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THE ROLE OF KLF4 AND PREGNANCY UNDER HFD ON ENERGY HOMEOSTASIS

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The role of KLF4 and pregnancy under HFD on energy homeostasis

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The role of KLF4 and pregnancy under HFD on energy homeostasis

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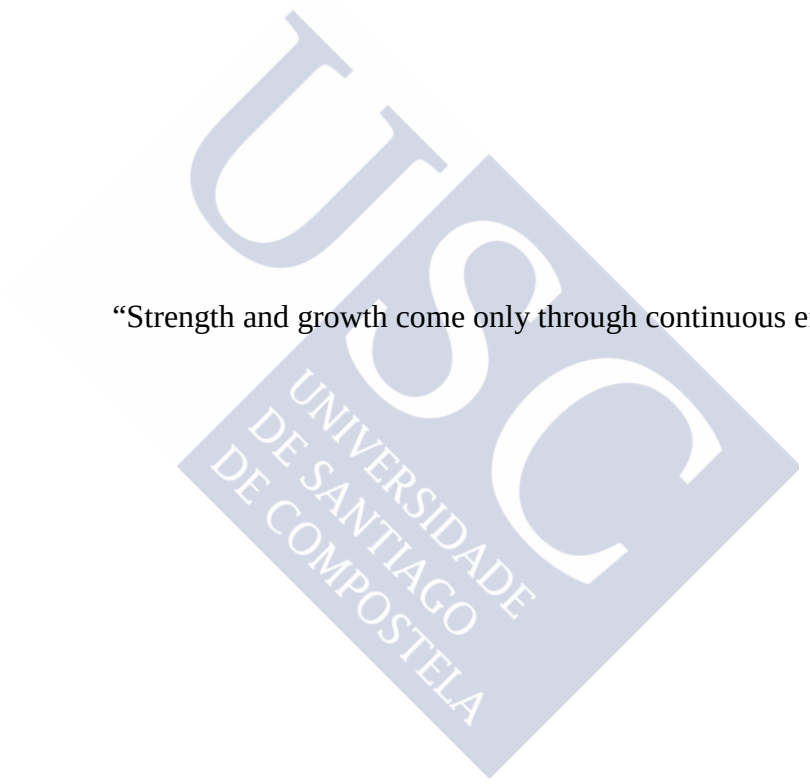

“Strength and growth come only through continuous effort and struggle”

Napoleon Hill

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A mis padres

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RESUMO

Nesta tese, mostramos que no hipotálamo **KLF4** representa un novo factor de transcripción que modula específicamente a expresión da proteína relacionada co AgRP *in vivo.* O KLF4 hipotalámico localízase xunto coas neuronas AgRP e está modulado polo estado nutricional e a leptina. A sobreexpresión de KLF4 no núcleo arcuato é suficiente para incrementar a inxesta e inhibir a acción anorexixénica da leptina dunha maneira independente de FoxO1. Ademáis o KLF4 hipotalámico é regulado pola leptina en ratas baixo dieta normal pero non en ratas con obesidade inducida pola dieta, e KLF4 non afecta á resistencia á leptina inducida por dieta alta en graxa. **O embarazo** é un estado metabólico especial onde quixemos caracterizar o impacto da dieta antes e durante o embarazo. Atopamos que independentemente do tipo de dieta no último estadío da preñez: os niveis de glucosa séricos diminúen, hai un incremento nos niveis séricos dos triglicéridos, as temperaturas do tecido adiposo marrón e do corpo vense diminuídas, e a mobilización lipídica no tecido adiposo marrón vese incrementada. **Palabras chave:** KLF4, AgRP, inxesta, leptina, embarazo.

RESUMEN

En esta tesis, mostramos que el **KLF4** hipotalámico representa un nuevo factor de transcripción que modula específicamente la expresión de AgRP *in vivo*. El KLF4 hipotalámico se colocaliza con las neuronas AgRP y está modulado por el estado nutricional y la leptina. La sobreexpresión de KLF4 en el núcleo arcuato es suficiente para incrementar la ingesta e inhibir la acción anorexigénica de la leptina de una manera independiente de FoxO1. Además el KLF4 hipotalámico es regulado por laleptina en ratas bajo dieta normal pero no en ratas con obesidad inducida por la dieta, y KLF4 no afecta a la resistencia a la leptina inducida por dieta alta en grasa. **El embarazo** es un estado metabólico especial en donde hemos querido caracterizar el impacto de la dieta antes y durante el embarazo. Encontramos que independientemente del tipo de dieta en el último estadío de la preñez: los nivels de glucosa séricos están disminuídos, hay un incremento en los niveles séricos de triglicéridos, las temperaturas del tejido adiposo marrón y el cuerpo se ven disminuídas, y la mobilización lipídica en el tejido adiposo marrón se ve incrementada. **Palabras clave**: KLF4, AgRP, ingesta, leptina, embarazo.

ABSTRACT

In this thesis we report that hypothalamic **KLF4** represents a new transcription factor specifically modulating AgRP expression *in vivo*. Hypothalamic KLF4 colocalizes with AgRP neurons and is modulated by nutritional status and leptin. KLF4 over-expression in the arcuate nucleus is sufficient to increase food intake and to blunt the anorectic action of leptin in a FoxO1-independent manner. Moreover hypothalamic KLF4 is regulated by leptin in rats fed a chow diet but not in diet induced obese rats, and KLF4 does not affect high fat diet-induced leptin resistance. **Pregnancy** is a special metabolic state and we wanted to characterize the impact of diet before and during pregnancy. We found that independently of the type of diet in late pregnancy: glucose serum levels were decreased, there is an increase in triglyceride serum levels, brown adipose tissue and body temperature are decreased and lipid mobilization is increased in brown adipose tissue. **Keywords:** KLF4, AgRP, food intake, leptin, pregnancy.

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RESUMEN

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RESUMEN

La homeostasis energética promueve la estabilidad con respecto a la cantidad de energía corporal almacenada en forma de grasa. Esta estabilidad se basa en el equilibrio entre las calorías ingeridas y el gasto de energía. El éxito de la homeostasis depende de que el cerebro sea capaz de leer, interpretar e integrar una amplia gama de señales que describen el estado nutricional del animal y su entorno inmediato, y hacer ajustes apropiados en la ingesta de alimentos y el gasto energético como resultado de la información recibida. La ingesta de alimentos es regulada a dos niveles diferentes: señales periféricas y sistema nervioso central.

El control central de la ingesta de alimentos es regulado por múltiples áreas cerebrales, siendo la más estudiada el hipotálamo, que está compuesto por varios núcleos. Uno de ellos es el núcleo arcuato (ARC). Las neuronas ARC expresan el péptido relacionado con el agoutí (AgRP) y el neuropéptido Y (NPY), que son neuropéptidos orexigénicos y también expresan pro-opiomelanocoritna (POMC) y transcripción regulada por la anfetamina y la cocaína (CART), que son neuropéptidos anorexigénicos.

La leptina es una hormona circulante polipeptídica secretada por el tejido adiposo blanco (WAT) de tal manera que sus niveles plasmáticos se correlacionan con la cantidad de triglicéridos presentes en el tejido adiposo. Los niveles de leptina en la sangre disminuyen cuando el consumo de energía es limitado y las reservas energéticas en forma de grasa disminuyen. La caída de los nieveles de leptina durante la inanición promueve una mayor ingesta de energía para disminuir el gasto energético. Dentro del cerebro, la principal isoforma de los receptores de leptina es LEPRb y está implicada en la transducción de señales que regulan los efectos reductores de peso e ingesta de la leptina. Una de las vías más importantes en la regulación de la leptina a través de LEPRb es la del transductor de señales y el activador de la transcripción (STAT3), que es el principal regulador de la transcripción de genes relevantes como el que codifica POMC, AgRP o incluso al supresor de señalizacion de citoquinas 3 (SOCS3) que media la inhibición de la señalización de LEPRb-STAT3. También la fosfoinositida 3-quinasa (PI3K) juega un papel importante en el control central de la leptina, actuando de manera independiete, a parte de la vía de STAT3.

El tejido adiposo marrón se caracteriza y se diferencia del WAT por tener adipocitos con forma poligonal y pequeñas gotas lipídicas en el citoplasma, un núcleo central y un gran número de mitocondrias en el citoplasma que le proporciona ese color marrón característico. Los lípidos son su principal fuente de combustible para la fosforilación oxidativa y la producción de calor. Tiene una capacidad oxidativa muy alta, utilizando la oxidación de ácidos grasos a través del ciclo de ácido tricarboxílico para la producción de calor en lugar de la producción de ATP; esto se denomina desacoplamiento. UCP1 es una proteína de canal de protones que pertenece a la familia de transportadores mitocondriales, que es responsable del desacoplamiento del gradiente de protones.

El estrés del retículo endoplasmático (ER) consiste en una acumulación de proteínas mal plegadas en la luz del retículo endoplasmático. El retículo endoplasmático es el orgánulo responsable del plegamiento de las proteínas. Bajo estrés del ER, las proteínas dejan de doblarse adecuadamente. La acumulación de proteínas mal plegadas promueve la activación de la respuesta de proteína desplegada (UPR) que consiste en la degradación de proteínas mal plegadas y un aumento en la actividad de las chaperonas responsables del plegamiento de proteínas, como la proteína de inmunoglobulina de unión (BIP). Hay tres proteínas transmembrana responsables de activar el UPR: el factor de iniciación eucariota ER 2

dependiente del ARN quinasa dependiente de ARN (PERK), factor de transcripción activador 6 (ATF6) y proteína de señalización del ER al núcleo que requiere inositol 1 (IRE).

El factor 4 similar a Krüppel (KLF4) es un factor de transcripción que regula múltiples funciones biológicas, incluída la proliferación celular, la diferenciación, la apoptosis, el metabolismo, la inflamación, la embriogénesis y la tumorogénesis. KLF4 se expresa predominantemente en el tracto gastrointestinal (especialmente en el colon y el intestino delgado), pero también en el pulmón, la piel, los testículos, el timo, la córnea, los miocitos cardíacos, los linfocitos y las células madre embrionarias. En el cerebro, KLF4 se expresa en las células madre neuronales y es crítico para la diferenciación neuronal. Recientemente se demostró que KLF4 está implicado en la regulación de AgRP *in vitro.*

El embarazo es un estado de cambio fisiológico en el que las vías metabólicas se centran en ganar y no perder el excedente de energía. Es bien sabido que las ratas embarazadas son hiperfágicas y aumentan de peso durante el embarazo. El embarazo es un estado diabetogénico en el cual la glucosa y la diabetes tipo II están asociadas con el aumento de peso gestacional. Durante el embarazo, hay una acumulación materna de depósitos de grasa e hiperlipidemia debido a la utilización de estos depósitos, asociada con una mayor cantidad de triglicéridos, fosfolípidos y colesterol en plasma. En cuanto a la termorregulación, ni la hiperfagia ni el frío estimulan la termogénesis en ratas preañadas. La obesidad materna puede aumentar la posibilidad de tener diabetes mellitus gestacional (GMD), aborto espontáneo, muerte fetal intrauterina, enfermedad de hígado graso no alcohólica, parto por cesárea y preeclampsia.

En el capítulo 1, quisimos comprobar si KLF4 estaba involucrado en la regulación de la leptina, ya que esta hormona es conocida por modular la excitabilidad intrínseca de las neuronas AgRP/NPY en el hipotálamo. El estudio de esta hipótesis establece la relevancia de KLF4 como un factor de transcripción *in vivo* implicado en la homeostasis energética. Más específicamente, dos líneas de evidencia respaldan esta conclusión: primero, el KLF4 hipotalámico está regulado por el estado nutricional y por la leptina de manera independiente de FoxO1; y en segundo lugar, la sobreexpresión mediada por el virus de KLF4 desencadena un aumento en los niveles de AgRP y mitiga la acción anoréxica de la leptina en ratas delgadas. La importancia de las neuronas AgRP en el control del balance energético ha llevado a varias investigaciones de sus mecanismos de acción, proyecciones hacia y desde otras poblaciones neuronales, y las vías de señalización que modulan la expresión de AgRP. El factor de transcripción KLF4 es uno de los activadores de AgRP que se han descubierto más recientemente. Los estudios *in vitro* que implican la sobreexpresión o el silenciamiento de KLF4 han demostrado que se requiere KLF4 para la activación de AgRP. Se ha descrito que un compuesto llamado PMI-5011 activa tanto la expresión de KLF4 como la de AgRP *in vivo*; sin embargo, PMI-5011 también activa la orexina hipotalámica y MCH, estimulando la ingesta de alimentos por esa ruta. Dos cuestiones importantes han quedado sin respuesta: primero, en qué tipos de células KLF4 se encuentra dentro del hipotálamo y, segundo, si la manipulación específica de KLF4 *in vivo* es suficiente para controlar la ingesta de alimentos estimulada por AgRP, la partición nutricional y el peso corporal. Nosotros describimos que KLF4 se encuentra predominantemente en el ARC y, dentro de este lugar hipotalámico, se localiza en las neuronas AgRP. Nuestros datos también muestran que los niveles de proteína KLF4 en el ARC aumentan después del ayuno y que se regulan negativamente con la leptina.

Para demostrar aún más la capacidad de KLF4 para modular AgRP *in vivo*, inyectamos vectores virales que sobreexpresan KLF4 en el ARC, y descubrimos que los animales que

portan estos virus comían más y ganaban más peso que sus controles, pero que ni el gasto energético ni el cociente respiratorio cambiaron significativamente. Esta ingesta de alimentos inducida por KLF4 solo podría explicarse por niveles elevados de AgRP, porque los niveles de otros neuropéptidos relevantes conocidos en el ARC como NPY, CART o POMC, no se vieron afectados por la sobreexpresión de KLF4. Curiosamente, encontramos que los niveles de las enzimas que sintetizan GABA GAD65 y GAD67, así como el transportador vesicular GABA (VGAT) se redujeron en el ARC después de la sobreexpresión de KLF4. Dado que los ratones que carecen de transportador vesicular de GABA en las neuronas AgRP son delgados y resistentes a la obesidad, inicialmente esperábamos que la sobreexpresión de KLF4 aumentara la síntesis y/o el transporte de GABA. Sin embargo, los niveles disminuidos de GAD65, GAD67 y VGAT observados en el ARC después de la sobreexpresión de KLF4 sugieren que: a) las acciones de KLF4 en la alimentación y el peso corporal no implican la señalización de GABA; y b) la disminución en la síntesis y el transporte de GABA después de la sobreexpresión de KLF4 podría deberse a una respuesta compensatoria. Además, la administración de vectores virales que silenciaron KLF4 en el ARC no provocó diferencias en la ingesta de alimentos o el peso corporal, y la respuesta inducida por el ayuno observada en los animales control se mitigó. Además, este impedimento de respuesta al ayuno fue mediado específicamente por AgRP, ya que el silenciamiento de KLF4 provocó una disminución significativa en los niveles de AgRP hipotalámicos inducidos por el ayuno. Es importante destacar que la ingesta de alimentos inducida por el ayuno se vio comprometida en las ratas delgadas y DIO, lo que indica que KLF4 es un modulador importante de la respuesta de ayuno normal, independientemente del tipo de dieta. En conjunto, estos nuevos datos apoyan los previos resultados *in vitro* e indican que KLF4 es un activador específico de las neuronas AgRP, modulando sus acciones biológicas *in vivo*.

Anteriormente se sabía que las neuronas AgRP desempeñaban un papel fundamental en la mediación de las acciones de la leptina. También se hipotetizó a cerca de que el incremento del KLF4 hipotalámico inducido por el ayuno, un estado hipoleptinémico, probablemente estaban regulados por los niveles de leptina. Específicamente, mostramos que la inhibición de KLF4 causada por la leptina estaba mediada tanto por STAT3 como por PI3K, dos moduladores clave de su acción anorexígena. Estos datos indican que KLF4 forma parte de la ruta de señalización de la leptina, y que está situado debajo de STAT3 y PI3K en la cascada de señalización. Con el fin de investigar el papel funcional de KLF4 en la acción de la leptina, administramos leptina en ratas inyectadas previamente con un virus vacío o con un vector viral que sobreexpresaba KLF4, en el ARC. Nuestros resultados indican que la activación de KLF4 en el ARC es suficiente para eliminar el efecto anorexígeno de la leptina en ratas delgadas, y por lo tanto que KLF4 en el ARC es un componente esencial de la vía de señalización de la leptina que controla la ingesta de alimentos. Dado que FoxO1 media los efectos dependientes de AgRP de la leptina sobre la ingesta de alimentos, supusimos que KLF4 podría estar interactuando con FoxO1. Sin embargo, la leptina aún fue capaz de aumentar FoxO1 en el ARC de las ratas a las que se les inyectó el vector viral que sobreexpresa KLF4, y también fue capaz de estimular KLF4 cuando se inhibía FoxO1 en el ARC. Por lo tanto, nuestros resultados indican colectivamente que KLF4 no es una diana directa de FoxO1, y sugieren que cada factor de transcripción funciona de forma independiente para modular la sensibilidad a la leptina.

Es probable que la resistencia del SNC a la leptina contribuya al aumento de peso asociado con obesidad inducida por dieta (DIO), y se ha demostrado que existe una disminución de la señalización de la leptina en los roedores con DIO. Por lo tanto,

formulamos la hipótesis de que la disminución de KLF4 en el ARC de ratas con DIO, sería capaz de revertir la resistencia a la leptina inducida por HFD. De acuerdo con esta hipótesis, encontramos que la leptina administrada periféricamente no logró disminuir los niveles hipotalámicos de KLF4 en ratas con DIO, y esto podría explicar la falta de efecto de la leptina sobre la ingesta en ratas con DIO. Sin embargo, la leptina no fue capaz de reducir la ingesta de alimentos en ratas tratadas con un vector viral que silencia KLF4 en el ARC. Estos últimos datos muestran que KLF4 no mejora la resistencia a la leptina periférica inducida por HFD. Una posible explicación de este efecto podría ser que la resistencia a la leptina está mediada por multitud de factores que incluyen: a) alteraciones en el transporte de leptina a través de la barrera hematoencefálica; b) alteraciones en la expresión génica del receptor de la leptina, y en endocitosis y tráfico de receptores de superficie celular activados por ligando; y c) alteraciones en la vía de señalización de la leptina. Por lo tanto, no podemos descartar por completo la posibilidad de que KLF4 pueda restaurar algunos de los componentes implicados en la resistencia a la leptina inducida por HFD y también de que represente un objetivo potencial para restablecer la sensibilidad neuronal a la leptina.

Otra hipótesis nos lleva a investigar si KLF4 está involucrado en la vía de señalización de la ghrelina en el hipotálamo ya que estudios *in vitro* mostraron que KLF4 puede controlar directamente la actividad del promotor de AgRP y que la ghrelina es conocida por aumentar los niveles de AgRP y el peso corporal. Cuando inyectamos la ghrelina ICV, no vimos diferencias en los niveles proteícos hipotalámicos de KLF4, PPARγ (un activador de KLF4) o RXRα (que forma un heterodímero con PPARγ). Por lo tanto, llegamos a la conclusión de que KLF4 no participa en la señalización de la ghrelina en el hipotálamo.

En resumen, este estudio establece la relevancia de KLF4 como un factor de transcripción *in vivo* implicado en la homeostasis energética. Este papel se debe a que se expresa predominantemente en las neuronas AgRP. Curiosamente hemos demostrado que este factor de transcripción es un componente esencial de la vía de señalización de la leptina, aunque no es un objetivo directo de FocO1. Contrariamente a nuestra hipótesis, no pudimos documentar una mejora de la resistencia a la leptina inducida por HFD cuando modificamos KLF4 en el ARC.

En el capítulo 2 quisimos investigar la interacción entre la dieta y el embarazo en rutas metabólicas de la madre en hígado y BAT.

Con respecto a nuestro primer grupo experimental en donde las ratas gestantes ingirieron dieta estándar antes y durante el embarazo (SDSD), observamos una disminución en los niveles de glucosa sérica en ratas gestantes y también observamos que esta disminución es independiente del tipo de dieta, ya que disminuye en las ratas que han sido alimentadas con una dieta normal antes del embarazo y una dieta alta en grasa durante el embarazo en donde los controles son ratas no embarazadas bajo dieta estándar (SDHFD) y ratas alimentadas con dieta alta en grasas antes y durante el embarazo siendo sus controles, ratas no preñadas alimentadas con dieta alta en grasa (HFDHFD), ya que la glucosa es el principal combustible para el crecimiento del feto y sus necesidades energéticas son muy altas hacia el final de la gestación.

Los niveles de triglicéridos en el suero de ratas gestantes SDSD aumentan comparado con ratas no gestantes, y no se ven afectados por la dieta ya que los niveles séricos de TG también aumentan en las ratas SDHFD y HFDHFD. Las ratas gestantes SDSD no mostraron diferencias con las ratas control, respecto a los niveles de FFA séricos y los nivels de TG

hepáticos. También los datos de FFA en SDHFD y HFDHFD están correlacionados con los datos de TG ya que aumentan en ambas dietas.

. Observamos también un aumento en el peso del hígado de las ratas gestantes en los tres grupos experimentales alimentados con diferentes dietas. Los niveles de triglicéridos en el hígado de ratas preñadas SDSD no difirieron con los niveles en ratas control no preñasdas, viéndose incrementados en los hígados de ratas preñadas SDHFD y HFDHFD como resultado de una dieta alta en grasa.

Como la producción hepática de FGF21 aumenta en la última fase de la preñez del ratón, queríamos comprobar si sucedía lo mismo en la rata y para ello, medimos los niveles séricos de FGF21 y observamos un aumento significativo de los mismos en cada uno de los 3 grupos a estudio; siendo observados los niveles más altos en el grupo HFDHFD. Con respecto a la cascada de señalización PERK/eIF2α, observamos un aumento en ambos niveles de proteína en el hígado de ratas gestantes SDSD. No obstante, en las ratas preñadas SDHFD vemos un aumento en los niveles de pPERK en el hígado, pero no en los niveles de peIF2a/eIF2a. Con respecto a las ratas preñadas HFDHFD, no hay diferencias significativas en ninguna de las dos proteínas.

Con respecto a los niveles de estrés del retículo endoplasmático en el hígado, los niveles de caspasa 3 se vieron incrementaron en las ratas SDSD y HFDHFD preñadas, pero no en las ratas SDHFD preñadas. Sorprendentemente, detectamos una disminución en los niveles de CHOP, en ratas SDSD y SDHFD preñadas y un aumento cuando comparamos ratas no preñadas bajo HFD, con ratas HFDHFD preñadas. Observamos una disminución en los niveles de proteína hepática XBP1 y no detectamos diferencias respecto a los niveles de pIRE1α/IRE1α en ratas preñadas SDSD, mientra que ambos niveles proteicos se vieron disminuídos en ratas preñadas SDHFD y sólo XBP1 se vio disminuido en las ratas preñadas HFDHFD.

FGF21 es una diana de los PPARs ya que los agonistas de PPARα y PPARγ elevan los niveles de ARNm de FGF21 en el hígado. FXR es un factor de transcripción activado por ligando, que regula el metabolismo de los ácidos grasos, y su agonista el ácido obeticólico, reduce la inflamación hepática y la fibrosis. Además, los ligandos de FXR regulan la expresión del gen de PPARγ. Decidimos medir los niveles de ARNm de PPARα y encontramos un aumento de los niveles de ARNm en el hígado de las ratas gestantes lo cual cuadra con el aumento de los niveles de ARNm hepático y sérico de FGF21 en ratas preñadas SDSD y SDHFD. Sin embargo, estos niveles no se vieron aumentados en las ratas preñadas HFDHFD, aunque el ARNm hepático y los niveles séricos de FGF21 sí que se vieron aumentados en estas ratas. También se sabe que los activadores naturales y sintéticos de FXR aumentan la expresión y secreción del gen FGF21. En este sentido, encontramos una disminución en los niveles de ARNm de FXR en el hígado de los tres grupos a estudio. Esto, junto con el hecho de que FGF21 induce la activación del BAT y que encontramos que la temperatura tanto del BAT como corporal de las ratas gestantes se regula negativamente, nos conduce a la hipótesis de la existencia de una resistencia a FGF21 durante el embarazo.

Nuestros resultados sugieren que la lipogénesis de novo se activa en ratas preñadas SDSD y SDHFD para aumentar los depósitos de grasa materna. Con respecto al grupo HFDHFD, los marcadores de la lipogénesis de novo se encuentran regulados negativamente y el contenido de grasa epididimal y omental no difiere con respecto a las ratas no gestantes HFDHFD.

No hemos detectado diferencias en los niveles de proteína hepática LPL en ratas gestantes SDSD y cuando observamos los niveles de LPL en ratas preñadas SDHFD y HFDHFD, observamos una disminución. A este respecto, cabe destacar que los niveles proteicos del ratio pHSL/HSL disminuyeron en el hígado de ratas gestantes SDSD, lo que significa una disminución en la movilización y β-oxidación de triglicéridos en el hígado, ya que no existe tal acumulación de triglicéridos. Cuando observamos los niveles pHSL/HSL en el hígado de ratas preñadas SDHFD, vimos que se produce una disminución como sucedió en el grupo SDSD. Sin embargo, con respecto a los niveles de pHSL/HSL en las ratas preñadas HFDHFD, observamos un aumento probablemente debido a la mayor acumulación de grasas en este grupo.

No encontramos diferencias significativas entre los pesos de BAT de ratas gestantes y no gestantes en SDSD y encontramos que esto se produce independientemente del tipo de dieta que las ratas reciben antes y durante el embarazo. El hecho de que no haya diferencia en los datos de peso de iBAT podría ser controvertido ya que el BAT se inactiva en ratas gestantes, pero debemos tener en cuenta que el contenido de proteína y ARN de iBAT es menor en las ratas gestantes que en las no preñadas a pesar de tener el mismo peso. Observamos una disminución en la temperatura interescapular y corporal, disminución en los niveles de proteicos de UCP1 y β3 adrenérgico en BAT de ratas preñadas SDSD tal y como estaba descrito en la literatura. En las ratas preñadas SDHFD, UCP1 también disminuye. En las ratas preñadas HFDHFD ni los niveles proteicos en BAT de UCP1 ni de β3 adrenérgico se vieron modificados en comparación con los controles, aunque la temperatura en el BAT estuviese disminuída, implicando que otros mecanismos pueden modular la actividad BAT en las ratas preñadas que toman una dieta alta en grasas antes y durante el embarazo . En vista de estos resultados, podemos concluir que la disminución en la temperatura del BAT durante el embarazo es independiente de la ingesta de una dieta alta en grasas. PGC1α es inducido por el frío en la grasa parda y actúa como un regulador de la biogénesis mitocondrial y el metabolismo oxidativo. PGC1α también induce la expresión de UCP1, que es esencial para la activación termogénica inducida por agonistas β-adrenérgicos o el frío. En este sentido, no encontramos diferencias en los niveles proteicos de PGC1α en las ratas preñadas SDSD y HFDHFD, mientras que encontramos una disminución en SDHFD lo que significa que hay una desregulación de PGC1α en el embarazo. Con la actividad BAT disminuída en ratas preñadas, no esperábamos diferencias en los niveles de LPL y CD36 en BAT, y un aumento en los niveles séricos de TG como finalmente observamos en todos los grupos de preñadas, con la excepción del grupo HFDHFD en donde los niveles de LPL disminuyeron.

En nuestros datos no encontramos diferencias en los niveles de pAMPKα en el BAT de las ratas gestantes SDSD y sí encontramos una disminución en los niveles de pAMPKα en las ratas preñadas SDHFD, ya que el BAT se encuentra inactivado. Con respecto a las ratas preñadas HFDHFD, observamos un aumento de pAMPKα en el BAT como hemos observado en el hígado, por lo que parece que la dieta también está modulando la señalización de AMPK en el BAT. Con respecto a los niveles de FAS en SDSD y HFDHFD, no se vieron modificados mientras se encontraban disminuidos en SDHFD. HSL es un efector clave de la señalización β3-adrenérgica siendo su actividad fundamental para la oxidación de ácidos grasos en el BAT. Teniendo esto en cuenta, esperábamos una disminución en los niveles de pHSL/HSL en el BAT de las ratas gestantes. Sorprendentemente encontramos un aumento en los tres grupos de preñadas, esto puede deberse a una resistencia durante el embarazo. Todos estos datos nos llevan a pensar que existe una desregulación de la lipogénesis y las vías de la

lipólisis en el BAT delas ratas preñadas, haciendo posible su inactivación para ahorrar energía para el feto. Cabe destacar que una desregulación del metabolismo de los lípidos en otros tejidos como el hipotálamo ha sido mostrada con anterioridad durante la preñez.

El final del embarazo es la fase más precaria desde el punto de vista nutricional del ciclo reproductivo de las ratas, necesitando de una importante movilización de reservas maternas para satisfacer las demandas metabólicas crecientes de los compartimentos materno, placentario y fetal a pesar del aumento de la hiperfagia. Se han realizado una gran cantidad de estudios sobre el impacto de la dieta en la descendencia, pero se sabe poco sobre el impacto en la salud y el funcionamiento de la madre. El objetivo de este estudio es proporcionar una imagen integrada de la expresión de los diversos cambios que ocurren en la última etapa del embarazo y la influencia de la ingesta una dieta alta en grasas solo durante el embarazo o antes y durante el embarazo, con respecto a la salud de la madre. Aunque nuestro estudio no proporciona pruebas claras de los mecanismos moleculares implicados, plantea la necesidad de más investigación respecto a este tema.

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ABBREVIATIONS

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ABBREVIATIONS

ACCα (Acetyl-CoA Carboxylase)

ACS (Acyl-CoA Synthase)

ACTH (Adrenocorticotropic Hormone)

ADP (Adenosine Diphosphate)

AgRP (Agouti-Related Peptide)

AKT (Protein Kinase B)

ALT (Alanine Transaminase)

AMPK (AMP-activated Protein Kinase)

APo (Area Postrema)

AP (Anterior to the bregma)

ApoB (Apolipoprotein B)

APS (Ammonium Persulfate)

AR (Adrenergic Receptor)

ARC (Arcuate Nucleus)

ASCL1 (Achaete-Scute Homolog 1)

AST (Alanine Aminotransferase)

ATF (activating transcription factor)

ATGL (Adipose Triglyceride Lipase)

ATP (Adenosine Triphosphate)

BAT (Brown Adipose Tissue)

BBB (Blood Brain Barrier)

BCL2 (B-cell Lymphoma 2)

BDNF (Brain-derived Neurotrophic Factor)

BIP (Binding Immunoglobulin Protein)

BMI (Body mass index)

BSA (Bovine Serum Albumin)

BSX (Brain Specific Homeobox Protein)

CART(Cocaine and Amphetamine Regulated Transcript)

cAMP (Cyclic Adenosine Monophosphate)

CC3 (Cleaved Caspase 3)

CCK (Cholecystokinin)

cDNA(Complementary DNA)

CBP1 (Calcineurin Binding Protein 1)

C/EBP(CCAT/enhancer-binding protein)

CHOP (CCAAT-Enhancer-Binding Protein Homologous Protein)

CNS (Central Nervous System)

CPT1 (Carnitine-palmitoyl transferase-1)

CREB (cAMP Response Element-Binding Protein)

CRH (Corticotropin-Releasing Hormone)

CRP (C-Reactive Protein)

CSF (Cerebrospinal Fluid)

$\mathbf{C_t}$ (Threshold Cycle)

DAB (Diaminobenzidine)

DAMPS (Damage Associated Molecular Patterns)

DGAT (DiacylglycerolTranferase)

DIO (Diet Induced Obesity)

DEPC (Diethylpyrocarbonate)

DMBX1 (Diencephalon/Mesencephalon Homeobox 1)

DMN (Dorsomedial Hypothalamic Nucleus)

DNA (Deoxyribonucleic Acid)

DRD2 (Dopamine Receptor D2)

DV (Ventral from the surface of the skull)

DVC (Dorsal Complex of the Vagus Nerve)

DVN (Dorsal Motor Nucleus of the Vagus)

eIF2a (eukaryotic translation initiation factor-2α)

EE (Energy Expenditure)

ELISA (Enzyme-Linked ImmunoSorbent Assay)

ER (Endoplasmic Reticulum)

ERα (Estrogen Receptor Alpha)

ERAD (ER-associated protein degradation)

ERK (Extracellular Signal-Regulated Kinase)

EZF (Epithelial Zinc Finger)

FABP4 (Fatty Acid-Binding Protein 4)

FADH2 (Flavin Adenine Dinucleotide Hydrogen)

FAS (Fatty Acid Synthase)

FABP (Fatty Acid Binding Protein)

FATP (Fatty Acid Transport Protein)

FFA (Free Fatty Acids)

FGF (Fibroblast Growth Factor)

FoxO1 (Forkhead Box Protein O1)

FRET (Fluorescence Resonance Energy Transfer)

FXR (Farnesoid X Receptor)

GABA (γ-aminobutyric acid)

GAD (Glutamic Acid Decarboxylase)

GADD34 (Growth Arrest and DNA Damage-inducible Protein)

GFAP (Glial Fibrillary Acidic Protein)

GFP (Green Fluorescent Protein)

GLP1 (Glucagon-like peptide-1)

GLUT2 (Glucose Transporter 2)

GLUT5 (Glucose Transporter 5)

GRP17 (Uracil Nucleotide/Cysteinyl Leukotriene)

GPR78 (78-kDa glucose-regulated protein)

GTT (Glucose Tolerance Test)

HDL (High-density lipoprotein)

HFD (High Fat Diet)

HMGB1 (high mobility group protein B1)

HSC (Hepatic Stellate Cells)

HSL (Hormone Sensitive Lipase)

HPRT (Hypoxanthine Phosphoribosyltransferase 1)

iBAT (Interscapular Brown Adipose Tissue)

ICV (Intracerebroventricular Infusion)

IKK-β (Inhibitor of Nuclear Factor Kappa Beta)

IL (Interleukin)

IP (Intraperitoneally)

IRE1 (Inositol-Requiring ER-to-Nucleus Signaling Protein 1)

IRS (Insulin Receptor Substrate)

JAK (Janus Kinase)

JNK (c-Jun N-terminal)

KCs (Kupffer cells)

KLF4 (Kruppel-like Factor 4)

KO (Knock Out)

KOR (Kappa Opioid Receptor)

Krox20 (Early Growth Response Protein 2)

L (Lateral to the Sagittal Suture)

LHA (Lateral Hypothalamic Area)

Lhx8 (LIM Homeobox Protein 8)

LDL (Low-Density Lipoprotein)

LEPRb (Leptin Receptor b)

LPL (Lipoprotein Lipase)

LPS (Bacterial Lipopolysaccharide)

LA (Locomotor Activity)

LXR (Liver X Receptor)

MAPK (Mitogen-Activated Protein Kinase)

MC3R (Melanocortin Receptor 3)

MC4R (Melanocortin Receptor 4)

MCH (Melanin-Concentrating Hormone)

MCP-1 (Monocyte Chemotactic Protein 1)

Meox2 (Mesenchyme Homeobox 2)

MGAT (Monoacylglycerol Transferase)

MGL (Monoacylglycerol Lipase)

MGLL (Monoglyceride Lipase)

NADH (Nicotinamide Adenine Dinucleotide Hydrogen)

NAFLD (Non Alcoholic Fatty Liver Disease)

NASH (Non Alcoholic Steatohepatitis)

NCBI (National Center for Biothechnology Information)

NMR (Nuclear Magnetic Resonance)

NLS (Nuclear localization signal)

NPC1L1 (Niemann-Pick C1-Like1)

NPY (Neuropeptide Y)

NTS (Nucleus of the Solitary Tract)

NRS (Normal Rabbit Serum)

NTK (Natural Killer Cells)

PACAP (Pituitary Adenylate Cyclase Activating Polypeptide)

PBS (Phosphate Buffered Saline)

PERK (RNA-dependent protein kinase-like ER eukaryotic initiation factor-2α kinase)

PEST (Proline, glutamic acid, serine and threonine)

PGC1α (Peroxisome proliferator-activated receptor gamma coactivator 1 alpha)

PI3K (Phosphoinositide 3-kinase)

PKA/B (Protein Kinase A/B)

PMSF (Phenylmethylsulfonyl Fluoride)

PMV (Ventral Premammillary)

PNPLA (Patatin-Like Phospholipase Domain-Containing Protein)

POMC (Proopiomelanocortin)

PP (Pancreatic Polypeptide)

PP1 (Phosphatase 1)

PPAR (Peroxisome Proliferator-Activated Receptor)

PRDM16 (Protein Domain Zinc Finger Protein 16)

PTEN (Phosphatase and Tensin Homolog)

PTP1B (Protein tyrosine phosphatase 1B)

PUFAs (Polyunsaturated Fatty Acids)

PVDF (Polyvinylidene Polyfluoride)

PVN (Paraventricular Nucleus)

PYY (Peptide YY)

RD2 (Dopamine Receptor 2)

RNA (RiboNucleic Acid)

ROS (Reactive Oxygen Species)

RPa (Raphe Pallidus Nucleus)

RPTPe (Protein tyrosine phosphatase epsilon)

RQ (Respiratory Quotient)

RT-PCR (Real-time Polymerase Chain Reaction)

RXR (Retinoid X Receptor)

S1P (Site 1 Protease)

SC (Subcutaneous)

SD (Standard Diet)

SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylate Gel Electrophoresis)

SECs (Sinusoidal endothelial cells)

SF1 (Steroidogenic Factor 1)

SGLT1 (Sodium-glucose Linker Transporter 1)

SH2B1 (Src homology 1)

SIRT1 (Sirtuin 1)

SNS (Sympathetic Nervous System)

SOCS3 (Suppressor of Cytokine Signaling 3)

SREBP1 (Transcription Factor Steroid Regulatory Element-Binding Protein 1)

STAT (Signal Transducer and Activator of Transcription)

T2DM (Type 2 diabetes)

TAp63α (Transactivation Protein 63)

TBS-T (Tris Buffered Saline-Tween)

TCA (Tricarboxylic AcidCycle)

TCPTP (T-cell protein tyrosine phosphatase)

TEMED (N, N, N, N tetramethylethylenediamine)

TG (Triglyceride)

Th (T Helper)

TH (Tyrosine Hydroxylase)

TLR4 (Toll-like Receptor 4)

TNF-α (Tumor Necrosis Factor alpha)

TRH (Thyrotropin-releasing Hormone)

T3 (Triiodothyronine)

UCP (Uncoupling Protein)

UPR (Unfolded Protein Response)

VGAT (Vesicular Gamma Aminobutyric Acid Transporter)

VLDL (Very Low Density Lipoprotein)

VMN (Ventromedial Hypothalamus)

WAT (White Adipose Tissue)

XBP1 (X-Box-Binding Protein-1)

ZIC1 (Zic Family Member 1)

α-MSH (Alpha Melanocyte-Stimulating Hormone)

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INTRODUCTION

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1 DYSREGULATION OF ENERGY HOMEOSTASIS: OBESITY

Evolutionarily, our body has a more robust system to respond to deficiencies of energy intake and reserves than to excess energy [1]. The role of the accumulation of energy reserves has always been crucial in the survival of our species, and now begins to play against us. This is because our habits have become more sedentary; highly energetic food is easy to get and unconscious physical activity has decreased in an alarming way [2]. That is why we are facing the XXI century pandemic: obesity. According to the World Health Organization, overweight and obesity are defined as abnormal or excessive fat accumulation that presents a risk to health. A crude population measure of obesity is the body mass index (BMI): a person's weight (in kilograms) divided by the square of his or her height (in meters). A person with a BMI of 30 or more is generally considered obese and a person with a BMI equal to or more than 25 is considered overweight.

Behind obesity there are sensory, physiological, genetic, molecular, social, economic and psychological factors, making it a highly complex and expanding disease. Although obesity is more prevalent in developed countries, it also exists in least developed countries due to low cost of food with high energy density and great palatability. However, the obesogenic changes in contemporary human lifestyle do not affect all individuals equally, revealing that some individuals have an inherently greater predisposition to becoming overweight and increased adiposity [3]. In addition, it is a disease that favors the development of other diseases. According to the World Health Organization, obesity is an important cause of morbidity, disability and premature death. Obesity increases the risk for a wide range of chronic diseases such as type II diabetes, hypertension, cancer, vascular damage and respiratory problems among others [2, 4-6] (Diagram 1). Consequently, BMI is thought to account for about 60% of the risk of developing type 2 diabetes, over 20% of that for hypertension and coronary-heart disease and between 10 and 30% for various cancers with greater incidence in females. Other comorbidities include gallbladder disease, fatty liver, sleep apnea, and osteoarthritis [7].

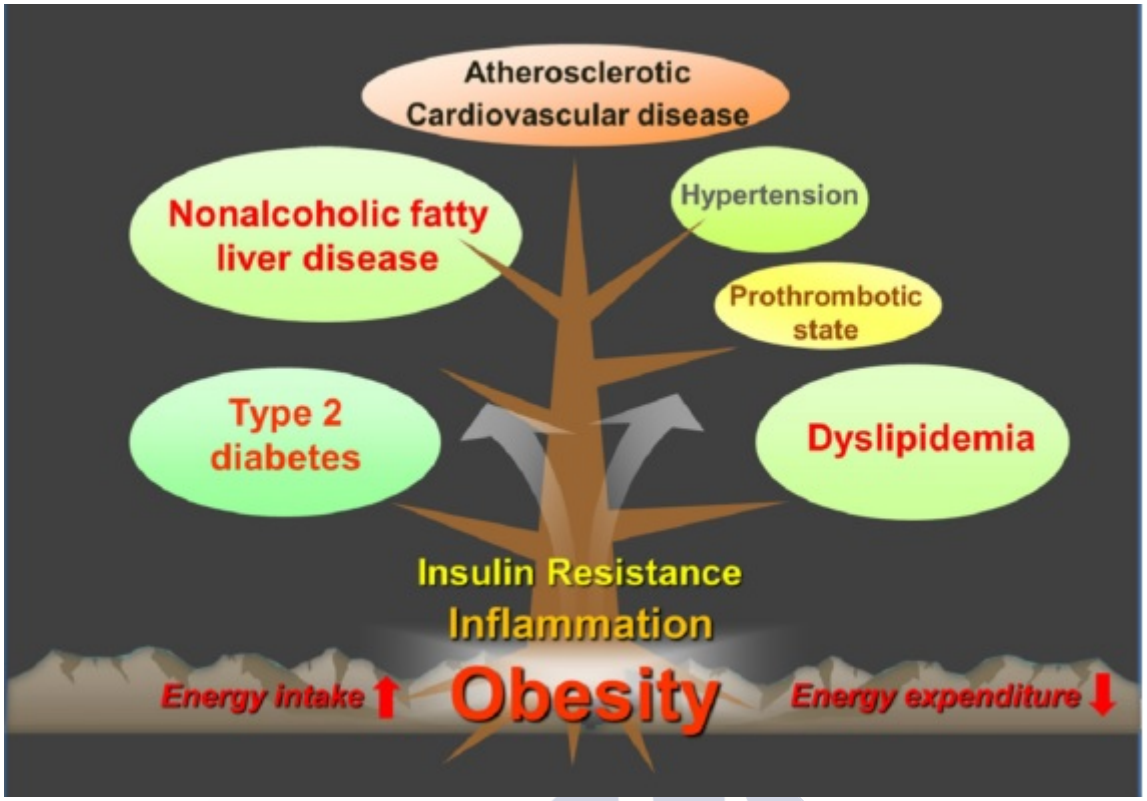

Diagram 1: The metabolic syndrome [10]. Creative commons license.

In 1953, Kennedy [8] proposed that the amount of energy stored in adipose mass represented the balance between ingested calories and energy expenditure (Diagram 2). Energy homeostasis promotes stability regarding the amount of body energy stored as fat [9]. This term refers to the body's ability to maintain energy balance taking into account the variability with respect to possible access to food and energy expenditure demand. Homeostasis has two parts: energy input (intake) and energy expenditure (60% resting metabolic rate, 10% food-induced thermogenesis and 30% associated with physical activity), which are both physiologically linked through hypothalamic circuits [6].

Homeostasis' success depends on the brain being able to read, interpret and integrate a wide range of signals that describe the animal's nutritional state and its immediate environment, and to make appropriate adjustments in food intake and energy expenditure (physical exercise, basal metabolism and thermogenesis) as a result of the information received [11].

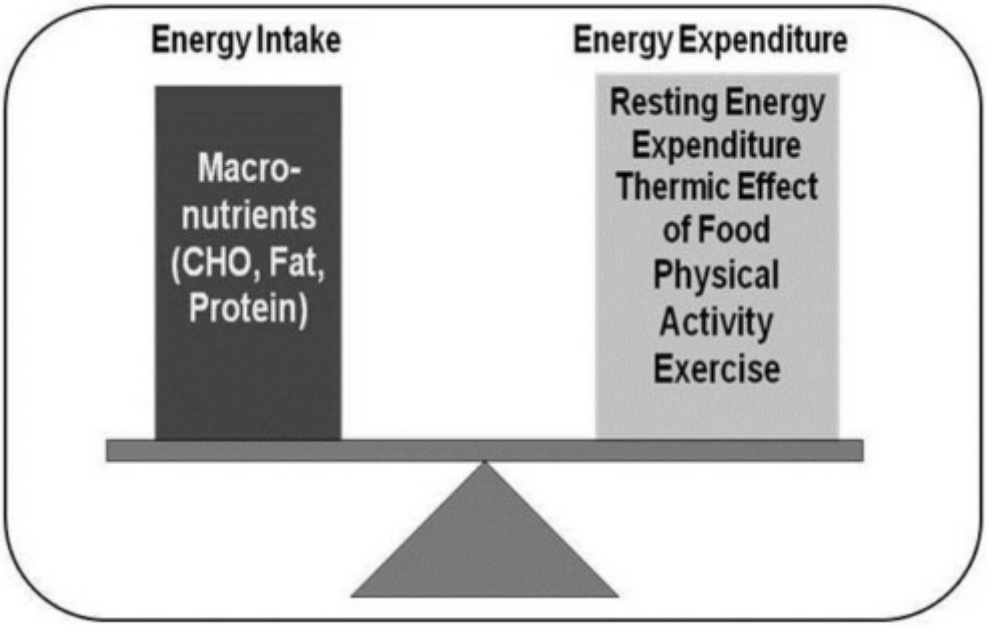

Diagram 2. Components of energy expenditure and energy intake [12]. Creative commons license

The first step that the body has to do to obtain energy for proper cell functioning is the nutritional partition. With a meal, we ingest mostly macromolecules such as complex carbohydrates, proteins and lipids. While they are moved through the intestinal tract, these molecules experience mechanical disruption and enzymatic hydrolysis so that they will separate into their constituent simpler molecules: monosaccharides, amino acids and fatty acids. Through the hepatic portal vein, monosaccharides fatty acids and amino acids are transported to the liver. Inside the enterocytes, some of the fatty acids and monoacylglycerols are re-esterified, making new triacylglycerols. Next they are packaged with apolipoprotein B, phospholipids and cholesterol to form chylomicrons. Chylomicrons get into the bloodstream via the lymphatic system, allowing fats and cholesterol to arrive at tissue capillaries. Once there, fatty acids with length below 12-14 carbons are not able to esterificate and penetrate the capillary plasma right away in a non-esterified fatty acid form. The cholesterol that is also added into the chylomicrons is taken into the enterocyte at the brush border membrane via the Niemann-Pick C1-Like1 (NPC1L1) (Diagram 3).

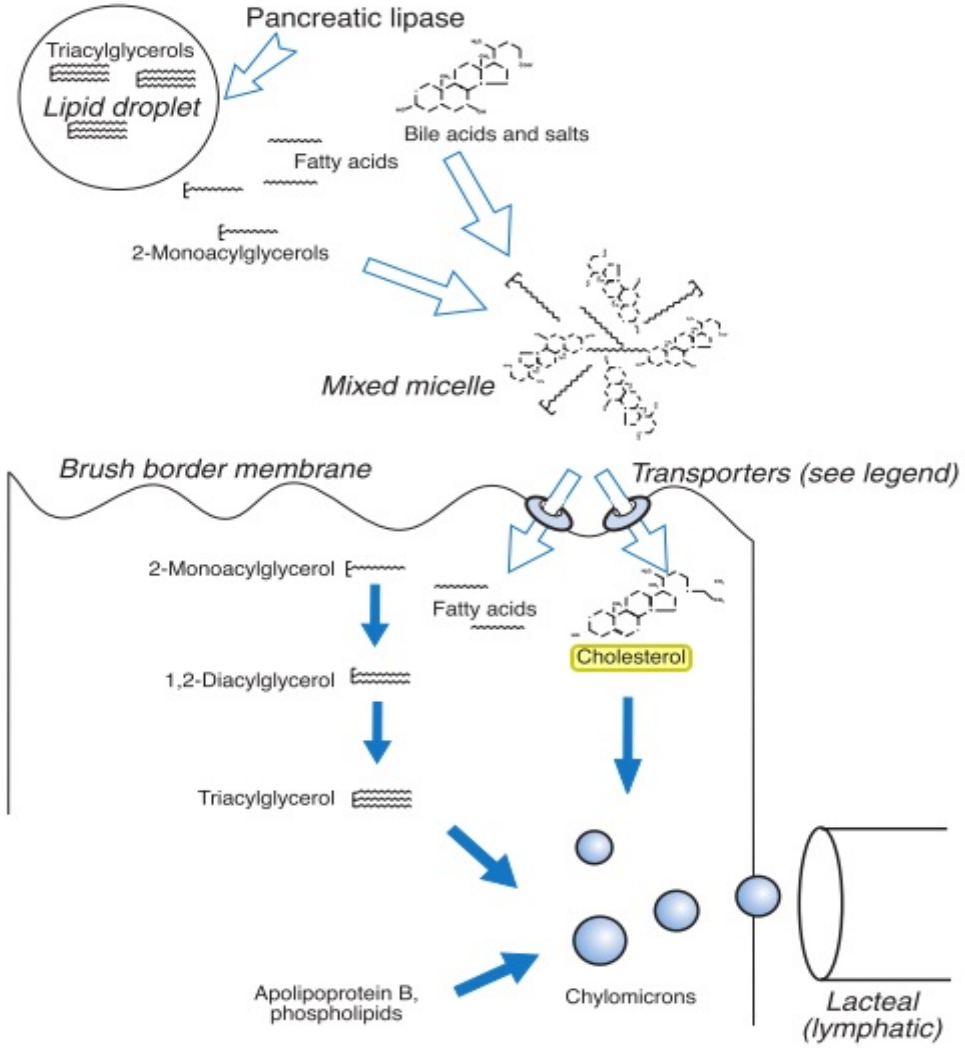

Diagram 3: Lipid absorption [13]. With permission of ELSEVIER.

Monosaccharides enter the enterocytes across the brush border and leave the cell by the basolateral membrane using specific transporter proteins such as glucose transporter 2 (GLUT2), glucose transporter 5 (GLUT5) and sodium-glucose linker transporter 1 (SGLT1) . Amino acids are transported by sodium-linked carriers, entering the epithelial cells and capillaries of the intestinal mucosa. Regarding fatty acids, there are two ways in which they can reach cells: one is in form of non-esterified fatty acids bound to albumin which allows them to be able to be transported through the plasma, and the other one is when fatty acids are liberated from triglyceride (TG) by lipoprotein lipase (LPL) which is located in the endothelial cells of the capillaries [13, 14].

Insulin and glucose controls *de novo* synthesis of fatty acids in liver. After food intake and in response to insulin, the liver acquires and stores glucose (as glycogen) for future use. During glycolysis, glucose is used as fuel leading to an increase in acetyl-CoA which will be transformed into malonyl-CoA via acetyl-CoA-carboxylase activity [15]. Gluconeogenesis works in a reciprocal manner to glycolysis and is directly correlated with oxidation of fatty acids: if there is a large amount of fatty acids in the liver, gluconeogenesis increases. Fatty

acids are substrates for acyl-CoA synthase (ACS). Acyl-CoA enters the mitochondrion mediated by carnitine palmitoyltransferase-1 (CPT-1) for oxidation. CPT-1 activity is regulated by the amount of malonyl-CoA available: if fuel is abundant, it inhibits CPT1-A and therefore β-oxidation [13, 14]. The function of Malonyl-CoA in the liver, was discovered by McGarry's team in 1977 [16]. Malonyl-CoA is a link between carbohydrate and fat metabolism as we can see in diagram 4, because both β-oxidation and glycolysis will generate acetyl-CoA. Furthermore, this molecule can either be used to form ketones, or enter into the tricarboxylic acid cycle (TCA), to be converted into fatty acids through *de novo* synthesis which will be packaged in the form of very low density lipoproteins (VLDL) and transported to the adipose tissue [17]. On the other hand, we have the adipocyte lipolysis in which stored triglycerides are metabolized to fatty acids and glycerol. [18]. In adipose tissue, LPL is situated in capillaries of endothelial cells, hydrolyzing the TG of the chylomicrons and VLDL into glycerol and fatty acids, and storing these lipids in the adipocytes in an insulin-dependent manner [19].

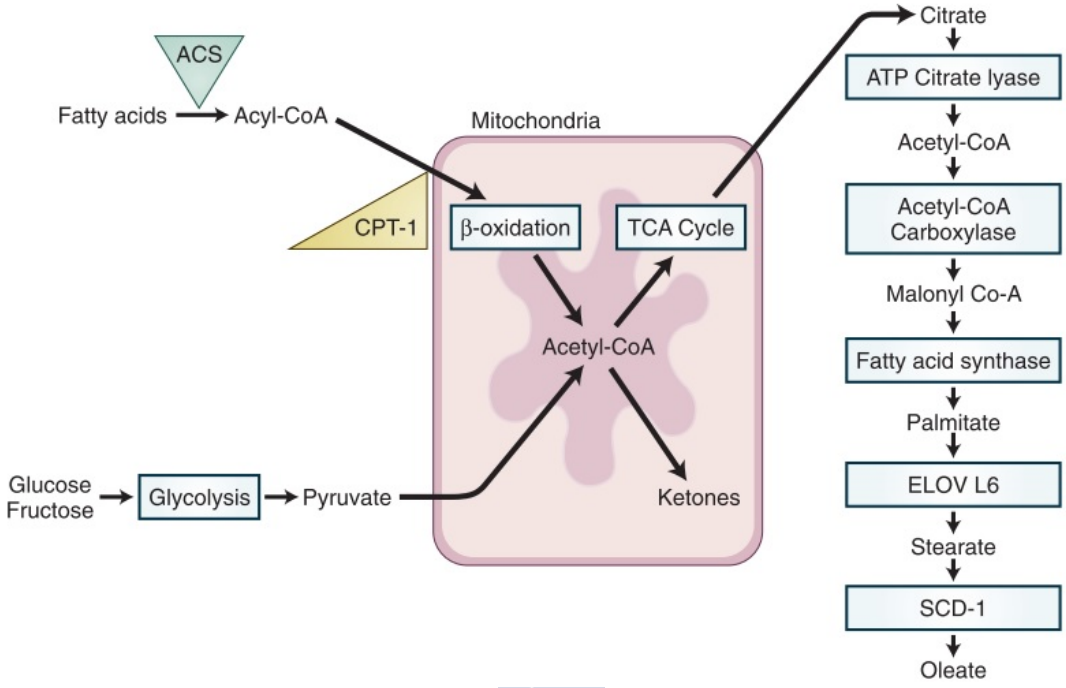

Diagram 4: Fatty acid metabolism. Abbreviations: ACS (acyl-CoA synthase); CPT-1 (carnitine palmitoyltransferase); ATP (adenosine triphosphate); CoA (coenzyme A); ELOVL6 (elongation of very-long-chain fatty acids protein 6); SCD-I (stearoyl-CoA desaturase I); TCA (tricarboxylic acid). [14]. With permission of Elsevier.

2 CENTRAL NERVOUS SYSTEM IN THE CONTROL OF ENERGY HOMEOSTASIS

Food intake is regulated at two different levels: peripheral signals and CNS (Scheme 5). Central control of food intake is regulated by multiple brain areas, but the most studied is the hypothalamus, where we will focus.

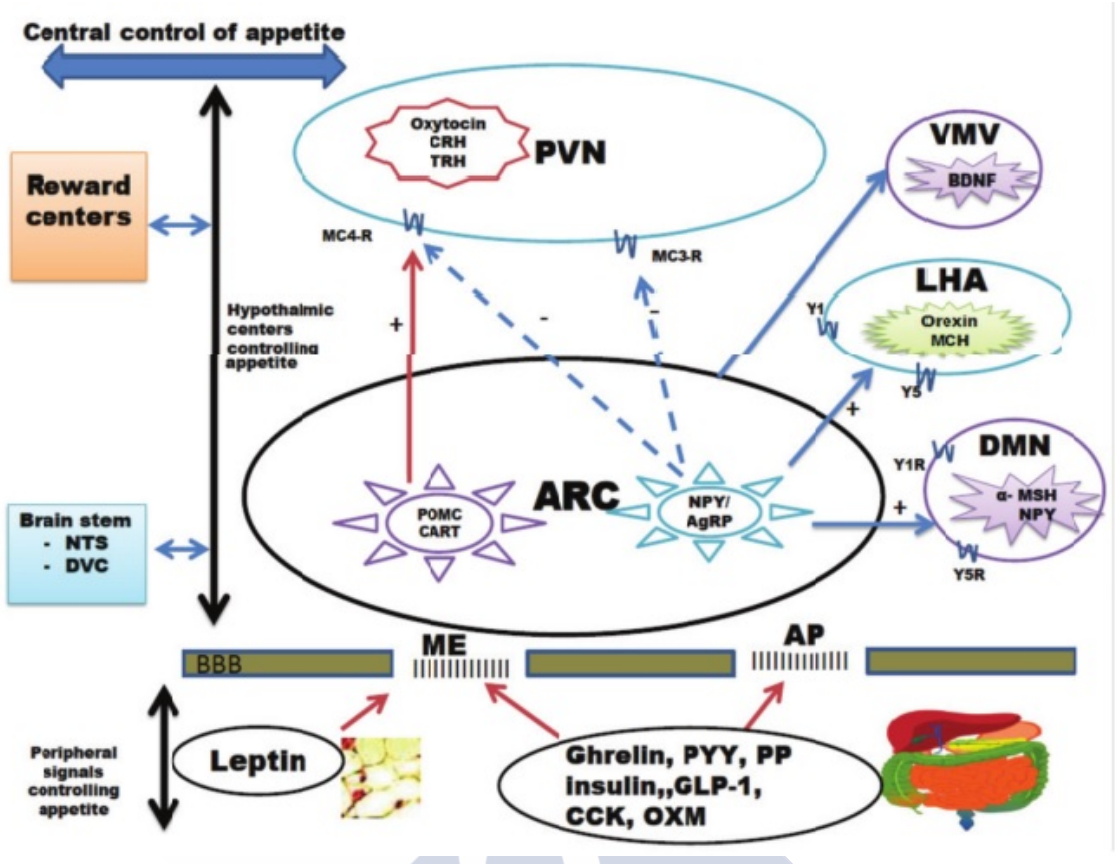

Diagram 5: Central and peripheral signals for food intake control. Abbreviations: ARC (arcuate nucleus); NPY/AgRP (neuropeptide Y and agouti related peptide); POMC/CART (pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript); PVN (paraventricular nucleus); LHA (lateral hypothalamic area); DMN (dorsomedial nucleus); VMN (ventromedial hypothalamic nucleus); ME (median eminence); AP (area postrema); GLP-1 (glucagon-like peptide-1); CCK (cholecystokinin); PP (pancreatic polypeptide); PYY (peptide YY); OXM (oxyntomodulin); BBB (blood brain barrier); MCH (melanin-concentrating hormone); α-MSH (alpha melanocyte stimulating hormone); BDNF (brain derived neurotrophic factor); CRH (corticotropin releasing hormone); NTS (nucleus tractus solitarius); DVC (dorsal vagal complex); TRH (thyroid releasing hormone); MC4-R (melanocortin receptor 4); MC3-R (melanocortin receptor 4); Y1R & Y5R (neuropeptide Y receptors 1 & 5). [20]. Creative commons license.

2.1 HYPOTHALAMIC AREAS

Hypothalamus plays a critical role in the regulation of energy balance receiving and integrating peripheral signals. It is composed of several neuronal populations (Diagram 6):

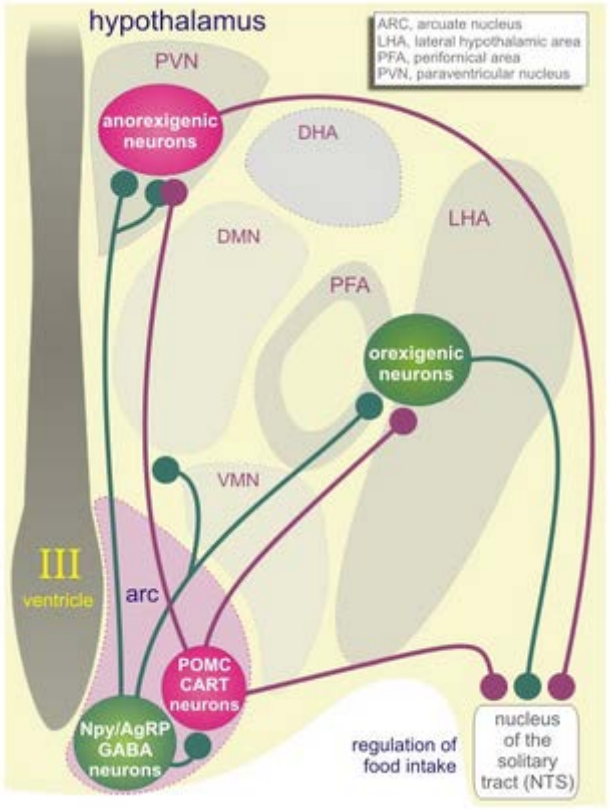

Diagram 6: Hypothalamic populations [21]. Creative commons license

2.1.1 **Arcuate nucleus (ARC)**

2.1.1.1 The role of ARC on food intake regulation

The arcuate nucleus is the main food intake regulator. It is located in the middle part of the hypothalamus, adjacent to the third ventricle and the median eminence. ARC neurons co-express AgRP and neuropeptide Y (NPY), which are orexigenic neuropeptides. Conversely, they also express pro-opiomelanocortin (POMC) and cocaine-amphetamine-regulated transcrpt (CART), which are anorexigenis neuropeptides [22-27]. Furthermore, NPY/AgRP neurons suppress the activity of CART/POMC expressing neurons in the ARC [28]. These neurons are regulated not only by humoral signals but also by excitatory local synaptic signals.

The hematoencephalic barrier is more permissive in the ARC than other brain regions and therefore the neurons of this nucleus are more easily exposed to circulating hormones such as leptin and ghrelin, nutrients and other peripheral factors that control the energy balance [29-31].

2.1.1.1.1 Agouti-related peptide (AgRP)

In 1997 and in parallel, Shutter and Ollmann laboratories, isolated the gene encoding a C-terminal fragment of AgRP, which when administered ICV into rats, increased food intake

over a 24-h period [32]. AgRP's RNA is expressed in the hypothalamus and its levels are significantly elevated in leptin-deficient mice, and AgRP is a selective antagonist of MC3R and MC4R involved in the control of energy balance [33]. MC3R and MC4R inhibition leads to an obese phenotype associated with hyperphagia, downregulation of thermogenesis and caloric efficiency [34].

AgRP is a neuropeptide produced in the brain by AgRP/NPY neurons. AgRP neurons, when activated, promote food intake by acting as a competitive MC4R antagonist [3]. AgRP possesses 132 amino acids encoded by the 16q22 chromosomal region in humans, while the rodent orthologue has 131 amino acids. It is found mostly in the hypothalamus, but also in the subthalamic nucleus, adrenal gland, testes, lung and dorsal root ganglia. Orthologues have also been found in the pig, sheep, pigeon and several fish where AgRP levels are overexpressed under fasting conditions, indicating that it has an evolutionarily conserved function with respect to energy homeostasis [35]

Specifically, it is the overexpression of the C-terminal fragment (Agrp 83-132), which causes the obese phenotype: rats treated with this fragment showed an increased intake in a similar way to when they are given MC3R and MC4R antagonists [32]. On the other hand, the blockade of these neurons by the diphtheria toxin carried out in adult mice [36], caused a marked decline in intake, and in the same year was demonstrated that by blocking NPY/AgRP arcuate neurons after embryogenesis, the mice showed a thin hypophagic phenotype [37]. These results support the idea that there are compensatory mechanisms that are expressed strongly when depleting these neurons at embryonic stages.

AgRP/NPY neurons are inhibited by leptin, insulin and peptide YY (PYY) while they are stimulated by ghrelin [6, 38]. At the same time, these neurons inhibit the anorexigenic functions of POMC neurons through GABA synaptic release (an inhibitory neurotransmitter). Fasting for 24 hours leads to an increase of hypothalamic mRNA of NPY and AgRP and to a decrease of the POMC mRNA in mice [39].

Simple knockouts (KO) for NPY or AgRP, do not show any change in the phenotype, and the double KO for AgRP and NPY does not have alterations in food intake or body weight either [29]. However, in the double KO the orexigenic effect of ghrelin is blunted [29], which indicates that they are critical factors mediating the action of ghrelin [29]. Furthermore, post-embryonic ablation of AgRP neurons leads to a marked decrease in food intake, probably due to the resulting dysregulation in GABA signaling [40, 41].

The *in vivo* regulation of AgRP neurons depends on several transcription factors including forkhead box protein 01 (FoxO1) [42], uracil nucleotide/cysteinyl leukotriene receptor (Gpr17)[43], the hypothalamic homeobox domain transcription factor brain specific homeobox protein (BSX) [44], signal transducer and activator of transcription 3 (STAT3) [45] and diencephalon/mesencephalon homeobox 1 (Dmbx1) [46]

2.1.1.1.2 Neuropeptide Y (NPY)

NPY is a 36-amino acid neuropeptide that acts as a neurotransmitter in the brain and in the autonomic nervous system of humans. Neurons expressing NPY are located in hypothalamic ARC. These neurons project to important appetite-regulating nuclei such as

PVN among others. Starvation stimulates ARC/NPY neurons while circulating leptin and insulin inhibits them, participating in the increased hunger associated with this and other conditions of energy deficit. The hyperphagia and obesity that we can observe in ob/ob, db/db mice and fa/fa rats (where leptin is inhibited through mutations affecting leptin or its receptor), are mediated by ARC/NPY neurons. Antagonists of the Y5 receptor (currently thought to be the NPY "feeding" receptor) have anti-obesity effects. MC4Rs are expressed in various hypothalamic regions, including the ventromedial and arcuate nuclei, and activation of these receptors by agonists such as α-MSH (a cleavage product of pro-opiomelanocortin which is expressed in ARC neurons) inhibits feeding and causes weight loss [11].

2.1.1.1.3 Cocaine and amphetamine regulated transcript (CART)

CART is a neuropeptide protein that in humans is encoded by the CARTPT gene. The neurons that secrete CART are associated with the reward and reinforcement systems, sensory processing, stress, endocrine regulation and intake, characterized by the inhibition of intake without modifying energy expenditure [47].

It's also well known that in rats ICV administration of CART peptide fragments decreases food intake. CART mRNA in the ARC is decreased in food-deprived animals, and intraperitoneal administration of leptin increased CART mRNA levels in the ARC [48]. On the other hand, CART KO mice are hyperphagic. However, only when the mice are fed with high fat diet do they experience an increase in body weight compared with the controls [49].

2.1.1.1.4 Pro-opiomelanocortin (POMC)

Pro-opiomelanocortin is a precursor polypeptide of the endogenous ligand of the melanocortin system (α-MSH), with 241 amino acid residues which is expressed at high levels in endocrine cells of the pituitary gland and neurons present in the ARC of the hypothalamus. The POMC gene encodes both the anorexigenic peptide alpha melanocyte-stimulating hormone (α-MSH), and the opioid peptide β-endorphin [50]. In the anterior pituitary, corticotrophs release ACTH, whereas in hypothalamic neurons the POMC prohormone is further processed to generate the melanocortins α, β and γ melanocyte stimulating hormones and the analgesic endogenous opioid β-endorphin. POMC-producing neurons promote satiety when activated, releasing melanocortin peptides that induce anorexia upon stimulation of Gs-coupled MC4R. Mutations in MC4R gene are the most common monogenic disorders causing obesity. In POMC KO mice, hypothalamic POMC neurons develop normally and project to the typical target areas, indicating that POMC-derived peptides are not critical for normal development and maintenance of the neuronal circuitry per se. POMC KO mice were hyperphagic and developed early-onset extreme obesity when consuming a standard low-fat chow, in contrast to high-fat or high-sucrose diet-induced obesity models that produce changes in dopaminergic reward centers [3]. POMC is converted into ACTH and then into α-MSH [51]. α-MSH reduces food intake [52] and when it is dispensed intracerebroventricularly, appetite and weight are reduced and when the α-MSH antagonist is administered, these effects are reversed [53].

2.1.1.2 The role of ARC on thermogenesis

As previously stated, ARC is known for being the main food intake regulator, in fact NPY and AgRP arcuate neurons expression is upregulated in fasting conditions [54]. But ARC also takes part on thermoregulation since the thermogenic potential of beige fat is downregulated by the activation of AgRP neurons [55]. Moreover ARC NPY regulation is related with downregulation of tyrosine hydroxylase expression, mediated by the Y1 receptor in the PVN which leads to a decrease in BAT UCP1 [56].

When CART peptide is increased in the ARC in a chronic way, it leads to an increase in BAT thermogenesis proposing a role of CART in cold adaptation. In fact, cold exposure extended on time raises CART mRNA concentration in the ARC [57].

POMC neurons are sensors of the energetic state of the body since they receive information about adiposity by means of leptin and insulin. It has been discovered that a combined action of leptin and insulin in POMC neurons promotes WAT browning, energy expenditure and weight loss. Deletion of the phosphatases PTP1B and TCPTP enhanced insulin and leptin signaling in proopiomelanocortin neurons and prevented dietary-induced obesity by increasing WAT browning and energy expenditure [58].

2.1.2 **Dorsomedial hypothalamic nucleus (DMN)**

2.1.2.1 The role of DMN on food intake regulation

This nucleus takes part on the regulation of food intake, thermogenesis, stress and circadian rhythms [59]. One of the first studies showing the importance of DMN in ingestive behavior was performed in sheeps and when they stimulated the DMN, food intake increased [60]. DMN is connected with PVN, LHA and specially ARC [61] [62]. It receives ARC projections and sends projections towards the paraventricular and lateral nuclei [63]. DMN neurons express NPY and its expression is higher under chronic conditions of hyperphagia like pregnancy or diet-induced obesity [64]. NPY neurons of the DMN present the incretin CCK receptor, whose administration in DMN reduces the intake [64, 65].

2.1.2.2 The role of DMN on thermogenesis

Injuries in DMN reduce the interscapular brown adipose tissue (iBAT) weight [66] while normally, iBAT increases its weight following exposure to cold circumstances [67]. Electrical stimulation of the DMN has also been documented to raise iBAT temperature [68]. DMN projects to the raphe pallidus nucleus (RPa) [69] and when glutamate receptor antagonists are injected in this area, thermogenesis is reversed [70]. The dorsomedial nucleus is an important rely receiving inputs from the preoptic region to regulate BAT metabolism.

Since the XIX century, the rostral hypothalamus or preoptic region, has been pointed as a key regulatory area of thermogenesis [71]. DMN has cold sensitive neurons in lower proportion [72] compared with warm-sensitive neurons. Regarding the warm-sensitive neurons, they show co-expression of the neuropeptides brain-derived neurotrophic factor

(BDNF) and pituitary adenylate cyclase activating polypeptide (PACAP), which are upregulated in response to environmental warmth [73]. The GABAergic neurons of the medial preoptic region provide a negative tonic inhibition toward glutamatergic neurons located in the DMN. DMN neurons project to the rostral ventromedial medulla apparently to the raphe pallidus, a main site of BAT SNS premotor neurons. NPY neurons of the DMN could apparently also negatively affect BAT thermogenesis, possibly by inhibiting glutamatergic neurons located in the DMN itself [74].

2.1.3 **Paraventricular nucleus (PVN)**

2.1.3.1 The role of PVN on food intake regulation

PVN is localized in the anterior hypothalamus close to the upper part of the third ventricle. It receives projections from AgRP and POMC neurons from ARC and projects to the nucleus tractus solitarius (NTS) [75], ventral tegmental area, Edinger-Westphal nucleus, ventrolateral periaqueductal gray matter, reticular formation, pedunculopontine tegmental nucleus, and dorsal raphe nucleus among others [76]. It takes part of the inhibitory control of food intake, appetitive response to sodium deficiency, changes in gastric and pancreatic functions, esophageal motor control, regulation of hypoglycemia, hyperventilation and parasympathetic bradycardia among others [76]. Corticotropin-releasing hormone (CRH) is the main physiological regulator of pituitary adrenocorticotropic hormone (ACTH) secretion and is released in PVN. When it is infused intracerebroventricularly (ICV), it inhibits food intake and decreases body weight in rats [77]. Also the thyrotropin-releasing hormone (TRH) is released in PVN [78] and when injected subcutaneously, it decreases food intake and increase water intake and body temperature in rats [79]. This nucleus also express high levels of melanocortin receptor 3 (MC3R) and melanocortin receptor 4 (MC4R) [59], which plays crucial roles in the regulation of energy homeostasis [80].

2.1.3.2 The role of PVN on thermogenesis

As previously mentioned, the PVN is closely linked with ARC-mediated thermogenic regulation shown by the synapses of ARC POMC and AgRP neurons with PVN neurons [81]. POMC, NPY and AgRP arcuate neurons have projections to γ-aminobutyric acid (GABA) interneurons in PVH coordinating GABA release which, in turn, takes part in the regulation of thermogenesis [82]. In addition, microinjection of NPY in PVN blocks the sympathetic nerve activity to BAT [83]. Melanocortin administration in this nucleus also regulates energy expenditure [82]. ICV injection of an MC3/4-R agonist fires sympathetic projections to iBAT, and when an MC3/4-R agonist is infused in the PVN, there is an increase in the functioning of BAT[84].

2.1.4 **Lateral hypothalamic area (LHA)**

2.1.4.1 The role of LHA on food intake regulation

The lateral hypothalamic area of the brain was commonly known as the “feeding centre”; when the LHA is damaged bilaterally, food intake decrease [85]. In this area two kind of peptides are synthesized: orexins and melanin-concentrating hormone (MCH). The expression of prepro-orexin is increased in fasting [86] and this effect is reversed when the rats are treated with leptin in the LHA [87]. When orexin is administered acutely in LHA, food intake increases [86]. Under starvation, orexins can mediate the attention response to stay awake, and and also the order for starting the meal. This makes orexins an interface for sleep and feeding regulation being potential targets for the treatment of insomnia [88]. It was also shown recently by our group that downregulation of KOR in LHA and not in other hypothalamic nuclei is sufficient to block acute central MCH-induced food intake [89]. LHA can also be a site of action for the endocannabinoid system since the orexigenic neurons of this zone (orexin and MCH) express CB1 an endocannabinoid receptor. It has been recently demonstrated that stimulation of CB1 activates the same intracellular route as orexin A, suggesting an orexigenic role of endocannabinoids through this neuronal population. In addition, taking into account that these neurons project towards zones of the VTA, it is possible that the LHA acts as an integrating point of orexigenic hypothalamic and extrahypothalamic signals, mediated by the endocannabinoid system [90].

2.1.4.2 The role of LHA on thermogenesis

When injuries have been caused in the lateral hypothalamus of rats, it has been seen that there is an increase in energy expenditure during 24 hours in part due to the increase in motor activity [91]. In addition, the lateral hypothalamus is related to the BAT activity since the disinhibition of neurons in this area causes a rise in BAT sympathetic activity and corresponding rise in iBAT temperature [92]. Moreover, mice with orexin depleted in the LHA, show an hypoactivation of BAT because the adipocytes are unable to differentiate. This failure to differentiate is followed by a decrease of total energy expenditure [93]. Moreover, the blockade of MCH in the LHA leads to a significant drop in body mass, accompanied by an increase in the mass of interscapular BAT and the expression of UCP1 [94].

2.1.5 **Ventromedial hypothalamus (VMN)**

2.1.5.1 The role of VMN on food intake regulation

The first studies that related the VMN with the regulation of the food intake, occurred in the decade of the 40s. Animals exhibiting lesions in this region, developed obesity [95]. Furthermore, lesions in VMN leads to an increase in food intake, morbid obesity and insulin resistance [23]. There are projections from agouti-related peptide (AgRP) and pro-opiomelanocortin (POMC) neurons of the ARC into the VMN [59]. The importance of VMN in the regulation of energy homeostasis has been supported by studies demonstrating the existence of ObRb and leptin-induced neuronal activation in the VMN. Selective injections of

leptin in the VMN reduce the intake and weight in thin rats, and leptin receptor depletion from VMN neurons causes mild obesity and insulin resistance in mice [96]. The steroidogenic factor (SF1) is a transcription factor whose expression is required for the development of the VMN. When SF1 neural activity is modified, changes in food intake and feeding-related behaviours such as locomotion, exploration and anxiety occurs. The activation of SF1 neurons suppress feeding and while the inhibition increased cumulative food intake. This neurons are also sensitive to energy stores so that an increase in SF1 activity impedes food intake [97].

2.1.5.2 The role of VMN on thermogenesis

VMN was one of the first hypothalamic nuclei to be associated with the regulation of thermogenesis [98]. However, electrical stimulation of VMN has controversial results both elevating or decreasing iBAT thermogenesis [98, 99]. Regarding the increase in the interscapular temperature of the BAT when electrically stimulated, this hypothesis is refuted by the fact that this phenomenon disappears when the β3-adrenergic receptors are blocked [100, 101]. In spite of these polemical results, there are strong evidences that support the important function of VMN in the control of energy expenditure. For instance, AMPK, a cellular energy sensor [102] plays a key role in the regulation of BAT thermogenesis. Also, signals such as estradiol, nicotine, BMP8b, thyroid hormones or GLP-1 inhibit AMPK in the VMN and results in activation of BAT thermogenesis through the SNS [103]. It is also demonstrated that thyroid hormones inhibits AMPK through the hypothalamus leading to an increase in thermogenesis and weight loss. Triiodothyronine (T3) act on the ventromedial hypothalamus to promote WAT browning which is mediated by AMPK activity in such a way that VMN genetic activation of AMPK reverse the browning provoked by T3 [104]. In addition, AMPKα1 in SF1 neurons of the VMN, mediates central T3 effect [105]. Finally, liraglutide (a glucagon-like peptide 1 (GLP1) agonist) catalyzes BAT thermogenesis and browning through VMN AMPK [106]. In addition, the inhibition of the lipogenic route in the VMN prevents the activation of BAT mediated centrally by nicotine or estrogen, reversing the weight loss associated with the central administration of these substances [103, 107].

3 WHITE ADIPOSE TISSUE AND ENERGY HOMEOSTASIS: ADIPOKINES

Adipose tissue is composed of different types of cells such as adipocytes, preadipocytes, endothelial- and immune-cells among others. The adipocytes present a single lipid drop, where the triacylglycerol is stored, of large size that displaces the cytoplasm and the nucleus towards the cellular periphery; they have a diameter over 100 μM and often take a hexagonal shape. When the homeostatic equilibrium is inclined towards the energetic gain, the white adipose tissue (WAT) keeps this energetic excess in form of triglycerides inside the lipid droplets of the adipocytes: a phenomenon that is known as hyperplasia [108]. Interestingly, the fat cell number is largely determined in childhood and adolescence, remaining almost invariable in adulthood [109].

As previously stated, it's well known the capacity of WAT as a fat store, but was not until the 50's that Kennedy started to hypothesize about the possibility of fat as a endocrine organ,

which sends signals through the bloodstream to several other organs [8]. With the studies of genetically modified mice, ob/ob (leptin deficient animals which are models of peripheral neuropathy, type 2, diabetes and obesity) and db/db (LEPRb-deficient non-insulin dependent diabetic animals which are models of diabetic dyslipidemia), this hypothesis of a circulating factor was confirmed [110-112]. Both models shared the same phenotype: mice were obese, hyperphagic, diabetic, with low physical activity and thermoregulation. Therefore, it is accepted that the WAT is not a mere energy depot, and it also has the capacity to release peptides called adipokines. These molecules participate through endocrine, paracrine, autocrine or juxtacrine crosstalking in a vast diversity of physiological and physiopathological processes.

3.1 LEPTIN

The first adipokine was discovered in 1994 by Friedman and collaborators. They isolated and characterized the product of the ob gene and they called it leptin from the Greek root *leptos*, meaning thin. Leptin is evolutionarily conserved across mammalian and non-mammalian species [113, 114]. Nowadays we know that the ob/ob mice can't synthesize leptin, while db/db mice and fa/fa rats (Zucker) can synthesize leptin but they are leptin receptor-deficient. Both ob/ob or db/db mice are obese, diabetic, infertile and hypoactive [111, 113, 115-118], while leptin ICV administration causes reduction of body weight and food intake on ob/ob mice only [119]. The weight loss produced by leptin is different from that produced by starvation because it only involves loss of fat mass, although when administered chronically at high doses can lead to animal death by depletion of energy stores [120].

Leptin is a 16 KDa single-chain polypeptide circulating hormone which holds 167 amino acids and is secreted by the WAT in such a way that, its plasma levels correlates with the amount of triglycerides present in the adipose tissue, but its synthesis is also regulated by inflammatory mediators [121]. Leptin is a measure of available energy stored as fat in the organism [3]. It suppresses appetite and stimulates energy expenditure through the stimulation of BAT and the expression of uncoupling protein 1 (UCP1) [77, 122]. Apart from WAT, leptin does exist with lower expression in BAT, the stomach, gut, epithelium of the mammary gland, placenta and skeletal muscle [13, 123]. In addition, leptin stimulates the activity of the sympathetic nervous system (SNS) and increases energy expenditure [58, 77, 124].

Proteins enter the brain through the blood-cerebrospinal fluid (CSF) barrier at the choroid plexus or through the blood brain barrier (BBB) at the cerebral endothelium [125]. However, simple diffusion is not enough for leptin to cross the blood brain barrier because of its size. Hence, it crosses the BBB using a saturable transport system [122, 126, 127] and may also be transported into the brain via cerebrospinal fluid [128], in order to access the central leptin receptors. The long form leptin receptor (LEPRb) is co-expressed with NPY, AgRP, POMC and CART in the ARC.

The expression of orexigenic peptides such as MCH and orexin/hypocretin in the lateral hypothalamic nucleus is regulated by leptin-sensitive neurons in the arcuate hypothalamic nucleus. Other intermediaries of leptin activity in the brain are CRH, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), urocortin, bombesin and serotonin. In states of leptin deficiency like ob/ob mice or fasting, MCH and NPY orexigenic peptide levels are increased, and decreased after leptin administration. Conversely, POMC and CART anorexigenic

peptide expressions are decreased in ob/ob mice and during fasting and increased in response to leptin administration. Leptin activates POMC/CART neurons through a neural network while deactivates AgRP/NPY in the ARC [25, 129-131]. (Diagram 7).

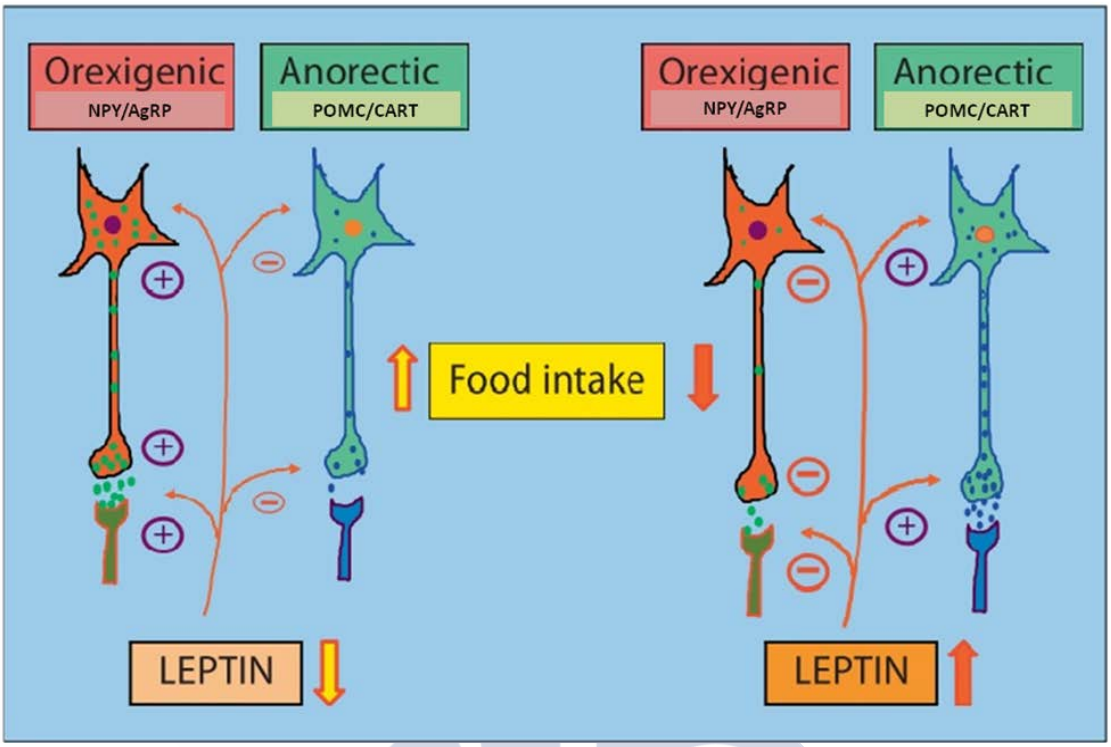

Diagram 7. Schematic presentation of leptin action on hypothalamic signals governing feeding. Abbreviations: NPY (neuropeptide Y); AgRP (agouti-related peptide); POMC (proopiomelanocortin); CART (cocaine and amphetamine regulated transcript). Modified from Sahu et al 2011 [132]. With permission of Karger.

The fact that blood leptin levels correlate with adipocyte size means that the obese have a greater amount of circulating leptin since hypertrophy of adipocytes leads to an increase in leptin production by individual cells. This increase can get as high as approximately twice the initial value of leptin. Leptin levels in the blood fall when energy intake is limited and fat energy stores are declining. Falling leptin during starvation promotes increased energy intake to decrease energy expenditure. One could think of this as being advantageous with leptin acting as an anti-obesity hormone: since the more leptin the more sense of satiety. Also animals with mutations in the ob gene are obese and lose weight when given leptin. But unfortunately, it is not the panacea against obesity. This is due to the fact that obese people are insensitive to endogenous leptin production meaning that there is a decreased sensitivity to leptin as a whole. Many factors, including limited leptin transport across the BBB, inflammation, endoplasmic reticulum stress (ER stress), and defective autophagy, contribute to the development of leptin resistance [129, 133-135].

The expression of leptin is controlled by several hormones such as insulin and glucocorticoids. Within the brain the main isoform of the leptin receptor that is implicated in the signal transduction which regulates weight and food-reducing effects of leptin is the long form of leptin receptors (LEPRb) [129, 136, 137]. This receptor has a full-length intracellular domain of approximate 300 amino acid residues and is the only receptor isoform that contains

active intracellular signaling domains. LEPRb is expressed in ARC, VMN, DMH and PMV (ventral premammillary) nuclei [138-142]. LEPRb does not contain intrinsic enzymatic activity, but instead, binds to and activates a cytoplasmic tyrosine kinase called Janus kinase 2 (JAK2) [129, 136, 137, 143]. Activation provoke autophosphorylation on multiple tyrosine residues: Tyr985, Tyr1077 and Tyr1138. Phospho-Tyr985, -Tyr1077 and -Tyr1138. After activating tyrosine sites then it bind to downstream molecules and activate the JAK2/STAT3, JAK2/STAT5, PI3K/IRS/AKT, and SHP2/ERK pathways. These pathways act in a coordinated fashion to regulate energy balance and body weight. LEPRb signaling is regulated both negatively by the suppressor of cytokine signaling 3 (SOCS3), protein-tyrosine phosphatase 1B (PTP1B), T-cell protein tyrosine phosphatase (TCPTP), phosphatase and tensin homolog (PTEN) and protein tyrosine phosphatase epsilon (RPTPe) and positively by src homology 1(SH2B1). [112, 129, 132].

One of the most important pathways in leptin regulation through LEPRb is signal transducer and activator of transcription (STAT3) pathway, which is the chief regulator of the transcription of relevant genes such as the one encoding POMC, AgRP or even suppressor of cytokine signaling 3 (SOCS3) which mediates the inhibition of LEPRb-STAT3 signaling [136, 144-146]. Also phosphoinositide 3-kinase (PI3K) plays an important part in central leptin control, acting in an independent way apart from STAT3 pathway. It regulates the expression and activity of forkhead boxO1 (FoxO1), a transcription factor which has functions in the control of liver metabolism and pancreatic β-cell, but also mediates AgRP-dependent effects of leptin on food intake [42, 129, 132, 147-150].

Leptin acting through LEPRb has also been shown to regulate insulin receptor substrate-1 and 2 (IRS 1 and 2), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), protein kinase b (AKT), and PI3K, raising the possibility of cross-talk between leptin and insulin [151].

Leptin inhibits glucose-responsive neurons in the hypothalamus and insulin secretion by pancreatic β-cells, through effects on adenosine triphosphate (ATP)- sensitive potassium channels [129]. Insulin is a glucose-dependent hormone which is secreted from pancreatic islets and is proportional to the amount of fat. In the ARC insulin downregulates NPY expression and upregulates POMC expression, leading to a reduction of food intake. After feeding, glucose levels are high and trigger the secretion of insulin, which stimulates glucose uptake and utilization, and subsequently promotes glycogen and fatty acid synthesis in the liver [152]. Furthermore, in 2005 Coppari *et al.* demonstrate that the ARC is important for the regulation of glucose homeostasis via leptin mediated pathways [153].

STAT3 and PI3K signaling pathways are essential modulators of the anorectic action of leptin. When the binding of leptin with its receptor (Ob-Rb) occurs, a cascade of signaling is activated by activating JAK2, with the subsequent activation of STAT3. This leads to a dimerization of STAT3 that translocates to the nucleus and transactivates target genes including SOCS3, NPY and POMC. On the other hand, leptin activates PI3K and PDE3B, reducing the levels of cAMP, which leads to the activation of STAT3 (Diagram 8).

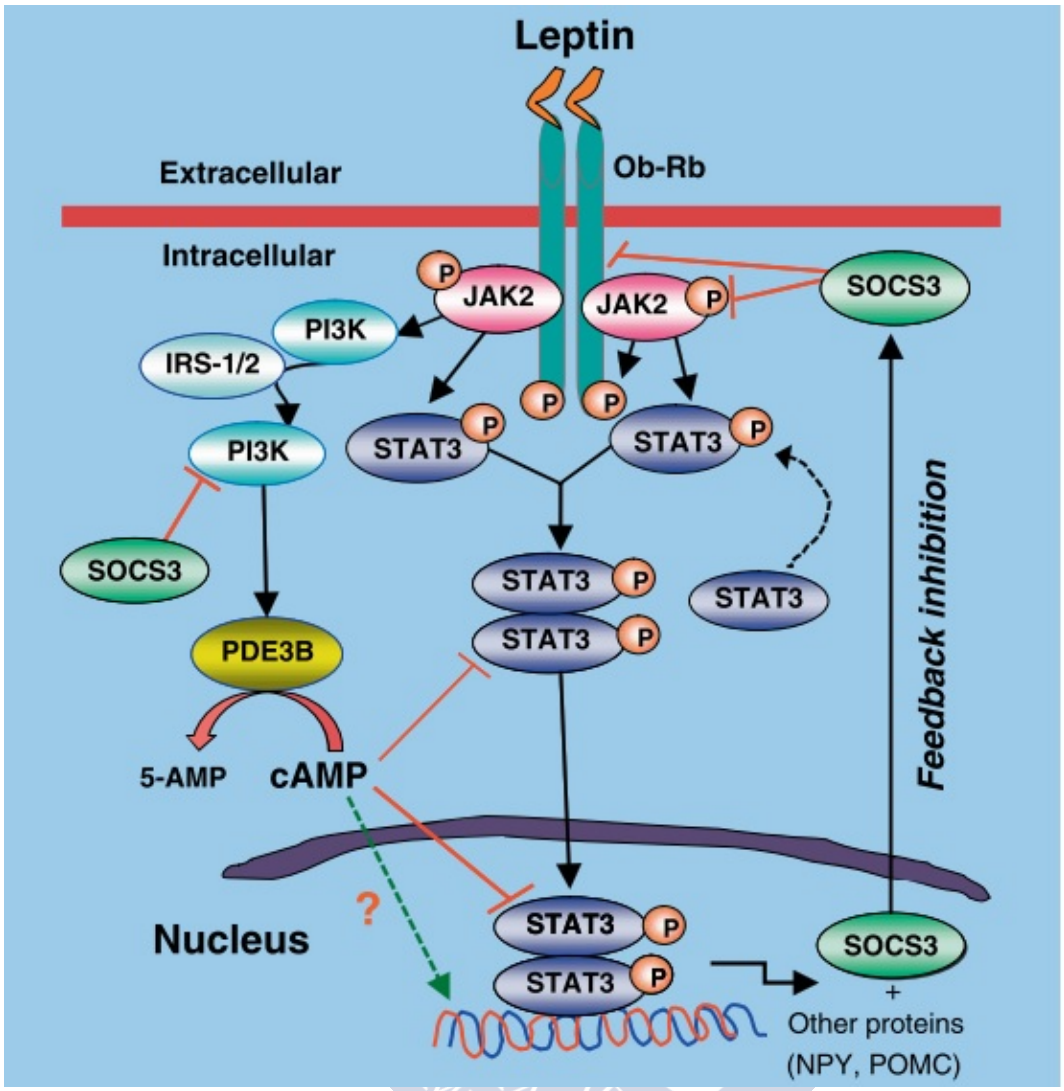

Diagram 8. Leptin intracellular signaling through the STAT3 and PI3K-PDE3B-cAMP pathways. Abbreviations: Ob-Rb (long form of the leptin receptor); JAK2 (janus kinase 2); PI3K (phosphoinositide 3-kinase); IRS-1/2 (insulin receptor substrate 1/2); STAT3 (signal transducer and activator of transcription 3); SOCS3 (suppressor of cytokine signaling 2); PDE3B (phosphodiesterase 3); cAMP (cyclic adenosine monophosphate); NPY (neuropeptide Y); POMC (proopiomelanocortin). [132]. With permission of Karger.

3.2 ADIPONECTIN

Another important adipokine is adiponectin, which is a 244 amino acid protein predominantly secreted from adipocytes and has an important role in regulating glucose and lipid metabolism as well as insulin sensitivity [154]. Circulating levels of adiponectin, are inversely correlated with obesity and its associated disorders such as Type II diabetes, hypertension, hypertriglyceridemia and cardiovascular diseases, whereas increased adiponectin serum levels are associated with improved insulin sensitivity [155]. It is known that after losing weight adiponectin secretion can be increased, proposing that the inflammation associated with diet-induced obesity could be affecting the expression and secretion of adiponectin. Adiponectin can cross the blood brain barrier and link to AdipoR1 and AdipoR2, which are expressed in NPY and POMC neurons within the ARC. There is

some controversy about if adiponectin is upregulating food intake or not. On one hand, intravenous administration of adiponectin, increases food intake in mice stimulating AMPK; [156]; on the other hand, when adiponectin was administered directly intracerebroventricularly, food intake was diminished [157]. Further studies are needed to fully clarify this point.

3.3 RESISTIN

Resistin is a 12.5 kDa cysteine-rich adipose-derived hormone which circulates in the serum [158] and is thus named because of its resistance to the action of insulin. This adipokine is also expressed in several tissues apart from WAT, including hypothalamus [159]. Its levels are affected by nutritional status, testosterone, glucocorticoids, endothelin-1, growth hormone, tumor necrosis factor-α, lipopolysaccharide and thyroid hormones [160]. Circulating resistin levels are increased in obese humans, playing an important role in the development of diabetes. Resistin secretion is stimulated by the inflammatory process (Interleukin 6, hyperglycemia and hormones such as growth hormone and gonadal hormones), and serum resistin is positively correlated with changes in BMI and body adipose mass [161].

Some other chemokines and cytokines such as interleukin (IL)-1, IL-6, IL-8, monocyte chemotactic protein (MCP)-1 and tumor necrosis factor alpha (TNF-α) contribute to the inflammation produced by obesity [162] and take part in the development of insulin resistance [163-165].

4 BROWN ADIPOSE TISSUE AND ENERGY HOMEOSTASIS: THERMOGENESIS

Brown adipose tissue is characterized and differentiated from WAT, by having adipocytes with polygonal shape and with multiple small cytoplasmic lipid droplets (multilocular), a central nucleus and a large number of mitochondria in the cytoplasm which gives it that characteristic brown color. Lipids are their primary source of fuel for oxidative phosphorylation and heat production. It has a very high oxidative capacity, using the oxidation of fatty acids via the tricarboxylic acid cycle for the production of heat instead of the production of ATP; this is named uncoupling [13, 159] .

The mitochondrion is found in all eukaryotic organisms and is composed of two compartments surrounded by external and internal membrane. The outer membrane is permeable to molecules of 5000 daltons or less through protein channels called porins, while the internal membrane is less permeable and is responsible for maintaining the electrochemical gradient generated by the respiratory chain. It has specific transporters for the interchange of ADP-ATP, phosphate, pyruvate, oxoglutarate, citrate, glutamate and malate [166]. In other tissues with mitochondria apart from the BAT, the electron transport chain is responsible for pushing hydrogen ions from the mitochondrial matrix into the space of the intermitochondrial membrane. In this way, the energy produced by the oxidation of the substrates is stored temporarily. Then adenosine triphosphate synthase (ATP synthase), which is the enzyme responsible for replacing these protons in the mitochondrial matrix, synthesize ATP from ADP and inorganic phosphate.

The same occurs in BAT, except when stimulated by the sympathetic nervous system. The signaling cascade that is prompted when the BAT is activated by an external stimulus such as cold or a hypercaloric diet, begins with the release of norepinephrine due to the signal transmitted by the sympathetic nervous system. Norepinephrine binds to the β-3 adrenergic receptors (β-3AR) present in brown adipocytes [167]. Intriguingly there is a significant decrease in β-3AR mRNA levels, in genetic models of obesity such as fa/fa rats and ob/ob mice. In humans, it was also shown that a mutation in these receptors is related with the metabolic syndrome, downregulation of energy expenditure and reduced insulin sensitivity [168, 169]. Those observations pointed the importance of β-3AR in thermogenesis [170, 171]. Furthermore, there are several types of adrenergic receptors. While the sympathetic activation of thermogenesis is due to β-3 adrenergic receptors, the β-1 adrenergic receptor is responsible for the noradrenergic action on the proliferation of brown preadipocytes [172]. The binding of norepinephrine with the β-3AR results in the activation of the adenylate cyclase through G protein which generates cAMP (cyclic adenosine monophosphate). The increased cAMP levels in turn activates protein kinase A (PKA) which accelerates fatty acid release from stored triglycerides through the phosphorylation and activation of hormone-sensitive lipase (HSL), adipose triglyceride lipase (ATGL) and monoacylglycerol lipase (MGL). The consequence, is that the number of free fatty acids (FFA) increases in the mitochondrial cytosol leading to increased β-oxidation and a subsequent increment of Acetyl-CoA which will enter in the citric acid cycle. This results in more nicotinamide adenine dinucleotide hydrogen (NADH) and flavin adenine dinucleotide hydrogen (FADH2) being released and entered into the electron transport chain leading to protons pumping out of the mitochondrial matrix [13, 166, 167, 173, 174]. When this occurs UCP1, which is a proton channel protein that belongs to the family of mitochondrial transporters, is responsible of the uncoupling of the proton gradient. Now the protons re-enter the mitochondrial matrix but without forming ATP, leaving this energy, that was produced through the oxidation of substrates, to be released as heat.

Several studies have demonstrated the thermogenic function of BAT. Mice with genetically reduced BAT are prone to develop obesity, however mice deficient in UCP1 are not prone to gain body mass [175, 176]. On the other hand, mice lacking UCP1 are extremely sensitive to cold [177] due to a global mitochondrial disruption [178]. This implies the possibility of a compensation mechanism between UCP1 and some other UCPs regarding the BAT regulation. Although UCP1 is mainly expressed in BAT [179], there are more UCPs that are expressed in other parts of the body (UCP2 in a wide range of tissues, UCP3 mainly in BAT and skeletal muscle [180]). These UCPs are involved in the transport of fatty acids. When there is an increase of the oxidation of fatty acids, these fatty acids start to accumulate in the mitochondrial matrix. UCPs pump the fatty acids outside the matrix to keep its concentration low [13] (Diagram 9).

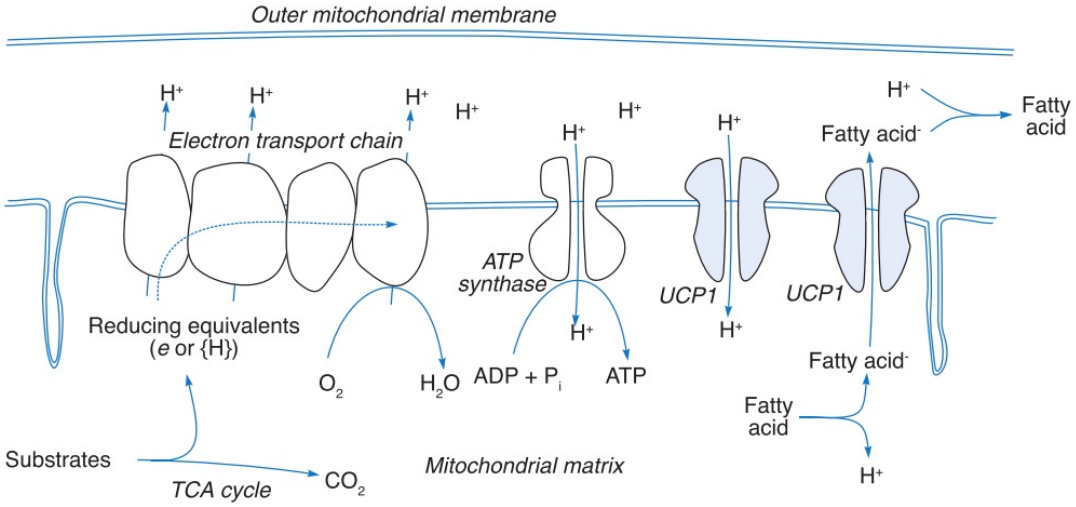

Diagram 9: Uncoupling of respiration in brown adipose tissue. Abbreviations: ATP (adenosine triphosphate); ADP (adenosine diphosphate); UCP1 (uncoupling protein 1); Pi (inorganic phosphate); TCA (tricarboxylic acid cycle) [13]. With permission of Elsevier.

With the activation of BAT through the sympathetic nervous system, the release of fatty acids from TGs is increased along with the blood supply through the many capillaries of this tissue. [13, 181, 182].This eventually causes the transportation of the heat generated through thermogenesis to other parts of the body through the blood [183].

The smaller the size of the body compared to the area, the more heat a mammal needs to generate. That's why BAT in babies is much more developed than in human adults. In fact it was thought that in the adult stage, there was no active BAT since the area/surface ratio is so low. But in 2007 [184] it became clear that human adults still have BAT, especially near the neck and shoulders [185, 186] (Diagram 10), and that its activity increases with exposure to cold [187].

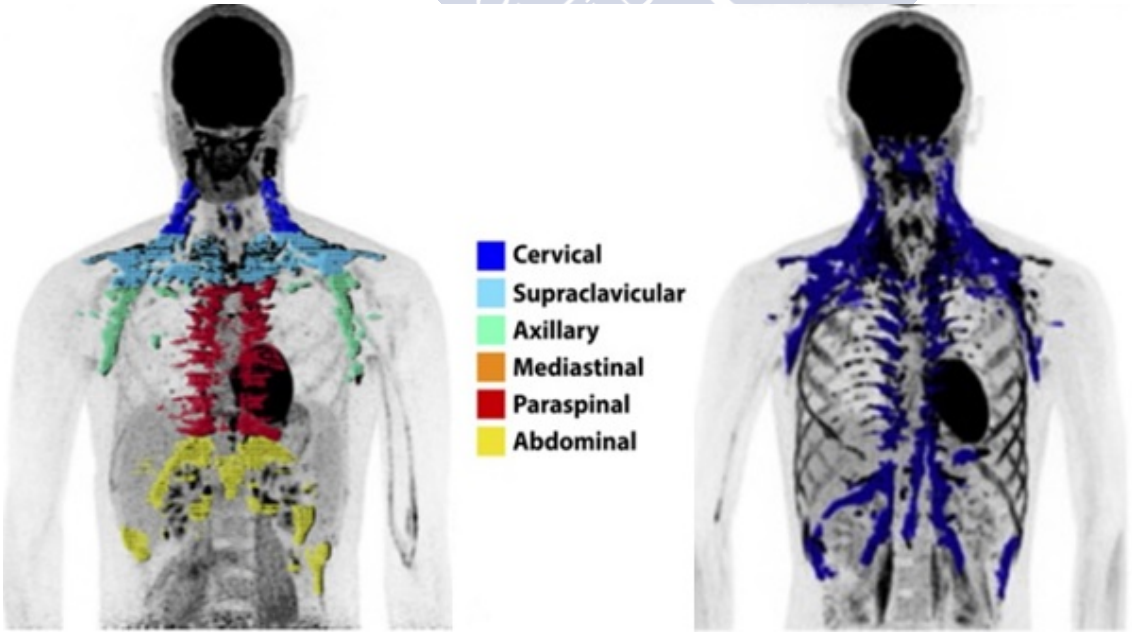

Diagram 10. Left: BAT distribution in a human adult. Right: Detected BAT with the axial method, shown in blue pixels [186]. Creative commons license

It has been seen that after exposure to cold, something similar to brown fat cells appear in WAT, which were then named beige or brite (brown in white) adipocytes. These unique cells are derived from precursor cells that differ from classic BAT and are closer to the cellular lineage of a white adipocyte (Diagram 11).

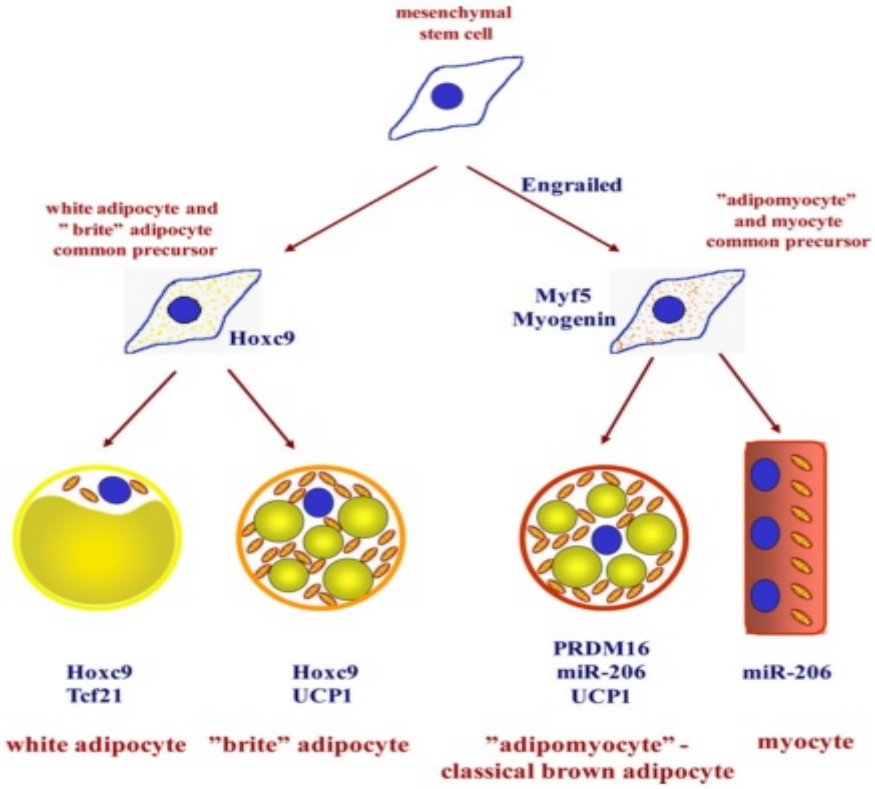

Diagram 11. Subtypes of adipocytes and their origins. Abbreviations: Hoxc9 (homeobox protein hox-c9); Myf5 (myogenic transcription factor 5); Tcf21 (transcription factor 21); UCP1 (uncoupling protein 1); PRDM16 (protein domain containing 16); miR-206 (microRNA-206). [188]. Creative commons license.

With the appropriate stimulus, white adipocytes would differentiate into brite adipocytes allowing them to develop thermogenic characteristics, but not expressing brown-fat specific markers such as protein domain zinc finger protein 16 (PRDM16), mesenchyme homeobox 2 (Meox2), LIM homeobox protein 8 (Lhx8) or zic family member 1 (Zic1). [188-191]. They are switched into brite adipocytes following a stimulus such as cold exposure which results in the required β3-adrenoceptor activation [192, 193]. Though the UCP1 of these adipocytes is functionally thermogenic, it has less thermogenic capacity than brown adipocyte UCP1 [194].

The warm/cold stimulus is first perceived by cutaneous warm and cold thermoreceptors, as well as thermoreceptors located within the body cavity, intestine and other general interoceptive means. The sensory receptors then send signals to peripheral sensory neurons which, in turn, relay the information to be conducted and integrated at a central level. The integration is carried out by the hypothalamus through many nuclei, such as the ARC, PVH, DMH, LH, VMN and the preoptic region [74, 195]. Additionally, peripheral signals such as ghrelin, leptin, thyroid hormones and estrogens have receptors in some of these nuclei, thus taking part in the thermoregulation process [159, 196, 197]. Leptin is also expressed in the

BAT [198] and both ob/ob and db/db mice have hypothermia apart from hyperphagia, thus affecting energy expenditure. This point is also seen when leptin is injected in dorsomedial hypothalamus, resulting in an increase in body temperature, heart rate and blood pressure [199]. Leptin increases glucose utilization in BAT and reduces TG utilization [200].

5 LIVER DISORDERS IN OBESITY: STEATOSIS, INFLAMMATION AND ER STRESS

The liver is located under the diaphragm and is irrigated by the hepatic artery and hepatic portal vein, providing blood from the intestinal tract as well as the stomach and spleen. In this way, the nutrients that come from the diet (mainly monosaccharides and amino acids) pass first through the liver before entering the general circulation. The liver is the first place where insulin and glucagon function takes part, since pancreatic veins are attached to the portal vein before they reach the liver. The liver is composed mainly of five types of cells: phagocytic Kupffer cells (KCs), sinusoidal endothelial cells (SECs), natural killer cells (NTK), hepatic stellate cells (HSCs) (involved in liver fibrosis) and hepatocytes. The hepatocytes are distributed hexagonally and are the main cell type of the liver taking care of metabolic biosynthesis, biliary secretion and detoxification. It has an important function in regard to the metabolism of amino acids, fats and mainly carbohydrates. It is not surprising then, that these cells are also where the glycogenolysis, glycogenesis, glycolysis and gluconeogenesis pathways exist in such a way that the concentration of glucose in blood is maintained despite of nutritional status [13].

Fibroblast growth factor 21 is a hormone chiefly produced by the liver that acts through a receptor complex consisting of a co-receptor (β-Klotho) and a canonical FGFR receptor located on the surface of the plasma membrane. FGF21 expression is highly regulated by feeding and also by fat and carbohydrate levels in diet so that prolonged fasting raises FGF21 circulating levels. It is also a direct target of PPAR since agonist treatment with PPARα and PPARγ, upregulate FGF mRNA levels in liver and adipocytes respectively. In obese patients with NAFLD, FGF21 levels are upregulated meaning that a FGF21 resistance exists, because its metabolic beneficial effects are impaired in these subjects. FGF21 is a cold-induced activator of BAT activity increasing energy expenditure, and plays an important role in fatty acid oxidation and glucose metabolism. In addition the activation of farnesoid X receptor (FXR) induces hepatic expression and secretion of FGF21 [201-208].

As for lipid metabolism, the liver through the autonomic regulation, synthesizes and degrades lipoproteins as well as converts excess carbohydrates into fatty acids for storage in the adipose tissue, through the autonomic nervous system [209]. Furthermore, our group has recently published that chronic central administration of MCH in the brain favors the lipid uptake and accumulation through the parasympathetic nervous system [210]. This particular MCH action in liver is mediated by kappa opioid receptor (KOR) in LHA. We had also shown that silencing KOR in the LHA attenuates ER stress, inflammation, steatohepatitis and fibrosis produced by the intake of methionine choline-deficient diet, choline-deficient diet and high fat diet [211].

Once the triglyceride accumulation capacity of the adipose tissue is exceeded, a phenomenon called ectopic accumulation of lipids occurs, whereby the lipids begin to

accumulate in other tissues [212]. This is the first step towards non alcoholic fatty liver disease.

5.1 NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) AND NONALCOHOLIC STEATOHEPATITIS (NASH).

Non alcoholic fatty liver disease (NAFLD) is defined as an illness that takes place when there is a fat accumulation in the liver exceeding 5% to 10% by weight, in absence of alcohol consumption. This term ranges from the hepatic accumulation of triglycerides in the cytoplasm of the hepatocyte (steatosis) without significant liver injury (NAFLD) to the development of NASH (nonalcoholic steatohepatitis) which is also hepatic steatosis with added inflammation (steatohepatitis), fibrosis and/or cirrhosis [213, 214]. As mentioned earlier, the first step towards NAFLD is the liver ectopic accumulation of triglycerides thus developing steatosis. However, the manifestation of some other elements are necessary to promote inflammation, cell death and fibrosis (which is an exaggerated wound healing response based in an increased deposition by the hepatic stellate cells of connective tissue (collagen) in hepatic cells) to develop NASH [215, 216]. (Diagram 12).

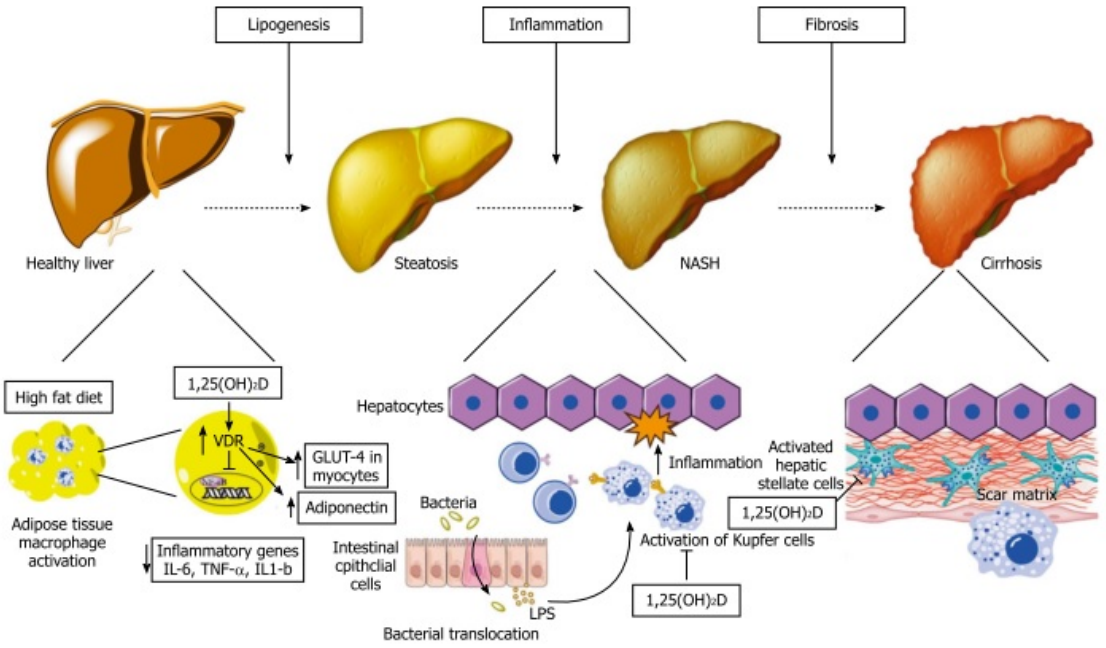

Diagram 12. Progression from a healthy liver to cirrhosis. Abbreviations: VDR (vitamin D receptor); GLUT-4 (glucose transporter 4); IL-6 (interleukin-6); TNF-a (tumor necrosis factor-alpha); IL1-b (interleukin 1-beta); LPS (lipopolysaccharides). [217]. Creative commons license

When there is an excessive accumulation of fat, especially saturated fatty acids, lipotoxicity occurs [218], producing the appearance of ER stress and inflammatory pathways activation.

5.2 ENDOPLASMIC RETICULUM STRESS (ER STRESS)

ER stress consists in the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum. The endoplasmic reticulum is the organelle responsible for the folding of proteins. Under ER stress the proteins stop folding properly. Accumulation of misfolded proteins promotes activation of the unfolding protein response (UPR) which consists in the degradation of the misfolded proteins, and an increase in the activity of the chaperones responsible for protein folding, such as the binding immunoglobulin protein (BIP). There are three transmembrane proteins responsible for activating the UPR: RNA-dependent protein kinase-like ER eukaryotic initiation factor-2α kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring ER-to-nucleus signaling protein 1 (IRE) [219].

Under normal conditions, these three effectors are linked to the BIP chaperone, also known as GPR78 (78-kDa glucose-regulated protein), causing these sensors to be deactivated under stress. In response to an increase in unfolded proteins in the endoplasmatic reticulum, however, BIP is detached from those proteins in order to start folding the problem proteins. This detachment, thus activate these ER stress detectors. PERK is a kinase that dimerizes and induces the phosphorylation of the α-subunit of eukaryotic translation initiation factor-2α (eIF2α). peIF2α attenuates translation to prevent the assembly of the 80s-ribosome that is required to perform protein translation halting the process of protein synthesis, while targeting the selective translation of ATF4 .This activation of the transcription factor ATF4, induces the expression of several genes: amino acid transporters, chaperones and CCAAT-enhancer-binding protein homologous protein (CHOP) in turn, induces the expression of growth arrest and DNA damage-inducible protein (GADD34) which is associated with protein phosphatase 1 (PP1) to dephosphorylate the eIF2a in a negative feedback loop, thus resuming translation. IRE1α is a serine-threonine protein kinase and an endoribonuclease. Upon ER stress this protein autophosphorylates via its kinase domain in turn dimerizing and switching on its endonuclease activity. Once activated, it works by degrading mRNA in the ER and also promotes the splicing of X-box-binding protein-1 (XBP1) mRNA. XBP1 is a transcription factor in charge of molecular chaperones as BIP/GPR78 and genes involved in ER-associated degradation. ATF6 is also a transcription factor that is anchored in the ER membrane which, upon UPR activation, is transported to the Golgi apparatus, where the transcription factor domain is released from the membrane by sequential action of site 1 and 2 protease (S1P and S2P). This enables its migration to the nucleus to activate transcription of UPR target genes. ATF4, calcineurin binding protein (CBP1) and ATF6 are responsible for uprising the transcription of components related with ER-associated protein degradation (ERAD) which are used to direct the degradation of misfolded proteins, as well as components involved in autophagy and apoptosis to re-establish ER homeostasis. If UPR signaling fails, it could lead to the onset of programmed cell death. [218, 220-228]. (Diagram 13).

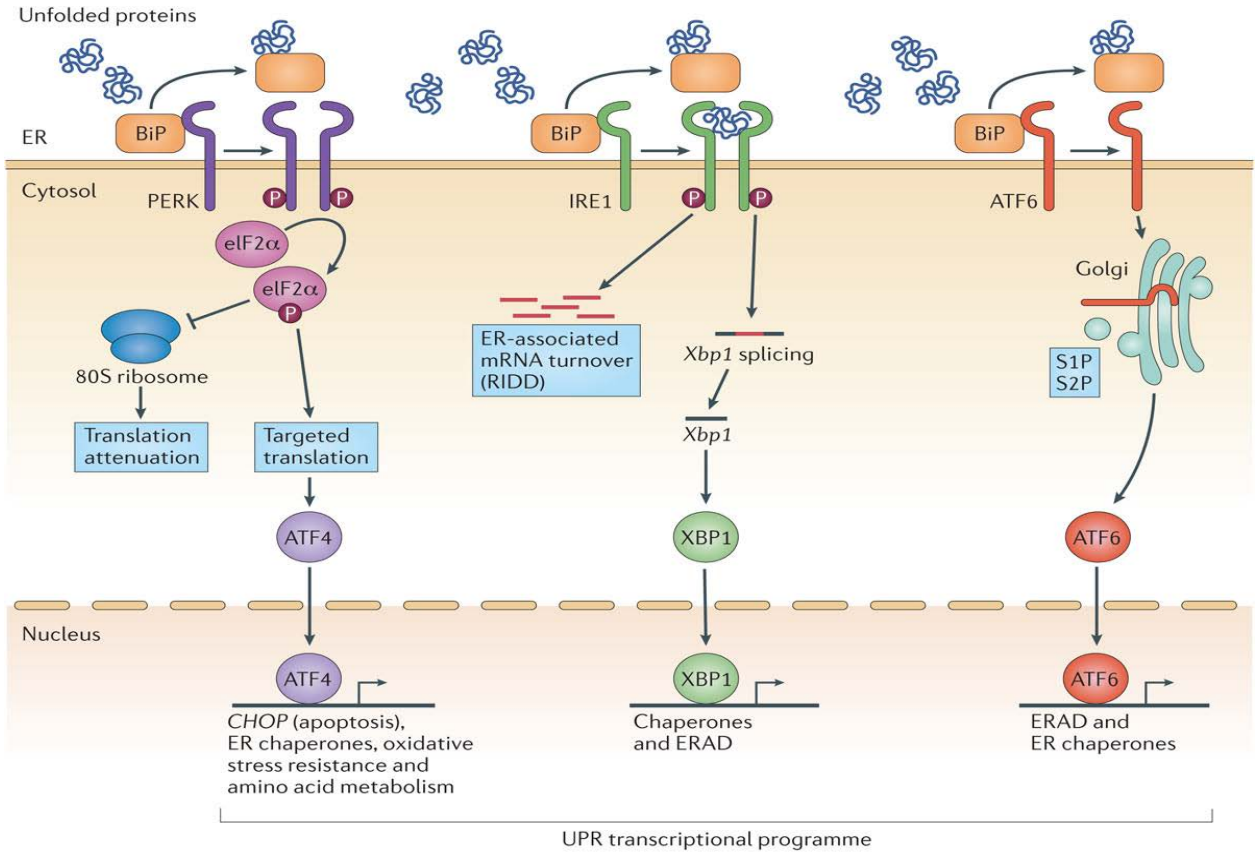

Diagram 13: Cellular responses to endoplasmic reticulum stress. Abbreviations: ER (endoplasmic reticulum); BIP (binding immunoglobulin protein); PERK (RNA-dependent protein kinase-like ER eukaryotic initiation factor-2α kinase); elF2α (eukaryotic translation intiation factor-2α); IRE1 (inositol-requiring ER-to-nucleus signaling protein 1); ATF6 and 4 (activating transcription factor 6 and 4); XBP1 (X-box-binding protein-1); S1 and 2 P (site 1 and 2 protease); ERAD (endoplasmic reticulum associated protein degradation); UPR (uncoupling protein response) [220]. With permission of Springer Nature.

The aims of UPR pathways are to increase the ER folding function, demote misfolded proteins and diminish the amount of new proteins getting in the ER [229]. Unresolved ER stress perturbs hepatic lipid homeostasis which is translated into apoptosis. This occurs through a high action of the proapoptotic transcription factor CHOP which leads to the inhibition of the transcription factor CCAT/enhancer-binding protein α (C/EBPα). C/EBPα is an activator of important regulators of lipid metabolism resulting in reduction of lipid oxidation, lipoprotein secretion and lipogenesis. This activation therefore participates in the generation of hepatic steatosis [230]. Also B-cell Lymphoma 2 (BCL-2) family proteins, calcium, redox homeostasis, caspase activation and activated protein kinase c-jun N-terminal (JNK) are other proteins that mediate ER-stress induced apoptosis [229].

Regarding mitochondrial dysfunction, the mitochondria of NASH patients has morphologic abnormalities [231], producing aberrations in the mitochondrial respiratory chain leading to an impairment in the mitochondrial β-oxidation [232]. Also, it was shown in an obese-rodent model that mitochondrial dysfunction is developed before insulin resistance and hepatic steatosis [233].

Reactive oxygen species formation (ROS) are very small, highly reactive molecules due to the presence of an unpaired valence electron layer and thus powerful oxidants [234], and

oddly enough, are intrinsic to oxygen metabolism [235]. The reduction in mitochondrial respiration rate results in an imbalance between oxidative species production and antioxidant defenses. This imbalance leads to decreases in ATP [236] levels, and also upregulates the generation of ROS. ROS causes lipid peroxidation which results from electrons of the membrane lipids being taken by these reactive oxygen damaging the cell integrity [237]. When peroxidation is achieved in polyunsaturated fatty acids (PUFAs), it leads to the proteolysis of the apolipoprotein B (ApoB) in the endoplasmic reticulum resulting in reduction of VLDL secretion and contributes to the rise of lipid stores in the liver [238].

5.3 INFLAMMATORY PATHWAYS ACTIVATION

When the production of triglycerides is excessive like in the case of diet induced obesity (DIO), there is an expansion of the tissue due to excessive storage. This expansion is a harmful stimuli for the body which, consequently, causes a protective response via the inflammation of the tissue. This can be seen by an increase in proinflammatory cytokines, a decrease in protective factors and an increase in communication between inflammatory and metabolic cells in order to repair the injured tissue. If the problem persists, it can result in a metabolic disease called meta-inflammation (metabolically triggered inflammation). In this case, instead of working as short-term solution, the inflammation is prolonged and its effects are not beneficial [239]. It has been seen that this inflammation precedes the insulin resistance characteristic of DIO [240].

The inflammation leads to the activation of the immune response: a process that includes the action of FFA, bacterial lipopolysaccharide (LPS), chemokines, cytokines and adipokines [241] .

When activated, neutrophils discharge proinflammatory cytokines and myeloperoxidase which produces reactive oxidants and radicals, resulting in oxidative damage to hepatocytes [242]. NK cells may have an anti-fibrotic effect in the liver through stimulation of HSC killing and the reduction in their levels participates in the tendency of cirrhosis progression in obesity [243]. NTK cells are a subset of lymphocytes that express NK cell markers (CD61 and CD94) as well as a T cell receptor. After stimulation, they secrete T helper 1 and 2 (Th1 and Th2) cytokines. NTK cell also appear to drive hedgehog ligands leading to fibrogenesis [244]. However, when there is moderate or severe steatosis, the NTK cell population decreases. The Kupffer Cells are mononuclear phagocytes that reside within the sinusoids of the liver. They can be activated by DAMPS (damage associated molecular patterns) such as DNA, RNA, high mobility group protein B1 (HMGB1) and FFAs, leading to the release of proinflammatory cytokines (TNFα, IL-6, IL-1β). These cytokines lead to T cell activation, induction to apoptosis and HSCs. Regarding macrophage cell types, there are mainly two: M1 or “classically activated” with a role in humoral immunity and response to pathogens, and M2 or “alternatively activated” with an anti-inflammatory property. In response to HFD and obesity, there is an increase in M1 relative to M2. Thus, this M1/M2 imbalance may have a role in NASH progression [245, 246].

At a metabolic level, when cytokines such as TNF-α and IL-1 are displayed, JNK is phosphorylated in the liver [247]. JNK is a well known mediator of cellular stress response, being activated in NAFLD and to which inactivation ameliorates NASH [248]. Intriguingly, our group described that JNK1 plays an important role in the MCH-induced hepatic steatosis [210]. Another critical cytokine is inhibitor of nuclear factor kappa- β (IKK-β), which is key

in the development of insulin resistance [249]. In fact our group has shown that transactivation protein p63 (TAp63) induces liver steatosis through IKK-β activation and the induction of ER stress [250]. TLR4 expression is increased under liver injury as a sensor of FFA levels with an anti-apoptotic result in HSCs. Furthermore, TLR4 leads to NF-ƙ B activation which in turn, protects the liver from the induction of cell death made by TNF-α [251].

The inflammation of the liver leads to a launch of necrotic and pro-apoptotic pathways, which consequently, stimulate hepatic stellate cells and Kupffer cells to drive the cell into fibrogenesis [252, 253].

6 GASTROINTESTINAL HORMONES AS REGULATORS OF ENERGY HOMEOSTASIS

There are gastrointestinal hormones that play an important role in appetite/satiety: mediating the crosstalk between the brain and the periphery. These hormones include ghrelin, cholecystokinin (CKK), gastric inhibitory polypeptide (GIP), glucagon like peptide-1 (GLP-1) and peptide YY (PYY) [106, 254, 255] among others.

6.1 GHRELIN

Ghrelin was discovered in 1999 by the team of Kojima M. as the endogenous ligand for G-protein-coupled receptor (GHS-R), which stimulates the secretion of GH. The sequences of human and rat ghrelin differ in only 2 amino acids [256]. The ghrelin protein is expressed mainly in the stomach [257] but can also be found in the small intestine, brain, cerebellum, pituitary gland, lung, pancreas, salivary and adrenal glands, ovaries and testes [38, 258]. Its plasma levels are strongly increased before meals and fall sharply afterwards [259]. Its action is antagonistic to that of leptin [260, 261] and insulin since it stimulates the intake: this lead to it being the first orexigenic peripheral hormone described in humans [29, 261, 262]. Its intracerebroventricular administration stimulates the intake in a dose-dependent manner and increases the weight gain in rats. Similarly, it has also been found that the total concentration of serum ghrelin (acylated and deacylated form) decreases with oral refeeding or glucose administration but not when only water is ingested [38]. In addition to an increase in intake, it also produces an increase in adiposity and reduces the utilization of fat in mice [263]. In studies with mice deficient for the ghrelin gene, it has been observed that these mice have a normal intake and body composition. Fasting produces the same decrease in serum leptin and insulin as control mice, and the administration of exogenous ghrelin stimulates appetite in these mutated mice [264, 265]. From all this, we can deduce the possible existence of compensatory mechanisms. However, in a 2005 study [266] with mice deficient for the ghrelin receptor fed a high-fat diet, the observed phenotype was lower intake and lower body weight and accumulation of adiposity compared to control mice. The same phenotype was observed in receptor-deficient females fed a normal fat diet content, indicating that ghrelin is essential for the development of obesity induced by the diet. The endogenous ghrelin plays an important role in determining the type of metabolic substrate to be used for homeostatic maintenance, especially in conditions of high-fat diet. Therefore, this protein is a sensor of the

availability of nutrients effectively modulating the efficiency of the accumulation of energy in periods of maximum availability [263, 264]. The regulation of the orexigenic action of ghrelin is carried out via activation of the orexigenic neuropeptides NPY/AgRP as well as through the suppression of the hypothalamic tone of the melanocortins. The latter is illustrated by the double KO mice for MC3R and MC4R, which have shown an attenuation of the effect of ghrelin [29]. At the molecular level, the central administration of ghrelin *in vivo*, results in a activation at the hypothalamic level of the SIRT1/AMPK/P53 pathway [267]. In addition, our group has shown that the orexigenic action of ghrelin is regulated by hypothalamic kappa opioid receptor (KOR) signaling since inhibition of KOR expression in the hypothalamic ARC is sufficient to blunt ghrelin-induced food intake. Dynorphin is the endogenous ligand of KOR and we also shown that ProDyn was increased in the ARC nucleus at 2 hours after central ghrelin administration [268]. Furthermore, the neurotransmitter dopamine also plays an important role in ghrelin regulation since specific central blockade of dopamine receptor 1, 2 and 3 (DRD1, 2 and 3), respectively reduces the orexigenic action of ghrelin [269]. Finally, the recent identification of the liver-expressed antimicrobial peptide 2 (LEAP2) as an endogenous non-competitive allosteric antagonist of the ghrelin receptor, offers new perspectives in the understanding of the role played by the ghrelin system in energy balance [270].

6.2 CHOLECYSTOKININ (CCK)

CKK is a peptide that is expressed throughout the digestive tract, although most of it is produced in I cells of the duodenum in response to dietary lipid and protein, where it is rapidly released into the circulation and surrounding tissues in response to the arrival of food in the intestine. The main effect of CCK is the reduction of food intake, delay of gastric emptying and stimulus of the secretion of pancreatic enzymes. The response of vagal afferent neurons to CCK are attenuated in obesity [271], [272, 273]. There are 2 receptors for CCK: the CCK-1 (or CCK-A) that is found throughout the entire gastrointestinal tract and the CCK-2R (or CCK-B) that is located in the brain. The administration of CCK inhibits the food intake by reducing meal size and duration and also stimulates pancreatic enzyme secretion and gallbladder contraction. At high doses, CCK causes nausea and taste aversion. This anorexigenic effect is mediated by the receptor CCK-1R and the signal to the autonomic nervous system is made through the vagus nerve, since it has been found a blockage in this effect after vagotomy. The CCK antagonists generate an increase in the amount of calories ingested as well as the reduction in the perception of satiety [274]. CCK has a very short half-life, in such a way that it will only have an effect when it is administered less than 15 minutes before ingestion. The chronic administration of CCK does not produce a weight decrease due to development of tolerance to its effect after the first 24 hours of administration. Interestingly, it has been described that the CCK and leptin treatment together, improves the effect on weight loss associated with leptin treatment [275, 276].

6.3 GASTRIC INHIBITOR POLYPEPTIDE (GIP)

GIP is a 42 amino acid hormone that is produced predominantly in the enteroendocrine K-cells in the proximal regions of the small intestine, which is released into the circulation in response to nutrient stimulation. It is also localized in the CNS favoring cell survival [277]. Circulating levels of GIP are low during periods of fasting but these levels increase a few

minutes after the intake of nutrients. The GIP receptor is member of the B-family of G protein-coupled receptors and has two isoforms, of 466 and 493 amino acids respectively, which are expressed in the β-pancreatic cells, adipose tissue, heart and brain. The GIP receptor is coupled to adenylate cyclase whose activation will increase intracellular calcium and the flow of arachidonic acid. Activation of the GIP receptor results in the stimulation of adenylate cyclase and Ca^{2+}-independent phospholipase A and activation of protein kinase A (PKA) and PKB [277, 278]. It has not been described as having an important anorectic effect, but it does promote energy storage, interacting with insulin to increase lipoprotein lipase activity and lipogenesis in adipose tissue. Furthermore, it has been shown that the association of GIP, GLP-1 and glucagon in a chimera generates a powerful triagonist with a potent anti-obesity action [279].

6.4 GLUCAGON-LIKE PEPTIDE-1 (GLP1)

GLP-1 is produced in the intestinal epithelial endocrine L cells of the small intestine , a peptide resulting from the differential processing of proglucagon. Proglucagon is a polypeptide of 160 amino acids that is expressed in the intestine (thin and thick), α cells of the pancreas, taste buds and regions of the central nervous system, including the hypothalamus [280, 281]. GLP-1 is released in response to meal intake in a two-phase pattern, a first phase at 10-15 minutes after ingestion and a second phase between 30-60 minutes. GLP-1 is extremely rapidly metabolized and inactivated by the enzyme dipeptidyl peptidase IV raising the possibility that the actions of GLP-1 are transmitted via sensory neurons in the intestine and the liver expressing the GLP-1 receptor [282]. The GLP-1 receptor (GLP-1R) is an heptameric protein of 463 amino acids, coupled to G proteins that is expressed in multiple tissues: pancreatic islets, the ducts of the pancreas, kidneys, lungs, skin, heart, immune cells , hypothalamus, hippocampus and cerebral cortex; the presence of GLP-1R in the liver, in WAT and in skeletal muscle is unclear, although specific effects of GLP-1 have been found in these tissues [283]. GLP-1 stimulates insulin secretion and inhibits glucagon secretion, by which contribute to limit postprandial glucose excursions. It also inhibits gastrointestinal motility and secretion. Central administration of GLP-1 acutely reduces food intake in fasted rats, ICV injection of the specific GLP-1 receptor antagonist exendin cause suppression of the inhibitory effect of GLP-1 on food intake [276, 284]. Furthermore, the central activation of GLP-1 stimulates the incorporation of glucose in the muscle and favors the formation of glycogen in the liver [285]. The central actions of GLP-1 also involve lipid metabolism in fatty adipose tissue, reducing TG storage independently of intake [286]. Pharmacological GLP1R activation controls energy balance [284] . BAT is also regulated by the central effects of GLP-1 [284]. Our group demonstrated that when liraglutide (a GLP1R agonist) is injected in mice, it increases the thermogenic program, also favoring the transformation of adipocytes from WAT to a browning adipocyte phenotype through the hypothalamic AMPK [287, 288].

6.5 PEPTIDE YY (PYY)

This peptide is a 36 amino acid polypeptide located throughout the intestine with a greater concentration in the colon and rectum. PYY is synthesized and released from specialized endocrine cells (L-cells) from the gut in proportion to the calories ingested, leading to an increased plasma levels at 15 minutes and a peak at 60 minutes [289]. Although

there is some controversy in this regard, form PYY1-36 is orexigenic while PYY 3-36 reduced food intake in rodents and in normal weight and obese humans.

Its post-prandial effects include delayed gastric emptying and reduced gastric secretion [290]. It is described that there are age-dependent changes in PYY levels through postnatal development, with the highest hormone levels achieved in early postnatal life (day 10) and decreasing thereafter. Is well described the interrelationship between thyroid status and energy homeostasis since patients with alterations in thyroid status showed alterations in food intake, energy expenditure, and body weight. In this regard, PYY levels are decreased in hyperthyroid rats [291].

7 KLF4 : A NEW TRANSCRIPTION FACTOR IN THE REGULATION OF ENERGY BALANCE

KLF4 was originally cloned by two separated groups, given it a different name: “Krüppel like factor” (KLF) is because its highly expressed in the intestine [292], however other study, named it “epithelial zinc finger” (EZF) due to its high expression in skin epithelium [293]. Finally, since the expression of this protein is also evident in other tissues (appendix, bone marrow, lung, testis, duodeno, placenta, among others), it was called Krüppel like factor 4 because of the strong homology with the Drosophila gene product Krüppel which is crucial in the segmentation of developing embryo [294, 295]. KLF4 gene is conserved among vertebrate species from zebrafish to human [296].

KLF4 is a zinc-finger-containing transcription factor that is part of the Krüppel-like factor family (from KLF1 to KLF17) that binds to GC-rich DNA with a consensus binding sequence of CACCC. KLF4 has three types of domains: an N-terminal transcriptional activation domain, a repression domain and a C-terminal DNA binding domain. Both the activation and repression domains interacts with p300/CBP. Once this takes place, the KLF4 raise the expression of their target genes. The DNA binding domain has three zinc fingers that identify GC/CACCC and get attached to them. Also there is a potential proline, glutamic acid, serine and threonine sequence (PEST sequence) between the activation and repression domains and a nuclear localization signal (NLS) between the DNA binding and repression domains, which function is to facilitate the transport of KLF4 into the nucleus [292, 297] (Diagram 14). KLF4 contains both transcriptional activation and repression domains, and thereby activates and represses gene expression [298]. KLF4 regulates multiple biological functions, including cell proliferation, differentiation, apoptosis, metabolism, inflammation, embryogenesis and tumorigenesis.

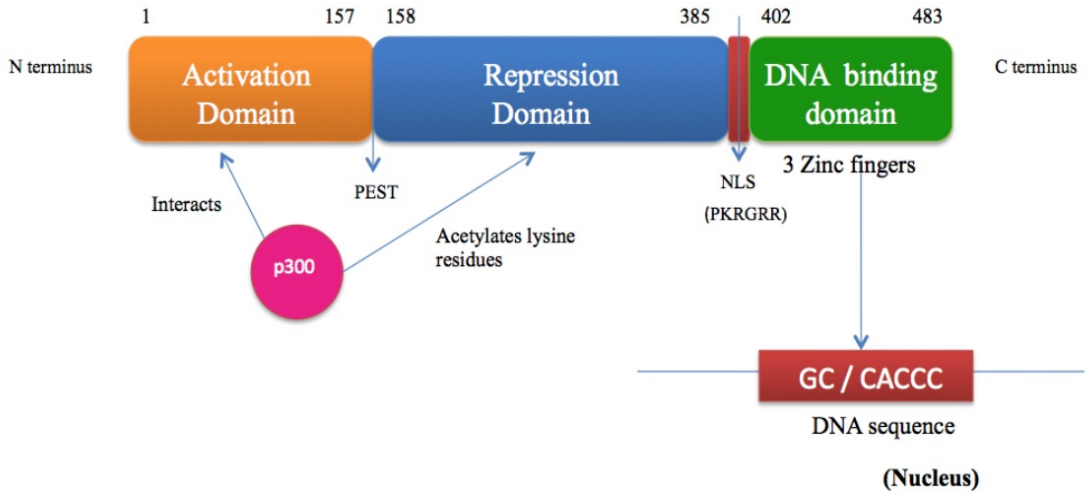

Diagram 14: Structure of KLF4. Abbreviations: PEST (proline, glutamic acid, serine and threonine sequence); NLS (nuclear localization signal)[294]. Creative commons license.

Its importance is highlighted by the fact that its germ-line deletion (exons 2 and 3 which produces a frameshift mutation in exon 4) leads to postnatal lethality due to a loss of skin barrier function even though the epidermis appeared morphologically normal. Regardless, it results in the penetration of external dyes and rapid loss of body fluids. KLF4-/- mice were born in a normal rate but they weren't able to feed themselves, dying within 15 hours of birth. [299]. These mice also show abnormal differentiation of goblet cells [300] [294]. The fact that the KLF4 KO are lethal because of the lost fatty layer of the skin, made Birsoy *et al.* think that KLF4 would be essential in the development of adipose tissue. KLF4 together with early growth response protein 2 (Krox20), activates CCAAT/enhancer-binding protein beta (C/EBPβ) promoter which in turns enhanced expression of PPARγ which is a very important factor in adipocyte differentiation [301]. Furthermore cyclic AMP (cAMP) which stimulates adipocyte differentiation [302] regulates KLF4 expression [301]. Altogether, this leads to the idea of KLF4 being a crucial factor in the developmental origins of adipose tissue [303].

KLF4 is expressed predominantly in the gastrointestinal tract (especially the colon and small intestine), but also in lung, skin, testis, thymus, cornea, cardiac myocytes, lymphocytes and embryonic stem cells [304, 305]. In the brain, KLF4 is expressed in neural stem cells [306] and is critical to neuronal differentiation [307, 308]. It is also implicated in self-renewal of embryonic stem cells and reprogramming of somatic cells to pluripotency [306]. Recent evidence suggests that KLF4 also plays an important role in the central regulation of energy balance [308].

It was shown that there is a colocalization of KLF4 and AgRP in GT1-7 hypothalamic cells. Also, overexpression of KLF4 in hypothalamic cells, leads to the activation of AgRP. Furthermore, after fasting, both proteins show a significant increase in mRNA levels. Algorithm analysis of AgRP human promoter, revealed the presence of two CACCC conserved boxes. The CACCC proximal box showed a specific binding to KLF4. PMI-5011 is a botanical extract of tarragon which activates AgRP *in vitro* and *in vivo* whose action is mediated via KLF4. Similarly, pharmacological activation of AgRP is abrogated when KLF4 is down-regulated [35]. Accordingly, GT1-7 cells transfected with a mouse KLF4 construct show a significant increase in AgRP message expression [309].

Similarly to ghrelin, KLF4 is highly expressed in the gastrointestinal tract. Butyrate treatment (an inducer of KLF4 expression [310]), stimulates the expression of ghrelin. Also, while fasting stimulates ghrelin expression, it also increases the expression of KLF4 in the stomach, liver, small intestine, kidney and liver. Also cAMP Response Element-Binding Protein (CREB), a transcription factor which activates several genes under fasting conditions, stimulates ghrelin expression under fasting in an indirect way through the interaction with KLF4 [311].

8 METABOLIC ALTERATIONS DURING PREGNANCY

Pregnancy is a physiological change state where the metabolic pathways are focused on gaining, not losing, energy surplus. It is well known that pregnant rats are hyperphagic and gain weight during pregnancy [312], increasing food intake 50% over non-pregnancy values at term [313]. This increase in food intake is explained at central levels in the ARC where there is an increase in NPY mRNA on day 13 and an increase in AgRP protein levels on day 18. At the same time it can be seen due to the abolition of appetite-decreasing α-MSH-ergic neural pathways [314-316]. In lateral hypothalamus, MCH and prepro-orexin mRNA levels are decreased [316]. Reproductive success is dependent on equitable sharing of resources, maintaining a healthy balance between the mother and the fetus, ensuring optimum fetal growth without endangering mother's health. This also effectively establishes a positive energy balance in preparation of the energy demands during lactation [317].

The main nutrients for a normal fetal growth are: glucose, FFAs and amino acids. The availability of these substrates for the fetus, is dependent on their concentration in the maternal bloodstream, and their transport through the placenta [318]. Each kind of nutrient has a specific transporter to cross the syncytium of the placenta [319, 320] (Diagram 15).

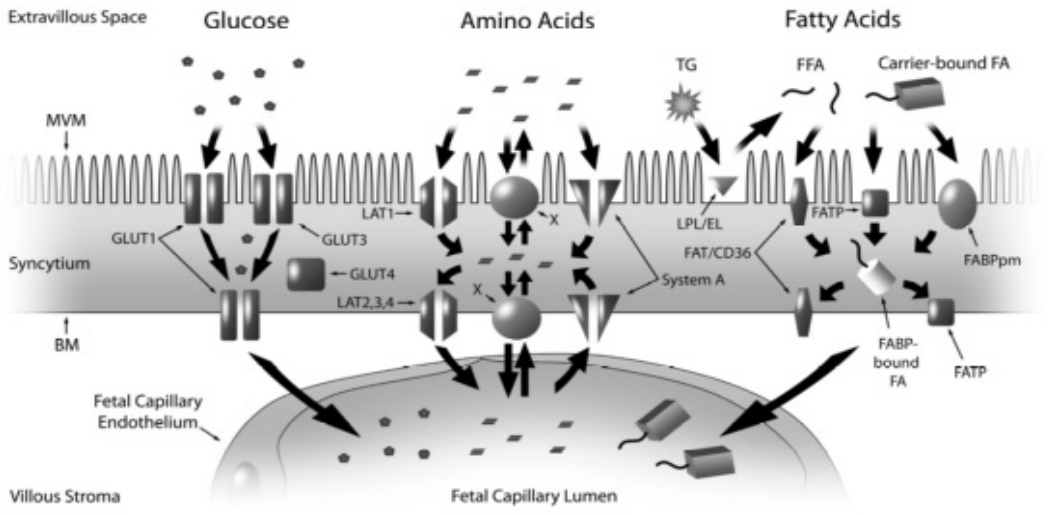

Diagram 15: Nutrient transport across the placenta. Abbreviations: BM (basal membrane); MVM (microvillous membrane); GLUT1, 3, 4 (glucose transporter 1, 3, 4); LAT (large neutral amino acid transport); FAT/CD36 (fatty acid translocase); FATP (fatty acid transport protein); LPL (lipoprotein lipase); FABPpm (fatty acid binding protein); FFA (free fatty acids); TG (triglyceride) [321]. Creative commons license

In pregnancy, plasma volume increases with a fall in hemoglobin and platelet concentration, but no changes occur in mean corpuscular volume or mean corpuscular hemoglobin concentration [322]. Also, the requirement for iron is increased [323]. In addition, there is peripheral vasodilatation, an increase in stroke volume, and an increase in heart rate. Blood pressure decreases at the beginning of pregnancy. Renal changes such as arterial underfilling, leads to the stimulation of arterial baroreceptors and an increase of renal plasma flow and glomerular filtration rate. These changes lead to sodium and water retention in the kidneys and create a hypoosmotic state [324]. It's also well known that pregnancy induce cholestasis which is the impairment of the bile flow [325]. Usually there is a feeling of breathlessness without hypoxia due to maternal hyperventilation. Another adaptive change of pregnancy is the nausea together with an increase in calcium absorption [326].

Regarding endocrine changes, there is an increase in estrogen and progesterone [327] which are steroid sexual hormones released by the ovarium and placenta during pregnancy. There is also a relative iodine deficiency while there is an increase in serum levels of adrenocorticotropic hormone (ACTH), cortisol and free cortisol, and oxytocin among others [324]. Common sense then tells us that it is logical that the sex hormones could be behind the increase of intake at the beginning of pregnancy [313, 328] and before the metabolic demand of the fetus increases. Without going any further, progesterone raises food intake when its expression is elevated in periovulatory stage, while estrogens decreases it in the curse of the luteal phase when progesterone levels are lofty [329]. Estradiol carry on both centrally and peripherally influencing in ingestion, metabolism, partitioning, storage and energy expenditure towards weight loss and progesterone rearrange all the estradiol effects towards weight gain [330].

It's also recognized that once the woman has passed through the menopause, the absence of estrogens causes increased intake and body weight [331]. Moreover, when the ovariectomy is performed with the subsequently loss of estradiol, it increases appetite and body weight a 10-30% [332, 333] and when estradiol is injected, food intake is decreased [334] and also the body weight [335]. Either way when the rats are treated with estradiol after ovariectomy, showed a decrease in NPY levels in the ARC [336], and normalize body weight and food intake [337]. Furthermore, estradiol is involved in the decrease of FAS in VMN observed in pregnancy [334].

8.1 GLUCOSE METABOLISM

Pregnancy is a diabetogenic state in which glucose and type II diabetes are associated with gestational weight gain [338]. There is a markedly hyperplasia in the insulin-secreting pancreatic beta-cells leading to an increase in insulin secretion and an increase in insulin resistance as pregnancy progresses, being greater in the second trimester than in the first and even higher in the third. The mechanisms behind this increased insulin resistance probably are different at the different stages of pregnancy but in general terms they can be ascribed to increased release of diabetogenic hormones like human placental lactogen, human placental growth hormone, glucagon, progesterone, cortisol and prolactin, resulting in a decrease in insulin sensitivity at the end of the pregnancy (50-70% lower). This resistance to insulin, originate alterations in postprandial concentrations of glucose, VLDL, amino acids etc., which is thought to ensure fetal feeding after a mother's meal by promoting greater use of carbohydrate substrate by the fetus [339, 340]. Either way, glucose levels in pregnancy are

decreased in fasting and basal state as a result of increased storage of tissue glycogen, increased peripheral glucose use, decrease in glucose production by the liver and uptake of glucose by the fetus. As a consequence of insulin resistance and hypoglycemia there is an increase in lipolysis, which favor pregnant mother to use fat for fuel, so that the glucose and amino acids will be almost completely accessible for the fetus, being glucose the principal oxidative substrate for the fetus [318, 341-343]. In postprandial situation, glucose levels are elevated but glucose uptake is reduced [344], together with an increase of basal endogenous hepatic glucose production which correlates with the increase of body weight [324] [345]. Furthermore, leptin effects on glucose homeostasis are blunted during pregnancy [346].

8.2 LIPID METABOLISM

During pregnancy, there is a maternal accumulation of fat depots and hyperlipidemia due to the breakdown of these depots, associated with a higher amount of plasma TG, phospholipids and cholesterol. This fat depot accumulation is increased in the first stage of pregnancy in part due to an increase in LPL activity leading to an upregulation of the uptake of circulating TG by the adipose tissue. In this stage, it's also seen the highest rate of fetal growth, which decrease towards the end [318]. Similar to some cases of fat enrichment like obesity, in early pregnancy there is a rise in glycerol kinase activity leading to metabolization of glycerol, which is not possible under a regular situation [347]. Thus together with the increase in insulin sensitivity, antilipolytic action of insulin in adipose tissue, lipogenesis and LPL activity (such as uptaking non-esterified fatty acids and glycerol), support the increase in fat accumulation during first part of pregnancy [318].

In the last third of pregnancy the situation changes from anabolic to catabolic with a higher adipose tissue activity, downregulation of LPL activity [348] being its lipolytic activity also enhanced [339], high plasma levels of lactogen (which produces lipolytic effects, increasing plasma non-esterified fatty acids and glycerol that goes to maternal liver in order to form VLDL-TG and to be released in the bloodstream [349]) and decrease in insulin sensitivity [344], which lead us to the fact that insulin is the main fat regulator in pregnancy [350] (Diagram 16). In addition, there is a rose in total serum cholesterol and serum triglyceride because the liver increases the synthesis of TG [339]. These changes are important to ensure mother's energy needs, decreasing the catabolism of adipose tissue. It's also due an increase in low-density lipoprotein (LDL) levels, which is crucial for the placental steroidogenesis which has an important function in menstrual cycle, pregnancy and embryogenesis. Regarding high-density lipoprotein (HDL), the levels are 15% higher than non-pregnant levels. [324] Insulin resistance together with increased estrogens in the last part of gestation contributes to enhance adipose tissue lipolysis and hyperlipidemia [318]. The diminution of LPL activity in WAT and liver together with the increment of its activity in the placenta provokes an alteration in VLDL clearance. All these changes favors fat mobilization in the last part of the pregnancy [340].

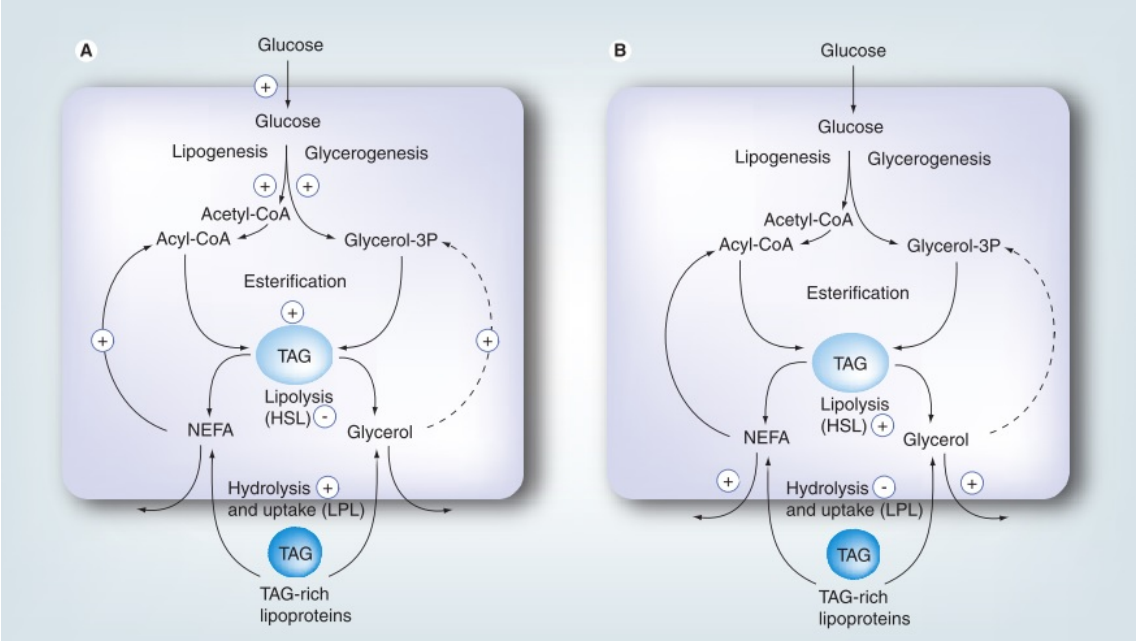

Diagram 16: Adipose tissue metabolism changes between early and late pregnancy. A) Early pregnancy B) Late pregnancy. Abbreviations: TAG (triacylglycerol); HSL (hormone sensitive lipase); NEFA (non esterified fatty acids) [318]. Creative commons license.

Leptin is released in both maternal placenta and fetal and maternal adipose tissue [351] and its levels are high at term [352-354]. This upregulation of leptin levels in pregnancy can be due to its lipid metabolism activation-function leading to a release and mobilization of maternal fat reserves with the following transplacental transfer of lipid constituents [355]. In pregnancy there is also a leptin resistance starting at 14th day of pregnancy [356], as we can see when pregnant rats are injected ICV with leptin, without reducing the food intake [357]. This resistance is due to a decrease in leptin transport through the BBB also due to a modification in STAT3 and PI3K hypothalamic signaling pathways [334]. This ensures the food intake in pregnancy in order to have energy substrates available for the fetus. Also it was demonstrated that placental lactogen production is essential for the development of leptin resistance in pregnancy [356] and that leptin correlates positively with estrogen concentrations [329]. Leptin is important for fetal growth and development, particularly organogenesis [358, 359] apart from its role in food intake regulation. The levels of leptin are correlated with adiposity in the fetus [360].

Regarding important gastrointestinal hormones involved in food intake, ghrelin is secreted by stomach and placenta during pregnancy [361] and basal ghrelin levels decreases [362]. Furthermore ghrelin functioning is crucial in fetal development [363]. There are contradictory results concerning CCK levels in pregnancy. Some studies found an increase [364] in CKK and another studies showed no difference compared with non-pregnant animals [273]. This being said, further studies are needed to clarify CCK role in pregnancy. In pregnancy there is a decrease in PYY sensitivity, while PYY circulating levels are increased in late gestation even under the typical pregnancy hyperphagia [291]. Pregnancy is also a α-MSH-resistant state. This melanocortin family neuropeptide in other situations, like obesity, produces a decrease in food intake which is not shown in pregnancy [365, 366]. This loss of sensitivity with α-MSH, is thought to be related with the hypothalamic response to leptin that occurs in pregnancy [367].

Hormones that take part in appetite regulation are related with sex hormones. As stated earlier, leptin and estrogen levels go hand in hand [329]. Satiation caused by CCK is increased by estrogens [368] which also augments the ghrelin-related hyperphagia [369]. There are progesterone receptors in some hypothalamic nuclei [370] as well as estrogen receptors [371]; in fact, ER (estrogen receptor) colocalizes with NPY-expressing neurons within the hypothalamus [372]. Therefore both could be implicated in food intake regulation. Actually, NPY and AgRP levels are reduced by estrogens [373], and ovariectomized rodents have higher expression of NPY in the ARC compared to sham animals [374]. It's hypothesized that AgRP and NPY levels could be regulated by progesterone since its levels are raised in pregnant rats [375]. However, when considering POMC expression in the ARC, the ovariectomy produces a rise in POMC levels, which is reversed when estrogen is provided [376].

8.3 THERMOREGULATION

Neither hyperphagia [377] nor cold [326], stimulate thermogenesis in pregnant rats. Furthermore, there is a decrease in UCP1 BAT levels [334].

At central levels, it was described that AMPK in VMN regulates BAT thermogenesis. This occurs through the SNS in such a way that the inactivation of AMPK by hyperthyroidism, leads to the activation of the BAT with the subsequent loss of weight [378]. Interestingly the decrease of AMPK that occurs in VMN fails to activate thermogenesis in pregnant rats [334]. Also, it was shown that estradiol administration produces a reduction in BAT activity [337]. In addition, estradiol administration acts to inactivate AMPK in the VMN via ERα (estrogen receptor alpha) and a subsequent increase in malonyl-CoA. Estradiol also regulates FAS expression in the VMN through the ERα. Pregnant rats showed a decrease in pAMPKα, ACCα and FAS with an increase in malonyl-CoA at hypothalamic level. More specifically the decrease of FAS, pAMPKα, pACCα and AMPKα1 is restricted to VMN [103]. It has been seen that hypothalamic malonyl-CoA is an indicator of energy status. Accordingly, in fasting mice, hypothalamic malonyl-CoA levels are low and when these mice are then fed, the levels increase [379]. Inhibition of FAS activity produces the increase in malonyl-CoA, decreasing food intake [380]. In pregnant rats malonyl-CoA levels are elevated despite the hyperphagia, as well as ACCα function and there is a decrease in pAMPKα, FAS and CPT1. This is characteristic of an anorexigenic state and in light of that it has been proposed that there is a malonyl-CoA resistance in pregnancy to assure mother's food intake [334].

8.4 GESTATIONAL DIABETES

Maternal obesity can result in several problems for mother's health. During pregnancy, obesity can increase the chance to have gestational diabetes mellitus (GMD), spontaneous abortion, intrauterine fetal demise, non-alcoholic fatty liver disease, cesarean delivery and preeclampsia (hypertension and high levels of proteins in the urine). After pregnancy, maternal obesity, can lead to heart problems, hypertension, later obesity and diabetes among others [381, 382] (Diagram 17).

Complication	OR (95% CI) or % vs Normal Weight	P
Early pregnancy		
Spontaneous abortion (miscarriage)		
After spontaneous conception	1.2 (1.1-1.5)	.04
After IVF conception	1.8 (1.1-3.0)	< .05
Recurrent miscarriage	3.5 (1.1-21.0)	.04
Congenital anomalies		
Neural tube defects	1.8 (1.1-3.0)	< .05
Spina bifida	2.6 (1.5-4.5)	< .05
Congenital heart disease	1.2 (1.1-1.3)	< .05
Omphalocele	3.3 (1.0-10.3)	< .05
Late pregnancy		
Hypertensive disorder of pregnancy		
Gestational nonproteinuric hypertension	2.5 (2.1-3.0)	< .0001
Preeclampsia	3.2 (1.8-5.8)	.007
Gestational diabetes mellitus	2.6 (2.1-3.4)	< .001
Preterm birth	1.5 (1.1-2.1)	< .05
Intrauterine fetal demise (stillbirth)	2.8 (1.9-4.7)	< .001
Peripartum		
Cesarean delivery	47.7% vs 20.7%	< .01
Decreased VBAC success	84.7% vs 66%	.04
Operative morbidity	33.8% vs 20.7%	< .05
Anesthesia complications		
Excessive blood loss		
Postpartum endometritis		
Wound infection/breakdown		
Postpartum thrombophlebitis		

Diagram 17: Obstetric complications in obese pregnant woman. Abbreviations:95% CI (95% confidence interval); EFW(estimated fetal weight); IVF (in vitro fertilization); OR (odds ratio); VBAC (vaginal birth after cesarean)[381]. With permission of Karger.

Gestational diabetes develops when the mother's pancreas stops working so that the insulin secretion is impaired, however insulin resistance associated with pregnancy can also occur [324, 383]. Usually, there is no diabetes before pregnancy and disappears after labor, but women with GMD have more chances to relapse with this disease in future pregnancies [384]. The cause of GDM in obese women could be a mismatch of production or secretion of certain adipokines (chemokines, cytokines and hormones) due to an expansion of adipose tissue together with a disproportion of pro-inflammatory and anti-inflammatory cytokines [10]. This could ultimately lead to type 2 diabetes (T2DM) if not attended to [385]. There are studies showing that in women with GMD, there is an upregulation of leptin, TNFα, resistin, fatty acid-binding protein 4 (FABP4) and C-reactive protein (CRP) levels, together with a decrease of adiponectin and chemerin [386-388]. Due to this upregulation, leptin and adiponectin levels can be used to predict the development of GDM in pregnant females [388-390].

Besides GDM, peripheral and hepatic insulin resistance is manifested during pregnancy in obese woman after midpregnancy, due to an increase of the fetus' glucose needs [391]. Also, because of the fetus' needs of glucose, there is a decreased glucose uptake with an increased level of hepatic gluconeogenesis and hepatic *de novo* lipogenesis. This all helps to facilitate the development of hyperglycemia in diabetic pregnant rats [392]

It is shown that there is a downregulation in LPL levels in women with GDM in adipose tissue compared with normal pregnancy [393]. This occurs together with a decreased expression of another genes involved in fatty acid uptake and intracellular transport (FATP2,

FATP6, FABPpm and ASCL1), TG biosynthesis (MGAT1,7 MGAT2 and DGAT1), lipogenesis (FAS), lipolysis (PNPLA2, HSL and MGLL), and other transcription factors related to lipid metabolism (LXRα, PPARα, PPARγ, PPARδ, RXRα and SREBP1c). Conversely, there is an increase in the mRNA of adipokines TNFα, IL-1β and leptin levels [394].

A high fat diet it's known to produce insulin resistance together with hyperinsulinemia. [395, 396]. In addition, high-calorie diets are seen to develop GDM [397, 398] in pregnant rats even after only 4 weeks of a high-calorie diet before mating. [384]. Due to the development of GDM, there are certain metabolic alterations that occur. These include an increase in the branched-chain amino acids, TG and FFA in the plasma, a deferment in clearance of fatty acids after a meal [393], and lower LDL-cholesterol concentrations [399].The presence of GMD also seems to lower the insulin-dependent suppression of gluconeogenesis in the liver [399]. Furthermore, gluconeogenesis in liver is less sensitive to hyperinsulinemia in GDM [340]. Finally, it was shown that is only possible to reverse this glucose intolerance in HFD fed pregnant mice with exercise [400].

In women with gestational diabetes, a wide range of dyslipidemic conditions are present. There is also a positive correlation between maternal TG concentrations and neonatal body weight or fat mass has been found even in normoglycemic conditions. Changes in the amounts and relative proportions of endocrine, paracrine and autocrine proteins secreted by WAT (collectively termed adipocytokines) have been observed in women with GDM and associated with adverse outcomes under normoglycemic conditions [355].

In GDM, it has been seen that high levels of glucose in the mother affect the related leptin levels [401]. Furthermore, the amount of glucose present in the mother is directly proportional to the BMI. It is also known that when GDM occurs, there are higher levels of leptin in both the mother [359] and the fetus [402]. This increase in leptin is also correlated with the increase in release of the other cytokines such as IL-6 or TNFα, which leads to a chronic inflammation which in turn, leads to more increase in leptin production [402].

OBJECTIVES

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OBJECTIVES

In this thesis, the following objectives were proposed:

1. To investigate the role of hypothalamic KLF4 in energy homeostasis and its potential relationship with ghrelin and leptin signaling.

2. To investigate the interactions between diet and pregnancy in the mother's metabolic pathways in liver and BAT.

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MATERIAL AND METHODS

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1 MICE, RATS AND ANIMAL CARE

All animal experiments were reviewed and approved by the ethics committee of the University of Santiago de Compostela (procedures of Prof. Rubén Nogueiras Pozo, 1500AE / 12 / FUN01 / FIS02 / RNP2 and 15005AE / 12 / FUN01 / FIS02 / RNP1), following the current regulations:

- Directive 2010/63/EU of the European Parliament and of the Council of September 22th on the protection of the animals used for scientific purposes.
- State law 32/2007 of November 7th, for the care of animals in their exploitation, transportation, experimentation and sacrifice.
- Royal decree 53/2013 of February 1st, which establishes the basic rules applicable for the protection of animals used in experimentation and other scientific purposes, including teaching.
- Decree 296/2008 of December 30th, on the protection of animals used for experimentation and other scientific purposes, including teaching, and by which the Register of breeding centers, suppliers and users, and the Galician Commision of Animal Welfare of Experimentation is created.

The aforementioned procedures continued in force during the realization of this thesis while the application for a new license of authorization of the projects is made with experimental animals under the current RD 53/2013 compliance, being the registration number of the animal facilities where the experiments took place: ES150780263401.

Energy homeostasis involves the communication of peripheral tissues (liver, gastrointestinal tract, adipose tissue) with the central nervous system (CNS) through signals that reaches the brain through the bloodstream. Therefore, replacement of the animal models by cellular models or isolated tissues was not considered feasible because the connections between the organs were needed. However, of the ethical considerations associated with the use of animals, the experimental designs were made taking into account the Royal decree 53/2013. RD 53/2013 has as its main purpose to ensure that the number of animals used in the procedures is minimized and that they are not unnecessarily caused pain (Article 4: Principle of replacement, reduction and refinement). Based on this, during the execution of this doctoral thesis, it was ensured that the procedures performed were those that involved the minimum use of animals to achieve the objectives, affecting as little as possible the capacity to feel pain, suffering, anguish or lasting damage and, therefore, were more likely to produce satisfactory results.

In all experimental procedures rats either male or female (Sprague-Dawley strain) or male (C57-BL6 strain or leptin-deficient (ob/ob) mice purchased from Charles River Laboratories International Inc.), were used between 8 and 10 weeks of age with weights ranging from 200-250 g in rats and between 25-30 g in mice and over 50 g in the case of ob/ob mice. Animals were housed under controlled light conditions (12 light hours/12 hours darkness) and temperature (23 ºC) with a period of adaptation between 5 to 7 days, prior to the experimental procedures. The animals had *ad libitum* access to water and standard laboratory chow, unless otherwise stated (High fat diet of 60% fat content, Research Diets D12492), as is detailed in the experimental designs.

In order to reduce the stress caused by the experimental procedure and to accustom them to the management by the laboratory staff, during the adaptation period the intake and weight were monitored periodically. When surgery involving an invasive or potentially painful procedure was required, animals were under the influence of general anesthesia. Therefore, an anesthetic solution dissolved in physiological saline, consisting of 80 mg/kg ketamine (Imalgene 1000, Merial, Barcelona, Spain) and 8 mg/kg xylacine (Rompun 2%, Bayer HealthCare, Berlin, Germany) was administered intraperitoneally (IP) to rats, and 100 mg/Kg ketamine, 20 mg/kg xylazine to mice. To verify the anesthesia status of each individual, the foot and eyelid reflex were checked, so that their absence is indicative that the surgical procedure can be performed. Saline was administered subcutaneously (SC) to avoid dehydration caused by anesthesia, and also Ketoprofen (Orudis 100 mg, Sanoli Aventis, Barcelona, Spain) at a dose of 2.5 mg/Kg as an analgesic for rat and mouse.

2 *IN VIVO* EXPERIMENTAL APPROACHES

2.1 INTRACEREBROVENTRICULAR INFUSION (ICV)

Intracerebroventricular cannulation is a surgical process in which a cannula is implanted in the skull of the animal, allowing the administration of treatments directly into the lateral ventricle, in order to reach the hypothalamus. Polyethylene (PE-20, Clay-Adams, Becton-Dickinson, New Jersey, USA) fine-gauge cannulas (1.09 mm outside diameter and 0.38 mm inner diameter) were used. At one end of the cannula, a stop is put and cut into a bevel about 3/4 mm apart. This part is the one inserted into the brain which allows the access to the lateral ventricle. The opposite end of the cannula is sealed until the day of the experiment.

Once the animals are anesthetized, a transverse cut is made in the skin of the head, at the level of the forehead and until the back of the eyes, exposing the subcutaneous tissue, which must be removed with a scalpel leaving the skull in sight. The location of bregma, which separates frontal from occipitals, is used as a reference point to make a hole (from bregma: -0.9 mm anteroposterior (AP), ± 1.5 mediolateral (L), -3.50 mm dorsoventral (DV)), through which the cannula is inserted (randomly implanted on the right or left side of the lateral ventricle). Subsequently, cyanoacrylate is added so that the cannula is perfectly fixed and the entire open area is sealed. The postoperative period was 4 days, during which time the animals had free access to food and water in individual cages.

In the ICV administration, is not necessary the anesthesia, so that the animal is fully conscious. With the aid of a rag the animal it's slightly immobilized by pressing it gently against the base of its cage, trying to avoid excessive stress. The cannula implanted is opened by the sealed end, with the aid of a scissors, and then a Hamilton syringe (Model 7001KH 25s, Hamilton) is introduced and the treatment is injected once it is correctly inserted. When withdrawing the needle, it is necessary to be careful by holding the cannula to avoid its displacement of the place of insertion.

2.2 INTRAPERITONEAL INJECTION (IP)

This *in vivo* technique does not require anesthesia. Is performed in the ventro-caudal part of the rat below the peritoneum. The animal is taken by the back with the help of a rag to be able to immobilize it gently oppressing the previous extremities. The animal's head was tilted

slightly down to minimize the risk of tear in the viscera and the needle (BD Microlance, 25G 5/8 "0.5x16mm, BD Medical Surgicals Systems) attached to a syringe (1 ml BD slip -tip syringe, bulk, non-sterile, BD Medical Surgicals Systems) was inserted practically perpendicular to it, preferably on a body side assigned randomly.

2.3 STEREOTAXIC MICROINJECTION OF ADENOVIRAL AND LENTIVIRAL EXPRESSION VECTORS

After being anesthetized the rats received a cut of approximately one centimeter in the skin of the head perpendicular to the skull sagittal suture, and the subcutaneous tissue was removed leaving the skull exposed. The next step was placing the rats in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). This instrument allows us to localize three-dimensionally, a brain zone to specifically administrate a substance. This injection was done bilaterally in the ARC with a 25-gauge needle (Hamilton, 700 serie, 7001 model, Vol. 1 µL). The bregma is the anatomical point on the skull at which the coronal suture is intersected perpendicularly by the sagittal suture. This spot is used as a reference point to assess the coordinates following the rat brain atlas. The coordinates used to reach the ARC were AP: -2.8, Lat: -0.3, DV: -10.2. The incision was closed with sutures and rats were kept warm until fully recovered.

2.4 METABOLIC PHENOTYPING: BODY COMPOSITION, LOCOMOTOR ACTIVITY AND INDIRECT CALORIMETRY

For metabolic phenotyping, two different types of apparatus were used:

- **NMR (Nuclear Magnetic Resonance)**, Echo Medical Systems: The first step of metabolic phenotyping is to determine body composition using this rodent nuclear magnetic resonance apparatus before and after the experimental procedures, in order to determine fat and lean mass variation.

- **Indirect calorimetry Systems**: This type of system allows us to reflect the nutritional status of the organism under different genetic, pharmacological or dietary conditions. The animals were adapted to the new type of environment by installing them in adaptation boxes for 48 hours before the measurements. Every 20 minutes, for 48 hours, the LabMaster takes measurements of intake, consumption of O_2 (vO_2), production of CO_2 (vCO_2), locomotor activity (LA) and energy expenditure (EE) among others. With the obtained values, the Respiratory Quotient (RQ) of each individual during each measurement was calculated by dividing the vCO_2 between vO_2 ($vCO2/vO_2$). The resulting energy expenditure (EE) (Kcal/48h) was also normalized to the lean mass, measured at the end of the NMR process (EE/g lean mass). Locomotor activity is detected using infrared light sensors that record horizontal and vertical movement. With this data we can establish patterns of nutritional partitioning following.

RQ allows us to detect which is the main oxidized fuel in the organism under study. The RQ is, in terms of measurement, the relation between the volume of CO_2 produced as an oxidative residue, between the O_2 consumed per respiration (vCO_2/vO_2). The values of the ratio will determine the preferred macronutrient to perform the oxidation [403](Table 1)

Values of RQ for each macronutrient		
Macronutrient	RQ	Energy released (kcal)
Carbohydrate	1	5.047
Protein	0.802	4.463
Fatty acid	0.718	4.735

Table 1: Values of RQ for each macronutrient.

2.5 TEMPERATURE MEASUREMENTS

Body temperature was recorded with a rectal probe connected to digital thermometer (BAT-12 Microprobe-Thermometer; Physitemp, Clifton, NJ). Skin temperature surrounding BAT was recorded with an infrared camera (E60bx: Compact-Infrared-Thermal-Imaging-Camera; FLIR, West Malling, Kent, U.K.). We used at least eight animals per group, and for each animal, three or four pictures were analyzed. The skin temperature surrounding BAT for one particular animal was calculated as the average temperature records by analyzing those pictures.

2.6 INTRACARDIAC PERFUSION

For the accomplishment of immunohistochemistry on animal tissue, it is necessary to have the tissue preserving its morphological and molecular characteristics as close as possible as when the animal was alive. After general anesthesia, the rib cage of the animal was opened in order to expose the heart. Prior to cardiac cannulation, 200 µL of heparin (5% Sodium Heparin, Mayne Pharma) were injected into the heart to prevent coagulation. After a small incision in the left ventricle, a cannula was inserted to the entrance of the aorta. After immobilizing the cannula, the right atrium was sectioned to open the circuit and isotonic saline (Physan) is pumped to remove blood from the circulatory system. When the liver became whiter, and the output of the right atrium only expelled saline serum, the fixing solution (10% formalin in phosphate buffer) was infused. The complete rigidity of the animal indicated total fixation. Finally, the tissues of interest were removed and put in fixing solution for 24 hours. After fixation, tissues were transferred to 70% alcohol for paraffin inclusion, or to phosphate solution for fresh immunohistochemistry cut with a vibratome.

3 ANALYTICAL METHODOLOGY

3.1 REAL-TIME PCR

3.1.1 Ribonucleic acid (RNA) extraction

Total extraction of RNA content was performed using Trizol reagent (Invitrogen), a guanidium thiocyanate-containing compound for protein degradation and phenol-chloroform for the isolation of nucleic acids. In order to deactivate RNAse, all the H_2O used in this protocol was pre-treated at 37 °C under agitation for one hour with 0.1 % diethyl

pyrocarbonate (DEPC) and finally autoclaved. The tissue previously frozen with dry ice, was cut and homogenized together with Trizol in an polypropylene tube of 2 mL with a steel ball on it, using a mechanical homogenization machine (Tissuelyser, Qiagen). For the extraction of liver RNA, 1 mL of Trizol was added, and for LHA 250 µl. After that, was centrifuged 5 minutes at 13200 rpm and the whole homogenate was passed to another 2 ml tube.

Then RNA was isolated by adding 250 µL of chloroform, stirred and allowed to stand 5 minutes at room temperature. Samples were centrifuged at 4 °C for 15 minutes to separate the aqueous phase (RNA) from the organic (proteins and lipids), leaving the deoxyribonucleic acid (DNA) at the interface. The enriched aqueous phase of RNA was taken and 3 more volumes of isopropanol were added. Then the sample was stirred and poured in at -20 ° C for 10 minutes. It was then centrifuged at 13200 rpm at 4 °C for another 10 minutes.

Once the supernatant was removed, the RNA precipitate was washed by adding 1 mL of 70% ethanol (dissolved with H_2O DEPC), shaked and centrifuged again for 5 minutes at 13200 rpm at 4 °C. The ethanol was removed and in order to purify the precipitate, another centrifuge of one minute is performed to remove all the residual ethanol. Finally, the precipitate was resuspended in H_2O DEPC (Liver RNA: 300µl; LHA RNA: 30µl) by heating it at 60 °C for 15 minutes.

To determine the purity of the RNA content of the sample, a spectrophotometer was used in order to obtain the ratio of the RNA absorbance (260 nm) and of proteins absorbance (280 nm). The 260/280 ratio of 1.8 to 2 is considered acceptable purity. Once the purity was determined, the absorbance value of 260 nm was taken to determine the amount of RNA extracted. Aliquots of each sample were made for the retrotranscription in a 0.01 ng/µL concentration.

3.1.2 **DNAse treatment**

In order to remove DNA contamination we performed a DNAse treatment:

RNA: 1 µg

Buffer 10X with $MgCl_2$ (Thermo Fisher Scientific, Waltham, MA, USA): 1 µL

DNase I, RNase-free (Thermo Fisher Scientific, Waltham, MA, USA): 1 µL

H_2O-MilliQ: up to 10 µL

We incubated the samples 30 minutes at 37ºC, then we added 1 uL of 50 mM EDTA to inativate the DNAse I, RNase-free and finally we incubated the samples 10 minutes at 65ºC

3.1.3 **Reverse transcription**

Retrotranscription consists of an amplification of RNA extracted as complementary DNA (cDNA). In this way, the amount of RNA contained in the sample is amplified, the RNA sequence is converted to DNA and the stability is ensured during real-time PCR. The reaction is performed in a final volume of 30 µL with the following proportions:

H2O DEPC: 3.58 µL

Reverse Transcription Buffer (5X first strand buffer; Invitrogen): 6 µL

Mixture of dNTPs (dTTp, dCTp, dGTp. dATp at 10 mM each): 6 µL 50 mM MgCl2: 1.5 µL

Random primers (Invitrogen): 0.17 µL

RNAseOUT (Invitrogen) inhibitor: 0.25 µL

Reverse Transcriptase (m-MLV; Invitrogen): 1 µL

Sample: 10 µL

The mixture is incubated in a thermocycler with a program of consecutive steps of 1 hour at 37 °C, 15 minutes at 42 °C and 5 minutes at 95 °C. At the end of the samples are stored at 4 °C.

3.1.4 **Real time-PCR (RT-PCR).**

3.1.4.1 Sybr green method

Once the complementary DNA was obtained, the mRNA levels of the genes of interest were determined by real-time PCR in the 7300 Real Time PCR System (Applied Biosystems). The results were expressed in relative terms comparing our gene of interest with a control gene. RT-PCR is based on the ability to detect the PCR product (amplicon) in each cycle, using fluorescence.

To determine the cDNA concentration in each sample, the threshold cycle (Ct) value is analyzed. Ct is the cycle from which half of the cDNA content is amplified. Ct values are inverse to the amount of nucleic acid that is in the sample, and correlate to the number of copies in the sample. Lower Ct values indicate high amounts of targeted nucleic acid, while higher Ct values mean lower (and even too little) amounts of your target nucleic acid. Typically, Ct values below 29 cycles show abundant nucleic acids, and Ct values above 38 cycles indicate minimal amounts, and possibly an infection or environmental contamination. Reading the fluorescence amount at the beginning the cycle's exponential phase is much more accurate than reading it at the reaction's endpoint.

SYBR™ Green master mixes are designed for quantitative real-time PCR using a set of two PCR primers that flank the target region. The master mixes contain buffer, dNTPs, thermostable hot-start DNA polymerase, and SYBR Green dye.

The Sybr Green reaction mixture used had a final reaction volume of 12µL. Components of the Sybr Green reaction mixture:

H_2O-MilliQ: 3.1 uL

Sybr Green mastermix (Thermo Fisher Scientific, Waltham, MA, USA): 6 uL

Sense oligonucleotide (10 µM) (Eurofins Genomics, Ebersberg, Germany): 0.36 µL

Antisense oligonucleotide (10 µM) (Eurofins Genomics, Ebersberg, Germany): 0.36 µL

Sybr Green Dye (Thermo Fisher Scientific, Waltham, MA, USA) (1:500): 0.18 uL

The protocol used for the amplification consisted of an initial step of 3 minutes at 95°C 4 cycles of 5 seconds at 95 °C and 32 seconds at 60 °C; then the dissociation phase consisting of 15 seconds at 95 °C, 1 minute at 60 °C, 15 seconds at 95 °C and 15 seconds at 95 °C is added. This process is done on a time PCR system real (7300 Real Time PCR System, Applied Biosystems, Foster City, CA, USES).

3.1.5 **Primer design**

For the design of real time PCR primers, Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA) informatic program was used. The designs are based in the available sequences in NCBI (National Center for Biotechnology Information) web page.

Table 2: Primers for Real Time PCR		
mRNA	Sequence	Supplier
FXR	Fw primer: CAGCCACAGATCTCCTCCTC Rv primer: TCTTTGTCACAGGCATCTCG	Eurofins Scientific (Luxemburgo)
PPARα	Fw primer: TCACACAATGCAATCCGTTT Rv primer: GGCCTTGACCTTGTTCATGT	Eurofins Scientific (Luxemburgo)
PPARγ	Fw primer: CCCTGGCAAAGCATTTGTAT Rv primer: CCCTGGCAAAGCATTTGTAT	Eurofins Scientific (Luxemburgo)
HPRT	Fw primer: AGCCGACCGGTTCTGTCAT Rv primer: GGTCATAACCTGGTTCATCATCAC	Applied Biosystems (Foster City, CA, USA)

Table 2: Primers for Real Time PCR (Fw: forward. Rv: reverse).

3.2 **IMMUNOBLOT ANALYSIS**

3.2.1 **Protein extraction**

Tissue samples were preserved at -80 ^{o}C, and a small portion of liver, epididymal WAT, omental WAT, BAT, hypothalamus, ARC or LHA were processed. Throughout the process the samples should be kept on ice to avoid degradation of the proteins. The tissue and the lysis buffer were placed together with a steel pellet in a 2 mL polypropylene tube and homogenized in the Tissue Lyser. The lysis buffer (Table 3) facilitates together with the mechanical homogenization, the breakage of the tissue and the cellular components, dissolving the proteins of the tissue therein.

Lysis buffer components (in distilled water)	
Tris-HCl pH 7.5	50 mM
EGTA	1 mM
EDTA	1 mM
Triton X-100	1% vol/vol
Sodium orthovanadate	1 mM
Sodium fluoride	50 mM

Sodium pyrophosphate	5 mM
Sucrose	0.27 M
Phenylmethylsulfonyl fluoride (PMSF)	1 mM
Protease inhibitor cocktail (ROCHE)	1 tablet/50 mL

Table 3: Lysis buffer components.

After homogenization, the samples were centrifuged at 13200 rpm at 4 ^{o}C for 30 minutes. Then the supernatant (protein extract) was aliquoted to a new tube, discarding the precipitate (nuclei and tissue artifacts) and the possible top layer of fat. In the case of samples of adipose tissue (BAT or WAT), several centrifugations were performed on the initial extract to discard the fat.

3.2.2 **Protein quantification**

To quantify the proteins contained in the extract we performed a colorimetric method with the Bradford solution (Bio-rad Protein Assay Kit, Hercules, CA, USA). The method is based on the change of the brown color of the blue dye Coomassie G250 in a medium with phosphoric acid (absorbance 466 nm) to blue, in the presence of proteins. The dye is hydrophobic and is introduced into the interior of the protein structures, avoiding its contact in the phosphoric acid of the solution taking its original blue hue (595 nm). For determination in our samples, we performed a standard curve of bovine serum albumin (BSA), making a serial dilution curve from the maximum concentration (1 mg/mL). We performed a dilution of the samples to be able to perform the quantification of 1:51 for the liver, 1:26 for the fat and hypothalamus, and 1:15 for the ARC and LHA. All samples were measured in duplicate along with the standard straight line, 0 point and Bradford's solution in a spectrophotometer at 550 nm. Before mixing the samples with the Bradford, it was incubated at 37 ^{o}C for 7 minutes.

3.2.3 **Sample preparation**

The proteins were separated by denaturing electrophoresis on polyacrylamide gels (SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylate Gel Electrophoresis). This technique is based on the separation of proteins by their molecular mass, based on their migration in an electric field. For that it is necessary to neutralize several characteristics of the proteins that would give rise to erroneous migration patterns and incorrect identifications. On the one hand, it is necessary to eliminate the quaternary structure of the protein in order to reproduce the gel migration as proportional as possible to its size. On the other hand, since the proteins are going to be subjected to an electric field, it is necessary to eliminate the intrinsic charge of the polypeptides, (denature them) and give them the same sign of charge. For this purpose, an aliquot of each sample was prepared with the same total protein concentration (10-20 μg) in protease inhibitor lysis buffer. To each sample was also added the same proportion of 5x loading solution (Table 4).

Loading buffer components (adjust to pH 6,8 with milli Q H_2O to 1 L)	
SDS	2 g
Trizma-base	8 mL
ß-mercaptoethanol	1 mL
Bromophenol blue	4 mg
Glycerol	10 mL

Table 4: Loading buffer components

This loading buffer provides the deletion of the protein structure (β-mercaptoethanol, Sigma-Aldrich, St. Louis, USA), uniforms its electric charge in proportion to its length (SDS, Sodium Dodecyl Sulfate, Sigma-Aldrich, St. Lous, USA) and also provide the sample of a high density (Glycerol, Sigma-Aldrich, St. Louis, USA) and a coloring to identify the migration front (Bromophenol blue, MERCK, Darmstadt, Germany). All together these compounds make the gel loading easier.

3.2.4 **Electrophoresis**

The electrophoresis was performed in a gel consisting of a three-dimensional network-like structure formed by the polymerization of acrylamide and bis-acrylamide (N, N-methylenebisacrylamide). The polymerization process is catalyzed by ammonium persulfate (APS) (Sigma-Aldrich, St. Louis, USA) and TEMED (N, N, N, N-tetramethylethylenediamine, Sigma-Aldrich, St. Louis, USA). The concentration of acrylamide and bis-acrylamide (30% Acrylamide/Bis Solution 29:1, Bio-Rad, Hercules, CA, USA) in the gel determines the pore size of the three-dimensional network and the degree of protein migration according to their size. The gel consists of two fractions, a concentrating fraction with 4% acrylamide/bis-acrylamide, in which the samples are to be loaded so that the separation of the proteins from the different samples occurs simultaneously, and a separating fraction with 6%, 8% or 10% depending on the size of the protein of interest. The gels were mounted in an electrophoresis kit (Mini-PROTEAN Tetra Cell, Bio-Rad, Hercules, CA, USA) and immersed in 1x running buffer. (Composition of 5X running buffer: 72 g glycine, 15 g Trizma base, 5 g SDS, distilled H_2O to 1 L). Prior to loading the samples onto the gel they were shaked and heated to 95 °C for 10 minutes. In each well of the gel, 16 µL of sample were charged and also 5 µL of a molecular weight marker (Precision Plus Protein Standard-Dual Color, Bio-Rad, Hercules, CA, USA) was loaded onto one of the lanes. Samples of all experimental groups were loaded onto each gel. After this, the samples were subjected to an electric field (constant voltage of 140V-120V and amperage of 180 mA), connecting the electrophoresis kit to a power source (PowerPac HC High Current Power Supply, Bio-Rad, Hercules, CA, USA), in which the migration of proteins to the cathode was induced. Electrophoresis was stopped when the migration front reached a distance of about 0.5 cm from the end of the gel.

	Resolving gel 8 %	Resolving gel 10 %	Resolving gel 12 %
Distilled H_2O	11.5 mL	9.9 mL	8.2 mL
Acrylamide/Bisacrylamide (30%)	6.7 mL	8.3 mL	10 mL
1,5 M Tris (pH 8.8)	6.3 mL	6.3 mL	6.3 mL
SDS 10%	0.25 mL	0.1 mL	0.1 mL
APS 10%	0.25 mL	0.25 mL	0.25 mL
TEMED	0.015 µL	0.01 µL	0.01 µL

Table 5: Electrophoresis gel composition.

Stacking gel 5%: distilled H_2O 4.1 mL, acrylamide/bisacrylamide (30%) 1 mL; 1.5 M Tris (pH 8,8) 0.75 mL; SDS 10% 0.06 mL; APS 10% 0.06 mL; TEMED 0.006 mL.

3.2.5 **Semidry Transfer**

Once the proteins were separated in the gel, they were transferred to a polyvinylidene polyfluoride (PVDF, Immobilon-P, Millipore) membrane fixing and immobilizing them, in order to be immunodetected. The process of transferring the proteins from the gel to the membrane is voltage-dependent. In this case it was made in a horizontal support where membrane and gel were placed in contact and bounded by absorbent paper soaked in transfer buffer (Table 6), which favors the transmission of the current in the process. The process is performed at constant amperage 0.8 mA per cm^2 for one hour and 40 minutes and at the end of the process, proteins passed from the acrylamide gel to the PVDF membrane.

Transfer buffer composition (in distilled H_2O)	
Tris-HCl	40 mM
Glycine	40 mM
SDS	0.36 % weight/vol
Methanol	20% vol/vol

Table 6: Transfer buffer composition.

3.2.6 **IMMUNODETECTION**

3.2.6.1 Blocking

The membrane was incubated with blocking solution of 5% bovine serum albumin dissolved in washer buffer (TBS-T: tris buffered saline with tween, table 7). BSA solution binds to membrane spots not occupied by the proteins transferred from the gel. In this way, the antibodies could only bind to their specific antigen, reducing the background noise and unespecificities. If the antibodies were more unspecific, the blocking solution was skimmed milk powder at 5 %.

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Washing buffer/TBS-T (in distilled H_2O)	
Tris-HCl	20 mM
NaCl	146 mM
Tween-20	0.1 % vol/vol

Table 7: Washing buffer composition

3.2.6.2 Primary antibody

The membrane was incubated with a specific primary antibody against the protein (see table 8). The antibody was diluted in 3 or 5% TBS-T and incubated while shaking one hour at room temperature or overnight at 4 ^{o}C depending on data sheet instructions. After the incubation, there are 3 washes of 5 minutes each one with TBS-T.

3.2.6.3 Secondary antibody

To detect the protein is necessary to add a secondary antibody so that it will bind to the primary antibody. This secondary antibody is associated with a Horseradish Peroxidase which binds with the constant part of the primary antibody. The secondary antibody was also diluted in 3 or 5% TBS-T at a 1:5000 concentration, and incubated one hour at room temperature. After the incubation, we performed 3 washes of 5 minutes each one with TBS-T (see table 8 for antibodies used).

Primary Antibody	Supplier	Dilution	Incubation time	Temperature
ACCα	Upstate	1:5000	Overnight	4ºC
pACCα	Upstate	1:2000	Overnight	4ºC
FOX01	Cell Signaling	1:1000	Overnight	4ºC
Phospho-FOX01	Cell Signaling	1:1000	Overnight	4ºC
KLF4	Cell Signaling	1:1000	Overnight	4ºC
STAT3	Cell Signaling	1:2000	Overnight	4ºC
Phospho-STAT3	Cell Signaling	1:2000	Overnight	4ºC
Phospho-HSL	Cell Signaling	1:1000	Overnight	4ºC
AgRP	Abcam	1:1000	Overnight	4ºC
GAD65	Abcam	1:1000	Overnight	4ºC
GAD67	Abcam	1:1000	Overnight	4ºC
GFAP	Dakopatts	1:1000	Overnight	4ºC
VGAT	Abcam	1:1000	Overnight	4ºC
NPY	Sigma-Aldrich	1:1000	Overnight	4ºC
ß-actin	Sigma-Aldrich	1:5000	One hour	25ºC

FAS	Santa Cruz Biotechnology	1:2000	Overnight	4ºC
HSL	Santa Cruz Biotechnology	1:5000	Overnight	4ºC
LPL	Santa Cruz Biotechnology	1:5000	Overnight	4ºC
SF1	Santa Cruz Biotechnology	1:1000	Overnight	4ºC
GFP	Abcam	1:5000	Overnight	4ºC
DAB	Dako	1:1000	Overnight	4ºC
α-Tubulin	Sigma-Aldrich	1:5000	One hour	25ºC
IgG-HPRT Rabbit	Dakocytomation	1:5000	One hour	25ºC
IgG-HPRT Mouse	Dakocytomation	1:5000	One hour	25ºC
pAMPKα	Cell Signaling	1:2000	Overnight	4ºC
AMPKα1	Millipore	1:1000	Overnight	4ºC
AMPKα2	Millipore	1:1000	Overnight	4ºC
UCP1	Abcam	1:10000	Overnight	4ºC
UCP3	Abcam	1:1000	Overnight	4ºC
β1-Adrenergic	Abcam	1:1000	Overnight	4ºC
β3-Adrenergic	Abcam	1:1000	Overnight	4ºC
PRDM16	Abcam	1:1000	Overnight	4ºC
CD36	Santa Cruz	1:1000	Overnight	4ºC
pHSL	Cell signaling	1:1000	Overnight	4ºC
HSL	Abcam	1:5000	Overnight	4ºC
PGC1α	Santa Cruz	1:1000	Overnight	4ºC
pJNK1	Cell Signaling	1:1000	Overnight	25 ºC
JNK1	Santa Cruz	1:1000	Overnight	4ºC
pIREα	Abcam	1:1000	Overnight	4ºC
IREα	Abcam	1:1000	Overnight	4ºC
peIF2α	Santa Cruz	1:500	Overnight	4ºC
eIF2α	Santa Cruz	1:1000	Overnight	4ºC
pPERK	Santa Cruz	1:1000	Overnight	25ºC
XBP1	Santa Cruz	1:500	Overnight	4ºC
CHOP	Santa Cruz	1:1000	Overnight	4ºC
Cleaved caspase 3	Cell Signaling	1:1000	Overnight	4ºC
RXRα	Santa Cruz	1:5000	Overnight	4ºC

Table 8: Primary antibodies used in immunoblot analysis or immunohistochemistry

3.2.6.4 Protein immunodetection

In order to detect the antibody, the membranes were incubated for 1 minute in darkness, with a developing substrate used to detect the Horseradish Peroxidase in a ratio of 1:1 (Pierce ECL Western Blotting Substrate, Cultek, Thermo Fisher, Waltham, USA). After the incubation time, the membranes were placed in a developing cassette (Hypercassette, Amersham Biosciences, Little Chalfont, UK) and waterproofed with the aid of clear paper. In a dark room, a developing sheet (Fuji Medical X-Ray Film Super RX, Fujifilm Corporation, Tokyo, Japan) was put on the top of the membranes and the cassette was closed. The chemiluminescence signal was allowed to expose for a few seconds/minutes depending on the protein. The film was then removed and immersed in a developing solution (1:10 dilution) (G150, Developer/Replenisher, Agfa-Gevaert Group, Dubendorf, Switzerland) until the signal was visualized and at that time the film was immersed in Fixing liquid (1:5 dilution) (G354, Manual Fixing Bath, Agfa-Gevaert Group, Dubendorf, Switzerland) for a couple of minutes in order to fix the signal. Finally, the film was washed with running water and allowed to dry.

3.2.6.5 Protein quantification

A high resolution scanner (800 ppp resolution, Canon Scan 9900F, Canon, Tokio, Japan) was used to digitize the developing sheets. The amounts of pixels of the images were quantified using the informatics software ImageJ 1.46r (Wayne Rasband, National Institutes of Health, USA). The measurement area must be the same for all samples. These values were relativized with respect to the control (β-actin/α-tubulin) which must be a protein expressed constantly and at high levels in all the cell types (one control per each gel). The final value was normalized and finally, the percentage relative to the control group of each experiment was shown in a graph.

3.3 **METABOLITE ANALYSIS**

3.3.1 **Hepatic fat extraction**

Fragments of 0.1 g of rat liver were homogenized in the TissueLyser in a 2:1 vol/vol solution of chloroform-methanol on a 2 mL polypropylene tube together with a steel ball. The ratio is 1 mL solution per 0.1 g liver. After homogenization they were kept in orbital rotation for three hours to facilitate the dissolution of the fat in the organic solvent. After rotation, 0.3 mL of distilled water was added and centrifuged at 13000 rpm for 30 minutes at room temperature. After centrifugation the upper organic phase was discarded and the lower organic phase was collected into a new 1.5 mL polypropylene tube. The organic solvent is allowed to evaporate, leaving the polypropylene tubes open under a gas extraction hood for about 8 hours, to obtain the pellet with the fat of the extracted tissue.

3.3.2 **Measurements of liver's triglycerides**

The detection of triglyceride is performed by colorimetric detection in a spectrophotometer (Thermo Fisher, Waltham, USA) using a specific kit (TG, Spinreact; FFA, Wako, Denmark). Concentration of TG of the fatty extract was determined after evaporation of a part of the organic phase obtained. The reagent was then added to the pellet, and was put

with duplicates in the spectrophotometer, after the samples were distributed with the reagent in a 96-well plate accompanied by the standard straight line. The final result was corrected by the weight of the fragment used in the extraction.

3.3.3 **Measurement of serum triglycerides, FFAs, glucose and cholesterol**

Serum levels of triglycerides, glucose, cholesterol and FFA (Free fatty acids), were determined by the use of specific kits in a colorimetric reaction, following the instructions of the commercial house (TG, glucose, cholesterol, Spinreact, Spain; FFA, WAKO, Denmark)

3.3.4 **Immunoassay measurement of serum's FGF21**

The concentration of circulating FGF21 in serum was determined by using a commercial kit highly specific for rat (Quantikine ELISA R&D systems a bio-techne brand, USA). The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat FGF21 has been pre-coated onto a microplate. Standards, control, and samples were pipetted into the wells and any FGF21 present was bounded by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat FGF21 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution was added. The intensity of the color measured was in proportion to the amount of FGF21 bounded in the initial step. The sample values were then read off the standard curve.

3.4 **HISTOLOGICAL ANALYSIS OF CENTRAL AND PERIPHERAL TISSUES**

3.4.1 **Oil-red O staining**

This staining colors the lipid droplets of the tissue in a reddish tone. For this, frozen sections of liver were cut in a cryostat (8 µm) and placed in slides. These slides were immersed in Oil-Red O stain for 10 minutes. They were then washed in distilled H_2O and counterstained with Mayer's hematoxylin for 3 minutes. The slides were mounted in aqueous mounting medium.

3.4.2 **Hematoxylin/eosin staining**

This technique stains the cellular structures in general showing the structure of the tissue. Hematoxylin is cationic in nature (basic) so it stains blue basophilic structures (acidic) like cell nuclei. On the other hand the eosin is an acid which stain pink basic structures (acidophilic) like the cytoplasm. The WAT was submerged in 10% formalin, following animal dissection. After this step, WAT was included in paraffin and was cut (4 µm) with a microtome and placed in slides. The cuts were then passed through a rehydration process, consisting of alcohols of decreasing concentration (100 °C, 95 °C and 70 °C). After washing with distilled H_2O it was immersed in hematoxylin for 10 minutes, washed and immediately

immersed in an acid alcohol. After washing it, it was immersed 30 seconds in eosin. In order to dehydrate the sample, the slides were passed through the same alcohols in increasing graduation and before mounting, the mixture was left submerged for 10 minutes in xylene.

3.4.3 **Immunofluorescence**

Perfused brains were immersed in 10% formalin for 24 hours and then transferred to 0.1M phosphate buffer. Sections of 40 µm thickness were cut with the vibratome. For immunofluorescence, sections were incubated in flotation overnight at 4°C with rabbit anti-GFP diluted 1: 1000 in PBS (Phosphate buffered saline). After two 5 minute washes in 0.1M phosphate buffer, the sections were incubated 1 hour at room temperature with AlexaFluor® 488 anti-F (ab') 2 fragment (Molecular Probes) at a 1:200 dilution. Sections were mounted on slides and covered with fluorescent mounting medium. Fluorescence was observed and photographed under a Provis AX70 fluorescence microscope (Olympus Corporation).

3.4.4 **Diaminobenzidine (DAB) immunohistochemistry**

For the immunohistochemical study, the rat brain was fixed using intracardiac perfusion as detailed above. Once the tissues of the animal were fixed, the brain was removed and kept immersed in the same fixed solution for 12 hours at 4 °C. They were then transferred to 0.1M phosphate buffer for 1 hour. Brain sections of 40 µm thick were made on a vibratome (Vibratome 1000 Series, The Vibratome Company, St. Louis, MO, USA). Sections were collected in PBS. For immunohistochemical detection of KLF4 expression, sections of the brain were incubated with KLF4 primary antibody (Cell Signaling) at 1:1000 dilution overnight at 4 °C. After that, 2 washes of 10 minutes were made. It was then incubated with 3% H_2O_2 in distilled water for 10 minutes followed by 2 washings of 10 minutes. Polyclonal EnVision (dextran polymer conjugated with anti-rabbit and peroxidase antibodies, Dako, Glostrup , Denmark) was used as the detection system, and we incubated the samples for 30 minutes. After that, 2 washes of 10 minutes were made and incubated with DAB (3,3'-Diaminobenzidine: Dako, Glostrup, Denmark) for 10 minutes, following the manufacturer's protocol (1 drop of DAB in 1 ml of supplied solvent). At the end of the reaction, the sections were spread on xylanized gates (Histobond, Marienfeld-Superior, Lauda-Konigshofen) allowing them to dry. Subsequently they were dehydrated in ethanol, rinsed in xylene and mounted with permanent mounting medium. The reaction was observed and photographed under the Provis AX70 fluorescence microscope (Olympus Corp., Tokyo, Japan). In addition, the immunoreactivity of KLF4 in ARC was quantified using ImageJ program. The number of cells stained in each section was used as representative numbers of cells with positive KLF4 immunoreactivity.

3.4.5 **Double labeling**

Double immunohistochemistry was performed, on previously perfused brains. To expose antigenic sites and reduce non-specific binding of immunohistochemical reagents, sections were treated with 0.5% H_2O_2 dissolved in 0.1 M PBS for 10 minutes, and with a solution of pepsin (10 mg/ml in 0.2 M HCl solution) for 5 minutes at 37 ^{o}C. Paraffin sections of 40 µm

were consecutively incubated with a primary antibody against KLF4 (Cell Signaling) and AgRP (Abcam). The cuts were mounted on the glass slides with a coverslip and visualized in a confocal microscope (Nikon A1R). The number of cells stained in each section were quantified using ImageJ program and used as representative numbers of cells with positive KLF4/AgRP immunoreactivity.

3.4.6 *IN SITU* hybridization

In situ hybridization assays were performed to visualize hypothalamic mRNA expression of KLF4, AgRP, NPY, POMC and CART.

Coronal hypothalamic sections (16 μm) were cut on a cryostat and immediately stored at −80 °C until hybridization. For KLF4, AgRP, NPY, POMC and CART detection, we employed the specific antisense oligodeoxynucleotides (Table 9). These probes were 3′-end labeled with [α-35S]deoxy-ATP using terminal deoxynucleotidyl transferase. The specificity of the probes was confirmed by incubating the sections with an excess of the unlabeled probes. The frozen sections were fixed with 4% paraformaldehyde in 0.1 m phosphate buffer (pH 7.4) at room temperature for 30 minutes. They were then dehydrated using 70%, 80%, 90%, 95%, and absolute ethanol (5 min each). The hybridization was carried out overnight at 37 °C in a moist chamber. Hybridization solution contained 5×10^5 (KLF4, AgRP, POMC and CART) or 1×10^6 cpm (NPY) per slide of the labeled probe, 4× standard saline citrate (SSC), 50% deionized formamide, 1× Denhardt's solution, 10% dextran sulfate, and 10 μg/ml sheared, single-stranded salmon sperm DNA. Afterward, the hybridization sections were sequentially washed in 1× SSC at room temperature, four times in 1× SSC at 42 ºC (30 min/wash), and once in 1× SSC at room temperature (1 h), and then rinsed in water and ethanol. Finally, the sections were air-dried and exposed to Hyperfilm β-Max (Amersham International, Little Chalfont, UK) at room temperature for 4–6 d for KLF4, AgRP, POMC and CART. After the films were developed, sections were dipped in Ilford K5 autoradiographic emulsion (Ilford, UK) and exposed for 2 weeks at 4 ºC. The slides were then developed in Kodak D-19 developer (Eastman Kodak Co., Rochester, NY), fixed (Kodak fixer), and counterstained with methylene blue.

mRNA	Antisense oligonucleotide sequence
AgRP	5'-CGACGCGGAGAACGAGACTCGCGGTTCTGTGGATCTAGCACCTCTGCC-3'
NPY	5'-AGATGAGATGTGGGGGGAAACTAGGAAAAGTCAGGAGAGCAAGTTTCATT-3'
KLF4	5'-TCAGCACAAACTTGCCCATCAGCCCGCCACCTGGCG-3'
CART	5'-CCGAAGGAGGCTGTCACCCCTTCACAGCA-3'
POMC	5'-CTTGATGATGGCGTTCTTGAAGAGCGTCACCAGGGGCGTCTGGCTCTT-3'

Table 9: *In situ* antisense oligonucleotide sequences.

4 EXPERIMENTAL DESIGN

4.1 KLF4

4.1.1 Effect of acute administration of ghrelin in KLF4 response in the hypothalamus

In order to investigate whether the orexigenic action of ghrelin is mediated by KLF4, we performed one experiment infusing ghrelin in the hypothalamus.

First of all, an ICV surgery was performed as was explained above. After the postoperative period of 4 days the administration of ghrelin was performed. The ghrelin (Bachem, Switzerland) dose was 1 µg/µL in a final volume of 5 µL with saline as vehicle. The rats were administered with chow diet and water *ad libitum*. Food intake and body weight was measured after 2 hours after the ICV injection in order to observe the acute effects of ghrelin.

4.1.2 The effect of nutritional status in KLF4 levels

To determine whether food deprivation influenced KLF4 protein levels, three groups of animals were used: a control group was fed *ad libitum*, and two additional experimental groups were deprived of food for 12 h or 48 h. Rats were killed by cervical dislocation and ARC was dissected and stored at -80ºC until further processing.

4.1.3 Leptin central pathway studies

ICV cannula were implanted in the lateral ventricle as described previously and when rats were fully recovered, they were randomly allocated into four groups:

Vehicle + Vehicle

Vehicle + Leptin

STAT3PI or LY294002 + Vehicle

STAT3PI or LY294002 + Leptin

For inhibition of the STAT3 pathway, rats were fasted overnight (12 h) and then given a single ICV infusion of either vehicle (saline) or 75 pmol STAT3 peptide inhibitor (STAT3 PI, Calbiochem). 30 min later, rats received either vehicle (10mM NaHCO3, pH 7.9) or 3 mg of leptin (provided by Dr A. F. Parlow, National Hormone and Peptide Program, Harbor- UCLA Medical Center, Torrance CA).

For inhibition of the phosphatidylinositide 3-kinase (PI3K) pathway, a similar protocol was followed, using either vehicle (DMSO) or 1 nmol PI3K inhibitor (LY294002; Sigma Aldrich). 19 min later, either vehicle or 3 mg of recombinant rat leptin (1 mg/kg) was administered.

Thirty minutes after leptin administration, rats were sacrificed and the ARC was dissected out using micropunches and a dissecting microscope. Accuracy of ARC dissection was assessed in terms of protein levels of the ARC-specific marker AgRP. Dissections of the VMN and the LHA were also performed, and their accuracy assessed by SF1 protein levels.

4.1.4 Leptin sensitivity assessment

IP leptin was injected as described previously, at 1 mg/kg body weight, a dose reported to reduce caloric intake and body weight in rats [404]. Vehicle (100 mM $NaHCO_3$, pH 7.9) was injected in control animals. To study central leptin pathways and their influence on KLF4, rats were sacrificed after 90 min; to assess peripheral effects of leptin, food intake and body weight were measured 24 h after IP administration.

4.1.5 Genetic overexpression of KLF4 in ARC

To test the capacity of KLF4 to modulate AgRP *in vivo*, we performed a stereotaxic adenoviral mediated overexpression of KLF4 in the ARC. The stereotaxy was performed as described above and after one week of animal accommodation in the animal facilities. The following vectors were injected with either adenoviral vectors over-expressing GFP (control) or KLF4 (1x1010 PFU/ml each; SignaGen Laboratories, MD, USA). Rats had *ad libitum* access to standard diet (SD) and water during the time of the experiment (5 days), and were sacrificed after body weight and food intake measuring.

4.1.6 KLF4 genetic silencing in ARC

To test if KLF4 would be modulating leptin resistance induced by high fat diet, we performed a stereotaxic lentiviral mediated overexpression of shRNA against KLF4 in the ARC. The stereotaxy was performed as described above and after one week of animal accommodation in the animal facilities. 1 µL of the following vectors were injected: either lentiviral vectors over-expressing shRNA against KLF4 (shRNA KLF4 clone ID: TRCN0000095370; 5.3 x 106 TU/mL), or GFP (control). After the surgery, rats were fed with SD or HFD (65% fat content, Research Diets) for 15 weeks and their body weight and food intake was measured weekly.

4.1.7 Leptin treatment after FOXO1 genetic silencing in ARC

To evaluate the possibility of FOXO1 regulating KLF4 expression in leptin signaling pathway we performed a stereotaxic lentiviral mediated overexpression of shRNA against FOXO1 (shRNA FOXO1 clone ID: TRCN0000054879, Sigma Aldrich Inc., Buchs, Switzerland) or GFP (control) in the ARC, at 1 µL volume.

After 3 weeks, IP leptin injection was performed in the following groups:

GFP + Vehicle

shRNA FOXO1 + Vehicle

GFP + Leptin

shRNA FOXO1 + Leptin

IP leptin was injected as described previously, at 1 mg/kg body weight. Vehicle (100 mM NaHCO3, pH 7.9) was used in control animals.

Rats had *ad libitum* access to standard diet and water during the time of the experiment, and were sacrificed after 7 days of body weight and food intake measuring.

4.2 PREGNANT RATS

4.2.1 MATING PROTOCOL

Female rats were housed for 1 week in the animal facilities, so that their cycle was regular. After this time, male bedding was put to the female rats for 2 days in order to make them to enter in oestrus. Then, the rats were crossed with 1 male rat for 1 night. The next morning the rats were separated and that day was counted as day 1 of gestation.

Three experimental groups were stated:

Rats with SD before pregnancy + SD during pregnancy (21 days)

Rats with SD before pregnancy + HFD (60% fat) during pregnancy (21 days)

Rats with HFD before pregnancy (6 weeks) + HFD during pregnancy (21 days)

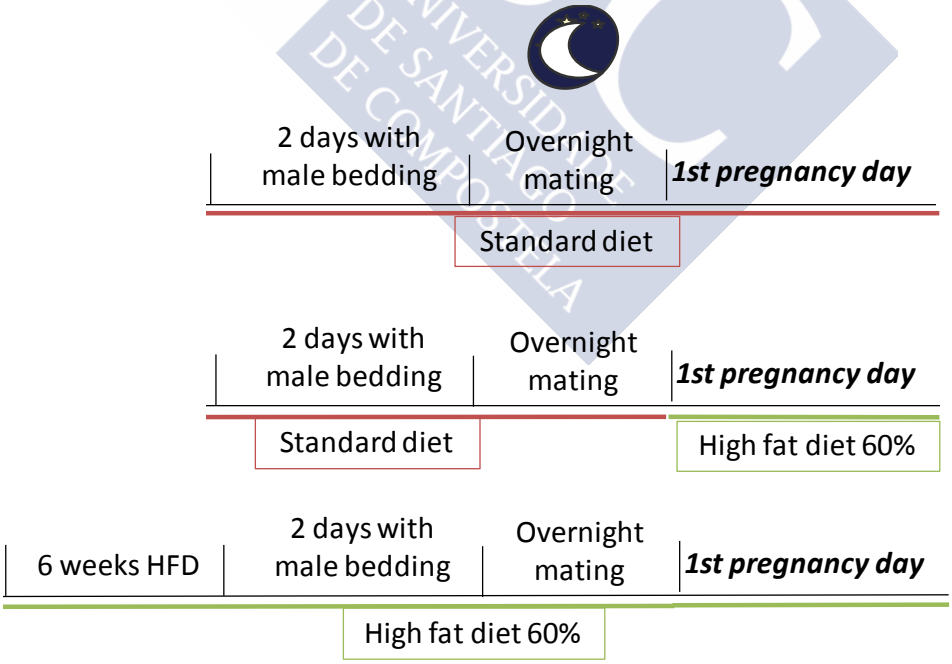

Diagram 18: Pregnant experimental groups.

When the rats arrived at 21 days of gestation were sacrificed for subsequent tissue harvesting. Pseudopregnant rats were used as controls: females that were crossed with a male but were not pregnant.

5 SACRIFICE AND TISSUE HARVESTING

The last step after each experiment was to sacrifice of the animals by decapitation, in order to study different parameters in BAT, epididymal and gonadal WAT, ARC, LHA, placenta, serum, fetus and liver. To harvest the brain they were made two cuts on the back of the skull with fine-tipped scissors, and with the help of some tweezers the top of the skull was lifted. Carefully all the tissues were harvested and saved in polyethylene tubes put in dry ice, and subsequently stored at -80 ºC in order to analyze protein, RNA, serum values etc. Regarding histological analysis, little pieces of tissues were introduced in 10% paraformaldehyde for later inclusion in paraffin for the hematoxylin/eosin while for oil red O the little pieces of tissue were wrapped in foil and conserved at -80ºC. For central-level studies, the rats were anesthetized and an intracardiac perfusion was performed first with saline to remove all blood from the torrent, and secondly with 10% paraformaldehyde to fix the brain as close to when they were alive as explained before.

6 STATISTICAL ANALYSIS AND DATA PRESENTATION

Data are expressed as mean ± standard error men (SEM). mRNA and protein data are expressed in relation (%) to control animals. The sample size of the populations of each experiment is detailed in the figure captions of the results. For the analysis, samples and/or animals whose value deviated ± 2 times the standard deviation were excluded [405], or when inadequate behavior is observed during the experimental procedure.

In the analysis, a normality test was first applied to contrast the normality of a data set. As a null hypothesis it was suggested that the sample of the data set comes from a normally distributed population; being the Shapiro-Wilks test used for sample sets of $n \leq 20$ and ≥ 7, and the Kolmogorov-Smirnov test when $n \leq 6$ [406]. If the analyzed populations show a normal distribution, parametric statistical tests were carried out. If the population did not follow a normal distribution, nonparametric statistical tests were carried out.

6.1 PARAMETRIC TESTS

They are performed on those experiments whose experimental groups follow a Gaussian (normal) distribution.

One-way Anova: determines if there is a difference between the means of three or more populations that follow a normal distribution and are independent of each other. Once the test has been carried out, in case of accepting the alternative hypothesis that indicates that there are differences between the means, itis necessary to perform a posteriori test to check which means are causing the significance. There are several tests that can be performed: one of them is the Tukey test that compares two simple means each time and executes all the possible

comparison; The Dunnett's test is performed when all the means are compared with the control group, so it is not an exhaustive procedure and finally, the Bonferroni test is the most powerful procedure; its use is recommended when comparisons are made between more than three experimental groups [407]. Throughout this thesis we carried out a posteriori test of Bonferroni and Tukey.

Student's t-test: compares the means of two unpaired populations assuming that they follow a normal distribution. It is a robust test, since it is able to compare populations with an n<5. In a contrast of bilateral hypothesis (2 tails), the inequality between means is studied, being the critical region of the statistic both regions corresponding to the lower and upper tail of the distribution. In a unilateral contrast (1 tail) the inequality in the hypothesis points to only one direction [408]. Throughout this thesis, one-tailed t-test was used.

6.2 NON PARAMETRIC TESTS

They were made on those experiments whose experimental groups do not follow a Gaussian (normal) distribution.

Kruskal-Wallis test: used to make comparisons between multiple populations that do not follow a normal distribution. As a Posteriori test, the Dunnett's test is used to determine between which populations the significance exists [409].

Mann-Whitney test: compares two populations, of which one does not follow a normal distribution [410].

Data was analyzed using GraphPad Instat (GraphPad Instat Software, v5, La Jolla, CA, USA) statistical software. $p<0.05$ was considered significant, $p<0.01$ very significant and $p<0.001$ highly significant.

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RESULTS

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1 CHAPTER 1: The role of KLF4 in leptin signaling pathway

This chapter has been published as “Imbernon, Sanchez-Rebordelo et al” in 2014 in the journal “Molecular Metabolism” which is refernced with the number 308.

1.1 KLF4 IS EXPRESSED IN THE HYPOTHALAMIC ARC

We first hypothesized that since KLF4 binds to a specific CACCC-box in the AgRP promoter [35], hypothalamic KLF4 should be modulated by nutritional status since Agrp is an orexigenic peptide considered one of the most important food intake regulators. AgRP is expressed in ARC neurons so we wanted to check first of all if there was any KLF4 expression in ARC.

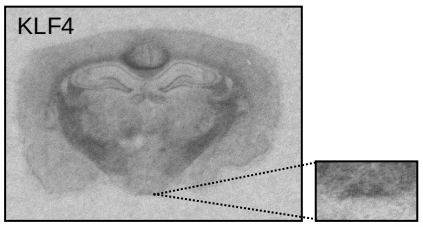

Figure 1. Representative *in situ* hybridization of KLF4 in rat brain.

Using *in situ* hybridization we observed that KLF4 mRNA was expressed in the hypothalamic ARC (Figure 1). [308].

1.2 NUTRITIONAL STATUS REGULATES KLF4 PROTEIN LEVELS IN THE HYPOTHALAMIC ARC

Immunohistochemistry (Figure 2 A, B) showed that within the ARC, KLF4 immunostaining intensity was increased after fasting 12 hours as compared with rats fed *ad libitum*. These data were corroborated by western blot, with an increasing in protein levels being observed in the ARC of fasted rats for 12 and 48 hours as compared with rats fed *ad libitum* (Figure 2C). To assess the specific hypothalamic ARC isolation, we measured AgRP (specific marker of ARC) in ARC (Figure 2D) and LHA while measuring SF1 (specific marker of VMN) in ARC and VMN (Figure 2E) [308].

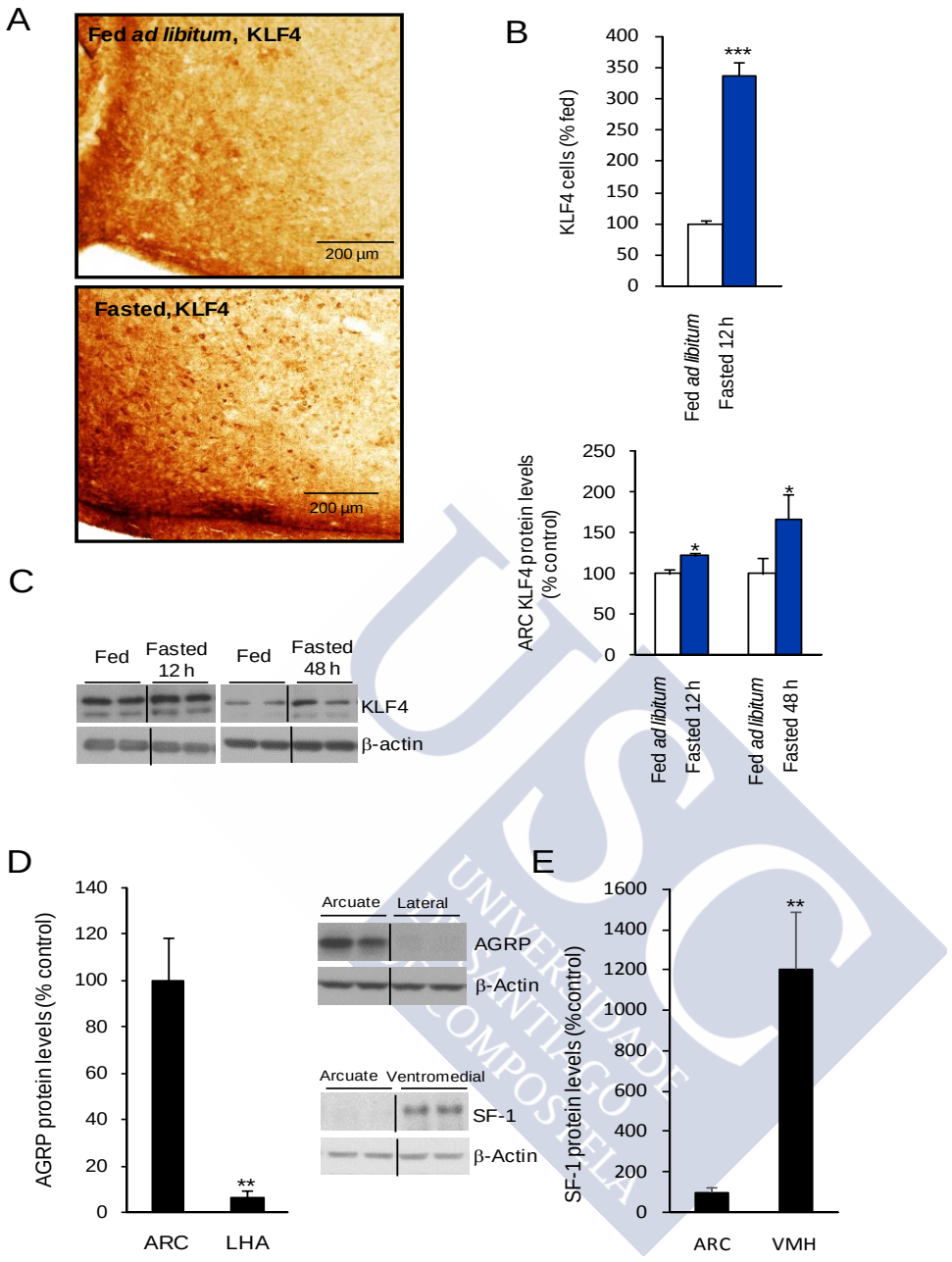

Figure 2. Effect of 12 hours fasting on KLF4 protein levels in the ARC measured by immunohistochemistry (A). Quantification of cells expressing KLF4 in the ARC of fasted rats (337±21%) compared the total KLF4 neurons in *ad libitum* rats (B). Effect of 12 and 48 hours of fasting on KLF4 protein levels in the ARC measured by western blot (C). Corroboration of specific hypothalamic nuclei isolation. AgRP protein levels in the ARC or LHA (D). SF1 protein levels in the ARC or VMN (E). Dividing lines indicate splicing of the same gel. Values are mean ± SEM of 8-10 animals per group. ß-actin was used to normalize protein levels. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

1.3 KLF4 AND AGRP ARE COLOCALIZED IN THE ARC NEURONS

Since *in vitro* was demonstrated that KLF4 binds to an AgRP promoter [35], we wanted to test if *in vivo* we could visualize a colocalization of both.

KLF4 and AgRP were colocalized in the hypothalamic ARC (Figure 3A). Quantification of co-expression of KLF4 and AgRP shows that 85 ± 0.2% of KLF4 neurons expressed AgRP and that 93 ± 2% of AgRP neurons expressed KLF4 (Figure 3B). However, KLF4 and glial fibrilar acidic protein (GFAP) did not show colocalization in the ARC (Figure 3C) [308].

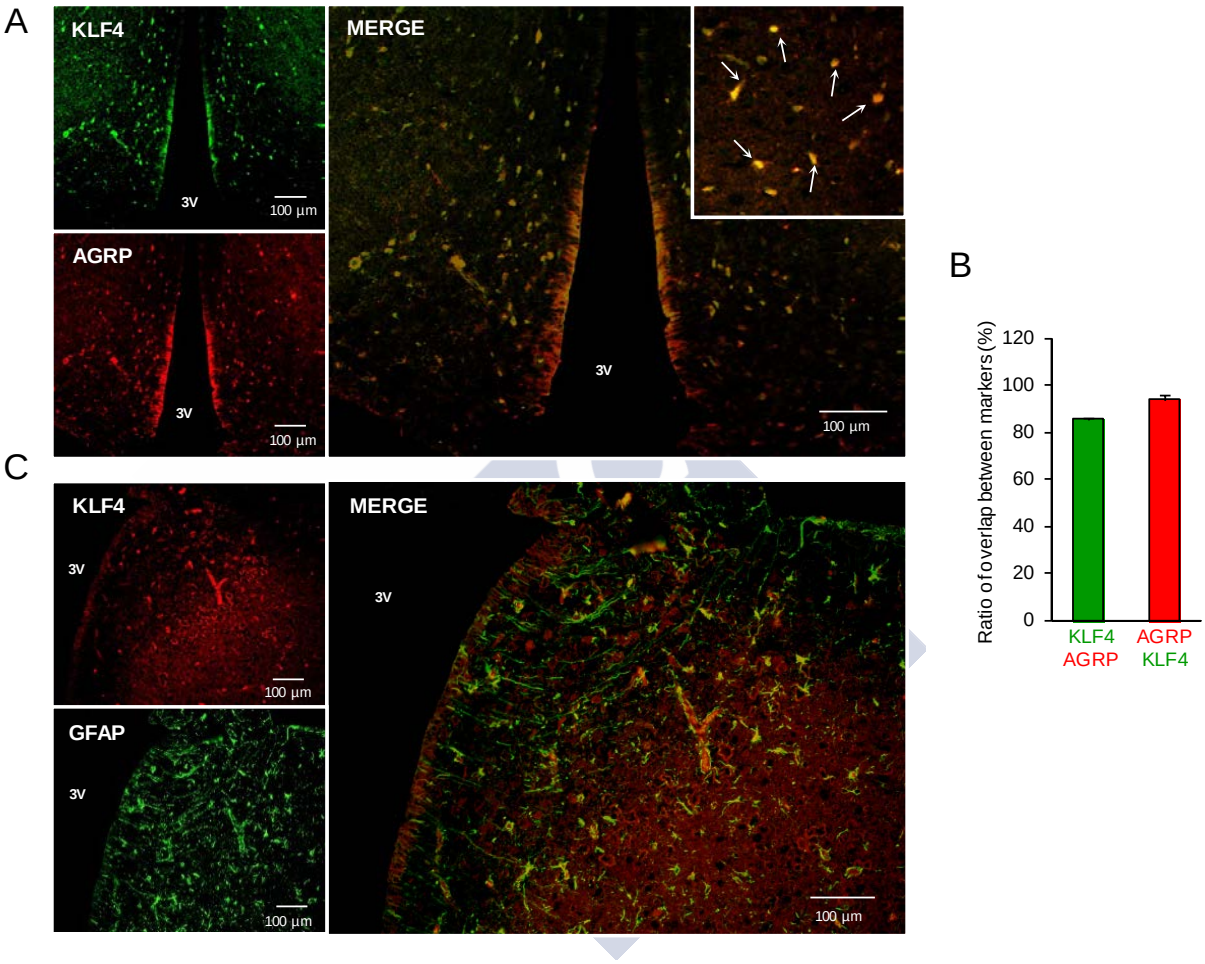

Figure 3. Colocalization (merge) of KLF4 (green) and AgRP (red) neurons in the hypothalamic arcuate nucleus (ARC) using double immunohistochemistry (A). Quantification of co-expression of KLF4 and AgRP shows that 85 ± 0.2 % of KLF4 neurons expressed AgRP (green bar) and that 93 ± 2 % of AgRP neurons expressed KLF4 (red bar) (B). Colocalization of KLF4 (red) and GFAP (green) in the ARC (C).

1.4 OREXIGENIC EFFECT OF GHRELIN IS NOT REGULATED BY KLF4

Ghrelin is regulated by nutritional status and increases AgRP peptide gene expression [411]. Since KLF4 is an activator of AgRP [35], we next wanted to test if KLF4 could be involved in the ghrelin signaling pathway in the hypothalamus. Thus, we performed an experiment injecting ghrelin ICV. Ghrelin increased food intake but did not affect protein

levels of KLF4 (Fig 4B), PPARγ (Fig 4C) or RXR (Fig 4D) suggesting that KLF4 does not take part of ghrelin signaling pathway in the hypothalamus.

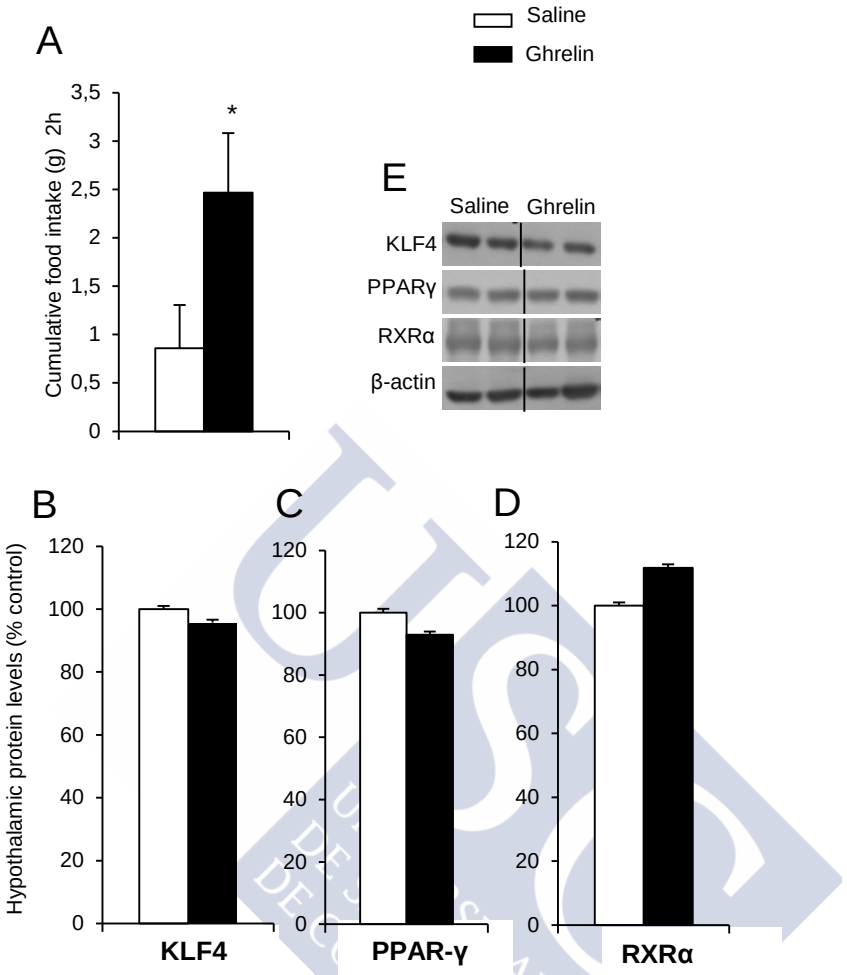

Figure 4. Two hours food intake of rats treated ICV with ghrelin (A). Effect of ghrelin infusion on KLF4 (B), PPARγ (C) and RXRα (D) hypothalamic protein levels measured by western blot (E). Dividing lines indicate splicing of the same gel. Values are mean ± SEM of 8-10 animals per group. β-actin was used to normalize protein levels. Error bars indicate SEM. *p<0.05 using one one-tailed Student's t-test.

1.5 LEPTIN REGULATES KLF4 PROTEIN LEVELS IN THE ARC VIA STAT3 AND PI3K PATHWAYS.

Because fasting is a hypoleptinemic state, we next tested whether leptin, one of the predominant hormones known to inhibit the activity of AgRP neurons, affected hypothalamic KLF4 levels. We found that IP administration of leptin decreased KLF4 levels in the ARC after 90 minutes in rats, but, by contrast, in leptin-deficient (ob/ob) mice ARC KLF4 protein levels were significantly increased (Figure 5A).

Since leptin seems to be an important modulator of hypothalamic KLF4 and both the STAT3 and PI3K signaling pathways are essential modulators of the anorectic action of leptin [132], we wanted to test if these effects were mediated by STAT3 and/or PI3K. To address

the role of central STAT3 signaling in mediating the effects of leptin on KLF4, we used a cell-permeable phosphopeptide-specific inhibitor of the STAT3 signaling pathway (STAT3 PI), which is normally activated by the long form of the leptin receptor [412]. Rats received, first, a single ICV infusion of either vehicle or STAT3 PI, and 30 minutes later, a second ICV infusion of either vehicle or leptin. Inhibition of the STAT3 signaling pathway in this way prevented the decrease in KLF4 protein levels caused by leptin in controls in the ARC (Figure 5B). Inhibition of the PI3K signaling pathway using the PI3K inhibitor LY294002 [413, 414] similarly prevented leptin-induced suppression of KLF4 protein expression (Figure 5C). Importantly, the inhibition of STAT3 (Figure 5D) or PI3K (Figure 5E) did not themselves cause any alteration in KLF4 protein levels in the ARC [308].

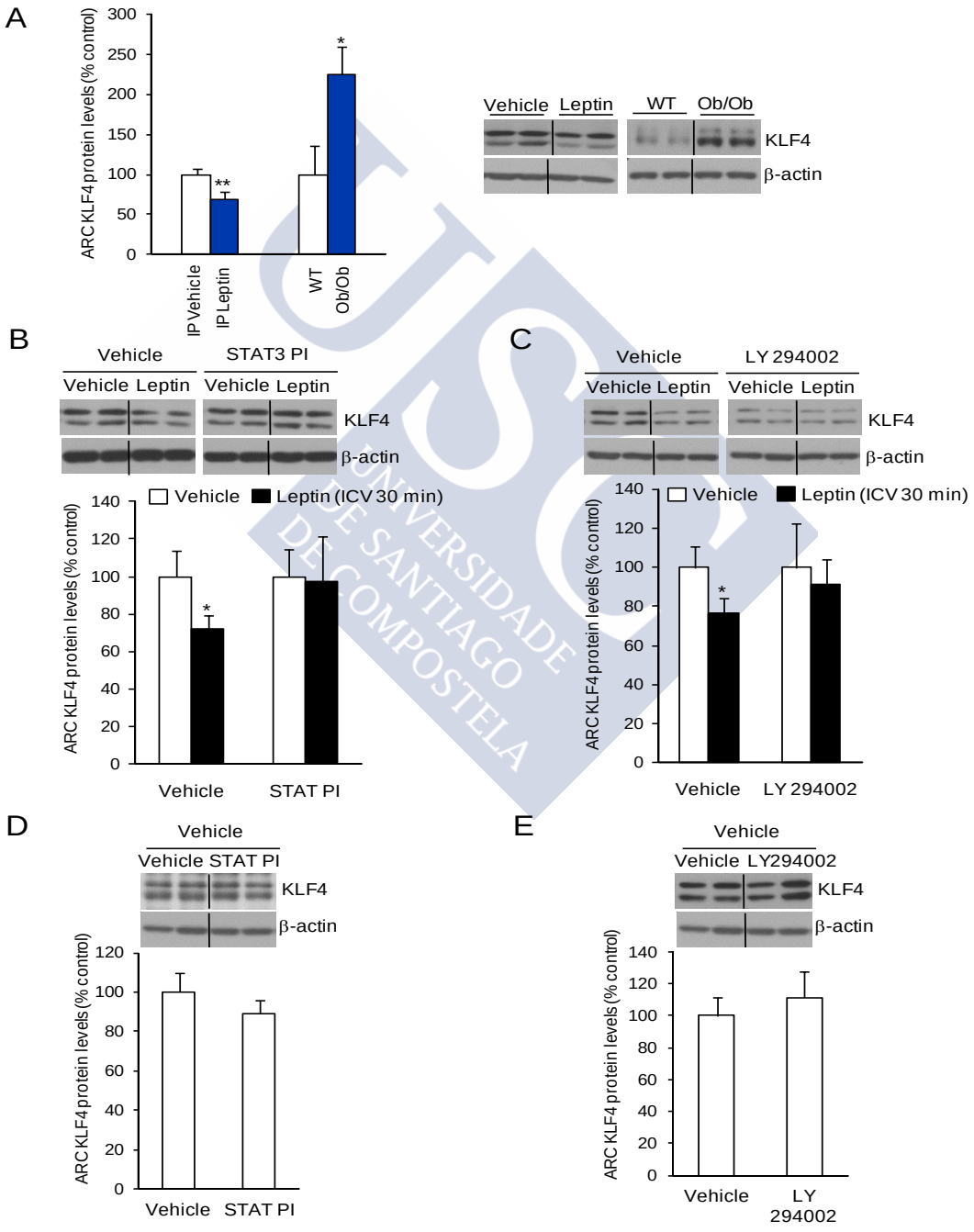

Figure 5. Effect of IP leptin treatment and leptin deficiency on KLF4 protein levels in the ARC measured by western blot (A). KLF4 protein levels in the hypothalamic arcuate nucleus of rats treated with a STAT3 inhibitor (B) or PI3K inhibitor (C) plus vehicle or leptin. Effect of the STAT3 inhibitor (D) or PI3K inhibitor (E) on KLF4 protein levels in the hypothalamic arcuate nucleus. Dividing lines indicate splicing of the same gel. Values are mean ± SEM of 8-10 animals per group. ß-actin was used to normalize protein levels. Error bars indicate SEM. *p<0.05, **p<0.01 versus controls using one-tailed Student's t-test.

1.6 OVER-EXPRESSION OF KLF4 IN THE HYPOTHALAMIC ARC INCREASES FOOD INTAKE AND BODY WEIGHT

Since KLF4 is located in AgRP neurons and is regulated by nutritional status and leptin, we next investigated whether specific over-expression of KLF4 in the ARC would be sufficient to affect food intake and body weight. Stereotaxic injection of adenoviruses over-expressing KLF4 into the ARC (Figure 6A) showed that the over-expression of KLF4 in this specific nucleus (Figure 6B) increased total food intake (Figure 6C) and body weight (Figure 6D) over a 5-day period. These transient effects on food intake and body weight are probably explained by the limited length of action of the adenoviruses. The gain of weight was consistent with an increased fat mass (Figure 6E) without any significant change in lean mass (Figure 6F) [308].

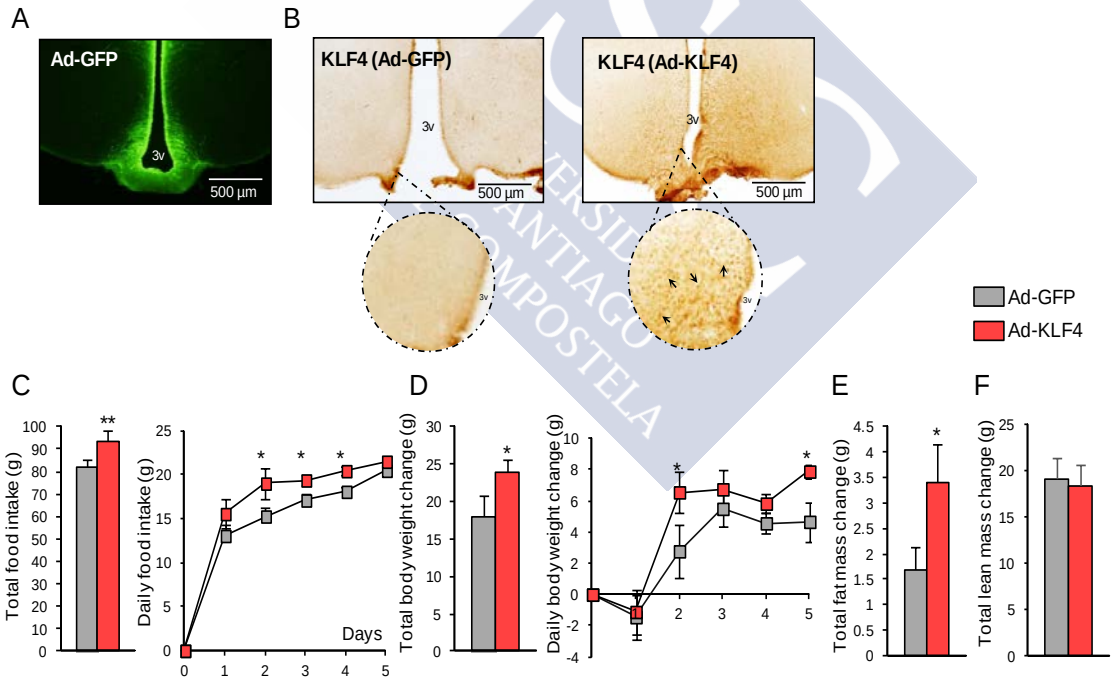

Figure 6. Immunofluorescence of GFP in the hypothalamic arcuate nucleus (A). Immunohistochemistry of KLF4 protein levels in the hypothalamic arcuate nucleus in Ad-GFP and Ad-KLF4 treated rats (B). Effect of the over-expression of KLF4 in the hypothalamic arcuate nucleus on food intake (C), body weight (D), fat mass (E) and lean mass (F). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. *p<0.05, **p<0.01, versus controls using one-tailed Student's t-test.

1.7 KLF4 ACTS SPECIFICALLY IN THE ARC

In rats in which adenoviral injections did not reach the ARC (Figure 7A), no differences in food intake (Figure 7B) or body weight (Figure 7C) were observed suggesting that KLF4 acts specifically in the ARC [308].

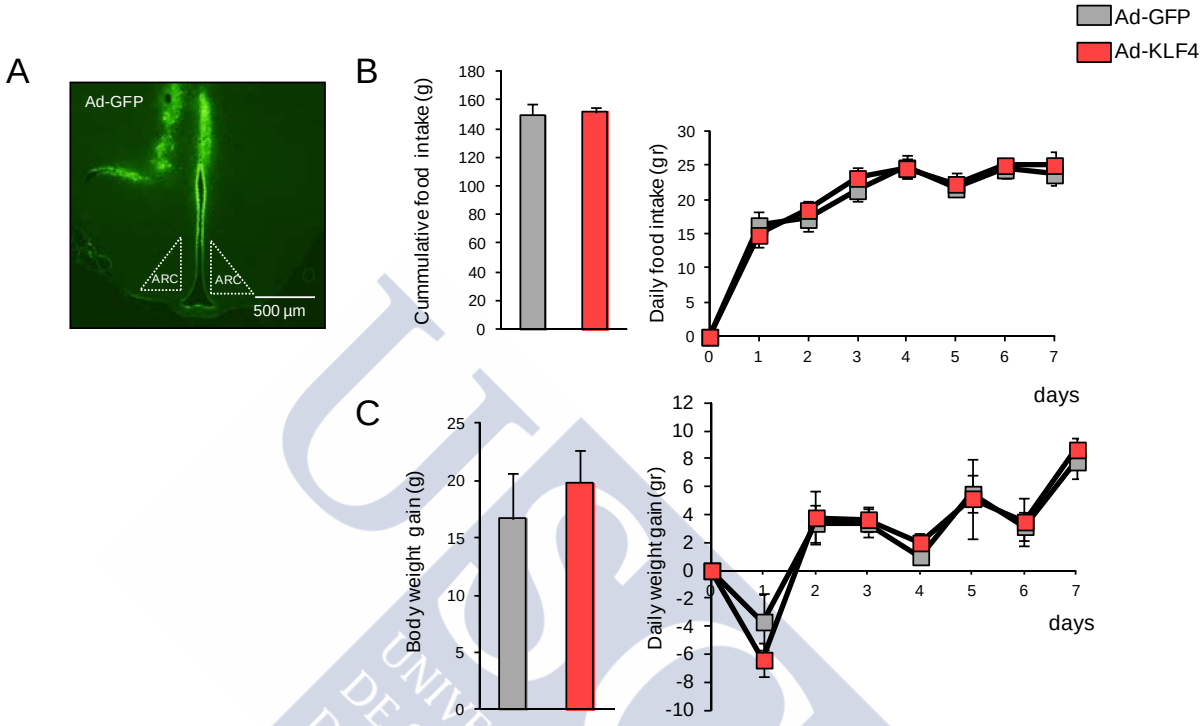

Figure 7. Lack of effect of a 7-day adenoviral injection expressing GFP or KLF4 respectively in a non-specific area using immunofluorescence (A) on food intake (B) and body weight (C). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. One-tailed Student's t-test was used for statistical analysis.

1.8 THE OVEREXPRESSION OF KLF4 DOES NOT AFFECT EE, LA OR RQ

The increase in body weight and fat mass when we injected KLF4 adenovirus in the ARC, is sufficiently explained by the hyperphagia, because energy expenditure (Figure 8A), locomotor activity (Figure 8B) and respiratory quotient (Figure 8C) remained unaltered in the same period following KLF4 over-expression [308].

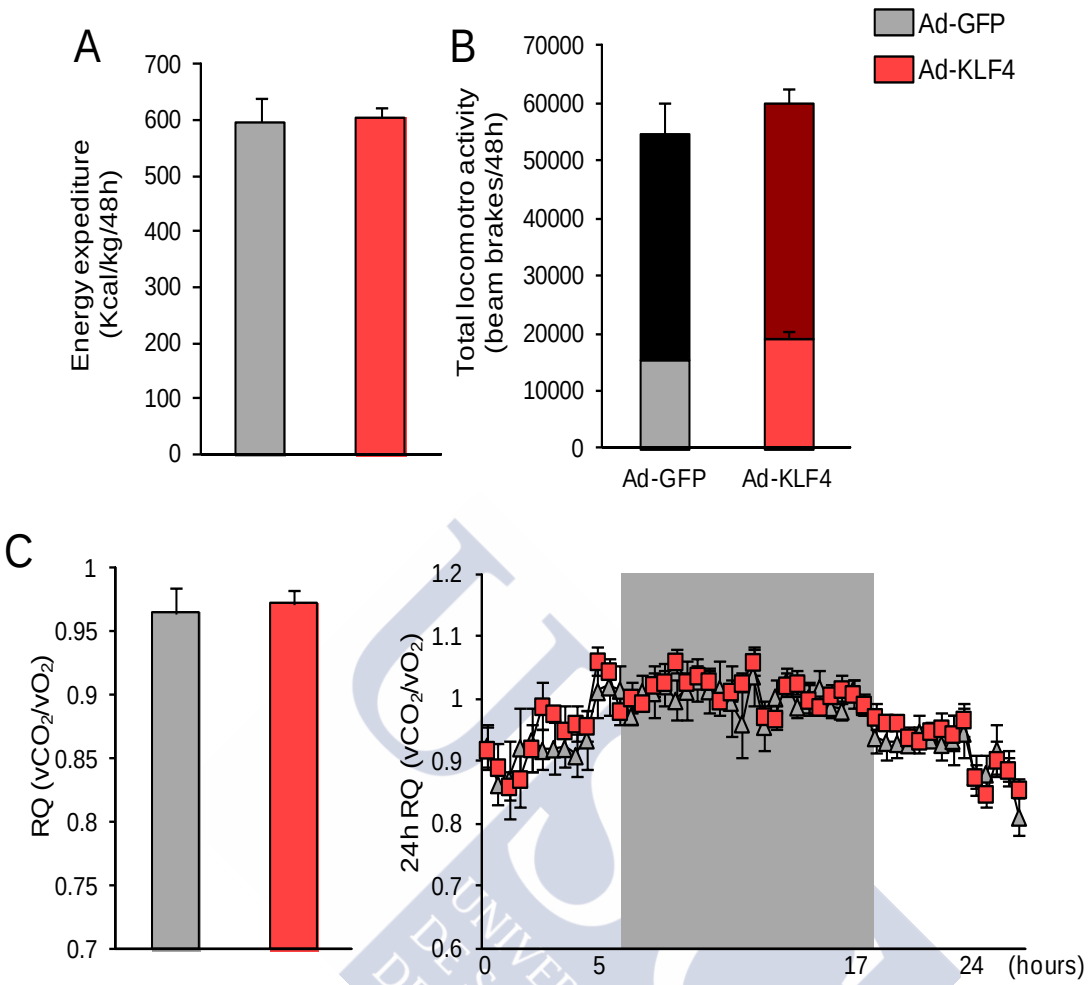

Figure 8. Effect of a 4-day adenoviral injection expressing GFP or KLF4 respectively on ARC on energy expenditure (A), locomotor activity (B) and respiratory quotient (C). Values are mean ± SEM of 8-10 animals per group. For statistics one-tailed Student's t-test was used.

1.9 THE OVEREXPRESSION OF KLF4 AFFECTS LIPID METABOLISM BUT NOT LIVER TG

Consistent with their increase in fat mass, rats injected in the ARC with adenoviruses over-expressing KLF4 also showed larger adipocytes (Figure 9A) and higher levels of FAS and lipoprotein (LPL) in WAT than rats injected with empty viruses (Figure 9B). No differences in hepatic TG levels were detected between the two groups (Figure 9C) [308].

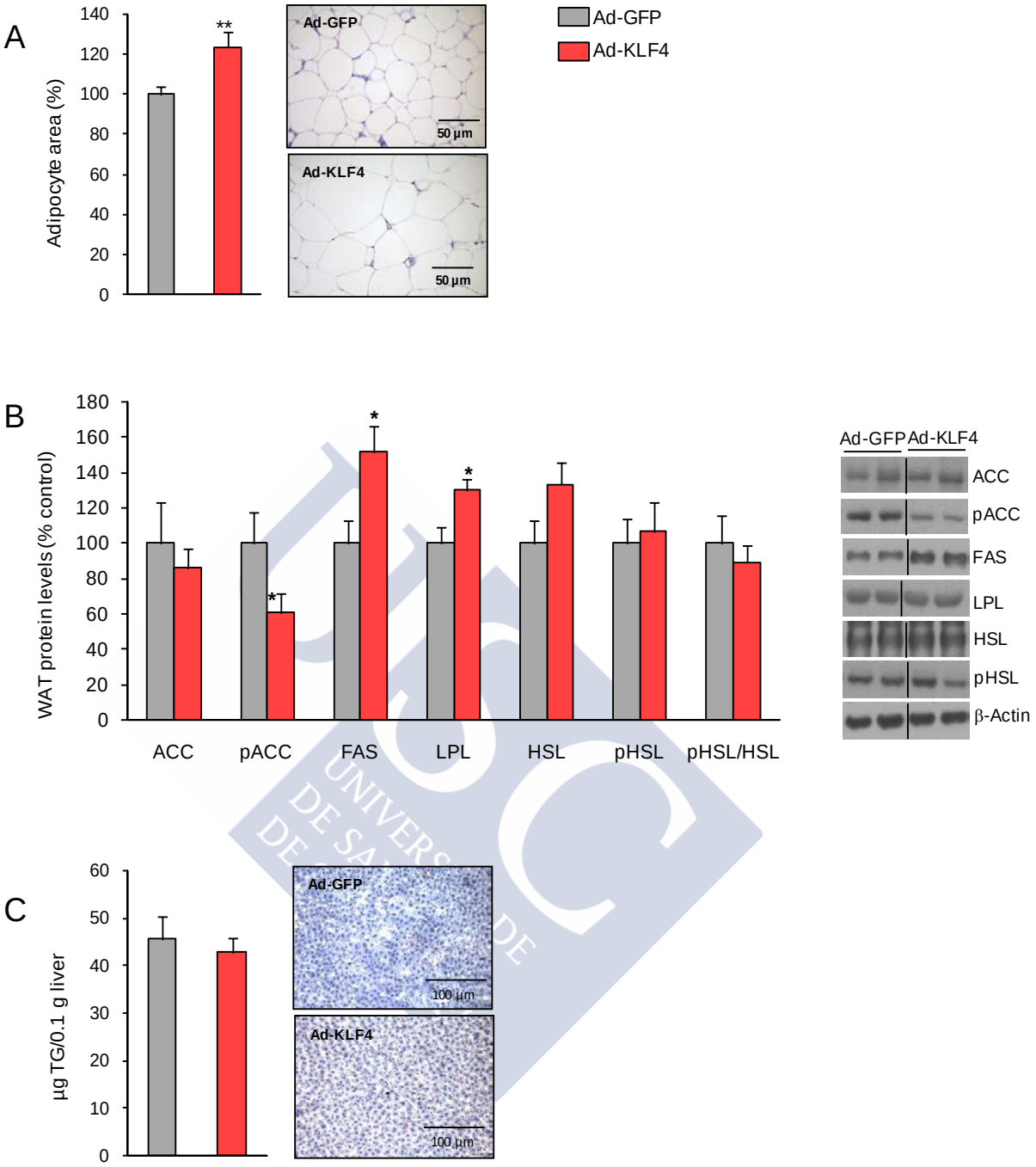

Figure 9. Effect of a 4-day adenoviral injection expressing GFP or KLF4 respectively in ARC on epididymal WAT adipocyte area (A), Epididymal WAT ACCα, pACCα, FAS, LPL, HSL and pHSL protein levels (B), and hepatic triglycerides (TG) content represented by red oil and total TG content (C). Dividing lines indicate splicing of the same gel. Values are mean ± SEM of 8-10 animals per group. ß-actin was used to normalize protein levels. Error bars indicate SEM. *p<0.05, **p<0.01 versus controls using one-tailed Student's t-test.

1.10 OVER-EXPRESSION OF KLF4 IN THE HYPOTHALAMIC ARC INCREASES FOOD INTAKE AND BODY WEIGHT THROUGH STIMULATION OF AGRP EXPRESSION

To ascertain which hypothalamic neuropeptides were triggered by over-expression of KLF4, we next assessed ARC levels of AgRP, NPY, POMC and CART. Thus, we found that the over-expression of KLF4 produced a significant up-regulation of AgRP mRNA expression, whereas other key neuropeptides regulating food intake, such as CART, NPY and POMC, were not affected (Figure 10A). These data, obtained by *in situ* hybridization, were corroborated by western blotting, with a significant increase being observed in AgRP but not NPY protein levels (Figure 10B). Additionally, we also found that the levels of GABA synthesizing enzymes glutamate decarboxylase 65 and 67 (GAD65 and GAD67) and vesicular GABA transporter (VGAT) were decreased in the ARC after over-expression of KLF4 (Figure 10B) [308].

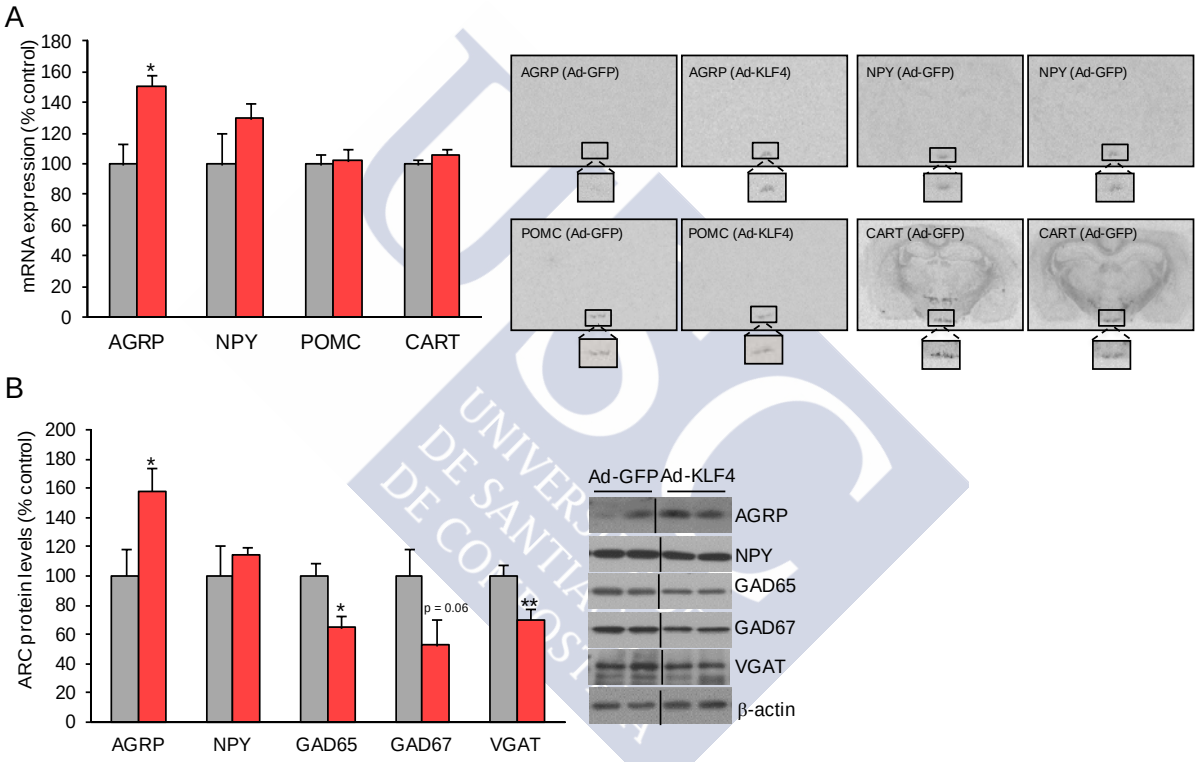

Figure 10. Hypothalamic mRNA expression of NPY, AgRP, POMC and CART (A), and arcuate protein levels of AgRP, NPY, GAD65, GAD67 and VGADT (B). Dividing lines indicate splicing of the same gel. Values are mean ± SEM of 8-10 animals per group. ß-actin was used to normalize protein levels. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

1.11 OVER-EXPRESSION OF KLF4 IN ARC BLUNTS LEPTIN SENSITIVITY IN RATS FED A CHOW DIET

Given the data thus far, showing KLF4 to be regulated by leptin and to stimulate both *in vivo* AgRP expression and food intake, we next hypothesized that hypothalamic KLF4 modulates leptin sensitivity. To test this, we used an adenovirus to over-express KLF4 in the ARC for 4 days and then delivered IP leptin or its vehicle, following a published protocol

[415]. As expected, in control rats treated with the empty virus the subsequent peripheral administration of leptin decreased 24 h-food intake (Figure 11A) and body weight (Figure 11B). However, over-expression of KLF4 in the ARC completely blocked leptin's subsequent actions on both feeding and body weight (Figure 11 A and B) [308].

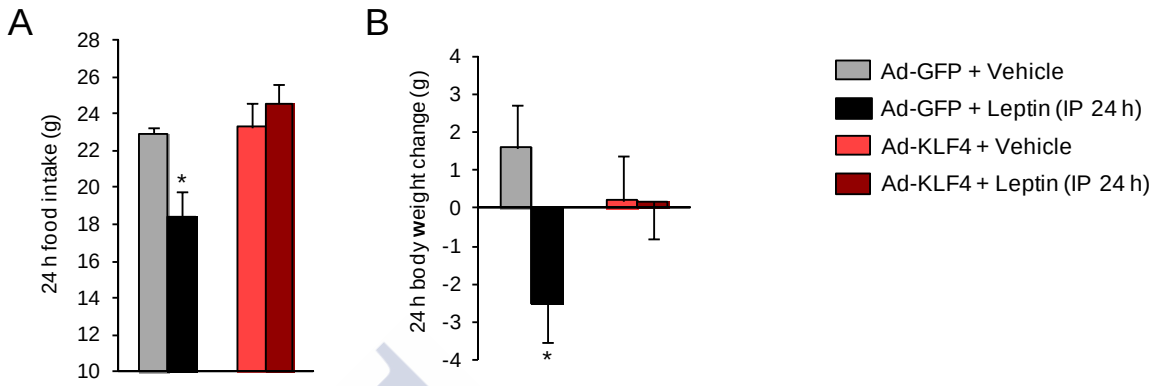

Figure 11. Effect of peripheral leptin (1 mg/Kg) or vehicle administration on rats after 4-day adenoviral injection expressing GFP or KLF4 respectively on food intake (A) and body weight (B) after 24 hours. Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. *p < 0.05 using one way ANOVA.

1.12 KLF4 IS NOT A DIRECT TARGET OF FOX01 IN LEPTIN PATHWAY

FoxO1 is a transcription factor that directly regulates AgRP expression [42], and its genetic ablation in AgRP neurons leads to reduced food intake and fat mass [43]. FoxO1 is, moreover, a critical modulator of leptin sensitivity [43, 416]. Therefore, we next evaluated any potential relationship between FoxO1 and KLF4 in modulating leptin sensitivity.

As expected, after 90 minutes peripheral administration of leptin increased phosphorylation of FoxO1 (pFoxO1) in the ARC of control rats (Figure 12A). Similarly, leptin also triggered pFoxO1 in rats over- expressing KLF4 in the ARC (Figure 12A), indicating that manipulation of KLF4 had not altered FoxO1 activity. Consistently, over-expression of KLF4 in the ARC did not modify FoxO1 protein levels in the ARC (Figure 12B). Next, we examined the effect of FoxO1 down-regulation in the ARC on leptin sensitivity, using lentiviral FoxO1 shRNA stereotaxically injected into the ARC and leptin administered peripherally. The efficiency of the stereotaxic injections in the ARC was corroborated by immunostaining of GFP (Figure 12C). FoxO1 protein levels were indeed decreased, whereas KLF4 protein levels remained unaffected in the ARC after the stereotaxic injection of the FoxO1 shRNA lentiviruses, (Figure 12D). The inhibition of FoxO1 blunted the anorexigenic action of leptin (Figure 12E). However, as shown in Figure 12F, leptin decreased KLF4 protein levels in the ARC of both control rats and in rats injected with lentiviral FoxO1 shRNA [308].

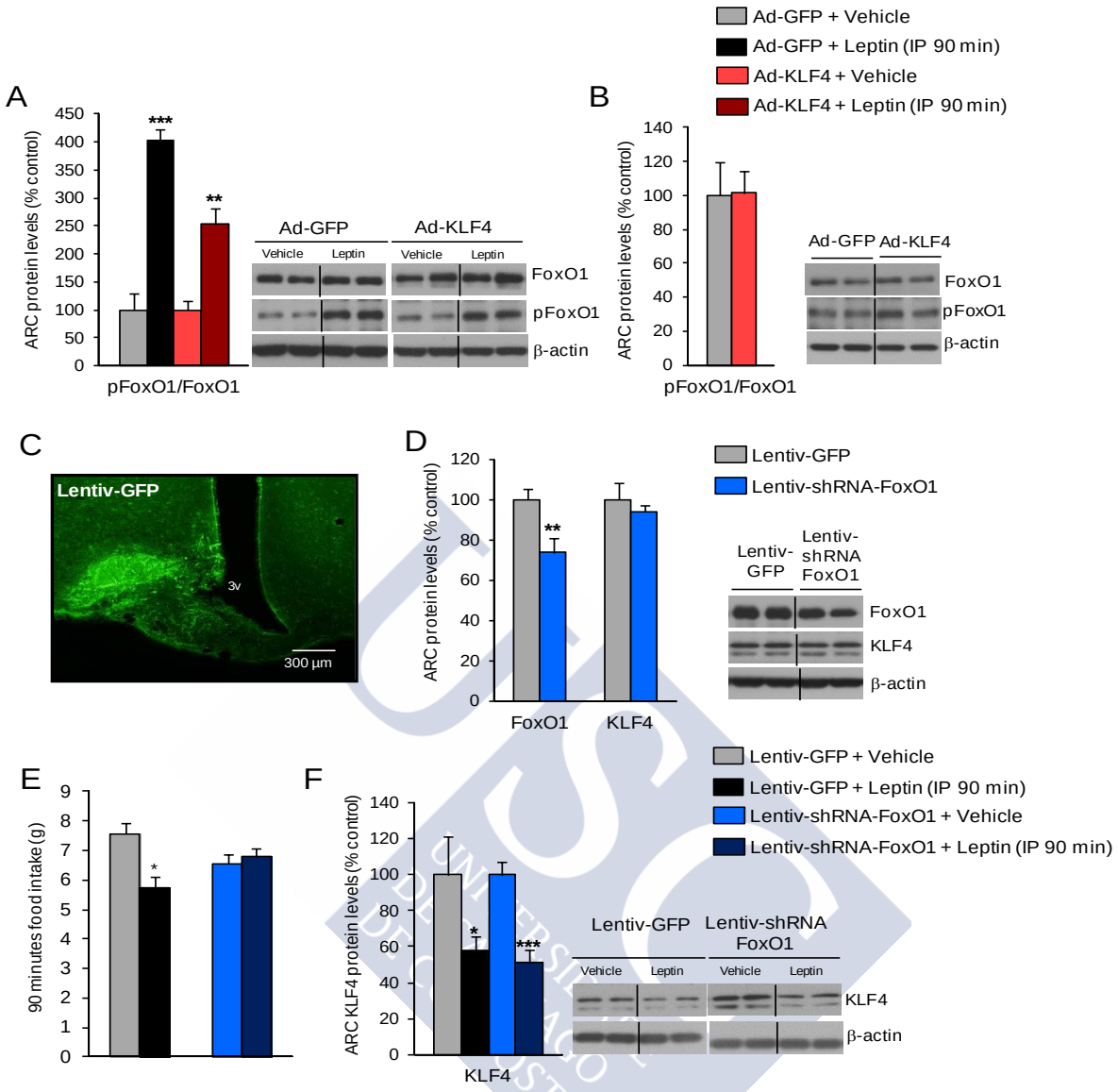

Figure 12. Effect of peripheral leptin (1 mg/kg) or vehicle administration on rats after 4-day adenoviral injection expressing GFP or KLF4 on FoxO1 levels in the hypothalamic arcuate nucleus (ARC) after 90 min (A). Effect of over-expression of KLF4 in the ARC on FoxO1 protein levels in the ARC (B). Localization studies of microinjection sites, showing immunofluorescence with anti-GFP antibody in the ARC (C). Protein levels of FoxO1 and KLF4 in the ARC of rats stereotaxically injected with scrambled or shFoxO1 lentiviruses (D). Effect of peripheral leptin (1 mg/kg) or vehicle administration on rats after 21-days of the lentiviral injection expressing GFP or FoxO1 shRNA on food intake (E) and KLF4 protein levels in the ARC (F) after 90 min of leptin injection. Dividing lines indicate splicing of the same gel. Values are mean ± SEM of 8-10 animals per group. ß-actin was used to normalize protein levels. Error bars indicate SEM. Figure E: one way ANOVA *p<0.05. The rest of the figures: *p<0.05, **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

1.13 HYPOTHALAMIC SILENCING OF KLF4 IN ARC NUCLEUS REDUCES HYPERPHAGIC RESPONSE DUE TO FASTING IN RATS FED A CHOW DIET

We next wanted to know what happens to the leptin signaling if we downregulate KLF4 in the ARC. To asses that, we used a lentivirus encoding a KLF4 shRNA to inhibit expression of KLF4 specifically within the ARC (Figure 13A). Chronic inhibition of KLF4 in the ARC of rats fed a chow diet did not cause any alteration in cumulative food intake, body weight or fat mass (Figure 13B and C). However, weekly measurements indicated that there was a significant decrease in the body weight gain after the third week (Figure 13C). We demonstrated that the lentiviral KLF4 shRNA was able to inhibit the hyperphagic response in rats pre-fasted overnight when the animals were fed a chow diet (Figure 13D). The reduced hyperphagia was consistent with a specific decrease in AgRP mRNA expression (Figure 13E) [308].

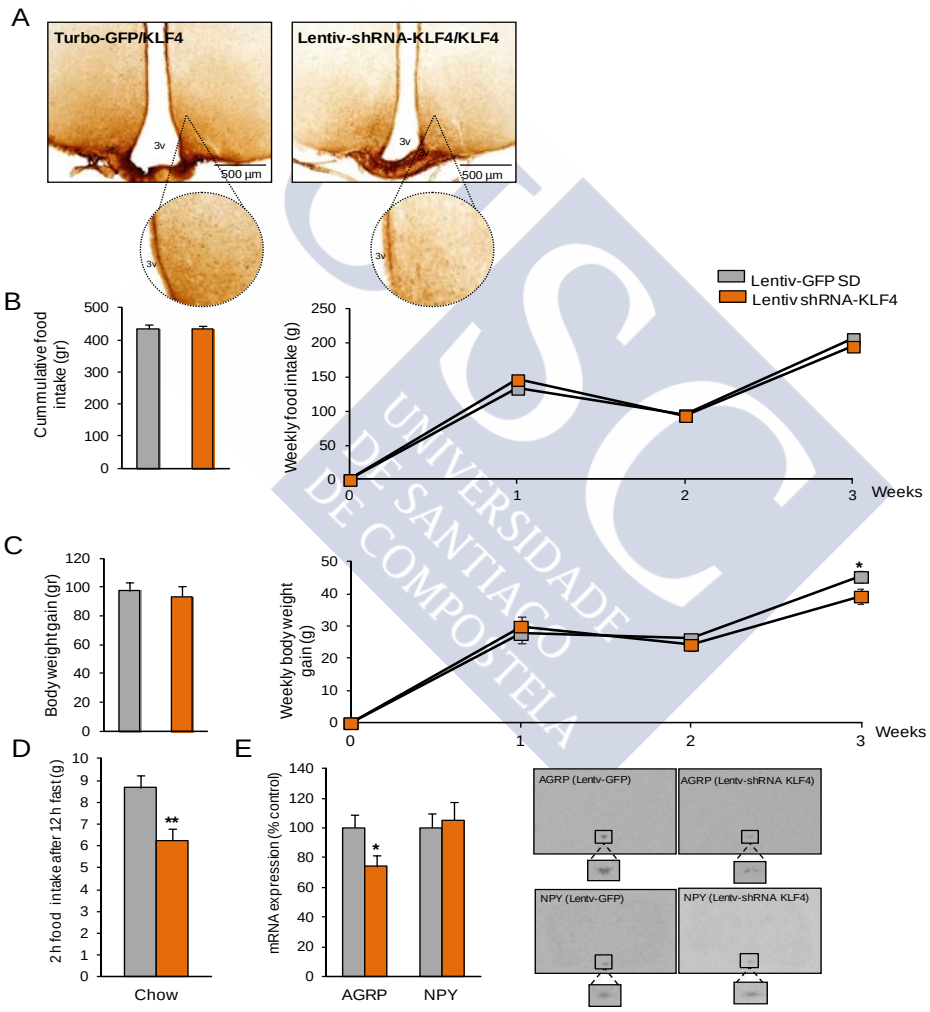

Figure 13. KLF4 protein levels in the ARC in lentivirus-GFP and lentivirus-shRNA KLF4 treated rats using immunohistochemistry (A). Effect of KLF4 down-regulation (using lentiviral shKLF4 injection) on food intake (B) and body weight (C) of rats fed standard diet. Effect of ARC KLF4 down-regulation on 2 h food intake after 12 h fasting (D) and hypothalamic levels of AgRP and NPY (E) in rats fasted for 12 hours and fed a chow diet for 2 hours. Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

1.14 HYPOTHALAMIC SILENCING OF KLF4 IN ARC NUCLEUS DOESN'T MODIFY LEPTIN SENSITIVITY

In order to investigate if the down-regulation of KLF4 in the ARC was able to modulate leptin sensitivity, we injected intraperitoneally leptin in rats 3 weeks after the stereotaxic delivery of lentiviral KLF4 shRNA into the ARC of rats fed a chow diet. However, our data indicated that the lower levels of KLF4 in the ARC did not modify leptin sensitivity, since rats injected with empty lentiviruses showed similar food intake (Figure 14A) and body weight (Figure 14B) to rats injected with lentiviral KLF4 shRNA [308].

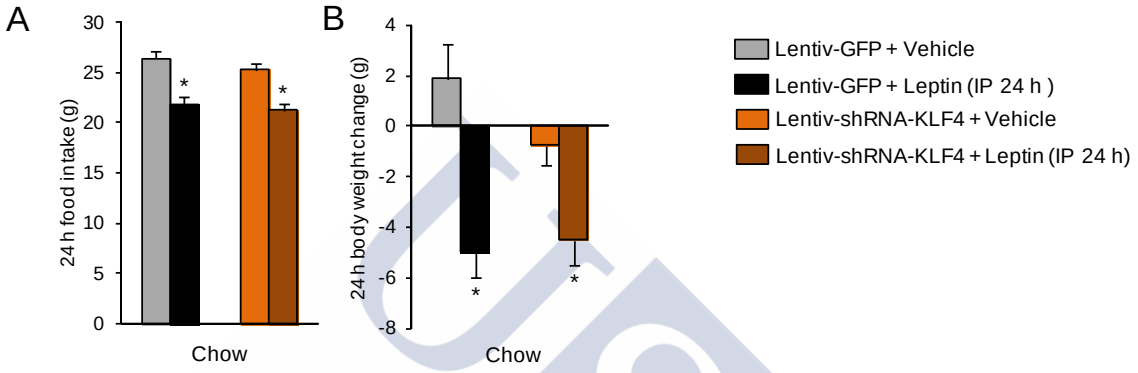

Figure 14. Effect of peripheral leptin (1 mg/Kg) or vehicle administration on rats after 21 days of injection with a lentivirus expressing GFP or KLF4 shRNA on food intake (A) and body weight (F) of rats fed a chow diet. Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. *p<0.05 one-way ANOVA.

1.15 HYPOTHALAMIC SILENCING OF KLF4 DOESN'T AMELIORATE HFD-INDUCED LEPTIN RESISTANCE IN HFD RATS

A hallmark of HFD-induced obesity is leptin resistance. Leptin signaling in the hypothalamus is blunted in rats fed a HFD [134]. Since KLF4 is a key modulator of leptin's actions on food intake and body weight, we next hypothesized that hypothalamic KLF4 contributes to the development of HFD-induced leptin resistance.

To test this, we initially measured KLF4 protein levels in the ARC of rats fed a chow diet and rats fed a HFD (Figure 15A) but we failed to detect significant differences between these two groups.

When we challenged DIO rats with exogenous IP leptin at a dose that reduced caloric intake and body weight in lean rats, leptin failed to inhibit hypothalamic KLF4 expression (Figure 15B).

Then, to study the role of KLF4 in DIO rats, we injected lentiviral KLF4 shRNA into the ARC of HFD-fed rats. Chronic inhibition of KLF4 in the ARC of HFD-fed rats also did not cause any alteration in food intake, body weight or fat mass (Figure 15C and D).

To explore whether silencing KLF4 in the ARC altered whole-body leptin sensitivity, we measured food intake 24 h after IP leptin administration. As expected, leptin failed to

suppress food intake in DIO rats treated with the empty lentivirus. We also found that DIO rats treated with the lentiviral KLF4 shRNA remained leptin resistant (Figure 15E) [308].

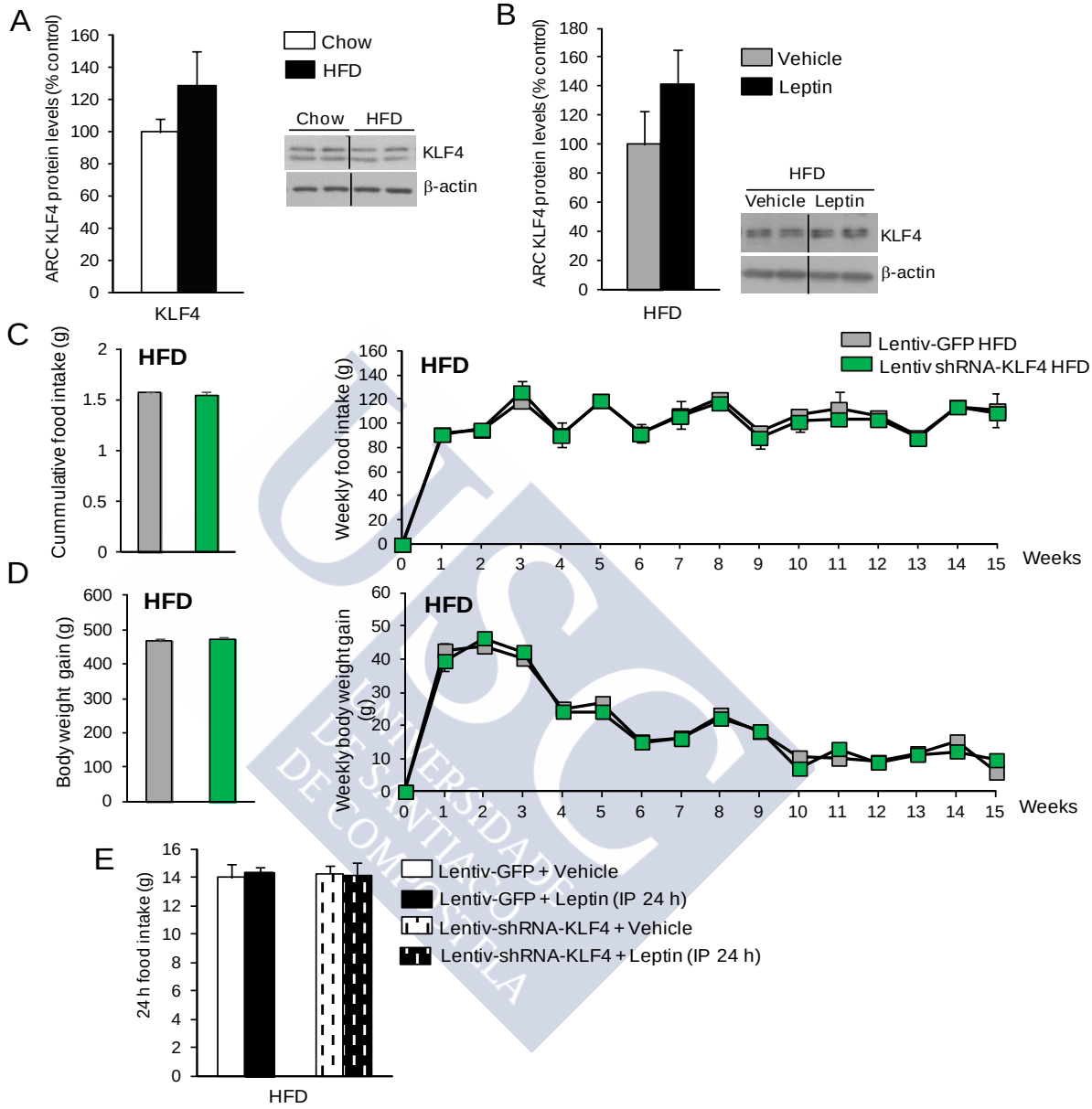

Figure 15. KLF4 protein levels in the hypothalamic arcuate nucleus (ARC) or rats fed a chow diet and high-fat diet (A). KLF4 protein levels in the ARC of DIO rats treated with leptin (B). Effect of KLF4 down-regulation (using lentiviral shKLF4 injection) on food intake (C) and body weight (D) of DIO rats. Effect of peripheral leptin (1 mg/Kg) or vehicle administration on rats treated with lentivirus-GFP and lentivirus shRNA KLF4 in rats fed a high fat diet. Dividing lines indicate splicing of the same gel. Values are mean ± SEM of 8-10 animals per group. ß-actin was used to normalize protein levels. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

1.16 HYPOTHALAMIC SILENCING OF KLF4 IN ARC REDUCES HYPERPHAGIC RESPONSE DUE TO FASTING IN HFD RATS

Similar to a chow diet rats fed a HFD and then administered lentiviral KLF4 shRNA in the ARC, showed a decreased hyperphagic response after overnight pre-fasting and three weeks after one single lentiviral injection (Figure 16) [308].

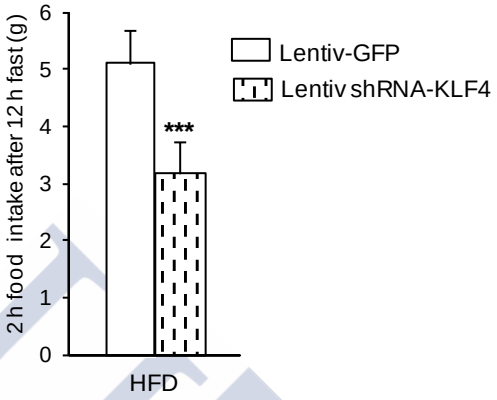

Figure 16. Effect of KLF4 down-regulation on 2 h food intake after 12 h fasting. Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. ***p<0.001 versus controls using one-tailed Student's t-test.

2 CHAPTER 2: The effect of diet before and during pregnancy

2.1 PREGNANCY PARAMETERS AND TISSUE WEIGHTS

In the SDSD group the controls are Sprague-Dawley rats that were fed with standard diet, mated with the male and non pregnant as a result, and on the other hand rats of 21 days pregnancy fed with standard diet before and during pregnancy.

In the SDHFD group the controls are rats that were fed with standard diet, mated with the male and non pregnant as a result, and on the other hand rats of 21 days pregnancy fed with standard diet before pregnancy and high fat diet during pregnancy.

In the HFDHFD group the controls are rats that were fed with high fat diet, mated with the male and non pregnant as a result, and on the other hand rats of 21 days pregnancy fed with high fat diet before pregnancy and high fat diet during pregnancy.

We can see the pregnancy weight gain, litter size, fetal weight and placental weight in table 10.

Type of diet	Days of gestation	Pregnancy weight gain (g)	Litter Size (n)	Fetal weight (g)	Placental weight (g)
SDSD	**21 days**	151.4 ±8.643	12.4 ±1.288	4.163 ± 0.311	0.550 ±0.030
SDHFD	**21 days**	156.4±7.655	14.4±1.179	3.955±0.086	0.547± 0.013
HFDHFD	**21 days**	130.9 ±4.347	13 ±1.021	3.904 ±0.036	0.547 ±0.018

Table 10. Pregnancy weight gain, litter size, fetal weight and placental weight at 21 days of pregnancy with SDSD, SDHFD and HFDHFD. Data presented as mean ± SEM.

We observed an increase in the weight of liver in pregnancy under all kind of diets (Figure 17A, B and C), no differences in BAT weight in all kind of diets (Figure 17A,B and C). There was an increase in omental and epididymal content in SDSD and SDHFD while no differences in HFDHFD pregnant rats (Figure 17A, B and C).

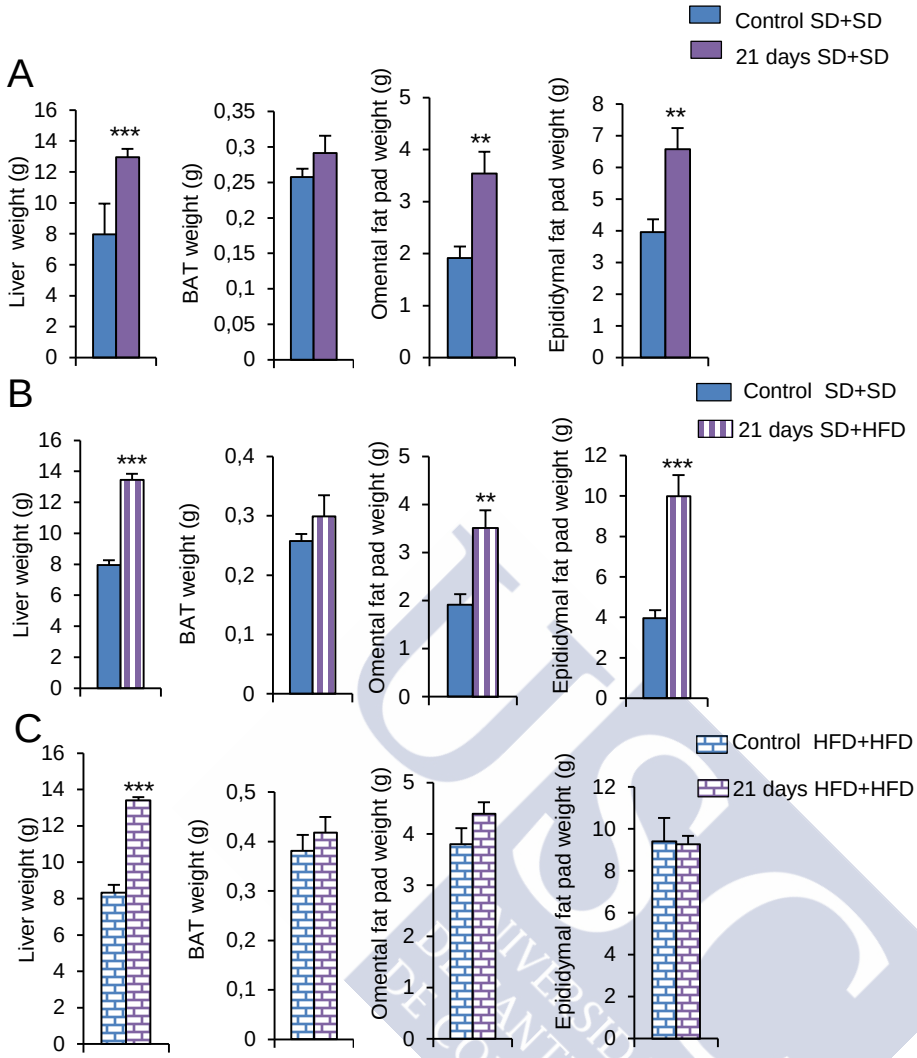

Figure 17. Liver, BAT, omental and epididymal fat pad weight in SDSD (A), SDHFD (B) and HFDHFD (C) pregnant rats. Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

2.2 SERUM PARAMETRES

We next decided to measure several parameters related with lipid metabolism, glucose metabolism and hepatic inflammation. As we can see in the charts, there is an increase in TG and FGF21 together with a decrease in glucose serum levels in pregnant rats under all kinds of diets (Figure 18 A, B and C). We also observed an increase in FFA and cholesterol serum levels in SDHFD and HFDHFD pregnant rats while those levels were unaffected in rats under standard diet before and during pregnancy (Figure 18A, B and C).

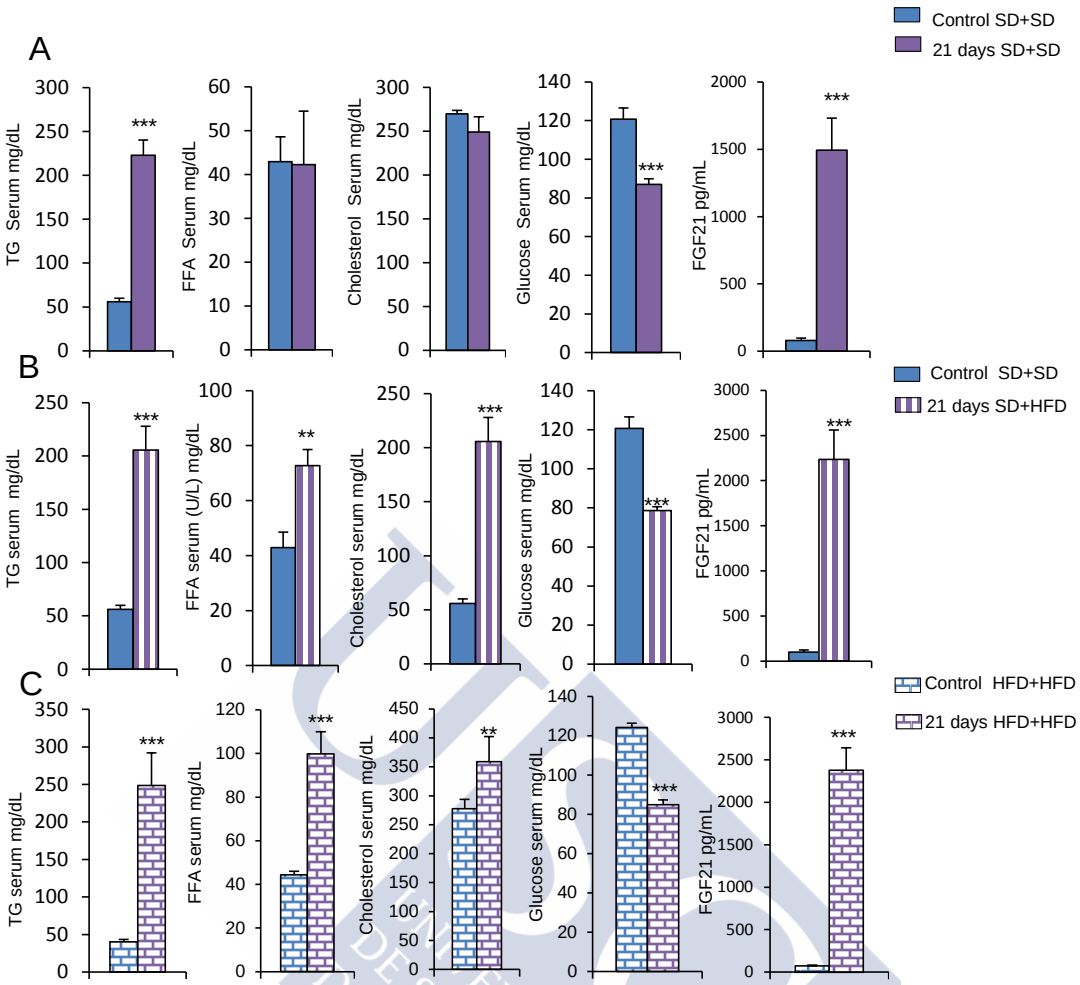

Figure 18. Serum concentration of TG, FFA, cholesterol, glucose and FGF21 in SDSD (A), SDHFD (B) and HFDHFD pregnant rats (C). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. ***p<0.001 versus controls using one-tailed Student's t-test.

2.3 LIVER PARAMETRES IN SDSD PREGNANT RATS

The liver plays a crucial role in metabolism of proteins, fats and carbohydrates. When fat is stored on the liver, several diseases such as NAFLD are more prone to appear. To asses fat levels in pregnant liver, we checked TG liver levels, and we haven't observed differences neither by colorimetric assay (Figure 19A) nor by oil red O staining (Figure 19B).

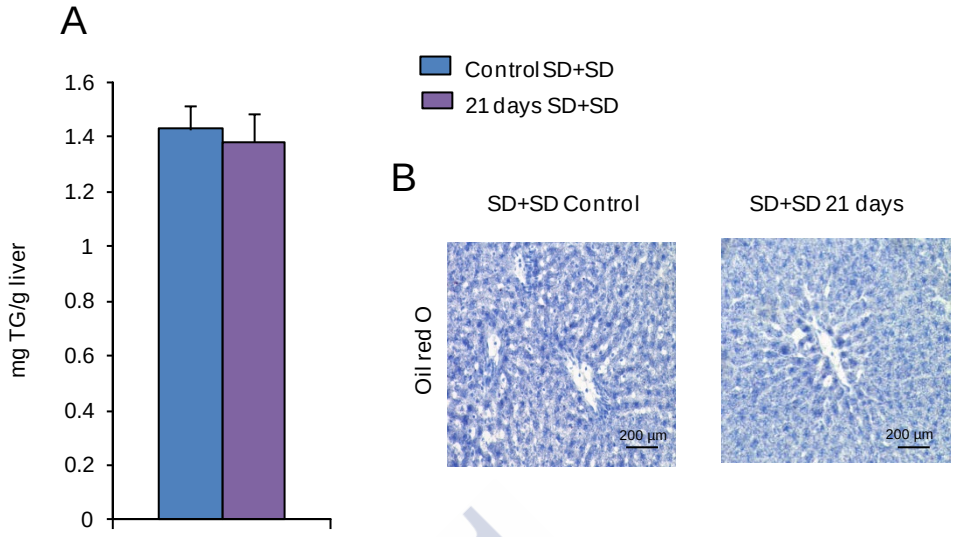

Figure 19. Liver triglyceride levels measured by a colorimetric assay (A) and represented in histological sections stained with Oil Red O (B). Non pregnant rats under standard diet (Control SD+SD) versus 21 days pregnant rats under standard diet before and during pregnancy (21 days SD+SD). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. One-tailed Student's t-test was used for statistical analysis.

To observe the effects at the molecular level in the liver of pregnant rats, we decided to measure by means of western blot the levels of different metabolic pathways.

Regarding lipid metabolism proteins, we haven't detected differences in LPL, AMPKα1 and ACCα. Western blot analysis also showed that pregnancy decreases the levels of pHSL/HSL, FAS, PGC1α , AMPKα2, pAMPKα, and there is also a tendency of decreasing on its downstream target pACCα (Figure 20A).

When we measured ER stress-related proteins, we observed no differences in pJNK1, an increase in peIF2α/eIF2α, pPERK and CC3 as well as a tendency of increasing in pIRE1α/IRE1α and a decrease in XBP1 and CHOP levels (Figure 20B).

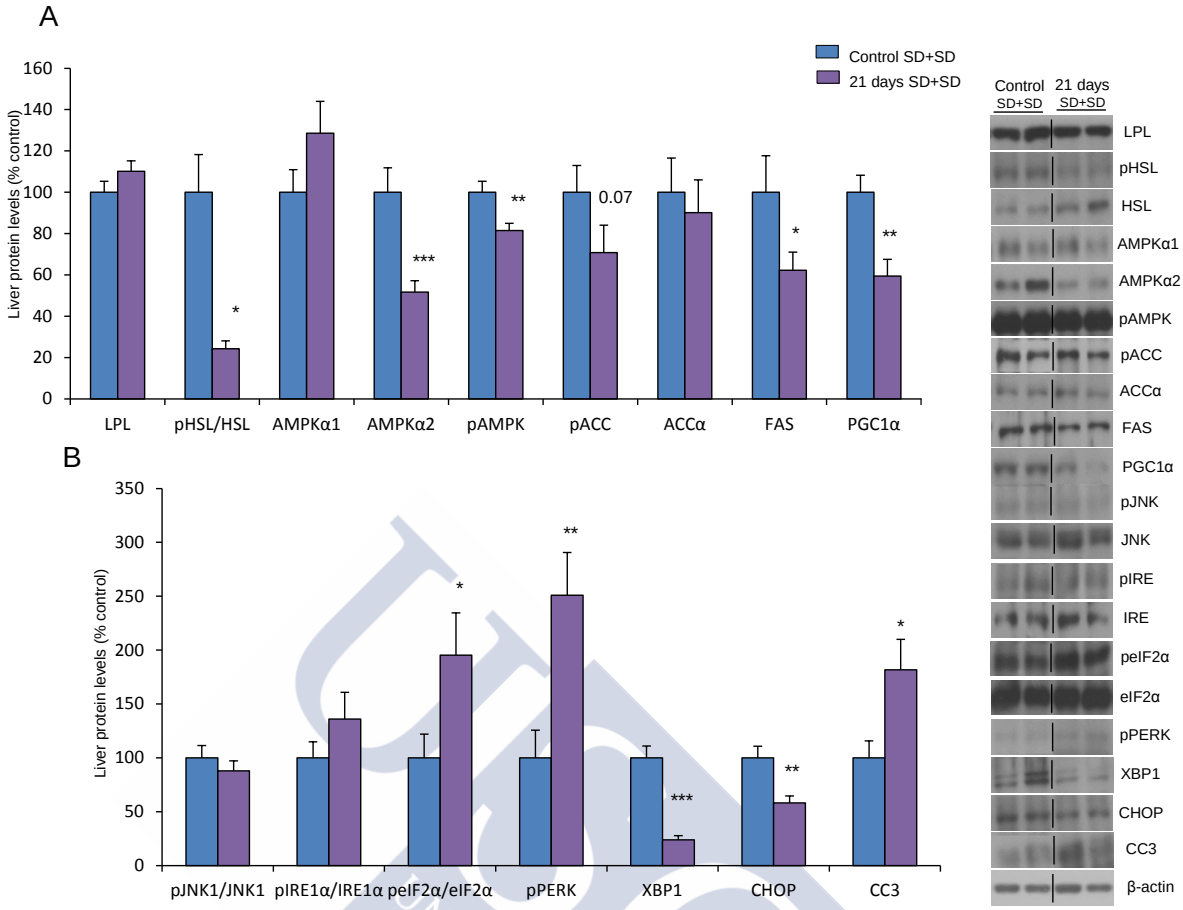

Figure 20. Western blot liver protein levels. A) Lipid metabolism proteins: LPL, pHSL, HSL, AMPKα1, AMPKα2, pAMPKα, pACCα, ACCα, FAS, PGC1α. B) ER stress proteins: pJNK1, JNK1, pIRE1α, IRE1α, peIF2α, eIF2α, pPERK, XBP1, CHOP, CC3. Non pregnant rats under standard diet (Control SD+SD) versus 21 days pregnant rats under standard diet before and during pregnancy (21 days SD+SD). Values are mean ± SEM of 8-10 animals per group. β-actin protein levels were used to normalize protein levels. Dividing lines indicate splicings within the same gel. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

Since FXR induces expression and secretion of FGF21 [202] and FXR also interacts with PPARγ and PPARα [417, 418], we wanted to measure its mRNA hepatic levels. As we can see in figure 21, there is a decrease in FXR levels and an increase in both PPARα and FGF21, with no differences in PPARγ.

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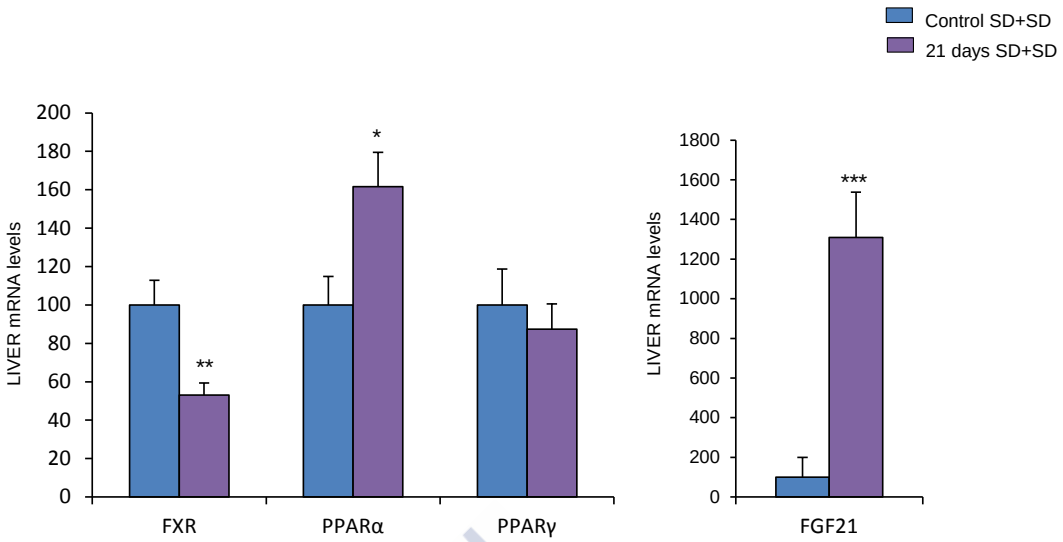

Figure 21. Liver mRNA levels of FXR, PPARα, PPARγ and FGF21. Non pregnant rats under standard diet (Control SD+SD) versus 21 days pregnant rats under standard diet before and during pregnancy (21 days SD+SD). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. *p<0.05, **p<0.01 versus controls using one-tailed Student's t-test.

2.4 BAT PARAMETERS IN SDSD PREGNANT RATS

Prior to measure what is going on at molecular level in BAT, we took thermographic photos (Figure 22C) and measured body temperature (Figure 22A) and interscapular BAT temperature (Figure 22B) to check the downregulation that is described in the literature [334].

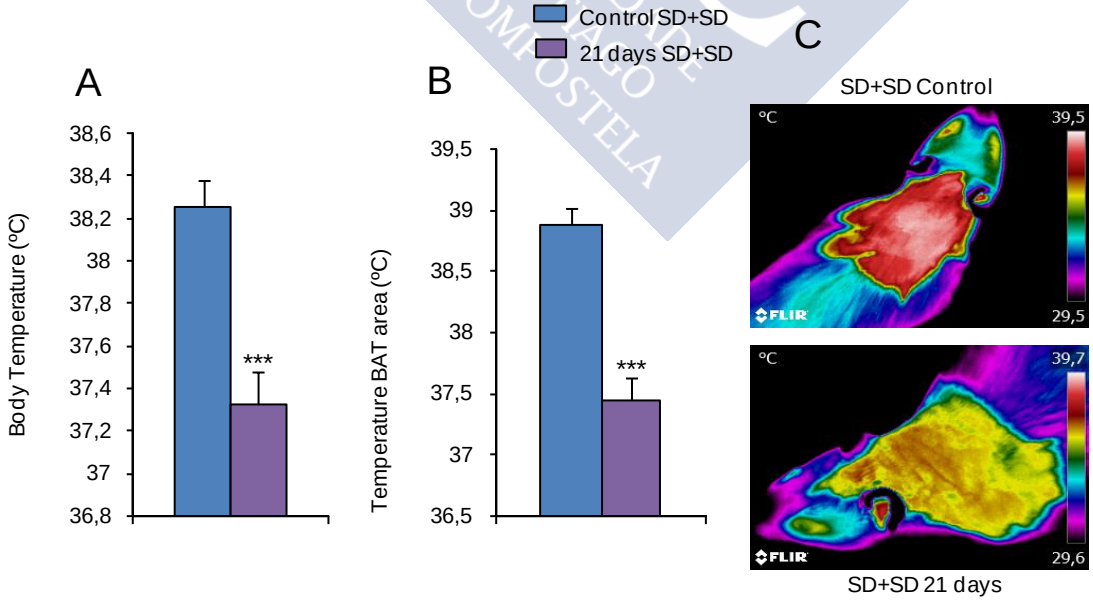

Figure 22. Body temperature (A), temperature BAT area (B) and infrared thermal images (C) in non pregnant rats under standard diet (Control SD+SD) versus 21 days pregnant rats under standard diet before and during pregnancy (21 days SD+SD). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

Next we studied BAT at molecular levels and we found a decrease in AMPKα2 levels but no differences were detected in FAS protein levels. As expected [334] we observed a decrease in UCP1 and β3-adrenergic interescapular BAT levels and also a tendency of UCP3 downregulation in pregnant rats. We haven't detected differences in pAMPKα, β1adrenergic receptor, CD36, LPL and PGC1α while there was a decrease in PRDM16 and an increase in pHSL/HSL BAT protein levels (Figure 23).

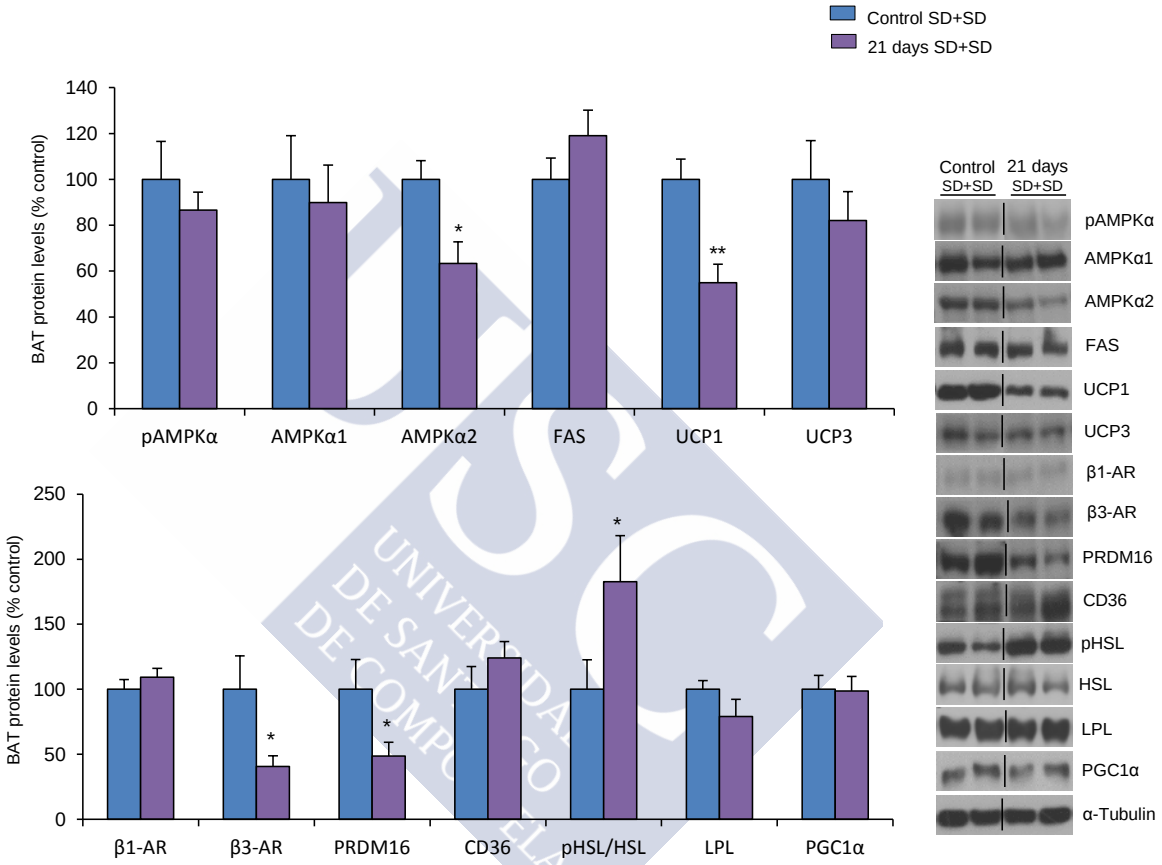

Figure 23. Western blot BAT protein levels of pAMPKα, AMPKα1, AMPKα2, FAS, UCP1, UCP3, β1-AR, β3-AR, PRDM16, CD36, pHSL/HSL, LPL, PGC1α. Non pregnant rats under standard diet (Control SD+SD) versus 21 days pregnant rats under standard diet before and during pregnancy (21 days SD+SD). Values are mean ± SEM of 8-10 animals per group. β-actin protein levels were used to normalize protein levels. Dividing lines indicate splicings within the same gel. Error bars indicate SEM. *p<0.05, **p<0.01 versus controls using one-tailed Student's t-test.

2.5 LIVER PARAMETRES IN SDHFD PREGNANT RATS

When we measured TG levels in the liver, we found an increase in pregnant rats that have consumed HFD only during pregnancy, compared with non-pregnant controls under standard diet either when we used a colorimetric assay (Figure 24A) and with oil red-O (Figure 24B).

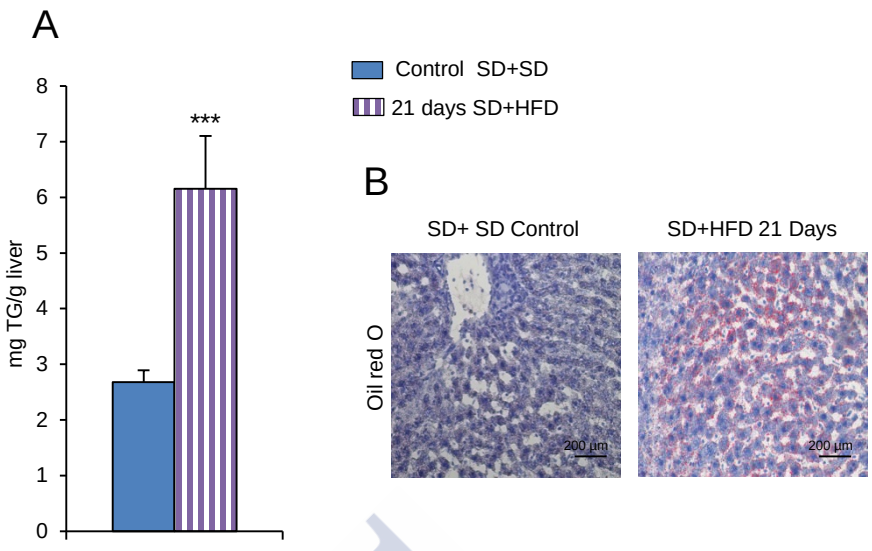

Figure 24. Liver triglyceride levels measured by a colorimetric assay (A) and represented in histological sections stained with Oil Red O (B). Non pregnant rats under standard diet (Control SD+SD) versus 21 days pregnant rats under standard diet before pregnancy and high fat diet during pregnancy (21 days SD+HFD). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. ***p<0.001 versus controls using one-tailed Student's t-test.

Regarding liver protein levels, we observed a decrease in LPL, pHSL/HSL, AMPK1α, AMPK2α, pAMPKα, ACCα, FAS, PGC1α, pIRE/IRE, CHOP and CC3; while an increase in pPERK. No differences were observed in pACCα, pJNK1/JNK1 and peIF2α/eIF2α (Figure 25A and B).

Finally, we observed again a decrease of XBP1 levels as happened in SD+SD pregnant rats (Figure 25A and B).

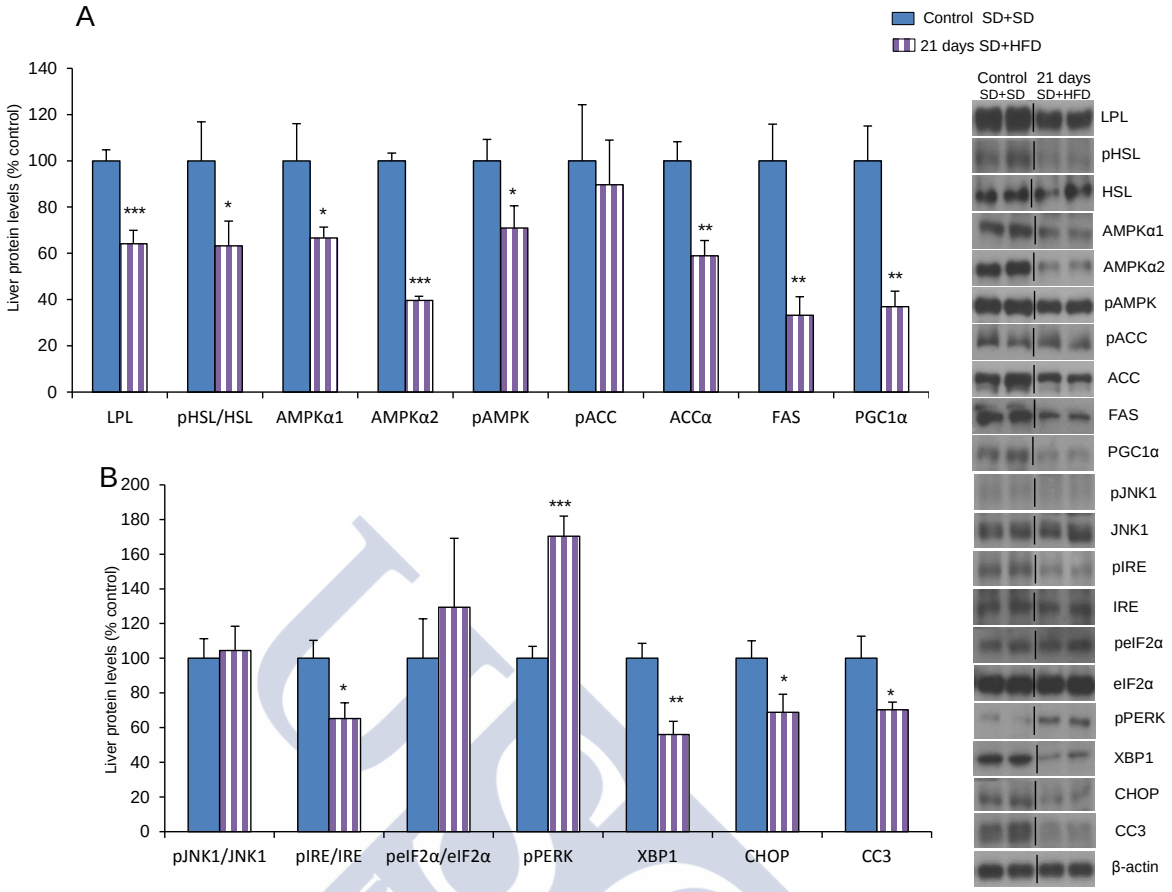

Figure 25. Western blot liver protein levels. A) Lipid metabolism proteins: LPL, pHSL, HSL, AMPKα1, AMPKα2, pAMPKα, pACCα, ACCα, FAS, PGC1α. B) ER stress proteins: pJNK1, JNK1, pIRE1α, IRE1α, peIF2α, eIF2α, pPERK, XBP1, CHOP, CC3. Non pregnant rats under standard diet (Control SD+SD) versus 21 days pregnant rats under standard diet before pregnancy and high fat diet during pregnancy (21 days SD+HFD). Values are mean ± SEM of 8-10 animals per group. β-actin protein levels were used to normalize protein levels. Dividing lines indicate splicings within the same gel. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

Regarding FXR levels in the liver, we observed a significant decrease in FXR, a significant increase in both PPARα and FGF21 levels and no differences in PPARγ mRNA levels in pregnant rats compared with non-gravid rats (Figure 26).

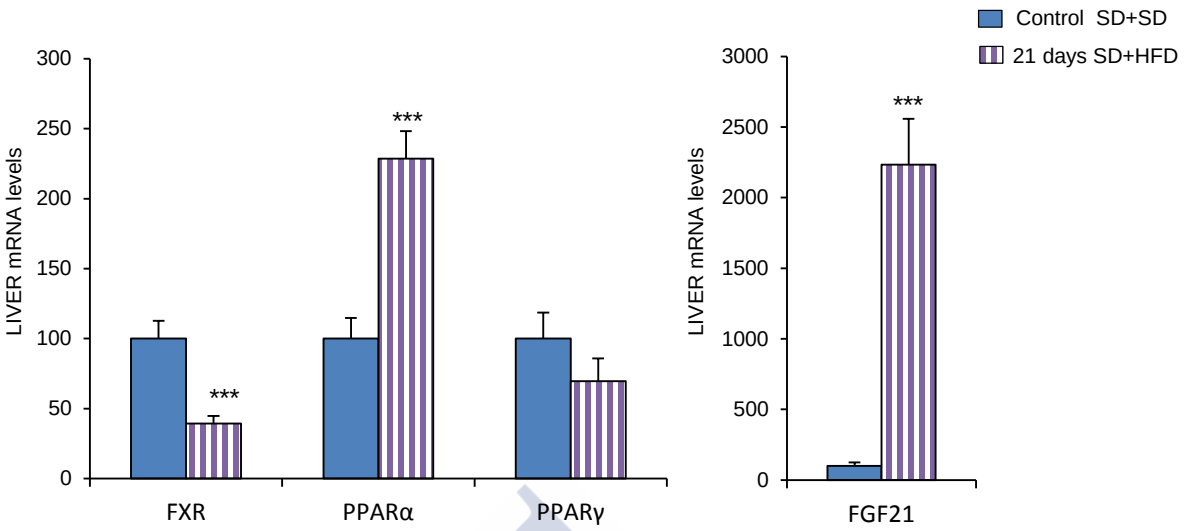

Figure 26. Liver mRNA levels of FXR, PPARα, PPARγ and FGF21. Non pregnant rats under standard diet (Control SD+SD) versus 21 days pregnant rats under standard diet before pregnancy and high fat diet during pregnancy (21 days SD+HFD). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. ***p<0.001 versus controls using one-tailed Student's t-test.

2.6 BAT PARAMETERS IN SDHFD PREGNANT RATS

Body temperature (Figure 27A) and interescapular temperature (Figure 27B and C) was decreased as it was shown before in SDSD rats despite the ingestion of HFD during pregnancy.

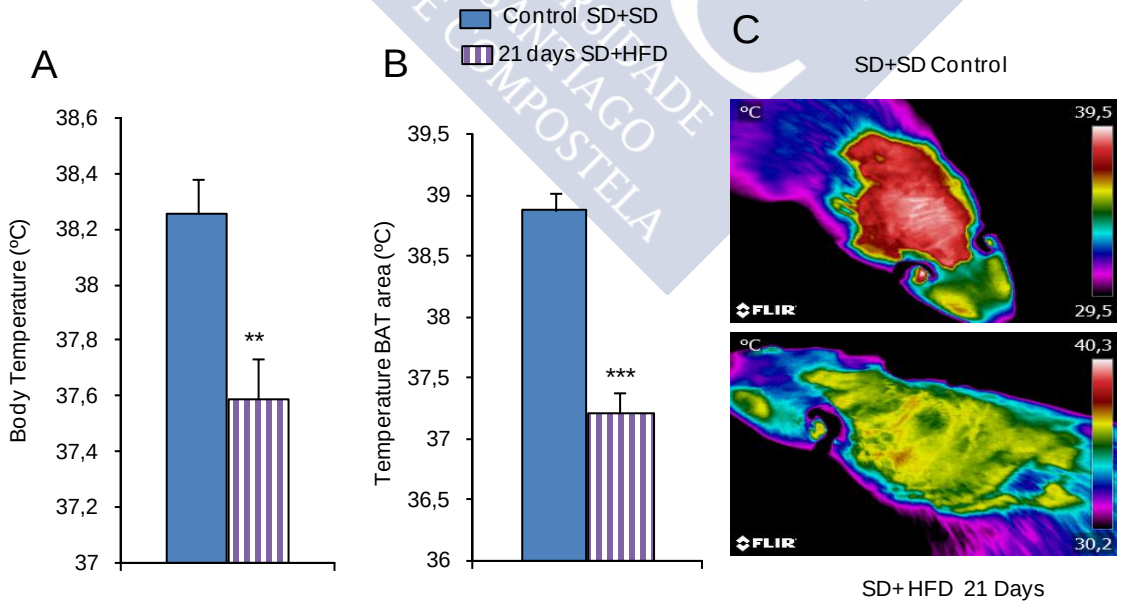

Figure 27. Body temperature (A), temperature BAT area (B) and infrared thermal images (C) in non pregnant rats under standard diet (Control SD+SD) versus 21 days pregnant rats under standard diet before pregnancy and high fat diet during pregnancy (21 days SD+HFD). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

When we measured BAT protein levels, we observed a decrease in pAMPKα, AMPKα1, AMPKα2, UCP1, UCP3, PRDM16 and PGC1α, while an increase in FAS and β3-AR. Finally, we haven't observed differences in β1-AR, CD36 and LPL. Interestingly pHSL/HSL levels were upregulated as we observed in SD+SD pregnant rats (Figure 28).

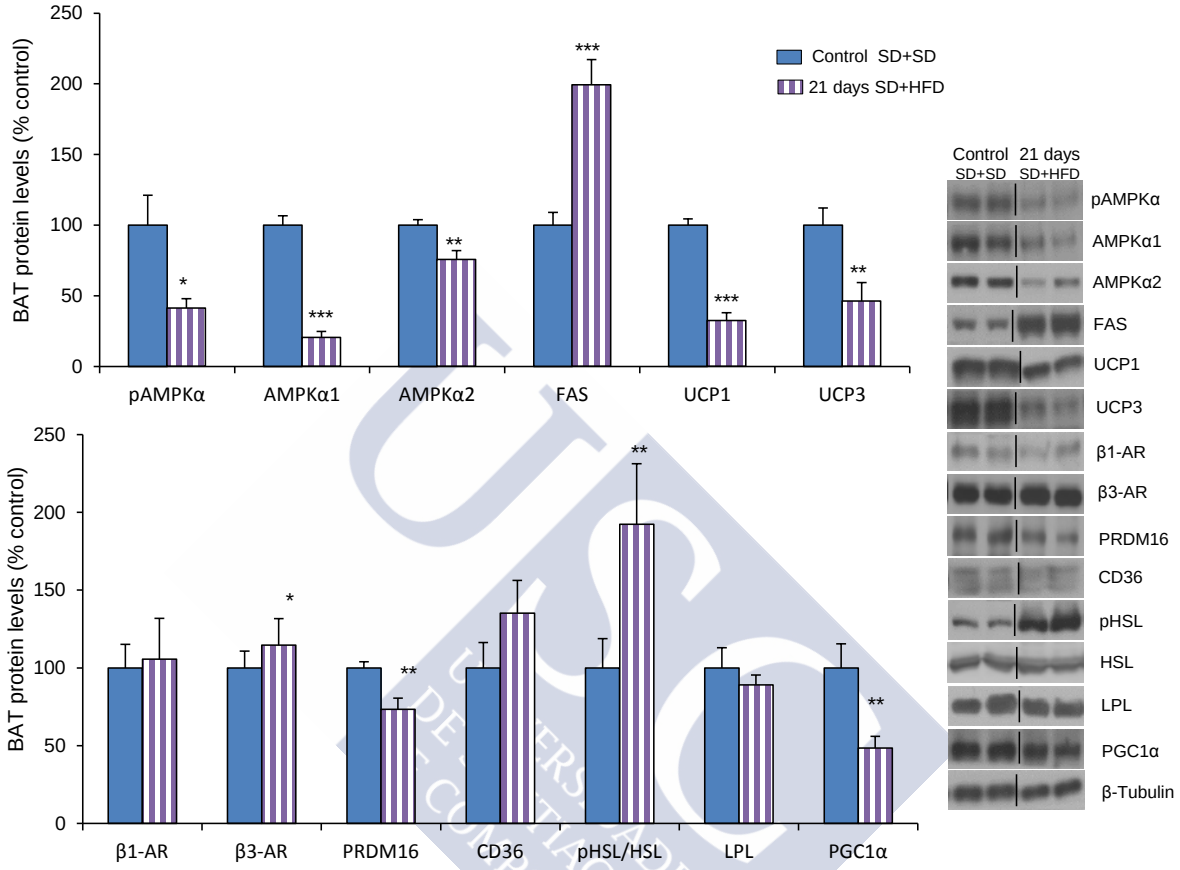

Figure 28. Western blot BAT protein levels of pAMPKα, AMPKα1, AMPKα2, FAS, UCP1, UCP3, β1-AR, β3-AR, PRDM16, CD36, pHSL/HSL, LPL, PGC1α. Non pregnant rats under standard diet (Control SD+SD) versus 21 days pregnant rats under standard diet before pregnancy and high fat diet during pregnancy (21 days SD+HFD). Values are mean ± SEM of 8-10 animals per group. β-actin protein levels were used to normalize protein levels. Dividing lines indicate splicings within the same gel. Error bars indicate SEM. *p<0.05, **p<0.01 versus controls using one-tailed Student's t-test.

2.7 LIVER PARAMETERS IN HFDHFD PREGNANT RATS

TG levels in the liver of pregnant rats under high fat diet before and during pregnancy were upregulated as we can see in the colorimetric measurement (Figure 33A) and Oil red O microscope picture (Figure 29B).

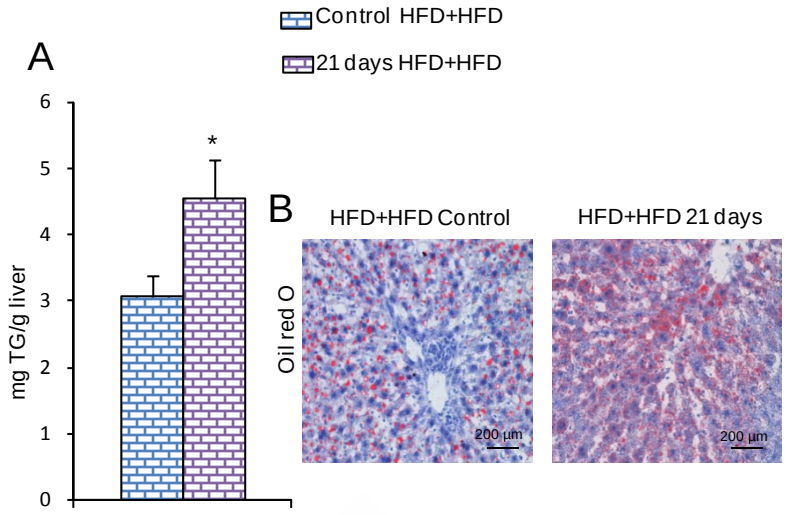

Figure 29. Liver triglyceride levels measured by a colorimetric assay (A) and represented in histological sections stained with Oil Red O (B). Non pregnant rats under high fat diet (Control HFD+HFD) versus 21 days pregnant rats under high fat diet before and during pregnancy (21 days HFD+HFD). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. ***p<0.001 versus controls using one-tailed Student's t-test.

Regarding levels of proteins implicated in lipid metabolism in the liver (Figure 30A), we observed a significant decrease in LPL with a tendency to decrease in AMPKα2. An increase in pHSL/HSL, pAMPKα, pACCα and FAS and no differences in AMPKα1, ACCα or PGC1α.

In respect to levels of proteins implicated in ER stress, we observed an increase in CHOP and C3C and no difference in pJNK1/JNK1, peIF2α/eIF2α and pPERK (Figure 30B).

Finally, we observed again a decrease of XBP1 levels as happened in SD+SD and SD+HFD pregnant rats (Figure 30 A and B).

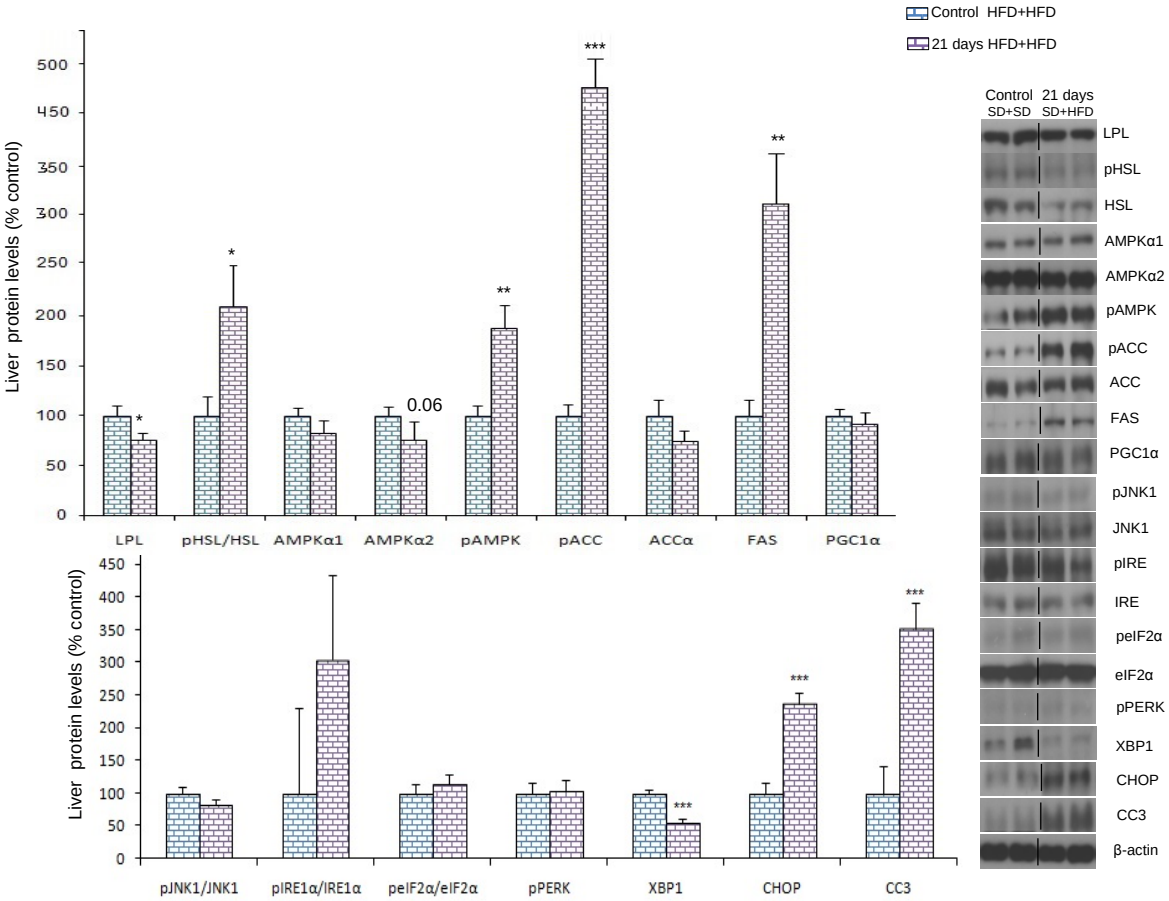

Figure 30. Western blot liver protein levels. A) Lipid metabolism proteins: LPL, pHSL, HSL, AMPKα1, AMPKα2, pAMPKα, pACCα, ACCα FAS, PGC1α. B) ER stress proteins: pJNK1, JNK1, pIRE1α, IRE1α, peIF2α, eIF2α, pPERK, XBP1, CHOP, CC3. Non pregnant rats under high fat diet (Control HFD+HFD) versus 21 days pregnant rats under high fat diet before and during pregnancy (21 days HFD+HFD). β-actin protein levels were used to normalize protein levels. Dividing lines indicate splicings within the same gel. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

When we measured mRNA hepatic levels, we found a decrease in FXR and PPARγ, and an increase in FGF21 while we haven't detected differences in PPARα mRNA levels as we can see in figure 31.

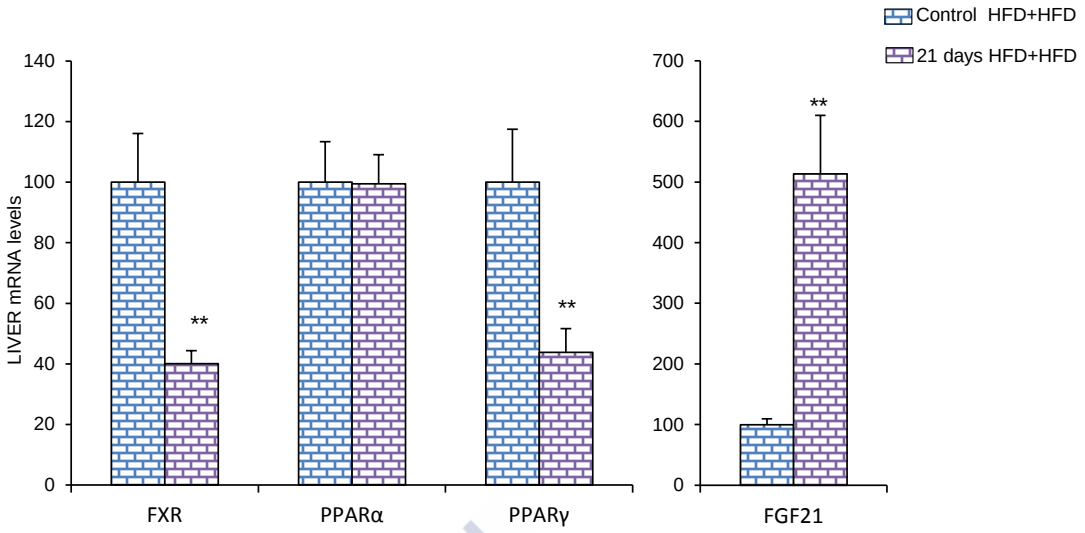

Figure 31. Liver mRNA levels of FXR, PPARα, PPARγ and FGF21. Non pregnant rats under high fat diet (Control HFD+HFD) versus 21 days pregnant rats under high fat diet before and during pregnancy (21 days HFD+HFD). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. *p<0.05, **p<0.01 versus controls using one-tailed Student's t-test.

2.8 BAT PARAMETERS IN HFDHFD PREGNANT RATS

When we measured both body temperature (Figure 32A) and interescapular temperature (Figure 36B and C) with an infrared camera, we observed a decrease in both in pregnant rats as what happened in SDSD and SDHFD pregnant rats.

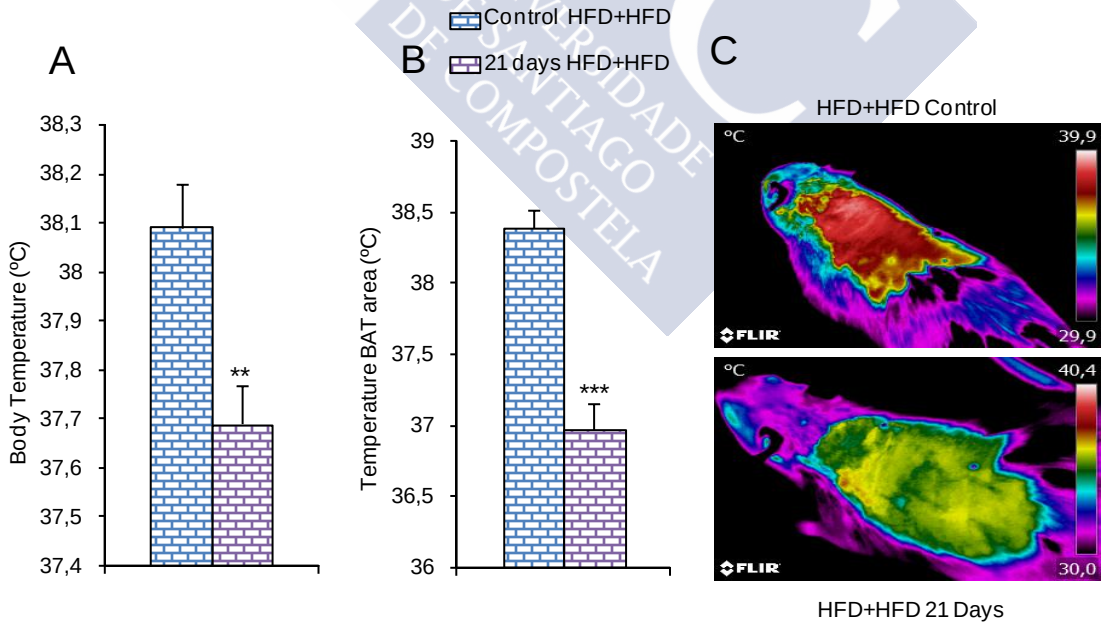

Figure 32. Body temperature (A), temperature BAT area (B) and infrared thermal images (C) in non pregnant rats under high fat diet (Control HFD+HFD) versus 21 days pregnant rats under high fat diet before and during pregnancy (21 days HFD+HFD). Values are mean ± SEM of 8-10 animals per group. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001 versus controls using one-tailed Student's t-test.

With regard to BAT protein levels measurement, we observed an increase in pAMPKα, UCP3 while a decrease in β1-AR and LPL. We haven't found any significant differences in AMPKα1, AMPKα2, FAS, UCP1, β3-AR, PRDM16, FGF21, CD36 and PGC1α. Interestingly we found again an increase in pHSL/HSL levels as we observed in SD+SD and SD+HFD pregnant rats (Figure 33).

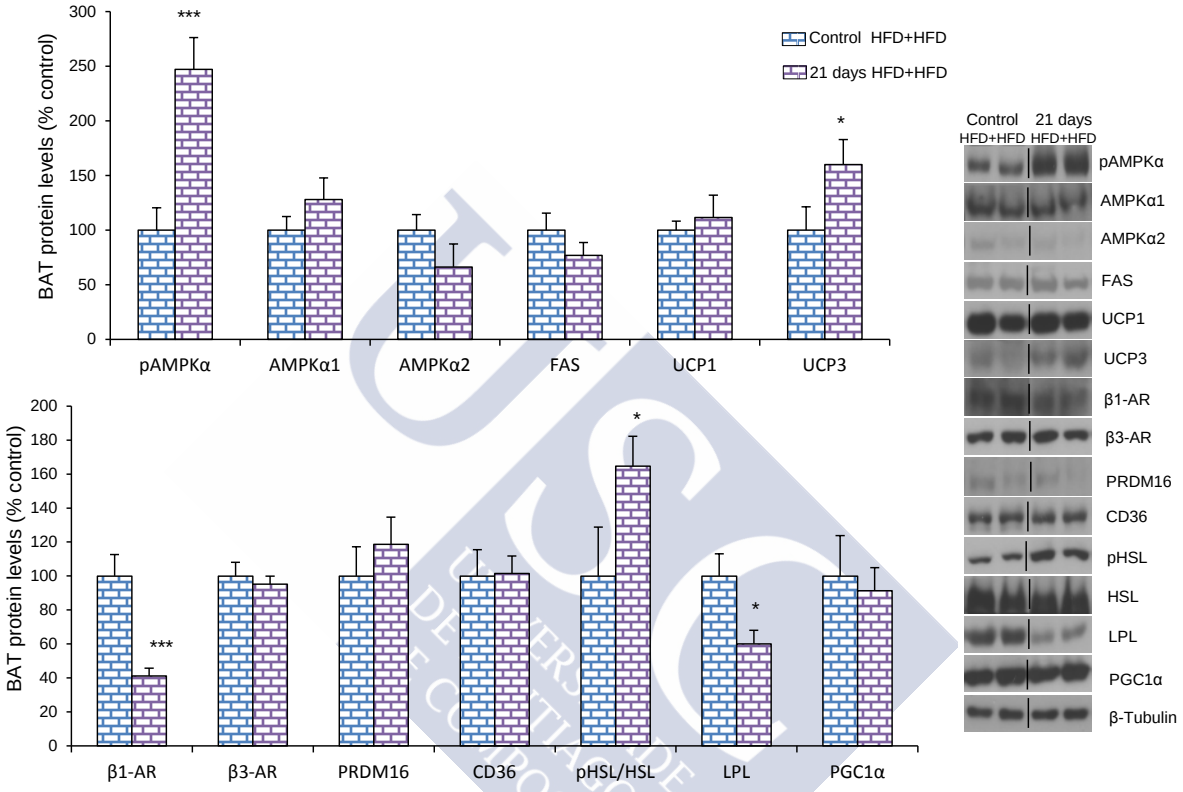

Figure 33. Western blot BAT protein levels of pAMPKα, AMPKα1, AMPKα2, FAS, UCP1, UCP3, β1-AR, β3-AR, PRDM16, CD36, pHSL/HSL, LPL and PGC1α. Non pregnant rats under high fat diet (Control HFD+HFD) versus 21 days pregnant rats under high fat diet before and during pregnancy (21 days HFD+HFD). Values are mean ± SEM of 8-10 animals per group. β-actin protein levels were used to normalize protein levels. Dividing lines indicate splicings within the same gel. Error bars indicate SEM. *p<0.05, **p<0.01 versus controls using one-tailed Student's t-test.

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DISCUSSION

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1 CHAPTER 1: The role of KLF4 in leptin signaling pathway

As is stated in our publication Imbernon, Sanchez-Rebordelo et al 2014 [308] we wanted to test if KLF4 was involved in the regulation of leptin since this hormone is known for modulate the intrinsic excitability of AgRP/NPY neurons in the hypothalamus [131]. The study of this hypothesis, establishes the relevance of KLF4 as an *in vivo* transcription factor involved in energy homeostasis. More specifically, two lines of evidence support this conclusion: first, hypothalamic KLF4 is regulated by nutritional status and by leptin in a FoxO1-independent manner; and second, viral-mediated over-expression of KLF4 triggers AgRP levels and blunts the anorectic action of leptin in lean rats. The importance of AgRP neurons in the control of energy balance has lead to several investigations of their mechanisms of action, projections to and from other neural populations, and the signaling pathways that modulate AgRP expression [419]. The transcription factor KLF4 is one of the most recently discovered activators of AgRP. *In vitro* studies involving over-expression or silencing of KLF4 have shown that KLF4 is required for activation of AgRP [35, 420]. *In vivo*, a compound named PMI-5011 has also been found to activate both KLF4 and AgRP expression; however, PMI-5011 also activated hypothalamic orexin and MCH, stimulating food intake by that route [35]. Two important issues have remained unanswered: first, in which cell types KLF4 is located within the hypothalamus and, second, whether specific manipulation of KLF4 *in vivo* is sufficient to control AgRP activity and AgRP-stimulated food intake, nutrient partitioning and body weight [34, 75, 421]. We now report that KLF4 is predominantly located in the ARC and, within this hypothalamic site, is localized in AgRP neurons. Consistent with its previously reported regulation of AgRP expression [422], our data also show that KLF4 protein levels in the ARC are increased after fasting and down-regulated by leptin [308].

To further test the capacity of KLF4 to modulate AgRP *in vivo*, we injected viral vectors that over-express KLF4 into the ARC, and found that animals carrying these viruses ate more and gained more weight than their controls, but that neither energy expenditure nor respiratory quotient changed significantly. This KLF4-induced food intake could only be explained by increased levels of AgRP, because levels of the other known relevant neuropeptides in the ARC, namely NPY, CART or POMC, were unaffected by KLF4 over-expression. Interestingly, we found that the levels of the GABA synthesizing enzymes GAD65 and GAD67 as well as the vesicular GABA transporter (VGAT) were decreased in the ARC after over-expression of KLF4. Since mice lacking vesicular GABA transporter in AgRP neurons are lean and resistant to obesity [423], we initially expected that the over-expression of KLF4 would increase the synthesis and/or transport of GABA. However, the decreased levels of GAD65, GAD67 and VGAT observed in the ARC after over-expression of KLF4 suggest that: a) KLF4 actions on feeding and body weight do not involve GABA signaling; and b) the decrease in synthesis and transport of GABA following over-expression of KLF4 might be due to a compensatory response. In addition, administering viral vectors that silenced KLF4 in the ARC did not elicit differences in either food intake or body weight, and the fasting-induced response seen in control animals was blunted. Moreover, this impaired response to fasting was specifically mediated by AgRP, since the silencing of KLF4 caused a significant decrease in fasting-induced hypothalamic AgRP levels. Importantly, fasting-induced food intake was compromised in both lean and DIO rats, indicating that KLF4 is an important modulator of the normal fasting response, independent of the type of diet.

Taken together, these new data that we have described in our paper Imbernon, Sanchez-Rebordelo et al 2014 [308] support previous *in vitro* results [35, 309] and indicate that KLF4 is a specific activator of AgRP neurons that modulates its biological actions *in vivo*.

AgRP neurons were previously known to play a critical role in mediating the actions of leptin [424]. It was also thought that increased levels of hypothalamic KLF4 induced by fasting, a hypoleptinemic state, were probably regulated by leptin levels. Specifically, the inhibition of KLF4 caused by leptin was known to be mediated by both STAT3 and PI3K, two key modulators of its anorectic action [132]. Those data thus indicated that KLF4 was part of the leptin signaling pathway, and that it was situated downstream of STAT3 and PI3K. In order to investigate the functional role of KLF4 in leptin's action, we therefore challenged leptin in rats injected either with an empty virus or with a viral vector over-expressing KLF4, into the ARC. Our results, discused in our publication Imbernon, Sanchez-Rebordelo et al 2014 [308], indicate that activation of KLF4 in the ARC is sufficient to abrogate the anorectic effect of leptin in lean rats, and therefore that KLF4 situated in the ARC is an essential component of the leptin signaling pathway that controls food intake. Since FoxO1 mediates AgRP-dependent effects of leptin on food intake [42] we hypothesized that KLF4 might be interacting with FoxO1. However, leptin was still able to increase FoxO1 in the ARC of rats injected with the viral vector over-expressing KLF4, and also able to stimulate KLF4 when FoxO1 was inhibited in the ARC. Thus, our results collectively indicate that KLF4 is not a direct target of FoxO1, and suggest that each transcription factor functions independently to modulate leptin sensitivity (Diagram 19).

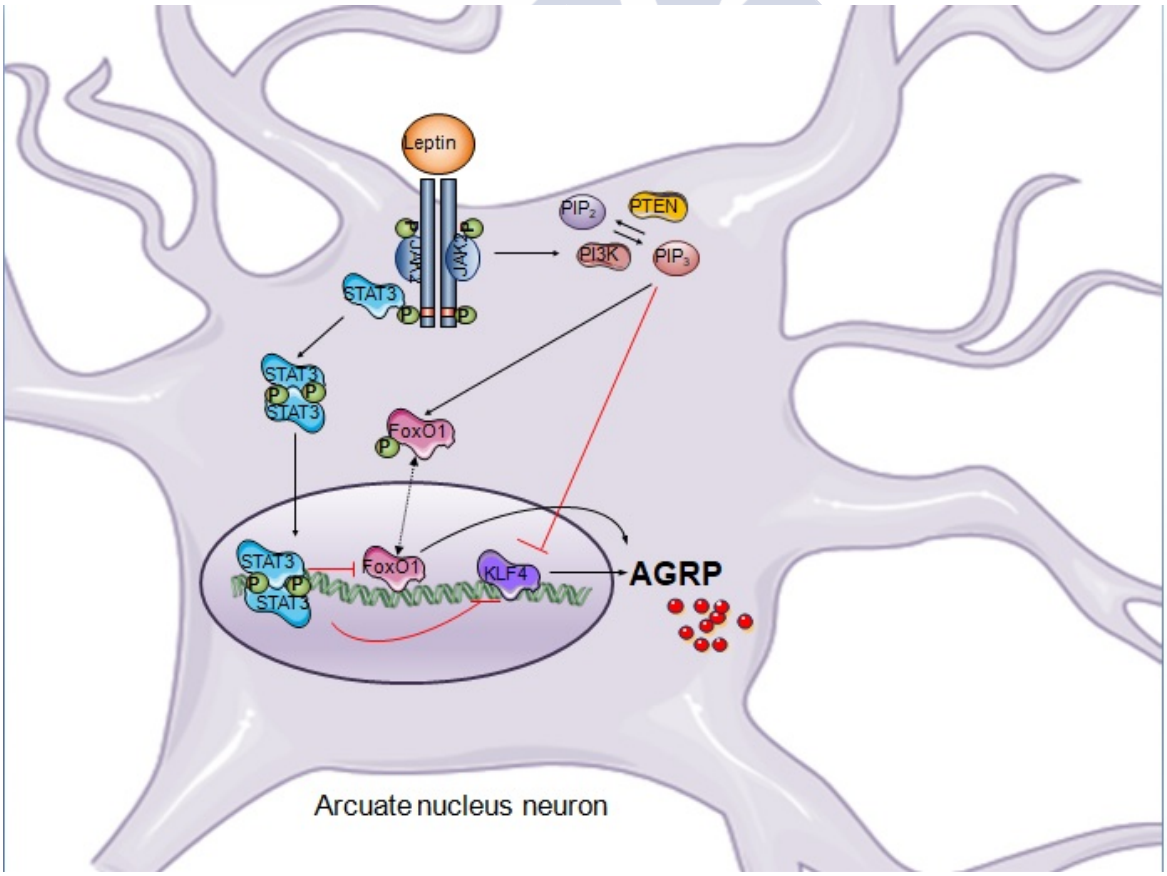

Diagram 19. Schematic overview of how KLF4 is regulated by leptin through both STAT3 and PI3K signaling pathways in a FoxO1 independent manner.

CNS resistance to leptin is likely to be an early contributor to the weight gain associated with DIO [425], and decreased leptin signaling has been demonstrated in DIO rodents [134, 426]. We therefore hypothesized that decreasing KLF4 in the ARC of DIO rats would be able to reverse HFD-induced leptin resistance. Consistent with that hypothesis, we found that peripherally administered leptin failed to decrease hypothalamic KLF4 levels in DIO rats, and this could explain the lack of effect that leptin had on feeding behaviour in DIO rats. Unexpectedly, the leptin was not able to reduce the food intake in rats treated with a viral vector silencing KLF4 in the ARC. These data published in our paper Imbernon, Sanchez-Rebordelo et al 2014 [308] shows that KLF4 does not ameliorate HFD-induced peripheral leptin resistance. The potential explanations for this effect might be that leptin resistance is mediated by a variety of factors including: a) alterations in leptin transport across the blood brain barrier; b) alterations in leptin receptor gene expression, and endocytosis and trafficking of ligand-activated cell surface receptors; and c) alterations in the leptin signaling pathway [427, 428]. Therefore, we cannot entirely rule out the possibility that KLF4 may be able to restore some of the components, albeit not all, involved in HFD-induced leptin resistance and that still represents a potential target to restore neuronal leptin sensitivity.

Another hypothesis leads us to investigate if KLF4 was involved in the ghrelin pathway in the hypothalamus since *in vitro* studies showed that KLF4 can directly control AgRP promoter activity [35] and ghrelin is known for increasing AgRP levels and body weight [429]. Using ghrelin via ICV, we failed to detect any differences in KLF4, PPARγ (an activator of KLF4 [430]) or RXRα (which forms an heterodimer with PPARγ [431]) in protein levels in the hypothalamus. Thus we concluded that KLF4 is not taking part on ghrelin signaling in the hypothalamus [308].

In sum this study, published in Molecular Metabolism in 2014 [308], established the relevance of KLF4 as an *in vivo* transcription factor involved in energy homeostasis. This role is due to being predominantly expressed in AgRP neurons. Interestingly we showed that this transcription factor is an essential component of the leptin signaling pathway although it is not a direct target of FoxO1. Contrary to our hypothesis we failed to document an amelioration of HFD-induced leptin resistance by targeting KLF4.

2 CHAPTER 2: The effect of diet before and during pregnancy

Pregnancy is a unique metabolic state in which each organ of the mother adapts metabolically to the demands of the fetus. With this study, we wanted to characterize the impact of diet before and during pregnancy on the metabolic pathways of the mother. In this discussion I have named SDSD the rats that have obtained a standard diet before and during pregnancy where the controls are rats not pregnant under standard diet, SDHFD to rats that have been fed a normal diet before pregnancy and diet high in fat during pregnancy where the controls are non pregnant rats under standard diet and finally with HFDHFD I refer to rats that were fed high-fat diet before and during pregnancy with the non pregnant controls being fed on a high-fat diet.

Regarding our first experimental group where pregnant rats ingested standard diet before and during pregnancy, we observed a decrease in serum glucose levels in pregnant rats as is assessed [432] and also observed that this decrease is independent of the type of diet since is decreased in SDHFD and HFDHFD pregnant rats too, because glucose is the main fuel for the growing fetus and their energetic needs are very high at the end of gestation. Moreover, hepatic glucose production is not altered in pregnancy [432].

Unexpectedly we failed to detect cholesterol increased levels in pregnant rats at term as described in literature in rats under standard diet [433] although other study showed that the increase in cholesterol in serum is less pronounced than that of the TGs [434]. However, we did find an increase in serum cholesterol levels in the group of pregnant rats SDHFD and HFDFHD; the present results are in agreement with the observations of Wang *et al.* [435], regarding that the high-fat diet increases serum cholesterol levels.

Measurement of pregnant TG levels in serum result in an increase in pregnant rats as expected in SDSD because the liver increases the synthesis of TG [436], and is not affected by diet as serum TG levels are increased also in SDHFD and HFDHFD pregnant rats. This is also probably a result of increased adipose tissue lipolysis as a consequence of insulin resistance. Triglycerides are used for maternal metabolic needs while saving glucose for the fetus since it is its main fuel.

Under normal circumstances, the FFAs contribute to the hepatic triglyceride reservoir [437]. This flexibility in the lipid storage, allows to neutralize the toxicity generated by the FFAs coming from the lipolysis of the white adipose tissue [438]. This make sense with the fact that SDSD pregnant rats have no differences when compared with controls, in serum FFA levels and hepatic TG levels. Also FFA data in SDHFD and HFDHFD is in agreement with TG data since its levels increases in both diets.

We observed an increase in liver weight of pregnant rats in the three experimental groups fed with different diets. Nevertheless, the levels of triglycerides in the SDSD pregnant rat livers did not differ with the levels in non-pregnant control rats as expected [439], and they are increased in SDHFD and HFDHFD pregnant livers as a result of high fat diet consumption.

FGF21 plays an important role in energy homeostasis: exogenous FGF21 leads to weight loss by increasing energy expenditure. Serum FGF21 levels increase in fasting or

consumption of a high fat diet, both conditions associated with increased lipid utilization, suggesting that it promotes the switch from glucose to lipid metabolism. Furthermore, in obesity it is described an increase in serum FGF21 as a result of increased fatty liver. Interestingly, obesity is a FGF21-resistant state since it responds poorly to the beneficial effects of exogenous FGF21 infusion [208, 440, 441]. As the hepatic production of FGF21 increases in late pregnancy in the mouse [442], we wanted to check if the same thing happened in the rat and for this we measured the serum levels of FGF21 and we observed a significant increase of them in each of the 3 groups under study; being the higher levels observed in the HFDHFD group. This increase could be due to liver production with little or no contribution from other known expressing tissues as happened in mice. In mouse plasma, FGF21 levels returned to virgin levels upon lactation indicating that the rise in FGF21 may be due as a consequence of maternal nutritional insufficiency. In this sense, it has been seen that FGF21 is induced by endoplasmic reticulum stress specifically by PERK/eIF2α signaling cascade [443]. This statement is refuted by our results since we observed an increase in both protein levels in SDSD pregnant rats' liver. Notwithstanding in SDHFD pregnant rats we still see an increase in pPERK levels in the liver, but not in peIF2a/eIF2a levels. With respect to HFDHFD pregnant rats, there are no significant differences in neither of the two proteins. These results lead us to consider that the increase of serum FGF21 observed in all study groups is not only directed by the signaling pathway PERK/eIF2a when rats are under diet high in fat, but necessarily other factors are involved.

Caspase 3 is one of the key executioners of apoptosis [444], which is related also with ER stress. Cleaved caspase 3 hepatic levels were increased in SDSD and HFDHFD pregnant rats but not in SDHFD pregnant rats. Surprisingly we detected a decrease in CHOP levels, a molecule involved in ER stress-induced apoptosis [445], in SDSD and SDHFD pregnant rats and an increase when we compared non pregnant rats under HFD with HFDHFD pregnant rats. IRE1α is a endoplasmic reticulum membrane resident stress sensor [446] and XBP1 is a key factor in the UPR and essential for hepatocyte differentiation [447]. In addition, both are important factors to achieve optimal insulin secretion and glucose control [448]. Taking into account that pregnancy is an insulin-resistant state, we observed a decrease in XBP1 liver protein levels and no differences regarding pIRE1α/IRE1α levels in SDSD pregnant rats while both protein levels were decreased in SDHFD pregnant rats and only XBP1 was decreased in HFDHFD pregnant rats. JNK1 is a member of MAPK proteins that has opposing roles depending on the stimulus, cell type or temporal aspects. In this sense it's been implicated in the induction of apoptosis and also in enhancing cell survival [449]. In our study, we haven't detected any differences between pregnant and non pregnant rats in any of the three groups under study.

FGF21 is a direct target of PPARs since PPARα and PPARγ agonists rise mRNA FGF21 levels in the liver [206]. FXR is a ligand-activated transcription factor that regulates fatty acid metabolism and its agonist, the obeticholic acid, reduces hepatic inflammation and fibrosis [450]. Furthermore FXR ligands regulate PPARγ gene expression [417]. We decided to measure PPARα mRNA levels and we found an increase of mRNA levels in the liver of pregnant rats which make sense with the increase in FGF21 serum and liver mRNA levels in SDSD and SDHFD pregnant rats. However these levels were not upregulated in HFDHFD pregnant rats although FGF21 liver mRNA and serum levels were increased in this rats. Regarding PPARγ there were no differences in mRNA levels in the liver of SDSD and SDHFD pregnant rats and a decrease in HFDHFD pregnant rats, showing that PPARγ is not involved in the increase of FGF21 serum levels. It is also known that natural and synthetic

activators of FXR increases FGF21 gene expression and secretion [202]. In this regard, we found a decrease in FXR mRNA levels in the liver in all of the three groups. This, together with the fact that FGF21 induces BAT activation [451] and that we found that BAT and body temperature of pregnant rats is downregulated, lead us to hypothesized about a FGF21 resistance in pregnancy.

PGC1α coactivates FXR to enhance FXR-target gene promoter activity and both PGC1α and FXR protein and mRNA levels were downregulated in the liver of SDSD and SDHFD pregnant rats compared with non-gravid rats while there were no differences in HFDHFD pregnant rats. Our results showed a decrease in XBP1 in pregnant rats regardless of the diet they took before and during pregnancy, while there is no increase in hepatic TG levels exclusively in the SDSD group. This makes us hypothesize a possible protection of the mother from the accumulation of fat in the liver, which loses effectiveness when the mother is fed a high-fat diet.

AMPK is a phylogenetically conserved fuel-sensing enzyme which plays a key role in lipid metabolism, being the fatty acid synthesis pathway one of the best characterized targets for AMPK in the liver and hypothalamus [452]. Our western blot analysis showed that pregnancy decreases the levels of pAMPKα and a tendency to decrease in its downstream target, pACCα, as well as a decrease in AMPKα2 and FAS in the liver of SDSD pregnant rats, just as it was described that it occurs in the hypothalamus of pregnant rats under normal diet [334].

Regarding to pregnant rats that ate a normal diet before pregnancy and a high fat diet during pregnancy, there is still a decrease in the levels of pAMPKα, AMPKa2 and FAS but not that of pACCα, although an increase in ACCα was observed when compared with non pregnant rats under normal diet. In the case of HFDHFD pregnant rats, there was an increase in the levels of pACCα, pAMPKα and FAS with which we can conclude that the diet is influencing the signaling cascade of AMPK in the liver of pregnant rats. Also these results suggest that *de novo* lipogenesis is activated in SDSD and SDHFD pregnant rats surely to increase mother fat depots [453, 454]. Regarding the HFDHFD group, *de novo* lipogenesis markers are downregulated and epididymal and omental fat content does not differ from HFDHFD non pregnant rats.

LPL is present in the vascular bed of extrahepatic tissues [339]. Although in normal conditions the LPL liver levels are low, there is a significant increase in its activity in the liver of patients with morbid obesity and non-alcoholic steatosis [455]. On day 21 of gestation there is a decrease in fat accumulation [456] together with a decrease LPL activity in the adipose tissue and a decline in hepatic lipase activity caused by the insulin-resistant condition of pregnancy [339]. This is in line with our results since we have not detected differences in LPL hepatic protein levels in SDSD pregnant rats and when we look at the levels of LPL in SDHFD and HFDHFD pregnant rats, we observed a decrease. In this regard it is noteworthy that pHSL/HSL protein ratio levels were decreased in the liver of SDSD pregnant rats, meaning that there is a decrease in the mobilization and β-oxidation of triglycerides in the liver, since there is not such accumulation of triglycerides. When we looked at pHSL/HSL ratio protein levels in the liver of SDHFD pregnant rats, we saw that there is a decrease as happened in the SDSD group. However, regarding pHSL/HSL protein levels in HFDHFD pregnant rats, we observed an increase probably due to the higher accumulation of fats in this group.

As previously described [457], we did not find significant differences between the BAT weights of pregnant rats and non-pregnant in SDSD and we found that this is regardless of the type of diet that rats receive before and during pregnancy. The fact that there is no difference in the iBAT weight data could be controversial since BAT is inactivated in pregnant rats, but we have to take into account that the protein and RNA content of iBAT pads is lower in pregnant rats than in non pregnant [458] despite having the same weight.

We observed a decrease in interescapular and body temperature, decrease in UCP1 and β3 adrenergic protein levels in SDSD pregnant BAT as it was described [334]. In SDHFD pregnant rats UCP1 is downregulated as well. In HFDHFD pregnant rats nor UCP1 neither β3 adrenergic BAT protein levels were modified compared with controls, although the temperature in the BAT is still diminished, implying that other mechanisms may be modulating BAT activity in pregnant rats that take a high fat diet before and during pregnancy. We have to take also into account that the intake of HFD upregulates UCP1 BAT levels in males but not in females [459]. It has been described that sympathetic stimulation of BAT thermogenesis in response to HFD is mediated through β1adrenergic receptors [460] and this could be the reason why it's diminished in HFDHFD rats while the levels of UCP1 are unchanged despite the decrease in interscapular temperature. In view of this results, we can conclude that the decrease in BAT temperature in pregnancy is independent of the intake of a high fat diet. PRDM16 is a zinc-finger protein that activates brown fat cell identity, causing a 85% reduction in UCP1 when is depleted from brown fat cells [461]. In this sense, we observed a correlation between PRDM16 and UCP1 BAT protein levels in all groups despite the type of diet.

Levels of UCP1 were addressed to be correlated with levels of UCP3 in BAT [462]. Furthermore in 2005 Frontera *et al.* described that at mid-pregnancy, UCP1 mRNA levels were not modified in BAT while UCP3 was highly increased [463], meaning that UCP1 and UCP3 levels are not always correlated in pregnancy being able to perform different function than thermogenesis in BAT. UCP3 is upregulated when the flux of fatty acids is high (during starvation) [13] and it is been suggested that it could be involved in the regulation of lipids as fuel substrate as well as in thermogenesis in BAT [464]. The increase of UCP3 levels in pregnant rats under HFD before and during pregnancy, could be due to an increase in fatty acid utilization by the BAT as a result of the impossibility of achieving glucose as substrate since is been used by the fetus.

PGC1α is induced by cold in brown fat and acts as a master regulator of mitochondrial biogenesis and oxidative metabolism. PGC1α also induces UCP1 expression being essential for thermogenic activation induced by cold or β-adrenergic agonists [465]. In this regard, there weren't differences in PGC1α protein levels in SDSD and HFDHFD pregnant rats, while we found a decrease in SDHFD meaning that there is a dysregulation of PGC1α in pregnancy. CD36 is a lipoprotein receptor [466] which translocation in rat cardiac myocytes is also mediated through AMPK [467]. It has also been demonstrated that exposure to cold increases BAT activity and also triglyceride uptake mediated by an increase in LPL and CD36 leading to an increase in TG plasma clearance [468]. We found no difference between non gravid and 21 days pregnant rats in standard diet regarding LPL protein levels, and the same result was observed in mRNA levels in a Giralt *et al.* study, whereas enzyme activity was lowered [469].With BAT activity downregulated in pregnant rats, we expected no differences in LPL and CD36 levels in BAT and an increase in TG serum levels as we finally observed in

all the pregnant groups, with the exception of the HFDHFD group where LPL levels were decreased.

In our data there is no difference in pAMPKα protein levels in the BAT of SDSD pregnant rats and ther is a decrease in pAMPKα levels in SDHFD pregnant rats, since BAT is inactivated. Concerning HFDHFD pregnant rats, we observed an increase in pAMPKα in BAT as we observed in liver, so it seems that diet is also modulating AMPK signaling in BAT. Regarding FAS levels in SDSD and HFDHFD, they are not modified while they are decreased in SDHFD. HSL is a key effector of β3-adrenergic signaling being its activity fundamental for fatty acid oxidation in BAT [470]. Taking this into account, we expected a decrease in pHSL/HSL levels in the BAT of pregnant rats but surprisingly we found an increase in all the three groups of pregnancy, this may be due to a resistance in pregnancy. All this data lead us to think that there is a dysregulation of lipogenesis and lipolysis pathways in the BAT of pregnant rats making possible its inactivation in order to save energy for the fetus. Noteworthy, a dysregulation of lipid metabolism in other tissues such as the hypothalamus have been previously reported under pregnancy [334].

Late pregnancy is the most nutritionally precarious phase of the rat reproductive cycle, needing a significant mobilization of maternal reserves to meet the increasing metabolic demands from maternal, placental and fetal compartments despite the increased hyperphagia. A large number of studies regarding impact of diet in the offspring have been made, but little is known about the impact in mother's health and functioning. The objective of this study is to provide an integrated picture of expression of the various changes that occur in the last stage of pregnancy and the influence of eating a high fat diet only during pregnancy or before and during pregnancy, with respect to the health of the mother. Although our study does not provide clear evidence of the molecular mechanisms involved, it raises the need of further investigation. In particular it would be quite interesting to study the lipid flux among different tissues as WAT, liver and BAT during pregnancy using labelled precursors.

CONCLUSIONS

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1 CHAPTER 1: The role of KLF4 in leptin signaling pathway

a) The transcription factor KLF4 is predominantly expressed in AgRP´s neurons in the hypothalamus. Its over-expression in the ARC is sufficient to increase food intake and to blunt the anorectic action of leptin in a FoxO1-independent manner.

b) Hypothalamic KLF4 is an essential component of the leptin signaling pathway in the hypothalamus of rats fed a chow diet. However KLF4 does not affect HFD-induced leptin resistance.

2 CHAPTER 2: The effect of diet before and during pregnancy

a) Energy homeostasis in pregnancy is associated to marked changes in the molecular set-point at tissue level as shown here by the finding of decreased BAT and body temperature during pregnancy in order to provide energy resources to the fetus.

b) Metabolic homeostasis undertakes also many adaptive changes as shown by increased liver mass in a diet-independent manner and decreased glucose and increased triglyceride serum levels. In addition there is a pregnancy-induced increase in fat mass that can be mimicked by exposure to HFD before pregnancy.

c) The changes in FGF21 and the likelihood of a pregnancy-induced FGF resistance highlights the adaptive maternal changes in this physiological setting.

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APPENDANT 1

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	Reference	Publisher	License number
Diagram 3 and 9	13	Elsevier	4391450908864
Diagram 4	14	Elsevier	4391420220695
Diagram 7	132	Karger	4391380658816
Diagram 8	132	Karger	4438880757512
Diagram 13	220	Springer Nature	4391400708908
Diagram 17	380	Karger	4391410392404

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