all from Biosystems, Barcelona, Spain). Lactate was measured with kit #1001330 (Spinreact, Sant Esteve d'en Bas, Spain) and non-esterified fatty acids with kit NEFA-HR (Wako, Neuss, Germany); 3-hydroxybutyrate and acetoacetate were estimated with a ketone bodies kit (Biosentec, Toulouse, France) based on 3-hydroxybutyrate dehydrogenase. Glycerol was estimated with kit #F6428 (Sigma-Aldrich, Darmstadt, Germany). Testosterone and oestradiol were determined by Elisa kits EIA1559 and EIA2693 (DRG International, Marburg, Germany). Glucose in plasma was measured with a glucose oxidase kit #11504 (Biosystems, Barcelona, Spain) supplemented with mutarotase (490 nkat/mL of reagent) (Calzyme, San Luis Obispo, CA, USA). Mutarotase 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 (Oliva *et al.,* 2015).

Liver and muscle determinations

Tissue lipid extraction was performed as described previously with slight modifications. In brief, samples of frozen liver and muscle (30-50 mg) were powdered under liquid nitrogen. A mix of chloroform-methanol solution (1 mL; 2:1 v/v) was added and incubated at room temperature for 1 h; occasional shaking of the tubs was applied to favour lipid extraction. H_2O (200 µL) was added to the tubes, vortexed and centrifugated at 3000g during 5 min. The upper phase was discarded and the rest of the content was dried with a nitrogen flow at room temperature. The lipid pellet was redissolved in a 2-methyl-2-propanol (60 µL) and Triton X-114-methanol (40 µL; 2:1 V/V) mix. Liver triacylglycerols and cholesterol were measured with the corresponding previously described kits for the plasma quantification of these metabolites.

Lactate dehydrogenase (LDH) activity in liver and muscle was estimated using a method previously described (Arriarán *et al*., 2015).

Gene expression analyses

Total tissue RNA was extracted from frozen samples (ca. 50 mg) using the Tripure reagent (Roche Applied Science, Indianapolis IN USA), and was quantified in a ND-1000 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. 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 300 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set at 0.15 for all runs. A semi-quantitative approach for the estimation of the concentration of specific gene mRNAs per unit of tissue weight was used. *Cyclophyllin A (ppia)* was used as the charge control gene. The data were expressed as the number of transcript copies per gram of protein in order to obtain comparable data between the groups, given the uniformity of the samples in that aspect. The analysed genes and their primers sequences are presented in Table 1.

Calculations

Energy intake was calculated from daily food consumption, which was converted using the energy equivalence of the different foods and components measured with the calorimeter bomb. Lean mass was calculated subtracting lipid content from total weight. Lean mass increase was estimated using reference data from previous studies using rats of the same stock, age and sex conducted by us (Romero *et al.,* 2013; Romero *et al.,* 2014). Fat and protein intake were calculated from total food intake corrected by the respective protein or lipid content.

Statistical calculations were done with two-way ANOVA analyses (sex and diet) and the *post hoc* Bonferroni test, using the Prism 5.0 program (GraphPad Software Inc, La Jolla CA, USA). Significant differences obtained with ANOVA or Bonferroni test were considered when p value was <0.05. Correlations were calculated with the same program, using the Pearson correlation test.

RESULTS

Table 2 shows the composition of the diets. Values for CAF diet were obtained from the actual consumption data. Both, crude and digestible energy were higher in the HF diet, since it contained more energy than SD and CAF diets. Cafeteria diet had the lowest crude energy value because of its abundant milk intake, although its digestible energy was akin to standard diet due to its low content of fibre. The fat content was similar for CAF and HF diets and was 3-

fold higher than that of SD diet. Polyunsaturated fatty acids predominated in standard diet, whereas the main component of HF diet was saturated medium-chain fatty acids. CAF diet contained an important monounsaturated fatty acid fraction together with a main saturated fraction.

Nutrient intake and balances are presented in Table 3. HF diet induced a greater lipid intake and decreased protein and carbohydrate intakes, being the effects more marked in males, which also showed significant decreases in protein balance and protein accretion efficiency. However, the total lipid content and the increase in lean mass were not different from SD fed animals, in spite of the decrease in male's protein content. CAF diet elicited increases in all parameters, except for protein content, percentage of lipid content, protein accretion efficiency and protein balance that were not different from SD group. All parameters showed differences caused by diet, and many showed differences caused by sex due to body size and food intake sex-related differences; all values were higher in males than in females, except when comparing the lipid and protein content expressed as a percentage of body weight or when considering the efficiency of lipid accumulation (similar between both sexes) and the cost of energy accrual, which was higher in females. Furthermore, total body lipid accrual correlated with protein intake (for all groups and sexes) ($r=0.589$, $p<0.0001$) whereas protein accrual did not correlate with lipid intake ($r=0.0766$, $p=0.6429$). Either lipid accrual ($r=0.6731$, $p<0.0001$) or protein accrual ($r=0.3893$, $p=0.0143$) correlated with carbohydrate intake.

Table 4 shows the weight of several tissues. CAF fed animals showed increases in all adipose tissue locations, and in total WAT values, whereas HF groups did not show differences with respect to SD fed groups. Interscapular brown adipose tissue weight did not show significant variations. Weights of male tissues were obviously higher than those of females, although relative values with respect to total body weight did not show differences.

Table 5 shows metabolite and hormone plasma levels. HF diet fed animals showed increased lactate and oestradiol levels, either in males or females. Cholesterol was decreased in both HF groups. However, only females presented a decrease in glucose and an increase in urea levels, whereas males showed increases in triacylglycerol and glycerol levels. CAF diet increased triacylglycerols and non-esterified fatty acids and decreased urea levels only in males. In CAF females, 3-hydroxybutyrate levels were decreased. Regarding testosterone and oestradiol, significant differences were obviously found between sexes, being those of testosterone lower in females. However, the effect of diet was significant for oestradiol, where HF groups showed the highest values, either in males or females.

The ectopic fat and cholesterol accumulation in liver is depicted in Figure 1. Male rats fed either HF or CAF diet showed higher triacylglycerol liver accumulation compared with SD fed animals (1.6-fold and 3-fold respectively). Cholesterol accumulation was only higher in CAF males. Females showed lower triacylglycerol and cholesterol values than males, without differences between groups. Significant correlations can be stablished, in males, between liver triacylglycerol content and lipid intake ($r=0.6615$, $p=0.0015$) and between liver cholesterol

content and lipid intake (r=0.6827, p=0.0013). Females did not show any correlation related with these parameters.

Figure 2 shows liver and muscle lactate dehydrogenase (LDH) activities and expressions. Liver lactate dehydrogenase activity was unaffected by diet, but its gene expression was increased in both CAF groups. Both male and female HF fed rats showed an increase of muscle lactate dehydrogenase gene expression, which was more marked in males; LDH activity was significantly increased in HF and CAF females. Furthermore, plasma lactate levels correlated with muscle LDH activity and expression, whereas there was no correlation with liver LDH activity (r=0.212, p=0.221) or expression (r=0.106, p=0.542).

Plasma oestradiol showed an inverse correlation with cholesterol levels and a direct correlation with plasma lactate and muscle LDH activity and expression (Figure 3).

The expression of different enzymes in liver is shown in Figure 4. Mitochondrial enzymes (*Cox4i1* and *Uqcrc1*) showed a clear different sex-pattern, being their expressions higher in female rats. Cytoplasmatic enzymes showed differences mainly caused by diet. Thus, *CPT1α* and *FAS* gene expressions showed opposite effects elicited by diet, increasing in the HF and CAF groups in the former, and decreasing in the latter. *Pgp* gene expression decreased in both male and female HF groups and only in males of the CAF group. HF groups showed also significant decreases in the *Srbf2f* expression whereas *PPARα* expression showed increases in HF and CAF groups.

DISCUSSION

The use of diets with high-fat content usually implies a potential increase in the energy consumption and therefore the increase of energy reserves facilitating obesity development (Buettner *et al.,* 2007; Martire *et al.,* 2013). This factor, depending on treatment extent, is counterbalanced by the increase in the satiation caused by lipid excess (Little *et al*., 2005), which can reduce the amount of food ingested, although not necessarily the energy intake (Buettner *et al.,* 2007), and consequently influence the weight gain extent (Buettner *et al.,* 2006). In this work, the ingestion of a standard diet enriched with saturated fats (coconut oil), with a fat percentage ten times higher than that of standard diet, does not cause "*per se*" an increase in the amount of the ingested energy nor a variation of the body weight. However, cafeteria diet causes a strong increase in body weight, especially in adipose tissue (Romero *et al*., 2014), even though the latter contained the same energy percentage from lipids as HF diet.

Thus, a high lipid intake does not automatically imply a higher lipid balance, as animals fed the standard diet were more efficient in the management of dietary lipids (efficiency of lipid accretion) when compared with high-fat fed groups. The different pattern followed by both high-fat diet fed animals (a HF with almost all saturated fatty acids, and CAF with notable monounsaturated fatty acids), indicates that crude energy factor does not predominate over

the individual lipid composition, pointing towards a restricted role for a ponderostat sensor system to regulate the body energy accrual as lipids in adipose tissue.

In fact, the increase in adipose mass (above the values of the SD fed groups) is only definite in CAF animals, implying that the excess of energy from lipids is not used up to generate fat accumulation in the HF group; it must partially replace the use of glucose for the maintenance of the organism's homeostasis. As a result, the fact that the lipid balance remained similar between the SD and HF groups confirmed a lower efficiency to store lipids in the HF group, which was also noticed in CAF groups. However, the excess of energy intake (either as lipid, carbohydrate or protein) in CAF groups allowed them to obtain a generous energy surplus that was devoted to increase both lean and adipose mass.

Because of the dilution of the standard diet with coconut oil, the protein balance of HF animals was lower than that of SD groups. This does not mean that they have a protein deficiency, since the maintenance of body weight and lean mass showed that the minimum protein requirements were achieved. Sex-related differences, manifested as lower efficiency in energy use by females, does not seem to be solely caused by oestradiol levels, since HF males exhibited high levels of oestradiol, and higher efficiency values than females. The hormonal context may also condition the resistance to accumulate triacylglycerols and cholesterol in liver (Pedram *et al*., 2013), since lipid intake and liver fat content correlated in males but not in females. This fact can be considered as a poor performance in the accretion of energy (Valle *et al.*, 2007) rather than because of differences in lipid storage performance, since the percentages of total body lipid accretion in males and females were in the same order. Our data confirm an increased expression of liver oxidative enzymes in females (*Cox4i* and *Uqrc1c)*, which can be responsible of the higher energy expenditure and the lower energy efficiency when compared with males. On the other hand, in treated males, the accumulation of triacylglycerols in liver may induce a decrease in the expression of lipogenic pathway (*FAS*) as an attempt to avoid excessive lipid accumulation.

The high levels of oestradiol found in the HF group may be caused by high levels of lauric acid in this diet (Cao and Gregoire, 2016). This fatty acid is not completely distributed into intestinal lipoproteins (Eyres *et al*., 2016) being able to easily reach peripheral tissues favouring the aromatase activity (Williams, 2012) and the increase of oestradiol synthesis. The decreasing effects of oestradiol in liver lipid content have been attributed to *Srbef1* sequestration into the membrane (Pedram *et al.,* 2013). However, our results indicate a substantial decrease in the expression of *Srbef2* and maintained levels of Srebf1c expression (data not shown). This fact may explain the lower synthesis of liver cholesterol in HF groups and, consequently, the inverse correlation between plasma cholesterol and oestradiol levels. In addition, it has been described that oestradiol interferes with the actions of PPARγ on adipogenesis by down-regulating adipogenesis-related genes (Jeong and Yoon, 2011).

It has been described that high-fat diets increase liver lipid content (Jones, 1997; Díaz Rúa *et al.,* 2016), a process that is mediated by *PPARα* signaling (Patsouris *et al.,* 2006), and that is in agreement with the increased expression found in HF and CAF fed animals. However,

this process seem to be selective and modulated by diet's fatty acid composition, since HF and CAF groups showed important differences in their ability to store triacylglycerols in liver despite the fact that both exhibited a lower capacity of fatty acid synthesis. This selective pattern can be partially explained by the increase in *CPT1* expression of HF groups, which contributes to a more oxidative pathway, opposing to the high storage ability of CAF groups.

The increased plasma levels of lactate and glycerol, in a metabolic normoglycemic condition, found in HF males are not consequence of a higher expression of the liver lactate dehydrogenase and glycerol phosphatase, or higher lactate dehydrogenase activity, since this is much greater (if we refer to total liver) in males than in females and plasma levels do not reflect this potential difference. Thus, the ability of other tissues to release/uptake lactate should be responsible for this increase. It is unlikely that WAT is responsible for excessive lactate production (Arriarán *et al.*, 2015) since neither its mass nor the glucose availability is increased. However, the high correlation found between oestradiol levels and lactate dehydrogenase activity (and expression) in muscle, indicates that this tissue must be responsible of the high lactate levels in plasma of HF groups, in a process controlled by estrogens through PGC 1-α (Summermatter *et al.*, 2013).

The use of two different high-fat diets, with similar energy content but dissimilar fatty acid composition, resulted in a substantially different energy partition. Furthermore, this different lipid composition affects the levels of basic metabolic parameters, such as lactate and glycerol, and hormones as oestradiol, modulating the liver ability to synthesize lipids and the expression of enzymes involved in their metabolism.

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Table 1. Primer sequences used for liver gene expression analysis.

Gene	Protein	Direction	Sequences	bp
Pgp	Phosphoglycolate phosphatase	5' >	CCTGGACACAGACATCCTCCT	100
		> 3'	TTCCTGATTGCTCTTCACATCC	
FAS	Fatty Acid Synthetase	5' >	CCCGTTGGAGGTGTCTTCA	117
		> 3'	AAGGTTCAGGGTGCCATTGT	
Ldha	L-lactate dehydrogenase a	5' >	AAAGGCTGGGAGTTCATCCA	125
		> 3'	CGGCGACATTCACACCACT	
Hmgcs2	Hydroxymethylglutaryl-CoA synthase 2, mitochondrial	5' >	CAACCTCTTCCCAGGCACTT	108
		> 3'	CCGGGGAATGGTTGTATGGA	
Cpt1a	Carnitine O-palmitoyltransferase 1, liver isoform	5' >	CCGCTCATGGTCAACAGCA	105
		> 3'	CAGCAGTATGGCGTGGATGG	
Srebf2	Sterol regulatory element-binding protein 2	5' >	ACCGTTTAGCAGCCACAGCA	121
		> 3'	CCACAACCCTGACCAACACC	
mt-Cox1	Cytochrome c oxidase I, mitochondrial	5' >	AGATGTAGACACCCGAGCCT	172
		> 3'	TGTTAGGCCCCCTACTGTGA	
PPARα	Peroxisome Proliferator Activated Receptor Alpha	5’ >	GCACAATCCCCTCCTGCAAC	124
		> 3’	TTCAATGCCCTCGAACTGGA	
Uqcrc1	Ubiquinol-Cytochrome C Reductase Core Protein 1	5’ >	TCGCAGCCTCCTGACTTATG	78
		> 3’	ATCTGGGCATCCACCTCCT	
PPIA	Peptidylprolyl isomerase A (cyclophillin A) [housekeeping gene]	5' >	CTGAGCACTGGGGAGAAAGGA	87
		> 3'	GAAGTCACCACCCTGGCACA	

Table 2. Diet composition

	Standard diet	High-fat diet	Cafeteria diet*
Crude energy content (kJ/g)	16.5	18.8	12.4 ± 0.2
Digestible energy content	12.1	14.6	12.0 ± 0.1
% energy intake			
Protein	19.3 ± 0.4	14.5 ± 0.3	11.7 ± 0.4
Fat	12.5 ± 0.2	36.8 ± 0.7	39.5 ± 0.9
Carbohydrate	67.1 ± 0.9	48.6 ± 0.6	48.5 ± 0.5
Sugars (as % of carbohydrates)	<1%	<1%	24.1 ± 0.5
Fat/Protein ratio	0.625	2.53	3.39 ± 0.2
Mean Fatty acid content (%)			
Saturated	25	74	55
C12:0 + C14:0		53	
Monoinsaturated	14	19	35
C18:1			33
Polyunsaturated	61	5	12
C18:2	56		

*Data obtained from the food consumption data of the animals fed cafeteria diet (mean values). Intake differences between male and female animals were not statistically significant.

Table 3. Weight increase, energy, lipid and protein intakes. Lipid and protein balances, efficiencies and costs of energy accrual.

	MALES			FEMALES			
	SD diet	**HF diet**	**CAF diet**	**SD diet**	**HF diet**	**CAF diet**	**ANOVA**
Weight increase (%)	21.2 ± 2.1^{A}	23.3 ± 1.6^{A}	34.8 ± 0.9^{B}	16.7 ± 1.7^{a}	12.4 ± 0.4^{a}	34.1 ± 3.2^{b}	D, S, I
Lean mass increase (g)	56.2 ± 4.1^{A}	59.6 ± 5.6^{A}	93.8 ± 4.7^{B}	24.3 ± 4.6^{a}	22.2 ± 1.1^{a}	39.3 ± 2.1^{b}	D, S, I
Energy							
Intake (MJ)	8.70 ± 0.44^{A}	8.43 ± 0.21^{A}	18.8 ± 0.3^{B}	6.32 ± 0.2^{a}	5.98 ± 0.1^{a}	16.1 ± 0.3^{b}	D, S
Accretion (MJ)	0.83 ± 0.16^{A}	0.92 ± 0.21^{A}	1.95 ± 0.18^{B}	0.41 ± 0.15^{a}	0.29 ± 0.07^{a}	1.56 ± 0.24^{b}	D, S
Cost of accrual (kJ/g)	106 ± 5^{A}	104 ± 7^{A}	153 ± 6^{B}	167 ± 12^{a}	220 ± 12^{a}	236 ± 17^{b}	D, S
Carbohydrate intake (kJ/d)	197 ± 5^{A}	136 ± 3^{B}	309 ± 4^{C}	141 ± 6^{a}	96.5 ± 1.5^{b}	274 ± 6^{c}	D, S
Lipid							
Intake (kJ/d)	36.7 ± 1.0^{A}	100 ± 2.4^{B}	267 ± 8.4^{C}	26.3 ± 1.0^{a}	71.1 ± 1.0^{b}	214 ± 7^{c}	D, S, I
Content (g)	74.2 ± 6.5^{A}	69.7 ± 3.7^{A}	92.4 ± 5.2^{B}	47.7 ± 4.7^{a}	39.1 ± 1.9^{a}	64.6 ± 4.5^{b}	D, S
Content (% bw)	15.7 ± 0.8^{A}	15.9 ± 0.5^{A}	17.9 ± 0.9^{A}	17.3 ± 1.4^{a}	14.9 ± 0.7^{a}	22.1 ± 1.2^{b}	D, I
Balance	23.9 ± 5.1^{A}	23.3 ± 2.8^{A}	39.5 ± 4.9^{B}	14.8 ± 4.1^{a}	6.44 ± 1.83^{a}	34.5 ± 6.51^{b}	D, S
Accretion efficiency (%)	42.4 ± 8.5^{A}	29.5 ± 2.2^{AB}	17.1 ± 2.3^{B}	47.6 ± 5.8^{a}	11.5 ± 3.3^{b}	19.9 ± 3.3^{b}	D, S
Protein							
Intake (kJ/d)	58.6 ± 1.7^{A}	40.7 ± 1.0^{B}	78.5 ± 0.6^{C}	42.1 ± 1.7^{a}	28.7 ± 0.4^{b}	63.1 ± 1.1^{c}	D, S
Content (g)	85.6 ± 4.7^{A}	74.1 ± 1.4^{B}	87.5 ± 3.0^{A}	51.1 ± 1.4^{a}	47.7 ± 1.0^{a}	46.9 ± 2.4^{a}	D, S, I
Content (% bw)	19.6 ± 0.2^{A}	16.8 ± 0.1^{B}	17.1 ± 0.6^{B}	18.6 ± 0.1^{a}	18.2 ± 0.2^{a}	16.2 ± 0.2^{b}	D, I
Balance	17.7 ± 1.8^{A}	6.45 ± 0.91^{B}	15.0 ± 3.3^{A}	4.94 ± 0.8^{a}	1.99 ± 0.41^{a}	5.40 ± 1.31^{a}	D, S, I
Accretion efficiency (%)	15.3 ± 1.8^{A}	8.98 ± 1.22^{B}	10.8 ± 2.0^{AB}	6.88 ± 1.18^{a}	3.88 ± 1.11^{a}	5.00 ± 1.82^{a}	D, S

Data are expressed as mean ± SEM. Statistical analysis: two-way ANOVA significant p-values for diet (D), sex (S) or Interaction (I): $p < 0{:}05$. Statistical significance (Bonferroni's post-hoc test) between diets is represented by different superscript letters.

Table 4. Weight of different tissues after dietary treatment.

	MALES			FEMALES			ANOVA
	SD diet	**HF diet**	**CAF diet**	**SD diet**	**HF diet**	**CAF diet**	
Liver	15.9 ± 1.6^{A}	13.1 ± 0.3^{B}	16.7 ± 0.9^{A}	8.56 ± 0.51^{a}	8.14 ± 0.21^{a}	8.16 ± 0.24^{a}	S
Perigonadal WAT	7.88 ± 0.74^{A}	6.28 ± 0.42^{A}	11.1 ± 0.5^{B}	6.97 ± 0.31^{a}	5.11 ± 0.27^{b}	11.0 ± 0.9^{c}	D
Retroperitoneal WAT	8.90 ± 1.03^{A}	7.47 ± 0.64^{A}	12.6 ± 1.0^{B}	4.21 ± 0.56^{ab}	3.49 ± 0.41^{a}	6.10 ± 0.52^{b}	DS
Mesenteric WAT	5.41 ± 0.59^{A}	5.75 ± 0.35^{A}	7.19 ± 0.11^{B}	4.42 ± 0.42^{a}	4.07 ± 0.21^{a}	5.94 ± 0.53^{b}	DS
Total WAT	22.2 ± 2.1^{A}	19.5 ± 1.3^{A}	30.9 ± 1.3^{B}	15.6 ± 1.2^{a}	12.6 ± 0.7^{a}	23.1 ± 1.8^{b}	DS
Interscapular BAT	0.55 ± 0.13^{A}	0.56 ± 0.05^{A}	0.68 ± 0.03^{A}	0.65 ± 0.07^{a}	0.46 ± 0.03^{a}	0.60 ± 0.04^{a}	ns

Data are expressed in grams as mean ± SEM. Statistical analysis: two-way ANOVA significant p-values for diet (D) or sex (S): $p < 0:05$. Statistical significance (Bonferroni's post-hoc test) is represented by different superscript letters.

Table 5. Metabolite and hormone plasma values of rats after dietary treatment**.**

	MALES			FEMALES			
	SD diet	**HF diet**	**CAF diet**	**SD diet**	**HF diet**	**CAF diet**	**ANOVA**
Glucose (mM)	9.85 ± 0.31^{A}	9.65 ± 0.51^{A}	10.5 ± 0.7^{A}	10.9 ± 0.71^{a}	8.43 ± 0.31^{b}	10.4 ± 0.6^{a}	D
Lactate (mM)	2.07 ± 0.08^{A}	4.71 ± 0.49^{B}	2.66 ± 0.30^{A}	2.27 ± 0.31^{a}	4.01 ± 0.28^{b}	2.70 ± 0.33^{a}	D
Glycerol (mM)	0.16 ± 0.02^{A}	0.34 ± 0.06^{B}	0.18 ± 0.03^{A}	0.15 ± 0.03^{a}	0.21 ± 0.01^{a}	0.22 ± 0.02^{a}	D, I
Cholesterol (mM)	2.66 ± 0.21^{A}	1.65 ± 0.08^{B}	2.24 ± 0.11^{AB}	2.64 ± 0.11^{a}	1.58 ± 0.21^{b}	2.48 ± 0.31^{a}	D
Non-esterified fatty acids (mM)	0.32 ± 0.05^{A}	0.38 ± 0.04^{AB}	0.55 ± 0.08^{B}	0.32 ± 0.05^{a}	0.35 ± 0.03^{a}	0.44 ± 0.07^{a}	D
Triacylglycerols (mM)	1.34 ± 0.04^{A}	1.94 ± 0.11^{B}	1.96 ± 0.18^{B}	1.01 ± 0.09^{a}	1.04 ± 0.21^{a}	0.98 ± 0.08^{a}	D, S, I
Urea (mM)	2.98 ± 0.31^{A}	3.55 ± 0.11^{A}	1.98 ± 0.15^{B}	2.26 ± 0.18^{a}	3.43 ± 0.31^{b}	1.88 ± 0.31^{a}	D
3OH butyrate (µM)	31.2 ± 5.0^{A}	48.9 ± 4.5^{A}	31.1 ± 5.8^{A}	47.1 ± 7.1^{ab}	62.4 ± 11.1^{a}	31.4 ± 6.78^{b}	D
Acetotacetate (µM)	190 ± 44^{A}	159 ± 32^{A}	128 ± 16^{A}	145 ± 59^{a}	183 ± 31^{a}	202 ± 49^{a}	ns
Oestradiol (ng/L)	29.1 ± 5.2^{A}	100 ± 8^{B}	35.9 ± 3.8^{A}	57.2 ± 4.6^{a}	106 ± 7^{b}	59.7 ± 6.1^{a}	D, S
Testosterone (ng/L)	3.18 ± 0.28^{A}	3.51 ± 0.44^{A}	3.69 ± 0.49^{A}	1.16 ± 0.07^{a}	1.72 ± 0.08^{a}	1.26 ± 0.11^{a}	S

Data are expressed as mean ± SEM. Statistical analysis: two-way ANOVA significant p-values for diet (D), sex (S) or Interaction (I): $p < 0:05$. Statistical significance (Bonferroni's post-hoc test) between diets is represented by different superscript letters.

FIGURE 1

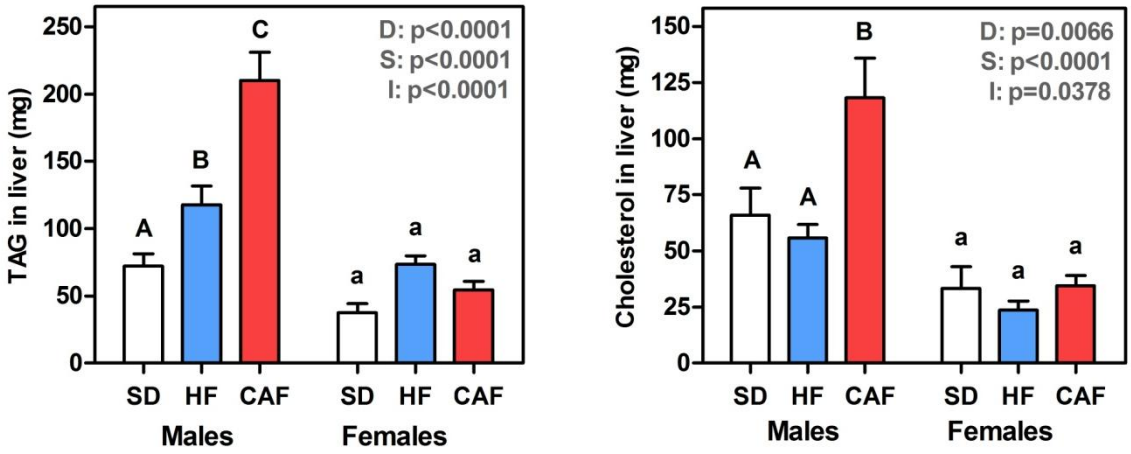

Figure 1. Triacylglycerol and cholesterol liver content. Data are the mean ± SEM of six to eight animals per group. White bars: standard diet (SD); blue bars: high-fat diet (HF) and red bars: cafeteria diet (CAF). Statistical differences between groups: two-way ANOVA (D, diet; S, sex; I, their interaction). Bonferroni post-hoc test: different letters represent statistically significant differences between groups of the same sex.

FIGURE 2

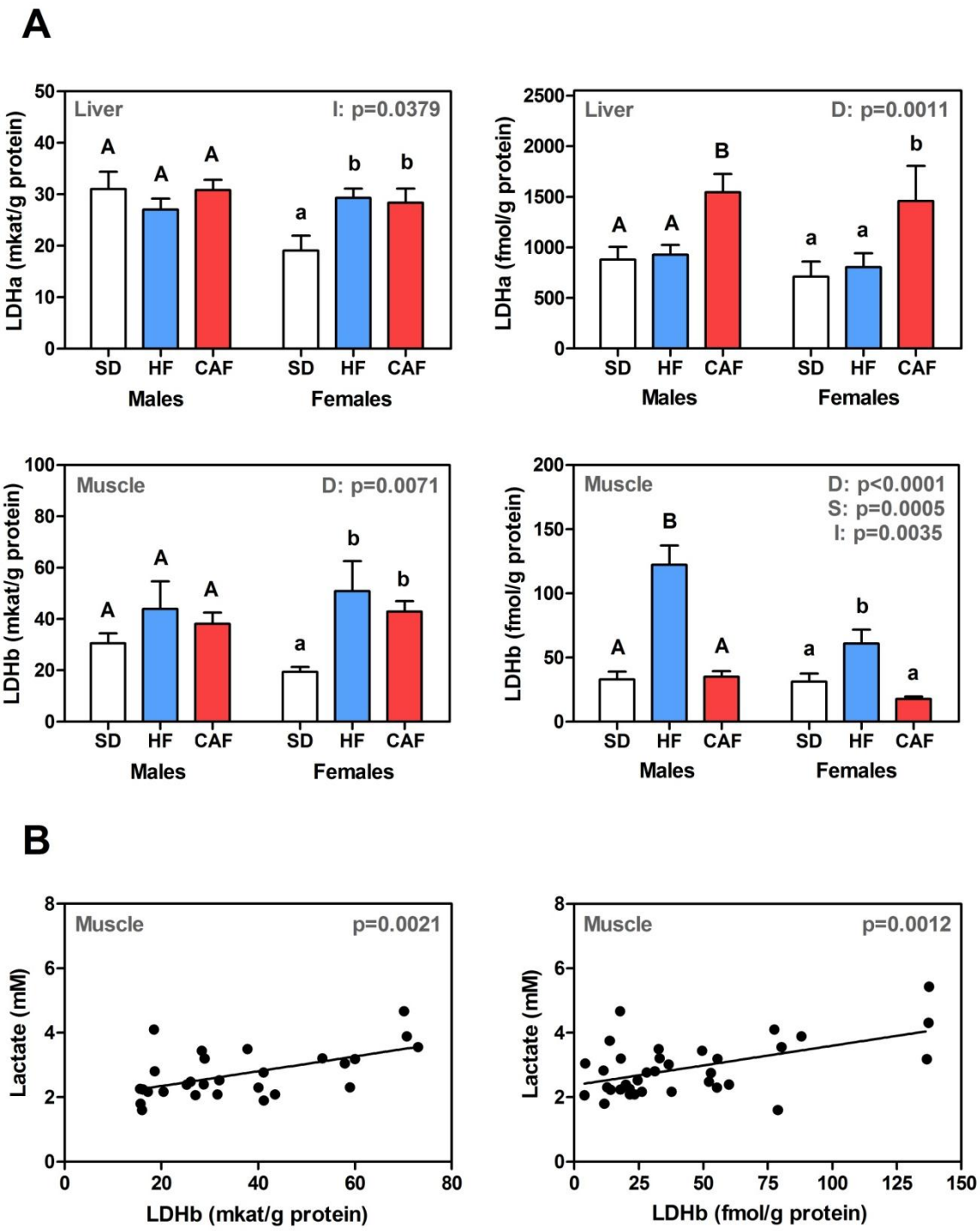

Figure 2. Lactate dehydrogenase (LDH) activities and expressions in liver and muscle (A) and correlation between plasma levels and muscle LDH activity and expression of all treated animals (B). Data are the mean ± SEM of six to eight animals per group. White bars: standard diet (SD); blue bars: high-fat diet (HF) and red bars: cafeteria diet (CAF). Statistical differences between groups: two-way ANOVA (D, diet; S, sex; I, their interaction). Bonferroni post-hoc test: different letters represent statistically significant differences between groups of the same sex. Values of statistical significance of correlations are also incorporated as p values.

FIGURE 3

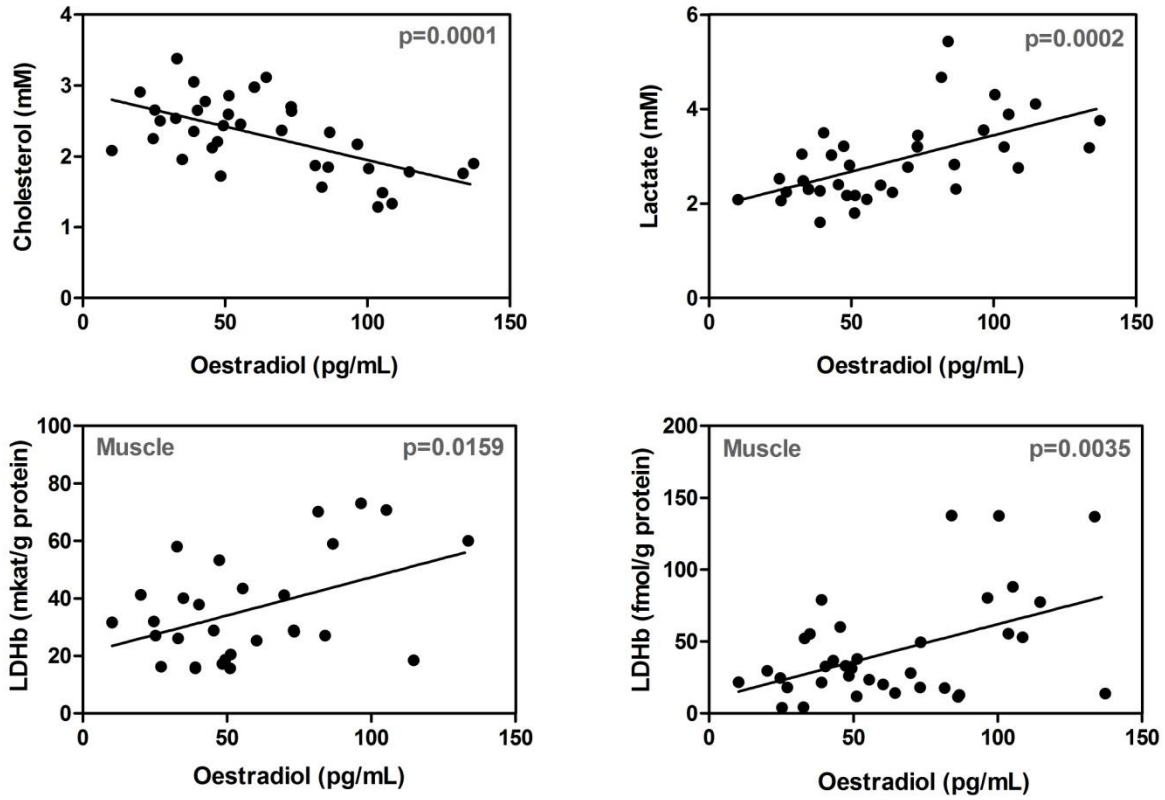

Figure 3. Correlation of plasma oestradiol values with plasma cholesterol, plasma lactate and the expression and activity of muscle lactate dehydrogenase (LDH) of all treated animals. Values of statistical significance of correlations are incorporated as p values.

FIGURE 4

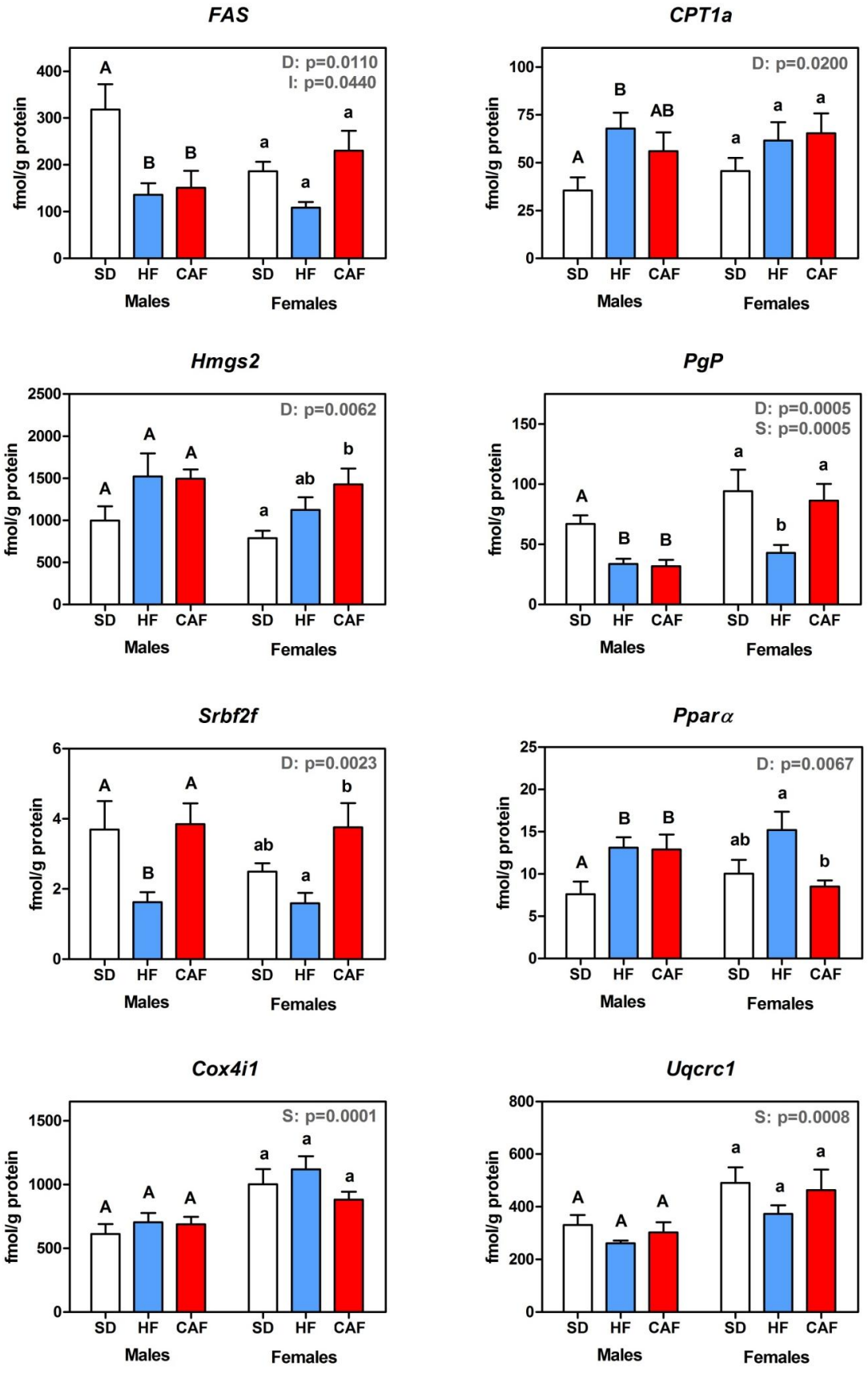
FAS
D: p=0.0110
I: p=0.0440
CPT1a
D: p=0.0200
Hmgs2
D: p=0.0062
PgP
D: p=0.0005
S: p=0.0005
Srbf2f
D: p=0.0023
Pparα
D: p=0.0067
Cox4i1
S: p=0.0001
Uqcrc1
S: p=0.0008
fmol/g protein
SD HF CAF SD HF CAF
Males
Females

Figure 4. Liver expression of different liver enzymes or transcription factors: Fatty Acid Synthetase (*FAS*), Carnitine O-palmitoyltransferase 1 (*CPT1α*), Hydroxymethylglutaryl-CoA synthase 2 (*Hmgs2*), Phosphoglycolate phosphatase (*PgP*), Sterol regulatory element-binding protein 2 (*Srbf2f*), Peroxisome Proliferator Activated Receptor α (*Pparα*), Cytochrome c oxidase I (*Cox4i1*) and Ubiquinol-Cytochrome C Reductase Core Protein 1 (*Uqcrc1*). Data are the mean ± SEM of six to eight animals per group. White bars: standard diet (SD); blue: high-fat diet (HF) and red: cafeteria diet (CAF). Statistical differences between groups: two-way ANOVA (D, diet; S, sex; I, their interaction). Bonferroni post-hoc test: different letters represent statistically significant differences between groups of the same sex.

REFERENCES

Agnelli S, Arriarán S, Oliva L, Remesar X, Fernández-López J-A, Alemany M. 2016. Modulation of rat liver urea cycle and related ammonium metabolism by sex and cafeteria diet. *RSC Advances* 6: 11278-11288.

Arriarán S, Agnelli S, Sabater D, Remesar X, Fernández-López JA, AlemanyM. 2015. 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.

Bensaïd A, Tomé D, Gietzenb D, Evena P, Morens C, Gausseresc N, Fromentin G. 2002. Protein is more potent than carbohydrate for reducing appetite in rats. *Physiology & Behavior* 75: 577– 582.

Buettner R, Parhofer KG, Woenckhaus M, Wrede CE, Kunz-Schugart LA, Schölmerich, Bollheimer LC. 2006. Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. *Journal of Molecular Endocrinology* 36: 485–501.

Buettner R, Schölmerich J, Bollheimer LC. 2007. High-fat Diets: Modeling the metabolic disorders of human obesity in rodents. *Obesity* 15: 798-808.

Cao JJ, Gregoire BR. 2016. A high-fat diet increases body weight and circulating estradiol concentrations but does not improve bone structural properties in ovariectomized mica. *Nutrition Research* 36: 320-327.

Davis C, Loxton NJ. 2014. A Psycho-Genetic Study of Hedonic Responsiveness in Relation to "Food Addiction". *Nutrients 6 :* 4338-4353.

Díaz-Rúa R, van Schothorst EM, Keijer J, Palou A, Oliver P. 2016. Isocaloric high-fat feeding directs hepatic metabolism to handling of nutrient imbalance promoting liver fat deposition. *International Journal of Obesity* 40: 1250–1259

Dieudonne MN, Sammari A, Dos Santos E, Leneveu MC, Giudicelli Y, Pecquery R. 2006. Sex steroids and leptin regulate 11-hydroxysteroid dehydrogenase 1 and p450 aromatase expressions in human preadipocytes: sex specificities. *Journal of Steroid Biochemistry and Molecular Biology* 99: 189–196.

Esteve M, Rafecas I, Fernández-López JA, Remesar X, Alemany M. 1992a. Fatty acid utilization by young Wistar rats fed a cafeteria diet. *Molecular and Cellular Biochemistry* 118: 67-74.

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 16:237-44.

Eyres L, Eyres MF, Chisholm A, Brown RC. 2016. Coconut oil consumption and cardiovascular risk factors in humans. *Nutrition Reviews* 74:267–280.

Fisler JS, Drenick EJ, Blumficld DE, Swendseid ME. 1982. Nitrogen economy during very low calorie reducing diets: quality and quantity of dietary protein. *American Journal of Clinical Nutrition* 35: 471-486.

Folch J, Lees M, Sloane-Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry* 226: 497-509.

Ghibaudi L, Cook J, Farley C, van Heek M, Hwa JJ. 2002. Fat intake affects adiposity, comorbidity factors, and energy metabolism of Sprague-Dawley rats. *Obesity Research* 10: 956-963.

Hariri N, Thibault L. 2010. High-fat diet-induced obesity in animal models. *Nutrition Research Reviews* 23: 270-299.

Jeong S, Yoon M. 2011. 17β-estradiol inhibition of PPARγ-induced adipogenesis and adipocyte-specific gene expression. *Acta Pharmacologica Sinica* 32:230-238, 2011.

Jones PJH. 1997. Regulation of cholesterol biosynthesis by diet in humans. American Journal of Clinical Nutrition 66:438-446.

Lissner L, Levitsky DA, Strupp BJ, Kalkwarf HJ, Roe DA. 1987. Dietary-Fat and the Regulation of Energy-Intake in Human-Subjects. American Journal of Clinical Nutrition 46: 886-892.

Little TY, Feltrin KL, Horowitz M, Smout AJPM, Rades T, Meyer JH, Pilichiewicz AN, Wishart J, Feinle-Bisset C. 2005. Dose-related effects of lauric acid on antropyloroduodenal motility, gastrointestinal hormone release, appetite, and energy intake in healthy men. American Journal of Physiology 289: R1090–R1098, 2005.

Martire SI, Holmes N, Westbrook RF, Morris MJ. 2013. Altered feeding patterns in rats exposed to a palatable cafeteria diet: increased snacking and its implications for development of obesity. PLoS One 8: e60407.

Oliva L, Baron C, Fernández-López JA, Remesar X, Alemany M. 2015. Marked increase in rat red blood cell membrane protein glycosylation by one-month treatment with a cafeteria diet . PeerJ 3:e1101.

Oliva L, Aranda T, Caviola G, Fernández-Bernal A, Alemany M, Fernández-López JA, Remesar X. 2017. In rats fed high-energy diets, taste, rather than fat content, is the key factor increasing food intake: a comparison of a cafeteria and a lipid-supplemented standard diet. PeerJ 5: e3697.

Palmisano BT, Zhu L, Stafford JM. 2017. Estrogens in the Regulation of Liver Lipid Metabolism. Advances in Experimental Medicine and Biology 1043: 227–256.

Patsouris D, Reddy JK, Müller M, Kersten S. 2006. Peroxisome proliferator-activated receptor alpha mediates the effects of high-fat diet on hepatic gene expression. Endocrinology 147:1508-1516.

Pedram A, Razandi M, O'Mahony F, Harvey H, Harvey BJ, Levin ER. 2013. Estrogen Reduces Lipid Content in the Liver Exclusively from Membrane Receptor Signaling. Science Signaling 6: 276 ra36.

Prats E, Monfar M, Iglesias R, Castellà J, Alemany M. 1989. Energy intake of rats fed a cafeteria diet. Physiology & Behavior 45: 263-272.

Rafecas M, Esteve M, Fernández-López JA, Remesar X, Alemany M. 1993. Individual amino acid balances in young lean and obese Zucker rats fed a cafeteria diet. Molecular and Cellular Biochemistry 121: 45-58.

Remesar X, Antelo A, Llivina C, Albà E, Berdié L, Agnelli S, Arriarán S, Fernández-López JA, Alemany M. 2015. Influence of a hyperlipidic diet on the composition of the non-membrane lipid pool of red blood cells of male and female rats. Peer J 3:e1083.

Romero MM, Holmgren F, Grasa MM, Esteve M, Remesar X, Fernández-López JA, Alemany M. 2013. Modulation in Wistar rats of blood corticosterone compartmentation by sex and prior exposure to a cafeteria diet. PLOS One 8: e57342.

Romero MM, Roy S, Pouillot K, Feito M, Esteve M, Grasa MM, Fernández-López JA, Alemany M, Remesar X. 2014. 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.

Rothwell NJ, Stock MJ. 1984. The development of obesity in animals: The role of dietary factors. Clinical Endocrinology and Metabolism 13: 437-449

Rothwell NJ, Stock MJ. 1988. The cafeteria diet as a tool for studies of thermogenesis. 1988. Journal of Nutrition 118, 925-928.

Sclafani A, Gorman AN. 1977. Effects of age, sex, and prior body weight on the development of dietary obesity in adult rats. Physiology & Behavior 18: 1021-1026.

Schemmel R, Mickelsen O, Tolgay Z. 1969. Dietary obesity in rats: influence of diet, weight, age, and sex on body composition. American Journal of Physiology 216: 373-379.

Scalfi L, Conlaldo F, Borrelli R, de Caterina M, Spagnuolo G, Alficri R, Mancini M. 1987. Protein balance during very-low-calorie diets for the treatment of severe obesity. Annals of Nutrition and Metabolism 31: 154-159.

Summermatter S, Santos G, Pérez-Schindler J, Handschin C. 2013. Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A. Proceedings of the National Academy of Sciences 110: 8738–8743.

Tsukahara S, Ohno M, lkeda Y. 1988. Dieteing using a very lo carlorie diet. In: Diet and Obesity.Bray GA. LeBlanc L, lnoue S, Suzuki M (eds.) Japan Scientific Societies Press/Karger. Tokyo/Basel. 1988. pp 205-217.

Valle A, Guevara R, García-Palmer FJ, Roca P, Oliver J. 2007. Sexual dimorphism in liver mitochondrial oxidative capacity is conserved under caloric restriction conditions American Journal of Physiology 293: C1302–C1308.

Watson CS, Alyea RA, Jeng YJ, Kochukov MY. 2007. Nongenomic actions of low concentration estrogens and xenoestrogens on multiple tissues. Molecular and Cellular Endocrinology 274:1–7.

Williams GW. 2012. Aromatese up-regulation, insulin and raised intracellular oestrogens in men, induce adiposity, metabolic syndrome and prostate disease, via aberrant ER-α and GPER signalling. Molecular and Cellular Endocrinology 251: 269-278.

Woods SC, Seeley RJ, Rushing PA, D'Alessio D, Tso P. 2003. A controlled high-fat diet induces an obese syndrome in rats. The Journal of Nutrition 133: 1081-1087.

3. VARIACIONES EN LA COMPOSICIÓN DE LA DIETA: METABOLISMO NITROGENADO

La intervención nutricional suele ser la primera opción para el control de peso o el abordaje de algunas patologías como la obesidad. Sin embargo, los efectos que generan algunas dietas, principalmente con elevado contenido proteico, sobre el metabolismo nitrogenado son todavía desconocidos. En ratas, la funcionalidad del ciclo de la urea hepático, y en consecuencia la biosíntesis de urea, se ve reducida ante la ingesta de una dieta hiperenergética como la dieta de cafetería, poniendo en paradero desconocido parte del destino del exceso proteico ingerido. Recientemente, se ha descrito la presencia del ciclo de la urea, plenamente funcional, en distintas localizaciones del tejido adiposo, sugiriendo la capacidad del tejido para contribuir en la eliminación del exceso proteico y, a la vez, poniendo en evidencia la necesidad de ahondar en el estudio de la regulación de la eliminación del nitrógeno. En los estudios presentados a continuación se evaluaron los efectos de cuatro dietas con distinta composición de macronutrientes sobre el metabolismo nitrogenado hepático y del tejido adiposo blanco y marrón de ratas de ambos sexos. Las diferentes dietas consistieron en, por un lado, una dieta estándar (grupo control) y por otro, dos dietas con un 40% de la energía derivada de lípidos aunque con distinta composición (dieta de cafetería y dieta estándar enriquecida con aceite de coco rico en ácidos grasos saturados). Por último, también se utilizó una dieta hiperproteica, con un 40% de la energía derivada de proteínas.

3.1. The food energy/protein ratio regulates the rat urea cycle but not total nitrogen losses

Laia Oliva, Marià Alemany, Xavier Remesar and José Antonio Fernández-López.

En este estudio se ha podido comparar la regulación de la capacidad ureogénica del hígado ante dietas con proporciones de macronutrientes y densidades energéticas distintas. Se cuantificó la ingesta energética y de macronutrientes, la composición corporal, las concentraciones plasmáticas de urea y aminoácidos, y la funcionalidad del ciclo de la urea hepático mediante la determinación de las actividades enzimáticas correspondientes. Los resultados obtenidos mostraron que, a pesar de las diferencias en la ingesta de proteína, no hubo cambios significativos en los niveles circulantes de aminoácidos. Sin embargo, la concentración de urea plasmática exhibió diferencias importantes y correlacionó significativamente con las actividades enzimáticas de la argininosuccinato liasa y sintetasa sugiriendo que estas son las principales enzimas reguladoras del ciclo. También se observó que la funcionalidad del ciclo de la urea depende de la relación proteína/energía de la dieta. Este es un factor importante en una situación como la dieta de cafetería, en la que a pesar del exceso de proteínas de la dieta (debido al todavía mayor exceso de energía), el ciclo de la urea se ve inhibido. Puesto que el balance proteico y la eliminación de nitrógeno amínico no dependen de la carga energética de la dieta tienen que existir mecanismos alternativos para eliminar el superávit de nitrógeno en situaciones, como la dieta de cafetería, en las que también hay una elevada disponibilidad energética.

3.2. Urea cycle enzyme activities are partially present in brown adipose tissue, contrarily to white adipose tissue

Laia Oliva, Sofía Arriarán, Marià Alemany, Xavier Remesar and José Antonio Fernández-López.

El tejido adiposo marrón utiliza aminoácidos como sustrato. Por ello, y ante la presencia de un ciclo de la urea funcional en el tejido adiposo blanco, en este estudio nos planteamos evaluar la capacidad ureogénica del tejido adiposo marrón en ratas macho alimentadas con una dieta estándar. Se evaluaron las expresiones génicas y las actividades enzimáticas del ciclo de la urea en el tejido adiposo marrón interescapular y en el tejido adiposo blanco subcutáneo. Otras enzimas fueron analizadas como marcadores de la capacidad metabólica, en concreto la lactato deshidrogenasa, la AMP deaminasa y la óxido nítrico sintasa. Los resultados obtenidos mostraron que en el tejido adiposo marrón, en comparación con el tejido adiposo blanco, y a pesar de tener una mayor actividad metabólica, la expresión y actividad de las enzimas del ciclo de la urea fueron menores. De hecho, en el tejido adiposo marrón, solo se encontraron actividades significativas de la argininosuccinato liasa y la argininosuccinato sintetasa, lo que indica la incapacidad del tejido adiposo marrón para contribuir a la eliminación del nitrógeno amínico mediante la síntesis de urea. Sin embargo, la presencia de dichas actividades apunta a una funcionalidad parcial del ciclo de la urea relacionada, principalmente, con la síntesis de arginina.

nutrients

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Article

The Food Energy/Protein Ratio Regulates the Rat Urea Cycle but Not Total Nitrogen Losses

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Abstract: Nitrogen balance studies have shown that a portion of the N ingested but not excreted is not accounted for. We compared several diets (standard, high-fat, high-protein, and self-selected cafeteria) to determine how diet-dependent energy sources affect nitrogen handling, i.e., the liver urea cycle. Diet components and rat homogenates were used for nitrogen, lipid, and energy analyses. Plasma urea and individual amino acids, as well as liver urea cycle enzyme activities, were determined. Despite ample differences in N intake, circulating amino acids remained practically unchanged in contrast to marked changes in plasma urea. The finding of significant correlations between circulating urea and arginine-succinate synthase and lyase activities supported their regulatory role of urea synthesis, the main N excretion pathway. The cycle operation also correlated with the food protein/energy ratio, in contraposition to total nitrogen losses and estimated balance essentially independent of dietary energy load. The different regulation mechanisms observed have potentially important nutritional consequences, hinting at nitrogen disposal mechanisms able to eliminate excess nitrogen under conditions of high availability of both energy and proteins. Their operation reduces urea synthesis to allow for a safe (albeit unknown) mechanism of N/energy excess accommodation.

Keywords: urea cycle; nitrogen balance; protein/energy ratio; rat

1. Introduction

Dietary excess of amino acid N, an obvious consequence of high-protein diets, necessarily induces its oxidation for energy, thus increasing urea synthesis [1]. This function is mainly carried out by the liver, which plays an active role in the adaptation of whole-body nitrogen homoeostasis to dietary protein, possibly via glucagon [2]. The control role of liver on the disposal of ammonium-N and excess amino-N is a critical process for the maintenance of body–N homeostasis. Liver urea production is both a tight control system to prevent the loss of valuable amino acid N but also the best-controlled outlet for excretion of excess N. This way, daily dietary variations in amino acid availability are counteracted in significant part through adjustments in liver metabolic function. This mechanism, used to maintain N homeostasis, is complemented by other tissues and pathways, which are in part practically unknown.

A quantitative analysis of nitrogen balances has shown that a significant portion of the N ingested but not excreted as urea (or other N-containing catabolites) is not accounted for [3]. It has been postulated that the differences may be justified (at least in part) by respiratory loss of nitric oxide [4], or even by the direct release of nitrogen gas [5], but so far no definitive explanation has been found for this "nitrogen gap". Although this deficit has been observed with different diet types, its extent

is higher when using high-energy cafeteria diets [6]. Cafeteria diets are made up of palatable foods in which the range and variety of offered food taste, energy content, and texture induces a marked hedonic-driven increase in food consumption [7,8]. Contrary to high-protein diets, cafeteria diets decrease the operation of the liver urea cycle. The consequence is a lower overall urinary excretion of N [9], not paralleled by a compensatory increase in protein accrual [10], in spite of maintained or increased protein intake.

In this study, we compared several diets with well-established differences in their content of protein, lipids, and overall energy. In addition to a standard diet (the usual rat chow), we used a cafeteria diet and a high-fat diet matched in composition to the standard rat chow but supplemented with oil rich in saturated fat, which has a moderate obesogenic capacity [11,12]. The fat content of the high-fat diet (ca. 40%) was selected to coincide with the percentage of fat self-selected by rats using our simplified cafeteria diet model [13,14]. Finally, we used an isoenergetic, high-protein diet model also matched in composition (except for protein) to the standard rat chow.

The match in nutrients, except lipids or protein, allowed us to establish comparisons based only on these aspects of diet, limiting possible interference by other dietary components [15]. By using this array of partially superimposable diet comparisons, we intended to analyze the paradox of decreased urea synthesis of rats fed a cafeteria diet and also determine how diet energy may affect nitrogen partition, including hepatic operation and overall efficiency of the urea cycle.

2. Materials and Methods

2.1. Diets

Table 1 presents the composition of the diets used. The standard diet (Teklad 2014, Teklad diets, Madison, WI, USA) contained 20% of digestible energy derived from proteins, 13% from lipids, and 67% from carbohydrates (including 0.10% from oligosaccharides). This diet contained essentially plant-derived aliments.

Table 1. Diet energy and macronutrients content.

	Standard Diet	Cafeteria Diet *	High-Fat Diet	High-Protein Diet
Crude energy (kJ/g)	16.5	12.4 ± 0.2	18.8	17.4
Digestible energy (kJ/g)	12.1	12.0 ± 0.1	14.6	12.4
Theoretical energy derived from (%):	67.0	47.3 ± 1.2	48.7	47.7
Carbohydrates	20.1	11.9 ± 0.3	14.5	40.4
Protein	13.0	40.3 ± 0.6	36.8	11.6
Lipids				

* The data for the rats fed the cafeteria diet were the mean ± SEM of six pairs of rats; no significant differences between sexes were observed.

The high-fat diet was prepared by the addition of coconut oil (Escuder, Rubí, Spain) to the standard chow coarsely ground. The initial mixture contained 33 parts (by weight) of standard chow, 4 of coconut oil, and 16 of water (added to favor the mixture and kneading of the paste). The high-protein diet was prepared in a similar way, although in this case the mix contained 16.5 parts (by weight) of standard chow, 2.35 of casein, 2.05 of fish gelatin, 0.20 of sunflower oil, and 17 parts of water. In both cases, the "dough" was thoroughly kneaded to form a rough paste that was extruded using cut-end syringes to form 1 × 6 cm cylindrical pellets, which were dried at 40 °C until achieving the required consistency [15]. Aversion tests to these diets gave negative results, i.e., they were not different from the standard diet.

The simplified cafeteria diet was formed by excess offering of the standard chow pellets, plain cookies spread with liver pâté, bacon, water, and milk supplemented with 300 g/L sucrose and 30 g/L of a mineral and vitamin supplement (Meritene©, Nestlé, Esplugues de Llobregat, Spain) [13,14].

All components were kept fresh (i.e., renewed daily). From the analysis of the ingested items and diet composition, we calculated that approximately 40% of ingested energy was derived from lipids, 12% from protein, and 47% from carbohydrates (23% from oligosaccharides and 24% starch).

2.2. Animals and Experimental Setup

All animal handling procedures and the experimental setup were devised and 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 (# DAAM 6911). The assumedly excessive suffering of the animals when placed in metabolic cages, due to their necessary isolation, formally prevented the direct measurement of the loss of nitrogen in urine and feces along the period studied. Since previous published data from our group [3,16,17] using animals fed a standard, cafeteria, or high-protein diet, when combined, showed the existence of a close linear correlation between circulating urea levels and global nitrogen elimination ($r = 0.9621$), plasma urea concentration was used as an indirect indicator of overall nitrogen loss in urine and feces.

Ten-week-old male and female Wistar rats (Janvier, Le Genest Saint-Isle, France) were used ($n = 52$). The animals were randomly divided into four groups ($n = 6$–8 for each sex) and were fed ad libitum either the standard, high-fat, high-protein, or cafeteria diets for 30 days. All animals had free access to water. The rats were housed (in same-sex pairs) in solid-bottom cages with wood shards as bedding material and were kept in a controlled environment (lights on from 08:00 to 20:00, temperature 21.5–22.5 °C, and 50%–60% humidity). Body weight and food consumption were recorded daily. The calculation of ingested food in cafeteria diet-fed rats was done as previously described by weighing the differences in food offered and debris left (corrected for desiccation) [8].

On day 30, at the beginning of the light cycle, the rats were anesthetized with isoflurane and then killed by exsanguination through the exposed aorta using a dry-heparinized syringe. Plasma was obtained by centrifugation and kept at −20 °C until processed. The liver was dissected, weighed, and a liver sample was frozen with liquid nitrogen and maintained at −80 °C until processed. The content of the gastrointestinal tract was cleaned, and the carcass (and remaining blood, liver, and debris) was sealed in polyethylene bags, which were subsequently autoclaved at 120 °C for 2 h [6]: The bag contents were weighed and then minced to a smooth paste with a blender, thus obtaining a "total rat" homogenate. The constituents of the diets given to the animals were also ground and homogenized, and then subjected to the same analytical procedures.

2.3. Analytical Procedures

Nitrogen content was measured with a semiautomatic Kjeldahl procedure using a ProNitro S system (JP Selecta, Abrera, Spain). Initial rat nitrogen content was estimated from the percentage of N previously published (at day 0) of control animals from the same stock (and provider), age, and sex [18,19], and was adjusted using the initial body weight of each animal used in this experiment. Total accrued N was calculated from the values of body nitrogen obtained at the end of the experiment and discounting the estimated initial nitrogen content. The conversion of rat N content to rat protein content was done using the specific 5.5 conversion factor for whole rat protein content previously measured experimentally by us [20].

Carcass and food lipid content were measured by weight using a classical solvent homogenization-extraction method with trichloromethane/methanol 2:1 (v/v) [21]. Water body content was measured from carcass samples by differential weighing, before and after 24 h at 110 °C. The energy content of diet components was determined using a bomb calorimeter (C7000, Ika, Staufen, Germany). Energy intake was calculated from daily food consumption converted with the energy equivalence of the different foods and components measured with the bomb calorimeter.

Plasma urea was measured with kit #11537 (Biosystems, Barcelona, Spain). Individual amino acids were analyzed with a Biochrom 30 (Biochrom Ltd., Cambridge, UK) amino acid analyzer, using plasma samples deproteinized with 100 g/L of trifluoroacetic acid.

2.4. Enzyme Activity Analyses

Frozen liver samples were homogenized in buffer using a tissue disruptor (Ultraturrax IKA-T10, Ika, Russia) at 2–4°C. Homogenates for carbamoyl-P 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, 50 mM KCl, 1 g/L Triton X-100, and 1 g/L lipid-free bovine serum albumin (all from Sigma-Aldrich, St Louis, MO, USA). 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 Triton X-100, 1 mM dithiothreitol, and 1 g/L lipid-free bovine serum albumin. The homogenates were coarsely filtered through a 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 [22], using the corresponding homogenization buffer (i.e., containing albumin) as a blank. Enzyme activities were expressed per unit of protein weight and total liver content.

Carbamoyl-P synthase activity was estimated from the incorporation of ^{14}C-bicarbonate into carbamoyl-P, and was converted to hydroxyurea [23]. Diluted (1:4 v/v) homogenates (50 µL) were mixed with a reaction buffer containing ATP-Na_2, N-acetyl glutamate, and magnesium acetate (all from Sigma-Aldrich): The final concentrations were 20 mM, 5 mM, and 20 mM, respectively. The reaction was started with 50 µL of ammonium bicarbonate and a sodium-^{14}C-bicarbonate (PerkinElmer, Bad Neuheim, Germany) mixture (final concentration: 50 mM, 5 kBq/tube), and was carried out at 37 °C for 0, 8, or 16 min. The reaction was stopped by introducing 200 µL of the reaction mixture into tubes kept on ice containing 30 µL of 2-M hydroxylamine-HCl and was rapidly put at 95 °C until total evaporation. All labeled carbamoyl-P formed was converted to labeled hydroxyurea and remained in the tube. The whole-tube contents were counted.

Ornithine carbamoyl transferase activity was measured from the reaction of condensation of carbamoyl-P and ornithine to yield citrulline (adapted from [24]). Aliquots (40 µL) of diluted homogenates (1:49 v/v) were mixed with urease S (Boehringer Mannheim, Mannheim, Germany) and a reaction buffer containing hepes, KCl, $MgCl_2$, and ornithine (all from Sigma-Aldrich): The final concentrations were, respectively, 100 µkat/L, 50 mM, 33 mM, 4.5 mM, and 10 mM. The reaction was started by adding 15 µL of lithium carbamoyl-P (Sigma-Aldrich) (final concentration 6.7 mM) and was carried out at 37 °C for 0, 5, or 10 min. The reaction was stopped by introducing 200 µL of the reaction mixture into chilled tubes containing 600 µL of reaction buffer: Diacetyl monoxime (59.3 mM), antipyrine (7.2 mM), and $FeCl_3$ (0.3 mM) diluted with 3.75% acetic acid (v/v) and 30% H_2SO_4, prepared as previously described [24] (all products were from Sigma-Aldrich). The color reaction was developed at 100 °C for 30 min in a boiling water bath. Absorbances (including standards and blanks) were measured at 450 nm using a plate reader spectrophotometer (ELx808 Ultra Microplate Reader, Biotek, Winooski, VT, USA).

The methods used for arginino-succinate synthase and lyase and arginase enzyme activities have been previously described in detail [25].

2.5. Statistical Procedures

Statistical comparisons were done using two-way ANOVA analyses (factors: Sex and diet) and the post hoc Bonferroni test, using the Prism 5.0 program (GraphPad Software Inc, La Jolla, CA, USA). Differences were considered significant when the p-value was <0.05.

3. Results

3.1. Body Balance and Nutrients Intake

Table 2 shows body weight, body composition, and energy, as well as macronutrients intake, in all groups during the 30-day study. As expected, males showed a higher food intake, which also induced higher energy and macronutrient intake than in females. When diets were compared, rats fed the cafeteria diet had higher values for both weight gain and energy, carbohydrates, and lipid

consumption. Cafeteria diet-fed rats showed a higher percentage of body fat. The rise in lipid content was compensated by lower percentages (but not in absolute values) of water and protein compared to the other diet groups. Again, as expected, the high-fat diet group showed the second-highest lipid intake, while the high-protein diet group had the highest protein intake. The freedom to select food items according to the individual whims of the rats fed the cafeteria diet resulted in only slight variations in the protein/energy consumption ratios between different cafeteria-fed animals (showing no significant differences between sexes: 11.3% ± 0.4% for females and 12.3% ± 0.5% for males), as depicted in Table 1. In all the other groups, there could not be such variation because the diet composition was fixed.

Table 2. Body weight increase and composition, energy, and macronutrients intake.

	Standard Diet		Cafeteria Diet		High-Fat Diet		High-Protein Diet	
	Female	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
Weight increase (g)	39.5 ± 4.3^{A}	79.2 ± 8.2^{a}	73.6 ± 6.9^{B}	126 ± 3^{b}	27.5 ± 1.7^{A}	82.8 ± 6.3^{a}	27.7 ± 3.8^{A}	68.7 ± 1.5^{a}
Body composition (%)								
Water	59.6 ± 1.1^{A}	61.2 ± 0.6^{a}	54.1 ± 1.5^{B}	59.0 ± 0.5^{a}	61.2 ± 0.37^{A}	61.3 ± 0.4^{a}	61.2 ± 0.6^{A}	61.4 ± 0.7^{a}
Lipids	17.3 ± 0.1^{A}	15.7 ± 1.0^{a}	22.1 ± 1.7^{B}	17.5 ± 0.9^{a}	14.9 ± 0.7^{AC}	15.9 ± 0.5^{a}	13.1 ± 0.6^{C}	12.7 ± 0.5^{b}
Proteins	18.6 ± 0.1^{A}	19.6 ± 0.7^{a}	16.2 ± 0.2^{B}	17.1 ± 0.5^{b}	18.2 ± 0.2^{A}	16.8 ± 0.1^{b}	18.3 ± 0.1^{A}	18.7 ± 0.3^{a}
Intake (MJ)								
Energy	6.33 ± 0.35^{A}	8.76 ± 0.25^{a}	16.8 ± 0.3^{B}	19.2 ± 0.6^{b}	5.95 ± 0.10^{A}	8.43 ± 0.22^{a}	5.40 ± 0.30^{A}	7.91 ± 0.01^{a}
Carbohydrates	4.24 ± 0.17^{A}	5.91 ± 0.17^{a}	8.40 ± 0.18^{B}	9.28 ± 0.13^{b}	2.90 ± 0.04^{C}	4.11 ± 0.10^{c}	2.54 ± 0.11^{C}	3.72 ± 0.00^{c}
Lipids	0.79 ± 0.03^{A}	1.10 ± 0.03^{a}	6.42 ± 0.20^{B}	8.02 ± 0.25^{b}	2.13 ± 0.03^{C}	3.02 ± 0.07^{c}	0.63 ± 0.03^{A}	0.92 ± 0.01^{a}
Proteins	1.26 ± 0.05^{A}	1.76 ± 0.05^{a}	1.90 ± 0.03^{B}	2.36 ± 0.02^{b}	0.86 ± 0.01^{C}	1.22 ± 0.03^{c}	2.18 ± 0.03^{D}	3.19 ± 0.01^{d}

Note: Data expressed during the whole 30-day period studied as mean ± SEM. Statistical analysis was two-way ANOVA: In all cases, *p*-values both for diet and for sex were $p < 0.0001$, except for protein accumulation ($p = 0.0003$ for diet comparison), water content ($p = 0.0049$ for sex), lipid content ($p = 0.0283$ for sex), and protein content (not significant for sex). Bonferroni's post hoc test of statistical significance, established at $p < 0.05$, is represented by different superscript letters.

3.2. Nitrogen Balance

Table 3 shows the nitrogen balance of the different groups of animals. The N content at the end of the experiment among the rats fed with different diets did not show significant differences in spite of wide differences for ingested nitrogen. Nevertheless, the males showed higher N content than females due to their larger size. Since the amount of accrued N was only a small proportion of the total N ingested, both ingested N and N losses presented similar profiles: Higher values in males, but showing marked differences between groups. The highest intakes and losses of nitrogen corresponded to the high-protein diet, followed by the cafeteria diet, with the high-fat diet showing the lowest values. The groups fed the standard and cafeteria diets had the highest values for protein accrual.

Table 3. Nitrogen balance of rats fed a control, cafeteria, high-fat, or high-protein diet for 30 days.

	Standard Diet		Cafeteria Diet		High-Fat Diet		High-Protein Diet		ANOVA
	Female	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	
Initial body N (g)	8.40 ± 0.17	12.7 ± 0.8	7.72 ± 0.13	12.7 ± 0.5	8.31 ± 0.11	12.3 ± 0.2	8.13 ± 0.41	13.0 ± 0.1	S
Final body N (g)	9.30 ± 0.25	15.6 ± 0.9	8.53 ± 0.20	15.1 ± 0.5	8.67 ± 0.19	13.3 ± 0.9	8.46 ± 0.30	15.1 ± 0.2	S
Ingested N (g)	13.5 ± 0.5	18.8 ± 1.0	20.3 ± 0.3	25.2 ± 0.2	9.22 ± 0.14	13.1 ± 0.3	23.3 ± 0.9	34.1 ± 0.1	D,S,I
Accrued N (g)	0.90 ± 0.14	2.84 ± 0.35	0.82 ± 0.18	2.37 ± 0.58	0.36 ± 0.11	1.03 ± 0.18	0.33 ± 0.13	2.15 ± 0.23	D,S
Accrued N (% of ingested)	6.58 ± 0.94	15.4 ± 1.8	4.08 ± 0.92	9.46 ± 2.0	3.89 ± 1.11	7.85 ± 1.10	1.37 ± 0.64	6.29 ± 0.58	D,S
Excreted N * (g)	12.6 ± 0.5	16.0 ± 1.0	19.5 ± 0.5	22.8 ± 0.7	8.86 ± 0.14	12.1 ± 0.3	23.0 ± 0.9	32.0 ± 0.2	D,S,I

Note: Data are expressed as mean ± SEM, and are represented as g of N in 30 days. * Excreted N was calculated as the difference between the ingested N and the accumulated N. Statistical analysis was two-way ANOVA. Only significant *p*-values are shown: Diet (D), sex (S), or their interaction (I).

3.3. Plasma Metabolites

Table 4 shows the plasma amino acid levels in the four diet groups studied at the end of the experiment. Several amino acids did not show significant concentration changes (Asp, Thr, Arg, citrulline, Met, Trp, Tyr), although in others there were differences related to sex, with higher values in males (Asn, Glu, Ala, His, Phe, Gly, ornithine): The only amino acid with higher plasma levels in

females was Lys. When comparing the effects of diets, the high-protein group showed higher plasma levels (Gly, Pro, Val, Leu, Ile, Lys, ornithine, and Tyr). In the high-fat diet group, only higher Gln and Tyr levels were observed.

Table 4. Plasma amino acid levels in rats fed a standard, cafeteria, high-fat, or high-protein diet for 30 days.

	Standard Diet		Cafeteria Diet		High-Fat Diet		High-Protein Diet		ANOVA
	Female	Male	Female	Male	Female	Male	Female	Male	
Ala	535 ± 53	517 ± 35	462 ± 28	575 ± 23	496 ± 37	560 ± 17	543 ± 37	589 ± 23	S
Ser	302 ± 33	248 ± 22	353 ± 39	403 ± 44	327 ± 15	340 ± 20	348 ± 18	351 ± 13	D
Thr	286 ± 42	242 ± 27	280 ± 42	296 ± 30	366 ± 31	255 ± 9	253 ± 23	291 ± 20	
Gly	210 ± 26	262 ± 25	271 ± 33	460 ± 55	262 ± 8	346 ± 25	408 ± 23	511 ± 19	D,S
Pro	279 ± 46	299 ± 6	240 ± 24	286 ± 8	247 ± 34	265 ± 16	410 ± 26	480 ± 46	D
Asp	21.0 ± 3.9	13.5 ± 1.2	13.2 ± 1.4	15.2 ± 0.8	17.0 ± 1.6	21.2 ± 1.6	15.1 ± 1.6	18.2 ± 1.3	I
Asn	63.3 ± 11.1	76.0 ± 6.3	59.2 ± 8.0	86.1 ± 11.0	58.3 ± 13.2	73.9 ± 6.6	79.6 ± 8.6	101 ± 7.7	S
Glu	82.1 ± 9.4	104 ± 10.8	89.7 ± 7.5	121 ± 7.4	98.7 ± 14.0	134 ± 7.5	90.6 ± 6.9	128 ± 10.2	S
Gln	682 ± 69	735 ± 79	535 ± 36	718 ± 38	792 ± 41	890 ± 30	632 ± 29	656 ± 23	D,I
Val	177 ± 25	164 ± 9	142 ± 19	166 ± 14	107 ± 21	116 ± 10	211 ± 11	258 ± 19	D
Leu	207 ± 24	189 ± 10	155 ± 14	186 ± 7	151 ± 17	156 ± 11	197 ± 10	242 ± 11	D
Ile	110 ± 10	102 ± 4	100 ± 11	113 ± 8	76.4 ± 9.3	84.0 ± 4.7	116 ± 6	144 ± 7	D
Arg	155 ± 21	162 ± 16	174 ± 17	202 ± 12	136 ± 23	152 ± 10	138 ± 11	158 ± 23	
Ornithine	49.5 ± 10.8	60.9 ± 5.7	38.9 ± 4.4	68.5 ± 6.5	74.6 ± 9.9	78.8 ± 8.8	87.6 ± 8.1	143 ± 24	D,S
Citrulline	104 ± 11	91.0 ± 9.3	87.3 ± 6.0	93.7 ± 3.7	92.1 ± 3.7	104 ± 4	94.6 ± 4.0	102 ± 10	
Met	73.9 ± 10.7	67.7 ± 5.6	57.9 ± 3.6	71.2 ± 4.5	61.4 ± 6.6	70.9 ± 2.9	66.0 ± 2.4	80.9 ± 6.6	
Phe	71.8 ± 6.2	68.9 ± 4.1	56.8 ± 3.4	70.4 ± 2.6	66.4 ± 7.3	79.8 ± 3.6	67.8 ± 3.7	81.6 ± 4.3	S
Tyr	55.9 ± 11.9	66.5 ± 6.6	42.5 ± 7.2	79.5 ± 3.2	60.7 ± 8.7	111 ± 5	60.7 ± 4.9	76.7 ± 5.9	D,S,I
His	69.5 ± 8.9	65.4 ± 5.3	55.8 ± 2.2	71.9 ± 5.4	53.3 ± 6.0	63.6 ± 2.9	55.7 ± 1.8	67.4 ± 1.4	S
Lys	382 ± 44	284 ± 19	346 ± 41	344 ± 36	293 ± 22	190 ± 10	461 ± 22	405 ± 17	D,S
Trp	127 ± 15	106 ± 16	98.8 ± 10.5	123 ± 7	126 ± 6	130 ± 10	139 ± 4	131 ± 8	

Note: The data (μM) correspond to the mean ± SEM of 6–8 different animals. Statistical analysis was two-way ANOVA: *p*-values are for diet (D), sex (S), or interaction (I).

The concentrations of total amino acids and plasma urea are shown in Figure 1. Changes between groups for total amino acid levels were minimal, with the differences reflecting the data presented for individual amino acids, i.e., slightly higher overall concentrations in the high-protein diet group. However, plasma urea levels changed much more widely, with higher values for the high-protein diet and the lowest for cafeteria-fed animals.

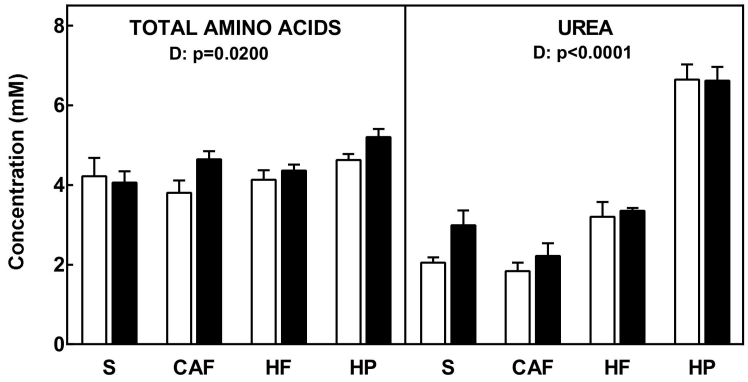

Figure 1. Plasma total amino acids and urea levels of female and male rats fed a standard, cafeteria, high-fat, or high-protein diet for 30 days. The data correspond to the mean ± SEM of 6–8 different animals. Females are represented by white bars, and males by black. Abbreviations: S is standard diet, CAF is cafeteria diet, HF is high-fat diet, and HP is high-protein diet. Statistical analysis was done using a two-way ANOVA program for diet (D) and sex (S). Only significant values are represented.

3.4. Enzyme Activities

Figure 2 shows liver urea cycle-related enzyme activities. All enzyme activities (except arginase) were affected by diet. However, while the lowest values, in the case of carbamoyl-P synthase, were observed in the high-fat diet group, in the case of arginine-succinate synthase (especially in males) and

lyase, the lowest activities were those of the cafeteria diet-fed rats. In general, high protein intakes were paralleled also by higher urea cycle enzymatic activities. The only enzymatic activity significantly affected by sex was arginase activity, with lower values in females in both the cafeteria and high-fat diet groups.

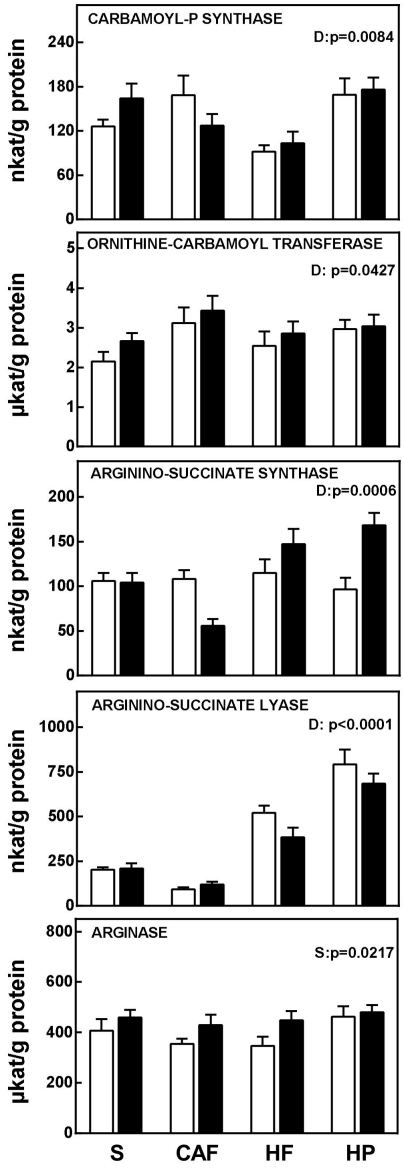

Figure 2. Enzyme activities related to the urea cycle in the liver of male and female rats fed a standard, cafeteria, high-fat, or high-protein diet for 30 days. The data correspond to the mean ± SEM of 6–8 different animals, and are all expressed per gram of tissue protein. Females are represented in white bars, and males in black. Abbreviations: S is standard diet, CAF is cafeteria diet, HF is high-fat diet, and HP is high-protein diet. Statistical analysis was done using a two-way ANOVA program for diet (D) and sex (S). Only significant values are represented.

3.5. Correlations

Total liver activities (calculated as a measure of global catalytic liver capacity) of arginino-succinate synthase and lyase were significantly correlated with plasma urea levels: Correlation values were $r = 0.322$ for arginino-succinate synthase ($p = 0.0200$) and $r = 0.762$ for arginino-succinate lyase ($p < 0.0001$). Arginino-succinate lyase activity and plasma urea levels were correlated with the diet protein/energy ratio (Figure 3).

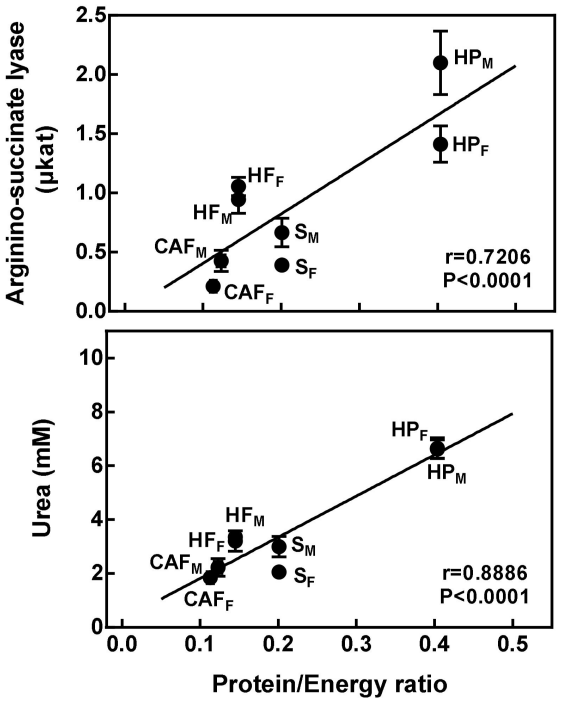

Figure 3. Relationship between the diet protein/energy ratio with total liver arginine-succinate lyase activity and plasma urea levels. The protein/energy ratio refers to the energy ingested derived from proteins versus total energy ingested. Lines of regression, correlations, and *p*-values were calculated using all individual data (n = 52). Abbreviations: S is standard diet, CAF is cafeteria diet, HF is high-fat diet, HP is high-protein diet, F means female rats, and M means male rats.

4. Discussion

We studied the effects of diets with different contents of energy, lipids, and protein, but which were essentially uniform in most of their other components. Using this approach, we were able to show the importance of the food protein/energy ratio in the control of urea cycle operation, in contraposition to total nitrogen losses and nitrogen balance, which were essentially independent of the diet energy content. The main limitation of the study was the impossibility of obtaining direct measures of nitrogen losses in urine and feces along the period studied: To overcome this limitation, we used an indirect measure of total N excretion, plasma urea levels.

The markedly different regulation observed between nitrogen losses and urea cycle operation was apparent by the lack of direct relationship between urea synthesis and obvious excess N, with energy playing a key role in the mode of disposal of excess dietary N. These differences have potentially important consequences, since they support the existence of an additional nitrogen disposal pathway able to eliminate excess nitrogen under situations of high availability of both energy and proteins. The urea cycle—in fact, all of the N disposal mechanisms for noncarnivore mammals—is centered in a tight control of N losses, which becomes a problem in the "rare" cases when both energy and N are in excess. The existence of an alternative pathway overcomes this question, with the apparent contradictory decrease in the operation of the urea cycle, which can be assumed to be a direct correlate of body–N homeostasis.

The quantitative importance of such an additional mechanism is considerable and grants the need for further study of its consequences in the maintenance of body–nitrogen balance under normal conditions and under pathological situations such as metabolic syndrome.

Homoeostatic maintenance of glycaemia and the preservation of body protein via the supply of amino acids (both general amino-N and essential ones) are critical for survival [26]. In our experimental model, the animals had no problems with amino acid availability in any of the diets tested. In fact, final body N was maintained and was unaffected regardless of diet: The only plausible question related to

amino acids was the need to dispose of their possible excess. Neither was there any deficit in dietary energy availability, since the digestible food energy density of the cafeteria and high-protein diets was similar to that of the standard diet, and that of the high-fat diet was even higher: The main differences between diets rested in the proportion of nutrients and the relationships between them and with total energy intake.

The fact that there was no energy deficit in these animals was confirmed by the weight gain of animals fed the high-fat and high-protein diets, similar to that of control animals. These results agreed with data previously described [12,27], although in both cases (higher or normal body weight gain for the high-fat diet and lower or normal body weight gain for the high-protein diet), the magnitude of the changes depended on the particular diet composition used [12,27].

The known obesogenic effects of cafeteria diets were confirmed by a significant increase in body weight [18], more marked in males and largely caused by the accumulation of fat, mainly in adipose tissue, although an increase in fat content affects all tissues [13]. This increase is accompanied by parallel, but less extensive, increases in lean body mass [28], with enhanced protein deposition (in absolute terms) [29]. This is in part driven by higher amino-N availability, paralleled by lower urea N excretion [9].

Despite the important differences in N intake, circulating amino acids showed only minor differences between groups, remaining practically unchanged, especially when compared to the marked variability of plasma urea. Food intake increases plasma amino acids, which stimulates insulin release and mTOR-dependent protein synthesis in muscle [1]. Most excess amino acids are oxidized (especially the non-essential or the highly available essential ones) [1,30]. These mechanisms are well regulated, resulting in remarkably maintained plasma amino acid levels even under conditions of protein restriction [31]. The liver is, at least in part, responsible for the limited effect of nutrient intake on plasma amino acid levels, since it disposes of most of the excess amino acids provided by a normal diet [31]. However, the extent to which amino acids are used by the liver is different for alanine and glutamine [32], which act as inter-organ vectors carrying amino-N and ammonium to the liver essentially for disposal [33], than for branched-chain amino acids, most of which are oxidized elsewhere [34].

Estimated enzyme activities are not direct approximations of in vivo enzyme function, but are generally taken as a correlate of the total amount of functional enzyme (i.e., that corresponding to V_{max}) and, consequently, of overall enzyme ability to carry out its physiological function. Since this study was done using different diet conditions on rats of both sexes, the finding of significant correlations between plasma urea concentrations and arginine-succinate synthase and lyase activities reinforce their assumedly important regulatory role in the control of liver urea cycle operation [35,36]. This correlation, akin to control of hepatic urea production, also suggests that circulating urea may be considered a fair index of urea cycle operation. This is strengthened by the fact that plasma urea levels vary in parallel to urinary losses: High plasma urea levels parallel high urine urea excretion following a high-protein diet [37–39], and both lower plasma urea and limited urinary losses have been found in rats fed cafeteria diets [6,9,39,40].

Increased protein intake, obvious in high-protein diets, initiates at least two major metabolic responses: An increase in protein synthesis (largely in muscle) driven by insulin [41,42] paralleled by an increased hepatic production of urea [43]. The urea cycle operates in part to prevent porta or hepatic metabolism-derived ammonium from entering systemic circulation [44]. However, a cafeteria diet, also characterized by a higher intake of protein-derived amino acids, resulted in both low plasma urea and excretion. As far as we know, the diet fat content did not affect per se the functionality of the urea cycle. This is a direct conclusion of the present study, and also confirms previous reports [25], since although the cafeteria diet lowered urea cycle operation, the high-fat diet did not.

It can be assumed that the relatively low contribution of protein-derived energy in relation to the total energy budget of the cafeteria diet could induce strongly unwanted protein-sparing mechanisms similar to those observed in situations of amino acid scarcity: Starvation [45] or diluted diets [46]. In all

these cases, amino acid oxidation is diminished with a parallel decrease in urea synthesis [47], but circulating amino acids are maintained by the well-balanced equilibrium between protein synthesis and proteolysis [48]. There is a conflict between the setting of amino acid sparing mechanisms (lower urea production, higher intestinal absorption, decreased urinary N excretion, etc. [49]) and their higher availability derived from the ingestion of roughly the same amount of amino-N and the effectiveness of the amino-N sparing mechanisms. Thus, the only outlets available for disposal of the excess amino-N are a shift to protein turnover favoring higher protein accrual and the unexplained and not yet understood mechanisms that have been defined as a "nitrogen gap" [3,6] and that have been described as a direct production of nitrogen gas [5]. It has been postulated that this "nitrogen gap" excretion process is related to the metabolism of arginine [39] and is influenced by sex [25]. It may be speculatively suggested that a lower operation of the guanidine-handling enzymes of the urea cycle in the liver of cafeteria diet-fed rats may allow an increased derivation of intermediates toward this so far unknown path for N excretion.

The factors that regulate the fate of N, described here, may also be important in humans since, western societies are characterized by an excessive intake of both protein and energy. The excess of protein causes an overload of N, whose elimination is hindered by the consequent excess of energy. The main question is how metabolic machinery can override the strong protective mechanisms preventing N-wasting under conditions of excess energy [50]. It is possible to venture that not only the excess of nutrients, but also the imbalance in the proportion of nutrients, can have important metabolic consequences: Part of the dysregulation found in the metabolic syndrome may be an unwanted consequence of this N disposal conflict.

5. Conclusions

In conclusion, the food protein/energy ratio has potentially important consequences in the control of urea cycle operation, since high-energy diets tend to inhibit urea cycle function: The different regulation observed hints at the existence of an additional nitrogen disposal pathway capable of eliminating excess nitrogen in situations of high availability of both energy and proteins.

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References

1. Bröer, S.; Bröer, A. Amino acid homeostasis and signalling in mammalian cells and organisms. *Biochem. J.* **2017**, *474*, 1935–1963. [CrossRef] [PubMed]
2. Petersen, K.; Vilstrup, H.; Tygstrup, N. Effect of dietary protein on the capacity of urea synthesis in rats. *Horm. Metab. Res.* **1990**, *22*, 612–615. [CrossRef] [PubMed]
3. Esteve, M.; Rafecas, I.; Remesar, X.; Alemany, M. Nitrogen balance discrepancy in wistar rats fed a cafeteria diet. *Biochem. Int.* **1992**, *26*, 687–694. [PubMed]
4. Bucca, C.; Cicolin, A.; Guida, G.; Heffler, E.; Brussino, L.; Rolla, G. Exhaled nitric oxide (FENO) in non-pulmonary diseases. *J. Breath Res.* **2012**, *6*, 27104. [CrossRef] [PubMed]
5. Costa, G.; Ullrich, L.; Kantor, F.; Holland, J.F. Production of elemental nitrogen by certain mammals including man. *Nature* **1968**, *218*, 546. [CrossRef] [PubMed]

6. Esteve, M.; Rafecas, I.; Remesar, X.; Alemany, M. Nitrogen balances of lean and obese Zucker rats subjected to a cafeteria diet. *Int. J. Obes. Relat. Metab. Disord.* **1992**, *16*, 237–244. [PubMed]
7. Sclafani, A.; Gorman, A.N. Effects of age, sex, and prior body weight on the development of dietary obesity in adult rats. *Physiol. Behav.* **1977**, *18*, 1021–1026. [CrossRef]
8. Prats, E.; Monfar, M.; Castellà, J.; Iglesias, R.; Alemany, M. Energy intake of rats fed a cafeteria diet. *Physiol. Behav.* **1989**, *45*, 263–272. [CrossRef]
9. Barber, T.; Viña, J.R.; Viña, J.; Cabo, J. Decreased urea synthesis in cafeteria-diet-induced obesity in the rat. *Biochem. J.* **1985**, *230*, 675–681. [CrossRef]
10. Sabater, D.; Agnelli, S.; Arriarán, S.; Fernández-López, J.A.; Romero, M.M.; Alemany, M.; Remesar, X. Altered nitrogen balance and decreased urea excretion in male rats fed cafeteria diet are related to arginine availability. *Biomed. Res. Int.* **2014**. [CrossRef]
11. Hariri, N.; Thibault, L. High-fat diet-induced obesity in animal models. *Nutr. Res. Rev.* **2010**, *23*, 270–299. [CrossRef] [PubMed]
12. Buettner, R.; Parhofer, K.G.; Woenckhaus, M.; Wrede, C.E.; Kunz-Schughart, L.A.; Schölmerich, J.; Bollheimer, L.C. Defining high-fat-diet rat models: Metabolic and molecular effects of different fat types. *J. Mol. Endocrinol.* **2006**, *36*, 485–501. [CrossRef] [PubMed]
13. Esteve, M.; Rafecas, I.; Fernández-López, J.A.; Remesar, X.; Alemany, M. Fatty acid utilization by young Wistar rats fed a cafeteria diet. *Mol. Cell Biochem.* **1992**, *118*, 67–74. [CrossRef] [PubMed]
14. Rafecas, I.; Esteve, M.; Fernández-López, J.A.; Remesar, X.; Alemany, M. Individual amino acid balances in young lean and obese Zucker rats fed a cafeteria diet. *Mol. Cell Biochem.* **1993**, *121*, 45–57. [CrossRef]
15. Oliva, L.; Aranda, T.; Caviola, G.; Fernández-Bernal, A.; Alemany, M.; Fernández-López, J.A.; Remesar, X. In rats fed high-energy diets, taste, rather than fat content, is the key factor increasing food intake: A comparison of a cafeteria and a lipid-supplemented standard diet. *Peer. J.* **2017**, *5*, e3697. [CrossRef] [PubMed]
16. Masanés, R.M.; 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*, 168–174. [CrossRef] [PubMed]
17. Masanés, R.M. Repercussions de les variacions del contingut de proteïnes en la dieta sobre el metabolisme nitrogenat de la rata: Aplicació a un model d'obesitat genètica. Ph.D. Thesis, Universitat de Barcelona, Barcelona, Spain, 2000.
18. Romero, M.M.; Roy, S.; Pouillot, K.; Feito, M.; Esteve, M.; Grasa, M.M.; Fernández-López, J.A.; Alemany, M.; Remesar, X. 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* **2014**, *9*. [CrossRef]
19. Romero, M.M.; Holmgren-Holm, F.; Grasa, M.M.; Esteve, M.; Remesar, X.; Fernández-López, J.A.; Alemany, M. Modulation in Wistar rats of blood corticosterone compartmentation by sex and a cafeteria diet. *PLoS ONE* **2013**, *8*, e57342. [CrossRef]
20. Rafecas, I.; Esteve, M.; Fernandez-Lopez, J.A.; Remesar, X.; Alemany, M. Whole-rat protein content estimation: Applicability of the N. x 6.25 factor 73. *Br. J. Nutr.* **1994**, *72*, 199–209. [CrossRef]
21. Folch, J.; Lees, M.; Stanley, G.H.S. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509. [CrossRef]
22. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin-Phenol Reagent. *J. Biol. Cemistry* **1951**, *193*, 265–275. [CrossRef]
23. Pierson, D.L. A rapid colorimetric assay for carbamyl phosphate synthetase I. *J. Biochem. Biophys. Methods* **1980**, *3*, 31–37. [CrossRef]
24. Sugawara, K.; Yoshizawa, Y.; Tzeng, S.; Epstein, W.L.; Fukuyama, K. Colorimetric determination of citrulline residues in proteins. *Anal. Biochem.* **1998**, *265*, 92–96. [CrossRef] [PubMed]
25. Agnelli, S.; Arriarán, S.; Oliva, L.; Remesar, X.; Fernández-López, J.-A.; Alemany, M. Modulation of rat liver urea cycle and related ammonium metabolism by sex and cafeteria diet. *RSC Adv.* **2016**, *6*, 11278–11288. [CrossRef]
26. Stumvoll, M. Control of glycaemia: From molecules to men. Minkowski Lecture 2003. *Diabetologia* **2004**, *47*, 770–781. [CrossRef] [PubMed]
27. Liisberg, U.; Myrmel, L.S.; Fjære, E.; Rønnevik, A.K.; Bjelland, S.; Fauske, K.R.; Holm, J.B.; Basse, A.L.; Hansen, J.B.; Liaset, B.; et al. The protein source determines the potential of high protein diets to attenuate obesity development in C57BL/6J mice. *Adipocyte* **2016**, *5*, 196–211. [CrossRef]

28. Maxwell, G.M.; Fourie, F.; Bates, D.J. The effect of restricted and unrestricted "cafeteria" diets upon the energy exchange and body-composition of weanling rats. *Nutr. Rep. Int.* **1988**, *37*, 629–637.
29. Barr, H.G.; McCraken, K.J. High efficiency of energy utilization in "cafeteria"-and force-fed rats kept at 29 degrees. *Br. J. Nutr.* **1984**, *51*, 379–387. [CrossRef]
30. Schutz, Y. Protein turnover, ureagenesis and gluconeogenesis. *Int. J. Vitam. Nutr. Res.* **2011**, *81*, 101–107. [CrossRef]
31. Cynober, L.A. Plasma amino acid levels with a note on membrane transport: Characteristics, regulation, and metabolic significance. *Nutrition* **2002**, *18*, 761–766. [CrossRef]
32. Häussinger, D.; Lamers, W.H.; Moorman, A.F. Hepatocyte heterogeneity in the metabolism of amino acids and ammonia. *Enzyme* **1992**, *46*, 72–93. [CrossRef] [PubMed]
33. Brosnan, J.T. Interorgan amino acid transport and its regulation. *J. Nutr.* **2003**, *133*. [CrossRef] [PubMed]
34. Skeie, B.; Kvetan, V.; Gil, K.M.; Rothkopf, M.M.; Newsholme, E.A.; Askanazi, J. Branch-chain amino acids: Their metabolism and clinical utility. *Crit. Care Med.* **1990**, *18*, 549–571. [CrossRef] [PubMed]
35. Husson, A.; Brasse-Lagnel, C.; Fairand, A.; Renouf, S.; Lavoinne, A. Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. *Eur. J. Biochem.* **2003**, *270*, 1887–1899. [CrossRef] [PubMed]
36. Latasa, M.U.; Carretero, M.V.; García-Trevijano, E.R.; Torres, L.; Mato, J.M.; Avila, M.A. Identification of argininosuccinate lyase as a hypoxia-responsive gene in rat hepatocytes. *J. Hepatol.* **2000**, *33*, 709–715. [CrossRef]
37. Morens, C.; Gaudichon, C.; Metges, C.C.; Fromentin, G.; Baglieri, A.; Even, P.C.; Huneau, J.F.; Tomé, D. A high-protein meal exceeds anabolic and catabolic capacities in rats adapted to a normal protein diet. *J. Nutr.* **2000**, *130*, 2312–2321. [CrossRef] [PubMed]
38. Morens, C.; Gaudichon, C.; Fromentin, G.; Marsset-Baglieri, A.; Bensaïd, A.; Larue-Achagiotis, C.; Luengo, C.; Tomé, D. Daily delivery of dietary nitrogen to the periphery is stable in rats adapted to increased protein intake. *Am. J. Physiol. Endocrinol. Metab.* **2001**, *281*, E826–E836. [CrossRef] [PubMed]
39. Rotondo, F.; Sanz, T.; Fernández-López, J.-A.; Alemany, M.; Remesar, X. Stable isotope analysis of dietary arginine accrual and disposal efficiency in male rats fed diets with different protein content. *RSC Adv.* **2016**, *6*. [CrossRef]
40. Esteve, M.; Rafecas, I.; Fernandez-Lopez, J.; Remesar, X.; Alemany, M. Dietary amino acid balances in young Wistar rats fed a cafeteria diet. *Biochem. Mol. Biol. Int.* **1993**, *29*, 1069–1081.
41. Castellino, P.; Luzi, L.; Simonson, D.C.; Haymond, M.; DeFronzo, R.A. Effect of insulin and plasma amino acid concentrations on leucine metabolism in man. Role of substrate availability on estimates of whole body protein synthesis. *J. Clin. Invest.* **1987**, *80*, 1784–1793. [CrossRef]
42. Malmberg, S.E.; Adams, C.M. Insulin signaling and the general amino acid control response: Two distinct pathways to amino acid synthesis and uptake. *J. Biol. Chem.* **2008**, *283*, 19229–19234. [CrossRef] [PubMed]
43. Waterlow, J.C. The mysteries of nitrogen balance. *Nutr. Res. Rev.* **1999**, *12*, 25–54. [CrossRef] [PubMed]
44. Fernandez-Lopez, J.A.; Casado, J.; Esteve, M.; Rafecas, I.; Argilés, J.M.; Remesar, X.; Alemany, M. Intestinal and hepatic nitrogen balance in the rat after the administration of an oral protein load. *Br. J. Nutr.* **1993**, *69*, 733–742. [CrossRef] [PubMed]
45. Cahill, G.F. Starvation in man. *Clin. Endocrinol. Metab.* **1976**, *5*, 397–415. [CrossRef]
46. Booth, D.A. Food intake compensation for increase or decrease in the protein content of the diet. *Behav. Biol.* **1974**, *12*, 31–40. [CrossRef]
47. Sapir, D.G.; Walser, M. Nitrogen sparing induced early in starvation by infusion of branched-chain ketoacids. *Metabolism* **1977**, *26*, 301–308. [CrossRef]
48. Hoffer, L.J.; Forse, R.A. Protein metabolic effects of a prolonged fast and hypocaloric refeeding. *Am. J. Physiol.* **1990**, *258*, E832–E840. [CrossRef]
49. Waterlow, J.C. Metabolic adaptation to low intakes of energy and protein. *Annu. Rev. Nutr.* **1986**, *6*, 495–526. [CrossRef]
50. Alemany, M. The problem of nitrogen disposal in the obese. *Nutr. Res. Rev.* **2012**, *25*, 18–28. [CrossRef]

Urea cycle enzyme activities are partially present in brown adipose tissue, contrarily to white adipose tissue

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Short title: Absence of a complete urea cycle in brown adipose tissue

Keywords: urea cycle, brown adipose tissue, white adipose tissue, rat

ABSTRACT

Recently we described the existence of a complete urea cycle in different white adipose tissue (WAT) locations. Since amino acids are used as metabolic substrates by brown adipose tissue (BAT), we considered studying whether BAT presents an active urea cycle, allowing it to eliminate the potential excess of ammonium produced in situations of high metabolic activity.

Interscapular BAT and subcutaneous WAT samples from adult male Wistar rats were used for the estimation of all urea cycle enzyme activities and gene expressions. AMP deaminase and lactate dehydrogenase gene expressions and enzyme activities were also measured, as well as eNOS gene expression.

Although, in general, BAT is metabolically more active than WAT, as evidenced by the greater activity of lactate dehydrogenase and AMP deaminase, gene expressions of urea cycle enzymes are lower, suggesting a lower metabolic capacity of the urea cycle enzymes in this tissue. BAT is characterized by the presence of significant expressions and activities of argininosuccinate lyase and synthase, but no significant expressions and activities of arginase and ornithine transcarbamylase. Therefore, BAT, differently to WAT, does not present a complete and functional urea cycle. The main function of the partial urea cycle operation in the tissue seems to be related to arginine production.

INTRODUCTION

Adipose tissue plays an essential role in the body's energy storage processes. Uniquely positioned to take up, store, and release free fatty acids depending on systemic nutritional status, it can cope with high concentrations of lipids without experiencing lipotoxicity [1]. Adipose tissue is also important in metabolic regulation, acting as a paracrine and endocrine organ [2], with a key role in energy partition processes.

There are, at least, three different types of adipose tissue, according to its specialized function, cell structure, main metabolic pathways, and anatomical placement [3]. White adipose tissue (WAT) is the most abundant type, made up of large cells, usually with a single fat-filled vacuole; WAT cells are largely glycolytic with low oxygen consumption, as a consequence of the limited number of mitochondria they contain [4]. Brown adipose tissue (BAT), highly vascularized and innervated, and formed by plurivacuolar cells, with a large number of mitochondria, is a highly oxidative tissue, specialized in thermogenic function [5]. Finally, beige (or brite) adipose tissue, found interspersed in white adipose tissue, shares mixed characteristics of brown and white adipose tissue; its function is also mainly related to thermogenesis [6].

Despite the sparse metabolic activity of white adipose tissue, recently we described the existence of a complete urea cycle in different WAT locations (mesenteric, epididymal, retroperitoneal and subcutaneous fat masses) [7]. The main role of WAT urea cycle does not seem to be the production of urea, but the generation of citrulline [7]. These results, however,

do not preclude the possibility of WAT to carry out a full urea cycle, eliminating excess N via a "peripheral" urea cycle.

The available data hint at WAT acting not only locally and peripherally, but also complementing the function of the splanchnic bed organs in the regulation of – at least— arginine metabolism. Hepatic and adipose tissue urea cycles are probably regulated differently. An example of this is the limited effect of cafeteria diet on WAT's urea cycle, in contrast with the marked effects observed in liver [8]. In addition, although urea cycle function was common to the four WAT sites studied, there were marked specific differences related to location. Subcutaneous WAT showed the highest activities/expressions for most enzymes, suggesting that it is the most probable WAT site to produce urea, eventually, in significant amounts [7]. The large mass of adipose tissue makes the potential capacity of WAT to perform the urea cycle quantitatively important, and only comparable to that of the liver.

The function of BAT is to oxidase substrates, transferring energy from nutrients into heat, by means of selective uncoupling of ATP synthesis from the respiratory chain [9]. This function is regulated via noradrenaline released by the sympathetic nervous system [10]. When the tissue is active, high amounts of lipids[5] and glucose are consumed [11,12]. Amino acids are also used as metabolic substrates by BAT [12]. BAT seems to be a net glutamine user and alanine releaser, at least during cold-acclimation [12]; its ability to take up glutamine stresses the similarities with intestine, an organ that takes up glutamine, releases alanine and produces free NH_4^+ [13,14]. The utilization of amino acids as energetic substrates poses the problem of nitrogen disposal. For this reason, we considered studying whether brown adipose tissue presents an active urea cycle, similarly to white adipose tissue, allowing it to eliminate the potential excess of ammonium produced in situations of high metabolic activity.

MATERIALS AND METHODS

Animal handling

Nine week old male Wistar rats (Harlan Laboratory Models, Sant Feliu de Codines, Spain) were used. The rats (N = 6) were housed in two-rat cages with wood shards for bedding, had free access to water and ate *ad libitum* normal rat chow (type 2014, Teklat diets, Madison, WI). They were kept in a controlled environment (lights on from 08:00 to 20:00; 21.5–22.5 C; 50–60% humidity) for at least one month.

The rats were killed by exsanguination (by aortic puncture), under isoflurane anesthesia, at the beginning of a light cycle; then, they were rapidly dissected, taking the interscapular BAT (iBAT) and a sample of subcutaneous WAT (scWAT), that were immediately blotted, weighed and frozen in liquid nitrogen. Finally, samples were stored at -80ºC until processed.

Enzyme activity analysis

Frozen tissue samples were homogenized, using a tissue disruptor (Ultraturrax IKA-T10, Ika Werke, Staufen, Germany), in 5 volumes of chilled 70mM hepes buffer pH 7.4 containing 1 mM

dithiothreitol, 50 mM KCl, 1 g/L Triton X-100 and 1 g/L lipid-free bovine serum albumin (all from Sigma-Aldrich, St Louis MO, USA). The homogenates were centrifuged for 10 min at 5000 x g; the floating fat layer and gross debris precipitate were discarded. The homogenates were kept on ice, and were used for the estimation of protein and for enzymatic analyses within 2 h after their preparation. They were diluted with buffer, as needed, in the reaction mixtures for the estimation of enzyme activities.

Tissue protein content was estimated with the Lowry method [15]. After the development of color, turbidity was eliminated with small amounts of finely powdered solid MgO (which adsorbed the remaining suspended fat), and centrifuging the tubes before reading the absorbance. In the measurements of homogenate protein content, homogenization buffer containing albumin (1 g/L) was used as blank.

Carbamoyl-P synthase activity was estimated from the incorporation of ^{14}C-bicarbonate into carbamoyl-P, and converted to hydroxyurea [16]. Diluted (1:1 v/v) homogenates (50 µL) were mixed with a reaction buffer containing ATP-Na_2, N-acetyl glutamate and magnesium acetate (all from Sigma-Aldrich); final concentrations were 20 mM, 5mM and 20 mM, respectively. The reaction was started with 50 µL of ammonium bicarbonate and a sodium-^{14}C-bicarbonate (PerkinElmer, Bad Neuheim Germany) mixture (final concentration: 50 mM, 5 kBq), and carried out at 37°C during 0, 8 or 16 minutes. The reaction was stopped by introducing 200 µL of the reaction mixture into tubes, kept on ice, containing 30 µL of 2 M hydroxylamine-HCl, and rapidly put at 95°C until total evaporation. The whole tube contents were counted.

Ornithine carbamoyl transferase activity was measured from the reaction of condensation of carbamoyl-P and ornithine to yield citrulline (adapted from [17]). Aliquots (40 µL) of homogenates were mixed with urease S (Boehringer Mannheim, Mannheim Germany) and a reaction buffer containing hepes, KCl, $MgCl_2$ and ornithine (all from Sigma-Aldrich); final concentrations were 100 µkat/L, 50 mM, 33 mM, 4.5 mM, and 10 mM. The reaction was started by adding 15 µL of lithium carbamoyl-P (Sigma-Aldrich) (final concentration 6.7 mM) and carried out at 37°C during 0, 5 or 10 minutes. The reaction was stopped by introducing 200 µL of the reaction mixture into tubes, kept on ice, containing 600 µL of reaction buffer: diacetyl monoxime (59.3 mM), antipyrine (7.2 mM) and $FeCl_3$ (0.3 mM) diluted with 3.75% acetic acid (v/v) and 30% H_2SO_4, prepared as previously described [17] (all products were from Sigma-Aldrich). The colorimetric reaction was developed at 100°C for 30 minutes in a boiling water bath. Absorbances (including standards and blanks) were measured at 450 nm using a plate reader spectrophotometer (ELx808 Ultra Microplate Reader, Biotek, Winooski, VT USA).

The methods used for arginino-succinate synthase and lyase, and arginase enzyme activities have been previously described in detail [18]. Lactate dehydrogenase enzyme activity was determined by a modified UV method [19], as previously described [20]. 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 [21]. The ammonium evolved was estimated with the classical Berthelot indophenol reaction [22], in which indophenol was formed by reaction of

ammonium with phenol in the presence of an oxidative agent (hypochlorite) and nitroprusside as catalyzer.

Gene expression analysis

Total tissue RNA was extracted from the frozen tissue samples using the Tripure reagent (Roche Applied Science, Indianapolis IN USA), 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.

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), 4 ng of reverse-transcribed RNA and 150 nM of primers. 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 [23]. Rplp0 was the charge control gene [24]. The genes analyzed, and a list of primers used, are presented in Table 1.

Statistics

t-Student comparisons between groups, correlations and curve fitting (including Vi estimations) were done with the Prism 5 program (GraphPad Software, San Diego CA USA).

RESULTS

The rats weighed 446 ± 30 g. Protein concentration values for iBAT and scWAT were respectively 67.3 ± 10.6 and 20.9 ± 0.3 mg of protein/g of tissue.

Fig. 1 shows the expression of urea cycle genes and the Vmax enzyme activities for the same enzymes, measured in both scWAT and iBAT; all data were expressed per gram of protein, for direct comparison between sites. Since no significant carbamoyl-P 1 expression was detected in neither scWAT nor iBAT, we can assume that carbamoyl-P synthase activity correspond only to that of the type 2, cytosolic enzyme.

Expression profile of urea cycle enzymes showed significantly smaller values per gram of protein in brown adipose tissue. iBAT showed an extremely low expression of ornithine carbamoyl transferase, arginino-succinate synthase and arginase, compared to the higher expression of the carbamoyl-P synthase and arginino-succinate lyase in this tissue. These last two expressions showed specially high levels in scWAT. Since BAT presents a greater percentage of active tissue, less fat reserves, smaller cells and, consequently, a greater percentage of proteins, when expression data are corrected per gram of tissue, similar expression values were found when comparing both tissues (although with slightly higher values for arginino-succinate synthase and arginase in WAT; data not shown).

When enzyme activities are considered, the main difference between tissues was the lack of detectable enzyme activities for ornithine carbamoyl transferase and arginase in the iBAT, in accordance with the very low expression values found in the tissue. This was not the case of scWAT, were specially ornithine carbamoyl transferase, but also arginase showed the greatest enzymatic activities in reference to the other enzymes of the cycle. When corrected per gram of tissue, iBAT arginino-succinate synthase and lyase activities were significantly higher than those of scWAT (39.4 ± 16.9 *vs.* 2.78 ± 0.51; and 19.8 ± 7.2 *vs.* 5.88 ± 0.98, respectively). Additionally, the presence of measurable enzyme activities for all the enzymes of urea cycle in scWAT, backed by the expression of the corresponding genes, confirms the presence of a complete and functional urea cycle in scWAT.

In order to compare the potential importance of urea cycle enzymes with other well-established enzymes of intermediate metabolism in adipose tissues, Table 2 shows the gene expression and enzyme activity of AMP deaminase, a regulatory enzyme of amino acid metabolism, and lactate dehydrogenase, the responsible of pyruvate conversion to lactate in glycolysis. The expression of endothelial NO synthase is also shown, there being no significant differences between tissues. The activity and expression of AMP deaminase showed values similar to those of the most active enzymes of the urea cycle, whereas in the case of lactate dehydrogenase, the values were an order of magnitude higher. It is worth noting the greater efficiency of brown adipose tissue when comparing the activity / expression ratio of these enzymes in both tissues, since similar expression values end up giving higher activities in BAT.

DISCUSSION

WAT is characterized by the existence of a complete and functional urea cycle, with a significant activity of all of the enzymes of the cycle [7,8]. This activity is common to all the WAT sites studied, although there were marked specific differences related to location [7]. In contrast, brown adipose tissue is characterized by the presence of significant expressions and activities of argininosuccinate lyase and synthase, but no significant expressions and activities of arginase and ornithine transcarbamylase. Therefore, BAT, differently to WAT, does not present a complete and functional urea cycle.

Despite the limited expressions and the absent activities of ornithine transcarbamylase and arginase in BAT, the expression profile of the different enzymes of the cycle is quite similar between both tissues. On the contrary, there are very important differences in the profile of enzymatic activities; while in WAT the greatest activities are those found for ornithine transcarbamylase and arginase, which were absent in BAT, in the latter tissue the greatest enzyme activities are those of argininosuccinate synthetase and lyase, suggesting a very different role of the cycle enzymes in both tissues. The discordance between similar gene expressions and different enzyme activities in WAT and BAT agrees with a possible post-transcriptional modulation of cycle enzymes and is a clear indication of powerful and important additional mechanisms of regulation, as is their turnover rates, which establish the real active life of the enzyme molecules.

Additionally, although, in general, BAT is metabolically more active than WAT, as evidenced by the greater activity of lactate dehydrogenase and AMP deaminase, gene expressions of urea cycle enzymes are lower, suggesting a lower metabolic capacity of nitrogen disposal in BAT. These minor expressions also agree with the slightly (although no statistically significant) lower expression of the endothelial NO synthase.

A possible cause of such different profiles may be related to the different origin of both tissues. Although all adipose tissues arise from a mesodermal origin, white and brown adipocytes are now believed to originate from different mesenchymal stem cell lineages [3]. Brown adipocytes are more closely related to skeletal muscle precursor cells than to white adipocyte precursors [3,25,26]. In fact, brown adipocytes and myocytes share a common precursor that expresses the myogenic lineage marker Myf5, in contrast with white adipocytes that come from Myf5-negative cells [3,25].

BAT seems to be a net glutamine user and alanine releaser [12], stressing the similarities with intestine, that takes up glutamine, releases alanine and produces free NH_4^+ [13,14]. However, enterocytes, similarly to WAT, are capable to synthetize urea from ammonia, glutamine and arginine [27]. A high ornithine transcarbamylase activity in enterocyte mitochondria favors the intestinal synthesis of citrulline, whereas the low activities of cytosolic argininosuccinate lyase and synthase minimize the conversion of citrulline into arginine and therefore, its recycling [28].

Urea cycle BAT metabolic profile is totally different from that of WAT and intestine. Contrary to these tissues, BAT seems to be a net user of citrulline, being its main destination the synthesis of arginine, an amino acid with multiple possible metabolic fates. Not only it is metabolically interconvertible with the amino acids proline and glutamate, but it also serves as a precursor for the synthesis of protein, nitric oxide, creatine, polyamines, agmatine, and urea [29]. However, most of these activities (synthesis of proline, glutamate, polyamines and urea) require the action of arginase, which is not present in brown adipose tissue. Therefore, part of the arginine synthesized in BAT may be exported to other tissues.

Arginine can be oxidized by the tissue itself through the action of the nitric oxide synthase (NOS) family of enzymes [30,31]. Nitric oxide has emerged as a critical regulator of cell and tissue function [30]. It has multiple biological actions [32], being blood flow regulation the most well-known function. eNOS-derived nitric oxide increases the abundance of mitochondria and stimulates substrate oxidation capacity in adipose tissue, promoting “browning” of white adipocytes. Although nitric oxide bioavailability is diminished in obese and diabetic states [32], NOS2-derived nitric oxide can promote insulin resistance and inflammation in key peripheral tissues such as liver, skeletal muscle, and white and brown adipose tissues. Nitric oxide also promotes thermogenesis in BAT.

Although expression levels of endothelial nitric oxide synthase in white and brown adipose tissue are similar, we must bear in mind that there are other nitric oxide synthases in the tissue, and that the lack of arginase in BAT but not in WAT probably allows a higher production

of nitric oxide in brown adipose tissue. This has been confirmed experimentally in other models, since the inhibition of arginase activity results in increased nitric oxide production [33,34].

In conclusion, although brown adipose tissue presents part of the enzymes of the urea cycle, it does not present a complete and functional urea cycle, unlike white adipose tissue. Consequently, brown adipose tissue cannot contribute to nitrogen disposal by producing urea; the main function of the incomplete urea cycle in the tissue seems to be related to arginine production.

Table 1. Enzymes and their corresponding primer sequences used in the analysis of scWAT and iBAT genes of adult male Wistar rats

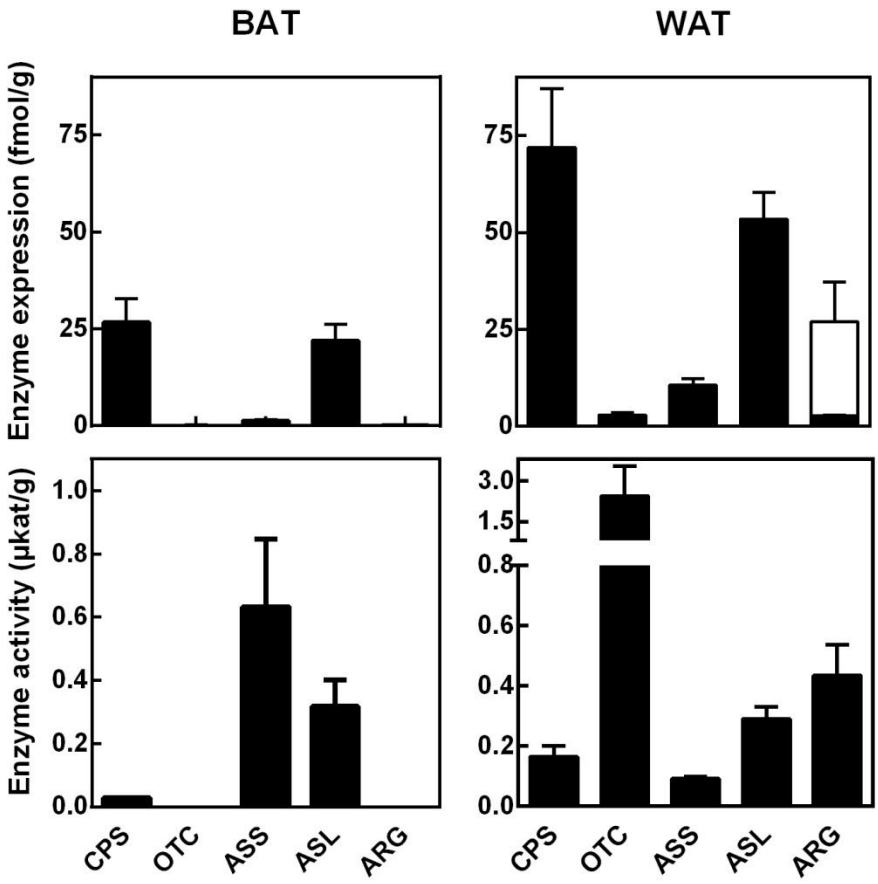
Enzyme	EC number	Gene	5' > 3'	BP
Carbamoyl-phosphate synthase [ammonia], mitochondrial type 1	6.3.4.16	*Cps1*	ACCCATCATCCCCTCTGACT	118
Glutamine-dependent carbamoyl-phosphate synthase, type 2	6.3.5.5	*Cad*	AGTTGGAGGAGGAGGCTGAG	90
Ornithine carbamoyltransferase	2.1.3.3	*Otc*	CTTGGGCGTGAATGAAAGTC	126
Arginino-succinate synthase 1	6.3.4.5	*Ass1*	CAAAGATGGCACTACCCACA	100
Arginino-succinate lyase	4.3.2.1	*Asl*	CCGACCTTGCCTACTACCTG	104
Arginase, liver (type 1)	3.5.3.1	*Arg1*	GCAGAGACCCAGAAGAATGG	126
Arginase-2	3.5.3.1	*Arg2*	GCAGCCTCTTTCCTTTCTCA	122
Adenosine monophosphate deaminase 2	3.5.4.6	*Ampd2*	CGGCTTCTCTCACAAGGTG	78
L-Lactate dehydrogenase B chain (heart type)	1.1.1.27	*Ldhb*	CCAGGAACTGAACCCAGAGA	131
Nitric oxide synthase, endothelial	1.14.13.39	*eNos*	CAAGTCCTCACCGCCTTTT	138
60S acidic ribosomal protein 0 (housekeeping gene)	--	*Rplp0*	GAGCCAGCGAAGCCACACT	62

Table 2. Gene expression and enzyme activity of AMP deaminase and lactate dehydrogenase in scWAT and iBAT sites of adult male Wistar rats

	iBAT	scWAT
Gene expression (fmol/g)		
AMPD2	59.5 ± 13.5	61.8 ± 15.8
LDHb	846 ± 152	842 ± 188
eNOs	22.9 ± 4.3	63.3 ± 19.8
Enzyme activity (µkat/g)		
AMPD	1.01 ± 0.13	0.44 ± 0.16 *
LDH	63.8 ± 13.6	3.11 ± 0.55 *

The data are the mean ± sem of 6 animals per group. Gene expression and enzyme activity values are expressed in fmol and µkat per g of protein respectively. * indicates statistically significant differences between iBAT and scWAT values.

Figure 1. Gene expression and enzyme activity of urea cycle enzymes in scWAT and iBAT sites of adult male Wistar rats.



The data are the mean ± sem of 6 animals per group. Gene expression and enzyme activity values are expressed in fmol and µkat per g of protein respectively. CPS: Carbamoyl-P synthase; OTC: Ornithine carbamoyl transferase; ASS: Arginino-succinate synthase; ASL: Arginino-succinate lyase; ARG: Arginase. The expression data for arginase are the sum of Arg1 (in black) and Arg2 (in white) for subcutaneous WAT. In all cases, there were statistically significant differences between iBAT and scWAT values, except for ASL activity.

REFERENCES

1. Rutkowski JM, Stern JH, Scherer PE. The cell biology of fat expansion. *J Cell Biol* **208**, 501-512 (2015). doi: 10.1083/jcb.201409063.
2. Scherer PE. Adipose tissue: from lipid storage compartment to endocrine organ . *Diabetes* **55**, 1537-1545 (2006). doi: 10.2337/db06-0263.
3. Giralt M, Villarroya F. White, brown, beige/brite: different adipose cells for different functions? *Endocrinology* **154**, 2992–3000 (2013). doi: 10.1210/en.2013-1403.
4. Cedikova M, Kripnerová M, Dvorakova J, Pitule P, Grundmanova M, Babuska V, Mullerova D, Kuncova J. Mitochondria in white, brown, and beige adipocytes. *Stem Cells Int* (2016). doi:10.1155/2016/6067349.
5. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* **84**, 277-359 (2004). doi:10.1152/physrev.00015.2003.
6. Wu J, Boström P, Sparks LM, Ye L, Choi JH, Giang AH, Khandekar M, Virtanen KA, Nuutila P, Schaart G, Huang K, Tu H, van Marken Lichtenbelt WD, Hoeks J, Enerbäck S, Schrauwen P, Spiegelman BM. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* **150**, 366–376 (2012). doi: 10.1016/j.cell.2012.05.016.
7. Arriarán S, Agnelli S, Remesar X, Fernández-López JA, Alemany M. The urea cycle of rat white adipose tissue. *RSC Adv* **5**, 93403-93414 (2015). doi: 10.1039/c5ra16398f.
8. Arriarán S, Agnelli S, Remesar X, Alemany M, Fernández-López JA. 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). DOI: 10.1039/c5fo01503k.
9. Nicholls DG. Brown adipose tissue mitochondria. *Biochim Biophys Acta - Rev Bioenerg* **549**, 1–29 (1979).
10. Himms-Hagen J. Neural control of brown adipose tissue thermogenesis, hypertrophy, and atrophy. *Front Neuroendocrinol* **12**, 38-93 (1991).
11. Ferré P, Burnol AF, Leturque A, Terretaz J, Penicaud L, Jeanrenaud B, Girard J. Glucose utilization in vivo and insulin-sensitivity of rat brown adipose tissue in various physiological and pathological conditions. *Biochem J* **233**, 249-252 (1986). doi:10.1042/bj2330249.
12. López-Soriano FJ, Fernández-López JA, Mampel T, Villarroya F, Iglesias R, Alemany M. Amino acid and glucose uptake by rat brown adipose tissue. Effect of cold-exposure and acclimation. *Biochem J* **252**, 843-849 (1988).
13. Brosnan JT, Man KC, Hall DE, Colbourne SA, Brosnan ME. Interorgan metabolism of amino acids in streptozotocin-diabetic ketoacidotic rat. *Am J Physiol Metab* **244**, E151–E158 (1983). doi: 10.1152/ajpendo.1983.244.2.E151.
14. Windmueller HG, Spaeth AE. Uptake and metabolism of plasma glutamine by the small intestine. *J Biol Chem* **249**, 5070-5079 (1974). doi:10.1074/jbc.M601576200.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin-Phenol Reagent. *J Biol Chem* **193**, 265–275 (1951).

16. Arriarán S, Agnelli S, Fernández-López JA,Remesar X, Alemany M. A radiochemical method for carbamoyl-phosphate synthetase-I: Application to rats fed a hyperproteic diet. *J Enzym Res* **3**, 29-33 (2012).

17. Sugawara K, Yoshizawa Y, Tzeng S, Epstein WL, Fukuyama K. Colorimetric determination of citrulline residues in proteins. *Anal Biochem* **265**, 92–96 (1998). doi: 10.1006/abio.1998.2925.

18. Arriarán S, Agnelli S, Remesar X, Fernández-López JA, Alemany M. Effects of sex and site on amino acid metabolism enzyme gene expression and activity in rat white adipose tissue. *PeerJ* **3**, e1399 (2015). doi: 10.7717/peerj.1399.

19. Bergmeyer HU, Bernt E. UV-Assay with pyruvate and NADH. In *Methods of Enzymatic Analysis* (1974). doi:10.1016/B978-0-12-091302-2.50010-4.

20. Arriarán S, Agnelli S, Sabater D, Remesar X, Fernández-López JA, Alemany M . 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). doi: 10.1371/journal.pone.0119572.

21. Ogasawara N, Goto H, Watanabe T, Kawamura Y, Yoshino M. Multiple forms of AMP deaminase in various rat tissues. *FEBS Lett* **44**, 63–66 (1974).

22. Fawcett JK, Scott JE. A rapid and precise method for the determination of urea. *J Clin Pathol* **13**, 156–159 (1960).

23. Romero MM, Grasa MM, Esteve M, Fernández-López JA, Alemany M. Semiquantitative RT-PCR measurement of gene expression in rat tissues including a correction for varying cell size and number. *Nutr Metab* **4**, 26 (2007). doi: 10.1186/1743-7075-4-26.

24. Ragni E, Viganò M, Rebulla P, Giordano R, Lazzari L. What is beyond a qRT-PCR study on mesenchymal stem cell differentiation properties: how to choose the most reliable housekeeping genes. *J Cell Mol Med* **17**, 168–180 (2013). doi: 10.1111/j.1582-4934.2012.01660.x.

25. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scimè A, Devarakonda S, Conroe HM, Erdjument-Bromage H, Tempst P, Rudnicki MA, Beier DR, Spiegelman BM. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* **454**, 961-967 (2008). doi: 10.1038/nature07182.

26. Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, Hamilton DL, Gimeno RE, Wahlestedt C, Baar K, Nedergaard J, Cannon B. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. *Proc Natl Acad Sci* **104**, 4401-4406 (2007). doi: 10.1073/pnas.0610615104.

27. Wu G. Urea synthesis in enterocytes of developing pigs. *Biochem J* **312**, 717–723 (1995).

28. Davis PK, Wu G. Compartmentation and kinetics of urea cycle enzymes in porcine enterocytes. *Comp Biochem Physiol - B Biochem Mol Biol* **119**, 527-537 (1998). doi:10.1016/S0305-0491(98)00014-5.

29. Morris SM, Jr. Arginine metabolism: boundaries of our knowledge. *J Nutr* **137**, 1602S-1609S (2007). doi: 10.1093/jn/137.6.1602S.

30. Hill BG, Dranka BP, Bailey SM, Lancaster JR Jr, Darley-Usmar VM. What part of NO don't you understand? Some answers to the cardinal questions in nitric oxide biology. *J Biol Chem* **285**, 19699-19704 (2010). doi:10.1074/jbc.R110.101618.

31. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J* **357**, 593-615 (2001). doi:10.1042/0264-6021:3570593.

32. Sansbury BE, Hill BG. Regulation of obesity and insulin resistance by nitric oxide. *Free Radic Biol Med* **73**, 383-399 (2014). doi:10.1016/j.freeradbiomed.2014.05.016.

33. Johnson FK, Johnson RA, Peyton KJ, Durante W. Arginase inhibition restores arteriolar endothelial function in Dahl rats with salt-induced hypertension. *Am J Physiol Regul Integr Comp Physiol* **288**, R1057-R1062 (2004). doi:10.1152/ajpregu.00758.2004.

34. Tenu JP, Lepoivre M, Moali C, Brollo M, Mansuy D, Boucher JL . Effects of the new arginase inhibitor N(ω)-hydroxy-nor-L-arginine on NO synthase activity in murine macrophages. *Nitric Oxide* **3**, 423-438 (1999). doi:10.1006/niox.1999.0255.

Discusión

1. GLUCEMIA Y GLUCOSILACIÓN NO ENZIMÁTICA

La medida de la glucemia es un parámetro bioquímico de gran importancia para el diagnóstico y el manejo de condiciones fisiopatológicas como la diabetes de tipo 2, una complicación frecuente en la obesidad y en el síndrome metabólico. Por esta razón y tras haber tropezado con algunas inconsistencias en la reproducibilidad de la obtención de datos durante la primera fase experimental, parte de esta tesis se ha enfocado en el análisis de la metodología utilizada para la cuantificación de la glucosa y su reactividad no enzimática, aspectos ampliamente utilizados en el ámbito clínico y de investigación.

La naturaleza química de la glucosa implica que en el plasma y los tejidos existan dos formas anoméricas, hecho que puede conllevar algunas diferencias a nivel de reactividad y de uso metabólico de la glucosa por parte de los tejidos. Aunque las proporciones anoméricas α/β de la glucosa tienden a estar en equilibrio, en las muestras biológicas la forma β tiende a encontrarse en cantidades ligeramente superiores a la forma α (63% *vs*. 37%) [355], hecho que *a priori* sugiere una mayor utilización del anómero α. Varios estudios mostraron diferencias en las preferencias por parte de los tejidos para el transporte y la metabolización de un anómero u otro, resaltando la posible existencia de un transporte de glucosa específico y dependiente del tipo de transportador GLUT que expresa el tejido [356]. No obstante, pocas investigaciones han centrado sus esfuerzos y recursos en el estudio de la especificidad anomérica en el transporte facilitado de la glucosa, probablemente debido a la presencia generalizada de la enzima mutarrotasa (EC.5.1.3.3) en plasma y en algunos tejidos o células como los eritrocitos [357]; la acción de la mutarrotasa facilita y acelera la interconversión espontánea entre el anómero α y β [358], poniendo la glucosa en condiciones para su uso metabólico [359].

En los estudios realizados y presentados como parte de esta tesis, y de acuerdo con la evidencia disponible desde la década de los 70 (aunque frecuentemente ignorada) [360, 361], se ha podido corroborar que la adición de mutarrotasa en el medio de incubación, al utilizar el método enzimático de la glucosa oxidasa / peroxidasa, acelera la interconversión anomérica de la glucosa y mantiene su equilibrio a medida que el sustrato de la reacción (β-D-glucosa) se va consumiendo. Así pues, la presencia de mutarrotasa permite la oxidación de la totalidad de la glucosa presente en las muestras de plasma y de los patrones de glucosa pura en un periodo de tiempo corto. Por ello, y como consecuencia de la oxidación completa de la glucosa, los valores de glucemia obtenidos en este estudio fueron significativamente superiores a los considerados fisiológicos (establecidos alrededor de 5-6 mM, en ratas). Parte de este incremento podría justificarse por el efecto hiperglucemiante del isoflurano, utilizado como agente anestésico [342]; no obstante, el análisis de muestras obtenidas previamente a la administración de anestesia, utilizando mutarrotasa en la valoración, también mostraron valores claramente superiores (alrededor de 7-9 mM) a los considerados fisiológicos. Todo ello sugiere la existencia de una posible infraestimación sostenida y sistemática de los valores reales de glucemia.

La dieta de cafetería es una dieta ampliamente utilizada por la comunidad científica; se caracteriza por inducir hiperfagia, y como consecuencia, obesidad e hiperglucemia crónica [170] [237, 239]. El análisis de la glucemia en ausencia de mutarrotasa mostró los resultados esperados; glucemias dentro del rango fisiológico para los animales alimentados con dieta estándar e hiperglucemias para los alimentados con dieta de cafetería. Sin embargo, al realizar la valoración en presencia de mutarrotasa, asegurando la oxidación completa de la glucosa, los valores de glucemia obtenidos fueron similares para ambos grupos experimentales, encontrándose ambos, sorprendentemente, dentro del rango considerado de hiperglucemia. Dicha situación no solo indica que en ausencia de mutarrotasa parte de la α-glucosa permanezca sin oxidar sino que además sugiere, al no mantenerse las diferencias entre los dos grupos de intervención, que la proporción anomérica inicial difiere de manera significativa entre ambos grupos, apuntando a una posible alteración del equilibrio anomérico dependiente de la dieta que, en consecuencia, podría interferir en la correcta medición de la glucemia. Asimismo, un desequilibrio en las proporciones anoméricas añadiría un factor de variabilidad desconocida al extender la problemática a las soluciones estándar y a los patrones de glucosa pura. Dichos hallazgos nos sitúan en una situación un tanto preocupante, pues todo ello en conjunto podría estar comprometiendo los criterios de diagnóstico establecidos en la actualidad.

Las situaciones fisiopatológicas como el síndrome metabólico, la diabetes, la obesidad, y en general cualquier condición en la que hay una hiperglucemia sostenida en el tiempo conllevan, también, un incremento de la glucosilación no enzimática de las proteínas del plasma [362] [323]. Por ello, aunque se ha propuesto el grado de glucosilación de la albúmina plasmática como indicador retrospectivo de la exposición prolongada a elevadas concentraciones de glucosa a lo largo del tiempo [362], el indicador más utilizado y establecido para ello es la glucosilación de los grupos amino terminal de la hemoglobina glucosilada (Hb_{1AC}). La determinación de la Hb_{1AC} es actualmente uno de los parámetros estándar para el control y la evaluación retrospectiva de la diabetes y la hiperglucemia sostenida (frecuentemente asociada a la obesidad y al síndrome metabólico) [330]. Sin embargo, la hemoglobina se encuentra en el interior de los eritrocitos, por lo que no está en contacto directo con la glucosa plasmática.

La glucosa entra en el eritrocito a través del transportador de glucosa GLUT1 [363], siendo principalmente metabolizada a lactato en el proceso de glucolisis anaeróbica, que constituye la única fuente de obtención de energía en forma de ATP por el eritrocito. La expresión de GLUT1 está modulada por la concentración de glucosa circulante, la insulina y los glucocorticoides, entre otros factores [364].

Dada su rápida utilización, y de acuerdo con los datos obtenidos, la concentración de glucosa que permanece en el interior del eritrocito es baja, lo que implica una baja exposición y baja capacidad para glucosilar la hemoglobina. Además, se da la paradoja que, ante el aporte excesivo y continuado de nutrientes que ocurre en los animales sujetos a la dieta de cafetería, la concentración intracelular de glucosa en los eritrocitos es significativamente inferior

respecto al grupo alimentado con la dieta estándar, mientras que la glucosa en plasma y en la fracción acuosa de la sangre son similares. Dichas observaciones, ponen en evidencia la existencia de un proceso de compartimentación de la glucosa sanguínea que puede constituir un potencial mecanismo de regulación del transporte/transferencia de glucosa entre el conjunto plasma/sangre y los tejidos.

En cuanto al grado de glucosilación de la hemoglobina y de las proteínas plasmáticas, los datos obtenidos muestran una ausencia de cambios significativos provocados por la dieta, que aunque estaría de acuerdo con los valores similares de glucemia descritos entre los animales alimentados con la dieta estándar y la de cafetería, contrasta con la mayor cantidad de proteínas glucosiladas descritas en el plasma de humanos y roedores con hiperglucemia o patología asociada al síndrome metabólico [365]. Además, aunque un incremento en el recambio celular inducido por la condición fisiopatológica [366] sostendría la ausencia de correlación, posiblemente esta esté limitada por el modelo utilizado, con una intervención dietética relativamente corta, que situaría el desarrollo del síndrome metabólico en sus estadios iniciales, donde los marcadores patológicos no están completamente establecidos.[367] Por otro lado, la presencia de un grado de glucosilación elevado en las membranas celulares de los eritrocitos muestra que los pequeños cambios generados en la glucemia basal, en este modelo de estudio, son suficientes para inducir cambios en la actividad glucosilante de la glucosa plasmática.

La reactividad de la glucosa con las proteínas (glucosilación no enzimática) se da únicamente cuando la glucosa se encuentra en forma de cadena abierta. Aunque la forma abierta de la glucosa plasmática se encuentra en cantidades insignificantes en solución [355], está presente al producirse la interconversión entre anómeros para restablecer el equilibrio α/β. Al mismo tiempo, la presencia y la acción de la mutarrotasa facilita y acelera la conversión y, con ello, la disponibilidad de la forma reactiva de la glucosa. De hecho, la existencia del equilibrio anomérico podría tener un significado biológico pues el sistema tendría la ventaja de limitar la duración de los periodos en los que la glucosa tiene reactividad máxima y puede causar daños en la estructura y funcionalidad de las proteínas.

2. VARIACIONES EN LA COMPOSICIÓN DE MACRONUTRIENTES DE LA DIETA

2.1. Efectos sobre la ingesta, el peso corporal y el metabolismo lipídico

El uso de dietas con un contenido energético elevado está ampliamente extendido en el ámbito científico con el objetivo de generar modelos animales que permitan estudiar la obesidad, el síndrome metabólico y otras patologías metabólicas. En muchos casos, el incremento energético de la dieta corresponde a un incremento en el contenido de lípidos, aunque es habitual (comúnmente en las dietas formuladas por las casas comerciales) que dicho incremento se acompañe de una proporción elevada de mono y disacáridos, principalmente sacarosa. El contenido elevado de carbohidratos simples suele desencadenar efectos similares a los de las dietas esencialmente hiperlipídicas, por lo que el análisis y la comparativa de los efectos inducidos en diferentes estudios pueden verse obstaculizados [171, 173]. Sin embargo, en la gran mayoría de ocasiones, el agravante es la falta de atención prestada a la composición específica de la dieta utilizada para generar un modelo específico, hecho que genera una gran variabilidad en la composición de las dietas y, en consecuencia, mayor dificultad para comparar los datos obtenidos. Por ello, y con el objetivo de facilitar la comparación de los efectos generados por las distintas dietas estudiadas en esta tesis, se ha optado por hacer la puesta a punto de una dieta que implicase una variación mínima de la dieta estándar, en este caso, utilizándola como factor común y suplementándola con un compuesto lipídico concreto y teniendo en cuenta las proporciones de cada uno de los macrocomponentes. De este modo, se ha desarrollado una dieta hiperlipídica a partir de pienso estándar enriquecido con aceite de coco (rico en ácidos grasos saturados), en la que se ha ajustado el porcentaje energético procedente de lípidos para que sea equivalente al de la dieta de cafetería (alrededor del 40%).

La dieta de cafetería es una dieta hiperenergética de referencia que permite inducir un modelo de obesidad y síndrome metabólico en rata [176, 237, 239]. Una dieta rica en ácidos grasos saturados, como en el caso del aceite de coco, tiene efectos obesogénicos, hiperglucemiantes, proinflamatorios [173, 192 368] y modula a la baja la inducción de la termogénesis [369]. Sin embargo, y a pesar de ajustar el contenido lipídico entre ambas dietas, los efectos exhibidos sobre el peso corporal son muy diferentes; los animales alimentados con la dieta estándar enriquecida únicamente con lípidos muestran un patrón de incremento de peso similar al grupo estándar, de acuerdo con lo descrito en otros estudios [172, 370]. Además, el perfil de incremento de peso exhibido por las distintas dietas muestra diferencias notables entre sexos; las diferencias entre los grupos de hembras son menos marcadas con respecto a las variaciones mostradas en los machos, siendo los machos más eficientes energéticamente y las hembras más eficientes termogénicamente [234].

En el caso de la dieta de cafetería, los efectos obesogénicos están bien establecidos, y se deben principalmente a un incremento de la acumulación de grasa en el tejido adiposo, aunque dicho incremento también se da de forma generalizada en otros tejidos [97]. Conforme

con ello, todos los animales sujetos a la dieta de cafetería mostraron un incremento de grasa corporal significativamente superior a aquellos que fueron alimentados con la dieta estándar o la hiperlipídica; las diferencias entre dietas fueron consecuencia esencialmente de la cantidad de energía ingerida. Aunque la dieta hiperlipídica tiene un contenido de energía digerible ligeramente mayor que las otras dietas, este no parece ser un factor influyente en la ingesta energética diaria, dado que ambos grupos de animales alimentados con la dieta estándar y la dieta hiperlipídica exhiben una ingesta energética equivalente. Por otro lado, dado que la ingesta energética depende de la densidad energética y es independiente del contenido de fibra [371], las diferencias observadas sobre dicha ingesta en los grupos de dieta hiperlipídica y de dieta de cafetería no son atribuibles a las diferencias en el contenido de fibra. Asimismo, el contenido y la calidad de la proteína de las tres dietas son similares, hecho que descarta un efecto saciante de las proteínas que pueda influir negativamente en la ingesta [82].

Las diferencias mostradas en la ingesta energética entre grupos se atribuyen principalmente a una alteración en el control de la ingesta y, en consecuencia, a la inducción de hiperfagia, que es característica de la dieta de cafetería debido a que la presencia de ingredientes sabrosos anula los mecanismos de control de la ingesta [372] y disminuye el umbral de saciedad [231]. Además, la presencia de ingredientes y sabores variados induce la ingesta (tanto en ratas como en humanos) propiciando la estabilización de patrones de alimentación emocional como mecanismo para combatir situaciones de estrés o, simplemente como acto placentero [167, 168].

La estimación del gasto energético presenta valores similares entre los grupos alimentados con dieta hiperlipídica y con dieta estándar, mientras que para el grupo alimentado con dieta de cafetería estos son significativamente más elevados, principalmente en hembras. En relación a la acumulación de grasa corporal, el grupo alimentado con la dieta hiperlipídica, a pesar de tener una ingesta de lípidos elevada, muestra una acumulación similar al grupo de dieta estándar, sugiriendo que en estos animales hay una elevada tasa de oxidación de lípidos que compensaría la disminución en la utilización de carbohidratos. La textura que aportan los lípidos a la dieta es atractiva para ratas y humanos [373, 374]; a pesar de ello, los patrones de ingesta de los animales sujetos a la dieta estándar y a la dieta enriquecida con lípidos fueron similares, revelando que las propiedades organolépticas de los lípidos no fueron un factor crítico para la inducción de hiperfagia. El análisis de los componentes nutricionales de ambas dietas hiperlipídicas (estándar enriquecida y dieta de cafetería) apunta a que las diferencias observadas en la ingesta y la cantidad de energía ingerida son atribuibles principalmente a la presencia de grandes cantidades de ingredientes sabrosos (azúcar y sal). El sabor dulce de la sacarosa genera sensaciones placenteras [87] que estimulan su consumo; un incremento en la ingesta de sacarosa puede contribuir a la acumulación de lípidos y al desarrollo de obesidad [375], por lo que se asume que en la dieta de cafetería el sabor dulce complementa a la textura grasa para inducir el exceso de ingesta.

Por otro lado, la sal es un claro contribuyente al incremento del sabor de los alimentos y, en consecuencia, de la inducción del apetito mediante los circuitos de recompensa que, como

cuando se ingiere azúcar, su respuesta está mediada por la acción de los opioides endógenos [376]. En humanos, el contraste dulce/salado dado por la amplia variedad y disponibilidad de alimentos, es uno de los factores que incita a la ingesta [377]. En la dieta humana actual es prácticamente inevitable el consumo de una cantidad mínima de sal. Inicialmente la ingesta de sal no está considerada como un factor inductor de hiperfagia, aunque en la dieta de cafetería parece tener un papel relevante, probablemente a través de un incremento de la secreción de corticoesteroides [378], que podrían comportar cambios metabólicos favorecedores de la acumulación de lípidos y del desarrollo del síndrome metabólico [379].

Las dietas con elevado contenido lipídico favorecen la acumulación ectópica de lípidos en el hígado [380], proceso que está mediado por la señalización de PPARα [381] y que está de acuerdo con lo observado en los machos alimentados con ambas dietas hiperlipídicas (enriquecida y cafetería). Sin embargo, la presencia de triacilgliceroles en el hígado de los machos alimentados con la dieta hiperlipídica es inferior y, a la vez, se corresponde con una mayor expresión hepática de la carnitina palmitoiltransferasa 1 que contribuye a una mayor oxidación de lípidos. Todo ello sugiere que la composición de ácidos grasos en la dieta podría tener un papel modulador de la capacidad para acumular triacilgliceroles en el hígado. En este sentido, la dieta estándar enriquecida con ácidos grasos saturados parece contribuir a la oxidación de lípidos mientras que en la dieta cafetería se propiciaría su almacenamiento, aunque en ambos casos habría una menor capacidad para la síntesis de ácidos grasos, probablemente como tentativa para evitar una acumulación excesiva.

Puesto que la cantidad de lípidos ingeridos correlaciona positivamente con la acumulación hepática de triacilgliceroles solo en los machos, parece que el contexto hormonal (estradiol) podría estar influyendo el proceso [382]. No obstante, la acumulación de triacilgliceroles en hígado no parece estar únicamente orquestada por el estradiol, ya que los machos alimentados con la dieta de cafetería son los que muestran una mayor acumulación de triacilgliceroles en hígado y, a la vez, concentraciones de estradiol circulante similares a los machos alimentados con dieta estándar. La relación con el estradiol es más clara en el caso del contenido hepático de colesterol, de tal forma que el estradiol parece desempeñar un papel protector; existe una correlación inversa, siendo los animales alimentados con dieta hiperlipídica los que presentan mayores niveles de estradiol plasmático y a la vez presentan menores concentraciones hepáticas de colesterol. El incremento de estradiol que se produce con la dieta hiperlipídica, podría deberse a la presencia elevada de ácido láurico en la dieta [383], el cual puede alcanzar fácilmente los tejidos periféricos [384] y favorecer la actividad aromatasa [385] propiciando y estimulando la síntesis de estradiol.

Las diferencias en la composición lipídica y de macronutrientes de las dietas estudiadas también influyen en la concentración de los metabolitos plasmáticos. En los animales alimentados con la dieta hiperlipídica, la concentración de lactato circulante es significativamente elevada, aunque dicho incremento no se corresponde con el patrón de expresión y actividad de la lactato deshidrogenasa hepática, sugiriendo la contribución de

otros órganos y tejidos. Por un lado el tejido adiposo blanco tendría dicha capacidad [386], aunque parece poco probable al no presentar un crecimiento de su masa ni tener la disponibilidad de glucosa incrementada. En este caso, el contribuyente principal parece ser el músculo, pues tanto la actividad como la expresión de la lactato deshidrogenasa muscular correlacionan con los valores plasmáticos de lactato y, adicionalmente, con los de estradiol. De nuevo, el estradiol parece tener un papel importante en la regulación de los niveles circulantes de lactato, en un proceso mediado por PGC 1-α [387].

2.2. Metabolismo nitrogenado en el hígado y en los tejidos adiposos

De acuerdo con lo discutido hasta el momento, la alteración en las proporciones de los macrocomponentes de la dieta tiene un impacto significativo en el funcionamiento de los procesos fisiológicos, como consecuencia de la tentativa del organismo por mantener el equilibrio homeostático [380]. En la actualidad el uso de dietas hiperproteicas es una de las opciones más empleadas para el control del peso corporal [388]. Sin embargo, el conocimiento y el estudio de los aspectos relacionados con el metabolismo nitrogenado y la eliminación del nitrógeno son limitados. Por ello, surge la necesidad de profundizar en la comprensión de los mecanismos a través de los cuales se elimina el exceso de nitrógeno amínico del organismo, en concreto, la regulación del ciclo de la urea. Parte de esta tesis se ha dedicado al estudio de dichos aspectos bajo la intervención nutricional con dietas de distinta composición de macronutrientes y, a la vez, ajustadas entre ellas para facilitar la comparación de sus efectos. Junto con las tres dietas comentadas hasta ahora, se ha añadido una dieta hiperproteica (dieta estándar enriquecida con caseína, una proteína de elevado valor biológico, y gelatina), en la que las proteínas representan el 40% de la energía de la dieta, mientras que la proporción del resto de componentes se mantiene uniforme. Dichas dietas nos han permitido mostrar la importancia de la relación entre la cantidad de proteína y de energía en el control del funcionamiento del ciclo de la urea.

En ningún caso los animales expuestos a las distintas dietas experimentaron déficit energético, puesto que el contenido energético de las dietas fue suficiente para que todos los grupos incrementasen el peso corporal, aunque en menor o mayor magnitud en función de la composición particular de cada una de las dietas. Además, no se evidenciaron problemas en cuanto a la disponibilidad de aminoácidos, pues el contenido de masa magra, en este estudio, no varió substancialmente por efecto de la dieta. En el caso de la dieta de cafetería, los animales experimentaron un incremento del peso corporal significativo debido, esencialmente, al aumento de la adiposidad [97], aunque paralelamente mostraron un ligero incremento de la masa magra favorecido, en parte, por una mayor disponibilidad y una menor excreción de nitrógeno amínico [240].

El ciclo de la urea está supeditado a un control estricto de las pérdidas de nitrógeno amínico [271, 272]. Sin embargo, ante la ingesta de una dieta con elevado contenido energético y proteico (debido a la hiperfagia inducida por la misma dieta) como ocurre en el caso de la dieta de

cafetería, el ciclo de la urea se ve paradójicamente alterado disminuyendo su actividad y, por lo tanto, la producción de urea [240, 242, 245, 281, 286]. Este hecho pone en evidencia la existencia necesaria de vías alternativas para la eliminación del exceso de nitrógeno, y a la vez, la necesidad de ahondar en sus implicaciones, ya sea en condiciones fisiológicas como patológicas.

La concentración de aminoácidos en plasma está fuertemente regulada, aunque tras la ingesta se produce un aumento de los niveles circulantes, que, a su vez, inducirán la liberación de insulina y la síntesis de proteínas en el músculo [389]. En caso de exceso, gran parte de los aminoácidos son oxidados, principalmente los no esenciales pero también los esenciales si su disponibilidad es elevada [389], hecho que contribuye a que los niveles de aminoácidos permanezcan estables. Los resultados obtenidos muestran que, a pesar de las diferencias en relación al contenido proteico de cada una de la dietas, las variaciones en la concentración de aminoácidos circulantes fueron mínimas, evidenciando la estrecha regulación de los mecanismos encargados de preservar la homeostasis proteica, incluso en situaciones de restricción [390]. Sin embargo, se hallaron diferencias considerables en los valores de urea plasmática, los cuales ponen en evidencia la capacidad ureogénica del hígado para la eliminación del exceso amínico y amónico [391]. Cabe comentar que el estudio realizado tiene una limitación significativa, pues la medida directa de la pérdida de nitrógeno en orina y heces no pudo llevarse a cabo debido a los requerimientos éticos del procedimiento experimental para tal evaluación. Por ello, se ha considerado la valoración de urea plasmática como una medida indirecta de la capacidad del organismo para eliminar el exceso de nitrógeno amínico.

La evaluación de la capacidad ureogénica del hígado se realizó mediante la estimación de las actividades enzimáticas del ciclo de la urea, que aunque no nos permiten hacer una aproximación de la funcionalidad enzimática *in vivo*, sí que nos proporcionan una idea de la cantidad de enzima funcional y de su capacidad para llevar a cabo su función fisiológica. En cuanto al control del ciclo, la argininosuccinato sintasa y argininosuccinato liasa tienen un papel regulador destacable [257], lo que se ve reforzado tras observar una correlación positiva de estas actividades con las concentraciones de urea plasmática de los distintos grupos dietéticos. Por otro lado, y dado que la urea circulante está directamente relacionada con la pérdida de nitrógeno por la orina [392], se sugiere que la urea plasmática puede ser un indicador fiable de la funcionalidad del ciclo.

Ante la ingesta elevada de energía y proteína, se inician dos respuestas fisiológicas básicas: el incremento de la síntesis de proteína muscular por efecto de la insulina [393] y el incremento de la producción hepática de urea [394]. No obstante, el efecto de la dieta de cafetería es paradójico, pues la concentración de urea plasmática y la funcionalidad del ciclo de la urea en el hígado disminuyen, probablemente debido a una menor disponibilidad de ornitina y de arginina. [241] Por otro lado, la arginasa y la óxido nítrico sintasa compiten por su sustrato (L-arginina), hecho que indica un posible papel regulador de la arginasa en la síntesis de óxido nítrico. Sin embargo, el descenso en la producción de urea no se traduce en un incremento en

la producción y eliminación de óxido nítrico cuando la ingesta de proteína y energía es elevada [241].

El estudio del efecto que ejercen las dietas con diferente composición sobre la producción de urea, ha permitido observar que la cantidad de lípido de la dieta por sí mismo no afecta a la funcionalidad del ciclo de la urea. Sin embargo, la relación proteína/energía de la dieta, parece tener un papel potencial en el control de la producción de urea; cuando la relación entre el contenido de proteína/energía en la dieta es elevada la actividad de la principal enzima reguladora del ciclo de la urea (argininosuccinato liasa) y la concentración de urea en plasma incrementan significativamente, y viceversa. Este hecho sugiere que el destino del exceso de nitrógeno amínico, y en definitiva su eliminación, se ve comprometido por el exceso energético.

En la dieta de cafetería, la contribución energética de las proteínas es relativamente baja teniendo en cuenta el exceso energético derivado de lípidos y carbohidratos, por lo que se podría asumir la inducción de mecanismos de ahorro de proteína, de manera similar a lo ocurrido en situaciones de ayuno o de carencia proteica. En dichas situaciones la oxidación de aminoácidos y la producción de urea disminuyen, mientras que hay un mantenimiento del equilibrio entre la síntesis de proteínas y la proteólisis [395]. Ante el exceso de proteína y energía, junto con la menor producción de urea, solo hay dos opciones disponibles para eliminar el exceso de nitrógeno, un aumento del anabolismo proteico que favorezca una mayor acumulación de proteínas, o bien, mediante vías de eliminación alternativas y todavía desconocidas; algunos autores han descrito la producción directa de nitrógeno gas en situaciones parecidas [299, 300]. Todo ello, pone en evidencia la importancia de las consecuencias metabólicas no solo ante un exceso de nutrientes sino también ante un desequilibrio en su proporción.

Por otro lado, recientemente se ha descrito la funcionalidad del ciclo de la urea en el tejido adiposo blanco, con presencia de actividad enzimática significativa para todos los enzimas del ciclo [251]. Dicha actividad se ha descrito en todas sus localizaciones anatómicas, a pesar de que sus funciones fisiológicas son ligeramente distintas [132]. En cambio, el tejido adiposo marrón, que se caracteriza por tener una mayor capacidad metabólica que el tejido adiposo blanco incluso sin estímulos termogénicos, presenta una expresión y actividad incompleta del sistema enzimático del ciclo de la urea, con ausencia de las actividades de la ornitina transcarbamilasa y la arginasa. A pesar de ello, el perfil de expresión génica de las diferentes enzimas es muy similar entre ambos tejidos, aunque el perfil de actividad enzimática difiere significativamente. Este hecho sugiere que dichas enzimas podrían desempeñar roles diferentes en ambos tejidos, que podrían estar, en parte, relacionados con un origen celular distinto [303, 302].

El tejido adiposo marrón tiene ciertas similitudes con el intestino. Este último es capaz de captar glutamina, exportar alanina y producir amonio [310], a partir del cual es capaz de sintetizar urea, igual que el tejido adiposo blanco [396]. El perfil enzimático observado en el tejido adiposo

marrón sugiere que su función podría ser, principalmente, la de utilizar la citrulina disponible para la síntesis y exportación de arginina, un aminoácido con múltiples destinos metabólicos. Además, la ausencia de actividad arginasa podría favorecer una mayor disponibilidad de la arginina para sintetizar óxido nítrico [264], con diversas funciones, entre ellas la de estimular la oxidación de sustratos promoviendo la termogénesis.

En definitiva, mientras que el tejido adiposo blanco parece tener un ciclo de la urea complementario al del hígado y la capacidad para contribuir a la eliminación de un exceso de proteína, incluso en condiciones de elevada ingesta energética [397], la presencia parcial de las enzimas del ciclo de la urea en el tejido adiposo marrón parece estar implicada principalmente en la producción de arginina.

Conclusiones

CONCLUSIONES

1. La adición de mutarrotasa cuando se utiliza el método de la glucosa oxidasa/peroxidasa, permite cuantificar la totalidad de la glucosa presente en las muestras independientemente de su forma anomérica inicial.

2. El equilibrio anomérico α/β de la glucosa se altera en función de los patrones nutricionales y de la situación fisiopatológica de los individuos, pudiendo interferir sustancialmente en la medición de la glucemia y, en consecuencia, comprometer los criterios diagnósticos establecidos.

3. El grado de glucosilación de la hemoglobina y de las proteínas plasmáticas es independiente de la dieta y del sexo. En cambio, el grado de glucosilación de las proteínas de membrana de los eritrocitos incrementa en ratas expuestas a la dieta de cafetería, pudiendo ser un indicador sensible del desarrollo temprano de la hiperglucemia relacionada con el síndrome metabólico.

4. El contenido elevado de azúcar y sal induce de manera efectiva un exceso de ingesta energética y un aumento del peso y de la adiposidad corporal, probablemente como consecuencia de una alteración en los mecanismos de la saciedad.

5. Las dietas con una elevada densidad energética, causada únicamente por su elevado contenido lipídico, no alteran los mecanismos del control de la ingesta y no generan incrementos en el peso y la adiposidad corporal.

6. La composición lipídica de la dieta influye en el metabolismo de lípidos y carbohidratos. Una dieta rica en ácidos grasos saturados de cadena media afecta los niveles de parámetros metabólicos básicos, como el lactato y el glicerol, y de hormonas como el estradiol. El estradiol modula la capacidad hepática para sintetizar y almacenar lípidos limitando la síntesis de colesterol y de triacigliceroles.

7. El contenido lipídico de la dieta, por sí solo, no afecta en la funcionalidad del ciclo de la urea.

8. La relación proteína/energía de la dieta tiene consecuencias sustanciales en el control del ciclo de la urea, pues es esta relación y no la cantidad total de proteínas ingeridas la que regula el funcionamiento del ciclo.

9. La dieta de cafetería, con una disponibilidad elevada de proteínas, pero sobre todo de energía, provoca un funcionamiento limitado del ciclo de la urea. Esto sugiere la existencia de una vía adicional de eliminación del exceso de nitrógeno en estas situaciones.

10. El tejido adiposo marrón, a diferencia del tejido adiposo blanco, presenta una funcionalidad parcial del ciclo de la urea.

Referencias

1. Alemany M. The metabolic syndrome, a multifaceted disease of affluence. Journal of Endocrinology and Metabolism, 2012; 2(4-5):155-165.

2. Evangelopoulos AA, Vallianou NG, Panagiotakos DB, *et al.* The association between uric acid and hepatic function markers with the metabolic syndrome in middle-aged, overweight, and obese people. The Endocrinologist. 2010;20(6):312-5.

3. Singh S, Goyal R, Pratyush D. Is hypoandrogenemia a component of metabolic syndrome in males? Experimental and clinical endocrinology & diabetes. 2011;119(01):30-5.

4. Vogelzangs N, Suthers K, Ferrucci L, *et al.* Hypercortisolemic depression is associated with the metabolic syndrome in late-life. Psychoneuroendocrinology. 2007;32(2):151-9.

5. Marchesini G, Brizi M, Bianchi G, *et al.* Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. Diabetes. 2001;50(8):1844-50.

6. Kumar A, Woods KS, Bartolucci AA, *et al.* Prevalence of adrenal androgen excess in patients with the polycystic ovary syndrome (PCOS). Clinical endocrinology. 2005;62(6):644-9.

7. Coughlin SR, Mawdsley L, Mugarza JA, *et al.* Obstructive sleep apnoea is independently associated with an increased prevalence of metabolic syndrome. European heart journal. 2004;25(9):735-41.

8. Hahn BH, Grossman J, Chen W, *et al.* The pathogenesis of atherosclerosis in autoimmune rheumatic diseases: roles of inflammation and dyslipidemia. Journal of autoimmunity. 2007;28(2-3):69-75.

9. Russo A, Autelitano M, Bisanti L. Metabolic syndrome and cancer risk. European Journal of Cancer. 2008;44(2):293-7.

10. Braun S, Bitton-Worms K, LeRoith D. The link between the metabolic syndrome and cancer. International journal of biological sciences. 2011;7(7):1003.

11. Reaven GM. Role of insulin resistance in human disease. Diabetes. 1988;37(12):1595-607.

12. Reaven GM. Syndrome X: a short history. The Ochsner Journal. 2001;3(3):124-5.

13. Kaplan NM. The deadly quartet: upper-body obesity, glucose intolerance, hypertriglyceridemia, and hypertension. Archives of internal medicine. 1989;149(7):1514-20.

14. Grundy SM, Cleeman JI, Daniels SR, *et al.* Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute scientific statement. Circulation. 2005;112(17):2735-52.

15. Melanson EL, Astrup A, Donahoo WT. The relationship between dietary fat and fatty acid intake and body weight, diabetes, and the metabolic syndrome. Annals of Nutrition and Metabolism. 2009;55(1-3):229-43.

16. Bruce KD, Hanson MA. The Developmental Origins, Mechanisms, and Implications of Metabolic Syndrome. The Journal of nutrition. 2010;140(3):648-52.

17. Ogden CL, Yanovski SZ, Carroll MD, *et al.* The epidemiology of obesity. Gastroenterology. 2007;132(6):2087-102.

18. James WPT. WHO recognition of the global obesity epidemic. International journal of obesity. 2009;32(S7):S120.

19. Collaborators GO. Health effects of overweight and obesity in 195 countries over 25 years. New England Journal of Medicine. 2017;377(1):13-27.

20. Nguyen DM, El-Serag HB. The epidemiology of obesity. Gastroenterology Clinics. 2010;39(1):1-7.

21. Collaboration PS. Body-mass index and cause-specific mortality in 900 000 adults: collaborative analyses of 57 prospective studies. The Lancet. 2009;373(9669):1083-96.

22. Kopelman PG. Obesity as a medical problem. Nature. 2000;404(6778):635.

23. Piché M-E, Poirier P, Lemieux I, *et al.* Overview of Epidemiology and Contribution of Obesity and Body Fat Distribution to Cardiovascular Disease: An Update. Progress in cardiovascular diseases. 2018.

24. Chalk MB. Obesity: addressing a multifactorial disease. The Case Manager. 2004;15(6):47-9.

25. Kurtz TW, Morris RC, Pershadsingh HA. The Zucker fatty rat as a genetic model of obesity and hypertension. Hypertension. 1989;13(6_pt_2):896-901.

26. Andreasen CH, Andersen G. Gene–environment interactions and obesity—further aspects of genomewide association studies. Nutrition. 2009;25(10):998-1003.

27. Dietz WH, Gortmaker SL. Do we fatten our children at the television set? Obesity and television viewing in children and adolescents. Pediatrics. 1985;75(5):807-12.

28. Prentice AM, Jebb SA. Obesity in Britain: gluttony or sloth? British Medical Journal. 1995;311(7002):437-9.

29. Kant AK, Graubard BI. Secular trends in patterns of self-reported food consumption of adult Americans: NHANES 1971-1975 to NHANES 1999–2002. The American journal of clinical nutrition. 2006;84(5):1215-23.

30. Stunkard AJ, Harris JR, Pedersen NL, *et al.* The body-mass index of twins who have been reared apart. New England Journal of Medicine. 1990;322(21):1483-7.

31. Sørensen T. Genetic and environmental influences on obesity assessed by the adoption method. Revue d'epidemiologie et de sante publique. 1989;37(5-6):525-31.

32. Bouchard C, Perusse L. Heredity and body fat. Annual review of nutrition. 1988;8(1):259-77.

33. Montague CT, Farooqi IS, Whitehead JP, *et al.* Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature. 1997;387(6636):903.

34. Clement K, Vaisse C, Lahlou N, *et al.* A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature. 1998;392(6674):398.

35. Halaas JL, Gajiwala KS, Maffei M, *et al.* Weight-reducing effects of the plasma protein encoded by the obese gene. Science. 1995;269(5223):543-6.

36. Farooqi IS, Keogh JM, Yeo GS, *et al.* Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. New England Journal of Medicine. 2003;348(12):1085-95.

37. Krude H, Biebermann H, Luck W, *et al.* Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. Nature genetics. 1998;19(2):155.

38. Phillips C. Nutrigenetics and metabolic disease: current status and implications for personalised nutrition. Nutrients. 2013;5(1):32-57.

39. Frayling TM, Timpson NJ, Weedon MN, *et al.* A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity. Science. 2007;316(5826), 889-94.

40. Scuteri A, Sanna S, Chen W-M, *et al.* Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. PLoS genetics. 2007;3(7):e115.

41. Loos RJ, Lindgren CM, Li S, *et al.* Common variants near MC4R are associated with fat mass, weight and risk of obesity. Nature genetics. 2008;40(6):768.

42. Speakman JR. Evolutionary perspectives on the obesity epidemic: adaptive, maladaptive, and neutral viewpoints. Annual review of nutrition. 2013;33:289-317.

43. Jéquier E, Tappy L. Regulation of body weight in humans. Physiological reviews. 1999;79(2):451-80.

44. Spiegelman BM, Flier JS. Obesity and the regulation of energy balance. cell. 2001;104(4):531-43.

45. Bleich S, Cutler D, Murray C, *et al.* Why Is the Developed World Obese? National Bureau of Eeconimic Research Working Paper No. 12954. National Bureau of Economic Research, Cambridge, MA; 2007.

46. Swinburn BA, Sacks G, Hall KD, *et al.* The global obesity pandemic: shaped by global drivers and local environments. The Lancet. 2011;378(9793):804-14.

47. Zellweger H, Schneider HJ. Syndrome of hypotonia-hypomentia-hypogonadism-obesity (HHHO) or Prader-Willi syndrome. American Journal of Diseases of Children. 1968;115(5):588-98.

48. Ledbetter DH, Riccardi VM, Airhart SD, *et al.* Deletions of chromosome 15 as a cause of the Prader–Willi syndrome. New England Journal of Medicine. 1981;304(6):325-9.

49. Weaver JU. Classical endocrine diseases causing obesity. Obesity and Metabolism. 36: Karger Publishers; 2008. p. 212-28.

50. Pansini F, Cervellati C, Guariento A, *et al.* Oxidative stress, body fat composition, and endocrine status in pre-and postmenopausal women. Menopause. 2008;15(1):112-8.

51. Howie G, Sloboda D, Kamal T, *et al.* Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet. The Journal of physiology. 2009;587(4):905-15.

52. Palou M, Priego T, Sánchez J, *et al.* Moderate caloric restriction in lactating rats protects offspring against obesity and insulin resistance in later life. Endocrinology. 2010;151(3):1030-41.

53. Flier JS. Obesity wars: molecular progress confronts an expanding epidemic. Cell. 2004;116(2):337-50.

54. Castaner O, Goday A, Park Y-M, *et al.* The Gut Microbiome Profile in Obesity: A Systematic Review. International journal of endocrinology. 2018; 4095789.

55. Keys A, Fidanza F, Karvonen MJ, *et al.* Indices of relative weight and obesity. Journal of chronic diseases. 1972;25(6-7):329-43.

56. Poirier P, Alpert MA, Fleisher LA, *et al.* Cardiovascular evaluation and management of severely obese patients undergoing surgery: a science advisory from the American Heart Association. Circulation. 2009;120(1):86-95.

57. Blundell JE, Dulloo AG, Salvador J, *et al.* Beyond BMI-phenotyping the obesities. Obesity facts. 2014;7(5):322-8.

58. Goossens GH. The metabolic phenotype in obesity: fat mass, body fat distribution, and adipose tissue function. Obesity facts. 2017;10(3):207-15.

59. Snijder M, Zimmet PZ, Visser M, *et al.* Independent and opposite associations of waist and hip circumferences with diabetes, hypertension and dyslipidemia: the AusDiab Study. International journal of obesity. 2004;28(3):402.

60. Stefan N, Häring H-U, Hu FB, *et al.* Metabolically healthy obesity: epidemiology, mechanisms, and clinical implications. The lancet Diabetes & endocrinology. 2013;1(2):152-62.

61. Kloting N, Fasshauer M, Dietrich A, *et al.* Insulin-sensitive obesity. Am J Physiol Endocrinol Metab. 2010;299(3):E506eE15.

62. Neel JV. The" thrifty genotype" in 1998. Nutrition Reviews. 1999;57(5):S2.

63. Neel JV. Diabetes mellitus: a “thrifty” genotype rendered detrimental by “progress”? American journal of human genetics. 1962;14(4):353.

64. Alemany M. Utilization of dietary glucose in the metabolic syndrome. Nutrition & metabolism. 2011;8(1):74.

65. Blundell J, Gibbons C, Caudwell P, *et al.* Appetite control and energy balance: impact of exercise. Obesity reviews. 2015;16:67-76.

66. Silvestri E, Schiavo L, Lombardi A, *et al.* Thyroid hormones as molecular determinants of thermogenesis. Acta Physiologica Scandinavica. 2005;184(4):265-83.

67. Richard D. Cognitive and autonomic determinants of energy homeostasis in obesity. Nature Reviews Endocrinology. 2015;11(8):489.

68. King NA, Caudwell P, Hopkins M, *et al.* Metabolic and behavioral compensatory responses to exercise interventions: barriers to weight loss. Obesity. 2007;15(6):1373-83.

69. Atrens DM, Siviy SM, Holmes LJ, *et al.* Hypothalamic modulation of thermogenesis and energy substrate utilization. Brain research bulletin. 1987;18(3):303-8.

70. Bartness T, Vaughan C, Song C. Sympathetic and sensory innervation of brown adipose tissue. International journal of obesity. 2010;34(S1):S36.

71. Ricquier D, Bouillaud F, Toumelin P, *et al.* Expression of uncoupling protein mRNA in thermogenic or weakly thermogenic brown adipose tissue. Evidence for a rapid beta-adrenoreceptor-mediated and transcriptionally regulated step during activation of thermogenesis. Journal of Biological Chemistry. 1986;261(30):13905-10.

72. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiological reviews. 2004;84(1):277-359.

73. Schneeberger M, Gomis R, Claret M. Hypothalamic and brainstem neuronal circuits controlling homeostatic energy balance. Journal of endocrinology. 2014;220(2):T25-T46.

74. Halford JC, Blundell JE. Separate systems for serotonin and leptin in appetite control. Annals of medicine. 2000;32(3):222-32.

75. Badman MK, Flier JS. The gut and energy balance: visceral allies in the obesity wars. Science. 2005;307(5717):1909-14.

76. Blundell JE. Perspective on the central control of appetite. Obesity. 2006;14(S7):160S-3S.

77. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. Nature. 1998;395(6704):763.

78. Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. Annual review of immunology. 2011;29:415-45.

79. Dubern B, Clement K. Leptin and leptin receptor-related monogenic obesity. Biochimie. 2012;94(10):2111-5.

80. Field BC, Chaudhri OB, Bloom SR. Bowels control brain: gut hormones and obesity. Nature Reviews Endocrinology. 2010;6(8):444.

81. Air EL, Benoit SC, Smith KAB, *et al.* Acute third ventricular administration of insulin decreases food intake in two paradigms. Pharmacology Biochemistry and Behavior. 2002;72(1-2):423-9.

82. Bensaïd A, Tomé D, Gietzen D, *et al.* Protein is more potent than carbohydrate for reducing appetite in rats. Physiology & behavior. 2002;75(4):577-82.

83. Anderson GH, Moore SE. Dietary proteins in the regulation of food intake and body weight in humans. The Journal of nutrition. 2004;134(4):974S-9S.

84. Potier M, Fromentin G, Lesdema A, *et al.* The satiety effect of disguised liquid preloads administered acutely and differing only in their nutrient content tended to be weaker for lipids but did not differ between proteins and carbohydrates in human subjects. British journal of nutrition. 2010;104(9):1406-14.

85. Bellissimo N, Akhavan T. Effect of macronutrient composition on short-term food intake and weight loss. Advances in Nutrition. 2015;6(3):302S-8S.

86. Blundell JE, Finlayson G. Is susceptibility to weight gain characterized by homeostatic or hedonic risk factors for overconsumption? Physiology & behavior. 2004;82(1):21-5.

87. Peciña S, Smith KS, Berridge KC. Hedonic hot spots in the brain. The Neuroscientist. 2006;12(6):500-11.

88. Zheng H, Patterson LM, Berthoud H-R. Orexin signaling in the ventral tegmental area is required for high-fat appetite induced by opioid stimulation of the nucleus accumbens. Journal of Neuroscience. 2007;27(41):11075-82.

89. Johnson PM, Kenny PJ. Dopamine D2 receptors in addiction-like reward dysfunction and compulsive eating in obese rats. Nature neuroscience. 2010;13(5):635.

90. Leigh S-J, Lee F, Morris MJ. Hyperpalatability and the generation of obesity: roles of environment, stress exposure and individual difference. Current obesity reports. 2018;7(1):6-18.

91. Hammarstedt A, Gogg S, Hedjazifar S, *et al.* Impaired Adipogenesis and Dysfunctional Adipose Tissue in Human Hypertrophic Obesity. Physiological reviews. 2018;98(4):1911-41.

92. Rosen ED, MacDougald OA. Adipocyte differentiation from the inside out. Nature reviews Molecular cell biology. 2006;7(12):885.

93. Farmer SR. Transcriptional control of adipocyte formation. Cell metabolism. 2006;4(4):263-73.

94. Marques BG, Hausman DB, Martin RJ. Association of fat cell size and paracrine growth factors in development of hyperplastic obesity. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 1998;275(6):R1898-R908.

95. Van Harmelen V, Skurk T, Röhrig K, *et al.* Effect of BMI and age on adipose tissue cellularity and differentiation capacity in women. International journal of obesity. 2003;27(8):889.

96. Rydén M, Andersson DP, Bergström IB, *et al.* Adipose tissue and metabolic alterations: regional differences in fat cell size and number matter, but differently: a cross-sectional study. The Journal of Clinical Endocrinology & Metabolism. 2014;99(10):E1870-E6.

97. 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. 2014;9(3):e90995.

98. Tchernof A, Després J-P. Pathophysiology of human visceral obesity: an update. Physiological reviews. 2013;93(1):359-404.

99. Lim S, Meigs JB. Links Between Ectopic Fat and Vascular Disease in Humans. Arteriosclerosis, Thrombosis, and Vascular Biology. 2014;34(9):1820-6.

100. Catanzaro R, Cuffari B, Italia A, *et al.* Exploring the metabolic syndrome: Nonalcoholic fatty pancreas disease. World journal of gastroenterology. 2016;22(34):7660.

101. Hall JE. The kidney, hypertension, and obesity. Hypertension. 2003;41(3):625-33.

102. Lonardo A, Ballestri S, Marchesini G, *et al.* Nonalcoholic fatty liver disease: a precursor of the metabolic syndrome. Digestive and Liver Disease. 2015;47(3):181-90.

103. Donnelly KL, Smith CI, Schwarzenberg SJ, *et al.* Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. The Journal of clinical investigation. 2005;115(5):1343-51.

104. Tchkonia T, Thomou T, Zhu Y, *et al.* Mechanisms and metabolic implications of regional differences among fat depots. Cell metabolism. 2013;17(5):644-56.

105. Celsus AC, ab Almeloveen TJ. Aur. Corn. Celsi De medicina: libri 8; brevioribus Rob. Constantini, Is. Casauboni aliorumque scholiis ac locis parallelis illustrati: Cominus; 1722.

106. Rather L. Disturbance of function (functio laesa): the legendary fifth cardinal sign of inflammation, added by Galen to the four cardinal signs of Celsus. Bulletin of the New York Academy of Medicine. 1971;47(3):303.

107. Alemany M. Relationship between energy dense diets and white adipose tissue inflammation in metabolic syndrome. Nutrition research. 2013;33(1):1-11.

108. van Dijk SJ, Feskens EJ, Bos MB, *et al.* A saturated fatty acid–rich diet induces an obesity-linked proinflammatory gene expression profile in adipose tissue of subjects at risk of metabolic syndrome–. The American journal of clinical nutrition. 2009;90(6):1656-64.

109. Alemany M. The defense of adipose tissue against excess substrate-induced hyperthrophia: Immune system cell infiltration and arrested metabolic activity. Journal Of Clinical Endocrinology and Metabolism, 2011; 96(1): 66-8.

110. Meijer K, de Vries M, Al-Lahham S, *et al.* Human primary adipocytes exhibit immune cell function: adipocytes prime inflammation independent of macrophages. PloS one. 2011;6(3):e17154.

111. Weisberg SP, McCann D, Desai M, *et al.* Obesity is associated with macrophage accumulation in adipose tissue. The Journal of clinical investigation. 2003;112(12):1796-808.

112. Skurk T, Alberti-Huber C, Herder C, *et al.* Relationship between adipocyte size and adipokine expression and secretion. The Journal of Clinical Endocrinology & Metabolism. 2007;92(3):1023-33.

113. Martins AR, Nachbar RT, Gorjao R, *et al.* Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function. Lipids in health and disease. 2012;11(1):30.

114. Baffy G. Kupffer cells in non-alcoholic fatty liver disease: the emerging view. Journal of hepatology. 2009;51(1):212-23.

115. Cai D, Yuan M, Frantz DF, *et al.* Local and systemic insulin resistance resulting from hepatic activation of IKK-β and NF-κB. Nature medicine. 2005;11(2):183.

116. Crandall DL, DiGirolamo M. Hemodynamic and metabolic correlates in adipose tissue: pathophysiologic considerations. The FASEB Journal. 1990;4(2):141-7.

117. West DB, Prinz WA, Francendese AA, *et al.* Adipocyte blood flow is decreased in obese Zucker rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 1987;253(2):R228-R33.

118. Alemany M. Regulation of adipose tissue energy availability through blood flow control in the metabolic syndrome. Free Radical Biology and Medicine. 2012;52(10):2108-19.

119. Kabon B, Nagele A, Reddy D, *et al.* Obesity decreases perioperative tissue oxygenation. The Journal of the American Society of Anesthesiologists. 2004;100(2):274-80.

120. Dijkers PF, O'Farrell PH. Dissection of a hypoxia-induced, nitric oxide–mediated signaling cascade. Molecular biology of the cell. 2009;20(18):4083-90.

121. 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. 2009;54(6):1384-92.

122. Yin J, Gao Z, He Q, *et al.* Role of hypoxia in obesity-induced disorders of glucose and lipid metabolism in adipose tissue. American Journal of Physiology-Endocrinology and Metabolism. 2009;296(2):E333-E42.

123. Solaini G, Baracca A, Lenaz G, *et al.* Hypoxia and mitochondrial oxidative metabolism. Biochimica et Biophysica Acta (BBA)-Bioenergetics. 2010;1797(6-7):1171-7.

124. Regazzetti C, Peraldi P, Grémeaux T, *et al.* Hypoxia decreases insulin signaling pathways in adipocytes. Diabetes. 2009;58(1):95-103.

125. Hosogai N, Fukuhara A, Oshima K, *et al.* Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. Diabetes. 2007;56(4):901-11.

126. Hotamisligil GS. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell. 2010;140(6):900-17.

127. Goossens GH, Blaak EE. Adipose tissue oxygen tension: implications for chronic metabolic and inflammatory diseases. Current Opinion in Clinical Nutrition & Metabolic Care. 2012;15(6):539-46.

128. Goossens GH, Blaak EE. Adipose tissue dysfunction and impaired metabolic health in human obesity: a matter of oxygen? Frontiers in endocrinology. 2015;6:55.

129. Ho-Palma AC, Rotondo F, del Mar Romero M, *et al.* A method for the measurement of lactate, glycerol and fatty acid production from 14 C-glucose in primary cultures of rat epididymal adipocytes. Analytical Methods. 2016;8(44):7873-85.

130. Ho-Palma AC, Rotondo F, Romero MdM, *et al.* Use of 14C-glucose by primary cultures of mature rat epididymal adipocytes. Marked release of lactate and glycerol, but limited lipogenesis in the absence of external stimuli. Adipocyte. 2018;7(3)204-217.

131. Rotondo F, Ho-Palma AC, Remesar X, *et al.* Glycerol is synthesized and secreted by adipocytes to dispose of excess glucose, via glycerogenesis and increased acyl-glycerol turnover. Scientific Reports. 2017;7(1):8983.

132. Rotondo F, Ho-Palma AC, Remesar X, *et al.* Effect of sex on glucose handling by adipocytes isolated from rat subcutaneous, mesenteric and perigonadal adipose tissue. PeerJ. 2018;6:e5440.

133. Cnop M, Foufelle F, Velloso LA. Endoplasmic reticulum stress, obesity and diabetes. Trends in molecular medicine. 2012;18(1):59-68.

134. Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nature reviews Molecular cell biology. 2007;8(7):519.

135. Özcan U, Cao Q, Yilmaz E, *et al.* Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science. 2004;306(5695):457-61.

136. Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nature cell biology. 2011;13(3):184-190.

137. Zhang K, Shen X, Wu J, *et al.* Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. Cell. 2006;124(3):587-99.

138. Flamment M, Hajduch E, Ferré P, *et al.* New insights into ER stress-induced insulin resistance. Trends in Endocrinology & Metabolism. 2012;23(8):381-90.

139. Barbosa-da-Silva S, Bringhenti Sarmento I, Bargut L, *et al.* Animal Models of Nutritional Induction of Type 2 Diabetes Mellitus. International Journal of Morphology. 2014;32(1)279-293.

140. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. Nature. 2001;414(6865):799-807.

141. Pessin JE, Saltiel AR. Signaling pathways in insulin action: molecular targets of insulin resistance. The Journal of clinical investigation. 2000;106(2):165-9.

142. Wilcox G. Insulin and insulin resistance. Clinical Biochemist Reviews. 2005;26(2):19-39.

143. Cross DA, Alessi DR, Cohen P, *et al.* Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature. 1995;378(6559):785-789.

144. Dent P, Lavoinne A, Nakielny S, *et al.* The molecular mechanism by which insulin stimulates glycogen synthesis in mammalian skeletal muscle. Nature. 1990;348(6299):302-8.

145. Brady M, Nairn A, Saltiel A. The regulation of glycogen synthase by protein phosphatase 1 in 3T3-L1 adipocytes. Evidence for a potential role for DARPP-32 in insulin action. The Journal of biological chemistry. 1997;272(47):29698.

146. Pilkis SJ, Granner D. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. Annual review of physiology. 1992;54(1):885-909.

147. Yecies JL, Zhang HH, Menon S, *et al.* Akt stimulates hepatic SREBP1c and lipogenesis through parallel mTORC1-dependent and independent pathways. Cell metabolism. 2011;14(1):21-32.

148. Fazakerley DJ, Krycer JR, Kearney AL, *et al.* Muscle and adipose tissue insulin resistance: malady without mechanism? Journal of lipid research. 2018:jlr. R087510.

149. Entingh-Pearsall A, Kahn CR. Differential roles of the insulin and insulin-like growth factor-I receptors in response to inuslin and IGF-I. Journal of Biological Chemistry. 2004;279(36): 38016-24.

150. Janssen I, Heymsfield SB, Ross R. Low relative skeletal muscle mass (sarcopenia) in older persons is associated with functional impairment and physical disability. Journal of the American Geriatrics Society. 2002;50(5):889-96.

151. Pan D, Lillioja S, Kriketos A, *et al.* Skeletal muscle triglyceride levels are inversely related to insulin action. Diabetes. 1997;46(6):983-8.

152. Adams JM, Pratipanawatr T, Berria R, *et al.* Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. Diabetes. 2004;53(1):25-31.

153. Itani SI, Ruderman NB, Schmieder F, *et al.* Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IκB-α. Diabetes. 2002;51(7):2005-11.

154. Samuel VT, Shulman GI. Mechanisms for insulin resistance: common threads and missing links. Cell. 2012;148(5):852-71.

155. Stratford S, Hoehn KL, Liu F, *et al.* Regulation of insulin action by ceramide dual mechanisms linking ceramide accumulation to the inhibition of Akt/protein kinase B. Journal of Biological Chemistry. 2004;279(35):36608-15.

156. Gao Z, Hwang D, Bataille F, *et al.* Serine phosphorylation of insulin receptor substrate 1 by inhibitor κB kinase complex. Journal of Biological Chemistry. 2002;277(50):48115-21.

157. Zhang J, Gao Z, Yin J, *et al.* S6K Directly Phosphorylates IRS-1 on Ser-270 to Promote Insulin Resistance in Response to TNF-α Signaling through IKK2. The Journal of Biological Chemistry. 2008;283(51):35375.

158. Dandona P, Aljada A, Mohanty P, *et al.* Insulin inhibits intranuclear nuclear factor κB and stimulates IκB in mononuclear cells in obese subjects: evidence for an anti-inflammatory effect? The Journal of Clinical Endocrinology & Metabolism. 2001;86(7):3257-65.

159. Summers SA, Garza LA, Zhou H, *et al.* Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide. Molecular and cellular biology. 1998;18(9):5457-64.

160. Yu C, Chen Y, Cline GW, *et al.* Mechanism by which fatty acids inhibit insulin activation of IRS-1 associated phosphatidylinositol 3-kinase activity in muscle. Journal of Biological Chemistry. 2002;277(52):50230-36.

161. Kelley DE, He J, Menshikova EV, *et al.* Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes. 2002;51(10):2944-50.

162. Houstis N, Rosen ED, Lander ES. Reactive oxygen species have a causal role in multiple forms of insulin resistance. Nature. 2006;440(7086):944.

163. Anderson EJ, Lustig ME, Boyle KE, *et al.* Mitochondrial H 2 O 2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. The Journal of clinical investigation. 2009;119(3):573-81.

164. Lionetti L, Mollica MP, Lombardi A, *et al.* From chronic overnutrition to insulin resistance: the role of fat-storing capacity and inflammation. Nutrition, Metabolism and Cardiovascular Diseases. 2009;19(2):146-52.

165. Eaton SB, Konner M. Paleolithic Nutrition: A Consideration of Its Nature and Current Implications. New England Journal of Medicine. 1985;312(5):283-9.

166. Rolls E. Understanding the mechanisms of food intake and obesity. Obesity reviews. 2007;8:67-72.

167. Dallman MF, Pecoraro N, Akana SF, *et al.* Chronic stress and obesity: a new view of "comfort food". Proceedings of the National Academy of Sciences. 2003;100(20):11696-701.

168. Sominsky L, Spencer SJ. Eating behavior and stress: a pathway to obesity. Frontiers in psychology. 2014;5:434.

169. Rothwell NJ, Stock MJ. The development of obesity in animals: The role of dietary factors. Clinics in endocrinology and metabolism. 1984;13(3):437-49.

170. Mašek J, Fabry P. High-fat diet and the development of obesity in albino rats. Experientia. 1959;15(11):444-5.

171. Oakes ND, Cooney GJ, Camilleri S, *et al.* Mechanisms of liver and muscle insulin resistance induced by chronic high-fat feeding. Diabetes. 1997;46(11):1768-74.

172. Buettner R, Schölmerich J, Bollheimer LC. High-fat diets: modeling the metabolic disorders of human obesity in rodents. Obesity. 2007;15(4):798-808.

173. Buettner R, Parhofer K, Woenckhaus M, *et al.* Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. Journal of molecular endocrinology. 2006;36(3):485-501.

174. Warden CH, Fisler JS. Comparisons of diets used in animal models of high fat feeding. Cell metabolism. 2008;7(4):277.

175. Crescenzo R, Bianco F, Mazzoli A, *et al.* Fat quality influences the obesogenic effect of high fat diets. Nutrients. 2015;7(11):9475-91.

176. Sclafani A, Gorman AN. Effects of age, sex, and prior body weight on the development of dietary obesity in adult rats. Physiology & behavior. 1977;18(6):1021-6.

177. Schemmel R, Mickelsen O, Tolgay Z. Dietary obesity in rats: influence of diet, weight, age, and sex on body composition. American Journal of Physiology--Legacy Content. 1969;216(2):373-9.

178. Samuels LT, Reinecke RM, Ball HA. Effect of diet on glucose tolerance and liver and muscle glycogen of hypophysectomized and normal rats. Endocrinology. 1942;31(1):42-5.

179. Belzung F, Raclot T, Groscolas R. Fish oil n-3 fatty acids selectively limit the hypertrophy of abdominal fat depots in growing rats fed high-fat diets. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 1993;264(6):R1111-R8.

180. Youngren JF, Paik J, Barnard RJ. Impaired insulin-receptor autophosphorylation is an early defect in fat-fed, insulin-resistant rats. Journal of Applied Physiology. 2001;91(5):2240-7.

181. Ahren B, Mansson S, Gingerich RL, *et al.* Regulation of plasma leptin in mice: influence of age, high-fat diet, and fasting. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 1997;273(1):R113-R20.

182. Barnea M, Shamay A, Stark AH, *et al.* A high-fat diet has a tissue-specific effect on adiponectin and related enzyme expression. Obesity. 2006;14(12):2145-53.

183. Tepperman HM, Dewitt J, Tepperman J. Effect of a high fat diet on rat adipocyte lipolysis: responses to epinephrine, forskolin, methylisobutylxanthine, dibutyryl cyclic AMP, insulin and nicotinic acid. The Journal of nutrition. 1986;116(10):1984-91.

184. Yaqoob P, Sherrington EJ, Jeffery NM, *et al.* Comparison of the effects of a range of dietary lipids upon serum and tissue lipid composition in the rat. The international journal of biochemistry & cell biology. 1995;27(3):297-310.

185. Todoric J, Löffler M, Huber J, *et al.* Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n−3 polyunsaturated fatty acids. Diabetologia. 2006;49(9):2109-19.

186. Lee JY, Ye J, Gao Z, *et al.* Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. Journal of Biological Chemistry. 2003;278(39):37041-51.

187. Weigert C, Brodbeck K, Staiger H, *et al.* Palmitate, but not unsaturated fatty acids, induces the expression of interleukin-6 in human myotubes through proteasome-dependent activation of nuclear factor-κB. Journal of Biological Chemistry. 2004;279(23):23942-52.

188. Kennedy A, Martinez K, Chuang C-C, *et al.* Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: mechanisms of action and implications. The Journal of nutrition. 2008;139(1):1-4.

189. Basu A, Devaraj S, Jialal I. Dietary factors that promote or retard inflammation. Arteriosclerosis, thrombosis, and vascular biology. 2006;26(5):995-1001.

190. Sekiya M, Yahagi N, Matsuzaka T, *et al.* Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. Hepatology. 2003;38(6):1529-39.

191. Buckley JD, Howe PR. Long-chain omega-3 polyunsaturated fatty acids may be beneficial for reducing obesity—a review. Nutrients. 2010;2(12):1212-30.

192. Hariri N, Thibault L. High-fat diet-induced obesity in animal models. Nutrition research reviews. 2010;23(2):270-99.

193. Gentilcore D, Chaikomin R, Jones KL, *et al.* Effects of fat on gastric emptying of and the glycemic, insulin, and incretin responses to a carbohydrate meal in type 2 diabetes. The Journal of Clinical Endocrinology & Metabolism. 2006;91(6):2062-7.

194. Moraes JC, Coope A, Morari J, *et al.* High-fat diet induces apoptosis of hypothalamic neurons. PloS one. 2009;4(4):e5045.

195. Cordner ZA, Tamashiro KL. Effects of high-fat diet exposure on learning & memory. Physiology & behavior. 2015;152:363-71.

196. Shaw MA, Rasmussen KM, Myers TR. Consumption of a high fat diet impairs reproductive performance in Sprague-Dawley rats. The Journal of nutrition. 1997;127(1):64-9.

197. Chan P, Dao TL. Enhancement of mammary carcinogenesis by a high-fat diet in Fischer, Long-Evans, and Sprague-Dawley rats. Cancer research. 1981;41(1):164-7.

198. Cloetens L, Ulmius M, Johansson-Persson A, *et al.* Role of dietary beta-glucans in the prevention of the metabolic syndrome. Nutrition reviews. 2012;70(8):444-58.

199. Schwartz GJ, McHugh PR, Moran TH. Integration of vagal afferent responses to gastric loads and cholecystokinin in rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 1991;261(1):R64-R9.

200. Nilsson AC, Ostman EM, Holst JJ, *et al.* Including indigestible carbohydrates in the evening meal of healthy subjects improves glucose tolerance, lowers inflammatory markers, and increases satiety after a subsequent standardized breakfast. The Journal of nutrition. 2008;138(4):732-9.

201. Mayer J. Glucostatic mechanism of regulation of food intake. New England Journal of Medicine. 1953;249(1):13-6.

202. Walker CG, Zariwala MG, Holness MJ, *et al.* Diet, obesity and diabetes: a current update. Clinical science. 2007;112(2):93-111.

203. Storlien LH, Higgins J, Thomas T, *et al.* Diet composition and insulin action in animal models. British journal of nutrition. 2000;83(S1):S85-S90.

204. Pagliassotti MJ, Prach PA. Quantity of sucrose alters the tissue pattern and time course of insulin resistance in young rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 1995;269(3):R641-R6.

205. Eiffert KC, McDonald RB, Stern JS. High sucrose diet and exercise: effects on insulin-receptor function of 12-and 24-mo-old Sprague-Dawley rats. The Journal of nutrition. 1991;121(7):1081-9.

206. Malik VS, Hu FB. Sweeteners and risk of obesity and type 2 diabetes: the role of sugar-sweetened beverages. Current diabetes reports. 2012;12(2):195-203.

207. Schultz A, Neil D, Aguila MB, *et al.* Hepatic adverse effects of fructose consumption independent of overweight/obesity. International Journal of Molecular Sciences. 2013;14(11):21873-86.

208. Bray GA, Nielsen SJ, Popkin BM. Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. The American Journal of Clinical Nutrition. 2004;79(4):537-43.

209. Felig P, Owen OE, Wahren J, *et al.* Amino acid metabolism during prolonged starvation. The Journal of Clinical Investigation. 1969;48(3):584-94.

210. Goodman M, Lowell B, Belur E, *et al.* Sites of protein conservation and loss during starvation: influence of adiposity. American Journal of Physiology-Endocrinology And Metabolism. 1984;246(5):E383-E90.

211. Schaafsma G. The protein digestibility–corrected amino acid score. The Journal of nutrition. 2000;130(7):1865S-7S.

212. 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. British Journal of Nutrition. 2007;97(6):1099-108.

213. Lacroix M, Gaudichon C, Martin A, *et al.* A long-term high-protein diet markedly reduces adipose tissue without major side effects in Wistar male rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2004;287(4):R934-R42.

214. Azzout-Marniche D, Gaudichon C, Blouet C, *et al.* Liver glyconeogenesis: a pathway to cope with postprandial amino acid excess in high-protein fed rats? American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2007;292(4):R1400-R7.

215. Pesta DH, Samuel VT. A high-protein diet for reducing body fat: mechanisms and possible caveats. Nutrition & metabolism. 2014;11(1):53.

216. Westerterp-Plantenga M, Luscombe-Marsh N, Lejeune M, *et al.* Dietary protein, metabolism, and body-weight regulation: dose–response effects. International Journal of Obesity. 2006;30(S3):S16.

217. Frank H, Graf J, Amann-Gassner U, *et al.* Effect of short-term high-protein compared with normal-protein diets on renal hemodynamics and associated variables in healthy young men. The American journal of clinical nutrition. 2009;90(6):1509-16.

218. Morell P, Fiszman S. Revisiting the role of protein-induced satiation and satiety. Food Hydrocolloids. 2017;68:199-210.

219. Weigle DS, Breen PA, Matthys CC, *et al.* A high-protein diet induces sustained reductions in appetite, ad libitum caloric intake, and body weight despite compensatory changes in diurnal plasma leptin and ghrelin concentrations. The American journal of clinical nutrition. 2005;82(1):41-8.

220. Karhunen L, Juvonen K, Huotari A, *et al.* Effect of protein, fat, carbohydrate and fibre on gastrointestinal peptide release in humans. Regulatory peptides. 2008;149(1-3):70-8.

221. Veldhorst MA, Westerterp KR, Westerterp-Plantenga MS. Gluconeogenesis and protein-induced satiety. British Journal of Nutrition. 2012;107(4):595-600.

222. Piatti P, Monti L, Magni F, *et al.* Hypocaloric high-protein diet improves glucose oxidation and spares lean body mass: comparison to hypocaloric high-carbohydrate diet. Metabolism. 1994;43(12):1481-7.

223. Brinkworth G, Noakes M, Keogh J, *et al.* Long-term effects of a high-protein, low-carbohydrate diet on weight control and cardiovascular risk markers in obese hyperinsulinemic subjects. International Journal of Obesity. 2004;28(5):661.

224. Due A, Toubro S, Stender S, *et al.* The effect of diets high in protein or carbohydrate on inflammatory markers in overweight subjects. Diabetes, obesity and metabolism. 2005;7(3):223-9.

225. Usami M, Seino Y, Seino S, *et al.* Effects of high protein diet on insulin and glucagon secretion in normal rats. The Journal of nutrition. 1982;112(4):681-5.

226. Jeganathan S, Abdullahi A, Zargar S, *et al.* Amino acid-induced impairment of insulin sensitivity in healthy and obese rats is reversible. Physiological reports. 2014;2(7).

227. Stengel A, Goebel-Stengel M, Wang L, *et al.* High-protein diet selectively reduces fat mass and improves glucose tolerance in Western-type diet-induced obese rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2013;305(6):R582-R91.

228. Denke MA. Metabolic effects of high-protein, low-carbohydrate diets. American Journal of Cardiology. 2001;88(1):59-61.

229. Peckrham S, Entenman C, Carroll H. The influence of a hypercaloric diet on gross body and adipose tissue composition in the rat. Journal of Nutrition. 1962;77:187-97.

230. Shafat A, Murray B, Rumsey D. Energy density in cafeteria diet induced hyperphagia in the rat. Appetite. 2009;52(1):34-8.

231. Reichelt AC, Morris MJ, Westbrook R. Cafeteria diet impairs expression of sensory-specific satiety and stimulus-outcome learning. 2014;5:1-11.

232. Esteve M, Rafecas I, Fernández-López J, *et al.* Effect of a cafeteria diet on energy intake and balance in Wistar rats. Physiology & behavior. 1994;56(1):65-71.

233. Muntzel MS, Al-Naimi OAS, Barclay A, *et al.* Cafeteria Diet Increases Fat Mass and Chronically Elevates Lumbar Sympathetic Nerve Activity in RatsNovelty and Significance. Hypertension. 2012;60(6):1498-502.

234. Prats E, Monfar M, Castella J, *et al.* Energy intake of rats fed a cafeteria diet. Physiology & behavior. 1989;45(2):263-72.

235. Sampey BP, Vanhoose AM, Winfield HM, *et al.* Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet. Obesity. 2011;19(6):1109-17.

236. Davidson M, Garvey D. Studies on mechanisms of hepatic insulin resistance in cafeteria-fed rats. The American journal of physiology. 1993;264(1 Pt 1):E18.

237. Gomez-Smith M, Karthikeyan S, Jeffers MS, *et al.* A physiological characterization of the Cafeteria diet model of metabolic syndrome in the rat. Physiology & behavior. 2016;167:382-91.

238. Sclafani A, Springer D. Dietary obesity in adult rats: similarities to hypothalamic and human obesity syndromes. Physiology & behavior. 1976;17(3):461-71.

239. Rothwell NJ, Saville ME, Stock MJ. Effects of feeding a" cafeteria" diet on energy balance and diet-induced thermogenesis in four strains of rat. The Journal of nutrition. 1982;112(8):1515-24.

240. Barber T, Viña J, Viña J, *et al.* Decreased urea synthesis in cafeteria-diet-induced obesity in the rat. Biochemical Journal. 1985;230(3):675-81.

241. 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 research international. 2014.

242. Agnelli S, Arriarán S, Oliva L, *et al.* Modulation of rat liver urea cycle and related ammonium metabolism by sex and cafeteria diet. RSC Advances. 2016;6(14):11278-88.

243. Murphy C, Newsholme P. Importance of glutamine metabolism in murine macrophages and human monocytes to L-arginine biosynthesis and rates of nitrite or urea production. Clinical science. 1998;95(4):397-407.

244. Rafecas I, Esteve M, Fernández-López JA, *et al.* Methodological evaluation of indirect calorimetry data in lean and obese rats. Clinical and experimental pharmacology and physiology. 1993;20(11):731-42.

245. Esteve M, Rafecas I, Remesar X, *et al.* Nitrogen balances of lean and obese Zucker rats subjected to a cafeteria diet. International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity. 1992;16(4):237-44.

246. Alemany M. The problem of nitrogen disposal in the obese. Nutrition research reviews. 2012;25(1):18-28.

247. Abumrad NN, Williams P, Frexes-Steed M, *et al.* Inter-organ metabolism of amino acids in vivo. Diabetes/metabolism reviews. 1989;5(3):213-26.

248. Christensen HN. Role of amino acid transport and countertransport in nutrition and metabolism. Physiological reviews. 1990;70(1):43-77.

249. Wang H, Ran J, Jiang T. Urea. Urea Transporters Subcellular Biochemistry. 73. Dordrecht: Springer; 2014:7-29.

250. Davis PK, Wu G. Compartmentation and kinetics of urea cycle enzymes in porcine enterocytes. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 1998;119(3):527-37.

251. Arriarán S, Agnelli S, Remesar X, *et al.* The urea cycle of rat white adipose tissue. RSC Advances. 2015;5(113):93403-14.

252. Rubio V, Ramponi G, Grisolia S. Carbamoyl phosphate synthetase I of human liver. Purification, some properties and immunological cross-reactivity with the rat liver enzyme. Biochimica et Biophysica Acta (BBA)-Enzymology. 1981;659(1):150-60.

253. Chabas A, Grisolia S, Silverstein R. On the mechanism of carbamoylphosphate synthetase. European journal of biochemistry. 1972;29(2):333-42.

254. Heibel SK, Lopez GY, Panglao M, *et al.* Transcriptional regulation of N-acetylglutamate synthase. PloS one. 2012;7(2):e29527.

255. Makoff A, Radford A. Genetics and biochemistry of carbamoyl phosphate biosynthesis and its utilization in the pyrimidine biosynthetic pathway. Microbiological reviews. 1978;42(2):307.

256. Neill MA, Aschner J, Barr F, *et al.* Quantitative RT-PCR comparison of the urea and nitric oxide cycle gene transcripts in adult human tissues. Molecular genetics and metabolism. 2009;97(2):121-7.

257. Husson A, Brasse-Lagnel C, Fairand A, *et al.* Argininosuccinate synthetase from the urea cycle to the citrulline–NO cycle. European Journal of Biochemistry. 2003;270(9):1887-99.

258. Jenkinson CGWCS. Comparative properties of arginases. Comp Biochem Physiol B Biochem Mol Biol. 1996;114:107-32.

259. Morris Jr SM. Regulation of enzymes of the urea cycle and arginine metabolism. Annual review of nutrition. 2002;22(1):87-105.

260. Hill BG, Dranka BP, Bailey SM, *et al.* What part of NO don't you understand? Some answers to the cardinal questions in nitric oxide biology. Journal of Biological Chemistry. 2010;285(26):19699-704.

261. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochemical Journal. 2001;357(3):593-615.

262. Sansbury BE, Hill BG. Regulation of obesity and insulin resistance by nitric oxide. Free radical Biology and Medicine. 2014;73:383-99.

263. Guoyao W, Morris SM. Arginine metabolism: nitric oxide and beyond. Biochemical Journal. 1998;336(1):1-17.

264. Johnson FK, Johnson RA, Peyton KJ, *et al.* Arginase inhibition restores arteriolar endothelial function in Dahl rats with salt-induced hypertension. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2005;288(4):R1057-R1062.

265. Tenu J-P, Lepoivre M, Moali C, *et al.* Effects of the new arginase inhibitor Nω-hydroxy-nor-L-arginine on NO synthase activity in murine macrophages. Nitric oxide. 1999;3(6):427-38.

266. Brosnan JT, Brosnan ME. Hepatic glutaminase—a special role in urea synthesis? Nutrition. 2002;18(6):455-7.

267. Nissim I, Horyn O, Luhovyy B, *et al.* Role of the glutamate dehydrogenase reaction in furnishing aspartate nitrogen for urea synthesis: studies in perfused rat liver with 15N. Biochemical Journal. 2003;376(1):179-88.

268. Palmieri L, Pardo B, Lasorsa F, *et al.* Citrin and aralar1 are Ca2+-stimulated aspartate/glutamate transporters in mitochondria. The EMBO journal. 2001;20(18):5060-9.

269. Watford M. Hepatic glutaminase expression: relationship to kidney-type glutaminase and to the urea cycle. The FASEB journal. 1993;7(15):1468-74.

270. Dimski DS. Ammonia metabolism and the urea cycle: function and clinical implications. Journal of veterinary internal medicine. 1994;8(2):73-8.

271. Emmanuel B. Autoregulation of urea cycle by urea in mammalian species. Comparative Biochemistry and Physiology Part A: Physiology. 1981;70(1):79-81.

272. Young VR, El-Khoury AE, Raguso CA, *et al.* Rates of urea production and hydrolysis and leucine oxidation change linearly over widely varying protein intakes in healthy adults. The Journal of nutrition. 2000;130(4):761-6.

273. Schimke RT. Differential effects of fasting and protein-free diets on levels of urea cycle enzymes in rat liver. J Biol Chem. 1962;237(6):1921-4.

274. Hayase K, Yokogoshi H, Yoshida A. Effect of dietary proteins and amino acid deficiencies on urinary excretion of nitrogen and the urea synthesizing system in rats. The Journal of nutrition. 1980;110(7):1327-37.

275. Takiguchi M, Mori M. Transcriptional regulation of genes for ornithine cycle enzymes. Biochemical Journal. 1995;312(Pt 3):649.

276. Hansen BA, Krog B, Vilstrup H. Insulin and glucose decreases the capacity of urea-N synthesis in the rat. Scandinavian journal of clinical and laboratory investigation. 1986;46(6):599-603.

277. Li Z, Yarmush ML, Chan C. Insulin concentration during preconditioning mediates the regulation of urea synthesis during exposure to amino acid-supplemented plasma. Tissue engineering. 2004;10(11-12):1737-46.

278. Hamberg O, Vilstrup H. Regulation of urea synthesis by glucose and glucagon in normal man. Clinical Nutrition. 1994;13(3):183-91.

279. Vilstrup H, Hansen BA, Almdal TP. Glucagon increases hepatic efficacy for urea synthesis. Journal of hepatology. 1990;10(1):46-50.

280. Grøfte T, Wolthers T, Jørgensen JOL, *et al.* Hepatic amino-to urea-N clearance and forearm amino-N exchange during hypoglycemic and euglycemic hyperinsulinemia in normal man. Journal of hepatology. 1999;30(5):819-25.

281. Schimke RT. Studies on factors affecting the levels of urea cycle enzymes in rat liver. J Biol Chem. 1963;238:1012-8.

282. Wolthers T, Hamberg O, Grøfte T, *et al.* Effects of budesonide and prednisolone on hepatic kinetics for urea synthesis. Journal of hepatology. 2000;33(4):549-54.

283. Grøfte T, Jensen DS, Grønbæk H, *et al.* Effects of growth hormone on steroid-induced increase in ability of urea synthesis and urea enzyme mRNA levels. American Journal of Physiology-Endocrinology And Metabolism. 1998;275(1):E79-E86.

284. Grøfte T, Jensen DS, Greisen J, *et al.* Growth hormone and insulin-like growth factor-I counteracts established steroid catabolism in rats by effects on hepatic amino-N degradation. Journal of hepatology. 2001;35(6):700-6.

285. Jean Cm, Rome S, Mathé Vr, *et al.* Metabolic evidence for adaptation to a high protein diet in rats. The Journal of nutrition. 2001;131(1):91-8.

286. Esteve M, Rafecas I, Fernández-López J, *et al.* Dietary amino acid balances in young Wistar rats fed a cafeteria diet. Biochemistry and molecular biology international. 1993;29(6):1069-81.

287. Goodman M, Lowenstein J. The purine nucleotide cycle. Studies of ammonia production by skeletal muscle in situ and in perfused preparations. Journal of Biological Chemistry. 1977;252(14):5054-60.

288. Tornheim K, Lowenstein J. The purine nucleotide cycle. Control of phosphofructokinase and glycolytic oscillations in muscle extracts. Journal of Biological Chemistry. 1975;250(16):6304-14.

289. Wu G. Intestinal mucosal amino acid catabolism. The Journal of nutrition. 1998;128(8):1249-52.

290. Lowenstein J. Ammonia production in muscle and other tissues: the purine nucleotide cycle. Physiological Reviews. 1972;52(2):382-414.

291. McGivan J, Chappell J. On the metabolic function of glutamate dehydrogenase in rat liver. FEBS letters. 1975;52(1):1-7.

292. Schoolwerth AC, Nazar BL, LaNoue KF. Glutamate dehydrogenase activation and ammonia formation by rat kidney mitochondria. Journal of Biological Chemistry. 1978;253(17):6177-83.

293. de Castro Ghizoni CV, Gasparin FRS, Júnior ASM, *et al.* Catabolism of amino acids in livers from cafeteria-fed rats. Molecular and cellular biochemistry. 2013;373(1-2):265-77.

294. Serra F, Gianotti M, Palou A, *et al.* Dietary obesity shows adaptations of amino-acid metabolism on enzyme activities to save amino nitrogen. Biochemistry international. 1991;24(4):769-76.

295. Casado J, Fernández-López J, Esteve M, *et al.* Rat liver amino acid balances after the administration of an oral protein load. Biochemistry international. 1991;25(5):869-77.

296. Asl SZ, Ghasemi A, Azizi F. Serum nitric oxide metabolites in subjects with metabolic syndrome. Clinical biochemistry. 2008;41(16-17):1342-7.

297. Maniscalco M, de Laurentiis G, Zedda A, *et al.* Exhaled nitric oxide in severe obesity: effect of weight loss. Respiratory physiology & neurobiology. 2007;156(3):370-3.

298. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. Nature reviews Drug discovery. 2008;7(2):156.

299. Cissik JH, Johnson R, Hertig B. Production of gaseous nitrogen during human steady state exercise. Aerospace medicine. 1972;43(11):1245-50.

300. Costa G, Ullrich L, Kantor F, *et al.* Production of elemental nitrogen by certain mammals including man. Nature. 1968;218(5141):546.

301. Cinti S. The adipose organ at a glance. Disease models & mechanisms. 2012;5(5):588-94.

302. Park A, Kim WK, Bae K-H. Distinction of white, beige and brown adipocytes derived from mesenchymal stem cells. World journal of stem cells. 2014;6(1):33.

303. Bartelt A, Heeren J. Adipose tissue browning and metabolic health. Nature Reviews Endocrinology. 2014;10(1):24.

304. Deveaud C, Beauvoit B, Salin B, *et al.* Regional differences in oxidative capacity of rat white adipose tissue are linked to the mitochondrial content of mature adipocytes. Molecular and cellular biochemistry. 2004;267(1-2):157-66.

305. Frayn KN, Humphreys SM, Coppack SW. Fuel selection in white adipose tissue. Proceedings of the Nutrition Society. 1995;54(1):177-89.

306. Frayn K, Coppack S, Smith U. Evidence for lactate production by human adipose tissue in vivo. Diabetologia. 1990;33(12):740-1.

307. Snell K, Duff DA. Alanine release by rat adipose tissue in vitro. Biochemical and biophysical research communications. 1977;77(3):925-31.

308. Markan KR, Jurczak MJ, Brady MJ. Stranger in a strange land: roles of glycogen turnover in adipose tissue metabolism. Molecular and cellular endocrinology. 2010;318(1-2):54-60.

309. Ho-Palma AC, Toro P, Rotondo F, *et al.* Insulin Controls Triacylglycerol Synthesis through Control of Glycerol Metabolism and Despite Increased Lipogenesis. Nutrients. 2019;11(3):513.

310. Windmueller HG, Spaeth AE. Source and fate of circulating citrulline. American Journal of Physiology-Endocrinology And Metabolism. 1981;241(6):E473-E80.

311. Coelho M, Oliveira T, Fernandes R. Biochemistry of adipose tissue: an endocrine organ. Archives of medical science: AMS. 2013;9(2):191.

312. Lean MEJ. Brown adipose tissue in humans. Proceedings of the Nutrition Society. 1989;48(2):243-57.

313. Klaus S, Casteilla L, Bouillaud F, *et al.* The uncoupling protein UCP: a membraneous mitochondrial ion carrier exclusively expressed in brown adipose tissue. International Journal of Biochemistry. 1991;23(9):791-801.

314. Nicholls DG, Locke RM. Thermogenic mechanisms in brown fat. Physiological reviews. 1984;64(1):1-64.

315. Blondin DP, Labbé SM, Tingelstad HC, *et al.* Increased brown adipose tissue oxidative capacity in cold-acclimated humans. The Journal of Clinical Endocrinology & Metabolism. 2014;99(3):E438-E46.

316. López-Soriano F, Alemany M. Activities of enzymes of amino acid metabolism in rat brown adipose tissue. Biochemistry international. 1986;12(3):471-8.

317. López-Soriano F, Fernandez-Lopez J, Mampel T, *et al.* Amino acid and glucose uptake by rat brown adipose tissue. Effect of cold-exposure and acclimation. Biochemical Journal. 1988;252(3):843-9.

318. Vargas-Castillo A, Fuentes-Romero R, Rodriguez-Lopez LA, *et al.* Understanding the biology of thermogenic fat: is browning a new approach to the treatment of obesity? Archives of medical research. 2017;48(5):401-13.

319. Group NDD. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. Diabetes. 1979;28(12):1039-57.

320. Yoo E-H, Lee S-Y. Glucose biosensors: an overview of use in clinical practice. Sensors. 2010;10(5):4558-76.

321. Vashist SK. Non-invasive glucose monitoring technology in diabetes management: A review. Analytica chimica acta. 2012;750:16-27.

322. O'Sullivan J, Mahan C. Glucose tolerance test. Variability in pregnant and nonpregnant women. The American journal of clinical nutrition. 1966;19(5):345.

323. Ulrich P, Cerami A. Protein glycation, diabetes, and aging. 2001;56(1):1-22.

324. Henning C, Smuda M, Girndt M, *et al.* Molecular basis of Maillard amide-advanced glycation end product (AGE) formation in vivo. Journal of Biological Chemistry. 2011;286(52):44350-6.

325. Rondeau P, Bourdon E. The glycation of albumin: structural and functional impacts. Biochimie. 2011;93(4):645-58.

326. Brownlee M, Vlassara H, Cerami A. Nonenzymatic glycosylation and the pathogenesis of diabetic complications. Annals of internal medicine. 1984;101(4):527-37.

327. Kilhovd BK, Berg TJ, Birkeland KI, *et al.* Serum levels of advanced glycation end products are increased in patients with type 2 diabetes and coronary heart disease. Diabetes care. 1999;22(9):1543-8.

328. Giuffrida FMA, Sallum CFC, Gabbay MAL, *et al.* Relationship between glycated hemoglobin and metabolic syndrome of type 1 and type 2 diabetes. A factor analysis study. Diabetes Care. 2010;33(6):e80-e.

329. Koga M, Kasayama S. Clinical impact of glycated albumin as another glycemic control marker. Endocrine journal. 2010;57(9):751-62.

330. Abe M, Matsumoto K. Glycated hemoglobin or glycated albumin for assessment of glycemic control in hemodialysis patients with diabetes? Nature Clinical Practice Nephrology. 2008;4(9):482-3.

331. Benedict SR. The analysis of whole blood. 2. The determination of sugar and of saccharoids (non-fermentable copper-reducing substances). Journal of Biological Chemistry. 1931;92:141-59.

332. Nelson N. A photometric adaptation of the Somogyi method for the determination of glucose. J biol Chem. 1944;153(2):375-80.

333. Carroll JJ, Smith N, Babson AL. A colorimetric serum glucose determination using hexokinase and glucose-6-phosphate dehydrogenase. Biochemical medicine. 1970;4(2):171-80.

334. Trinder P. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Annals of clinical Biochemistry. 1969;6(1):24-7.

335. Wilson CM. Quantitative determination of sugars on paper chromatograms. Analytical chemistry. 1959;31(7):1199-201.

336. Lopes JF, Gaspar EM. Simultaneous chromatographic separation of enantiomers, anomers and structural isomers of some biologically relevant monosaccharides. Journal of Chromatography A. 2008;1188(1):34-42.

337. Heller A. Amperometric biosensors. Current opinion in biotechnology. 1996;7(1):50-4.

338. Fulati A, Ali SMU, Asif MH, *et al.* An intracellular glucose biosensor based on nanoflake ZnO. Sensors and Actuators B: Chemical. 2010;150(2):673-80.

339. Carstensen B, Lindström J, Sundvall J, *et al.* Measurement of blood glucose: comparison between different types of specimens. Annals of clinical biochemistry. 2008;45(2):140-8.

340. Bruns DE, Knowler WC. Stabilization of glucose in blood samples: why it matters. Clinical Chemistry; 2009;55(5):850-52.

341. Meites S, Saniel-Banrey K. Modified glucose oxidase method for determination of glucose in whole blood. Clinical chemistry. 1973;19(3):308-11.

342. Zuurbier CJ, Keijzers PJ, Koeman A, *et al.* Anesthesia's effects on plasma glucose and insulin and cardiac hexokinase at similar hemodynamics and without major surgical stress in fed rats. Anesthesia and Analgesia. 2008;106(1):135-42.

343. Ollerton RL, Playle R, Ahmed K, *et al.* Day-to-day variability of fasting plasma glucose in newly diagnosed type 2 diabetic subjects. Diabetes care. 1999;22(3):394-8.

344. Stahl M, Jørgensen LGM, Petersen PH, *et al.* Optimization of preanalytical conditions and analysis of plasma glucose. 1. Impact of the new WHO and ADA recommendations on diagnosis of diabetes mellitus. Scandinavian journal of clinical and laboratory investigation. 2001;61(3):169-79.

345. Maillard L, Gautier M. The reaction of amino acids with sugars: Mechanisms of melanoid formation. CR Seances Acad Sci. 1912;3:66-8.

346. Nursten HE. The Maillard reaction: chemistry, biochemistry and implications: Royal Society of Chemistry; 2007.

347. Shaklai N, Garlick RL, Bunn HF. Nonenzymatic glycosylation of human serum albumin alters its conformation and function. Journal of Biological Chemistry. 1984;259(6):3812-7.

348. Wu CH, Huang HW, Huang SM, *et al.* AGE-induced interference of glucose uptake and transport as a possible cause of insulin resistance in adipocytes. Journal of Agricultural and Food Chemistry. 2011;59(14):7978-84.

349. Guo CX, Zeng XJ, Song JJ, *et al.* A soluble receptor for advanced glycation end-products inhibits hypoxia/reoxygenation-induced apoptosis in rat cardiomyocytes via the mitochondrial pathway. International Journal of Molecular Sciences. 2012;13(9):11923-40.

350. Poulsen MW, Bak MJ, Andersen JM, *et al.* Effect of dietary advanced glycation end products on postprandial appetite, inflammation, and endothelial activation in healthy overweight individuals. European Journal of Nutrition. 2014;53(2):661-72.

351. Gragnoli G, Tanganelli I, Signorini AM, *et al.* Non-enzymatic glycosylation of serum proteins as an indicator of diabetic control. Acta Diabetologica Latina. 1982;19:161-6.

352. Miller JA, Gravallese E, Bunn HF. Nonenzymatic glycosylation of erythrocyte membrane proteins. Relevance to diabetes. Journal of Clinical Investigation. 1980;65(4):896-901.

353. Bunn HF, Gabbay KH, Gallop PM. The glycosylation of hemoglobin: Relevance to diabetes mellitus. Science. 1978;200:21-7.

354. Borg R, Kuenen J. HbA1c levels can be expressed as an estimated Average glucose (eAG). Clinical Laboratory International. 2009;33(1):14-6.

355. Miwa I, Maeda K, Okuda J. Anomeric compositions of D-glucose in tissues and blood of rat. Cellular and Molecular Life Sciences. 1978;34(2):167-9.

356. Leitch JM, Carruthers A. α-and β-Monosaccharide transport in human erythrocytes. American Journal of Physiology-Cell Physiology. 2009;296(1):C151-C61.

357. Okuda J, Taguchi T, Tomimura A. Mutarotation of d-glucose in body fluids and perfused rat liver. Chemical and pharmaceutical bulletin. 1987;35(10):4332-7.

358. Okuda J, Miwa I. Mutarotase effect on micro determinations of D-glucose and its anomers with β-D-glucose oxidase. Analytical Biochemistry. 1971;43(1):312-5.

359. Carruthers A, Melchior D. Transport of alpha- and beta-D-glucose by the intact human red cell. Biochemistry. 1985;24(15):4244-50.

360. Miwa I, Okuda J, Maeua K, *et al.* Mutarotase effect on colorimetric determination of blood glucose with β-D-glucose oxidase. Clinica Chimica Acta. 1972;37:538-40.

361. Okuda J, Miwa I, Maeda K, *et al.* Rapid and sensitive, colorimetric determination of the anomers of D-glucose with D-glucose oxidase, peroxidase, and mutarotase. Carbohydrate research. 1977;58(2):267-70.

362. Roohk HV, Zaidi AR. A review of glycated albumin as an intermediate glycation index for controlling diabetes. Journal of Diabetes Science and Technology. 2008;2(6):1114-21.

363. Graybill C, van Hoek AN, Desai D, *et al.* Ultrastructure of human erythrocyte GLUT1. Biochemistry. 2006;45(26):8096-107.

364. Kahn BB, Flyer JS. Regulation of glucose-transporter gene expression in vitro and in vivo. Diabetes Care. 1990;13:548-64.

365. Gornik O, Lauc G. Glycosylation of serum proteins in inflammatory diseases. Disease Markers. 2008;25(4-5):267-78.

366. Cohen RM, Franco RS, Joiner CH. Is poor glycemic control associated with reduced red blood cell lifespan? Diabetes Care. 2004;27(4):1013-4.

367. Ferrer-Lorente R, Cabot C, Fernández-López JA, *et al.* Combined effects of oleoyl-estrone and a b3-adrenergic agonist (CL316,243) on lipid stores of diet-induced overweight male Wistar rats. Life Sciences. 2005;77(16):2051-8.

368. Wallace TC. Health Effects of Coconut Oil—A Narrative Review of Current Evidence. Journal of the American College of Nutrition. 2019;38(2):97-107.

369. Takeuchi H, Matsuo T, Tokuyama K, *et al.* Diet-induced thermogenesis is lower in rats fed a lard diet than in those fed a high oleic acid safflower oil diet, a safflower oil diet or a linseed oil diet. The Journal of nutrition. 1995;125(4):920-5.

370. 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. 2013;8(4):e60407.

371. Ramirez I, Friedman MI. Dietary hyperphagia in rats: role of fat, carbohydrate, and energy content. Physiology & Behavior. 1990;47(6):1157-63.

372. Radcliffe J, Webster A. Regulation of food intake during growth in fatty and lean female Zucker rats given diets of different protein content. British Journal of Nutrition. 1976;36(03):457-69.

373. Hamilton C. Rat's preference for high fat diets. Journal of comparative and physiological psychology. 1964;58(3):459.

374. Kant AK, Andon MB, Angelopoulos TJ, *et al.* Association of breakfast energy density with diet quality and body mass index in American adults: National Health and Nutrition Examination Surveys, 1999–2004. The American journal of clinical nutrition. 2008;88(5):1396-404.

375. Bocarsly ME, Powell ES, Avena NM, *et al.* High-fructose corn syrup causes characteristics of obesity in rats: increased body weight, body fat and triglyceride levels. Pharmacology Biochemistry and Behavior. 2010;97(1):101-6.

376. Nascimento AIR, Ferreira HS, Saraiva R, *et al.* Central kappa opioid receptors modulate salt appetite in rats. Physiology & behavior. 2012;106(4):506-14.

377. Low YQ, Lacy K, Keast R. The role of sweet taste in satiation and satiety. Nutrients. 2014;6(9):3431-50.

378. Lewicka S, Nowicki M, Vecsei P. Effect of sodium restriction on urinary excretion of cortisol and its metabolites in humans. Steroids. 1998;63(7):401-5.

379. Moosavian SP, Haghighatdoost F, Surkan PJ, *et al.* Salt and obesity: a systematic review and meta-analysis of observational studies. International journal of food sciences and nutrition. 2017;68(3):265-77.

380. Díaz-Rúa R, Van Schothorst E, Keijer J, *et al.* Isocaloric high-fat feeding directs hepatic metabolism to handling of nutrient imbalance promoting liver fat deposition. International Journal of Obesity. 2016;40(8):1250.

381. Patsouris D, Reddy JK, Müller M, *et al.* Peroxisome proliferator-activated receptor α mediates the effects of high-fat diet on hepatic gene expression. Endocrinology. 2006;147(3):1508-16.

382. Pedram A, Razandi M, O'Mahony F, *et al.* Estrogen reduces lipid content in the liver exclusively from membrane receptor signaling. Science signaling. 2013;6(276):ra36.

383. Cao JJ, Gregoire BR. A high-fat diet increases body weight and circulating estradiol concentrations but does not improve bone structural properties in ovariectomized mice. Nutrition research. 2016;36(4):320-7.

384. Eyres L, Eyres MF, Chisholm A, *et al.* Coconut oil consumption and cardiovascular risk factors in humans. Nutrition reviews. 2016;74(4):267-80.

385. Williams G. Aromatase up-regulation, insulin and raised intracellular oestrogens in men, induce adiposity, metabolic syndrome and prostate disease, via aberrant ER-α and GPER signalling. Molecular and Cellular Endocrinology. 2012;351(2):269-78.

386. 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. 2015;10(3):e0119572.

387. Summermatter S, Santos G, Pérez-Schindler J, *et al.* Skeletal muscle PGC-1α controls whole-body lactate homeostasis through estrogen-related receptor α-dependent activation of LDH B and repression of LDH A. Proceedings of the National Academy of Sciences 2013;110:8738-43.

388. 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. British Journal of Nutrition. 2013;110(5):781-9.

389. Bröer S, Bröer A. Amino acid homeostasis and signalling in mammalian cells and organisms. Biochemical Journal. 2017;474(12):1935-63.

390. Cynober LA. Plasma amino acid levels with a note on membrane transport: characteristics, regulation, and metabolic significance. Nutrition. 2002;18(9):761-6.

391. Brosnan JT. Interorgan amino acid transport and its regulation. The Journal of nutrition. 2003;133(6):2068S-72S.

392. Rotondo F, Sanz T, Fernández-López J-A, *et al.* Stable isotope analysis of dietary arginine accrual and disposal efficiency in male rats fed diets with different protein content. RSC Advances. 2016;6(73):69177-84.

393. Malmberg SE, Adams CM. Insulin Signaling and the General Amino Acid Control Response Two distinct pathways to amino acid synthesis and uptake. Journal of Biological Chemistry. 2008;283(28):19229-34.

394. Waterlow J. The mysteries of nitrogen balance. Nutrition Research Reviews. 1999;12(1):25-54.

395. Hoffer LJ, Forse RA. Protein metabolic effects of a prolonged fast and hypocaloric refeeding. American Journal of Physiology-Endocrinology And Metabolism. 1990;258(5):E832-E40.

396. Wu G. Urea synthesis in enterocytes of developing pigs. Biochemical Journal. 1995;312(3):717-23.

397. 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 & function. 2016;7(3):1554-63.

Anexo

1.1 MÉTODO ALTERNATIVO PARA LA DETERMINACIÓN DE LA CARBAMOIL FOSFATO SINTETASA 1 EN TEJIDOS DE RATA

Introducción

La carbamoil fosfato sintetasa de tipo 1 es una enzima crítica en el control de la eliminación de nitrógeno amínico del organismo a través del ciclo de la urea; es la encargada de aportar uno de los dos nitrógenos que se incorporarán al ciclo para la biosíntesis de urea [1]. La carbamoil fosfato sintetasa 1 (EC 6.3.4.16) se localiza en la matriz mitocondrial [2] y usa NH_3 y CO_2 como sustrato para sintetizar el carbamoil fosfato, gastando 2 moles de ATP por mol de carbamoil fosfato producido [3]. Su actividad enzimática depende de la presencia de N-acetil-glutamato que actúa como activador alostérico [4].

A lo largo de los años se han descrito diversos métodos para la determinación de la actividad de la carbamoil fosfato sintetasa. El carbamoil fosfato es un compuesto muy inestable, que se convierte o degrada fácilmente a cianato. Por ello, la gran mayoría de métodos se han centrado en el acoplamiento de la reacción de síntesis del carbamoil fosfato con la de la ornitina transcarbamilasa para la producción de citrulina, cuya cuantificación se lleva a cabo por métodos radioquímicos [5] o colorimétricos [6]. En el pasado también se han usado métodos de análisis de subproductos como el ADP o mediante el acoplamiento a deshidrogenasas [3, 7]. Generalmente, el paso clave para la determinación de la actividad recae en la separación del carbamoil fosfato marcado radioactivamente de los productos resultantes, también marcados, o bien, de la ornitina generada en reacciones acopladas [8]. Sin embargo, dichos procesos generan problemas relacionados con la necesidad de utilizar diversas reacciones acopladas y con la disponibilidad de enzimas complementarias y sustratos marcados, lo que contribuye a la generación de variabilidad adicional así como a un incremento del tiempo necesario para la valoración al ser necesario utilizar procedimientos de separación.

Recientemente, hemos puesto a punto un método radioquímico que permite la determinación directa de la formación de carbamoil fosfato sin la necesidad de acoplar la reacción a la ornitina transcarbamilasa. La determinación se basa en eliminar el exceso del bicarbonato marcado del medio, el cual podría interferir en la cuantificación del carbamoil fosfato, generando un flujo controlado de gas $^{14}CO_2$ a partir de HCl y mármol ($CaCO_3$) con un generador de gas (Kipp) [9, 10]. Dicho método permite evaluar varias muestras de manera simultánea y con elevada sensibilidad, pues la cantidad de muestra requerida es pequeña. No obstante, requiere instrumentación específica y actuar a gran velocidad. Para evitar estos problemas se ha tratado de establecer un método que permita la determinación directa de la formación del carbamoil fosfato sin que se produzcan interferencias en la cuantificación y mediante la generación de un compuesto más estable que el carbamoil fosfato.

Materiales y Métodos

Esquema del método

Los homogenados de las muestras de tejido fueron incubados con cantidades en exceso de ATP, N-acetil-glutamato (activador alostérico), amonio y bicarbonato marcado con ^{14}C.

$$H^{14}CO_3^- + NH_4^+ + 2\ ATP \longrightarrow [CP\ Sintetasa\ 1] \longrightarrow {}^{14}CP + 2\ ADP + PO_3^{2}-$$

La reacción genera carbamoil fosfato marcado (inestable) y un exceso de bicarbonato marcado en el medio de incubación. La adición de hidroxilamina y la exposición a un medio ligeramente ácido y a temperaturas elevadas permite la conversión del carbamoil fosfato a hidroxiurea, un compuesto altamente estable a la temperatura elevada y pH ácido utilizado.

$$^{14}CP + hidroxilamina \longrightarrow 95ºC \longrightarrow {}^{14}hidroxiurea + H_2O$$

El exceso de bicarbonato marcado, que podría interferir en la cuantificación, es evaporado en su totalidad mediante la exposición de los tubos de reacción a temperaturas cercanas a la ebullición. Por ello, y ante la rápida evaporación del CO_2 marcado, es necesario extremar las medidas de protección, trabajando bajo campana extractora de gases. La estabilidad de la hidroxiurea permite su cuantificación y, de esta manera, estimar la cantidad de carbamoil fosfato formado tras la incubación de la muestra.

Preparación de la muestra

Se homogenizaron muestras de 50 mg de tejido de rata (congeladas a -80ºC hasta su utilización) en 500 µL de tampón Hepes 70 mM pH 7,4 usando un disruptor celular (IKA, Stauffen, Alemania). Una vez se obtiene el homogenado, y para llevar a cabo la incubación, puede ser necesario hacer una dilución adicional, concretamente 1/5 para el hígado y 1/2 para el tejido adiposo blanco; en el caso del tejido adiposo marrón y el intestino no se requiere ninguna dilución adicional.

Incubación

Las incubaciones se llevaron a cabo a 37ºC, usando un termobloque, en tubos de polietileno (tubos Eppendorf) de 1,5 mL de capacidad durante 0, 8 y 16 minutos. La mezcla de incubación contiene ATP-Na_2, N-acetil glutamato y acetato de magnesio (Sigma-Aldrich, St Louis, MO, EEUU). Las concentraciones finales fueron, respectivamente, 20 mM, 5 mM y 20 mM. Con el objetivo de eliminar la variabilidad intrínseca de la manipulación, cada muestra se incubó en un solo tubo, que contenía un volumen de la mezcla de incubación suficiente para cubrir cada uno de los tiempos de incubación (0, 8, 16 minutos).

Tras mezclar 200 µL de homogenado de tejido con 400 µL de medio de incubación, se inició la reacción al añadir 200 µL de una solución de bicarbonato amónico y ^{14}C-bicarbonato sódico (Perkin Elmer, Bad Neuham, Alemania); la concentración final de bicarbonato fue de 50mM, con una cantidad de radioactividad de 5kBq / tubo. La reacción se detuvo a cada uno de los tiempos de incubación establecidos al pasar 200 µL de reacción a tubos mantenidos en hielo que contenían 30 µL de hidroxilamina-HCl 2 M. Rápidamente se agitaron y se llevaron a 95ºC hasta la total evaporación de la fase líquida. En este proceso, todo el carbamoil fosfato marcado generado se convirtió a hidroxiurea marcada, que permaneció en el tubo en forma de precipitado. La cantidad de radiactividad de los tubos se cuantificó tras dejarlos reposar durante 12h (para eliminar el exceso de fluorescencia) en líquido de centelleo. Se incluyeron tubos de control en los que el homogenado de tejido se sustituyó por el mismo volumen de tampón de homogenado.

Cálculos de la actividad enzimática

Los tubos correspondientes a los blancos y a los tiempos cero, proporcionaron contajes extremadamente bajos, que fueron considerados como ruido de fondo y descontados de los valores obtenidos en las muestras incubadas. La cantidad de carbamoil fosfato producido fue estimada a partir de las cuentas netas obtenidas y de la actividad específica del bicarbonato marcado.

Resultados

En la figura 1 se muestra la correlación positiva entre la presencia de radiactividad (dpm) correspondiente a la síntesis de carbamoil fosfato marcado, y el tiempo de incubación de las muestras de homogenados de distintos tejidos.

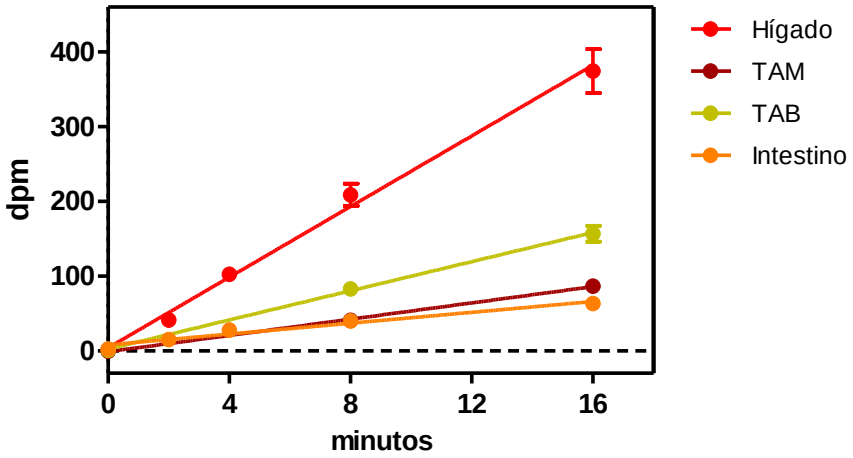


Figura 1. Relación entre la síntesis de carbamoil fosfato y el tiempo de incubación en muestras de hígado, tejido adiposo marrón, tejido adiposo blanco e intestino de rata. Los datos representados equivalen al promedio ± error estándar de triplicados. Linealidad: hígado (dilución 1/50; R^2=0,996, *p*=0,0001); tejido adiposo marrón (TAM) (dilución 1/10; R^2=0,999 X, *p*=0,0175), tejido adiposo blanco (TAB) (dilución 1/10; R^2=0,0999; *p*=0,0188) intestino (dilución 1/10; R^2=0,965, *p*=0,0028).

El hígado presenta una síntesis de carbamoil fosfato mayor respecto a los tejidos adiposos y al intestino, y en consecuencia mayor actividad. Los datos obtenidos se corresponden con la mayor actividad y funcionalidad del ciclo de la urea descrita en el hígado.

Discusión

El método presentado tiene la ventaja de ser simple, rápido y sensible; se requieren pocos mg de tejido para el análisis y, en caso de ser necesario, permite incrementar la sensibilidad mediante una menor dilución de las muestras o un incremento de la actividad específica del bicarbonato de la mezcla de reacción. Además, se caracteriza por proporcionar una linealidad y repetitividad buena en los diferentes tejidos testados, requiriendo en todos los casos una manipulación mínima del medio de incubación.

Las condiciones de incubación y homogeneización de este método se asemejan a las descritas en otros métodos previamente publicados, en las que la mayor limitación es la estabilidad a largo plazo de los homogenados.

La inestabilidad del carbamoil fosfato es una de las principales limitaciones para la cuantificación de la actividad de la carbamoil fosfato sintetasa. En el método aquí descrito, dicha limitación se ha solventado mediante la conversión del carbamoil fosfato a otro producto más estable [11], puesto que ante la presencia de hidroxilamina y de temperaturas elevadas más del 98% del carbamoil fosfato se convierte a hidroxiurea. [12] Además, aunque el 2% restante del carbamoil fosfato se degrada a cianato, este también se convierte en hidroxiurea mediante la acción de la hidroxilamina [11, 13] permitiendo, por consiguiente, la determinación completa y directa de todo el carbamoil fosfato formado en la reacción, sin pérdidas del mismo debido a los procesos de degradación.

Las principales ventajas del método son, que no requiere acoplar la reacción de la carbamoil fosfato sintetasa a la de la ornitina transcarbamilasa (evitando así una medición indirecta mediante la producción de citrulina [5] [6]), y que tampoco depende de reacciones complejas que impliquen instrumentos específicos para la producción controlada de gas marcado radiactivamente. [10]

Finalmente, los niveles de actividad encontrados en los tejidos de rata usando el método descrito aquí son similares a los encontrados cuando se ha utilizado el método radioquímico de determinación directa mediante la generación de un flujo controlado de CO_2 marcado.

Referencias

1. Chabas, A., S. Grisolia, and R. Silverstein, On the mechanism of carbamoylphosphate synthetase. European journal of biochemistry, 1972. **29**(2): p. 333-342.
2. Clarke, S., A major polypeptide component of rat liver mitochondria: carbamyl phosphate synthetase. Journal of Biological Chemistry, 1976. **251**(4): p. 950-961.
3. Rubio, V., G. Ramponi, and S. Grisolia, Carbamoyl phosphate synthetase I of human liver. Purification, some properties and immunological cross-reactivity with the rat liver enzyme. Biochimica et Biophysica Acta (BBA)-Enzymology, 1981. **659**(1): p. 150-160.
4. Cheung, C.-W. and L. Raijman, The regulation of carbamyl phosphate synthetase (ammonia) in rat liver mitochondria. Effects of acetylglutamate concentration and ATP translocation. Journal of Biological Chemistry, 1980. **255**(11): p. 5051-5057.
5. Lof, C., R. Wanders, and A. Meijer, Activity of carbamoyl-phosphate synthetase (ammonia) in isolated rat-liver mitochondria: cycling of carbamoyl phosphate in the absence of ornithine. European journal of biochemistry, 1982. **124**(1): p. 89-94.
6. Pierson, D.L., A rapid colorimetric assay for carbamyl phosphate synthetase I. Journal of biochemical and biophysical methods, 1980. **3**(1): p. 31-37.
7. Guthöhrlein, G. and J. Knappe, Structure and Function of Carbamoylphosphate Synthase: 1. Transitions between two Catalytically Inactive Forms and the Active Form. European journal of biochemistry, 1968. **7**(1): p. 119-127.
8. Christopherson, R.I. and L.R. Finch, A radioisotopic method for the assay of aspartate carbamoyltransferase and carbamoyl phosphate. Analytical biochemistry, 1976. **73**(2): p. 342-349.
9. Griffin, J.J., Chemical reactions: A popular manual od experimental chemistry. Second division: Non-metallic elements. 1860, London: Griffin J.J. and Griffin R.
10. Arriarán, S., et al., A radiochemical method for carbamoyl-phosphate synthetase-I: Application to rats fed a hyperproteic diet. Journal of Enzyme Research, 2012. **3**(1): p. 29-33.
11. Kofod, H., On the Isomerism of Hydroxyurea. Acta Chemica Scandinavica, 1955. **9**(3): p. 455-470.
12. Levine, R.L., Kretchmer, N., Conversion of carbamoyl phosphate to hydroxyurea: An assay for carbamoylphosphate synthetase. Analytical biochemistry, 1971. **42**(2): p. 324-337.
13. Kofod, H., Furfuryl Mercaptan. Acta Chemica Scandinavica, 1953. **7**: p. 1302-1306.

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

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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.

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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

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[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.

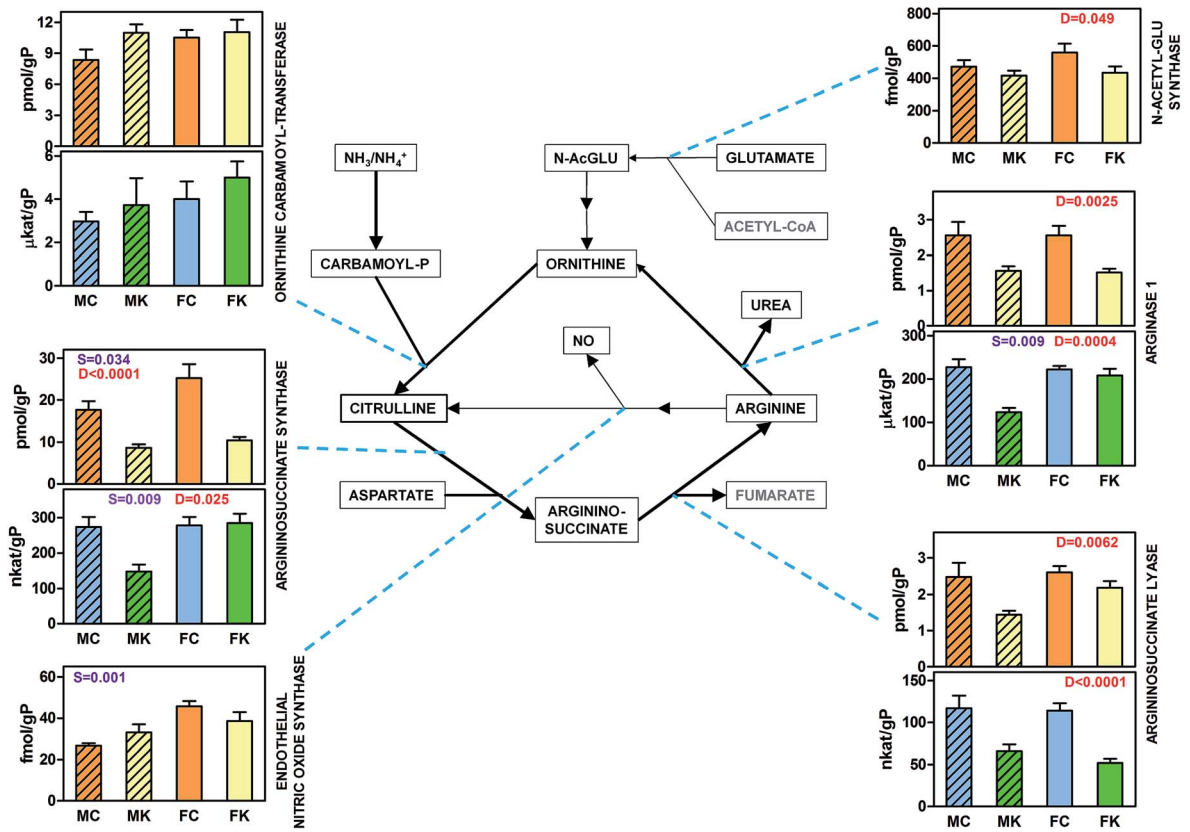

Fig. 1 Gene expressions and enzyme activities of the urea cycle in the liver of male and female rats fed control or cafeteria diet for 30 days. The data correspond to the mean ± sem of 6 different animals, and are all expressed per gram of tissue protein (gP). Statistical analysis of the differences 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

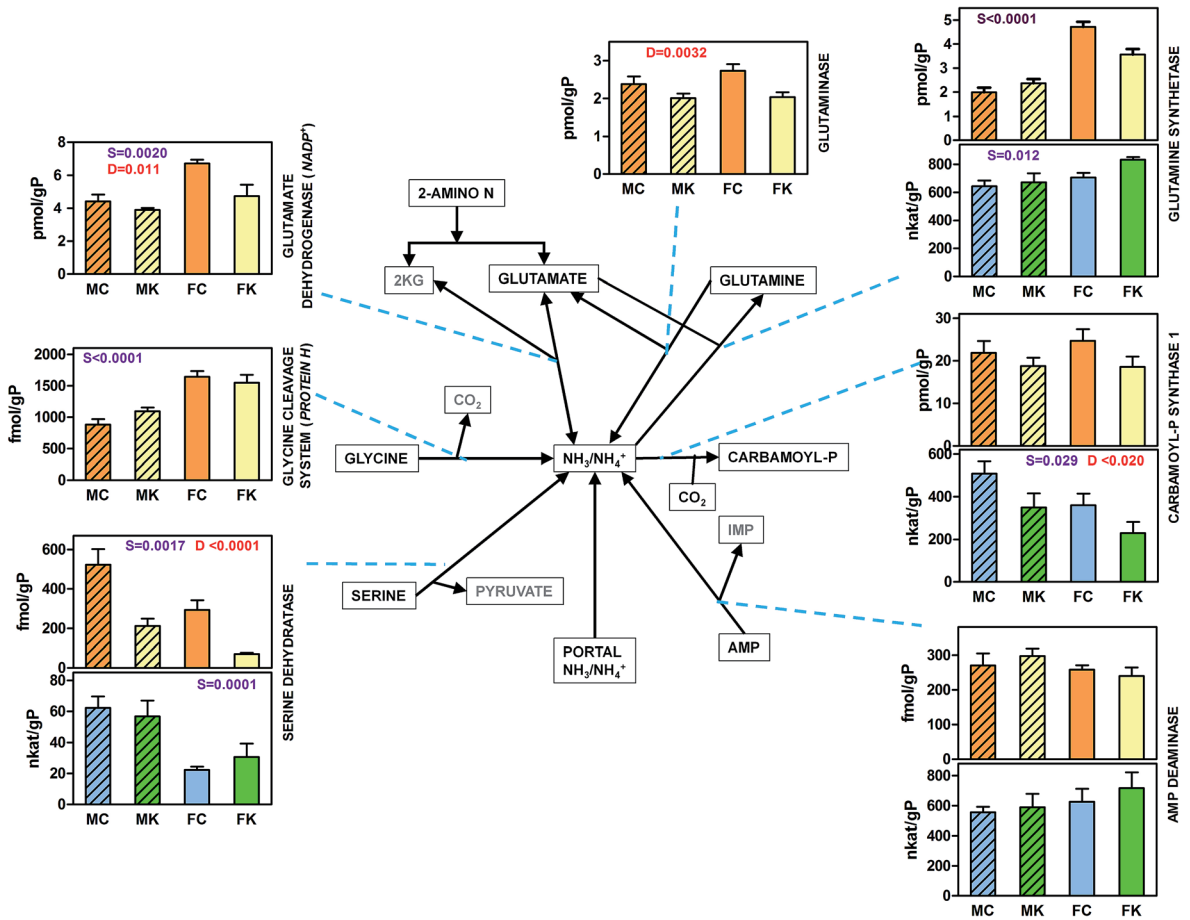

Fig. 2 Gene expressions and enzyme activities of enzymes related to ammonia/ammonium metabolism in the liver of female and male rats fed control or cafeteria diet for 30 days. The data correspond to the mean ± sem of 6 different animals, and are expressed per gram of tissue protein (gP). Statistical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

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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.