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Estudo das consequências da desnutrição perinatal sobre a plasticidade energética e metabólica do músculo esquelético em termos bioquímicos e moleculares

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Recife,

2012

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	Português	Français
11β-HSD	11β-hidroesteroide desidrogenase	11β- hydro stéroïde déshydrogénase
AG	Ácido Graxo	Acide Gras
AGL	Ácido Graxo Livre	Acide Gras Libre
AgRP	Peptídeo relacionado ao Agouti	Peptide corrélé à agouti
AICAR	5-aminoimidazolo-4-carboxamido-1-β-D-ribofuranosido	5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AIG	Adequado para Idade Gestacional	Appropriée pour l'Âge Gestationnel
AMP	Adenosina monofosfato	L'adénosine monophosphate
AMPK	Proteína quinase ativada por AMP	Protéine Kinase activée par l'AMP
ATP	Adenosina trifosfato	L'adénosine triphosphate
CaMK	Proteína quinase dependente de Ca^{2+}/Calmodulina	Protéine kinase Ca^{2+}/calmoduline-dépendante
ChREBP	Proteína de ligação de elementos responsivos a carboidratos	Proteine de liaison aux éléments de réponse aux carbohydrates
CoA	Coenzima A	Coenzyme A
COXIV	Citocromo C oxidase subunidade IV	Cytochrome c oxydase sub-unité IV
CPM	Células Precursoras Musculares	Cellules précurseurs musculaire
CPT	Carnitina Palmitoil Transferase	Carnitine palmitoyltransférase
CREB	Proteína de ligação em resposta a cAMP	Protéines de liaison en réponse au cAMP
CS	Citrato Sintase	Citrate synthase
DNA / ADN	Ácido desoxirribonucleico	Acide désoxyribonucléique
EDL	Extensor Longo dos Dedos	Extenseur long des doigts
EDTA	Ácido etilenodiamino tetra-acético	Acide éthylène diamine tétraacétique
ERO / DRO	Espécie Reativa de Oxigênio	Dérivés réactifs de l'oxygène
FBPase	Frutose -1,6-bifosfatase	Fructose-1,6-bisphosphatase
FGF	Fator de crescimento de fibroblastos	Facteur de croissance des fibroblastes
FOXO1	*Forkhead box protein O1*	
G-6-Pase	Glicose-6-fosfatase	Glucose-6-phosphatase
GC	Glicocorticoides	Glucocorticoïdes
GK	Glicoquinase	Glucokinase
Glut4	Transportador de Glicose 4	Transporteur de glucose 4
GR	Receptor de Glicocorticoides	Récepteur de Glucocorticoïdes
HDAC	Histona desacetilase	Histone deacétylases
HDL-c	Lipoproteína de baixa densidade - colesterol	Lipoprotéine de haute densité - Cholestérol
HGF	Fator de crescimento de hepatócitos	Facteur de croissance des

		hépatocytes
HK	Hexoquinase	Hexokinase
IDF	Federação Internacional de Diabetes	Fédération Internationale de Diabetes
IGF	Fator de crescimento semelhante à Insulina	Facteur de croissance semblable à l'Insuline
IGF2r	Receptor do Fator de crescimento semelhante à Insulina 2	Récepteur du Facteur de croissance semblable à l'Insuline 2
IL-6	Interleucina 6	Interleukine 6
IMC	Índice de Massa Corporal	Indice de masse corporelle
IMTG	Triglicerídeos Intramusculares	Triglycérides Intramusculaires
IR	Receptor de Insulina	Récepteur de l'Insuline
IRS	Substrato de Receptor de Insulina	Substrat du Récepteur de l'Insuline
IκBα	*Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha*	
LDH	Lactato Desidrogenase	Lactate Déshydrogénase
MAPK – p38	Proteína quinase ativada por mitógeno	Protéine kinase activée par mitogène
mATPase	Miosina miofibrilar adenosina trifosfatase cálcio ativada	Myosine myofibrillaire adénosine triphosphatase activé par calcium
MCAD	Desidrogenase das acil-CoA de cadeia média	Déshydrogénase des acyl-CoA à chaîne moyenne
MEF2	Fator potenciador de miócitos 2	Facteur amplificateur de myocyte 2
miRNA	Micro RNA	Micro ARN
MLC	Cadeia Leve de Miosina	Chaîne légère de myosine
MRF/Myf	Fator de transcrição miogênica	Facteur de transcription myogénique
mRNA/mARN	RNA mensageiro	ARN messagers
mTOR	Alvo da rapamicina em mamíferos	Cible de la rapamycine chez les mammifères
MyHC	Cadeia Pesada de Miosina	Chaîne lourde de myosine
MyoD	*Myogenic differentiation 1*	
NAD+	Dinucleótido de nicotinamida e adenina oxidado	Nicotine adénine dinucléotide oxydé
NADH	Dinucleótido de nicotinamida e adenina reduzido	Nicotine adénine dinucléotide réduit
NFAT	Fator nuclear de células T ativadas	Facteur nucléaire des cellules T activés
NFκB	Fator nuclear kappa B	Facteur nucléaire kappa B
NPY	Neuropeptídeo Y	Neuropeptide Y
Pax3/Pax7	*Paired box*	
PDK	Piruvato desidrogenase quinase	Pyruvate déshydrogénase kinase
PEPCK	Fosfoenolpiruvato carboxiquinase	Phosphoénolpyruvate carboxykinase
PFK	Fosfofrutoquinase	Phosphofructokinase
PGC1α	Coativador-1 alfa do receptor gama	Coactivateur-1 alpha du récepteur

	ativado por proliferador de peroxissomo	gamma activé par les proliférateurs des peroxysomes
PI3K	Fosfatidilinositol-3-quinase	Phosphatidylinositol-3-Kinase
PIG / PAG	Pequeno para Idade Gestacional	Petit pour l'Âge Gestationnel
PKC	Proteína quinase	Protéine kinase C
POMC	Pro-opiomelanocortina	Pro-opiomélanocortine
PPAR	Receptor ativado por proliferador de peroxissomo	Récepteur activé par les proliférateurs de peroxysomes
RCIU	Retardo de Crescimento Intrauterino	Retard de la croissance intra-utérine
SC	Células Satélites	Cellules Satellites
SM	Síndrome Metabólica	Syndrome métabolique
UCP3	Proteína desacopladora 3	Protéine découplant 3
β-HAD	Beta-hidroxiacil-CoA desidrogenase	Beta-Hydroxyacyl-CoA déshydrogénase

Resumo

O ambiente nutricional pobre durante o desenvolvimento precoce pode programar o organismo para o desenvolvimento de doenças metabólicas na vida adulta. O músculo esquelético é um órgão de grande importância para o corpo em termos metabólicos, além de apresentar grande capacidade de adaptação na utilização de energia em resposta à ingestão de alimentos ou à demanda energética do organismo. Esta tese tem como objetivo analisar as repercussões, em termos bioquímicos e moleculares, da desnutrição proteica materna sobre a função metabólica e a plasticidade do músculo esquelético em ratos. Ratos Wistar-Han machos foram divididos inicialmente em dois grupos, de acordo com a dieta recebida pelas progenitoras durante a gestação e lactação: Controle (proteína a 17%) e Desnutrido (proteína a 8%). Aos 110 dias, todos os animais foram submetidos ao teste de tolerância à glicose. Aos 120 dias, foi realizado o sacrifício em duas condições nutricionais distintas: *ad libitum* ou jejum (48 horas de privação alimentar). Foram retirados os músculos sóleo e EDL para análises. Foi observado que os animais do grupo desnutrido apresentaram menor peso corporal ao desmame, o que perdurou até o sacrifício, apesar de terem apresentado maior ganho de peso pós-desmame. Não foi observada mudança na resposta à glicose entre os dois grupos. A comparação entre animais controles e desnutridos sacrificados *ad libitum* deu origem ao primeiro artigo da tese. Foram observados menores níveis de triglicerídeos no grupo desnutrido. No sóleo, além de apresentar redução na porcentagem de fibras oxidativas, também foi observada redução da atividade da hexoquinase, no grupo desnutrido. Houve aumento da expressão de PGC1α, CPT1a e UCP3, no músculo sóleo de animais desnutridos. No EDL, foi observado aumento da expressão de ChREBP e PPARδ, e diminuição de MCAD. O segundo artigo abrange o estudo da resposta adaptativa do músculo esquelético ao jejum, onde os animais controle e desnutridos foram comparados com seus pares *ad libitum*. Os animais do grupo controle apresentaram aumento de AGL em resposta ao jejum, enquanto que os desnutridos apresentaram maior perda de peso corporal. No músculo sóleo, ambos os grupos apresentaram aumento de mRNA de UCP3 e diminuição de PGC1α, PPARδ, MHCI, em resposta á privação alimentar de 48 horas. Entretanto o grupo desnutrido não apresentou aumento na expressão de CPT1a em resposta ao jejum. No EDL, ambos os grupos apresentaram aumento da expressão de CPT1a. Considerando todos os dados em conjunto, a desnutrição proteica perinatal programou o músculo esquelético modificando o metabolismo de lipídios. Além disso, os dois músculos são diferentemente afetados pela desnutrição. Ademais, estes animais apresentaram prejuízo na resposta adaptativa ao jejum de 48 horas. Estes resultados podem indicar maior propensão ao desenvolvimento de distúrbios metabólicos, visto que eles apresentam prejuízos na flexibilidade metabólica muscular.

Palavras-chaves: Programação nutricional, músculo esquelético, metabolismo, jejum.

Résumé

Il est clairement établi que la dénutrition périnatale augmente le risque de développement d'obésité et de maladies métaboliques à l'âge adulte. Cependant, les mécanismes cellulaires et moléculaires qui sous-tendent cette susceptibilité physiopathologique restent largement inconnus. L'objectif de cette thèse a été d'évaluer les conséquences d'une restriction en apports protéiques à la mère pendant la gestation et la lactation sur les propriétés structurales et métaboliques du muscle squelettique chez la descendance. Nous avons observé que les rats adultes nés et allaités par de mères nourries avec un aliment contenant un faible teneur en protéines (8%), présentent une majeur densité de fibres musculaires non oxydatives par rapport aux animaux nés de mères nourries avec un aliment standard. Ces altérations structurales s'accompagnent d'une diminution du taux d'oxydation des acides gras et de la glycolyse dans le muscle soléaire mais pas dans l'extenseur commun des orteils (EDL). La dénutrition périnatale entraîne également une modification différentielle de l'expression de gènes dans le soléaire par rapport à l'EDL et une diminution de la capacité d'adaptation métabolique en réponse au jeûne. Dans leur ensemble, ces changements correspondent à un état de déficience énergétique et sont présents chez les animaux qui ne montrent, pas encore, aucun signe obésité ou de d'intolérance au glucose. Nous concluons que la restriction an apports protéiques pendant le développement périnatal modifie sur long terme les propriétés structurales et métaboliques du muscle squelettique en fonction du type de muscle. Ces modifications pourraient avoir un rôle causal dans le développement à l'âge adulte d'une obésité et des troubles métaboliques associés.

Mots-clés: programmation métabolique, muscle squelettique, métabolisme, jeûne.

Abstract

It is clearly established that perinatal malnutrition increases the risk of developing obesity and metabolic disease in adulthood. However, the cellular and molecular mechanisms underlying this enhanced disease susceptibility remain largely unknown. The aim of this thesis was to evaluate the effects of perinatal malnutrition on the metabolic and structural properties of skeletal muscle. Adult rats born to dams fed a low-protein diet (8%), exhibited a higher density of non-oxidative muscle fibers along with decreased rate of fatty acids oxidation and glycolysis in soleus muscle but not in the extensor digitorum longus (EDL). Perinatal protein-restriction induced also a differential gene expression profile in soleus in relation to EDL and decreased metabolic flexibility in response to fasting. Taken together, these changes correspond to a state of energy deficiency and are present in animals that do not show yet any sign of obesity or glucose intolerance. We conclude that perinatal protein-restriction alters in the long term the structural and enzymatic properties of skeletal muscle in a fibre type-dependent manner. These alterations might have a causative role in the development of obesity and related metabolic disorders later in life.

Keywords: metabolic programming, skeletal muscle, metabolism, fasting.

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Sumário / Sommaire

RÉSUMÉ DE LA THÈSE EN FRANÇAIS

Présentation

1.1 Présentation

De très nombreuses études indiquent qu'il existe un lien direct entre une croissance fœtale et infantile diminuée et le risque de développer de maladies cardiovasculaires et métaboliques à l'âge adulte. Pour expliquer cette corrélation, il a été proposé que chez un organisme sous-alimenté en voie de développent, le métabolisme est modifié de telle sorte que ses chances de survie sont maximisées. Il y a tout d'abord une distribution sélective des nutriments de façon à préserver la croissance cérébrale au détriment des autres organes. Ensuite, il y a une « programmation » conduisant à des adaptations métaboliques lui permettant de survivre dans des conditions de restriction alimentaire. Cependant, si par la suite cet organisme est suralimenté ou exposé à un déséquilibre nutritionnel, l'incompatibilité entre sa programmation physiologique et les nouvelles conditions nutritionnelles génère le syndrome métabolique qui se traduit par de l'obésité, une diminution de la tolérance au glucose, de l'hypertension et un taux augmenté de triglycérides. Ces adaptations physiologiques seraient sous-tendues par des modifications épigénétiques qui « s'imprimeraient » dans le génome du fœtus ou du nouveau-né modifiant de façon permanente son métabolisme

L'hypothèse de programmation métabolique est confortée par les résultats de multiples études expérimentales. En fait, les expérimentations réalisées sur diverses espèces animales, démontrent que les individus nés des mères exposées à une diminution en apports nutritionnels au cours de la gestation et/ou de la lactation, soit en restreignant la quantité de nourriture soit en diminuant la quantité de protéines dans les aliments, présentent des altérations physiopathologiques propres du syndrome métabolique. Toutefois, les mécanismes physiologiques et moléculaires qui sous tendent le lien entre un environnement nutritionnel appauvri au cours des premières étapes du développement et le risque accru de développer de maladies métaboliques à l'âge adulte, restent largement à déterminer.

Ainsi, **les questions qui ont guidé** cette thèse ont été: 1) Quels sont les changements que la malnutrition protéique maternelle programme en termes biochimiques et moléculaires, sur le métabolisme du muscle squelettique qui peuvent sous-tendre le développement ultérieur du syndrome métabolique? ; 2) Le muscle squelettique «programmé» conserve-t-il sa flexibilité métabolique lorsqu'il est soumis à une forte demande énergétique?

L'objectif central de ce de projet de thèse, a été de définir les conséquences en termes biochimiques et moléculaires de la dénutrition périnatale sur la plasticité métabolique du muscle squelettique et de déterminer dans quelle mesure les éventuelles altérations de la physiologie musculaire induites par la dénutrition périnatales contribuent au développement d'obésité et d'insulino-résistance généralement observées chez les individus exposés à un environnement nutritionnel appauvri ou déséquilibré au cours de leur développement précoce.

Les objectifs spécifiques ont été d'analyser, chez des rats adultes de 120 jours, les conséquences de la malnutrition protéique maternelle sur:

- La masse corporelle et le profil biochimique dans le plasma;

- La proportion des différents types de fibres musculaires;

- Les propriétés enzymatiques des muscles squelettiques;

- L'expression globale des gènes associés au métabolisme lipidique et glucidique;

- La plasticité musculaire en réponse à une forte demande énergétique.

La réalisation de cette thèse a réposée sur **l'hypothèse de travail** selon laquelle la restriction en nutriments pendant la phase critique du développement périnatal altère la capacité du muscle squelettique de s'adapter à l'utilisation des substrats énergétiques (lipides, glucose), en réponse aux apports nutritionnels et les demandes énergétiques de l'organisme et que ces modifications sont dépendantes du type musculaire.

Les résultats obtenus au cours de la réalisation de cette thèse sont présentés sous forme d'articles. Ceux-ci ont été envoyés pour publication. Le premier article, intitulé « **Differential developmental programming by early protein restriction of rat skeletal muscle according to its fibre type composition** », est sous publication dans journal journal Acta Physiologica (Annexe A). Le deuxième article, intitulé « **Nutritional programming impairs metabolic flexibility of skeletal muscle before any sign of obesity or glucose intolerance**», a été soumis à Obesity (Annexe B).

En plus des articles soumis pour publication, les résultats de cette thèse ont été présentés dans des colloques nationaux et internationaux.

- **RAQUEL DA SILVA ARAGÃO**; OMAR GUZMÁN-QUEVEDO; GEORGINA PEREZ-GARCIA; RAUL MANHÃES-DE-CASTRO; FRANCISCO BOLAÑOS-JIMENEZ. PERINATAL PROTEIN RESTRICTION ALTERS IN THE LONG TERM THE METABOLIC PLASTICITY OF SKELETAL MUSCLE. 6th Congress of the International Society of Nutrigenetics/Nutrigenomics. São Paulo – SP. 2012.
- **ARAGÃO, R. S.**, Guzmán-Quevedo, O., Pérez García, G, Manhães de Castro, R, Bolaños-Jímenez, F Perinatal undernutrition impairs the metabolic plasticity of skeletal muscle, In: I Simpósio Internacional de Mitocôndria e Metabolismo, 2012, Vitória de Santo Antão.
- **ARAGÃO, R. S.,** Guzmán-Quevedo, O., Pérez García, G, Toscano, A. E., Manhães de Castro, R, BOLAÑOS-JIMENEZ, F. Protein restriction during early life induces long-lasting changes on the functional and structural properties of rat skeletal muscle In: XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental, 2012, Águas de Lindóia.
- **da Silva Aragão, R.,** Guzmán-Quevedo, O., Pérez García, G, Lopes de Souza, S, Manhães de Castro, R, Bolaños-Jímenez, F Analysis of the consequences of perinatal undernutrition on the metabolic plasticity of skeletal muscle In: 11th European Nutrition Conference (FENS), 2011, Madri. Annals of Nutrition and Metabolism. Karger, 2011. v.58. p.1 - 444

Des recherches supplémentaires non directement liées à ce travail de thèse ont été également développés en parallèle. Ces recherches ont donné lieu aux publications suivantes:

- Elizabeth Nascimento, Omar Guzman-Quevedo, Nellie Delacourt, **Raquel da Silva Aragão,** Georgina Perez-Garcia, Sandra Lopes de Souza, Raul Manhães-de-Castro, Francisco Bolaños-Jiménez, Bertrand Kaeffer. Long-Lasting Effect of Perinatal Exposure to Ltryptophan on Circadian Clock of Primary Cell Lines Established from Male Offspring Born from Mothers Fed on Dietary Protein Restriction. PLoS One. 2013;8(2):e56231.
- **Aragão, Raquel da Silva**, Rodrigues, Marco Aurélio Benedetti, de Barros, Karla Mônica Ferraz Teixeira, Silva, Sebastião Rogério Freitas, Toscano, Ana Elisa, de Souza, Ricardo Emmanuel, Manhães-de-Castro, Raul Automatic system for analysis of locomotor activity in rodents - A reproducibility study. Journal of Neuroscience Methods. , v.195, p.216 - 221, 2011.

- MAGALHAES, C. P., CAMPINAS, R. C. F., BORBA, T. K. F., LIRA, L. A., **ARAGÃO, R. S**., LOPES DE SOUZA, S., MANHÃES-DE-CASTRO, R.Programação Perinatal e o Comportamento Emocional em Ratos. Neurobiologia (Recife. Impresso). , v.74, p.83 - 95, 2011.
- Pereira, K.N.F., VITORIANO, I. L. S., Melo, M.P.P., **ARAGÃO, R. S.,** TOSCANO,A.E., TOSCANO,A.E., Silva, H.J., CASTRO, R. M. Effects of malnutrition and/or neonatal inhibition of serotonin reuptake in neuromuscular development of the gastrointestinal tract: review of literature. Neurobiologia (Recife. Impresso). , v.72, p.145 - 154, 2009.
- FERRAZ-PEREIRA, K. N., VITORIANO, I. L. S., MELO, M. P. P., **ARAGÃO, R. S.,** Toscano, A. E., SILVA, H. J., MANHÃES-DE-CASTRO, R. Repercussões da Desnutrição e/ou da Inibição Neonatal da Recaptação de Serotonina no Desenvolvimento Neuromuscular do Trato Gastrintestinal: Revisão da Literatura. Neurobiologia (Recife. Impresso). , v.72, p.145 - 154, 2009.

Des articles ont été soumis :

- Omar Guzmán-Quevedo; **Raquel Da Silva Aragão**; Georgina Pérez García; Rhowena Jane Barbosa Matos; Raul Manhães de Castro; Francisco Bolaños-Jiménez (Soumis à PLoS ONE). Impaired hypothalamic mTOR activation in the adult rat offspring born to mothers fed a low-protein diet.
- Georgina Pérez-García, Omar Guzmán-Quevedo, **Raquel da Silva Aragão**, Raul Manhães de Castro, Francisco Bolaños-Jiménez (Pour summision à Journal of Physiology). THE COGNITIVE DEFICITS INDUCED BY PERINATAL UNDERNUTRITION ARE ASSOCIATED WITH ALTERED NEUROGENESIS IN RESPONSE TO A LEARNING TASK

Des résumés ont été présentés lors de conférences nationales et internationales :

- Guzmán-Quevedo, O., **da Silva Aragão, R.,** Pérez García, G, Lopes de Souza, S, Bolaños-Jímenez, F. Analysis of the impact of perinatal under-nutrition on hypothalamic nutrient sensing involving mTOR In: 11th European Nutrition Conference (FENS), 2011, Madri. Annals of Nutrition and Metabolism. Karger, 2011. v.58. p.1 – 444.
- Pérez García, G, Guzmán-Quevedo, O., **da Silva Aragão, R.,** Lopes de Souza, S, Bolaños-Jímenez, F. Effects of perinatal protein-restriction on hippocampal

neurogenesis in respose to a learning task In: 11th European Nutrition Conference (FENS), 2011, Madri. Annals of Nutrition and Metabolism. Karger, 2011. v.58. p.1 - 444

- SANTOS, P. C. P., **ARAGÃO, R. S.,** BARROS, K. M. F. T., MANHÃES-DE-CASTRO, R. INFLUÊNCIA DA ADMINISTRAÇÃO DE AGONISTAS 5-HT1A SOBRE A LOCOMOÇÃO EM RATOS HIPERNUTRIDOS In: XXV Reunião Anual Federação de Sociedades de Biologia Experimental (FeSBE), 2010, Águas de Lindóia.
- **ARAGÃO, R. S.,** SANTOS, P. C. P., ARAUJO-FILHO, J. C., RODRIGUES, M. A. B., BARROS, K. M. F. T., MANHÃES-DE-CASTRO, R. A MANIPULAÇÃO NEONATAL COM BUSPIRONA ALTERA O DESENVOLVIMENTO DA LOCOMOÇÃO ESPONTÂNEA EM RATOS? In: XXIV Reunião Anual da FeSBE, 2009, Águas de Lindóia.
- LEITE, R. M. P., ESTABILE, P. C., **ARAGÃO, R. S.,** FIDALGO, M. A., ARAUJO-FILHO, J. C., DANTAS, M. L. M., SANTOS, P. C. P., VITORIANO, I. L. S., LEITE, S. P., MANHÃES-DE-CASTRO, R. Estudo morfométrico de células epiteliais do proestro em ratas desnutridas In: XXIV Reunião Anual da FeSBE, 2009, Águas de Lindóia.
- **ARAGÃO, R. S.,** SANTOS, P. C. P., ARAUJO-FILHO, J. C., VITORIANO, I. L. S., RODRIGUES, M. A. B., BARROS, K. M. F. T., MANHÃES-DE-CASTRO, R. HÁ PROGRAMAÇÃO DA RESPOSTA À BUSPIRONA EM RATOS ADULTOS SUBMETIDOS À MANIPULAÇÃO FARMACOLÓGICA NEONATAL? In: XXIV Reunião Anual da FeSBE, 2009, Águas de Lindóia.

Avant d’exposer les résultats et les conclusions issues de ce travail, nous ferons un rappel bibliographique du concept de programmation métabolique et des changements structurels et fonctionnels induits par la dénutrition périnatale sur divers organes.

Cette thèse est écrite dans le format de «règles et règlements de soutenance de thèse » du Programa de Pós-graduação em Nutrição, Université Fédérale de Pernambuco. Il est à noter que dans ce cas, s’agissant d'une thèse en cotutelle avec l'Université de Nantes, est inclut uniquement un résumé de la thèse en français suivant ainsi les normes de la défense et l'octroi du titre de Docteur d’après la convention de cotutelle signée entre l'Université Fédérale de Pernambuco et l'Université de Nantes.

Rappel Bibliographique

1.2 Rappel Bibliographique

1.2.1 L'obésité. Un problème de santé mondiale

L'obésité est devenue un problème majeur de santé dans le monde car elle constitue l'un des facteurs de risque le plus important pour le développement du syndrome métabolique caractérisé par tout un ensemble de troubles métaboliques comprenant intolérance au glucose, résistance à l'insuline, dyslipidémie, hypertension et une risque élevé de développement de maladies cardiovasculaires (ECKEL, GRUNDY e ZIMMET, 2005; JUNIEN *et al.*, 2005).

En Europe le pourcentage de la population affectée par le syndrome métabolique est de l'ordre de 10 à 25% en fonction du sexe et de l'âge (BALKAU *et al.*, 2002b). La situation est encore plus préoccupante aux USA, où 26% de la population active et plus de la moitié des américains âgés de plus de 60 ans en sont atteints (ZIMMET, SHAW e ALBERTI, 2003). En France, la dernière enquête nationale ObEpi réalisée en 2003 indique que la proportion des personnes en surpoids ou obèses a progressé de 36,7% à 41,6% entre 1997 et 2003, soit une augmentation de 13%. Cette enquête a également révélé que de plus en plus d'enfants et de personnes âgées sont touchées par l'obésité. Ainsi, 19% des enfants français seraient obèses ou en surpoids et après 65 ans la prévalence de l'obésité serait de 15%. L'obésité constitue un problème de santé publique non seulement dans les pays développés mais aussi dans les pays en voie de développement où elle ne cesse d'augmenter. Par exemple, les derniers chiffres publiés par l'institut statistique national et le ministère de la santé brésiliens en décembre 2004, montrent que 38,8 millions de Brésiliens adultes sont en surcharge pondérale soit 40 % de la population. D'après cet organisme, 13 % de femmes et 10 % d´hommes brésiliens sont obèses à proprement parler. Enfants et adolescents ne sont pas épargnés. Les données épidémiologiques recueillies pas les sociétés savantes chiffrent la prévalence de l'obésité dans ces dernières populations à 10%. Il est à noter que dans la plupart des pays émergents, les maladies métaboliques se développent à une vitesse beaucoup plus rapide que dans les régions industrialisées (POPKIN, 2002). Ceci est en particulier le cas du diabète de type 2 dont le nombre de personnes affectées, selon les prévisions, sera de 300 millions vers l'année 2025 (KIBERSTIS, 2005).

D'après l'Organisation Mondiale de la Santé, l'obésité correspond à un Indice de Masse Corporelle (IMC, poids en kg divisé par le carré de la taille en mètres), supérieur à 30 kg/m^2, et le surpoids à un IMC > 25. Cependant, en termes cliniques, plus que l'excès de

poids en soi, c'est l'accumulation du tissu adipeux, surtout au niveau viscéral et abdominal, qui est le facteur de risque le plus important pour le développement du syndrome métabolique. Cette accumulation de masse grasse est le résultat d'un déséquilibre du métabolisme énergétique, c'est-à-dire, d'un excès de calories ingérées par rapport à la dépense énergétique. L'importance de la prise alimentaire dans le développement d'obésité est soulignée par les études épidémiologiques qui montrent que l'obésité morbide (IMC supérieur à 40), est associée dans un certain nombre de cas à des mutations des gènes impliqués dans la régulation de la prise alimentaire (BARSH, FAROOQI e O'RAHILLY, 2000). Il est toutefois important de signaler que la proportion de patients soufrant d'obésité d'origine génétique est très faible. En fait, plusieurs auteurs s'accordent pour dire que la pandémie actuelle d'obésité est due à la combinaison des facteurs génétiques, environnementaux et sociaux.

Tableau 1. Prévalence du syndrome métabolique dans plusieurs pays d'Amérique Latine. Récupérée de (CUEVAS, ALVAREZ e CARRASCO, 2011).

Author	Country	Sample, *n* (women/men)	Age range (years)	Criteria diagnosis	Prevalence (%, overall/men/women)
Valenzuela *et al.* [20]	Chile	1833	>17	Updated ATP III/IDF	31.6 overall (ATP) 36.8 overall (IDF)
Rojas *et al.* [22]	Mexico	6021	>20	IDF AHA/NHLBI ATP III	49.8 overall/46.4 m/52.7 w 41.6 overall/34.7 m/47.4 w 36.8 overall/30.3 m/42.2 w
Flores *et al.* [23]	Venezuela	3108	>20	ATP III	31.2 overall
Sempértegui *et al.* [24]	Ecuador	352	≥65	IDF	40 overall /19 m/81 w
Medina-Lezama *et al.* [25]	Perú	1878 (1011/867)	>20	AHA/NHLBI	14.3 m/23.2 w
Pérez *et al.* [26]	Puerto Rico	859	21–79	NCEP ATP	43.3 overall/45.3 m/42.2 w
Rodrigues *et al.* [27]	Brasil	1655 (896/759)	25–64	ATP III	32.8 overall/33 m/32.7 w
Caceres *et al.* [28]	Bolivia	61	5–18 (obese)	NCEP/ATP III for children	36 overall/40 boys/32.2 girls
Bustos *et al.* [29]	Chile	461	10–18	NCEP/ATP III for children	37.5 overall/43.7 m/33 w
Escobedo *et al.* [30••]	Seven cities from Latin America	Total = 11 502	26–64	ATP III	23 m /22.7 w (Barquisimeto) 14.7 m/18.2 w (Bogota) 17.3 m/9.7 w (Buenos Aires) 13.2 m/17.6 w (Lima) 22.4 m/22.2 w (Mexico City) 5.5 m/16.4 w (Quito) 15.3 m/19 w (Santiago)

ADP, Adult Treatment Panel; IDF, International Diabetes Federation; m, men; NCEP, National Cholesterol Education Program; w, women.

Parmi les facteurs sociaux impliqués dans le développent d'obésite, plusieurs études ont mis en évidence l'importance du changement du style de vie lié au développement industriel caractérisé par la consommation de nourriture riche en graisses et en sucres hautement raffinées associée à une vie sédentaire (PRENTICE, 2006). L'exemple type est la société américaine où plus du 70% de la population s'adonne à moins de 30 min d'activité physique modérée par jour (CHAKRAVARTHY e BOOTH, 2004). Ce changement de style de vie est également intervenu dernièrement dans les pays en voie de développent où les maladies liées aux conditions socio-économiques et géographiques défavorables, comme les

maladies infectieuses et parasitaires, ont perdu de l'importance face au fléau des maladies métaboliques. Dans ces pays, la pandémie d'obésité s'explique par la migration d'une grande partie de la population des régions rurales vers des régions hautement urbanisés où, grâce au développement économique et industriel, les individus peuvent trouver, comme dans les pays développés, des aliments riches en énergie à des prix concurrentiels (MISRA e KHURANA, 2008). Le cas de la Gambie illustre bien ce phénomène. Dans ce pays de l'Afrique de l'ouest, la migration constante des jeunes des régions rurales vers les zones urbanisées à la recherche d'éducation et du travail, s'est traduite par un taux d'obésité très important qui, de surcroît, est bien supérieur chez la population d'origine rurale.

Les taux d'obésité variables au sein d'une population de différentes origines (urbain ou rural), soumise aux mêmes influences nutritionnelles, comme l'illustre le cas de la Gambie, révèle que non seulement la suralimentation et le manque d'activité physique détermine le développement du syndrome métabolique. Le changement accéléré à partir d'un environnement pauvre nutritionnellement vers des conditions de suralimentation, est l'un des facteurs qui conditionne aussi l'apparition d'obésité et qui peut expliquer l'apparition accéléré des maladies liées au syndrome métabolique dans les pays en voie de développement au cours de ces dernières années.

1.2.2 Dénutrition périnatale et obésité, le concept de programmation métabolique

Au cours des dernières années il a été également établi que des stimuli environnementaux, dont la nutrition périnatale et le stress, contribuent de façon importante au dérèglement du métabolisme énergétique associé à l'obésité.

De multiples études épidémiologiques chez l'homme indiquent en effet que les nouveau-nés de petit poids de naissance (poids corporel à terme < 2,5 kg), présentent un risque accru de développer une obésité, un diabète de type 2, et des maladies cardiovasculaires (HALES e BARKER, 2001b). Ces observations épidémiologiques, ont donné lieu à l'hypothèse de « programmation » métabolique (HALES e BARKER, 2001b; OZANNE e HALES, 2002). D'après cette hypothèse, chez un organisme sous-nourri en voie de développent le métabolisme est modifié de telle sorte que ses chances de survie sont maximisées. Il y a tout d'abord une distribution sélective des nutriments de façon à préserver la croissance cérébrale au détriment des autres organes. Ensuite, il y a une « programmation » conduisant à des adaptations métaboliques lui permettant de survivre dans des conditions de

restriction alimentaire. Cependant, lorsque cette pression de sélection disparaît si l'organisme est suralimenté ou soumis à un déséquilibre nutritionnel, l'incompatibilité entre sa programmation physiologique et les nouvelles conditions nutritionnelles génère le syndrome métabolique qui se traduit par une altération de la tolérance au glucose, de l'hypertension et un taux augmenté de triglycérides. Un processus de programmation des circuits nerveux a également été évoqué pour expliquer la susceptibilité à l'obésité chez des individus soumis à des stimuli stressants au cours de leur développement pré- et/ou postnatal (LEVIN, 2000).

Les enfants nés avec un faible poids, le plus souvent comme conséquence d'un apport nutritionnel réduit pendant la gestation lié à une réduction du flux sanguin placentaire, constituent entre 2 et 10% des 800.000 naissances annuelles en France, et sont exposés également à un risque d'altérations cérébrales qui se traduisent par des déficits cognitifs et des réponses altérées au stress.

Bien que la corrélation entre l'environnement nutritionnel périnatal et le développement d'une obésité chez l'adulte soit mois évident que d'autres types de perturbations métaboliques, plusieurs études épidémiologiques ont montré que des individus nées avec un poids de naissance élevé tendent à avoir un BMI plus élevé à l'âge adulte (ERIKSSON *et al.*, 2003a; ROGERS, 2003). Ces individus ont tendance également à accumuler de la masse grasse abdominale (OKEN e GILLMAN, 2003; ROGERS, 2003). Il est aussi connu que le fait de fumer pendant la gestation et corrélé avec le développement d'une obésité chez les enfants et adolescents probablement dû au faible poids de la naissance (POWER e JEFFERIS, 2002; TOSCHKE *et al.*, 2002).

1.2.3 Mécanismes physiologiques de la programmation métabolique

Les résultats de multiples expérimentations chez l'animal confortent l'hypothèse de programmation métabolique et, de fait, cette hypothèse sert comme support conceptuel à la plupart des recherches réalisées actuellement pour essayer d'expliquer les taux élevé d'obésité dans les pays européennes mais aussi dans les pays émergentes. Ainsi, des études réalisées chez le mouton, la souris ou encore chez le rat, ont démontré que les animaux nés des mères qui ont été soumises à une restriction nutritionnelle, soit multi- calorique soit protéique, pendant la gestation et la lactation, présentent diverses altérations physiologiques liées au syndrome métabolique dont résistance à l'insuline, insensibilité aux effets de la leptine, accumulation de graisse dans le foie (stéatose), hypertension et hyperlipidémie.

Grace aux études effectuées dans divers espèces d'animaux, les mécanismes physio-pathologiques de la programmation métabolique ont pu être appréhendés. Plusieurs modèles animaux de programmation métabolique ont été établis. Et tous ces modèles sont basés sur une modification en apports nutritionnels pendant différentes périodes du développement dont la restriction nutritionnelle multi calorique, la diminution du contenu en protéines de l'aliment, la ligature de l'artère utérine, le diabète gestationnel, et une alimentation hyper-calorique. Concernant les espèces animales, les rongeurs, et en particulier le rat, sont de loin les plus utilisés. D'autres espèces, qui possèdent l'avantage de présenter des taux de croissance pré-et postnatale similaires à ceux de l'homme, dont le mouton, et le porc, ont été également utilisées. Sur chacun de ces modèles, des altérations du développement des organes et les conséquences physio-pathologiques à long-terme ont été répertoriées (BERTRAM e HANSON, 2001a; REUSENS e REMACLE, 2001; HOLEMANS, AERTS e VAN ASSCHE, 2003). Les résultats de toutes les expérimentions chez les animaux confortent l'existence d'un lien cause-effet entre la malnutrition et les conditions de stress *in utero* ou pendant la période précoce du développement et le risque élevé de développement des maladies chroniques et dégénératives liées au syndrome métabolique à l'âge adulte. Ainsi, il a été observé que plusieurs tissus sont des « cibles » privilégiées de la programmation métabolique dont le pancréas, le muscle, le foie, le rein, le tissue adipeux, l'axe HPA, et l'hypothalamus (REMACLE *et al.*, 2007).

Il est cependant à remarquer que, si bien toutes les expérimentions chez l'animaux confortent l'existence d'un lien cause-effet entre la malnutrition *in utero* ou pendant la période précoce du développement et le risque élevé de développement du syndrome métabolique à l'âge adulte, les bases cellulaires et moléculaires des adaptations métaboliques induites par l'environnement nutritionnel précoce restent encore largement à déterminer. En particulier, à ce jour, il n'existe aucune étude intégrale des conséquences de la dénutrition périnatale sur la structure et/ou la fonction du muscle squelettique, ni de comment les altérations métaboliques éventuellement induites sur ce tissu par l'exposition à un environnement nutritionnel appauvri pendant la gestation et les premières étapes de la vie post-natale, contribuent au développement d'obésité à l'âge adulte. Pourtant, étant donné que le muscle est le site majeur d'utilisation du glucose postprandiale, on peut raisonnablement imaginer que des altérations de la masse musculaire, du type, du profile de croissance et des caractéristiques fonctionnelles des cellules musculaires et des fibres squelettiques établies pendant la période du développement, soient importantes pour la programmation de la

sensibilité à l'insuline et au diabète. La figure 2 résume des altérations physiologiques induites par la dénutrition périnatale et de leurs contributions au développement du syndrome métabolique.

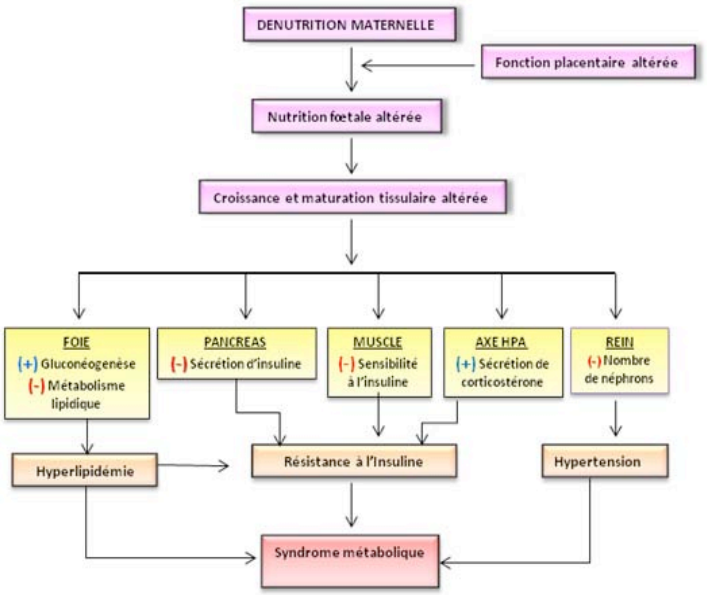

Figure 1. Résumé des altérations physiologiques induites par la dénutrition périnatale et de leurs contributions au développement du syndrome métabolique.

1.2.4. Rôle du muscle squelettique dans la régulation du métabolisme énergétique

Le muscle squelettique est l'organe le plus important de l'organisme aussi bien en termes de masse corporelle que de capacité métabolique. Chez l'adulte, 30-40% de l'activité métabolique au repos a lieu dans cet organe et l'oxydation du glucose et des acides gras a lieu majoritairement dans les cellules musculaires. En fait, 80% du glucose circulant est capté par muscle squelettique sous l'action de l'insuline et les cellules squelettiques sont responsables de 20 à 30% de la dépense énergétique globale de l'organisme.

Par ailleurs, le muscle squelettique possède une remarquable capacité d'adaptation pour utiliser l'énergie en réponse aux apports caloriques et les demandes énergétiques de l'organisme. Ainsi, les niveaux circulants élevés de triglycérides et d'acides gras libres, consécutifs à l'ingestion de nourriture, favorisent l'utilisation de lipides par le muscle squelettique comme source d'énergie au dépend de l'oxydation du glucose (SILVEIRA *et al.*, 2008b). Ce phénomène, lorsqu'il est associé à la détérioration de la capacité oxydative de la mitochondrie et des mécanismes de découplage de la phosphorylation oxydative au sein de la

matrice mitochondriale, serait à l'origine de l'accumulation intracellulaire de lipides et des radicaux libres dérivés de leur peroxydation (SCHRAUWEN e HESSELINK, 2004c; SILVEIRA LR 2008) conduisant au développement d'obésité et de diabète de type 2.

En effet, l'augmentation des lipides circulants (acides gras libres et triglycérides) et leur accumulation dans de nombreux tissus, dont le muscle squelettique, sont impliquées dans l'altération de la sécrétion pancréatique d'insuline, dans la modification de l'expression des gènes clés du métabolisme glucidique et lipidique et dans l'instauration de la résistance à l'insuline (Silveira et al., 2008). Ces dysfonctionnements du métabolisme lipidique résultent en partie d'une altération de l'oxydation mitochondriale des acides gras à chaîne longue (AGCL) associée à une production exacerbée d'espèces réactives à l'oxygène (ROS ou radicaux libres). En fait, lorsque les acides gras sont en excès par rapport aux capacités oxydatives de la mitochondrie, ceux-ci se retrouvent à l'intérieur de la matrice mitochondriale sous forme d'anions d'acide gras où leur accumulation est potentiellement toxique car ils sont très sensibles à la peroxydation (SCHRAUWEN e HESSELINK, 2004c; HIRABARA *et al.*, 2006b; SILVEIRA LR 2008) L'accumulation d'acides gras à l'intérieur de la mitochondrie est évitée en découplant la phosphorylation oxydative de la production d'ATP (découplement mitochondrial). Dans le muscle squelettique, cette fonction est accomplie par la protéine découplante mitochondriale de type 3 (UCP3) qui transporte les anions d'acide gras de la matrice mitochondriale vers le cytosol afin d'augmenter la capacité oxydative de la mitochondrie et de limiter leur peroxydation (HIRABARA *et al.*, 2006a). Cependant, les acides gras activent l'expression d'UCP3 établissant ainsi une boucle de rétrocontrôle permettant de prévenir la production excessive de radicaux libres et des dommages à la mitochondrie (SCHRAUWEN e HESSELINK, 2004c; HIRABARA *et al.*, 2006b). Le fait que l'expressiond'UCP3 est diminue de 50% chez les patients diabétiques (SCHRAUWEN *et al.*, 2001), a emmené divers auteurs à proposer que l'exposition prolongée aux acides gras entraîne un défaut du mécanisme de rétrocontrôle de la production de radicaux libres via le découplage de la phosphorylation oxydative et que cette altération contribue au développent du diabète(SILVEIRA *et al.*, 2008b).

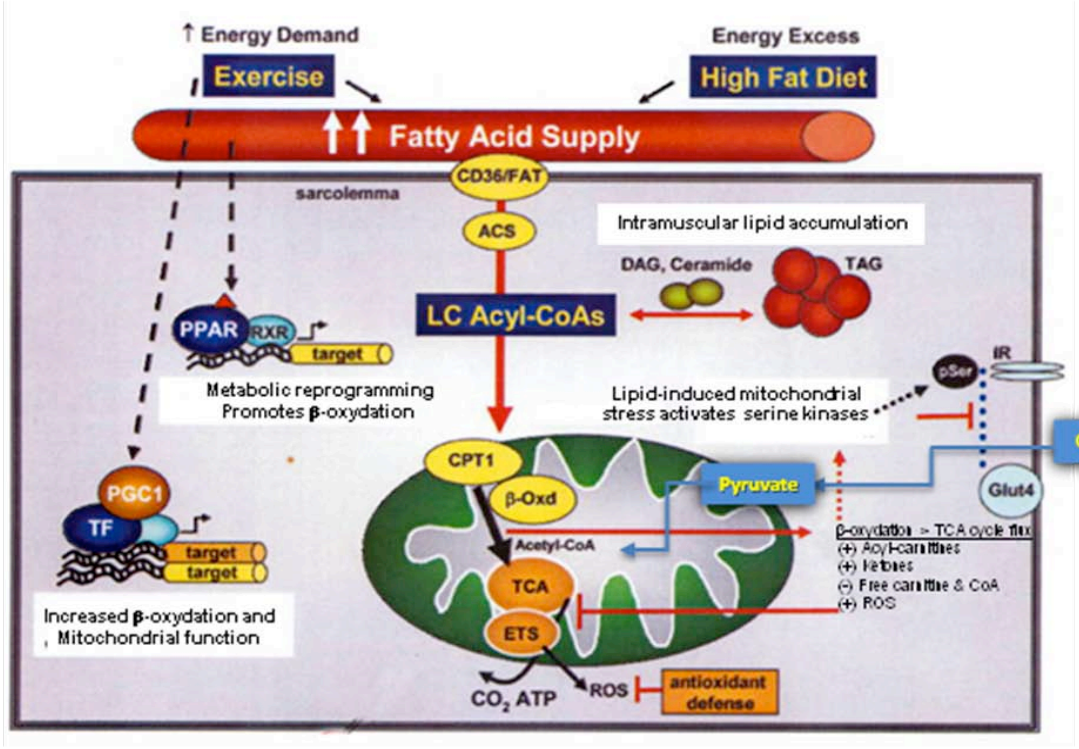

Figure 2. Représentation schématique des voies énergétiques oxydative et glycolytique dans le muscle squelettique. Le glucose rentre à l'intérieur des cellules squelettiques via le transporteur Glut4 dont la translocation au niveau de la membrane est régulée par l'insuline. A l'intérieur de la cellule, le glucose est métabolisé pour donner deux molécules de pyruvate qui seront à leur tour transformées en acétyl-coenzyme A (Acétyl-CoA). Cette dernière molécule, qui est également formée lors de la b-oxydation des lipides, sert à la production des intermédiaires énergétiques (dont la forme oxydée de la coenzyme NADH), qui serviront à la production d'ATP dans la chaine respiratoire via le cycle des acides tricarboxyliques (TCA) ou cycle de Krebs. Lors d'un excès de lipides, la capacité d'oxydation de la mitochondrie est dépassée ayant comme conséquence une accumulation de lipides à l'intérieur de la cellule, une augmentation des taux NADH/NAD et ATP/ADP et une accumulation de d'Acétyl-CoA au sein de la matrice mitochondriale. Cet environnement entraîne la production de radicaux libres (ROS) qui, en inhibant des enzymes du cycle de Krebs, bloquent l'oxydation des substrats énergétiques. Les radicaux libres peuvent également activer de sérine kinases qui vont bloquer le transport du glucose et/ou l'action de l'insuline. L'exercice augmente la capacité oxydative du muscle, via la reprogrammation métabolique des fibres squelettiques, et stimule la fonction mitochondriale. Ces deux processus aboutissent à la réduction de la production de radicaux libres et du stress mitochondrial et restaurent la sensibilité à l'insuline (adapté de (MUOIO e KOVES, 2007).

1.2.5. Plasticité métabolique du muscle squelettique

Dans certaines conditions physiologiques, le muscle adapte son métabolisme de façon à favoriser l'utilisation des lipides comme source énergétique. Cette adaptation métabolique s'accompagne, d'une part, d'une augmentation de l'expression des gènes impliqués dans le

métabolisme de lipides, la phosphorylation oxydative et la biogénèse mitochondrial et, d'autre part, d'une réorganisation structurale et morphologique (WANG *et al.*, 2004a; RODGERS *et al.*, 2008b). Le muscle squelettique es composé en effet de divers types de fibres possédant des propriétés métaboliques et contractiles différentes dont les fibres de type I et les fibres de type II. Les fibres de type I utilisent essentiellement les lipides comme substrat énergétique, leur vitesse de contraction est relativement lente et sont résistantes à la fatigue. En revanche, les fibres de type II, utilisent le glucose comme source d'énergie, se contractent rapidement et ne possèdent pas une grande résistance à la fatigue (STARON *et al.*, 1999).

Tableau 2. Résume des caractéristiques des différentes fibres musculaires.

Caractéristique	Fibre Type I	Fibre Type IIA	Fibre Type IIB
Chain lourde de myosine	MyHCI/β	MyHCIIa	MyHCIIb
Vitesse de contraction	Lent	Rapide	Rapide
Métabolisme prédominant	Oxydative	Oxydative-Glycolytique	Glycolytique
Résistance à la fatigue	Haute	Intermédiaire	Baisse
Production de force	Baisse	Intermédiaire	Haute
Stocks d'IMTG	Haute	Intermédiaire	Baisse
Stocks de glycogénie	Baisse	Intermédiaire	Haute
Capture de glucose	Plus Grand	Grand	Baisse

Or, sous l'effet de l'exercice ou de la restriction calorique, le muscle squelettique est remodelé de telle sorte que le phénotype métabolique de fibres de type II évolue progressivement vers un phénotype métabolique caractéristique des fibres de type I conduisant à une augmentation du nombre de fibres musculaires oxydatives (PIETER DE LANGE *et al.*, 2007). Au niveau moléculaire, cette reprogrammation met en jeu un complexe mécanisme de régulation de la transcription impliquant les récepteurs nucléaires PPARδ, le co-régulateur de la transcription PGC1α et les senseurs nutritionnels SIRT1 et AMPK (LIN *et al.*, 2002a; BASSEL-DUBY e OLSON, 2006a; JORGENSEN, RICHTER e WOJTASZEWSKI, 2006; ROCKL *et al.*, 2007b; RODGERS *et al.*, 2008b). Ainsi, la contraction musculaire induit l'expression de PGC1α a, via une voie de signalisation calcium-dépendante et l'activation du facteur de transcription CREB (CHOWANADISAI *et al.*, 2009). L'exercice augmente aussi l'expression de PPARδ via des mécanismes et des voies de signalisation qui restent encore à déterminer. L'activité élevée de PPARδ entraîne à son tour une augmentation de l'expression de PGC1α (WANG *et al.*, 2004a; SCHULER *et al.*, 2006a).

Ce dernier facteur, en formant un complexe avec les récepteurs nucléaires NRF1 et NRF2, facilite l'expression des gènes mitochondriaux et, de façon simultanée, régule la reprogrammation des fibres de type II en fibres de type I via la co-activation du Myosine Enhancer Factor 2 (LIN *et al.*, 2002a; BRUNMAIR *et al.*, 2006). En dehors des récepteurs nucléaires PPARδ, l'expression de PGC1α est aussi augmentée par SIRT1 et AMPK (JORGENSEN, RICHTER e WOJTASZEWSKI, 2006; LEE *et al.*, 2006; REZNICK e SHULMAN, 2006; CLARET *et al.*, 2007). Ces deux facteurs sont activés dans des conditions physiologiques qui demandent une forte quantité d'énergie comme la restriction nutritionnelle ou l'exercice. PGC1α joue donc un rôle central dans le processus d'adaptation métabolique du muscle squelettique en réponse aux besoins énergétiques de l'organisme et aux apports nutritionnels.

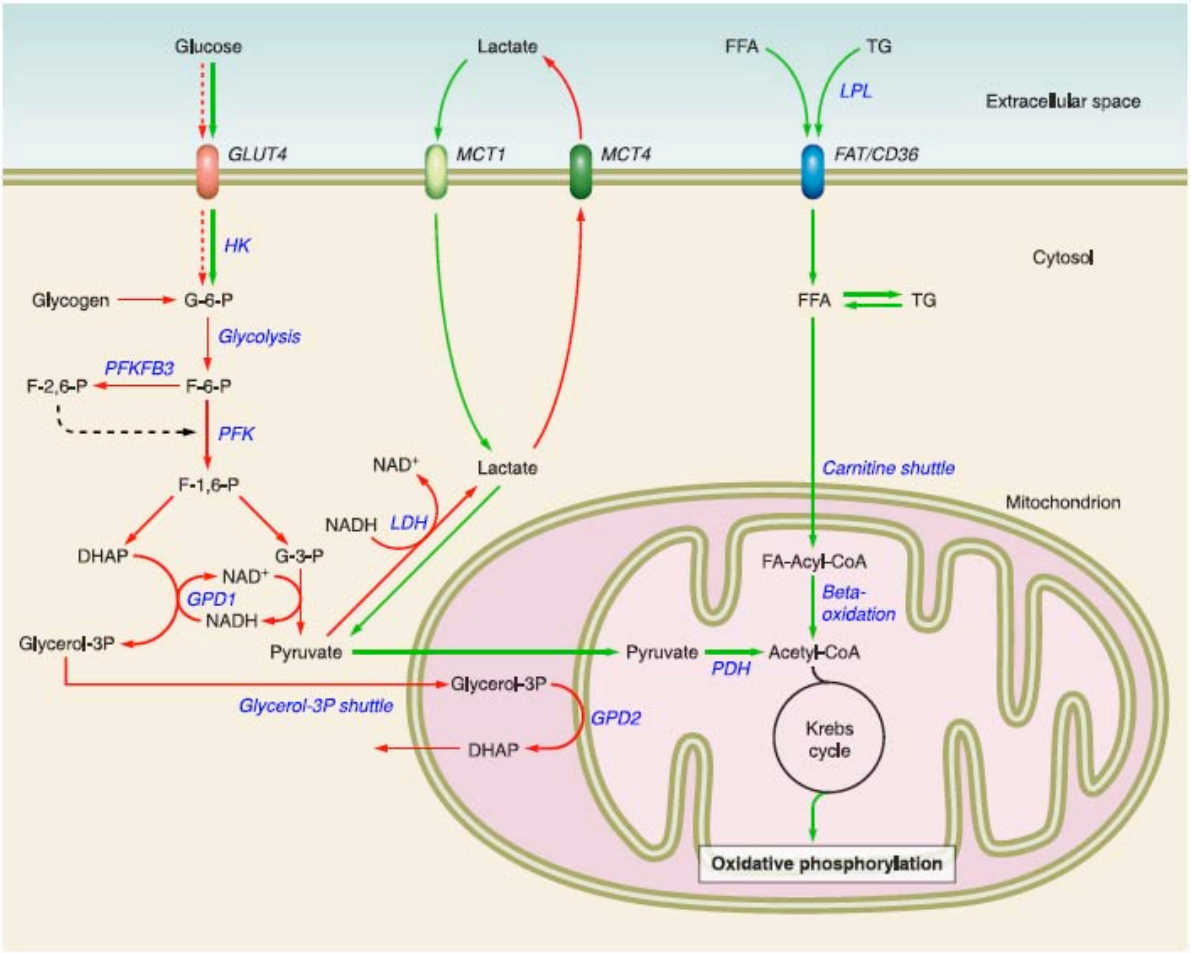

Figure 3. Schéma illustratif des différentes voies métaboliques de chaque type de fibre musculaire. Fibres lentes sont représentés en vert et fibres rapides en rouge. Récupérée de (SCHIAFFINO e REGGIANI, 2011).

Des différences dans le nombre de fibres musculaires de type I versus type II sont associées à l'obésité et au diabète de type 2. Ainsi, chez les patients obèses et/ou diabétiques, les capacités oxydatives du muscle squelettique sont réduites alors que les capacités glycolytiques sont augmentées et ce profil métabolique s'accompagne d'une diminution du nombre de fibres de type I (HICKEY *et al.*, 1995b; TANNER *et al.*, 2002b). (LILLIOJA *et al.*, 1987; HICKEY *et al.*, 1995b; ZIERATH e HAWLEY, 2004). Figure 4

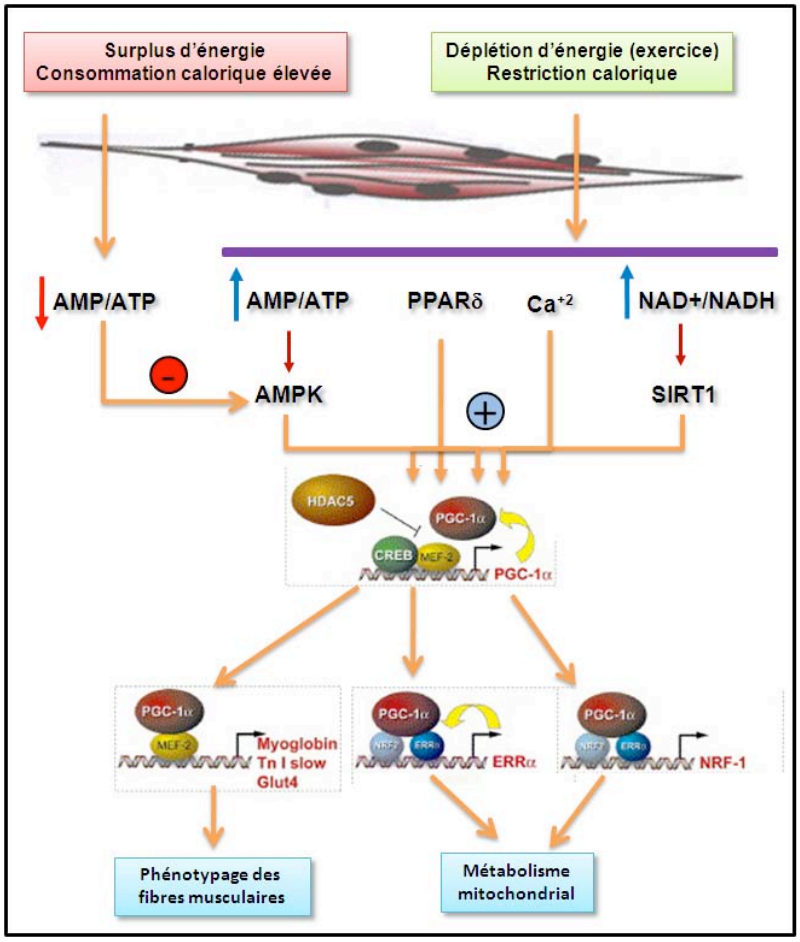

Figure 4. Représentation schématique de la plasticité métabolique des fibres squelettiques. Dans des conditions de déficience ou de forte demande d'énergie, les senseurs nutritionnels AMPK et SIRT1 sont activés. Ces facteurs, ainsi que l'augmentation intracellulaire de calcium et les récepteurs nucléaires PPAR**δ**, activent à leur tour le co-régulateur de la transcription PGC1α qui, en formant des dimères avec divers facteurs de transcription augmente l'expression des gènes impliqués dans la b-oxydation et dans la reprogrammation métabolique des fibres musculaires de type II vers un phénotype de type I. Un surplus d'énergie exerce l'effet contraire en raison de l'effet négatif qui exerce la diminution du ratio AMP/ATP sur l'activité de l'AMPK.

1.2.6. Altérations du métabolisme musculaire induites par la dénutrition périnatale

En dépit du rôle majeur du muscle squelettique dans l'utilisation du glucose et des lipides, et par conséquent, dans la régulation du métabolisme énergétique, peu d'études ont cherché à déterminer les conséquences de la dénutrition périnatale sur la masse musculaire, le type, le profil de croissance, et les caractéristiques fonctionnelles des cellules musculaires et des fibres squelettiques. En utilisant un modèle de programmation métabolique basé sur la l'occlusion de l'artère utérine pendant la dernière semaine de la gestation, il a été montré que le contenu de glycogène, et la capture de 2-deoxyglucose à la suite de la stimulation par l'insuline est diminuée chez des rats nés avec un faible poids. Dans cette étude il a été également rapporté que les muscles des animaux programmés métaboliquement, présentent au niveau de la mitochondrie de défets associés à une réduction chronique de l'apport d'ATP à partir de la phosphorylation oxydative, ce qui suggère une diminution de la synthèse d'ATP dans le muscle. Ceci se traduirait par la réduction du recrutement du transporteur du glucose GLUT4 au niveau de la surface cellulaire et, par conséquent, par une diminution du transport de glucose et de la biosynthèse de glycogène (SELAK *et al.*, 2003). D'autre part, les jeunes animaux adultes dénutris pendant leur développement périnatal, présentent une meilleure tolérance au glucose que leurs congénères contrôles du même âge ainsi qu'une sensibilité plus importante à l'insuline. Toutefois, vers 15 mois de vie, les animaux dénutris deviennent insulino-résistants et présentent des modifications dans le niveau d'expression de diverses molécules impliquées dans la signalisation de l'insuline dont les protéines PKCzeta, GLUT4 et p85 (PETRY CJ, 2001; OZANNE *et al.*, 2003b; OZANNE *et al.*, 2005).

Concernant les effets de la dénutrition périnatale sur le nombre et le phénotype des fibres musculaires, les études réalisées sur diverses espèces animales indiquent que, d'une façon générale, la dénutrition périnatale augmente la proportion des fibres de type I et réduit la proportion des fibres de type II (BAYOL, SIMBI e STICKLAND, 2005; ZHU *et al.*, 2006; COSTELLO *et al.*, 2008). Cependant, ces effets semblent dépendre, au moins chez le rat, aussi bien du type de muscle que du sexe. Ainsi, chez des rats femelles de 4 semaines soumises à une restriction en apports protéiques pendant la gestation, le nombre de fibres de type I et de type II dans le muscle est diminué alors que dans le muscle gastrocnemius seulement le nombre des fibres de type I est réduit (MALLINSON *et al.*, 2007). En revanche, chez les animaux mâles soumis au mêmes conditions expérimentales, le nombre de fibres de type I est augmenté (MALLINSON *et al.*, 2007). Il doit être également signalé que, jusqu'à

présent, les effets de la dénutrition périnatale sur le nombre et le phénotype des fibres musculaires ont été examinés uniquement sur de très jeunes animaux. En fait, pendant la période de gestation ou jusqu'au sevrage. Il reste donc à déterminer les conséquences à l'âge adulte de la restriction nutritionnelle précoce sur les caractéristiques phénotypiques des fibres musculaires. De même, il reste à déterminer les éventuelles modifications induites par la dénutrition périnatale sur la plasticité métabolique du muscle squelettique et/ou sur sa capacité de différentiation.

1.2.7. Mécanismes moléculaires de la programmation métabolique

Le support conceptuel de la programmation métabolique est la notion selon laquelle le manque de nutriments pendant le développement pré- ou post-natal entraîne des modifications fonctionnelles qui conditionnent la susceptibilité à développer des maladies métaboliques à l'âge adulte. L'un des défis majeurs relevés par cette hypothèse est donc d'identifier les mécanismes moléculaires qui sous-tendent ce lien physiopathologique. Des données récentes de la littérature suggèrent que la modulation épigénétique de l'expression des gènes par les nutriments pourrait constituer le lien entre un environnement nutritif périnatal appauvri et la production d'un phénotype physiopathologique dans des étapes postérieures de la vie.

Le terme épigénétique a été formulé originalement par Conrad Waddigton dans les années 40 pour définir « la branche de la biologie qui étudie les relations de cause à effet entre les gènes et leurs produits, faisant apparaître le phénotype » (JABLONKA e LAMB, 2002). Au cours des années, le concept d'épigénétique s'est affiné et il est utilisé à l'heure actuelle pour indiquer l'ensemble de processus d'expression et de transmission de l'information génique, y compris de façon trans-générationnelle (CHONG e WHITELAW, 2004b), via des mécanismes qui n'affectent pas la séquence de l'ADN mais l'organisation structurale de la chromatine (TURNER, 2002; EGGER *et al.*, 2004).

La régulation épigénétique de l'expression des gènes joue un rôle fondamental dans transduction fonctionnelle de l'information contenue dans le génome. Toutes les cellules de notre organisme contiennent la même information génétique, c'est à dire le même génome. Pourtant les caractéristiques morphologiques et fonctionnelles d'une cellule du pancréas, par exemple, sont très différentes de celles d'une cellule musculaire. Ce qui rend une cellule différente d'une autre ce n'est donc pas l'information génétique qu'elles contiennent mais l'information génétique qu'elles expriment. L'expression différentielle d'une population de

gènes d'une cellule à une autre, et même d'un stade de développement à un autre, est contrôlée par des mécanismes épigénetiques qui comprennent principalement deux mécanismes : la méthylation de l'ADN et les modifications post-transductionnelles des histones (Figure 4).

Le génome se présente en effet à l'intérieur du noyau cellulaire sous forme d'un complexe nucléoprotéique compact, la chromatine, qui, outre l'ADN, contient des protéines globulaires dénommées histones. C'est grâce à son interaction avec les histones que l'ADN d'une cellule long de 2 mètres peut être stocké à l'intérieur d'un noyau d'à peine quelques microns de diamètre. L'unité de base de la chromatine est le nucléosome, qui est composé d'un octamère comprenant 2 copies de chacune des histones H2A, H2B, H3 et H4, autour duquel s'enroule un fragment de 147 paires de bases d'ADN. Dans certaines régions du génome, les histones H2A, H2B et H3 peuvent être remplacés par des "variants" d'histones, dont les séquences sont différentes des histones canoniques. Or, pour qu'un gène puisse être transcrit, et en conséquence exprimé, il est tout d'abord nécessaire que la région de l'ADN correspondant à son promoteur soit accessible aux facteurs de transcription. Cette accessibilité est contrôlée, outre la méthylation de l'ADN, par les modifications post-traductionnelles des histones. Les histones peuvent en effet subir plusieurs modifications post-traductionnelles dans leur partie N-terminale dont l'addition des groupes méthyl, acétyl, phosphate, ubiquitine (JENUWEIN e ALLIS, 2001). Ces modifications modifient leur affinité pour l'ADN conduisant à un changement de la conformation de chromatine qui favorise (euchromatine) ou, au contraire, empêche (heterochromatine), l'accès des facteurs de transcription aux régions promotrices des gènes (BORRELLI *et al.*, 2008).

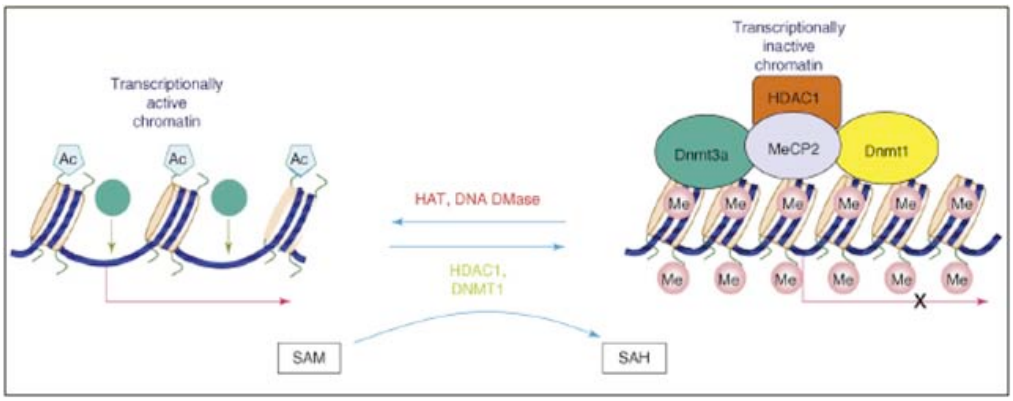

Figure 5. Représentation schématique des mécanismes épigénétiques de régulation de l'expression des gènes. La chromatine oscille entre une forme décondensée (euchromatine), et une forme compacte (hétérochromaine), qui permet l'accès des facteurs de transcription aux sites promoteurs des gènes, et en conséquence l'expression

génique, et une forme fermée (hétérochromatine), qui en rendant inaccessibles les sites promoteurs des gènes, bloque leur transcription.

1.2.7.1 Modifications post-traductionnelles des histones

Contrairement à la méthylation de l'ADN conduisant généralement à l'inhibition de la transcription, les modifications post-traductionnelles des histones peuvent donc mener soit à une augmentation soit à une diminution de l'expression d'un gène en fonction du profil des modifications post-traductionnelles des histones, ou code histone (JENUWEIN e ALLIS, 2001; WATERLAND e MICHELS, 2007), associées à son promoteur.

Il a été ainsi montré, par exemple, que la phosphorylation de l'histone H3 sur la serine 10 est associée à une augmentation de la transcription tandis que la méthylation de la même histone sur la lysine 9 est caractéristique de la chromatine inactive. Cependant, l'effet d'un type de modification post-transductionnelle des histones sur la transcription n'est pas figé. Il peut varier en fonction du contexte cellulaire. Par exemple, bien que la tri-méthylation de l'histone H3 en lysine 4 (H3K4me3) soit typiquement associée avec l'activation de la transcription (SIMS *et al.*, 2007), lorsque l'ADN est endommagé, cette même modification inhibe la transcription via le recrutement du complexe répressif constitué par mSin3a et l'histone déacétylase 1 (SHI *et al.*, 2006). Des études récentes ont également montré que la tri-méthylation de l'histone H3 en lysine 4 permet la reconnaissance et facilite l'assemblage des complexes antigène-anticorps en interagissant avec la recombinase RAG1/2 V(D)J (MATTHEWS *et al.*, 2007). Aussi, la modification épigénétique H3K4me3 peut avoir des effets positifs ou négatifs sur la transcription de telle sorte qu'elle n'a pas de valeur prédictive si l'on ne considère pas le contexte cellulaire. Dans ce sens, le code histone est « contexte spécifique » de façon analogue à la régulation de la transcription (LEMON e TJIAN, 2000).

1.2.7.2 La méthylation de l'ADN

La méthylation de l'ADN est l'addition d'un groupe méthyle aux résidus cytosine de l'ADN. Cette modification a lieu généralement dans des cytosines qui sont suivies d'une guanosine (dinucléotides CpG), et conduit le plus souvent à l'inhibition de l'expression des gènes en empêchant directement la liaison des facteurs de transcription ou bien en recrutant des complexes protéiques qui à leur tour empêcheront la fixation des facteurs de transcription sur l'ADN. Dans les génomes des mammifères, les séquences CpG sont majoritairement localisées à l'intérieur ou à proximité des sites promoteurs ou des premiers exons des gènes

dans des régions du génome que l'on dénomme des îlots CpG. Chez l'homme, ces îlots couvrent environ 0.7% du génome et 60% des régions promotrices sont associeés aux îlots CpG. Cependant, en termes globaux le pourcentage des séquences CpG est très faible dû à la transformation spontanée de la 5-méthyl cytosine en thymine.

La méthylation de l'ADN joue un rôle essentiel dans l'empreinte parentale, mécanisme par lequel seul l'allèle de l'un des parents est exprimé, et l'inactivation du chromosome X (ROBERTSON, 2005), mécanisme par lequel chez les femelles des mammifères l'expression des gènes à partir de l'un des chromosomes X est abolie afin de compenser la différence de dosage génique entre mâles (XY) et femelles (XX). En fait, après la fécondation, l'ADN du zygote est soumis à une deméthylation massive, de telle sorte que l'ADN de l'embryon ne contient pratiquement pas de groupe méthyl. Cette hypométhylation est à l'origine de la totipotence des cellules souches embryonnaires. Cette vague de déméthylation massive est suivie quelques temps après par la méthylation de novo du génome établissant un profil de méthylation spécifique dans chaque cellule ce qui a comme effet de bloquer l'expression de certains gènes et, par conséquent, d'orienter la différentiation cellulaire et tissulaire (REIK, DEAN e WALTER, 2001). Dans la plupart des cas, le profil de méthylation de l'ADN établi pendant l'embryogénèse est maintenu avec une haute fidélité au cours de la vie (WATERLAND e MICHELS, 2007).

Il est important de remarquer que la méthylation de l'ADN et les modifications post-traductionnelles des histones ne sont pas des processus indépendants. Diverses évidences expérimentales montrent en effet que ces deux mécanismes épigénétiques sont couplés et agissent souvent en concert. En fait, les répresseurs à domaine MBD (methyl-CpG-binding domain), qui se fixent spécifiquement sur l'ADN méthylé, sont capables de recruter les histone-désacétylases (HDAC) au niveau des séquences génomiques méthylées, ce qui a pour effet de désacétyler les histones et de maintenir une structure condensée de la chromatine, réprimant ainsi la transcription (JONES *et al.*, 1998; NAN *et al.*, 1998). Il a été également démontré que les enzymes DNA méthylases Dnmt1, Dnmt3a et Dnmt3b, interagissent avec les histone-désacétylases et que leur effet répresseur est levé par la trichostatine A, un inhibiteur spécifique des HDAC (FUKS *et al.*, 2000; ROBERTSON *et al.*, 2000; ROUNTREE, BACHMAN e BAYLIN, 2000; FUKS *et al.*, 2001). Enfin, la méthylation de la lysine 9 par les histone-méthyltransférases (HMT) crée un site de fixation pour le répresseur HP1 (heterochromatin protein 1), une protéine associée à la formation de l'hétérochromatine.

Chez les mammifères, Dnmt1 et Dnmt3a interagissent avec la protéine HP1 et sont associées à une activité de méthylation de l'histone H3 sur la lysine 9. Ces travaux indiquent que la méthylation de l'ADN est dépendante de la méthylation des histones. Notons cependant que la relation inverse semble également vraie, c'est-à-dire que la méthylation de l'ADN pourrait faciliter la méthylation des histones. Ainsi, des expériences d'immunoprécipitation de la chromatine (ChIP) réalisées sur des cellules inductibles pour le répresseur MeCP2, qui se fixe spécifiquement sur l'ADN méthylé, indiquent que la présence de MeCP2 est requise pour la méthylation de la lysine 9 de l'histone H3 dans le cas de l'imprinting d'un gène (FUKS *et al.*, 2003). Dans leur ensemble, ces dernières études suggèrent l'existence d'une boucle de régulation entre la méthylation de l'ADN et la méthylation des histones, qui aurait pour conséquence de maintenir et de propager les états épigénétiques répressifs de la chromatine au cours des divisions cellulaires successives.

1.2.8 Modifications épigénétiques induites par l'environnement nutritionnel précoce.

On savait depuis longtemps que les nutriments pouvaient potentiellement interférer avec les mécanismes de régulation épigénétique de l'expression des gènes mais ce n'est qu'en 2000 qu'il a été montré que des manipulations nutritionnelles au cours du développement périnatal peuvent affecter le niveau d'expression d'un gène via des modifications épigénétiques. Le premier exemple, et aussi le plus étudié, d'un gène soumis à des modifications épigénétiques par la nutrition est l'allée A^{vy} du gène agouti chez la souris. Le gène agouti code une molécule qui stimule la production de phaeomélanine jaune à partir des mélanocytes folliculaires à la place de l'eumélanine noire. La transcription du gène agouti a lieu uniquement dans la peau et ce de façon transitoire au cours du développement de telle sorte que les poils des souris contiennent une bande de phaeomélanine jaune mélangée à une bande de eumélanine noire ce qui donne des poils de couleur marron caractéristiques des souris sauvages. Chez la souris mutante A^{vy}, l'insertion d'une sequence d'ADN transposable (Intracisternal A sequence, IAP), en amont du site promoteur du gène agouti déclenche son expression de façon ectopique conduisant à la génération des souris qui, outre le pelage jaune, sont obèses, diabétiques et présentent de nombreuses tumeurs. Le niveau de méthylation de la particule IAP varie d'une souris isogènique A^{vy} à une autre ce qui a comme conséquence l'existence de souris avec une couleur du pelage allant du jaune (particule IPA non méthylée) au marron (particule IAP méthylée). Or, l'addition à la nourriture des mères gestantes d'acide folique (WOLFF *et al.*, 1998) ou de génistéine (DOLINOY *et al.*, 2006), un phytoestrogène

présent dans le soja, augmente la proportion de souris avec le phénotype marron (pseudo agouti) et ce changement de phénotype est directement lié à l'augmentation du niveau de méthylation des particules IAP. Ces observations ont été les premières à montrer que le régime alimentaire de la mère au cours de la gestation peut altérer le phénotype du nouveau-né et que ces altérations phénotypiques sont directement liées à des modifications épigénétiques.

Plus récemment, les modifications épigénétiques induites par la déficience de nutriments au cours de la gestation ont été analysées par le groupe de R.H. Lane aux Etats Unis. Dans une série d'articles, ces auteurs ont utilisé le modèle d'insuffisance utero-placentaire pour examiner les conséquences du retard de croissance intra-utérin sur le profil global de méthylation de l'ADN et les modifications post-transductionnelles de l'histone H3 dans le rein, le foie et le cerveau (PHAM *et al.*, 2003; FU *et al.*, 2004; MACLENNAN *et al.*, 2004; KE *et al.*, 2005; FU *et al.*, 2006). Dans leur ensemble ces études ont montré que, chez le rat, le retard de croissance intra-utérin entraîne une diminution globale du niveau de méthylation du génome et augmente le taux d'acétylation de l'histone H3. Dans le foie, ces modifications épigénétiques sont observées dès la naissance, persistent à 21 jours chez les mâles mais pas chez les animaux femelles et ont lieu dans le promoteur du gène « dual specificity Phosphatase-5 », un gène impliqué dans la cascade de signalisation intracellulaire des MAP kinases ainsi que dans le promoteur du gène p53 dans le rein. L'importance physiologique de cette dernière observation réside dans le fait que le gène P53 régule positivement le développent rénal et qu'une réduction dans le nombre de néphrons pendant les premières étapes de la vie semble être associée au développement d'hypertension à l'âge adulte (INGELFINGER, 2004).

Une diminution du niveau de méthylation du promoteur des récepteurs aux glucocorticoïdes et du promoteur du PPARα (peroxysomal proliferator-activated receptor), dans le foie a également été observée chez des animaux soumis à une restriction en apport protéique pendant leur gestation (LILLYCROP *et al.*, 2005a). De façon intéressante, ces modifications épigénétiques peuvent être abolies par l'addition d'acide folique dans la diète et/ou persistent chez les animaux de la deuxième génération (BURDGE *et al.*, 2007).

En conclusion, il est maintenant clairement établi chez le rat que la restriction nutritionnelle pendant la gestation entraîne une diminution du niveau de méthylation de l'ADN et une augmentation du taux d'acétylation de l'histone H3 dans le foie et le cerveau,

deux organes clé du métabolisme. Ces modifications épigénétiques, qui globalement se traduisent par une augmentation de l'expression de gènes, pourraient donc sous-tendre la susceptibilité accrue à développer une obésité et un diabète généralement observée chez les personnes de faible poids de naissance. Il est cependant important de signaler que la plupart des études sur les conséquences épigénétiques de la dénutrition périnatale ont été effectuées chez le rat nouveau-né ou chez des animaux juvéniles et en utilisant des manipulations expérimentales qui sont loin de reproduire les conditions nutritionnelles des nouveaux-nés humains de faible poids de naissance. La pertinence physiopathologique chez l'homme des modifications épigénétiques induites par la nutrition chez le rat reste donc à déterminer.

Méthodes

1.3. Méthodes

1.3.1 Modèle expérimental

Nous avons utilisé le rat et la restriction en apports protéiques pendant la gestation et la lactation comme modèle de programmation métabolique ainsi qu'il est décrit dans nos précédents travaux (LOPES-DE-SOUZA *et al.*, 2008; OROZCO-SÓLIS *et al.*, 2009). En bref. Des rates gestantes ont été nourries avec un aliment contenant soit 20 % (groupe contrôle) soit 8% (groupe dénutri) de protéine dès le début de la gestation et jusqu'au sevrage des ratons. Les expériences ont été réalisées sur des ratons de 3 mois d'âge. Au cours du développement post-natal des ratons et jusqu'à leur sacrifice, leur poids corporel et leur consommation journalière de nourriture ont été évalués périodiquement.

Tableau 3. Composition de l'aliment contrôle et de l'aliment avec un faible teneur en protéines.

Ingrédients (g/Kg)	Diète Contrôle (17% Protéine)	Diète Protein-restricted (8% Protéine)
Caséine (>92,5 protéine)	183,8	84,3
Amidon de maïs	545,6	555,6
Saccharose	100	100
Huile de soja	70	70
Fibres	50	50
Mix de vitamines	10	10
Mix de minérales	35	35
Bitartrate de choline	2,5	2,5
L-methionine	3,0	3,0
BHT	0,1	0,1
Total	1000	1000

1.3.2 Analyse des conséquences de la dénutrition périnatale sur le nombre, le phénotype et les propriétés métaboliques des fibres musculaires

Des altérations dans le nombre et/ou le phénotype des fibres musculaires chez des animaux programmés métaboliquement en relation aux animaux contrôles ont été rapportées dans la littérature (BAYOL, SIMBI e STICKLAND, 2005; ZHU *et al.*, 2006; MALLINSON *et al.*, 2007; COSTELLO *et al.*, 2008) Cependant, les résultats issus de ces études étant très variables d'une étude à une autre, aucune conclusion ne peut être faite quant aux effets de la dénutrition périnatale sur la formation des fibres musculaires. De plus, aucune de ces études n'a examiné les capacités oxydatives du muscle à la suite de la dénutrition périnatale. Or, il

est fort probable que, plus que des altérations morphologiques, ce soient des modifications fonctionnelles qui se trouvent à la base des désordres métaboliques induites par la dénutrition périnatale. Dans cette première partie du projet, nous avons évalué le nombre total et la proportion des fibres de type I et de type II dans le muscle squelettique des animaux dénutris versus contrôles. Ces analyses ont été réalisées en utilisant deux types de muscles constitués, dans des conditions de repos, de différents types de fibres. A savoir, le muscle soleaire et le muscle *extensor digitorum longus* (EDL). Le premier muscle est constitué majoritairement de fibres de type I (oxydatives), tandis que les fibres de type II (glycolytiques), sont présentes de façon prépondérante dans le muscle EDL (STARON *et al.*, 1999).

Le phénotypage des fibres musculaires a été réalisé sur des coupes de muscle squelettique de 10 microns d'épaisseur en utilisant la méthode de marquage à l'ATPse après incubation dans du tampon à pH acide ou basique (STARON *et al.*, 1999). Ce type de marquage permet de différentier les fibres de type I, qui sont colorées en bleu après marquage, des fibres de type II qui ne sont pas colorées (apparaissent blanches).

Plusieurs approches expérimentales ont été utilisées afin de déterminer les conséquences de la dénutrition périnatale sur l'activité métabolique du muscle squelettique. Tout d'abord, les coupes de muscle ont été marquées avec la NADH-tetrazolium reductase. Cette enzyme est un marqueur de l'activité du complexe I de la chaine respiratoire mitochondriale et, en conséquence, est utilisée comme indicateur de la capacité oxydative des fibres musculaires squelettiques (SCHULER *et al.*, 2006a; MALLINSON *et al.*, 2007). Par la suite, les activités enzymatiques de la citrate synthase (CS, EC 2.3.3.1) de la b-hydroxyacyl-CoA dehydrogenase (b-HAD, EC 1.1.1.35) et de l'hexoquinase (HK, EC 2.7.1.1), dans des homogénats musculaires ont été déterminées par spectrométrie. Le niveau d'activité des deux premières enzymes reflète la capacité de production d'énergie par les fibres musculaires via les voies oxydatives aérobies (oxydation des acides gras, cycle de Krebs), tandis que le niveau d'activité de l'enzyme hexoquinase est un indicateur de la production d'énergie par la voie glycolytique. Enfin, les niveaux d'expression de plusieurs gènes impliqués dans la régulation de la lipolyse, la b-oxydation ou encore la chaine respiratoire mitochondriale (Tableau 4), ont été quantifiés par la méthode de PCR quantitative.

1.3.3. Evaluation des effets de la dénutrition périnatale sur la capacité de reprogrammation métabolique des fibres squelettiques

Ainsi qu'il a été mentionné, le muscle squelettique possède la remarquable capacité d'adapter son métabolisme en fonction des apports nutritionnelles et des demandes énergétiques de l'organisme. En particulier, dans des conditions de restriction nutritionnelle ou en réponse à l'exercice, le muscle utilise majoritairement les lipides comme source d'énergie. Cette adaptation métabolique s'accompagne de la reprogrammation phénotypique des fibres de type II (glycolytiques), dont les propriétés métaboliques évoluent progressivement vers un phénotype caractéristique des fibres de type I (oxydatives).

L'objectif de cette partie du projet, a été de déterminer si la plasticité métabolique du muscle squelettique à l'âge adulte est altérée par l'exposition à un environnement nutritionnel appauvri pendant la gestation et la lactation. Pour ce faire, des animaux de 3 mois contrôles et dénutris ont été privés de nourriture pendant 48h. A l'issue de ce période de jeûne, les animaux ont sacrifiés et leurs muscles soleaire et EDL disséqués. Sur les échantillons de muscle ainsi obtenus a été évaluée l'expression de plusieurs gènes impliqués dans l'oxydation lipidique, le métabolisme mitochondrial et le métabolisme de glucose (Tableau 4). Cette étude nous a permis de déterminer si les effets de la privation aigue de nourriture sur la re-programmation phénotypique des fibres squelettiques ont été altérés chez les animaux qui ont été dénutris pendant leur développement périnatal.

Au niveau moléculaire, la re-programmation phénotypique et métabolique des fibres musculaires met en jeu un complexe système de régulation de la transcription initié et coordonné par les senseurs nutritionnels AMPK et SIRT1 et le co-activateur de la transcription PGC1α. Nous avons quantifié les niveaux d'expression d'AMPK et de PGC1α dans des échantillons de muscle squelettique en provenance d'animaux contrôles et dénutris soumis ou non à une période de jeûne de 48h. Des expériences de western blot avec des anticorps dirigés contre la forme totale de l'AMPK ou contre la forme phosphorylée de la sous-unité alpha de l'AMPK (forme active), ont été également réalisées.

Tableau 4. Les gènes étudiés et leurs séquences

Gene	Symbol	Forward	Reverse	Gene Bank
Hexokinase II	HKII	GCGGTGCTGTGGCGAATC	AGCCTCCTCACTGCCTTATGG	NM_012735.2
Glucose Transporter type 4	Glut4	CAGCACTTTAGCCCTCTCTTCC	CCACAGCCTAGCCACAACAC	M25482.1
Carbohydrate response element binding protein	ChREBP	GTACTGTTCCCTGCCTGCTCTC	CCCTCTGTGACTGCCCTTGTG	FN432819.1
Carnitine palmitoyltransferase 1a	CPT1a	TGCCTGCCAGTTCCATTAAGC	GTCTCACTCCTCTTGCCAACAG	NM_031559.2
Medium chain acyl coenzyme A dehydrogenase	MCAD	GAGGCTACAAGGTCCTGAGAAGTG	TCTGCTGCTCCGTCAACTCG	NM_016986.2
Hydroxyacyl-Coenzyme A dehydrogenase	β-HAD	CTCCATGTCCTCCTCTTCCTCTGC	CAGCCCGCCGCCGATGAC	NM_057186.1
Pyruvate Dehydrogenase Kinase, Isozyme 4	PDK4	GGTGGCGGTGTTCCTCTGAG	TGAATTGTCCATCACAGGCGTTG	NM_053551.1
Citrate Synthase	CS	CTCTCTCTCCGATCCCTTCCC	AGGACGAGGCAGGATGAGTTCTTG	NM_130755.1
Cytochrome c oxidase subunit IV isoform 1	COX IV	GGCAGCAGTGGCAGAATGTTG	GAAGGCACACCGAAGTAGAAATGG	NM_017202.1
Uncoupling protein 3	UCP3	CCGTTAAGCCTTCAGCCTTCC	CGAGAGTCCATCCTGTCCTTCC	NM_013167.2
Peroxisome proliferator-activated receptor alpha	PPARα	CACGATGCTGTCCTCCTTGATG	ATGATGTCGCAGAATGGCTTCC	NM_013196.1
Peroxisome proliferator-activated receptor delta	PPARδ	ACTCTCCTTTCTTCCTGCCTGTG	TGTGCTGCTGCTCCTTCTGG	NM_013141.2
Peroxisome proliferator-activated receptor gamma, co-activator 1	PGC1α	ACACCGCACACATCGCAATTC	TTCGTCCCTCTTGAGCCTTTCG	NM_031347.1
Myosin Heavy chain 7	MHCI	ACAGAGGAAGACAGGAAGAACCTAC	GGGCTTCACAGGCATCCTTAG	NM_017240.1
Protein kinase AMP-activated alpha 1	AMPKα1	TTGCGTGTGCGAAGGAAGAAC	CCAAATCAGGGACTGCTACTCCA	NM_019142.1
Protein kinase AMP-activated alpha 2	AMPKα2	GATGATGAGGTGGTGGAGCAGAC	CACTGTCTGGCTCTCTCACTGC	NM_023991.1
Ribosomal 18S	18S	GATGCGGCGGCGTTATTC	CTCCTGGTGGTGCCCTTCC	M11188.1

Résultats

1.4. Résultats

Les résultats expérimentaux de cette thèse se sont traduits par la rédaction de deux manuscrits. Le premier, intitulé « **Differential developmental programming by early protein restriction of rat skeletal muscle according to its fibre type composition** » est sous publication dans journal Acta Physiologica et il est présenté dans son intégralité de la page 163 à page 176. Le deuxième article, intitulé « **Nutritional programming impairs metabolic flexibility of skeletal muscle before any sign of obesity or glucose intolerance** » a été soumis au journal Obesity et il est présenté dans son intégralité de la page 179 à la page 207

Discussion générale

1.5 Discussion Générale

La malnutrition protéique maternelle pendant la gestation et la lactation chez le rat est un modèle expérimental de programmation métabolique bien établit (OZANNE *et al.*, 1996a; OZANNE *et al.*, 1996b; OZANNE *et al.*, 1998; OZANNE e HALES, 1999; OZANNE *et al.*, 1999; OZANNE *et al.*, 2003a; OZANNE *et al.*, 2004; TOSCANO, MANHAES-DE-CASTRO e CANON, 2008; LEANDRO *et al.*, 2012). Nous avons utilisé ce modèle pour déterminer les effets à long terme de la malnutrition périnatale sur l'activité enzymatique, les caractéristiques structurales ainsi que sur la flexibilité métabolique du muscle squelettique. Deux muscles possédant de propriétés métaboliques et structurales différentes ont été étudiés : le muscle soléaire et le muscle EDL.

Il a été montré de façon répétée dans la littérature que la malnutrition protéique maternelle induit une réduction du poids corporel chez la descendance qui persiste après leur récupération nutritionnelle. C'est-à-dire, lorsque les animaux sont nourris avec un aliment standard après sevrage (TOSCANO, MANHAES-DE-CASTRO e CANON, 2008; LEANDRO *et al.*, 2012). En accord avec ces résultats de la littérature, nous avons observé une diminution significative du poids corporel chez les animaux adultes nés de mères nourries pendant la gestation et la lactation avec un aliment pauvre en protéines. En outre, ces animaux ont présenté un pourcentage plus faible de masse grasse abdominale associé à une diminution du taux plasmatique de triglycérides et d'insuline dans des conditions d'ingestion de nourriture *ad libitum*. En revanche, les animaux dénutris ont présenté la même tolérance au glucose que les animaux contrôles. Ces dernières résultats concordent avec les observations de Fidalgo et collaborateurs (2012) qui, en utilisant le même modèle expérimental, ont rapporté que les animaux de 145 jours programmés métaboliquement ne présentent pas d'intolérance au glucose ou de résistance à l'insuline. Ces dernières observations contrastent cependant avec les données obtenues par Ozanne et collaborateurs (1996) indiquant que le muscle squelettique des ratons nés de mères dénutries présente une meilleure sensibilité à l'insuline. Ces résultats contradictoires peuvent s'expliquer par les différences d'âge des animaux au moment des expériences. A savoir, 120 jours dans le cas du groupe d'Ozanne et collaborateurs *versus* 145 jours dans le cas du groupe de Fidalgo et collaborateurs. De fait, Ozanne et collaborateurs ont montré par la suite que les animaux de 15 mois d'âge qui ont été dénutris pendant leur développement périnatal présentent de la résistance à l'insuline et une

masse grasse abdominale beaucoup plus importante que les animaux contrôles (OZANNE *et al.*, 2003a).

Nos résultats montrent également que le phénotype du muscle squelettique est modifié par la malnutrition maternelle. De fait, nous avons observé une augmentation du nombre de fibres IIA et IIB (glycolytiques), dans le muscle soléaire, mais pas dans l'EDL, des animaux dénutris. De façon intéressante, il a été rapporté que le muscle squelettique des patients obèses possède un plus grand nombre de fibres de type IIB para rapport au muscle des personnes de poids normal et qu'il existe une corrélation positive entre l'IMC et le pourcentage de fibres de type IIB dans le muscle (TANNER *et al.*, 2002a). Aussi, le fait que la proportion de fibres glycolytiques dans le muscle squelettique des animaux dénutris soit augmentée avant toute manifestation d'obésité ou de résistance à l'insuline, indique que ces altérations peuvent constituer un indicateur précoce du risuae de développement du syndrome métabolique.

Nos résultats sur les effets induits par la dénutrition périnatale sur le nombre des fibres de type IIA, concordent avec les observations de Toscano et al., indiquant que la restriction en apports protéiques à la mère pendant la gestation augmente la proportion des fibres de type IIA dans le muscle soléaire de la progéniture dès l'âge de 25 jours (TOSCANO, MANHAES-DE-CASTRO e CANON, 2008). Toutefois, contrairement à ce que nous avons observé, Toscano et al., ont rapporté une diminution de la proportion des fibres de type IIB dans le muscle EDL des animaux dénutris. Par ailleurs, d'autres auteurs ont rapporté que la dénutrition au cours du développement précoce réduit le nombre de fibres squelettiques (BAYOL *et al.*, 2004; YATES *et al.*, 2012), (ZHU *et al.*, 2004; QUIGLEY *et al.*, 2005). Ces résultats contradictoires peuvent s'expliquer par le type de restriction nutritionnelle appliquée (multi-calorique ou protéique), la durée et la période pendant lesquelles les mères ont été exposées à la dénutrition (gestation uniquement ou gestation plus lactation), ainsi que par le type de muscle étudié. En effet, étant donné que la composition en type de fibres varie d'un type de muscle à un autre, les différents muscles peuvent être affectés différemment par des stimuli nutritionnels. A cet égard, il est intéressant de noter que, dans notre étude, le muscle le plus touché par la dénutrition périnatale a été le muscle soléaire. Ce muscle joue un rôle clé dans le maintien posturale aussi bien chez les bipèdes que chez les quadrupèdes. Il est par ailleurs très riche en fibres oxydatives et sa différentiation débute au stade embryonnaire et se poursuit lors des premiers jours de développement post-natal de telle sorte que la gestation et la lactation sont les périodes où le muscle soléaire est le plus sensible aux effets

programmateurs de l'environnement (BARBET *et al.*, 1991; BARBET, THORNELL e BUTLER-BROWNE, 1991; WIGMORE e DUNGLISON, 1998; MALTIN *et al.*, 2001; TE e REGGIANI, 2002). Le cursus temporaire de différentiation du soléaire pourrait donc expliquer pourquoi il est plus vulnérable à la malnutrition pendant la période périnatale.

Nous avons également évalué l'activité enzymatique de trois enzymes clé des différentes voies métaboliques. A savoir, l'héxokinase (HK), la citrate synthase (CS) et la beta-Hydroxyacyl-CoA déshydrogénase (β-HAD). La dénutrition périnatale n'a pas affecté l'activité basale de ces enzymes à l'exception de l'héxoquinase, dont l'activité a été réduite dans le muscle soléaire des animaux dénutris. Cependant, le rapport des activités enzymatiques HK/CS et β-HAD/CS ont été réduits dans le muscle soléaire des animaux dénutris indiquant que la carence en apport protéiques pendant la période précoce du développement entraîne une diminution de la capacité glycolytique et oxydative du muscle squelettique. Il est à noter que nous avons sacrifié les animaux une heure après la fin du cycle obscur dans des conditions d'ingestion de nourriture *ad libitum.* C'est-à-dire, pendant la phase postprandiale au cours de laquelle les niveaux plasmatiques d'insuline, et par conséquent l'absorption de glucose par le muscle, sont optimisées. Le fait que dans ces conditions les animaux dénutris présentent une diminution de l'activité de l'héxokinase indique que leur capacité à métaboliser le glucose est altérée. De façon intéressante, dans une étude en parallèle nous avons observé que les animaux dénutris sacrifiés dans des conditions d'ingestion de nourriture *ad libitum*, présentent un faible niveau d'activation d'Akt (Guzman-Quevedo, en préparation), indiquant une altération de la voie de signalisation de l'insuline. En relation avec ces résultats, il est intéressant de noter aussi que les patients obèses et diabétiques présentent une diminution des niveaux d'expression de l'héxokinase II et une moindre efficacité de l'insuline à stimuler l'activité de cette enzyme (PENDERGRASS *et al.*, 1998).

Dans le but de déterminer les mécanismes moléculaires pouvant être à l'origine des altérations enzymatiques induites par la dénutrition périnatale, nous avons analysé le profil d'expression de plusieurs gènes impliqués dans la glycolyse ou la beta-oxydation. Nous n'avons observé aucune différence significative entre les animaux contrôles et dénutris dans le niveau d'expression des gènes régulant le métabolisme du glucose. De façon similaire, le muscle squelettique des animaux contrôles et dénutris ont presenté le même niveau d'expression des ARNm codant pour la chaîne lourde de la myosine 7, un marqueur de fibres

de type I, ou pour l'AMPK, une kinase impliquée dans la détection des faibles quantités d'énergie. En revanche, la dénutrition périnatale a affecté de façon significative l'expression de plusieurs gènes impliqués dans l'oxydation des acides gras. En particulier, nous avons observé une expression accrue de CPT1a et d'UCP3. Le premier de ces gènes favorise le transport des acides gras à l'‘intérieur de la matrice mitochondriale où ils seront dégradés sous l'action de plusieurs enzymes dont la MCAD et la β-HAD. Pour ce qui est de l'UCP3, ce gène possède plusieurs fonctions. D'une part il permet de découpler l'oxydation des acides gras de la production d'ATP de façon à produire de la chaleur au lieu de l'énergie et, d'autre part, il réduit l'accumulation d'espèces réactives de l'oxygène (ERO), en expulsant de la matrice mitochondriale les anions issus de la beta-oxydation des acides gras (SCHRAUWEN *et al.*, 2010; VAN DEN BERG *et al.*, 2011). Grâce à ces actions l'UCP3 augmente la capacité oxydative de la mitochondrie et évite le dommage cellulaire induit par un excès d'espèces réactives de l'oxygène. L'augmentation de l'expression de CPT1a et d'UCP3 induite par la dénutrition périnatale indique donc que le muscle des animaux dénutris possède une plus grande capacité d'oxydation des acides gras que celui des animaux contrôles. Cependant, comme indiqué précédemment, l'activité de la β-HAD reste inchangée par la dénutrition périnatale. De plus, les muscles squelettiques des animaux contrôles et dénutris présentent le même niveau d'expression des ARNm codant pour la MCAD et la β-HAD.

Ces derniers résultats indiquent que l'augmentation du transport d'acides gras dans la matrice mitochondriale consécutive à l'accroissement de l'expression de CPT1a ne se traduit pas par une augmentation de la beta oxydation. En accord avec cette idée, l'activité de l'enzyme citrate synthase, un marqueur de la capacité de phosphorylation oxydative mitochondriale, n'a pas été affectée par la dénutrition périnatale. En fait, le taux d'oxydation des acides gras dans le muscle soléaire est en réalité réduit chez les animaux dénutris comme l'indiqué la diminution de la capacité d'oxydation des acides gras à fournir des substrats pour la phosphorylation mitochondriale (rapport β-HAD/CS).

Dans leur ensemble, les résultats de nos expériences de PCR quantitative et la mesure des activités enzymatiques favorisent un scénario dans lequel la restriction et apports protéiques pendant la grossesse et l'allaitement augmente l'utilisation des lipides comme source d'énergie dans le muscle soléaire en stimulant l'entrée des acides gras dans la matrice mitochondriale via une augmentation de l'expression de CPT1a. Cependant, comme de façon concomitante la dénutrition périnatale diminue les capacités oxydatives du muscle

squelettique, l'augmentation d'expression de CPT1a, et vraisemblablement de son activité, conduit à l'accumulation des produits intermédiaires du métabolisme des acides gras au sein de la matrice mitochondriale. Cette accumulation, à son tour, inhibe l'activité de l'héxokinase (THOMPSON e COONEY, 2000) et active l'expression d'UCP3 aboutissant à la translocation vers l'extérieur d'acides gras à partir de la matrice mitochondriale. Aussi, la restriction en apports protéiques pendant le développement précoce semble, paradoxalement, augmenter le transfert des acides gras dans la matrice mitochondriale sans que cette translocation accrue se traduise par une augmentation de la production d'énergie.

De façon intéressante, nous avons également observé une augmentation de l'expression de PGC1α qui, en agissant comme co-activateur transcriptionnel de plusieurs récepteurs nucléaires et d'autres facteurs de transcription (KNUTTI e KRALLI, 2001), joue un rôle clé dans la régulation du métabolisme énergétique y compris la stimulation de la biogenèse mitochondriale et la modulation de la glycolyse et de la beta-oxydation (FINCK e KELLY, 2006). D'ailleurs, l'augmentation de l'expression de PGC1α s'est accompagnée d'une expression accrue de ses gènes cibles CPT1a et UCP3. De variations d'expression de PGC-1a en réponse à la dénutrition périnatale ont été également rapportées par d'autres auteurs. Ainsi, le retard de croissance utérin chez le rat induit par l'occlusion de l'artère utérine pendant les derniers jours de gestation, diminue l'expression de PGC1α dans le muscle soléaire (LANE *et al.*, 2003) mais augmente son expression dans l'EDL (LANE *et al.*, 2003). Toutefois, il convient de préciser que, contrairement à notre étude, ces résultats ont été obtenus chez des très jeunes animaux (de rats de 21 jours), de telle sorte qu'il est ne peut pas être déterminé si ces variations différentielles de l'expression de PGC1α en fonction du type de muscle correspondent à une véritable programmation métabolique ou si elles sont le résultat de l'état de dénutrition dans lequel se trouvent les animaux au moment du sacrifice. Un autre facteur qui pourrait expliquer les différences entres les observations de Lane et al., et nos résultats est le modèle de programmation métabolique utilisé.

L'expression de PGC1α est régulée positivement par déacetylation via le senseur nutritionnel Sirt1 (NEMOTO, FERGUSSON e FINKEL, 2005), et cette désacétylation est nécessaire et concomitante à l'augmentation de son activité pendant le jeûne (GERHART-HINES *et al.*, 2007; RODGERS *et al.*, 2008a). L'activité de PGC1α est également régulée par phosphorylation (FERNANDEZ-MARCOS e AUWERX, 2011) soit via Akt soit via AMPK. Ces deux kinases phosphorylent toute fois des résidus différents au sein de la structure de

PGC1α et exercent des effets opposés sur son activité. Ainsi, alors que la phosphorylation de la sérine en position 570 de PGC1α par Akt réduit son activité, la phosphorylation de la sérine 538 ou de la thréonine 177 par AMPK augmente l'activité co-transcriptionnelle de PGC1α (FERNANDEZ-MARCOS e AUWERX, 2011). Grâce à des expériences réalisées en parallèle au sein de notre groupe de travail, nous avons constaté que le niveau de phosphorylation d'AMPK est augmentée dans le muscle soléaire des animaux adultes nés de mères nourris pendant la gestation et la lactation avec un aliment pauvre en protéines (Guzmán-Quevedo, soumis). Le niveau d'acétylation de PGC1α dans le muscle squelettique de ces animaux est également réduit (Guzmán-Quevedo, en préparation). Ces observations suggèrent que l'augmentation d'expression de PGC1α a chez les animaux dénutris pourrait être consécutive aux modifications de l'activité d'AMPK et de Sirt1. De façon intéressante, ces deux facteurs nutritionnels s'activent lorsque les apports énergétiques sont faibles (jeûne ou restriction calorique), où dans des conditions de forte demande énergétique (exercice). Sur la base de ces observations, on peut spéculer que la dénutrition périnatale altère de façon permanente l'activité des senseurs nutritionnels et que cette altération est l'un des mécanismes fondamentaux de la programmation métabolique.

Ainsi qu'il a déjà été mentionné, le muscle squelettique est l'un des organes le plus importants en termes de masse corporelle et de capacité métabolique. Chez l'adulte, 80% du glucose postprandial est captée par le muscle squelettique sous l'action de l'insuline et les cellules musculaires sont responsables de 20-30% de la dépense énergétique globale de l'organisme. En outre, le muscle squelettique a une remarquable capacité pour adapter son métabolisme en réponse à l'apport calorique et les exigences énergétiques de l'organisme. En fait, la privation de nourriture ou la consommation d'un aliment riche en graisses, favorise l'utilisation des lipides par le muscle squelettique comme source d'énergie au détriment de glucose, un phénomène connu sous le nom de flexibilité métabolique. Une altération de la flexibilité métabolique a été associée à l'obésité et l'insulino-résistance (KELLEY *et al.*, 1999; STORLIEN, OAKES e KELLEY, 2004; THYFAULT, RECTOR e NOLAND, 2006), mais les mécanismes qui établissent le lien entre l'inflexibilité métabolique du muscle squelettique et ces entités pathologiques ne sont pas connus. Nous ignorons également si le défaut d'adaptation du muscle aux variations énergétiques est la cause ou la conséquence de l'obésité où du diabète.

L'objectif des expériences réalisées au cours de la deuxième partie de cette thèse, a été de définir les conséquences de la restriction protéique pendant la gestation et la lactation sur la plasticité métabolique du muscle squelettique à l'âge adulte. Notre hypothèse de travail étant que la restriction en apports nutritionnels pendant la période critique de développement périnatal altère la capacité du muscle squelettique à s'adapter à l'utilisation des substrats énergétiques (lipides, de glucose) en réponse aux besoins nutritionnels et énergétiques de l'organisme. Pour évaluer la flexibilité métabolique du muscle squelettique nous avons subdivisé chacun de nos groupes expérimentaux (contrôles et dénutris), en trois sous-groupes. Le premier de ces sous-groupes a été sacrifié dans des conditions d'ingestion de nourriture *ad libitum* tandis que les autres deux sous-groupes ont été sacrifiés après une période de jeûne de 48h.

Nous avons choisi le jeûne comme stimulus parce que la privation de nourriture entraîne des adaptations métaboliques rapides dans le du muscle squelettique qui se traduisent par l'utilisation des acides gras comme source d'énérgie en lieu et place de l'oxydation du glucose. Ce phénomène d'adaptation métabolique est sous-tendu par un mécanisme de régulation transcriptionnel très complexe qui met en jeu les récepteurs nucléaires PPARα et PPARδ, le co-régulateur de la transcription PGC1α ainsi que les senseurs nutritionnels SIRT1 et l'AMPK (BASSEL-DUBY e OLSON, 2006b; JORGENSEN, RICHTER e WOJTASZEWSKI, 2006; RODGERS *et al.*, 2008a). Ces deux derniers facteurs, dont l'activation est déclenchée par des conditions physiologiques qui nécessitent une grande quantité d'énergie telles que la restriction alimentaire ou l'exercice, induisent l'expression de PGC1α et augmentent son activité soit par désacétylation (SIRT1), ou par phosphorylation (AMPK) (CANTO e AUWERX, 2009). L'expression de PGC1α peut également être augmentée par les récepteurs nucléaires PPARδ (SCHULER *et al.*, 2006b) ou via une voie de signalisation dépendante du calcium impliquant l'activation du facteur de transcription CREB (CHOWANADISAI *et al.*, 2010). Une fois activé, PGC-1α facilite l'expression de plusieurs gènes impliqués dans l'oxydation des acides gras et la biogenèse mitochondriale en formant un complexe avec les récepteurs nucléaires PPARα (VEGA, HUSS e KELLY, 2000) et, simultanément, régule la reprogrammation des fibres glycolytiques (type II), en fibres oxydatives (type I), en agissant en tandem avec le gène myosine Enhancer Factor 2 (LIN *et al.*, 2002b).

Les variations d'expression génique du muscle squelettique en réponse au jeûne sont dépendants du temps. Ainsi, l'expression de PPARδ et PGC1α augmente initialement avec la privation de nourriture, mais après 48h de jeûne leur expression diminue en dessous des niveaux observés chez les animaux contrôles (DE LANGE *et al.*, 2006). En accord avec ces résultats, nous avons observé que la privation de nourriture pendant 48h réduit l'expression de ces deux facteurs de transcription ainsi que celle de PPARα aussi bien dans le muscle soléaire des rats dénutris que des rats témoins. Cependant, nous avons constaté une nette différence entre les rats témoins et les rats dénutris dans le profil d'expression des gènes CPT1a, MCAD et β-HAD en réponse au jeûne. En fait, les niveaux des ARNm codant pour le premier gène ont été augmentés par la privation de nourriture chez les contrôles mais pas chez les rats dénutris. En outre, alors que les rats dénutris exposés au jeûne présentent une diminution de l'expression de MCAD et du β-HAD par rapport à leurs homologues nourris *ad libitum*, la privation de nourriture n'induit aucun changement dans l'expression de ces gènes chez les animaux témoins. Dans leur ensemble, ces changements d'expression indiquent que la capture et l'oxydation des acides gras en réponse à jeûne sont diminuées dans le muscle soléaire des rats dénutris.

Par ailleurs, le muscle soléaire et le muscle EDL ont réagi différemment à la privation de nourriture et leur flexibilité métabolique a été affectée de façon différentielle par la restriction en apports protéiques pendant le développement précoce. Ainsi, alors que le jeûne diminue l'expression des régulateurs de la transcription du métabolisme des lipides (PPARα, PPARδ, PGC1α) dans le muscle soléaire aussi bien des rats dénutris que des rats témoins, dans le muscle EDL ces changements ont été observés uniquement dans les rats souffrant de malnutrition périnatale. Par ailleurs, en contraste avec ses effets dans soléaire, le jeûne induit un profil d'expression génique propre d'une augmentation de la β-oxydation et d'une diminution de la glycolyse dans EDL des rats dénutris. Collectivement, ces résultats montrent que le jeûne modifie d'une manière très fine et sélective les propriétés métaboliques du muscle squelettique.

Kelly et al., ont été les premiers à proposer que l'inflexibilité métabolique du muscle squelettique, définie comme une diminution de l'oxydation des acides gras dans des conditions de jeûne ou comme une capacité réduite pour passer de l'oxydation des lipides à l'utilisation du glucose comme source énergétique pendant la transition de jeûne à la réalimentation, est associée à l'obésité et le diabète et prédit la sévérité de la résistance à

l'insuline (KELLEY *et al.*, 1999). D'autres auteurs ont par la suite corroboré que la capacité d'oxydation des acides gras pendant le jeûne ou l'exercice du muscle squelettique de patients obèses ou diabétiques est diminué de même qua sa capacité à basculer de l'oxydation de lipides à l'oxydation du glucose sous la stimulation de l'insuline (BLAAK *et al.*, 2000; THYFAULT, RECTOR e NOLAND, 2006). Cependant, il n'a pas été déterminé si l'inflexibilité métabolique est une manifestation supplémentaire de l'obésité et du diabète ou si elle la cause de ces troubles métaboliques.

Les résultats que nous avons obtenus pendant la deuxième partie de cette thèse montrent que les rats âgés de 4 mois qui ont été dénutris pendant la gestation et la lactation, présentent un profil d'expression génique modifié dans le muscle squelettique en réponse au jeûne. Le fait que, à cet âge, ces animaux ne montrent pas encore aucun signe d'intolérance au glucose ou d'obésité, suggère que l'inflexibilité métabolique pourrait être la cause et non la conséquence de l'obésité et du diabète. De plus, la restriction en apports protéiques au cours du développement précoce affecte de façon différentielle les réponses de muscles soléaire et EDL au jeûne indiquant que l'inflexibilité métabolique du muscle squelettique pourrait dépendre du type de fibre.

En conclusion, les résultats obtenus au long de la réalisation de cette thèse, indiquent que les rats adultes qui ont été dénutris pendant leur développement périnatal, présentent dans des conditions d'alimentation *ad libitum* un profil d'expression génique dans le muscle squelettique favorisant le transport des acides gras à longue chaîne dans la matrice mitochondriale sans une augmentation concomitante de l'oxydation des acides gras. En outre, le réseau transcriptionnel régulant la transition métabolique entre l'utilisation du glucose et l'oxydation des acides gras comme source énergétique en réponse au jeûne est clairement altéré chez les animaux dénutris qui, par ailleurs, présentent un profil métabolique normal.. Cette dernière observation indique que l'altération de la flexibilité métabolique du muscle squelettique précède et peut être à l'origine du développement d'obésité et de l'insulino-résistance induite par la dénutrition périnatale. Étant donné que les effets délétères de la malnutrition précoce se développent avec l'âge, d'autres études sont nécessaires afin de déterminer les mécanismes par lesquels les altérations de la plasticité métabolique du muscle squelettique rapportées dans cette mémoire contribuent au développement de l'obésité et de l'insulino-résistance.

Perspectives

1.6 Perspectives

Nous avons mis en évidence au cours de cette thèse que la réduction en apports protéiques à la mère pendant la gestation et l'allaitement induit des modifications sur le long terme des propriétés structurales et métaboliques du muscle squelettique chez la descendance. Ces modifications varient d'un muscle à un autre en fonction de leur composition en type de fibres. Ces résultats ouvrent plusieurs perspectives de travail. Par exemple, il serait intéressant d'identifier les modifications épigénétiques qui sous-tendent les différences d'expression génique dans le muscle des animaux dénutris versus contrôles. Ceci est particulièrement pertinent dans le cas du co-régulateur de la transcription PGC-1α qui joue un rôle clé dans la régulation du métabolisme du muscle squelettique et dans ses réponses adaptatives aux variations en apports nutritionnels et aux besoins énergétiques de l'organisme.

Par ailleurs, sachant qu'en dehors de cellules musculaires différenciées le muscle squelettique contient des cellules satellites (souches), qui restent dans un état quiescent pendant le développement mais dont la prolifération et la différentiation peuvent être déclenchées en réponse à des stimuli environnementaux, comme le croissance, le trauma, ou l'exercice, il serait extrêmement important de déterminer si ces cellules portent une empreinte épigénetique de la dénutrition périnatale et/ou si leur prolifération et différentiation, voir leur fonction, sont altérées par la programmation métabolique. Dans ce contexte, au moins trois perspectives de recherche se dégagent de ce travail :

- Des études des altérations épigénétiques induites par la dénutrition maternelle sur le muscle squelettique ;

- L'analyse comparative du profil et de la fonction métabolique des cellules satellites musculaires issues d'animaux contrôles et dénutris.

- Des études pharmacologiques *in vitro* sur l'action de l'insuline, des lipides ou des agonistes de récepteurs nucléaires qui modulent le métabolisme du muscle squelettique.

APRESENTAÇÃO

2 Apresentação

Numerosos estudos indicam ligação direta entre o retardo de crescimento fetal e infantil e o risco maior de desenvolver doenças cardiovasculares e metabólicas na vida adulta nos mamíferos. Para explicar essa correlação, foi proposto que um organismo quando submetido ao ambiente pobre em nutrientes durante o desenvolvimento, modifica o seu metabolismo de tal forma que suas chances de sobrevivência sejam aumentadas. De forma geral, o crescimento cerebral é preservado em detrimento do desenvolvimento de outros órgãos. Desta forma, o organismo estaria "programado" para desenvolver-se em um ambiente constantemente pobre em nutrientes. Entretanto, se após esse período, se o organismo for exposto a um ambiente rico ou desequilibrado nutricionalmente, a incompatibilidade entre a sua "programação" fisiológica e as novas condições nutricionais torna-o mais susceptível ao aparecimento de doenças cardiovasculares e da síndrome metabólica.

Vários estudos experimentais corroboram com a hipótese da programação metabólica. Estudos realizados em diversas espécies animais demonstram que descendentes de mães que sofreram algum tipo de restrição nutricional durante gestação e/ou lactação apresentam alterações próprias da síndrome metabólica, como obesidade, dislipidemias, entre outros. Entretanto, os mecanismos pelos quais o ambiente de desenvolvimento precoce interfere no metabolismo na vida adulta ainda não são totalmente esclarecidos.

O músculo esquelético é um órgão de grande importância para o organismo, seja em termos de massa corporal seja em sua capacidade metabólica. Ele é responsável pela maior parte da captação da glicose pós-prandial. Contudo, durante o desenvolvimento precoce, ele está entre os órgãos que têm seu metabolismo alterado para que o sistema nervoso central possa desenvolver-se normalmente. Desta forma, usualmente, tem-se como indicador de restrição de crescimento fetal o baixo peso ao nascer. Isso é devido, principalmente, à diminuição da massa muscular.

O sistema muscular também apresentada grande plasticidade pós-desenvolvimento, podendo mudar sua fonte de energia, entre glicose e ácidos graxos, dependendo das condições nutricionais que se apresentem. Assim, considera-se que este sistema teria flexibilidade para melhor atender as necessidades fisiológicas.

Desta forma, esta tese tem como **pergunta condutora:** Quais as alterações que a desnutrição proteica materna programa, em termos bioquímicos e moleculares, sobre o metabolismo do sistema muscular, relacionando-as ao posterior desenvolvimento da síndrome metabólica? Além disso, este sistema mesmo "programado" ainda guarda capacidade de flexibilidade metabólica quando submetido à alta demanda energética?

Assim, o **objetivo geral** deste trabalho foi analisar as repercussões, em termos histológicos, bioquímicos e moleculares, da desnutrição proteica materna sobre a função metabólica e a plasticidade do músculo esquelético em ratos.

Os **objetivos específicos** foram definir, na prole aos 120 dias, as possíveis consequências da desnutrição proteica materna sobre:

- o fenótipo corporal e parâmetros bioquímicos plasmáticos;

- a proporção dos diferentes tipos de fibras musculares;

- as propriedades enzimáticas do músculo esquelético;

- a expressão global de genes relacionados às diferentes vias metabólicas;

- e a capacidade plástica muscular em resposta à alta demanda energética.

Nossa **hipótese** é que desnutrição proteica materna programa o sistema muscular esquelético da prole para melhor aproveitamento de lipídios como fonte energética. Entretanto, essa desnutrição diminui a sua capacidade de resposta ao aumento da demanda energética. Estas alterações predispõem o organismo ao desenvolvimento de síndrome metabólica.

Para dar subsídios teóricos a essa hipótese, serão apresentados na revisão bibliográfica elementos do conceito de programação perinatal e alterações estruturais e funcionais já observadas em outros trabalhos com a mesma temática. Além da apresentação do desenvolvimento do músculo esquelético, suas propriedades fenotípicas e funcionais.

Para testar esta hipótese, optou-se por realizar a desnutrição por redução do aporte de proteínas na dieta materna durante a gestação e lactação. A prole, onde os efeitos da desnutrição foi estudada, apresentava desnutrição induzida não somente pela redução da

passagem de proteína durante a gestação, como também redução no conteúdo de proteína no leite materno e no volume do mesmo ofertado.

Os resultados oriundos deste trabalho são apresentados em forma de artigos. Estes foram enviados para publicação. O primeiro artigo, intitulado "**Differential developmental programming by early protein restriction of rat skeletal muscle according to its fibre type composition**", foi aceito para publicação no jornal Acta Physiologica (Anexo A). O segundo artigo, intitulado "**Nutritional programming impairs metabolic flexibility of skeletal muscle before any sign of obesity or glucose intolerance**", foi submetido ao jornal Obesity (Anexo B).

Além dos artigos submetidos à publicação, os resultados desta tese foram apresentados resumos em congressos de âmbito nacional e internacional.

- **Raquel da Silva Aragão**; Omar Guzmá-Quevedo; Georgina Perez-Garcia; Raul Manhães-de-Castro; Francisco Bolaños-Jímenez. Perinatal protein restriction alters in the long term the metabolic plasticity of skeletal muscle. 6th Congress of the International Society of Nutrigenetics/Nutrigenomics. São Paulo – SP. 2012.
- **ARAGÃO, R. S.**, Guzmán-Quevedo, O., Pérez García, G, Manhães de Castro, R, Bolaños-Jímenez, F Perinatal undernutrition impairs the metabolic plasticity of skeletal muscle, In: I Simpósio Internacional de Mitocôndria e Metabolismo, 2012, Vitória de Santo Antão.
- **ARAGÃO, R. S.,** Guzmán-Quevedo, O., Pérez García, G, Toscano, A. E., Manhães de Castro, R, Bolaños-Jímenez, F. Protein restriction during early life induces long-lasting changes on the functional and structural properties of rat skeletal muscle In: XXVII Reunião Anual da Federação de Sociedades de Biologia Experimental, 2012, Águas de Lindóia.
- **da Silva Aragão, R.,** Guzmán-Quevedo, O., Pérez García, G, Lopes de Souza, S, Manhães de Castro, R, Bolaños-Jímenez, F Analysis of the consequences of perinatal undernutrition on the metabolic plasticity of skeletal muscle In: 11th European Nutrition Conference (FENS), 2011, Madri. Annals of Nutrition and Metabolism. Karger, 2011. v.58. p.1 - 444

Ademais das pesquisas ligadas diretamente à tese, alguns trabalhos foram desenvolvidos em paralelo. Sendo publicados os seguintes artigos :

- Elizabeth Nascimento, Omar Guzman-Quevedo, Nellie Delacourt, **Raquel da Silva Aragão,** Georgina Perez-Garcia, Sandra Lopes de Souza, Raul Manhães-de-Castro, Francisco Bolaños-Jiménez, Bertrand Kaeffer. Long-Lasting Effect of Perinatal Exposure to Ltryptophan on Circadian Clock of Primary Cell Lines Established from Male Offspring Born from Mothers Fed on Dietary Protein Restriction. PLoS One. 2013;8(2):e56231.
- **Aragão, Raquel da Silva**, Rodrigues, Marco Aurélio Benedetti, de Barros, Karla Mônica Ferraz Teixeira, Silva, Sebastião Rogério Freitas, Toscano, Ana Elisa, de Souza, Ricardo Emmanuel, Manhães-de-Castro, Raul Automatic system for analysis of locomotor activity in rodents - A reproducibility study. Journal of Neuroscience Methods. , v.195, p.216 - 221, 2011.
- MAGALHAES, C. P., CAMPINAS, R. C. F., BORBA, T. K. F., LIRA, L. A., **ARAGÃO, R. S**., LOPES DE SOUZA, S., MANHÃES-DE-CASTRO, R.Programação Perinatal e o Comportamento Emocional em Ratos. Neurobiologia (Recife. Impresso). , v.74, p.83 - 95, 2011.
- Pereira, K.N.F., VITORIANO, I. L. S., Melo, M.P.P., **ARAGÃO, R. S.,** TOSCANO,A.E., TOSCANO,A.E., Silva, H.J., CASTRO, R. M. Effects of malnutrition and/or neonatal inhibition of serotonin reuptake in neuromuscular development of the gastrointestinal tract: review of literature. Neurobiologia (Recife. Impresso). , v.72, p.145 - 154, 2009.
- FERRAZ-PEREIRA, K. N., VITORIANO, I. L. S., MELO, M. P. P., **ARAGÃO, R. S.,** Toscano, A. E., SILVA, H. J., MANHÃES-DE-CASTRO, R. Repercussões da Desnutrição e/ou da Inibição Neonatal da Recaptação de Serotonina no Desenvolvimento Neuromuscular do Trato Gastrintestinal: Revisão da Literatura. Neurobiologia (Recife. Impresso). , v.72, p.145 - 154, 2009.

Os seguintes artigos foram submetidos ou estão em preparação:

- Omar Guzmán-Quevedo; **Raquel Da Silva Aragão**; Georgina Pérez García; Rhowena Jane Barbosa Matos; Raul Manhães de Castro; Francisco Bolaños-Jiménez

(Submetido à PLoS ONE). Impaired hypothalamic mTOR activation in the adult rat offspring born to mothers fed a low-protein diet.

- Georgina Pérez-García, Omar Guzmán-Quevedo, **Raquel da Silva Aragão**, Raul Manhães de Castro, Francisco Bolaños-Jiménez (A ser submetido ao Journal of Physiology). THE COGNITIVE DEFICITS INDUCED BY PERINATAL UNDERNUTRITION ARE ASSOCIATED WITH ALTERED NEUROGENESIS IN RESPONSE TO A LEARNING TASK

E resumos foram apresentados em congressos nacionais e internacionais :

- Guzmán-Quevedo, O., **da Silva Aragão, R.,** Pérez García, G, Lopes de Souza, S, Bolaños-Jímenez, F. Analysis of the impact of perinatal under-nutrition on hypothalamic nutrient sensing involving mTOR In: 11th European Nutrition Conference (FENS), 2011, Madri. Annals of Nutrition and Metabolism. Karger, 2011. v.58. p.1 – 444.
- Pérez García, G, Guzmán-Quevedo, O., **da Silva Aragão, R.,** Lopes de Souza, S, Bolaños-Jímenez, F. Effects of perinatal protein-restriction on hippocampal neurogenesis in respose to a learning task In: 11th European Nutrition Conference (FENS), 2011, Madri. Annals of Nutrition and Metabolism. Karger, 2011. v.58. p.1 - 444
- SANTOS, P. C. P., **ARAGÃO, R. S.,** BARROS, K. M. F. T., MANHÃES-DE-CASTRO, R. INFLUÊNCIA DA ADMINISTRAÇÃO DE AGONISTAS 5-HT1A SOBRE A LOCOMOÇÃO EM RATOS HIPERNUTRIDOS In: XXV Reunião Anual Federação de Sociedades de Biologia Experimental (FeSBE), 2010, Águas de Lindóia.
- **ARAGÃO, R. S.,** SANTOS, P. C. P., ARAUJO-FILHO, J. C., RODRIGUES, M. A. B., BARROS, K. M. F. T., MANHÃES-DE-CASTRO, R. A MANIPULAÇÃO NEONATAL COM BUSPIRONA ALTERA O DESENVOLVIMENTO DA LOCOMOÇÃO ESPONTÂNEA EM RATOS? In: XXIV Reunião Anual da FeSBE, 2009, Águas de Lindóia.
- LEITE, R. M. P., ESTABILE, P. C., **ARAGÃO, R. S.,** FIDALGO, M. A., ARAUJO-FILHO, J. C., DANTAS, M. L. M., SANTOS, P. C. P., VITORIANO, I. L. S., LEITE, S. P., MANHÃES-DE-CASTRO, R. Estudo morfométrico de células epiteliais do proestro em ratas desnutridas In: XXIV Reunião Anual da FeSBE, 2009, Águas de Lindóia.

- **ARAGÃO, R. S.,** SANTOS, P. C. P., ARAUJO-FILHO, J. C., VITORIANO, I. L. S., RODRIGUES, M. A. B., BARROS, K. M. F. T., MANHÃES-DE-CASTRO, R. HÁ PROGRAMAÇÃO DA RESPOSTA À BUSPIRONA EM RATOS ADULTOS SUBMETIDOS À MANIPULAÇÃO FARMACOLÓGICA NEONATAL? In: XXIV Reunião Anual da FeSBE, 2009, Águas de Lindóia.

Esta tese está escrita segundo o modelo para “Regulamentação e defesa e normas de tese” da Pós-graduação em Nutrição da Universidade Federal de Pernambuco. Vale salientar que neste caso, por se tratar de uma tese em cotutela com a Université de Nantes, a tese está sendo apresentada com um resumo em língua francesa. Desta forma, as normas de defesa e concessão de título se adequam à convenção de cotutela assinada entre a Universidade Federal de Pernambuco e a Université de Nantes.

REVISÃO DA LITERATURA

3 Revisão da literatura

3.1 Síndrome metabólica – um problema de saúde mundial

A síndrome metabólica (SM) pode ser caracterizada como a associação de numerosos fatores que aumentam o risco de doenças cardiovasculares e diabetes tipo 2, estando usualmente relacionados à deposição central de gordura e à resistência à insulina (BRANDÃO *et al.*, 2005). O estudo da SM tem sido dificultado pela ausência de consenso na sua definição e nos pontos de corte dos seus componentes, com repercussões para a prática clínica e as políticas de saúde (BRANDÃO *et al.*, 2005).

Segundo os critérios da Organização Mundial de Saúde, para o diagnóstico de SM há necessidade da presença de um dos seguintes fatores: "diabetes mellitus", intolerância à glicose, elevação da glicemia de jejum ou resistência à insulina, além de dois dos seguintes fatores: pressão arterial $\geq$ 140/90 mmHg; dislipidemia (triglicerídeos: $\geq$ 1,695 mmol/L e HDL-C $\leq$ 0,9 mmol/L (homens), $\leq$ 1,0 mmol/L (mulheres)); obesidade central (relação cintura/quadril > 0,90 (homens), > 0,85 (mulheres), ou índice de massa corpórea (IMC) > 30 kg/m^2); microalbuminuria (razão de excreção de albumina urinária $\geq$ 20 μg/min ou razão de albumina/creatinina $\geq$ 30 mg/g) (ALBERTI e ZIMMET, 1998). Este critério exige confirmações clínicas-laboratoriais, o que dificulta sua utilização mais ampla. Para o "National Cholesterol Education Program's Adult Treatment Panel III" (NCEP-ATP III), considera-se que um indivíduo apresenta SM quando há presença de, ao menos, três dos seguintes fatores: obesidade central (circunferência da cintura $\geq$ 102 cm (homens), $\geq$ 88 cm (mulheres)); hipertrigliceridemia ($\geq$ 1,7 mmol/L), baixos níveis de HDL-C < 1,0 mmol/L (homens), < 1,3 mmol/L (mulheres); pressão arterial $\geq$ 130/85 mmHg; glicemia de jejum $\geq$ 110 mg/dL (BONOW, 2002). Além dessas duas classificações, ainda existe a classificação da "International Diabetes Federation" (IDF), onde é necessária a presença de obesidade central, medida pela circunferência da cintura, respeitando valores específicos para cada etnia ($\geq$ 90 cm para homens e $\geq$ 80 cm para mulheres, sul-americanos), além da presença de dois dos quatro fatores seguintes: aumento dos triglicerídeos $\geq$ 1,7 mmol/L; diminuição do HDL-C < 1,03 mmol/L (homens) e < 1,29 mmol/L (mulheres); aumento da pressão arterial $\geq$ 130/85 mmHg; e glicemia de jejum aumentada $\geq$ 100 mg/dL (ALBERTI, ZIMMET e SHAW, 2007).

A não uniformidade nos critérios para a classificação da SM gera algumas distorções nos valores de prevalência da síndrome. As estimativas mostram que entre 20% e 25% da população mundial de adultos possivelmente tem SM. Na Europa, estima-se que a porcentagem da população afetada pela SM é da ordem de 10 a 25 %, em função do sexo e idade (BALKAU *et al.*, 2002a). Enquanto que torno de 30% da população americana apresentaria SM (GRUNDY, 2008). Em estudo com coorte da Malásia, foi observado presença de SM em 38,2 a 41,4% da população, dependendo do critério utilizado para o diagnóstico (MOY e BULGIBA, 2010). Em recente revisão, Cuevas, Alvarrez e Carrasco (2011) apresentaram o perfil epidemiológico da SM na América Latina. De acordo com o trabalho, observam-se prevalências que variam entre 5,5 a 52,7%, de acordo com o país estudado, o critério de diagnóstico utilizado, a idade da população, além de diferenças entre os gêneros (Tabela 1) (CUEVAS, ALVAREZ e CARRASCO, 2011). O acúmulo de adiposidade abdominal seria o fator de risco mais prevalente entre essas populações e o aumento da prevalência de SM foi observado de forma mais significativa nos últimos 10 anos (CUEVAS, ALVAREZ e CARRASCO, 2011).

Tabela 1. Prevalência da SM em diversos países da América Latina. Retirado de (CUEVAS, ALVAREZ e CARRASCO, 2011).

Author	Country	Sample, *n* (women/men)	Age range (years)	Criteria diagnosis	Prevalence (%, overall/men/women)
Valenzuela *et al.* [20]	Chile	1833	>17	Updated ATP III/IDF	31.6 overall (ATP) 36.8 overall (IDF)
Rojas *et al.* [22]	Mexico	6021	>20	IDF AHA/NHLBI ATP III	49.8 overall/46.4 m/52.7 w 41.6 overall/34.7 m/47.4 w 36.8 overall/30.3 m/42.2 w
Flores *et al.* [23]	Venezuela	3108	>20	ATP III	31.2 overall
Sempértegui *et al.* [24]	Ecuador	352	≥65	IDF	40 overall /19 m/81 w
Medina-Lezama *et al.* [25]	Perú	1878 (1011/867)	>20	AHA/NHLBI	14.3 m/23.2 w
Pérez *et al.* [26]	Puerto Rico	859	21–79	NCEP ATP	43.3 overall/45.3 m/42.2 w
Rodrigues *et al.* [27]	Brasil	1655 (896/759)	25–64	ATP III	32.8 overall/33 m/32.7 w
Caceres *et al.* [28]	Bolivia	61	5–18 (obese)	NCEP/ATP III for children	36 overall/40 boys/32.2 girls
Bustos *et al.* [29]	Chile	461	10–18	NCEP/ATP III for children	37.5 overall/43.7 m/33 w
Escobedo *et al.* [30••]	Seven cities from Latin America	Total = 11 502	26–64	ATP III	23 m /22.7 w (Barquisimeto) 14.7 m/18.2 w (Bogota) 17.3 m/9.7 w (Buenos Aires) 13.2 m/17.6 w (Lima) 22.4 m/22.2 w (Mexico City) 5.5 m/16.4 w (Quito) 15.3 m/19 w (Santiago)

ADP, Adult Treatment Panel; IDF, International Diabetes Federation; m, men; NCEP, National Cholesterol Education Program; w, women.

No Brasil, não foram encontrados trabalhos que estimem a prevalência de SM na população nacional. Entretanto, trabalhos no âmbito regional têm demonstrado variações nesta prevalência de 14,9 a 48,3% (SALAROLI *et al.*, 2007; VELASQUEZ-MELENDEZ *et al.*, 2007; PIMENTA, GAZZINELLI e VELASQUEZ-MELENDEZ, 2011; SILVA, PRATA e CUNHA, 2011; DUTRA *et al.*, 2012). Estudos em população adulta de área urbana têm

observado prevalência da SM variando entre 32 a 48,3% (SALAROLI *et al.*, 2007; GRONNER *et al.*, 2011; DUTRA *et al.*, 2012). Em todos os casos, a SM foi positivamente associada à idade e não foi associada ao gênero (SALAROLI *et al.*, 2007; GRONNER *et al.*, 2011; DUTRA *et al.*, 2012). Em relação ao grau educacional, os resultados diferem, sendo observado correlação em alguns estudos (SALAROLI *et al.*, 2007; GRONNER *et al.*, 2011; DUTRA *et al.*, 2012) e não correlação em outros (SALAROLI *et al.*, 2007; GRONNER *et al.*, 2011; DUTRA *et al.*, 2012). Estudos em adultos, da área rural do país, apresentaram variações de prevalência da SM de 14,9 a 21,6% (VELASQUEZ-MELENDEZ *et al.*, 2007; PIMENTA, GAZZINELLI e VELASQUEZ-MELENDEZ, 2011; SILVA, PRATA e CUNHA, 2011). Nestes estudos, a idade foi sempre relacionada à SM (VELASQUEZ-MELENDEZ *et al.*, 2007; PIMENTA, GAZZINELLI e VELASQUEZ-MELENDEZ, 2011; SILVA, PRATA e CUNHA, 2011), da mesma forma do observado para a população urbana (SALAROLI *et al.*, 2007; GRONNER *et al.*, 2011; DUTRA *et al.*, 2012). Entretanto, houve associação do gênero com o aparecimento da SM (VELASQUEZ-MELENDEZ *et al.*, 2007; PIMENTA, GAZZINELLI e VELASQUEZ-MELENDEZ, 2011). Coorte de adolescentes estudada por Stabeline-Neto e colaboradores (2011) apresentou prevalência de SM em 7,7% da população estudada, sendo esta prevalência associada ao nível de atividade física e aptidão cardiorrespiratória, sem associação com o gênero. Como observado nos estudos de coorte com adultos, podemos esperar que esta prevalência aumente com o decorrer da idade.

Observa-se que o aumento na prevalência da SM acontece em maior proporção em países subdesenvolvidos ou na população de zonas rurais que migraram para a zona urbana, mesmo em países desenvolvidos (CUEVAS, ALVAREZ e OLIVOS, 2009; CUEVAS, ALVAREZ e CARRASCO, 2011). Observa-se, não somente no Brasil (BATISTA FILHO e RISSIN, 2003), mas em toda América Latina (ALBALA *et al.*, 2001), mudanças nas prevalências de desnutrição e obesidade. Atualmente, considera-se que estes países estejam vivendo uma transição nutricional, com o aumento da prevalência de sobrepeso e obesidade, porém convivendo paralelamente com a presença da desnutrição (ALBALA *et al.*, 2001; BATISTA FILHO e RISSIN, 2003). Além de enfrentarem mudança nos hábitos de atividade física, estas pessoas que viveriam em um ambiente de carência nutricional, ao entrar em contato com um ambiente com excesso de nutrientes estariam mais propensas ao desenvolvimento da obesidade e de outros fatores de riscos associados à SM (CUEVAS, ALVAREZ e OLIVOS, 2009; CUEVAS, ALVAREZ e CARRASCO, 2011).

O aumento da prevalência da SM na população também estaria relacionado ao modo de vida atual. Estudos mostram que fatores como a ingestão aumentada de gordura saturada (DE OLIVEIRA *et al.*, 2012), presença de tabagismo, elitismo, estresse e baixo nível de atividade física (LEITAO e MARTINS, 2012) estão diretamente associados com o risco de desenvolver SM, enquanto que a ingestão de frutas e a variedade de itens durante as refeições são fatores protetores contra o seu diagnóstico(DE OLIVEIRA *et al.*, 2012). Levy-Costa e colaboradores (2005) observaram a presença da excessiva ingestão de açúcar e gordura saturada e insuficiente ingestão de frutas e vegetais na dieta dos brasileiros. Além disso, na comparação entre duas pesquisas feitas no Brasil em 1989 e 2005, com o IMC de jovens entre 10 e 15 anos, observou-se aumento nos percentis 85 e 95 de IMC em 2005 em relação à 1989, indicando aumento na prevalência de obesidade entre os adolescentes (CINTRA IDE *et al.*, 2007). Esses dados somados nos levam a inferir que a prevalência da SM deve aumentar nos próximos anos no Brasil.

3.2 Origem Desenvolvimentista da Saúde e da Doença (DOHaD – "Developmental Origins of Health and Disease") – evolução de conceitos

Um aporte nutricional adequado é necessário para o bom desenvolvimento e crescimento do organismo. Alterações neste aporte durante períodos críticos do desenvolvimento podem interferir de forma permanente sobre a estrutura e função dos órgãos, e, consequentemente, podem predispor o organismo ao aparecimento de doenças (MORGANE *et al.*, 1993; HALES e BARKER, 2001a). Dobbing (1965) formulou o conceito de "período crítico do desenvolvimento" que corresponde a janelas de tempo onde os tecidos e órgãos são susceptíveis a injúrias, desenvolvendo alterações permanentes na estrutura e função. Em seu trabalho, Dobbing focou-se no período crítico para o desenvolvimento do sistema nervoso central, porém, hoje sabe-se que todos os sistemas apresentam períodos de rápida proliferação e diferenciação celular onde estão mais vulneráveis às influências ambientais.

Grande número de evidências epidemiológicas e experimentais têm demonstrado correlação entre aporte nutricional deficiente e/ou desequilibrado, durante o desenvolvimento intrauterino e infantil, com o aparecimento de doenças cardiovasculares e metabólicas, na vida adulta (HALES e BARKER, 2001a; ARMITAGE *et al.*, 2004).

Estudo no coorte de Hertfordshire (Inglaterra) encontrou correlação entre baixo peso ao nascer e no primeiro ano de vida com altas taxas de mortes por doenças cardiovasculares na vida adulta (BARKER *et al.*, 1989). Nesta mesma coorte, Hales e colaboradores (1991) observaram correlação entre baixo peso ao nascer ou no primeiro ano de vida com intolerância à glicose na vida adulta. Estes e outros dados da época levaram à proposição de que o retardo no crescimento (representado pelo baixo peso) durante a gestação e a primeira infância estaria relacionado a maior predisposição ao desenvolvimento de doenças cardiovasculares e diabetes tipo 2, na vida adulta (HALES e BARKER, 1992). Esta proposição ficou conhecida como "*Thrifty phenotype hypothesis*" (hipótese do fenótipo poupador) (HALES e BARKER, 1992). Nesta hipótese, o organismo em desenvolvimento adaptar-se-ia às condições de baixo aporte nutricional, modificando seu metabolismo no sentido de melhor aproveitamento energético e maior capacidade de estocagem de energia (HALES e BARKER, 1992). Quando há aumento do aporte nutricional no período pós-natal, o organismo apresentaria alterações metabólicas que levaria a problemas cardiovasculares, diabetes tipo 2 e obesidade (Figura 1) (HALES e BARKER, 1992).

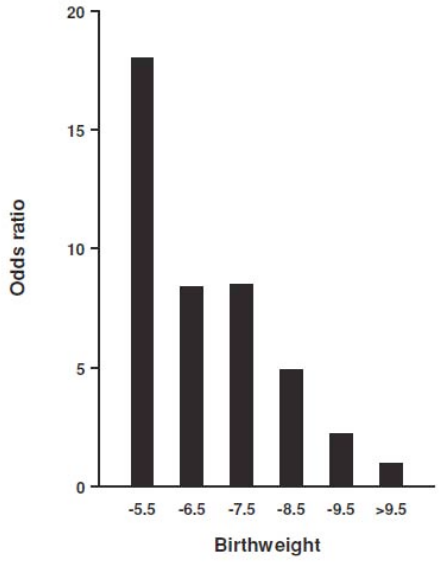

Figura 1. Odds ratio para SM de acordo com o peso ao nascer (em libras). Dados da coorte de Hertfordshire. Retirado de (HALES e BARKER, 2001a).

Lucas (1991) propôs o termo "programação" para designar as adaptações das funções fisiológicas que ocorreriam durante o período crítico do desenvolvimento para aumentar as chances de sobrevida. Entretanto, restava a esclarecer como esta "memória" precoce poderia ser "estocada" para desencadear alterações na vida adulta. As propostas iniciais de

mecanismos para esta nova “programação” do organismo incluíam efeitos adaptativos sobre a expressão gênica que seriam transmitidos para a progênie das células originalmente programada (LUCAS, 1991). Outra proposição seria que o ambiente nutricional precoce poderia estimular proliferação celular ou seleção clonal adaptativa de modo que a quantidade ou proporção de células em um tecido é permanentemente afetada (DESAI *et al.*, 1996).

Trabalhos publicados pelo grupo do pesquisador neozelandês Gluckman reforçam a ideia da programação fetal. Entretanto, estes autores se referem a este fenômeno como “resposta adaptativa preditiva” e baseiam seu conceito na plasticidade do desenvolvimento. A “resposta adaptativa preditiva” seria o mecanismo pelo qual o feto usa a sinalização ambiental para “prever” seu futuro e adotar a via de desenvolvimento que poderia melhor se encaixar no seu ambiente pós-natal esperado (GLUCKMAN *et al.*, 2005a). A plasticidade do desenvolvimento é definida como o fenômeno pelo qual um genótipo pode dar origem a uma gama de diferentes estados fisiológicos ou morfológicos em resposta a diferentes condições ambientais durante o desenvolvimento (WEST-EBERHARD, 2005). O organismo apresentaria a habilidade de mudanças na estrutura e funções em respostas a sinais do ambiente. Entretanto, essa resposta seria irreversível e apenas poderia ocorrer se as mudanças ambientais acontecessem durante os períodos críticos de desenvolvimento (GLUCKMAN e HANSON, 2004b). O objetivo maior das respostas adaptativas preditivas seria permitir, ao organismo em desenvolvimento, a utilização de sua capacidade de plasticidade para estabelecer seu “melhor” fenótipo pós-natal previsto, possibilitando a melhor chance de sobrevivência para posterior reprodução quando adulto e, assim, preservar a continuidade da espécie (GLUCKMAN e HANSON, 2004a).

A eficácia deste tipo de estratégia de plasticidade irá depender se a predição feita foi correta ou não (BATESON *et al.*, 2004). Porém, o organismo em desenvolvimento tem que fazer uma escolha, pois programar-se para viver em um ambiente pobre de nutrientes e terminar em um ambiente rico é menos perigoso do que o inverso (BATESON e GLUCKMAN, 2012). Desta forma, o aumento da prevalência da SM estaria relacionado à incompatibilidade do ambiente perinatal com a realidade após o nascimento (GLUCKMAN e HANSON, 2004a).

A evolução da capacidade de montar uma resposta plástica preditiva e adaptativa provavelmente dependerá de uma série de fatores, tais como a precisão do sinal e as frequências de vários estados ambientais, bem como as consequências da incompatibilidade

(GLUCKMAN *et al.*, 2005b). Este capacidade de resposta plástica do fenótipo levaria, com o passar das gerações, a adaptações evolutivas nas espécies, de acordo com a da robustez das alterações incorporadas (BATESON e GLUCKMAN, 2012). Devemos considerar que o organismo em desenvolvimento apresenta plasticidade e, dependendo do quão frequente seja este estímulo ou do quão permanentes sejam as alterações incorporadas, este novo perfil pode se tornar uma característica robusta da espécie (BATESON e GLUCKMAN, 2012).

Apesar do respaldo em estudos epidemiológicos e experimentais, estes conceitos de fenótipo poupador e/ou resposta adaptativa preditiva receberam várias críticas e contestações no meio acadêmico. Segundo alguns autores, interpretações estatísticas incorretas ou incompletas levam a conclusões errôneas sobre o problema observado (LUCAS, FEWTRELL e COLE, 1999). Sugere-se que o retardo do crescimento intrauterino *per si* não representaria dado preditivo para a correlação com doenças da vida adulta, mas sim a diferença entre o peso ao nascer e o peso na vida adulta, ou a velocidade do crescimento pós-natal que seriam preditores (LUCAS, FEWTRELL e COLE, 1999; SINGHAL e LUCAS, 2004). Além disto, sugeriu-se que o fenótipo materno seria responsável pela modificação no crescimento fetal e aquele seria o agente "programador" (WELLS, 2003). Considerando que o "instinto" materno de evolução levaria a mãe a diminuir sua utilização de nutrientes, em favor do adequado aporte energético para o feto, o que estaria sendo transmitido a este não seria a ação do ambiente, mas sim o fenótipo presente na mãe que viria de uma história de vida já em um ambiente pobre em nutrientes (WELLS, 2003). Desta forma, o feto estar-se-ia adaptando à realidade materna, sofrendo a influência/manipulação materna para que a gestação pudesse ser levada a termo (WELLS, 2007). Huxley, Neil e Collins (2002), em revisão de 55 trabalhos publicados que relacionavam peso ao nascer e a prevalência de hipertensão na vida adulta, puderam observar que alguns trabalhos superestimaram esta relação. Os autores citam erros de aquisição dos dados de peso ao nascer, além de ajuste inapropriado, ao peso atual e ignorância de variáveis atuais de confusão (como presença de tabagismo/elitismo, estado social atual e nível de atividade física) (HUXLEY, NEIL e COLLINS, 2002; JADDOE e WITTEMAN, 2006).

Segundo Barker (2003), deve-se considerar que mesmo uma criança que tenha nascido dentro das variações normais de peso pode ter sofrido programação no período fetal. Este achado estaria relacionado à dieta maternal e composição corporal durante a gestação, independente do peso ao nascer da prole (BARKER, 2003). Desta forma, estes dois fatores

maternos também apresentam papel importante na determinação da saúde futura da criança (BARKER, 2003).

Além das contestações sobre a real influência do ambiente perinatal sobre o surgimento de doenças na vida adulta, outro grande desafio para os defensores da origem desenvolvimentista da saúde e da doença é a identificação dos mecanismos moleculares que sustentam e agrupam todas as modificações observadas. Alguns dados da literatura são sugestivos de que a modulação epigenética da expressão de genes por nutriente poderia constituir a ligação entre o ambiente nutritivo perinatal carente e a produção do fenótipo patológico posterior (VAN SPEYBROECK, 2002; LILLYCROP e BURDGE, 2011). Segundo definição inicial de Conrad Waddington, epigenética é o estudo de como um genótipo pode originar diferentes fenótipos durante o desenvolvimento (VAN SPEYBROECK, 2002). Com o passar dos anos, a definição de epigenética tem variado, sendo considerado atualmente como o conjunto de processos de expressão e transmissão da informação genética, incluindo a transmissão transgeracional (CHONG e WHITELAW, 2004a) por mecanismos que não afetam a sequência de DNA, mas sim a organização estrutural da cromatina (TURNER, 2002; EGGER *et al.*, 2004). Assim, podemos considerar que as alterações epigenéticas representam as adaptações estruturais de regiões cromossômicas a fim de registrar, sinalizar ou perpetuar estados de atividade alterada (BIRD, 2007). Segundo Gluckman, Hanson e Pinal (2005), as mudanças epigenéticas podem ser a base primária da programação, e as alterações estruturais e regulatórias que são observadas nos diferentes tecidos seriam secundárias às alterações induzidas pelo ambiente sobre a expressão gênica.

A regulação epigenética da expressão genética desempenha um papel fundamental na transdução funcional da informação contida no genoma. Todas as células do nosso corpo contêm a mesma informação genética, isto é, o mesmo genoma. No entanto, as características morfológicas e funcionais de uma célula do pâncreas, por exemplo, são muito diferentes das de uma célula muscular. O que torna uma célula diferente da outra, portanto, não é a informação genética que eles contêm, mas a informação genética que eles expressam. A expressão diferencial de uma população de genes de uma célula para outra e até mesmo de um estágio de desenvolvimento para outro, é controlado por mecanismos epigenéticos que incluem dois mecanismos principais: a metilação do DNA e modificações pós-traducionais de histonas.

O exemplo mais comum de modificação epigenética é a metilação do DNA, ou seja, a adição de um grupo metil nos resíduos de citosina do DNA. Esta modificação ocorre geralmente nas citosinas que são seguidas de guaninas (dinucleotídeos CpG). Esta modificação suprime a expressão gênica por impedir diretamente a ligação de fatores de transcrição ou por recrutar proteínas que irão impedir a fixação dos fatores de transcrição sobre o DNA (BIRD, 2007). Estas proteínas recrutam outras proteínas que remodelam a cromatina, modificando as histonas e, portanto, formando uma cromatina compacta e inativa (BIRD, 2007). A metilação do DNA é uma alteração estável, resistindo a vários ciclos de mitose, desta forma, considerava-se incialmente que fatores ambientais não poderiam modificar de forma significante e perserverante o padrão de metilação do DNA no tecido normal de um adulto (BARRES e ZIERATH, 2011). Entretanto, estudo com gêmeos monozigóticos demostrou que os irmãos eram mais divergentes em relação ao padrão de metilação quando adultos do que quando crianças, isto validaria a influência de fatores ambientais sobre o epigenoma ao longo do tempo (FRAGA *et al.*, 2005).

O genoma existe dentro do núcleo da célula como um complexo de nucleoproteínas, a cromatina, que, além de DNA, contém proteínas globulares designadas histonas. A unidade básica da cromatina é o nucleossoma, que é composto de um octámero que consiste em 2 cópias de cada uma das histonas H2A, H2B, H3 e H4, em torno do qual é enrolado um fragmento de 147 pares de bases do DNA. Em certas regiões do genoma, as histonas H2A, H2B e H3 podem ser substituídas por "variantes" de histonas, que são diferentes das histonas canônicas. As histonas podem sofrer várias modificações pós-tradução em sua parte N-terminal, incluindo a adição de grupos metil, acetil, fosfato, ubiquitina (JENUWEIN e ALLIS, 2001). Essas alterações modificam sua afinidade pelo DNA levando a uma alteração na conformação da cromatina que promove (eucromatina) ou, pelo contrário, impede (heterocromatina) o acesso de factores de transcrição para as regiões de promotoras do gene (BORRELLI *et al.*, 2008).

Ao contrário a metilação do DNA, geralmente levando à inibição da transcrição, as modificações pós-traducionais das histonas podem levar ao aumento ou diminuição na expressão de um gene com base no perfil de modificações pós-traducionais das histonas, o código de histona (JENUWEIN e ALLIS, 2001; WATERLAND e MICHELS, 2007)

Assim, mostrou-se, por exemplo, que a fosforilação de histona H3 em serina 10 está associada com aumento na transcrição enquanto a metilação da mesma histona sobre a lisina 9

é característica da cromatina inativa. No entanto, o efeito de um tipo de modificação pós-tradução de histonas na transcrição não é fixo. Ela pode variar dependendo do contexto celular de forma análoga à regulação da transcrição, visto que uma mesma modificação pós-traducional pode levar à ativação ou inativação da cromatina dependendo do tecido (LEMON e TJIAN, 2000).

O estado nutricional materno também poderia induzir alterações da expressão de genes através da metilação de DNA e da modificação de histonas (metilação e acetilação) do genoma fetal (SMITH, GARFIELD e WARD, 2006), sendo esse mecanismo conhecido como "*imprinting*" genômico (SMITH, GARFIELD e WARD, 2006). Modificações na dieta podem afetar o padrão de metilação do DNA e o *imprinting* genômico. A deficiência de doadores de metil (ácido fólico e metionina) modifica o "*imprint*" de metilação do DNA do fator de crescimento semelhante à insulina 2 (IGF2) (WATERLAND, 2003). A privação de ácido fólico altera o padrão de metilação do DNA, porém de modo reversível, visto que este padrão é restaurado após administração de dieta normal (GHOSHAL *et al.*, 2006).

Além de fatores epigenéticos, a prole ainda está sujeita à influência da expressão preferencial de genes provenientes da mãe ou do pai. Esta influência é conhecida como "*imprint*" parental (MORISON e REEVE, 1998; MOORE, 2001). Alguns "*imprinted*" genes têm levantado interesse na área de origem desenvolvimentista da saúde e da doença devido a sua grande expressão e também por serem os primeiros a terem suas expressões relacionadas ao desenvolvimento fetal e placentário. São eles H19, IGF2 e IGF2r (MORISON e REEVE, 1998; MOORE, 2001). O IGF2 apresenta grande interesse por ser o maior fator de crescimento fetal (MOORE, 2001; SMITH, GARFIELD e WARD, 2006). O H19 é expresso pelo alelo materno, porém não codifica para nenhum RNA de função conhecida (MORISON e REEVE, 1998). O IGF2, tem expressão paterna e estaria sobre o controle do locus do H19 (MORISON e REEVE, 1998), pois a deleção da região promotora do gene H19 é suficiente para abolir o *imprinting* em ambos os genes (MOORE, 2001). O IGF2r tem *imprinting* materno e atua como inibidor do crescimento fetal e placentário (SMITH, GARFIELD e WARD, 2006). Desta forma, além da regulação epigenética sobre a expressão de genes, haveria a regulação pelo *imprint* parental (disputa entre a expressão de genes maternos ou paternos) ambos influenciando o fenótipo que será apresentado.

3.3 DOHaD – evidências epidemiológicas e experimentais

Como relatado anteriormente, estudos epidemiológicos observaram que baixo peso ao nascer e no primeiro ano de vida está associado com intolerância à glicose, diabetes tipo 2 (HALES *et al.*, 1991) e doenças cardiovasculares (BARKER *et al.*, 1989) na vida adulta. Diversos sistemas podem ser afetados pelo estado nutricional materno durante o desenvolvimento precoce e, desta forma, podem apresentar alterações futuras que levem ao desenvolvimento da SM (Figura 2).

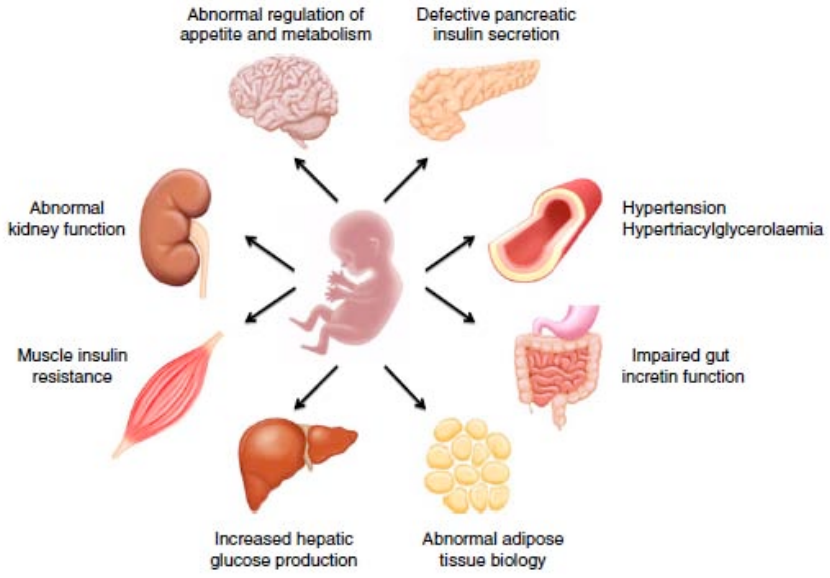

Figura 2. Órgãos que podem ser afetados pelo estado nutricional materno durante o desenvolvimento e posteriores alterações que podem levar ao desenvolvimento da SM. Retirado de (VAAG *et al.*, 2012).

Os estudos da coorte de Hertfordshire foram confirmados pela coorte de Helsinki, onde 8760 homens e mulheres tiveram seu crescimento acompanhado. Aqueles que apresentaram doenças coronárias e diabetes tipo 2 na vida adulta eram pequenos ao nascimento e na infância, porém apresentavam acelerado ganho de peso posteriormente (ERIKSSON *et al.*, 2001a; ERIKSSON *et al.*, 2001b; 2003b; ERIKSSON *et al.*, 2003c).

Ravelli e colaboradores (1998) também observaram correlação entre a resistência à insulina e alteração no metabolismo da glicose, em pessoas que nasceram de mães que foram expostas a períodos de grande restrição alimentar (*Dutch famine*), principalmente quando este coincidiu com o final da gestação. Esta correlação foi mais forte em pessoas que se tornaram obesas no período pós-natal (RAVELLI *et al.*, 1998). Em outro estudo, adultos-jovens que apresentaram baixo peso ao nascer tinham maior acúmulo de gordura abdominal, maior

glicemia e insulinemia de jejum, comparado a jovens de peso normal ao nascimento, apesar de não apresentarem maior peso corporal ou IMC, no momento do estudo (BRONS *et al.*, 2012). Pode-se observar nos diversos trabalhos descritos que o baixo peso ao nascer não seria uma marca evidente do fenótipo que levaria este indivíduo a desenvolver SM ou doenças correlacionadas na vida adulta (RAVELLI *et al.*, 1998; GLUCKMAN *et al.*, 2005a). Um melhor preditor seria a presença de rápido crescimento pós-natal ou, como ficou conhecido na literatura, *catch up* de crescimento (GLUCKMAN *et al.*, 2005a).

Trabalhos com gêmeos monozigóticos que eram discordantes para diabetes observaram que o gêmeo diabético era aquele que apresentou menor peso ao nascimento (BO *et al.*, 2001). Contudo, trabalho realizado com coorte de gêmeos monozigóticos e dizigóticos não reproduziu este achado, nem em relação à tolerância à glicose nem ao nível de pressão arterial (BAIRD *et al.*, 2001).

Parsons, Power e Manor (2001) estudaram uma coorte Britânica de nascidos no ano de 1958. Eles puderam observar que o peso ao nascer estava relacionado com o peso aos 33 anos de vida, porém esta associação seria explicada pelo peso (ou IMC) materno. Contudo, foi observado também forte correlação do IMC na vida adulta com a altura atingida aos 7 anos (PARSONS, POWER e MANOR, 2001).

Estudo sobre o aumento dos índices de hipertensão na população de descendentes africanos não observou correlação entre o ambiente perinatal e o desenvolvimento da hipertensão (FORRESTER, 2004). Este aumento nos índices estaria relacionado ao aumento da ingestão de sal e da obesidade, ou seja, os fatores de estilo de vida atual teriam maior influência sobre a prevalência de hipertensão do que a exposição a ambiente nutricionalmente pobre na vida perinatal (FORRESTER, 2004). Entretanto, não pode-se descartar completamente a importância do peso ao nascer, visto que pessoas que apresentam retardo do crescimento intrauterino (RCIU) também apresentam redução no número de células em órgãos que são chaves para o metabolismo, como o rim e o pâncreas. Segundo Brenner e Chertow (1993), a hipertensão arterial seria iniciada pela redução no número de glomérulos em pessoas que tiveram RCIU, favorecendo a hiper-filtração glomerular que levaria ao desenvolvimento de esclerose glomerular, culminando com o aumento da pressão arterial (BRENNER e CHERTOW, 1993).

Alterações epigenéticas também foram encontradas em estudos com humanos cujas mães estavam gestantes durante o período da *Dutch Famine*. Foi observado hipometilação do gene *imprinted* IGF2, em indivíduos cujas mães foram submetidas ao período de fome nos estágios periconcepcionais em comparação ao irmãos cuja gestação não ocorreu no período de fome (HEIJMANS *et al.*, 2008). Entretanto, quando a exposição ocorreu no final da gestação, não foram observadas alterações no padrão de metilação de IGF2 (HEIJMANS *et al.*, 2008).

Apesar das divergências em relação aos achados dos estudos epidemiológicos, a hipótese da programação metabólica no período perinatal tem encontrado grande suporte em estudos experimentais. Estes estudos são mais evidentes em relacionar o ambiente nutricional precoce com o desenvolvimento de diabetes tipo 2, obesidade e SM. Entretanto, não somente o ambiente pobre em nutrientes no período perinatal estaria relacionado com doenças na vida adulta, diferentes tipos de manipulações ambientais podem resultar em um mesmo fenótipo patológico.

Desai e colaboradores (1996) demonstraram, em ratos, que a dieta baixa em proteína (8% *versus* 20% do controle) durante a gestação e/ou lactação ocasionava redução seletiva do peso dos órgãos ao desmame sendo o músculo esquelético o único a permanecer reduzido aos 11 meses (DESAI *et al.*, 1996). O tipo de manipulação da dieta no período perinatal parece afetar o resultado no crescimento corporal desses animais. Passos, Ramos e Moura (2000), assim como De Moura e colaboradores (2007), observaram que animais cujas mãe foram restritas em proteína apresentavam menor peso ao desmame e permaneciam menores na vida adulta. Entretanto filhotes cujas mães foram submetidas à restrição calórica no mesmo período, eram menores ao desmame, porém maiores que os controles na vida adulta. Na análise do leite materno, foi observado que as mães restritas em proteína apresentavam menor concentração de proteína, enquanto que o leite das mães restritas em energia apresentava maior concentração de proteínas e lipídios (PASSOS, RAMOS e MOURA, 2000). Em paralelo a estes achados, foi observado que ambos os grupos apresentavam redução na expressão do hormônio do crescimento (GH) na hipófise ao desmame, enquanto que o grupo restrito em proteína continuava com este padrão na vida adulta, o grupo restrito em energia apresentava aumento dessa expressão (DE MOURA *et al.*, 2007). Estes achados nos levam a inferir que a alteração na composição do leite pode alterar a ingesta alimentar na vida adulta, além de que a programação perinatal pode levar a alterações na sinalização de hormônios que controlam o crescimento corporal (PASSOS, RAMOS e MOURA, 2000).

O tempo da programação também pode interferir no fenótipo demonstrado pela prole e na sua longevidade (OZANNE e HALES, 2004; OZANNE *et al.*, 2004). Foi observado que a restrição proteica durante o período *in utero* tornava os animais mais susceptíveis à obesidade induzida por dieta palatável, enquanto que aqueles desnutridos durante a lactação eram resistente ao aumento de peso induzido pela mesma dieta (OZANNE *et al.*, 2004). Além disso, o tempo de vida dos animais desnutridos no período pós-natal foi significativamente maior que o daqueles restritos *in utero* (OZANNE e HALES, 2004). Neste caso não somente o tempo da programação, mas também o tempo de *catch up* influenciariam no fenótipo observado (OZANNE e HALES, 2004; OZANNE *et al.*, 2004; COUPE *et al.*, 2012).

A restrição proteica no período perinatal leva à resistência à insulina na vida adulta (PETRY *et al.*, 2001). Entretanto, animais machos mais jovens cujas mães receberam dietas hipoproteica durante a gestação e lactação apresentam maior insulinemia ao jejum, porém sem diferenças na insulinemia e glicemia após teste de tolerância a glicose (CHAMSON-REIG *et al.*, 2009). Aos 17 meses, observa-se aumento na glicemia e insulinemia de jejum e intolerância à glicose, dados que indicam que estes animais apresentam diabetes nesta idade (PETRY *et al.*, 2001), enquanto que filhotes desnutridos somente durante a lactação apresentaram menor insulinemia e menor glicemia de jejum quando adultos-jovens (FAGUNDES *et al.*, 2007). Trabalho com ovelhas também observou alteração no metabolismo da glicose, principalmente relacionado com a sinalização da insulina, na prole cuja restrição calórica foi realizada no final da gestação (GARDNER *et al.*, 2005). As alterações que são observadas em relação à insulina podem variar em relação ao gênero, sendo os machos com maior tendência a serem insulinorresistentes enquanto que as fêmeas são insulino-deficientes (CHAMSON-REIG *et al.*, 2009), assim como o período da manipulação nutricional (GARDNER *et al.*, 2005; FAGUNDES *et al.*, 2007; CHAMSON-REIG *et al.*, 2009).

As repercussões da programação no período perinatal podem depender do tipo de dieta à qual o organismo será submetido após o período crítico de desenvolvimento (VICKERS *et al.*, 2003; THOMPSON *et al.*, 2007). Os machos parecem ser susceptíveis à desregulação metabólica desencadeada por uma dieta rica em gordura no período de pós-desmame, independente do regime dietético ao qual a mãe foi submetida (VAN STRATEN *et al.*, 2012). Porém, para as fêmeas, a dieta perinatal pobre em proteína torna-as mais susceptíveis ao desenvolvimento de SM apenas quando mantidas em dieta hiperlipídica (VAN STRATEN *et*

al., 2012). A ingestão de dieta rica em gordura no período pós-desmame leva ao aumento do peso corporal, da porcentagem de gordura peri-renal, da concentração de insulina e leptina plasmática (THOMPSON *et al.*, 2007). Além disso, observa-se redução da quantidade de glicogênio hepático e muscular e na glicose plasmática (THOMPSON *et al.*, 2007). Todas essas alterações foram mais evidentes em animais que sofreram desnutrição no período perinatal (THOMPSON *et al.*, 2007). Ademais, dieta hipercalórica no período pós-desmame exacerba o comportamento sedentário de animais desnutridos na gestação (VICKERS *et al.*, 2003).

A função de controle central do metabolismo e dos sensores nutricionais também pode ser alteradas por restrição alimentar materna. Em um modelo de restrição calórica, não foram observadas diferenças na expressão dos neuropeptídios hipotalâmicos neuropeptídeo Y (NPY) e pró-opiomelanocortina (POMC), em animais *ad libitum*, porém em resposta ao jejum essa expressão foi modificada em relação aos animais controles (BRETON *et al.*, 2009), enquanto que a restrição proteica perinatal resultou no aumento da expressão de NPY e peptídeo relacionado ao Agouti (AgRP) e diminuição na expressão de POMC (OROZCO-SOLIS *et al.*, 2009). Alterações nas concentrações basais e na resposta ao jejum de dois sensores nutricionais, o alvo da rapamicina nos mamíferos (mTOR) e a quinase dependente de adenosinamonofosfato (AMP) (AMPK), no hipotálamo, foram encontradas na prole de mães submetidas à restrição proteica (Guzmán-Quevedo et al., submetido). Também foram observadas alterações no ciclo de ingesta alimentar em animais cujas mães sofreram restrição proteica (OROZCO-SOLIS *et al.*, 2009) ou calórica (BRETON *et al.*, 2009) durante o período perinatal. Essa alteração foi pronunciada pelo aumento na quantidade da ingesta e atraso no aparecimento da saciedade (OROZCO-SOLIS *et al.*, 2009). Além disso, animais desnutridos no período perinatal apresentam hiperfagia no período após o desmame (LOPES DE SOUZA *et al.*, 2008; OROZCO-SOLIS *et al.*, 2009), preferência aumentada por dieta rica em lipídeos (BELLINGER e LANGLEY-EVANS, 2005; COUPE *et al.*, 2012), expressão hipotalâmica alterada de vários genes relacionados ao metabolismo de glicose e lipídeos e sensores do estado nutricional (OROZCO-SOLIS *et al.*, 2010) e diminuição na expressão de receptores à leptina (COUPE *et al.*, 2012). Ademais, apresentam redução na resposta a estímulos inibitórios da ingesta alimentar, como administração de agonistas serotoninérgicos (LOPES DE SOUZA *et al.*, 2008) e de leptina (COUPE *et al.*, 2012).

Modificações na expressão circadiana de alguns genes relacionados ao metabolismo, à ingesta alimentar e ao ciclo circadiano foram observadas em animais cujas mães foram submetidas à dieta baixa em proteínas no período de gestação e lactação (OROZCO-SOLIS *et al.*, 2011). Alteração no sistema de ciclo circadiano em camundongos mutantes levou estes ao desenvolvimento do diabetes (MARCHEVA *et al.*, 2010). A ablação seletiva do gene CLOCK nas células β desencadeia o aparecimento da diabetes, relacionando a importância do ciclo circadiano na liberação de insulina e o surgimento do diabetes (MARCHEVA *et al.*, 2010). Isto poderia indicar que alterações no controle circadiano da ingestão alimentar e da liberação de hormônios e substâncias relacionadas ao metabolismo energético poderiam contribuir para o desenvolvimento de SM (OROZCO-SOLIS *et al.*, 2011).

Restrição proteica no período perinatal diminui a massa das células β pancreáticas, com menores ilhotas pancreáticas e consequente menor quantidade de insulina (PETRIK *et al.*, 1999; THEYS *et al.*, 2009a). Estes achados estariam relacionados a maior taxa de apoptose nas ilhotas fetais e neonatais e redução na expressão de IGF2 (PETRIK *et al.*, 1999). Também foi observada redução da secreção de insulina após o estímulo com glicose ou aminoácidos, sendo esta alteração devido a prejuízos no mecanismo de exocitose da insulina (CHERIF *et al.*, 2001; THEYS *et al.*, 2009a). Entretanto, a restrição energética aumenta a liberação de insulina após estímulo com glicose (CHERIF *et al.*, 2001; THEYS *et al.*, 2009a). Porém, há aumento na produção de espécies reativas de oxigênio (EROs), alteração na expressão de genes relacionados à função e biogênese mitocondrial, como também ao metabolismo, em ambos os tipos de restrição (THEYS *et al.*, 2009a; THEYS *et al.*, 2009b; THEYS *et al.*, 2011). Estes achados podem indicar possível disfunção mitocondrial nas células β do pâncreas e que este seria fator comum para o desenvolvimento do diabetes (THEYS *et al.*, 2009a; THEYS *et al.*, 2009b; REUSENS, THEYS e REMACLE, 2011; THEYS *et al.*, 2011).

O fígado é um dos órgãos chaves para o estudo do desenvolvimento da SM devido a sua grande participação no metabolismo de glicose e lipídios. Sabe-se que a restrição proteica materna leva à alteração da localização da utilização de glicose, além de diminuição da atividade da hexoquinase (HK) e glicoquinase (GK) e aumento da atividade de fosfoenolpiruvato carboxiquinase (PEPCK) (BURNS *et al.*, 1997; DESAI e HALES, 1997). As alterações na atividade da GK e PEPCK entre os animais controles e os restritos em proteína permaneceram mesmo que esses animais tenham sido submetidos à dieta rica em

gordura após o desmame (BURNS *et al.*, 1997; DESAI e HALES, 1997). Além disso, observa-se aumento da expressão do receptor α ativado por proliferador de peroxissomo (do inglês *peroxisome proliferator–activated receptors*, PPARα) e do co-ativador 1 α do receptor gama ativado por proliferador de peroxissomo (do inglês, *peroxisome proliferator-activated receptor-gamma coactivator-1*, PGC1α) (LANE, 2002; BURDGE *et al.*, 2004). Aumento na expressão de RNAm e de proteína de PGC1α estaria relacionado com o concomitante aumento da expressão de RNAm da PEPCK, glicose-6-fosfatase (G-6-Pase) e frutose-1,6-bisfosfatase (FBPase), enquanto que a expressão de GK foi diminuída (LANE, 2002). Além disso, foi observada redução da atividade da Δ5 desaturase e relação inversa desta atividade com a concentração de insulina (OZANNE *et al.*, 1998). A restrição calórica gestacional foi capaz de alterar a expressão de genes no fígado da prole, predispondo estes animais à perturbações na habilidade de coordenar o metabolismo de glicose e ácidos graxos (MORRIS *et al.*, 2009). Em conjunto, esses achados estariam relacionados à modificações no metabolismo, havendo preferência pelo uso da gordura como fonte energética, o que levaria a disfunções oxidativas (BURNS *et al.*, 1997; LANE, 2002; BURDGE *et al.*, 2004; MORRIS *et al.*, 2009) pelo fígado, sendo este um dos mecanismos que pode contribuir para o desenvolvimento da SM.

Restrição em proteína apenas no período de gestação leva ao aumento da gordura visceral, aumentando a superfície dos adipócitos, com maior presença de adipócitos grandes e redução no número de adipócitos pequenos (COUPE *et al.*, 2012). Este aumento se correlaciona positivamente com a expressão de leptina e com a leptinemia plasmática (COUPE *et al.*, 2012), enquanto que, a ingestão de dieta baixa em proteína no período de gestação e lactação, reduz a quantidade de gordura visceral, reduz a área dos adipócitos e a expressão de leptina (COUPE *et al.*, 2012). Em animais submetidos à dieta baixa em proteína durante todo o período perinatal, há maior captação de glicose em estado basal ou depois de estimulação por insulina, sem haver diferença na lipólise basal e a estimulada por insulina, sendo menos efetiva em reduzir a lipólise (OZANNE *et al.*, 1999).

Além da restrição (proteica ou calórica) da dieta, outras manipulações no período perinatal podem induzir programação. A ingestão de dieta rica em gordura durante o período perinatal também pode predispor a prole ao desenvolvimento de SM. Foram observadas alterações no conteúdo de gordura corporal e de sensibilidade à insulina na prole de ratas submetidas à dieta rica em lipídeos no período gestacional (BUCKLEY *et al.*, 2005). A

redução da passagem de oxigênio para o feto na última semana de gestação também pode induzir programação metabólica, aumentando a susceptibilidade deste ao desenvolvimento de SM (CAMM *et al.*, 2011; DOLINSKY *et al.*, 2011).

A indução de hipóxia materna resultou em intolerância à glicose, resistência à insulina, maior acúmulo de gordura abdominal, diminuição das expressões proteicas de substrato do receptor de insulina (IRS) 1, proteína quinase B (PKB, também conhecida como Akt) 1 e 2 , fosfo-Akt e proteína quinase C ζ (PKC ζ), no fígado, além da redução de Akt-2 e do transportador de glicose (Glut) 4 no músculo esquelético (CAMM *et al.*, 2011; DOLINSKY *et al.*, 2011). Também foram observados aumento na quantidade de triglicerídeos, diacilglicerol e cerâmidas, tanto no fígado quanto no músculo esquelético (DOLINSKY *et al.*, 2011).

Neste mesmo sentido, a administração de dexametasona na terceira semana de gestação, também programa a prole para intolerância à glicose e resistência à insulina, assim como aumenta a expressão de PEPCK no fígado de ratos (NYIRENDA *et al.*, 1998). Tratamento com Bromocriptina (inibidor do hormônio prolactina) durante os três últimos dias de lactação induz aumento do peso corporal, do percentual de gordura, hiperleptinemia, além de induzir resistência à insulina na prole adulta de ratos (BONOMO *et al.*, 2007), enquanto que tratamento com leptina exógena nos últimos dez dias de lactação induz aumento do peso corporal, pressão sistólica e frequência cardíaca em ratos adultos (TREVENZOLI *et al.*, 2007).

Em estudo com o modelo de redução de proteína, o peso do rim dos neonatos foi pouco alterado. Entretanto observou-se redução de cerca de 30% no número de néfrons (MARCHAND e LANGLEY-EVANS, 2001). Quando observamos trabalhos que mostram alterações no tamanho, peso ou número de unidades funcionais de diferentes órgãos, podemos inferir sobre as alterações que o ambiente perinatal pode ocasionar sobre as estruturas dos organismos. Essas alterações podem ser um dos mecanismos pelo qual o período precoce da vida pode levar a maior susceptibilidade de desenvolver doenças na vida adulta (LANGLEY-EVANS, 2006).

Um outro possível mecanismo pelo qual a nutrição materna induziria programação metabólica na prole seria a exposição fetal a glicocorticoides (GC) (ALMOND *et al.*, 2012), pois a dieta baixa em proteína inibiria a expressão da enzima 11β-hidroesteroide

desidrogenase (11β-HSD) tipo 2 (BERTRAM e HANSON, 2001b; ERHUMA *et al.*, 2007). Esta enzima inativa a corticosterona, convertendo-a em cortisona (STEWART e KROZOWSKI, 1999). Dieta hipoproteica materna programa para o aumento da sensibilidade ao GC, provavelmente devido à baixa expressão de 11β-HSD2 na placenta (BERTRAM e HANSON, 2001). Ademais, sabe-se que animais desnutridos apresentam maiores concentrações de corticosterona e catecolaminas e maior expressão de receptores de glicocorticoides (GR) na vida adulta (BERTRAM e HANSON, 2001b; FAGUNDES *et al.*, 2007).

Alguns trabalhos têm relacionado o papel de fatores epigenéticos sobre o desenvolvimento de critérios envolvidos na SM (HENRY *et al.*, 1996; GASTER *et al.*, 2001; CARONE *et al.*, 2010; NG *et al.*, 2010; BARRES e ZIERATH, 2011). Padrão de metilação do promotor do PGC1α foi encontrado aumentado em indivíduos que tiveram baixo peso ao nascer (BRONS *et al.*, 2010), o que é sugestivo de que o período fetal pode estar relacionado a modificações epigenéticas. Além disso, aumento da ingestão alimentar por curto período de tempo alterou a metilação do promotor em PGC1α em indivíduos com peso normal ao nascer, porém sem efeitos nos que tiveram baixo peso ao nascimento (BRONS *et al.*, 2010).

Estudo que avaliou o estado de metilação do DNA de ratos que apresentaram RCIU evidenciou alterações na metilação de citosinas em aproximadamente 1400 *loci* em machos com 7 semanas (THOMPSON *et al.*, 2010). Modificações epigenéticas foram observadas principalmente em sequências intergênicas conservadas frequentemente próximas a genes que são conhecidos por regularem processos que são alterados em RCIU, demonstrando que a desregulação epigenética é um forte candidato para programação da “memória” dos eventos intrauterinos (THOMPSON *et al.*, 2010).

A prole de ratas que foram submetidas a restrição proteica durante a gestação apresenta hipometilação das regiões promotoras de PPARα e de receptores de glicocorticoides no fígado e no coração (BURDGE *et al.*, 2004; LILLYCROP *et al.*, 2005b; LILLYCROP e BURDGE, 2011). Além de terem sido observadas modificações na descendência da segunda geração (F2, netos dos ratos que foram inicialmente tratados) (BURDGE *et al.*, 2007). A hipernutrição neonatal induziu hipermetilação das regiões promotoras de POMC no hipotálamo, e este aumento foi inversamente correlacionado com a razão de expressão de POMC/insulina e POMC/leptina (PLAGEMANN *et al.*, 2009).

Restrição calórica durante a gestação resulta em redução da dimetilação da H3K4 de IGF1 em P1, em neonatos. Estas modificações permanecem até a idade adulta independente do regime alimentar durante o período de lactação (TOSH *et al.*, 2010). Enquanto que a hiperglicemia maternal induz diferentes níveis de metilação sobre o gene de IGF1, no fígado, sobretudo por reduzir a porcentagem de H3Me3K36 (ZINKHAN *et al.*, 2012). Sendo que essas diferenças são mais evidentes nos machos que nas fêmeas (ZINKHAN *et al.*, 2012).

Estudos com experimentais com primatas não-humanos observou aumento nos níveis de acetilação de H3K14 no fígado da prole de mães alimentadas com dieta rica em gordura (AAGAARD-TILLERY *et al.*, 2008). Estas modificações foram correlacionadas com alterações da expressão de diversos genes (AAGAARD-TILLERY *et al.*, 2008).

A desregulação epigenética não seria transmitida somente da mãe para a prole. Ng e colaboradores (2010) demonstraram que ratos machos submetidos à dieta rica em gordura antes do acasalamento transmitiam para as fêmeas da sua prole uma disfunção das células β pancreáticas. Este fenótipo foi associado à expressão alterada de genes pro-inflamatórios nas ilhotas pancreáticas e à variação do estado de metilação do promotor (NG *et al.*, 2010). Neste mesmo sentido, Carone e colaboradores (2010) demonstraram que a dieta paterna afetava a expressão de genes relacionados à oxidação de lipídeos e à proliferação, na prole, e que estas informações epigenéticas seriam carregadas no esperma em respostas as condições ambientais.

As alterações da programação metabólica sobre a musculatura esquelética serão discutidas posteriormente, após apresentação do desenvolvimento e propriedades (estruturais e metabólicas) do músculo esquelético, além de breve apresentação das alterações normalmente observadas, como manifestações da SM.

3.4 Sistema muscular esquelético – desenvolvimento

O desenvolvimento do tecido muscular inicia-se bastante cedo na embriogênese. Em ratos, as primeiras células miogênicas formam-se a partir do mesoderma, sendo que os eventos que definem as propriedades funcionais do músculo esquelético prolongam-se até o final do período neonatal, ou seja, as três primeiras semanas pós-natais, que correspondem ao período de aleitamento (HO *et al.*, 1983; PUNKT, NAUPERT e ASMUSSEN, 2004). Durante o desenvolvimento do músculo esquelético, células do somito se diferenciam em um

esclerótomo ventral e um dermomiótomo dorsal, do qual os precursores miogênicos se originam (BIRESSI, MOLINARO e COSSU, 2007). As primeiras células dão origem aosá miócitos mononucleares e as demais progridem nos processos de proliferação, diferenciação e formação das miofibras multinucleadas (Figura 3) (BUCKINGHAM, 2001; BIRESSI, MOLINARO e COSSU, 2007).

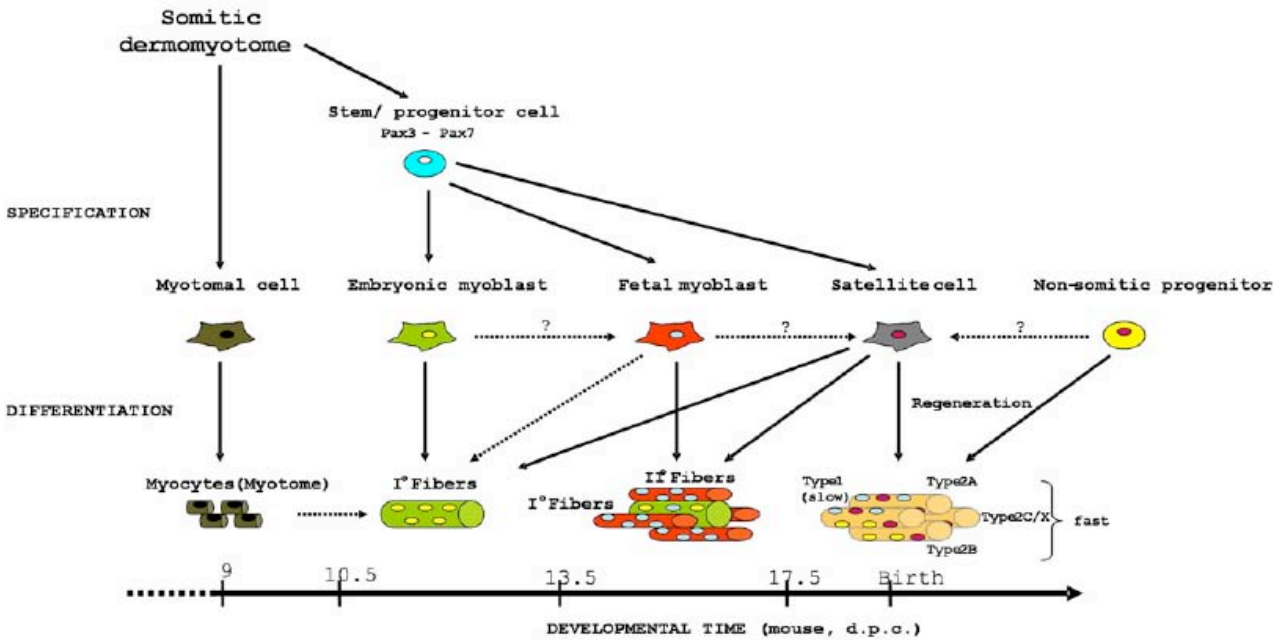

Figura 3. Esquema da linhagem de desenvolvimento do músculo esquelético. O somito dermomiótomo é a origem das células miotomais. Mioblastos embrionários e fetais dão origem, respectivamente, as 1ª e 2ª gerações de células. Células satélites surgem no final da gestação e são responsáveis pelo crescimento pós-natal e regeneração. Retirado de (BIRESSI, MOLINARO e COSSU, 2007).

O processos que levam à formação do tecido muscular são controlados por diversos fatores de transcrição específicos do músculo (Pax3/Pax7, MyoD e MEF2) que governam a miogênese muscular (TE e REGGIANI, 2002; KASSAR-DUCHOSSOY *et al.*, 2004; BUCKINGHAM e MONTARRAS, 2008). Pax3/Pax7 (*Paired Box* 3 e 7) determinam a entrada das células na linhagem miogênica e permitem a sua sobrevivência (BUCKINGHAM, 2007). Os fatores de transcrição da família dos MRF (fatores de regulação da miogênese) estão entre os mais importante para o desenvolvimento da linhagem miogênica (TE e REGGIANI, 2002). Esta família constitui-se de quatro membros: MyoD (*Myogenic differentiation 1*), Myf- (fator miogênico) 5, miogenina e MRF4/Myf-6 (TE e REGGIANI, 2002). Os dois primeiros fatores expressos são MyoD e Myf-5, devido à ativação pelo Pax3/Pax7 (TE e REGGIANI, 2002; BUCKINGHAM, 2007; BUCKINGHAM e MONTARRAS, 2008). A expressão da miogenina acontece na formação do miótomo,

enquanto que o MRF4 apresenta dois momentos de maior expressão, o primeiro logo após à expressão da miogenina e o segundo que coincide com a formação da segunda geração de fibras (TE e REGGIANI, 2002; BUCKINGHAM e MONTARRAS, 2008). MyoD, Myf-5 e MRF4 controlariam o entrada da célula na linhagem miogênica, porém de formas independentes. Enquanto que MyoD, miogenina e MRF4 controlariam a diferenciação celular (KASSAR-DUCHOSSOY *et al.*, 2004; BUCKINGHAM e MONTARRAS, 2008).

O desenvolvimento fetal do sistema muscular também passa por controles epigenéticos da expressão de genes (BAAR, 2010). Modificações estáveis como metilações de DNA de genes nas células mesenquimais evitam a transcrição de genes que não sejam da linhagem miogênica (BAAR, 2010). O padrão de acetilação também pode influenciar a formação dos diferentes tipos de fibras. Por exemplo, sabe-se que o padrão de desacetilação do fator potenciador de miócitos (do inglês *myocyte-enhancing factor*, MEF) 2 inibe a formação de fibras lentas (POTTHOFF, OLSON e BASSEL-DUBY, 2007). Além desses fatores, os micro-RNAs (miRNA) podem também modular a expressão de genes miogênicos, controlando a própria miogênese (GE e CHEN, 2011). Estes miRNAs teriam como alvos genes que estão relacionados a diversos processos chaves da formação muscular (proliferação, diferenciação, DNA polimerase, reguladores transcricionais, proteínas estruturais musculares, entre outros), sendo que estes genes regulariam, por retroalimentação, os miRNAs (GE e CHEN, 2011). A fosforilação de histonas desacetilases (HDAC), inibindo sua ação sobre o MEF2, aumenta tanto a expressão de genes mitocondriais quanto de genes de fibras lentas (LIN *et al.*, 2002b; POTTHOFF, OLSON e BASSEL-DUBY, 2007). Esta poderia ser a relação do porquê alterações na biogênese mitocondrial vêm acompanhadas de mudanças no padrão de fibras musculares (BAAR, 2010).

Sabe-se que a formação embrionária dos músculos estriados esqueléticos é originária de duas gerações distintas de fibras musculares (BARBET, THORNELL e BUTLER-BROWNE, 1991; WIGMORE e DUNGLISON, 1998). As fibras da primeira geração (primárias) desenvolvem-se mais cedo na miogênese, em ratos, em torno do 14° dia embrionário (WIGMORE e DUNGLISON, 1998) e, em humanos, entre a sexta e oitava semana de gestação (BARBET, THORNELL e BUTLER-BROWNE, 1991). As fibras primárias distribuem de através do membro em formação, aumentando rapidamente seu tamanho, estendendo-se de tendão a tendão e dando forma ao músculo (MALTIN *et al.*, 2001). Depois que a formação das fibras primárias está completa, uma segunda geração de

fibras forma-se sobre a superfície de cada fibra primária (MALTIN *et al.*, 2001). As fibras secundárias aparecem desincronicamente sobre um período de dias, do 17 ao 21° dia embrionário em ratos (WIGMORE e DUNGLISON, 1998) e entre a oitava e 18° semana de gestação em humanos (BARBET, THORNELL e BUTLER-BROWNE, 1991).

O sarcômero é uma unidade contrátil básica do músculo esquelético, e está constituído principalmente por filamentos de actina (parte do citoesqueleto) e por filamentos de miosina (proteína motora), além de outras proteínas. A miosina II é a maior proteína sarcomérica, sendo composta por duas cadeias pesadas (do inglês, *myosin heavy chain*, MyHC) e quatro cadeias leves (do inglês, *myosin light chain*, MLC). Várias isoformas MyHC foram descritas, entre elas: duas isoformas desenvolvimentais (embrionária, neonatal/perinatal), uma isoforma lenta (MyHC I/ β) e três rápidas (MyHC IIa, IIx/IId e IIb) (PETTE e STARON, 2000). Cada isoforma da MyHC é expressa por um gene em específico. O gene *MYH3* expressa a isoforma embrionária, o *MYH8* a isoforma neonatal, o *MYH7* a isoforma lenta e β cardíaca, os genes *MYH 2, 1 e 4* expressam as isoformas rápidas MyHC IIa, MyHC IIx/d e MyHC IIb, respectivamente (SCHIAFFINO e REGGIANI, 2011).

A primeira geração de fibras musculares expressa as isoformas lenta, embrionária e neonatal, a segunda expressa as isoformas embrionária, neonatal e rápida (BARBET, THORNELL e BUTLER-BROWNE, 1991). Com o desenvolvimento, a primeira geração vai expressar a isoforma lenta e este padrão não será modificado (BARBET, THORNELL e BUTLER-BROWNE, 1991). A segunda geração vai diminuir a expressão das fibras embrionárias ainda durante o período fetal, contudo as neonatais ainda restam no período pós-natal (BARBET, THORNELL e BUTLER-BROWNE, 1991; AGBULUT *et al.*, 2003).

Observa-se que no período gestacional ocorrem diversas modificações que são chaves para a disposição das fibras apresentadas ao nascimento. Sinais do ambiente onde o feto está em desenvolvimento também têm papel central na especificação do tipo de fibra muscular, sendo que esta influência depende da interação dos fatores internos e externos (KARDON, CAMPBELL e TABIN, 2002). As células precursoras musculares (CPM) não apresentam marcadores do tipo de fibra que dará origem quando ainda estão no somito, somente ao atingir o local de desenvolvimento do membro é que este tipo de marcador aparecerá (KARDON, CAMPBELL e TABIN, 2002). Entretanto, as células que dão origem as fibras lentas apresentam essa característica mais precocemente no desenvolvimento do que as que serão fibras rápidas, sugerindo que estas fibras podem ser programadas ainda quando nos somitos

(KARDON, CAMPBELL e TABIN, 2002). Além disso, o número de fibras musculares já está determinado ao nascimento (BUSCHER e IZPISUA BELMONTE, 1999), exceto em roedores onde este número pode variar no período pós-natal precoce (BRAMELD, 2004).

Em camundongos recém-nascidos, o padrão de expressão da MyHC é semelhante nos diferentes tipos musculares, porém a maturação e a aquisição do padrão adulto ocorrem em momentos cronológicos diferentes a depender da função do grupamento muscular (HO *et al.*, 1983; AGBULUT *et al.*, 2003). Nos músculos que desenvolverão padrão de contração rápida, as formas embrionária e neonatal são substituída logo após o nascimento pelas formas rápidas de MyHC, enquanto que nos músculos com programação para serem lentos e mais resistentes à fadiga, a forma neonatal persiste até os 21 dias pós-natal (AGBULUT *et al.*, 2003). Em humanos, no período fetal há a expressão de todos os tipos de MyHC, enquanto que aos 8 meses pós-natal, somente as MyHC lenta e rápidas são expressas no quadríceps (BARBET, THORNELL e BUTLER-BROWNE, 1991).

O desenvolvimento muscular pós-natal é resultado de mudanças na atividade neuromuscular, maturação do acoplamento excitação-contração e aumentos nos níveis de hormônios tireoidianos (PETTE e STARON, 2000; AGBULUT *et al.*, 2003). Estas mudanças no padrão de expressão das isoformas de MyHC coincidem com a eliminação da inervação polineural, que ocorre mais rapidamente em músculos de contração rápida do que nos lentos (JANSEN e FLADBY, 1990). Além disso, diferentes grupamentos funcionais musculares diferem no número de receptores aos hormônios tireoidianos (WHITE *et al.*, 2001), sendo sabido que o aumento nos níveis desses hormônios coincide com a diminuição da expressão das formas embrionária e neonatal (AGBULUT *et al.*, 2003). Estas informações nos mostram que o padrão de expressão de MyHC também pode ser alterado durante o começo da vida pós-natal (AGBULUT *et al.*, 2003).

A síntese proteica no músculo imaturo é altamente sensível às variações agudas de ingestão alimentar e a consequente flutuação dos níveis de insulina plasmática (DAVIS *et al.*, 1993). Embora a taxa de crescimento muscular seja altamente sensível a variações na ingestão de nutrientes no período neonatal, a maturação muscular tende a ser minimamente alterada (FIOROTTO e DAVIS, 1997). As maiores diferenças observadas são da taxa de crescimento corporal que está intimamente ligada a maior acreção de proteínas para a hipertrofia muscular (DAVIS e FIOROTTO, 2009).

Mauro (1961) reportou a observação de células mononucleadas e indiferenciadas localizadas na periferia das fibras musculares multinucleadas maduras, posicionadas entre a lâmina basal e a membrana plasmática das fibras, essas células foram denominadas células satélites (SC). As SC permanecem em um estado de quiescência, até serem ativadas por alguns estímulos, como crescimento ou trauma, entrando na linhagem miogênica, proliferando-se e dando origem, ou a novas SC ou a novas fibras musculares (Figura 4) (BUCKINGHAM e MONTARRAS, 2008). Estudo que realizou a remoção das SC por irradiação gama mostrou que estas células apresentam um papel ativo e obrigatório precoce sobre o processo curso temporal das mudanças no fenótipo das fibras induzidas por estímulos externos (Martins et al., 2009).

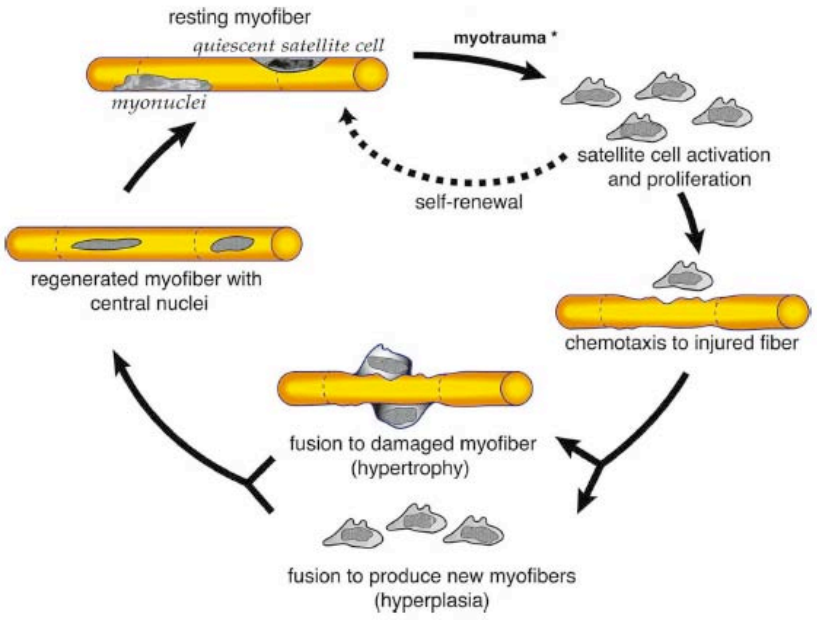

Figura 4. Células satélites ativadas em resposta ao miotrauma. *Trauma ou injúria de baixa intensidade (exercício) ou mais extenso (distrofias) (HAWKE e GARRY, 2001).

A expressão de algumas proteínas indica em qual fase miogênica a SC encontra-se. Pax7 é expresso em células quiescentes, ativadas, em proliferação e no começo da diferenciação (SEALE e RUDNICKI, 2000; BUCKINGHAM e MONTARRAS, 2008). Células ativadas e em proliferação também são positivas para Myf-5 (COOPER *et al.*, 1999; BEAUCHAMP *et al.*, 2000; BUCKINGHAM e MONTARRAS, 2008). SC em proliferação e em diferenciação, conhecidas como mioblastos, e já diferenciadas em miotubos expressam MyoD (COOPER *et al.*, 1999; BEAUCHAMP *et al.*, 2000; BUCKINGHAM e

MONTARRAS, 2008). Miogenina é expressa nos mioblastos e nos miotubos (Figura 5) (COOPER *et al.*, 1999; BEAUCHAMP *et al.*, 2000; BUCKINGHAM e MONTARRAS, 2008). Vários fatores (IGF-1 e 2, fator de crescimento de fibroblasto – FGF, fator de crescimento de hepatócito – HGF, interleucina – IL-6, macrófagos, entre outros) podem influenciar a atividade das SC aumentando ou diminuindo a sua taxa de diferenciação e proliferação (HAWKE e GARRY, 2001).

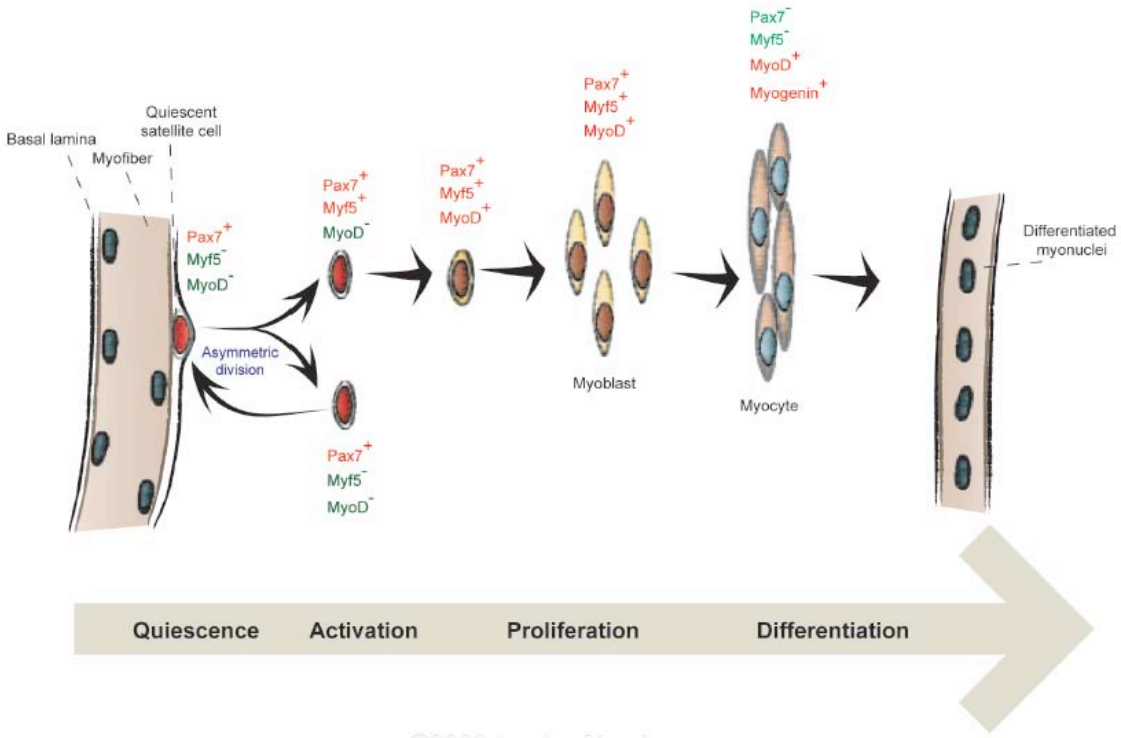

Figura 5. Miogênese a partir das células satélites. Fatores que são expressos durante a miogênese das células satélites até a célula muscular diferenciada. Algumas células quando ativadas escapam do ciclo celular e regeneram o pool de células satélites no músculo adulto (PERDIGUERO *et al.*, 2009).

O número de SC decresce com a idade, de 30% no período neonatal, para 4% no adulto e 2% nos idosos (SNOW, 1977; HAWKE e GARRY, 2001). Também é observada diferença no número de SC em relação ao tipo de fibras e de músculo (HAWKE e GARRY, 2001). As fibras oxidativas apresentam maior quantidade de SC do que as glicolíticas (GIBSON e SCHULTZ, 1982). Diferenças na taxa de proliferação e capacidade de diferenciação de SC provenientes de fibras lentas ou rápidas quando colocadas em cultura também foram observadas (LAGORD *et al.*, 1998). Além disso, SC isoladas de músculos predominantemente lentos apresentam maior potencial adipogênico do que as de músculos com predominância de fibras rápidas (YADA, YAMANOUCHI e NISHIHARA, 2006).

As SC musculares quando colocadas em cultura retém o perfil de expressão de isoformas de tropomiosina e da MLC que possuíam (MATSUDA, 1983). Esta observação indica que há um controle epigenético sobre as CPM, e talvez nos núcleos musculares, no sentido que estas mantenham um perfil característico de um tipo de fibra, rápida ou lenta. (BAAR, 2010). Caso este controle não existisse essas CPM apenas se diferenciariam em células musculares (BAAR, 2010).

3.5 Sistema muscular esquelético – propriedades

A massa e composição do músculo esquelético são críticos para suas funções; tais como, locomoção, manutenção da postura, gasto energético e metabolismo da glicose (BERCHTOLD, BRINKMEIER e MUNTENER, 2000). O músculo esquelético é um tecido heterogêneo (HO *et al.*, 1983; PETTE e STARON, 2000; PUNKT, NAUPERT e ASMUSSEN, 2004; BASSEL-DUBY e OLSON, 2006b), podendo ser classificado de várias formas, sendo a mais comum a classificação em relação ao tipo de MyHC predominante (PIEROBON-BORMIOLI *et al.*, 1981; PETTE e STARON, 2000; PUNKT, NAUPERT e ASMUSSEN, 2004). Existem fibras musculares que contém apenas uma isoforma de MyHC (fibras puras) e as que expressão mais de uma isoforma (híbridas) (PETTE e STARON, 2000). De acordo com a expressão de MyHC, o músculo esquelético de mamíferos adultos pode ser dividido nas seguintes fibras puras: tipo I ou lenta que expressa MyHCI/β, e três tipos de fibras rápidas, sendo elas, tipo IIA expressando MyHC IIa, tipo IID/X com MyHC tipo IId/x, e tipo IIB com MyHC IIb (PETTE e STARON, 2000). As fibras híbridas são classificadas baseadas na isoforma dominante de MyHC: tipo I/IIA, também conhecida como IC (MyHCIb>MyHCIIa); tipo IIA/I, conhecida como IIC (MyHCIIa>MyHCIb); tipo IIAD (MyHCIIa>MHyCIId); tipo IIDA (MyHCIId>MyHCIIa); tipo IIDB (MyHCIId>MyHCIIb), e tipo IIBD (MyHCIIb>MyHCIId) (PETTE e STARON, 2000).

Em ratos, no músculo sóleo, há predominância, aos 6 dias pós-natal de fibras tipo IIA, entretanto aos 21 dias, esta relação se inverte com predominância das fibras tipo I, que serão 70-80% da composição do músculo na vida adulta (HO *et al.*, 1983; PUNKT, NAUPERT e ASMUSSEN, 2004). Enquanto que no músculo plantar, as fibras IIA também são maioria no início da vida e aos 40 dias, sendo que, na vida adulta, sua proporção vai ser praticamente a mesma (47% tipo IIA, 43% tipo IIB) das fibras tipo IIB, sendo este músculo caracterizado como de contração rápida (HO *et al.*, 1983). No gastrocnêmio, observamos maior proporção de fibras tipo II indiferenciadas, na primeira semana de vida, e a predominância de fibras tipo

IIA no fim da lactação até a vida adulta (PUNKT, NAUPERT e ASMUSSEN, 2004). O extensor longo dos dedos (EDL), em ratos, apresenta cerca de 93% de fibras tipo II indiferenciadas na primeira semana de vida, enquanto que na vida adulta vai apresentar cerca de 55% de fibras IIA e 35% de fibras IIB (PUNKT, NAUPERT e ASMUSSEN, 2004). Essas modificação são observadas, não somente em colorações histoquímicas, mas também, quando se estuda as atividades das enzimas envolvidas, como a ATPase, SDH e GPDH (PUNKT, NAUPERT e ASMUSSEN, 2004).

Estudo com músculos de três diferentes tipo de mamíferos determinou histoquimicamente a porcentagem das fibras rápidas presentes em diferentes músculos (HAMALAINEN e PETTE, 1993). Em ratos, foi observada a seguinte relação de percentagem de fibras no adutor magno, na porção profunda do psoas e no tibial anterior: IIB>IID>IIA>I (HAMALAINEN e PETTE, 1993). Enquanto que, em camundongos, não foram observadas fibras IIA nem I nem tibial anterior, nem gastrocnêmios, nem vasto lateral (HAMALAINEN e PETTE, 1993). Diferentemente, as fibras IIA estavam presentes no vasto lateral de coelhos, e estes apresentavam somente tipo IID e IIA no tibial anterior (HAMALAINEN e PETTE, 1993).

Diferenças no perfil muscular também foram observadas por diferentes tipos de colorações e técnicas imunohistoquímicas (BROOKE e KAISER, 1970; PIEROBON-BORMIOLI *et al.*, 1981), onde se observa a predominância de diferentes isoformas de MyHC a depender do músculo estudado, correlacionando essas diferenças com as funções que estes músculos exercem (BROOKE e KAISER, 1970; PIEROBON-BORMIOLI *et al.*, 1981). Deve-se observar que necessita-se cuidado ao comparar um mesmo músculo em diferentes espécies, pois eles podem apresentar funções diferenciadas e portanto, composição diferenciada de fibras.

As mudanças nas isoformas tendem a seguir uma sequência geral e reversível de transição de rápida para lenta e de lenta para rápida: MyHCIb ⇿ MHCIIa ⇿ MHCIId ⇿ MHCIIb (PETTE e STARON, 2000). A transição entre os tipos de fibras ocorre em resposta à atividade neuromuscular, sobrecarga/descarga, alteração dos níveis de hormônio e envelhecimento (PETTE e STARON, 2000). Considerando sua heterogeneidade, o músculo esquelético pode responder diferentemente a um estímulo ambiental a depender da predominância do tipo de fibra, da mesma forma que a magnitude do estímulo para desencadear a transição de fibras também depende da isoforma predominante (PETTE e

STARON, 2000; PUNKT, NAUPERT e ASMUSSEN, 2004). A transição das fibras no indivíduo adulto, assim como no desenvolvimento, é controlado por fatores de sinalização e transcrição, como: MEF2, calcineurina, fator nuclear de células T ativadas (do inglês, nuclear factor of activated T cells, NFAT), proteína quinase dependente de cálcio/calmodulina (do inglês, Calcium/Calmodulin-dependent protein kinase, CaMK), PGC1α, mTOR, IGF1, entre outros (BASSEL-DUBY e OLSON, 2006b).

Nos mamíferos, os diferentes tipos de músculos também diferem em relação ao tipo de inervação recebida (SCHIAFFINO e REGGIANI, 2011). As unidades motoras que apresentam grande quantidade de impulsos, com séries de longa duração e baixa frequência de disparo são típicas de fibras lentas e resistentes à fadiga (SCHIAFFINO e REGGIANI, 2011). Em contraste, as fibras rápidas apresentam unidades motoras com alta frequência de disparo e podem ser divididas em dois subgrupos: o primeiro com baixa atividade diária, alta frequência de disparos e séries de curta duração (correspondendo as fibras tipo IIB), o outro subgrupo não difere na média de disparos, mas apresentam maior atividade diária e séries de longa duração (correspondendo as fibras tipo IIA e IIX) (SCHIAFFINO e REGGIANI, 2011).

Fibras lentas contraem mais lentamente, apresentam maior resistência à fadiga, menor potência mecânica, menor pico de força, mas também consomem menos ATP para gerar tensão (SCHIAFFINO e REGGIANI, 2011). Enquanto que as fibras rápidas produzem contração mais rapidamente, maior potência mecânica, maior pico de força, menor resistência à fadiga e maior consumo de ATP (SCHIAFFINO e REGGIANI, 2011). Estas características fazem das fibras lentas mais propícias a atividades de longa duração e baixa intensidade, enquanto que as rápidas são melhores para atividades de curta duração e de grande produção de força.

O tipo de atividade e a localização do músculo podem influenciar no perfil de distribuição do tipo de fibras (SILVADO e WERNECK, 2006; MATSUMOTO *et al.*, 2007). Os músculos bíceps e tríceps braquiais apresentam diferentes proporções de fibras a depender da região estudada (SILVADO e WERNECK, 2006; MATSUMOTO *et al.*, 2007). As porções mais superficiais, que sofrem menor ação da gravidade e apresentam menor irrigação, contém exclusivamente fibras tipo IIB, que também têm menor capacidade oxidativa e maior área de secção transversa quando comparadas com as porções mais profundas desses músculos (SILVADO e WERNECK, 2006; MATSUMOTO *et al.*, 2007). No músculo gastrocnêmio de ratos, a zona mais externa (superficial) é composta quase inteiramente de

fibras tipo IIA (94%), com apenas 6% de fibras tipo IIB, a região medial mostra predominância de fibras tipo IIA (69%), com as fibras do tipo IIB (28%) em segundo, e apenas 3% de fibras tipo I, na região mais profunda predominam fibras do tipo IIB (61%), juntamente com 25% de fibras do tipo I e 15% de fibras do tipo IIA (SILVADO e WERNECK, 2006; MATSUMOTO *et al.*, 2007).

Também são observadas diferenças na expressão das isoformas de α-actina no músculo esquelético a depender do tipo de fibra (ICHINOSEKI-SEKINE *et al.*, 2012). Todas as fibras do tipo IIB expressam a α-actina 3, enquanto que esta não é expressa nas fibras do tipo I, a isoforma α-actina 2 é expressa em todos os tipos de fibras, em ratos (ICHINOSEKI-SEKINE *et al.*, 2012). A expressão das isoformas de α-actina no músculo esquelético de ratos é considerada como sendo mais próxima da em humanos do que da expressão em camundongos (ICHINOSEKI-SEKINE *et al.*, 2012).

Apesar da cadeia pesada de miosina ser utilizada para caracterizar o tipo de fibras, ainda existem vários outros componentes do sarcômero (SCHIAFFINO e REGGIANI, 1996). As MLC também existem nas isoformas lenta e rápida, e em conjunto com as formas lenta e rápida da troponina, as isoformas de tropomiosinas, a proteína titina e outras proteínas fornecem ao músculo o seu potencial contrátil (SCHIAFFINO e REGGIANI, 1996).

Interessante trabalho apresentou análise transcriptômica em fibras únicas (microgenia) do tipo I e do tipo IIB (CHEMELLO *et al.*, 2011). As fibras foram extraídas do músculo sóleo e EDL de camundongos, tendo sua pureza certificada pela análise da cadeia pesada de miosina. Foram observadas diferenças na expressão de genes de via de sinalização e metabólicas entre os dois tipos de fibras (CHEMELLO *et al.*, 2011). No total, 1.505 genes diferencialmente expressos, não redundantes foram identificados em fibras tipo I *vs.* tipo IIB, dessas 930 sondas foram superexpressas em fibras tipo I e 602 em fibras tipo IIB (CHEMELLO *et al.*, 2011). Dentre estas, nas fibras tipo I estavam superexpressos genes envolvidos na oxidação fosforilativa e metabolismo de ácidos graxos, enquanto que no tipo IIB eram os envolvidos na via de sinalização de insulina, glicólise, gluconeogênese e proteólise (CHEMELLO *et al.*, 2011).

3.6 Sistema muscular esquelético – metabolismo e plasticidade

As diferenças entre as fibras musculares listadas no subitem anterior também podem ser observadas quando estudamos o metabolismo do músculo esquelético. Isto se reflete no metabolismo de todo o corpo, visto que o músculo esquelético é o maior reservatório de proteína do corpo, de onde pode-se liberar aminoácidos durante grandes períodos de fome para a manutenção da glicemia do sangue (CAHILL, 2006; HARRIDGE, 2007). Além de fonte de aminoácidos, o músculo esquelético é o maior captador da glicose pós-prandial (DEFRONZO, 1992), e durante o metabolismo basal e em atividades de leve à moderada intensidade é um grande consumidor de ácidos graxos (ABERNETHY, THAYER e TAYLOR, 1990).

Baseado nas diferenças metabólicas existentes entras os diferentes tipos de fibras, elas também tendem a ser classificadas como: fibras oxidativas (FO, correspondendo às fibras tipo I), fibras oxidativas-glicolíticas (FOG, correspondendo às fibras tipo IIA e IIX), fibras glicolíticas (FG, correspondendo às fibras tipo IIB) (PETER *et al.*, 1972).

Randle e colaboradores (1963) propuseram a existência de competição entre a utilização de ácidos graxos (AG) ou carboidratos como substrato para a produção de ATP no músculo esquelético. Em caso de aumento da disponibilidade de glicose, esta seria utilizada como principal fonte de produção de energia, caso houvesse mais ácidos graxos disponíveis, estes seriam utilizados em preferência aos carboidratos (Figura 6) (RANDLE *et al.*, 1963).

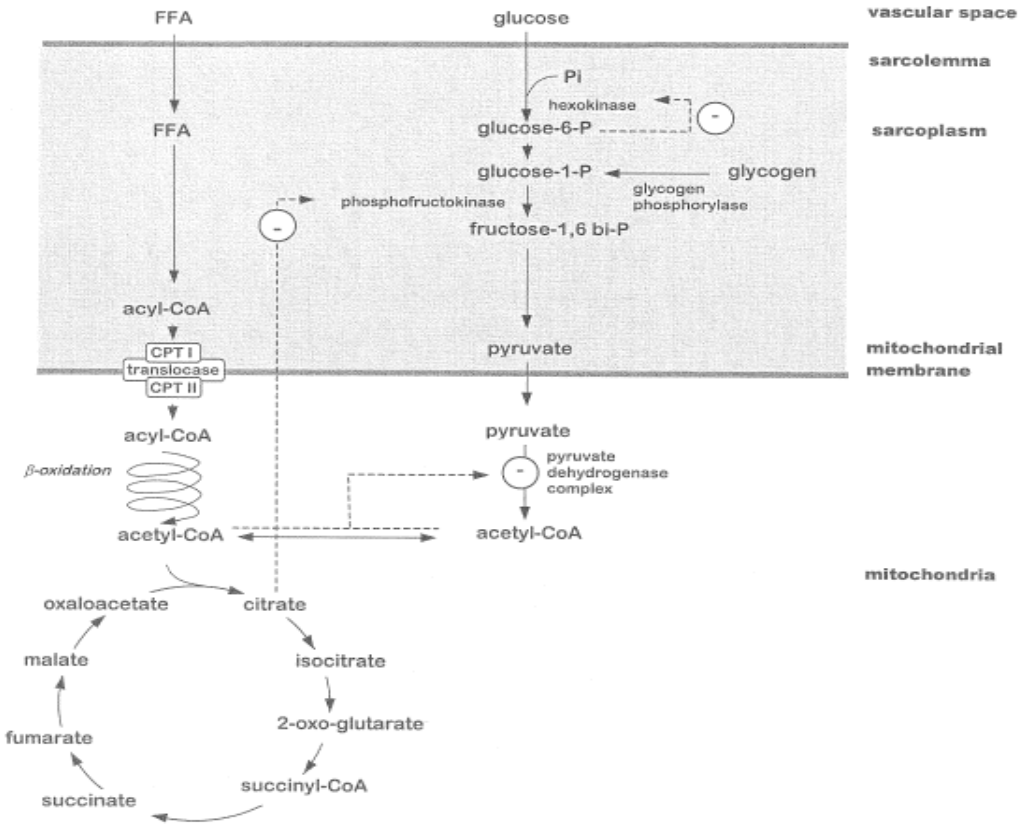

Figura 6. Ciclo de Randle ou ciclo glicose- ácidos graxos. Descrição dos mecanismos envolvidos na interação/regulação entre metabolismo de AG e glicose. Retirado de (JEUKENDRUP, 2002).

A oxidação da glicose até o piruvato se dá pela glicólise, que posteriormente necessita que o piruvato seja desidrogenado para formar acetil-coenzima A (acetil-CoA) ou carboxilado para formar oxalacetato, enquanto que os ácidos graxos podem ser oxidados pela β-oxidação diretamente à várias moléculas de acetil-CoA, a depender da quantidade de carbonos na molécula original de AG. Ambos os processos tem como finalidade a produção de substrato para ciclo de Krebs e, posterior, produção de energia pela cadeia transportadora de elétrons. Os triglicerídeos intramusculares (IMTG) são uma fonte rápida para o músculo de lipídios e consequentemente de energia, sendo a forma preferencial de AG utilizado durante os exercícios prolongados e de intensidade moderada(ABERNETHY, THAYER e TAYLOR, 1990). Sendo que as fibras do tipo I apresentam maior estoque de IMTG que as tipo II (ABERNETHY, THAYER e TAYLOR, 1990).

Músculos lentos geram todo ATP necessário via processos de oxidação mitocondrial, e, como não apresentam grande consumo de ATP durante a contração, eles conseguem manter a atividade por longo período de tempo sem apresentar fadiga (SCHIAFFINO e REGGIANI,

2011). Enquanto que os músculos rápidos geram ATP principalmente pela glicólise, que, apesar de ser uma fonte rápida de geração, não consegue ser mantida por muito tempo, o que é um dos limitantes da atividade desses músculos (SCHIAFFINO e REGGIANI, 2011).

As fibras musculares nas diversas espécies de mamíferos diferem na proporção de algumas organelas e conteúdos de substâncias, a depender do tipo de metabolismo predominante (SCHIAFFINO e REGGIANI, 2011). No rato, o volume ocupado pelas mitocôndrias nas células varia entre 2,2% em FG à 10% em FO (SCHIAFFINO, HANZLIKOVA e PIEROBON, 1970; GOLLNICK *et al.*, 1981; HOWALD *et al.*, 1985). O conteúdo de lipídios e triglicerídeos são maiores nas fibras lentas em relação às rápidas (GOLLNICK *et al.*, 1981; HOWALD *et al.*, 1985). Enquanto que o conteúdo de glicogênio é maior nas fibras rápidas comparadas com as lentas, além disso o conteúdo de glicogênio diminui mais rapidamente nas FO do que nas FG (VOLLESTAD, VAAGE e HERMANSEN, 1984).

Fibras lentas são mais eficazes na remoção da glicose do sangue dos que as rápidas, isto tem sugerido que alterações nas proporções das fibras podem contribuir para a resistência à insulina (PATTI *et al.*, 2003). A capacidade de captação de glicose é maior nas FO e FOG do que nas FG, com concomitante aumento da expressão de GLUT4 e atividade de hexoquinase II (HKII) nas primeiras fibras (BASS *et al.*, 1969; HENRIKSEN *et al.*, 1990; KONG *et al.*, 1994). Entretanto, as FG apresentam maior atividade da fosfofrutoquinase (PFK) e lactato desidrogenase (LDH), confirmando que estas fibras apresentam maior capacidade glicolítica (BASS *et al.*, 1969; LOWRY *et al.*, 1978). A atividade da piruvato desidrogenase quinase (PDK), enzima que inibe a conversão de piruvato em acetil-CoA, é maior nas FO do que nas FG (PETERS *et al.*, 2001). A expressão de duas lipases que contribuem para a disponibilidade de ácidos graxos, além da atividade da enzima β-hidroxiacil desidrogenase (β-HAD) (limitadora da β-oxidação) e da carnitina palmitoil transferase (CPT) I são maiores nas fibras lentas do que nas fibras rápidas (BASS *et al.*, 1969; LANGFORT *et al.*, 1999; KIM *et al.*, 2002). Além disso, a atividade da citrato sintase (CS), enzima limitadora do ciclo de Krebs, também é maior nos músculos oxidativos (BASS *et al.*, 1969).

A ação da insulina sobre a captação de glicose também ocorre de maneira específica para cada tipo de fibra, com maior resposta à insulina nas fibras do tipo I do que na de tipo IIA ou IIB (SNOW, 1977; HENRIKSEN *et al.*, 1990; SONG *et al.*, 1999). Ocorre também

diferenças na fosforilação do receptor de insulina (IR), IRS-1 e IRS-2; além de diferenças no aumento da fosfotirosina associada à atividade da fosfatidilinositol-3-quinase (PI3K), e aumento da fosforilação de Akt, todos em resposta ao estímulo da insulina (SONG *et al.*, 1999). Essas diferenças funcionais estão relacionadas a diferenças na expressão de proteínas intermediárias da cascata de sinalização de insulina, porém sem diferenças no curso temporal de ativação da cascata (SONG *et al.*, 1999).

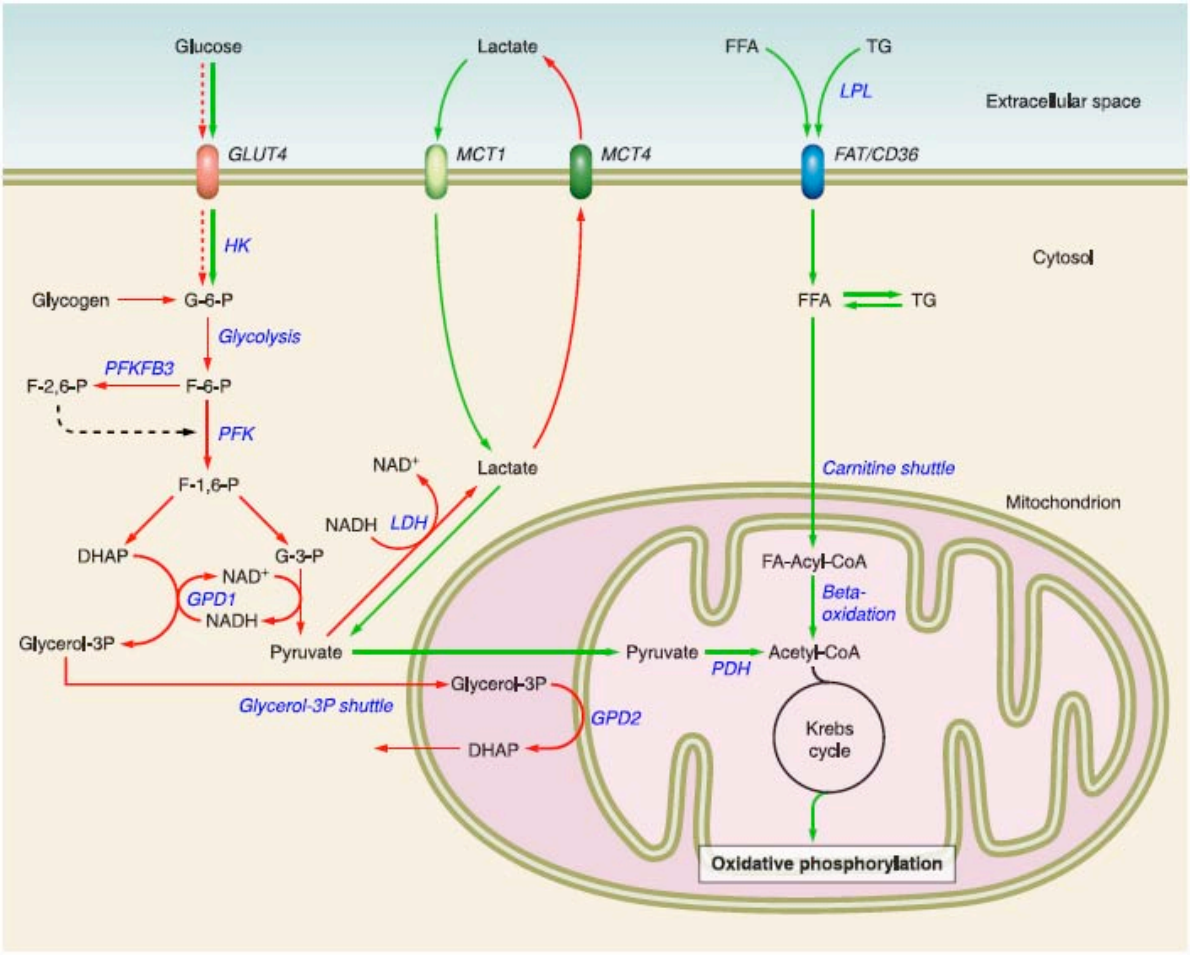

Figura 7. Esquema mostrando as diferentes vias metabólicas preferenciais em cada tipo de fibra muscular. Fibras lentas são representadas em verde e as fibras rápida em vermelho. Retirado de (SCHIAFFINO e REGGIANI, 2011).

O músculo esquelético também apresenta mecanismo de defesa da integridade de seu metabolismo, um desses mecanismo é executado pelas proteínas desacopladoras (principalmente a isoforma 3), conhecida pela sigla UCP3. A UCP3 é mais abundante no EDL, músculo rico em FG, do que no sóleo músculo rico em FO (HIRABARA *et al.*, 2006a). Caso o organismo, e principalmente o sistema muscular esquelético, seja submetido à excesso

de AG, acima da capacidade oxidativa das mitocôndrias, esses AG são armazenados na forma de ânions de AG que podem sofre mais facilmente peroxidação (SCHRAUWEN e HESSELINK, 2004b; HIRABARA *et al.*, 2006a; SILVEIRA *et al.*, 2008a), por este motivo a UCP3 é mais expressa em músculo com menor capacidade oxidativa. As UCP3s atuam, então, como carreadoras desses ânions de AG para fora da mitocôndria, limitando a produção de ERO e aumentando a capacidade oxidativa da mitocôndria (HIRABARA *et al.*, 2006a). É sabido que situações que aumentam a disponibilidade de AG para as células musculares, como ingestão de dietas ricas em lipídios ou infusão de lipídeos e jejum prolongado levam ao aumento da expressão de RNAm UCP3, servindo como um mecanismo de retro-controle da função mitocondrial (SCHRAUWEN e HESSELINK, 2004b; DE LANGE *et al.*, 2006; HIRABARA *et al.*, 2006a; SILVEIRA *et al.*, 2008a).

PPARs são receptores nucleares dependentes de ligantes que pertencem a superfamília de fatores de transcrição nucelares. PPARα é expresso no fígado, coração, rim e músculo esquelético e sua função está relacionada à regulação do catabolismo de AG, enquanto que o PPARγ é altamente encontrado em adipócitos e macrófagos estando envolvido na diferenciação de adipócitos, estocagem de lipídeos e homeostase da glicose (LEE, OLSON e EVANS, 2003). PPARβ/δ é o mais abundante PPAR no tecido muscular (MUOIO *et al.*, 2002b), sendo mais expresso nos músculos oxidativos do que nos glicolíticos e influenciando a formação de FO (WANG *et al.*, 2004b). O PPARβ/δ também é o maior regulador do metabolismo de ácidos graxos no tecido adiposo, o que indica uma possível relação entre tipo de fibras, metabolismo de lipídeos e obesidade (WANG *et al.*, 2004b), visto que o PPARβ/δ também está relacionado à determinação do fenótipo das fibras musculares (WANG *et al.*, 2004b).

Superexpressão constitutiva ou músculo-específica de PPAR β/δ leva a um perfil mais oxidativo das fibras, com aumento de DNA mitocondrial, aumento da expressão de genes de proteínas de contração lenta e aumento da resistência à fadiga, além de aumento no número de fibras oxidativas e aumento da resistência em corrida (GRIMALDI, 2003; WANG *et al.*, 2004b). A utilização de GW501516, um agonista do PPARδ, em cultura de células musculares levou ao aumento da oxidação de palmitato, redução na glicólise, na síntese de glicogênio e na liberação de lactato (BRUNMAIR *et al.*, 2006). Este achados não foram revertidos, mesmo após a estimulação com insulina, estando mais relacionados à disponibilidade de lipídios no meio (BRUNMAIR *et al.*, 2006). No mesmo sentido, a ablação

músculo-específica de PPARδ leva a redução do número de fibras tipo I e redução da expressão muscular de PGC1α (SCHULER *et al.*, 2006b).

PGC1α foi inicialmente identificado através da sua interação funcional com o receptor nuclear PPARγ (FINCK e KELLY, 2006). Atualmente, sabe-se que ele atua como co-ativador transcricional de vários receptores nucleares e de fatores de transcrição (KNUTTI e KRALLI, 2001) e também tem sua importância em diversas funções em relação ao metabolismo energético, incluindo indução de biogênese mitocondrial, regulação do metabolismo de glicose e oxidação de ácidos graxos (FINCK e KELLY, 2006).

Vários fatores de transcrição podem aumentar a expressão de RNAm PGC1α, esses fatores respondem a diferentes vias de sinalização como : insulina (atua via inibição da proteína Forkhead box O1 – FOXO1), exercícios e citocinas (ambas atuando via proteína quinase ativada por mitógeno, MAPK-p38, que ativa MEF2 e a proteína ligada a elemento responsivo à AMPc, CREB,) (FERNANDEZ-MARCOS e AUWERX, 2011). A atividade de PGC1α pode ser regulada pela acetilação/desacetilação. GCN5 acetila PGC1α no fígado e no músculo, o que diminui a sua atividade (LERIN *et al.*, 2006; GERHART-HINES *et al.*, 2007), enquanto que sirtuína 1 (Sirt1, uma deacetilase dependente de NAD) desacetila PGC1α *in vitro* (NEMOTO, FERGUSSON e FINKEL, 2005) e também é necessária para a desacetilação de PGC1α durante o jejum prologado, em ambos os casos a atividade de PGC1α é aumentada (GERHART-HINES *et al.*, 2007). PGC1α também pode ser regulado por fosforilação, mas ao contrário da acetilação, quando fosforilado PGC1α tende a aumentar sua atividade e sua estabilidade (FERNANDEZ-MARCOS e AUWERX, 2011). AMPK, MAPK p38, e Akt são proteínas quinases que podem fosforilar PGC1α, entretanto a fosforilação por Akt tem o efeito inverso na atividade de PGC1α, diminuindo sua atividade (FERNANDEZ-MARCOS e AUWERX, 2011).

Nos músculos lentos há maior expressão de PGC1α do que nos rápidos, quando há inativação do gene PGC1α de maneira músculo específica observa-se mudança parcial dos tipos de fibras tipo IIA para os tipo IIB e IIX e redução da tolerância ao exercício (HANDSCHIN *et al.*, 2007). Superexpressão de PGC1α no músculo esquelético acarreta efeitos sobre o metabolismo muscular similares aos efeitos observados quando se superexpressa uma forma ativada de PPARδ (WANG *et al.*, 2004b). Superexpressão muscular de PGC1α resulta em aumento de fibras tipo I nos músculos vasto lateral e plantar, além de maior resistência à fadiga e maior expressão de proteínas envolvidas na oxidação fosforilativa

(LIN *et al.*, 2002b; MIURA *et al.*, 2003), entretanto diminui a expressão de GLUT4 e prejudica o controle glicêmico após teste de resistência à insulina (MIURA *et al.*, 2003). Exercícios de resistência também induz aumento na expressão de RNAm e proteína PGC1α (BAAR *et al.*, 2002).

Superexpressão de PGC1α em culturas primárias de mioblastos induz rápida maturação dos miotubos, evidenciada pela redução da regulação da expressão das isoformas imaturas de MyHC, aumento das isoformas lentas e redução das isoformas rápidas (MORTENSEN *et al.*, 2010).

Apesar da sua função como regulador central de várias vias metabólicas, o PGC1α não é indispensável para as alterações induzidas pela restrição calórica na homeostase energética corporal total, mas sim, para as alterações causadas pela restrição na indução de biogênese mitocondrial (FINLEY *et al.*, 2012). Interessantemente, a indução da biogênese mitocondrial foi limitada aos músculos oxidativo, também requerendo a participação de PGC1α (FINLEY *et al.*, 2012).

O músculo como um órgão extremamente plástico, pode apresentar variações no tamanho, metabolismo e propriedades funcionais devido à condição fisiológica ou patológica (HARRIDGE, 2007; BAAR, 2010). Podendo variar o seu tamanho, mudar a expressão das isoformas de proteínas, sem mudar seu tamanho ou simplesmente mudar o substrato predominante do qual produz energia (HARRIDGE, 2007; BAAR, 2010). Além disso, é capaz de modificar o fenótipo das fibras em relação à MyHC devido atividade neuromuscular alterada, sobrecarga ou diminuição da carga mecânica, alterações no perfil hormonal e envelhecimento (PETTE e STARON, 2000).

A inervação apresenta um papel específico na manutenção do fenótipo muscular, estudos de inervação cruzada observaram que músculos rápidos se tornam lentos quando inervados por nervos lentos e vice-versa (BULLER, ECCLES e ECCLES, 1960). Da mesma forma, estudos com estimulação elétrica crônica com padrões de impulsos específicos de motoneurônios lentos ou rápidos também foram capaz de alterar o fenótipo muscular (PETTE e STARON, 2000). Treinamento físico também altera este perfil, mas parece estar mais restrito às fibras do tipo II (PETTE e STARON, 2000). Sobrecarga conduz a mudanças das fibras rápidas em lentas, enquanto que diminuição da carga (como em suspenção de membros, microgravidade, imobilização em posição encurtada do músculo) levam à mudança do

fenótipo em direção ao aumento de fibras rápidas (PETTE e STARON, 2000). A transição entre os diferentes tipos de fibras inclui mudanças nas proteínas sarcoméricas das diferentes isoformas e representa uma orquestrada mudança na expressão gênicas (PETTE e STARON, 2000). As mudanças nas isoformas de proteínas ou em suas quantidades é controladas nos níveis de transcrição, tradução e degradação proteica (PETTE e STARON, 2001). Sabe-se que os MEF2 podem ativar genes específicos musculares para induzir a miogênese, e que a via MEF2/HDAC tem papel importante na transformação de miofibras em resposta à flutuação intracelular de cálcio devido a fatores externos, como o exercício (BASSEL-DUBY e OLSON, 2006b).

Alteração na ingestão de proteínas e de energia, mesmo após o período de maior vulnerabilidade do sistema muscular, altera o fenótipo muscular e o tamanho das fibras, e esta alteração pode ter caráter permanente (PRESCOD, HALLIDAY e TAYLOR, 2011).

Em algumas situações, como a durante a privação alimentar e o exercício físico, o músculo adapta seu metabolismo de forma a favorecer a utilização de lipídios como fonte energética. A mudança para a utilização de lipídios requer, por um lado, o aumento da expressão de genes implicados no metabolismo lipídico, na oxidação fosforilativa e na biogênese mitocondrial, por outro há uma reorganização estrutural e morfológica muscular (WANG *et al.*, 2004b; RODGERS *et al.*, 2008a). Em nível molecular, esta adaptação implica a ativação de receptores nucleares como PPARδ, e do co-reguladores de transcrição PGC1α (RODGERS *et al.*, 2008a). Entretanto o mecanismo geral que controla esta bem orquestrada adaptação ainda não é totalmente entendido.

Sabe-se que as adaptações ao jejum seguem um curso temporal de acordo com as informações que são recebidas dos níveis dos hormônios e de moléculas que indicam o estado energético das células, como o nicotinamida adenina dinucleotído reduzida (NADH), trifosfato de adenosina (ATP) e AMP (DE LANGE *et al.*, 2006). Diferentes níveis de expressão de uma mesma molécula são observados durante o jejum. Por exemplo, PGC1α é um dos primeiros a ter sua expressão aumentada. Entretanto, após 48 horas de jejum, volta a seus níveis normais (DE LANGE *et al.*, 2006). As alterações observadas na expressão de PGC1α são acompanhadas por concomitante aumento da expressão de PPARδ 6, 24 e 48 horas após jejum (DE LANGE *et al.*, 2006) entretanto o mesmo achado não foi observado após jejum de 12horas (ESCHER *et al.*, 2001). Outra proteína que parece sempre ter sua expressão aumentada em resposta ao jejum é UCP3 (SAMEC *et al.*, 2002; TUNSTALL *et al.*,

2002; DE LANGE *et al.*, 2006; FRIER, JACOBS e WRIGHT, 2011). Os níveis de ATP/AMP são grandes responsáveis pela sinalização que controla a ativação do sensor nutricional AMPK (DE LANGE *et al.*, 2006).

Durante o jejum prolongado ou sob tratamento com glicocorticoides, as fibras de tipo IIB sofrem atrofia mais rapidamente do que as fibras de tipo I (LI e GOLDBERG, 1976). Esta proteção das fibras tipo I estaria relacionada a sua maior expressão de PGC1α (SANDRI *et al.*, 2012).

Diferenças nos níveis de síntese e degradação de proteínas também são observadas entres os tipos de fibras em resposta à privação alimentar (GOODMAN *et al.*, 2012). Fibras do tipo IIX e IIB de camundongos submetidos à 48h de jejum apresentaram maior redução na síntese de proteínas do que fibras tipo I e IIA, além de terem sido as únicas a presentar redução na secção de área transversa (GOODMAN *et al.*, 2012).

Além das situações fisiológica, manipulações farmacológicas também podem levar a adaptações metabólica e funcionais. Tratamento crônico com 5-aminoimidazolo-4-carboxamido-1-β-D-ribofuranosido (AICAR) um conhecido ativador da AMPK induz aumento da expressão da UCP3 e da atividade das enzimas CS e β-HAD, sem no entanto alterar a expressão da MyHC nem a atividade de enzimas ligadas a glicólise (PUTMAN *et al.*, 2003). Entretanto, a aplicação aguda de AICAR leva ao aumento da expressão de UCP3 e da HKII, enzima limitadora da glicólise, tanto na porção vermelha quanto na porção branca do músculo gastrocnêmio (STOPPANI *et al.*, 2002). A atividade da AMPK pode influenciar tanto o metabolismo glicolítico quanto lipídico no músculo esquelético, respondendo à um aumento na demanda energética (STOPPANI *et al.*, 2002; PUTMAN *et al.*, 2003).

Estimulação elétrica de baixa frequência de forma crônica também é capaz de alterar o metabolismo e o fenótipo das fibras (PUTMAN *et al.*, 2003). Observa-se ao final de 6 semanas de estimulação aumento da capacidade oxidativa, representada pelo aumento da atividade de enzimas como a β-HAD e CS, além de redução na expressão de UCP3 e aumento da expressão de MyHC I e IIa com concomitante redução de MyHC IIb (PUTMAN *et al.*, 2003).

Exercícios voluntários por algumas semanas em cicloergômetro promoveram aumento da percentagem de expressão de MyHC IIa e diminuição da MyHC IIb em músculos rápidos

de camundongos (ALLEN *et al.*, 2001). Camundongos mutantes onde a AMPK foi inibida ou ativada apresentaram diferentes expressões de MyHC em resposta ao exercício físico, indicando que esta enzima seria importante para a mudança no perfil de fibras (ROCKL *et al.*, 2007a).

3.7 Sistema muscular esquelético – modificações na síndrome metabólica

O transporte da glicose através da membrana plasmática é considerado o passo que controla a taxa de metabolismo da glicose em sujeitos normais e com diabetes tipo 2 (CLINE *et al.*, 1999). Resistência à insulina foi relacionada com alterações na glicólise, glicogênese, além de acúmulo de AG nas células e alterações na β-oxidação (DEL PRATO *et al.*, 1993). Além disso, resistência à insulina e diabetes tipo 2 tem sido associada com habilidade prejudicada de apresentar mudança entre o metabolismo de lipídios e de glicose (THYFAULT, RECTOR e NOLAND, 2006).

A resposta à insulina estimulada pela glicose estaria relacionada com a proporção de fibras tipo I, sugerindo que uma redução na proporção dessas fibras estaria envolvido nos processos multifatoriais de resistência à insulina (HICKEY *et al.*, 1995a). A proporção de fibras tipo I e tipo IIB tem sido associadas com obesidades e diabetes. Em pessoas obesas e/ou diabéticas, o número de fibras oxidativas (tipo I) estaria diminuído, enquanto que as glicolíticas (tipo IIB) estariam aumentadas (NYHOLM *et al.*, 1997). Em humanos, a captação de glicose corporal e o transporte muscular de glicose estão positivamente correlacionados com as fibras tipo I e negativamente com as fibras tipo IIB (NYHOLM *et al.*, 1997). A sinalização de insulina através da via IRS-1/PI3K está prejudicada em pacientes com diabetes tipo 2 (BJORNHOLM *et al.*, 1997). Indivíduos obesos apresentam capacidade oxidativa reduzida e capacidade glicolítica aumentada, além de diminuição da porcentagem de fibras tipo I (HICKEY *et al.*, 1995a).

Estudo com humanos em um acompanhamento rígido de perda de peso, revelou diferenças, no músculo esquelético, na expressão de genes relacionados à fosforilação oxidativa, metabolismo de glicose e de ácidos graxos (GERRITS *et al.*, 2010). Esses genes estavam superexpressos nos obesos que eram mais responsivos à dieta em comparação àqueles resistentes, além disso a obesidade foi relacionada à redução do conteúdo de mitocôndrias musculares (GERRITS *et al.*, 2010). Ademais os obesos menos responsivos à dieta apresentavam menor proporção de fibras tipo I comparados aos indivíduos magros e aos

obesos responsivos à dieta, ambos os grupos de obesos apresentavam maior acúmulo de IMTG que o grupo magro (GERRITS *et al.*, 2010).

Interessantemente, em ratos diabéticos e em humanos obesos e diabéticos, a taxa de transporte de ácidos graxos para o músculo esquelético é aumentada (CHAKKALAKAL *et al.*, 2004). Aumento nos níveis de ácidos graxos livres (AGL) no plasma e dentro das células e de IMTG podem atuar como limitadores da captação e oxidação da glicose (RODEN, 2004). No primeiro caso, os AGL dentro das células aumentaria os níveis de atividade de PKC e diminuiria a atividade de IκBα (nuclear factor of *kappa* light polypeptide gene enhancer in B-cells inhibitor alpha), um inibidor do NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), ou estimularia diretamente a fosforilação em serina do receptor de insulina e de seu substrato, o que levaria nos dois casos à redução da atividade do IRS associado à PI3K, levando a diminuição translocação do Glut4 para a membrana limitando o transporte de glicose (RODEN, 2004).

No caso da inibição da oxidação da glicose, entraria em ação o ciclo glicose-ácidos graxos proposto por Randle e colaboradores (1963), via aumento da relação NADH/NAD+, inibição de PDH, com consequente inibição de PFK, acúmulo de glicose-6-fosfato, com aumento da produção de glicogênio e inibição da atividade da HK, o que levaria a menor capacidade da glicose ficar dentro da célula, sendo liberada no plasma. Estas alterações provocadas pelo AGL são importantes como um dos mecanismos que pode levar à resistência a insulina em pessoas com grande ingestão de lipídios e em pessoas já obesas.

Foi observado que cultura de miotubos provenientes de pacientes obesos, são resistentes ao efeito deletério da incubação com palmitato sobre a sinalização de insulina, apesar de já apresentarem menores resposta a insulina em meio controle (BIKMAN *et al.*, 2010). Indicando que a alteração na resposta à incubação ao palmitato estaria relacionada à “habituação” destas células por estarem presentes em um ambiente com excesso de lipídios (BIKMAN *et al.*, 2010). Interessantemente, ambas as culturas, de obesos ou indivíduos controles, tiveram o decaimento da resposta na sinalização da insulina reduzido após a administração de AICAR, um ativador de AMPK (BIKMAN *et al.*, 2010).

Interessantemente, uma dieta rica em gordura comprometeu a captação de glicose estimulada pela insulina sem, no entanto, causar alterações na cascata inicial de insulina (BOUDINA *et al.*, 2012). Em ratos susceptíveis à obesidade, esse comprometimento da

captação de glicose foi precedido pela disfunção mitocondrial, enquanto que os ratos resistentes à obesidade aumentaram o desacoplamento mitocondrial via maior influxo de AG, porém isso também não foi suficiente para impedir o surgimento da resistência à insulina (BOUDINA *et al.*, 2012).

Alguns estudos mostram associação entre disfunção mitocondrial e resistência a insulina no músculo esquelético, porém os mecanismo não estão totalmente estabelecidos (PAGEL-LANGENICKEL *et al.*, 2010). O estresse oxidativo aumentado que atrapalharia o transporte de glicose e diminuiria a oxidação de ácidos graxos, assim como β-oxidação incompleta foram associados à resistência a insulina (KOVES *et al.*, 2008). Indivíduos insulinorresistentes e com diabetes tipo 2 apresentavam redução no conteúdo de mitocôndrias e menor atividade da cadeia transportadora de elétrons, o que corrobora com o conceito que a resistência à insulina pode ter como início redução no número e alteração na função das mitocôndrias (KELLEY *et al.*, 2002; PETERSEN e SHULMAN, 2002).

Civitarese e colaboradores (2006) observaram que ratos nocaute para adiponectina apresentavam alterações mitocondriais semelhantes às observadas em indivíduos com diabetes tipo 2. Ao tratar culturas primárias de células musculares humanas com adiponectina, eles observaram o aumento da bioenergética mitocondrial e redução na produção de ERO (CIVITARESE *et al.*, 2006).

A expressão de UCP3 (SCHRAUWEN e HESSELINK, 2004a) e de PPARβ/δ (MENSINK *et al.*, 2007) são diminuídas pela metade em pacientes diabéticos. Nesse mesmo sentido, PPARβ/δ foi observado está superexpresso em indivíduos obesos que eram responsivos à dieta em comparação aos resistentes (GERRITS *et al.*, 2010). Modesto aumento na expressão de PGC1α em ratos obesos leva ao aumento da sensibilidade e da sinalização de insulina (BENTON *et al.*, 2010). Sendo que diminuição na expressão de PGC1α foi observado em pacientes com diabetes tipo 2 (PATTI *et al.*, 2003).

Alguns trabalhos tem relacionado o papel de fatores epigenéticos sobre o desenvolvimento de critérios envolvidos na SM (HENRY *et al.*, 1996; GASTER *et al.*, 2001; CARONE *et al.*, 2010; NG *et al.*, 2010; BARRES e ZIERATH, 2011). Culturas de células musculares de pacientes com diabetes tipo 2, mantém o perfil de resistência à insulina, mesmo após vários ciclos celulares (HENRY *et al.*, 1996; GASTER *et al.*, 2001; CARONE *et al.*, 2010; NG *et al.*, 2010; BARRES e ZIERATH, 2011). Numerosos genes com padrões

diferentes de metilação foram observados no musculo esquelético de pacientes com diabetes tipo 2 quando comparado com pessoas normais, incluindo grupos de genes envolvidos nos processos metabólicos primários e função mitocondrial (HENRY *et al.*, 1996; GASTER *et al.*, 2001; CARONE *et al.*, 2010; NG *et al.*, 2010; BARRES e ZIERATH, 2011).

3.8 Sistema muscular esquelético – repercussões da programação metabólica

Uma ampla gama de intervenções e insultos durante o período periconcepcional e perinatal podem afetar o fenótipo e o metabolismo muscular (MALTIN *et al.*, 2001). Alguns trabalhos observaram que utilizando-se modelos de desnutrição materna perinatal pode-se induzir maior susceptibilidade de desenvolvimento de obesidade na prole (MALTIN *et al.*, 2001). Entretanto, esses efeitos são dependentes da natureza do insulto, da extensão e do tempo ao qual foi induzido, podendo afetar o desenvolvimento de fibras primária e secundárias ou somente uma dessas gerações (MALTIN *et al.*, 2001).

Insultos nutricionais no início ou no meio da gestação podem interferir na formação dos miotubos e reduzir a densidade de fibras musculares esquelética (YATES *et al.*, 2012). Restrição em 50% da dieta, em ovelhas, durante o período periconcepcional até o meio da gestação levou à redução em 20% do número de fibras, principalmente devido à redução no número de fibras secundárias, nos fetos, indicando prejuízo na miogênese (QUIGLEY *et al.*, 2005). Restrição de nutrientes entre o primeiro e o segundo trimestre de gestação, também em ovelhas, reduz o número de fibras secundárias na prole (ZHU *et al.*, 2004). A desnutrição materna com restrição de 40% da ingestão calórica, no período gestacional, também apresenta influência negativa sobre o desenvolvimento muscular em ratos, reduzindo o número de núcleos na prole, ao desmame, além de alterar negativamente a expressão de fatores de crescimento gerais e fatores que influenciam diretamente a proliferação e diferenciação muscular (BAYOL *et al.*, 2004).

Enquanto que dieta baixa em proteína somente durante a gestação aumentou a proporção de fibras tipo IIA no sóleo, aos 25 e 90 dias pós-natal, e no EDL, induziu redução das fibras IIA e aumento da tipo IIB aos 25 e 90 dias, com concomitante redução das fibras tipo I aos 90 dias (TOSCANO, MANHAES-DE-CASTRO e CANON, 2008). Entretanto, desnutrição por redução de proteína, nos períodos gestacional e lactacional, não alterou nem o número nem o fenótipo de fibras no músculo tibial anterior (OZANNE *et al.*, 1996b) nem no

EDL (LEANDRO *et al.*, 2012), mas reduziu o número de fibras tipo I e aumentou as fibras tipo IIA e intermediárias, no sóleo (LEANDRO *et al.*, 2012), na prole aos 3 meses de vida.

Bedi e colaboradores (1982) mostraram que o período gestacional e lactacional é o mais importante para o desenvolvimento de modificações permanentes no fenótipo muscular. Enquanto que a prole de mães que foram submetidas a redução de 50% da dieta durante a gestação e lactação apresentava redução na área de secção transversa e no número de fibras dos músculos sóleo e EDL aos 6 meses de vida, aqueles que foram desnutrido após o desmame não apresentavam alterações na musculatura após o período de recuperação nutricional (BEDI *et al.*, 1982).

Alguns estudos demonstraram que animais jovens desnutridos durante o período perinatal apresentam uma melhor tolerância à glicose e melhor sensibilidade à insulina que animais controles (OZANNE *et al.*, 1996b; CHAMSON-REIG *et al.*, 2009). Entretanto, ao atingirem 15 meses de vida, aqueles se tornam resistentes à insulina e apresentam diversas alterações de níveis de expressão de moléculas implicadas na sinalização de insulina (PETRY *et al.*, 2001; OZANNE *et al.*, 2003a). Os mecanismos pelos quais ocorre esta mudança de resposta metabólica ainda restam a esclarecer. Alguns trabalhos falam em alterações na sinalização de insulina (SHELLEY *et al.*, 2009). Enquanto outros afirmam que a capacidade de biogêneses mitocondrial e o metabolismo oxidativo podem ter um papel nesta mudança (BIKMAN *et al.*, 2010; BODEN, 2011).

Influência da dieta materna pode ser observada já em animais no período de desmame. Camundongos cuja mães receberam dieta baixa em proteína no período gestacional apresentam menor expressão de IRS, IRS1(Tyr612) , IRS1(Ser307), das subunidades de PI3K p85 e p110β, PKCζ e Akt (Ser473), no músculo vasto lateral, mesmo sem apresentar diferenças na insulinemia nem na glicemia de jejum, indicando menor sensibilidade à insulina e maior risco de desenvolver diabetes tipo 2 (CHEN *et al.*, 2009). Ademais, esses animais apresentam menor expressão de Sirt1, o que pode indicar menor tempo de vida (CHEN *et al.*, 2009). Entretanto, os camundongos que foram desnutridos somente durante a lactação apresentavam melhor sensibilidade à insulina, representada por maior expressão de IRS1 e PKCζ, apesar de apresentarem menor Akt (Ser473), IRS1(Tyr612) e IRS1 (Ser 307), mas provavelmente devido à redução da insulinemia (CHEN *et al.*, 2009).

Dieta materna baixa em proteína conduz a prole a uma maior transporte de glicose com concomitante maior quantidade de Glut4 na membrana plasmática, ambos em estado basal, no músculo tibial anterior, da prole aos 3 meses, além de melhor captação de glicose em resposta à estimulação de insulina (OZANNE *et al.*, 1996b). O ambiente nutricional precoce poderia então influenciar permanentemente a sensibilidade à insulina no músculo esquelético, sem modificar o fenótipo das fibras musculares (OZANNE *et al.*, 1996b).

Entretanto, animais adultos cujas mães foram restritas em proteína durante a gestação apresentam redução da atividade de Akt/PKB no músculo quando estimulada por infusão de insulina na via porta hepática, mas não há diferença em estado basal (CHAMSON-REIG *et al.*, 2009).

Foi observada redução na expressão de PGC1α, na 3° semana pós-natal de animais que apresentaram retardo de crescimento intrauterino (LANE *et al.*, 2003). Entretanto, outro trabalho observou o aumento da expressão de PGC1α (MUHLHAUSLER *et al.*, 2009). Superexpressão de PGC1α melhora a sensibilidade muscular à insulina, observada pelo aumento da fosforilação de Akt em resposta a um estímulo insulínico, além de aumentar a expressão de mioglobina e troponina I, indicando uma tendência à mudança no fenótipo muscular no quadríceps (LIANG *et al.*, 2009).

O aumento dos lipídios circulantes e sua acumulação nos tecidos, incluindo o músculo, também poderiam modificar a expressão de genes chaves do metabolismo de glicose e lipídios, favorecendo a instauração da resistência à insulina (SILVEIRA *et al.*, 2008a).

Em humanos; foi observado que adolescentes aos 12 anos que foram pequenos para a idade gestacional (PIG) apresentam taxas metabólicas basais comparáveis a adolescentes da mesma idade que foram adequados para idade gestacional (AIG) (JORNAYVAZ *et al.*, 2004). Entretanto, apresentam uma fração menor de energia produzida pela oxidação da glicose e maior fração a partir da oxidação de lipídeos (JORNAYVAZ *et al.*, 2004).

Não se pode ignorar o efeito da mudança da dieta entre o período perinatal e o período de crescimento (após o desmame). Ratas que cujas mães foram desnutridas durante a gestação e lactação e que continuaram a receber a mesma dieta materna até os 120 dias de vida apresentam melhor captação de 2-deoxi-d-glicose no músculo sóleo que animais controles

(BERLEZE *et al.*, 2009). Desta forma, animais que não foram submetidos durante o período de crescimento a uma dieta diferente da dieta materna apresentam melhor resposta à insulina, na vida adulta. Ratos que foram desnutridos pela técnica das grandes ninhadas, e submetidos depois do desmame à dieta controle ou dieta de cafeteria (rica em gordura) apresentam, às 15 semanas de vida, maior expressão, no gastrocnêmio, de CPT1a, PPARα e COXIV, independente da dieta pós-desmame (PRIOR *et al.*, 2008). Entretanto, o receptor 1 para adiponectina estava aumentado apenas nos animais que receberam dieta de cafeteria pós-desmame (PRIOR *et al.*, 2008). Indicando que mesmo que a dieta pós-desmame possa influenciar no perfil metabólico muscular adulto, o período de desenvolvimento ainda guarda grande relevância na determinação da sensibilidade à insulina e do metabolismo de ácidos graxos (PRIOR *et al.*, 2008).

MÉTODOS

4. Métodos

4.1. Animais

Para as avaliações realizadas foram utilizadas 18 ratas Wistar-Han virgens, com idade de 60 dias e pesando 210±20 g, provenientes do fornecedor Charles-River (França). As fêmeas foram acasaladas com machos de mesma idade, linhagem e fornecedor. Os animais foram mantidos em biotério de experimentação, com temperatura de 22±2°C, e ciclo claro-escuro de 12/12 horas (claro das 07:00 às 19:00 h, escuro das 19:00 às 07:00 h), e livre acesso à água e dieta padrão de biotério (Ração SAFE® - A04, 18% proteína).

O diagnóstico do estado de prenhez foi realizado através de esfregaço vaginal. Confirmada a gestação, as ratas passaram a receber as dietas experimentais dando origem a dois grupos: Controle (C), cujas ratas receberam dieta normoproteica (proteína à 17%) ou Desnutrido (PR – Protein Restricted), receberam dieta hipoproteica (proteína à 8%). As dietas eram isocalóricas e seguiam as recomendações da AIN-93G (REEVES, NIELSEN e FAHEY, 1993). Os regimes experimentais foram mantidos durante toda a gestação e lactação. Um dia após o nascimento, os filhotes foram pesados e selecionados, formando-se ninhadas com 8 filhotes e a mãe (5 machos e 3 fêmeas, quando possível). Após o desmame, todos os filhotes passaram a receber dieta de manutenção (Ração SAFE® - A04, 18% de proteína) e ficaram alojados em gaiolas com, no máximo, 3 animais.

Tabela 2. Ingredientes das dietas Controle e Protein-Restricted.

Ingredientes (g/Kg)	Controle (17% Proteína)	Protein-restricted (8% Proteína)
Caseína (>92,5% proteína)	183,8	84,3
Amido de milho	545,6	555,6
Sacarose	100	100
Óleo de soja	70	70
Fibras	50	50
Mix de vitaminas	10	10
Mix de minerais	35	35
Bitartarato de colina	2,5	2,5
L-metionina	3,0	3,0
BHT	0,1	0,1
Total	1000	1000

Durante a gestação o peso das ratas foi aferido a cada 3 dias, durante a lactação o peso das mães e dos filhotes também foi aferido a cada 3 dias (Balança Sartorius, sensibilidade 0,01g). Após o desmame o peso era aferido a cada 10 dias. Para todos os experimentos, foram utilizados 12 ratos do grupo Controle e 12 do grupo Desnutrido.

Todos os procedimentos realizados de acordo com o European Communities Council Directive of 24 November 1986 (86/609/EEC) em relação aos cuidados e uso de animais para procedimentos experimentais .

4.2. Teste de tolerância à glicose

O teste foi realizado aos 110 dias de vida nos filhotes provenientes de mães do grupo Controle ou Desnutrido. Após um jejum de 16 horas, foi realizada a primeira coleta de sangue (tempo 0). Em seguida, foi realizada a administração de glicose (dose de 1g/Kg de peso corporal) por gavagem e outras amostras de sangue foram coletadas após 20, 40, 80 e 120 minutos. As concentrações de glicose sanguínea foram identificadas por leitura através de glicosímetro (Accu Check Active). A partir dos valores de glicemia, foi calculada a área sob a curva para cada animal e posteriormente a média do grupo.

4.3 *Status* nutricional ao sacrifício

Aos 120 dias, os animais foram sacrificados para retirada de tecidos para análise. Para os experimentos descritos no primeiro artigo, todos os animais foram sacrificados em condições *ad libitum*. Para os experimentos descritos no segundo artigo, os animais foram sacrificados em condição *ad libitum* ou após jejum de 48 horas. No artigo, eles são referidos como sub-grupo *Ad Libitum* ou Fasting, respectivamente. A restrição alimentar foi realizada no início do ciclo claro, sendo retirada toda a ração e os animais permaneciam com livre acesso à água.

4.4 Análise do perfil bioquímico do plasma

Para o sacrifício, os animais foram anestesiados, via peritoneal, com pentobarbital sódico (50mg/Kg), em um dos *status* nutricional descritos no subitem anterior. Após comprovada a extinção de reflexos e nenhum sinal aparente de dor, foi realizada punção cardíaca e coleta de 2 a 3 ml de sangue em tubos contendo ácido etilenodiaminotetracético

(EDTA). Sendo imediatamente centrifugados (3500rpm, 10min, 4°C), sobrenadante coletado e guardado a -80°C até análise.

Os níveis de glicose, colesterol e triglicerídeos plasmáticos foram medidos colorimetricamente usando kit da Biomérieux (França). Os níveis de ácidos graxos livre foram medidos colorimetricamente com kit da Wako (França). Todos os procedimentos foram realizados em duplicado e seguindo as instruções dos fornecedores.

4.5 Coleta das amostras

Após os procedimentos descritos no subitem anterior, foi realizada uma incisão na região lateral da pata posterior para retirada do músculo EDL (*extensor digitorum longus*) e posteriormente foi retirado o músculo sóleo. Para as análises histológicas, ambos os músculos foram imediatamente imersos em isopentano à baixa temperatura, guardados em nitrogênio e depois transferidos para freezer a -80°C até análise. Para as análises de atividade enzimática e PCR, os músculos foram retirados e imediatamente congelados em nitrogênio líquido e posteriormente guardados a -80°C até análise. Após a coleta dos músculos, toda a gordura visceral foi retirada e pesada em balança (Balança Sartorius, sensibilidade 0,01).

4.6 Análise histológica

Os músculos foram fixados em um suporte com auxilio de Tissu Tek® e cortados em criostato (Microm HM560, Thermo Scientific), mantido à -20°C. Foram obtidas secções transversas de 10µm de espessura. Os cortes seriais foram cuidadosamente posicionados em diferentes lâminas e secos à temperatura ambiente por 2 horas.

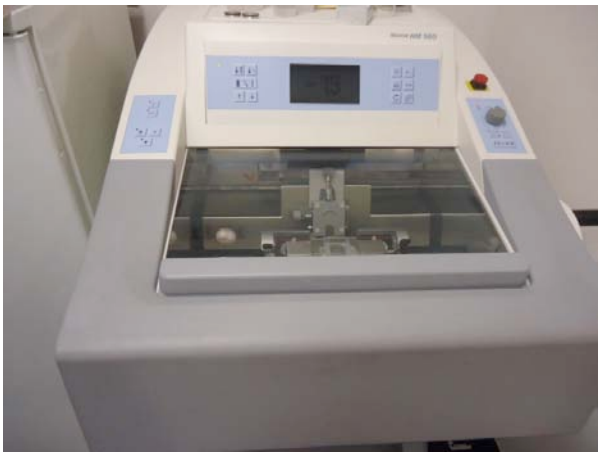

Figura 8. Criostato (Microm HM560, Thermo Scientific).

4.6.1 mATPase

Algumas das lâminas obtidas como descrito no subitem anterior foram coradas pela técnica da miosina miofibrilar adenosina trifosfatase cálcio ativada (mATPase). Esta técnica consiste numa pré-incubação em dois diferentes níveis de pH (4,3 e 4,55), com subsequente incubação em pH alcalino (9,4). Isto permite a distinção entre fibras rápidas (em seus dois subtipo, IIA e IIB) e lentas (tipo I) (BROOKE e KAISER, 1970; PIEROBON-BORMIOLI *et al.*, 1981).

Os cortes transversais dos músculos foram pré-incubados em temperatura ambiente por 10 minutos em solução contendo 140mM de acido acético e 60mM de acetato de sódio, com pHs ajustados para 4,3 ou 4,55. Em seguida, as lâminas foram lavadas em água destilada e incubadas a 37°C em uma solução, contendo 3mM ATP, 20mM barbital sódico e 20mM cloreto de cálcio (pH 9,4) por 40 minutos. Após o período de incubação, as lâminas foram novamente lavadas em água destilada e imersas em solução de cloreto de cobalto a 2% durante 5 minutos, seguidas por revelação em solução de sulfeto de amônio a 5%. Após a revelação, as lâminas foram desidratadas em uma bateria crescente de álcoois (70° a 100°), por fim foram imersas duas vezes em histolemon – Erba® e secas à temperatura ambiente. Após a secagem as lâminas foram recobertas por lamínulas utilizando resina EUKITT®. A pré-incubação ácida permite diferenciar as fibras tipo I (escuras) e tipo II (claras) no pH 4,3, e subdividir as fibras em tipo IIA (claras) e tipo IIB (coloração intermediária), no pH 4,55.

Os campos microscópicos foram obtidos através de microscópio óptico OLYMPUS modelo U-CMAD-2 (objetiva 4 X) acoplado a um programa para captação de imagens (TV Tuner Application – TelSignal Company Limited, Taiwan). Para contagem das células foi utilizado o software Mensurin Pro versão 3.2 (Jean-François Madre-Amiens, França). A composição muscular por tipos de fibras foi determinada através da contagem de aproximadamente 1500 fibras por músculo, em campos regularmente distribuídos sobre a amostra.

A

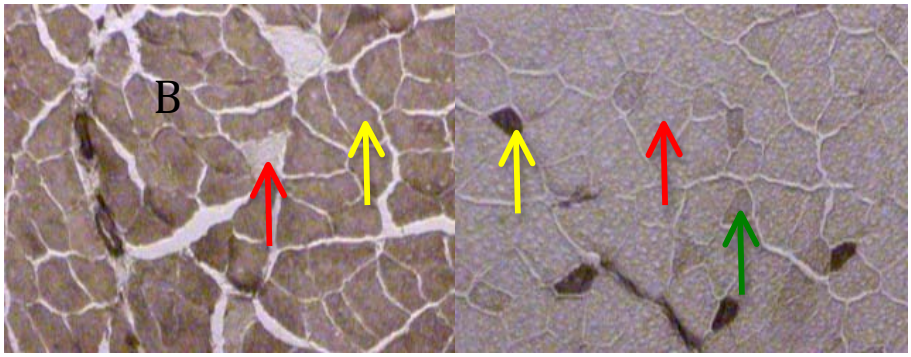

Figura 9. Exemplos de marcagem com mATPase. Setas amarelas indicam fibras tipo I, setas vermelhas, fibras tipo IIA, e setas verdes, fibras IIB.

4.6.2 NADH-TR

As outras lâminas obtidas como descrito no subitem 4.6, foram coradas pela técnica da NADH-TR (Nicotinamida Adenina Dinucleotídeo – Tetrazólio Redutase). Esta coloração permite a marcação da atividade do complexo I da cadeia respiratória sendo utilizada como indicador da capacidade oxidativa das fibras musculares. Assim sendo, é possível distinguir dois tipos de fibras: oxidativas (marcadas em azul escuro, incluindo fibras I e IIA) e não-oxidativas (marcadas em azul claro, fibras IIB).

Depois de terem sido secas durante 2h, à temperatura ambiente, as lâminas foram incubadas durante 1h em solução consistindo em 5ml de 0,1M tampão fosfato (pH 7,4), 4mg Nitro-blue tetrazolium e 3,2mg NADH (MARQUEZ, SWEAZEA e BRAUN, 2006). Após incubação, as lâminas foram lavadas em água destilada. Desidratação, montagem, aquisição de imagens e quantificação foram realizadas conforme descrito no subitem anterior.

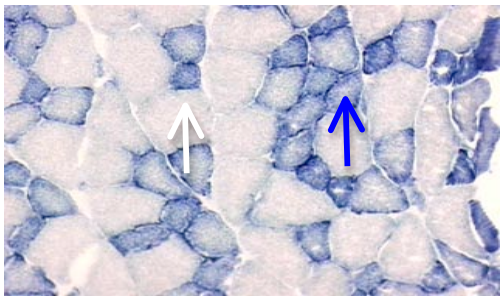

Figura 10. Exemplos de marcagem com NADH-TR. Seta azul indicam fibras oxidativas, setas branca, fibras não-oxidativas.

4.7 Análise da Atividade Enzimática

Pequenas amostras de músculos foram homogeneizadas com auxílio de um homogeneizador de vidro-teflon, em 1:25 (peso/volume) de tampão de extração contendo 50mM Tris (pH 7,4) e 1mM EDTA. Os homogenatos foram centrifugados a 10000g por 10 min à 4°C. Os pellets foram descartados e os sobrenadantes foram utilizados para quantificação de proteínas totais (DC Protein Assay Bio-Rad), tendo como padrão uma curva de albumina, e posteriormente para determinação da atividade enzimática. Todas as análises de atividade enzimática foram realizadas em placas de 96 poços, em triplicado. A variação da absorbância foi seguida durante 5 minutos, utilizando o espectofotômetro Varioskan 3.00.7(Thermo Scientific), e o SkanIt Software 2.2. A variação intra-ensaio foi de 5%. Os resultados foram expressos como atividade específica µmol/min/mg de proteína.

Figura 11. Varioskan 3.00.7(Thermo Scientific).

4.7.1 Hexoquinase (HK, EC 2.7.1.1)

A atividade foi determinada usando o método descrito por (REGMI, DIXON e OBA, 2008), com as seguintes modificações: solução (pH 8,5) de reação contendo 8,3mM glicilglicina, 17mM ATP, 0,001% vermelho de Cresol, 14mM cloreto de magnésio, 27mM glicose. A reação foi titulada com HCL. Foi observada a diminuição da absorbância a 560nm. Toda a leitura foi realizada a 25°C.

4.7.2 β-hidroxiacil-coenzima A desidrogenase (β-HAD, EC1.1.1.35)

Sua atividade foi analisada conforme descrito por (BASS *et al.*, 1969), com as seguintes modificações: solução de reação contendo 93mM tampão fosfato potássio (pH 7,3), 0,09mM acetoacetil-coenzima A e 0,1mM β -NADH. Foi registrada a diminuição da absorbância a 340nm, a 37°C.

4.7.3 Citrato Sintase (CS, EC 2.3.3.1)

Esta atividade foi analisada conforme descrito por (BASS *et al.*, 1969), com as seguintes modificações:46mM Tris (pH 8,0), 6,7mM ácido málico, 1,7 mM β-NAD, 0,13mM acetil-coenzima A, 56 unidades de malato desidrogenase. Foi observado o aumento da absorbância a 340nm, a 37°C.

4.8 Análise quantitativa de reação em cadeia de polimerase em tempo real (qRT-PCR)

Amostras de músculos sóleo e EDL foram homogeneizadas em reagente Trizol® (Invitrogen) para subsequente extração de RNA total. Os procedimentos de extração seguiram o recomendado pelo fabricante. Em resumo, 50-100mg de tecido foram homogeneizados em 1ml de Trizol®, depois de 5 minutos de incubação, 200µl de clorofórmio foram adicionados para permitir a separação das fases. Após centrifugação, a fase aquosa foi recuperada e adicionado 500µl de álcool isopropílico, para precipitar o RNA. Após centrifugação, o pelete foi lavado em etanol a 75% e redissolvido em água livre de RNases. Posteriormente, o RNA total foi tratado com DNase (RQ1 DNase Promega) por 45 min à 37°C, precipitado em acetato de sódio e etanol, e lavado em etanol. O RNA total foi quantificado utilizando-se Nanovue (GE) e sua pureza verificada pela relação A260/280 e por gel de agarose.

Figura 12. Nanovue (GE).

Em seguida, 1µg de RNA purificado foi transcrito reversamente usando a enzima M-MLV RT (Promega) em um volume total de 25µL. O cDNA resultante foi diluído 40 vezes em água livre de DNase e RNase. Por conseguinte, 5µL de cada cDNA diluído foi utilizado para amplificação em PCR usando o marcador fluorogênico SYBR Green (Quiagen, Courtboeuf, France) e um sistema de detecção do iCycler iQ Real-Time PCR (Bio-Rad, CA, USA). Os parâmetros de PCR utilizados foram: desnaturação inicial 5 min a 95°C, seguido por 45 ciclos de 30s a 95°C e 30s a 60°C.

Figura 13. iCycler iQ Real-Time PCR

As sequências de primers foram desenhadas no software Beacon® usando informações contidas na base se dados pública do GeneBank do National Center for Biotechnology Information (NCBI). Os genes estudados e suas sequências estão descrita na Tabela 3. Para análise, a quantidade relativa dos genes estudados foi normalizada pela expressão endógena de 18S e então se utilizou o método $2^{-\Delta\Delta CT}$ para os resultados (LIVAK e SCHMITTGEN, 2001).

Tabela 3. Genes estudados e suas sequências.

Gene	Symbol	Forward	Reverse	Gene Bank
Hexokinase II	HKII	GCGGTGCTGTGGCGAATC	AGCCTCCTCACTGCCTTATGG	NM_012735.2
Glucose Transporter type 4	Glut4	CAGCACTTTAGCCCTCTCTTCC	CCACAGCCTAGCCACAACAC	M25482.1
Carbohydrate response element binding protein	ChREBP	GTACTGTTCCCTGCCTGCTCTC	CCCTCTGTGACTGCCCTTGTG	FN432819.1
Carnitine palmitoyltransferase 1a	CPT1a	TGCCTGCCAGTTCCATTAAGC	GTCTCACTCCTCTTGCCAACAG	NM_031559.2
Medium chain acyl coenzyme A dehydrogenase	MCAD	GAGGCTACAAGGTCCTGAGAAGTG	TCTGCTGCTCCGTCAACTCG	NM_016986.2
Hydroxyacyl-Coenzyme A dehydrogenase	β-HAD	CTCCATGTCCTCCTCTTCCTCTGC	CAGCCCGCCGCCGATGAC	NM_057186.1
Pyruvate Dehydrogenase Kinase, Isozyme 4	PDK4	GGTGGCGGTGTTCCTCTGAG	TGAATTGTCCATCACAGGCGTTG	NM_053551.1
Citrate Synthase	CS	CTCTCTCTCCGATCCCTTCCC	AGGACGAGGCAGGATGAGTTCTTG	NM_130755.1
Cytochrome c oxidase subunit IV isoform 1	COX IV	GGCAGCAGTGGCAGAATGTTG	GAAGGCACACCGAAGTAGAAATGG	NM_017202.1
Uncoupling protein 3	UCP3	CCGTTAAGCCTTCAGCCTTCC	CGAGAGTCCATCCTGTCCTTCC	NM_013167.2
Peroxisome proliferator-activated receptor alpha	PPARα	CACGATGCTGTCCTCCTTGATG	ATGATGTCGCAGAATGGCTTCC	NM_013196.1
Peroxisome proliferator-activated receptor delta	PPARδ	ACTCTCCTTTCTTCCTGCCTGTG	TGTGCTGCTGCTCCTTCTGG	NM_013141.2
Peroxisome proliferator-activated receptor gamma, co-activator 1	PGC1α	ACACCGCACACATCGCAATTC	TTCGTCCCTCTTGAGCCTTTCG	NM_031347.1
Myosin Heavy chain 7	MHCI	ACAGAGGAAGACAGGAAGAACCTAC	GGGCTTCACAGGCATCCTTAG	NM_017240.1
Protein kinase AMP-activated alpha 1	AMPKα1	TTGCGTGTGCGAAGGAAGAAC	CCAAATCAGGGACTGCTACTCCA	NM_019142.1
Protein kinase AMP-activated alpha 2	AMPKα2	GATGATGAGGTGGTGGAGCAGAC	CACTGTCTGGCTCTCTCACTGC	NM_023991.1
Ribosomal 18S	18S	GATGCGGCGGCGTTATTC	CTCCTGGTGGTGCCCTTCC	M11188.1

4.9 Análises por Western Blotting

Para análises em western blotting, amostras de músculos foram homogeneizadas utilizando um homogeneizador de vidro, numa proporção de 1:8 (peso/volume). O tampão de extração continha: 50mM HEPES (pH 7,6), 50mM KCl, 50mM NaF, 5mM $Na_4P_2O_7$, 1mM

EDTA, 1mM EGTA, 1mM DTT, 5mM β-glicerofosfato, 1% NP-40, 1mM Na_3VO_4 e coquetel de inibidores (Sigma, P2714). Os dois últimos reagentes sempre eram adicionados no momento da homogeneização. O homogenato foi decantado por 30 min, no gelo, em seguida centrifugado 12000g, 10min, 4°C. Os pellets foram descartados e as proteínas totais do sobrenadante foram determinadas usando o kit DC Protein Assay Bio-Rad, com curva de albumina como padrão. As amostras foram desnaturadas à 95-100ºC por cinco minutos, em tampão de amostra contendo β-mercaptanol.

As proteínas foram separadas em gel de poliacrilamida 8 ou 10% (SDS-PAGE), utilizando-se 50-100µg de proteínas totais por *slot* do gel. As proteínas separadas no gel foram eletrotransferidas para membranas de PVDF (Fluoreto de polivinilideno, Immobilon-P, Millipore). Para inibir ligações inespecíficas dos anticorpos, as membranas foram incubadas por 1h, a temperatura ambiente, em solução de bloqueio que continha TBS (pH 7,5, 10 mM Tris–HCl, 150 mM NaCl) com 0,1% Tween-20 (T-TBS) acrescido de 5% de albumina bovina sérica (BSA), sob agitação constante. Posteriormente, as membranas foram incubadas, overnight (aproximadamente 16h), a 4°C, sob agitação constante, com um dos seguintes anticorpo primário anti-pAMPKα (Thr172, Cell Signalling, 1:1000) ou anti-PGC1α (H-300, Santa Cruz, 1:1000) diluído em solução de bloqueio. Após este período, as membranas foram lavadas em T-TBS e incubadas por 2h, a temperatura ambiente, com o anticorpo secundário Goat anti-rabbit conjugado com peroxidase (Jackson Immunoresearch, 1:40000), também diluído em solução de bloqueio. Após mais uma sequência de lavagens, as membranas foram tratadas com solução de revelação UptiLight™ HRP blot substrate, (Uptima, Interchim, França) por 1 min seguido de leitura da quimiluminescência em sistema G:BOX Chemi XL (Syngene, Frederick, Maryland, EUA), sendo as imagens adquiridas pelo programa GeneSnap (Syngene, Frederick, Maryland, EUA).

Após primeira revelação, as membranas foram desibridadas com solução de desibridação, pH6,8, (50mM tris-HCl, 2% de SDS, 0,7%β-mercaptanol), à 50°C, 10min, em agitação constante. As membranas foram lavadas em água e T-TBS e submetidas a novo bloqueio como descrito anteriormente. Após bloqueio, as membranas foram novamente incubadas em um dos anticorpos primários anti-AMPKα (Cell Signalling, 1:1000) e anti-α-tubulina (DM1A, Santa Cruz 1:100) e secundários conjugados com peroxidase Goat anti-rabbit e Goat anti-mouse (Jackson Immunoresearch, 1:40000), respectivamente, seguido por revelação como descrito anteriormente.

As imagens das bandas proteicas foram analisadas e quantificadas com auxílio do programa GeneTools (Syngene, Frederick, Maryland, EUA).

4.10 Análises Estatísticas

Inicialmente, foi aplicado o teste de normalidade (Kolmogorov-Smirnov). No primeiro artigo, para todos os parâmetros foi utilizado o teste T de Student, exceto para a variação da glicose no GTT, onde foi utilizado o ANOVA one-way seguido do teste *post-hoc* de Bonferroni. No segundo artigo, foi utilizado ANOVA one-way para a variação da glicose no GTT e ANOVA two-way (Dieta materna X Status ao sacrifício) para os parâmetros bioquímicos ao sacrifício, ambos seguidos do teste *post-hoc* de Bonferroni. Para todas as outras análises foi utilizado o teste T de Student. Os valores estão expressos em média e erro padrão da média (EPM). O nível de significância foi mantido em 5% ($p<0,05$) em todos os casos. As análises dos dados foram realizadas através do programa estatístico Graphpad Prisma 5® (GraphPad Software Inc., La Jolla, Califórnia, EUA).

RESULTADOS

Artigo 1

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Differential developmental programming by early protein restriction of rat skeletal muscle according to its fibre-type composition

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Abstract

Aims: Differences in fibre-type composition of skeletal muscle have been associated with obesity and insulin resistance. As a poor nutrient environment early in life is a predisposing factor for the development of obesity and related metabolic diseases at adulthood, this study aimed at determining the long-term consequences of maternal undernutrition on the structural and metabolic properties of two skeletal muscles characterized by their different fibre-type composition and metabolic properties.

Methods: The fibre-type composition and enzymatic activities of hexokinase (HK), beta-hydroxyacyl-CoA dehydrogenase (β-HAD) and citrate synthase (CS) were measured in soleus and extensor digitorum longus (EDL) muscles from adult rats born to dams fed a control (17% protein) or a low-protein [8% protein (PR)] diet throughout pregnancy and lactation. In addition, the expression levels of several genes regulating glycolysis, fatty acid oxidation and mitochondrial biogenesis were determined by real-time PCR.

Results: Protein rats exhibited enhanced density of type II fibres along with decreased rate of fatty acid oxidation and glycolysis in soleus but not EDL. Malnourished rats exhibited also a different gene expression profile in soleus and EDL. Altogether, these alterations correspond to a state of energy deficiency and are present in animals which do not show yet any sign of obesity or glucose intolerance.

Conclusion: We conclude that maternal protein restriction alters in the long term the structural and enzymatic properties of offspring skeletal muscle in a fibre-type-dependent manner. These alterations might have a causative role in the development of obesity and related metabolic disorders later in life.

Keywords developmental programming, metabolism, skeletal muscle.

It is now well established that an unbalanced nutritional environment *in utero* or during neonatal life induces a high risk of developing obesity and related metabolic disorders in adulthood (Fernandez-Twinn & Ozanne 2010, Wiedmeier *et al.* 2011). This enhanced disease susceptibility, named developmental or metabolic programming, has been associated with impaired structure and function of several organs

including brain (Grace *et al.* 2011), liver (Cianfarani *et al.* 2012), pancreas (Reusens *et al.* 2011) and skeletal muscle. In regard to this latter tissue, it has been shown that reduced nutrient supply during the last 3 days of gestation, induced by the ligation of the uterine artery, leads to decreased ATP production and reduced glucose transport and glycogen biosynthesis in red muscle (Selak *et al.* 2003). It has also been shown that young adult rats born to dams fed a low-protein diet during gestation and lactation exhibit better glucose tolerance and enhanced insulin sensitivity in soleus muscle than their control counterparts (Ozanne *et al.* 1996). However, aged animals exposed to the same nutritional insult become insulin resistant and present low expression levels of several molecules playing a key role in the insulin signalling pathway such as the zeta (ζ)-isoform of protein kinase C (PKCzeta) and the p85α regulatory subunit of the protein kinase PI3K (Ozanne *et al.* 2003, 2005). Impaired insulin sensitivity and altered expression of insulin signalling molecules have also been reported in quadriceps muscle from growth-restricted lambs (Muhlhausler *et al.* 2009), in the vastus lateralis of men born with low birth weight (Ozanne *et al.* 2005, Jensen *et al.* 2008) and in skeletal muscle from the offspring of obese dams (Yan *et al.* 2011, Shelley *et al.* 2009, Bayol *et al.* 2005).

Skeletal muscle is a heterogeneous tissue that contains different fibre types, namely type I and type II fibres, which differ in acid stability of their myofibrillar ATPase (mATPase) activity and their contractile and metabolic properties (Punkt *et al.* 2004, Schiaffino & Reggiani 2011). Type I fibres use mainly fat as energy substrate, their speed of contraction is relatively slow and are resistant to fatigue. In contrast, type II fibres use glucose as energy source, contract quickly and do not have a high resistance to fatigue. On the basis of their pH liability, type II fibres are further categorized as type IIa, which exhibit a mixture of glycolytic and oxidative properties, and IIb, which are exclusively glycolytic (Brooke & Kaiser 1970). Obese and diabetic individuals exhibit decreased oxidative capacity of skeletal muscle concomitantly with reduced amounts of type I (oxidative) fibres and enhanced number of type IIb (glycolytic) fibres (Hickey *et al.* 1995, Nyholm *et al.* 1997, MÅrin *et al.* 1994, Tanner *et al.* 2002). However, it is not known whether these defects are mechanistically linked or whether they are a cause or a consequence of obesity and diabetes.

Interestingly, several studies performed on various animal species indicate that perinatal malnutrition alters the fibre density of skeletal muscle although conflicting results exist about the exact nature of these changes. Thus, whereas Fahey *et al.* (2005) described decreased and increased numbers of, respectively, fast and slow-twitch fibres in the semitendinosus muscle from the offspring of calorie-restricted lambs, other authors reported no change in fibre density in this muscle using the same animal model and nutritional insult (Nordby *et al.* 1987). Moreover, decreased (Mallinson *et al.* 2007), increased (Toscano *et al.* 2008) or no change (Bayol *et al.* 2004) in the number of type II fibres have been documented in rat following maternal undernutrition during pregnancy or lactation. It should also be mentioned that most of the studies aimed to determine the effects of metabolic programming on the number and phenotype of muscle fibres have been performed using foetal or neonatal tissues. Therefore, the long-term consequences of early nutritional restriction on the phenotypic characteristics of skeletal cells remain to be fully determined. In addition, in spite of the fact that there are significant variations in the relative proportions of each fibre type between individual muscles of the same animal, and even between different regions of the same muscle, the impact of developmental programming on the metabolic properties of a given muscle in regard to its fibre-type composition has not been examined to date.

Based on the previous observations, we hypothesized that early nutrient restriction produces specific structural and metabolic alterations on different muscles depending on their fibre-type composition and that these defects might underpin the development of obesity and associated metabolic disorders in later life. To test this hypothesis, here we examined the long-term consequences of maternal protein restriction on the structural and metabolic properties of two skeletal muscles characterized by their different fibre composition and function. Specifically, we analysed the glycolytic and fatty acid activity, the fibre-type composition and expression profile of several genes regulating glucose and lipid metabolism in the soleus and extensor digitorum longus (EDL) muscles from adult offspring born to dams fed a low-protein diet. The soleus is a slow-twitch muscle involved in maintaining posture and contains a high percentage of type I fibres. In contrast, the EDL is a fast-twitch muscle which contains almost exclusively type IIA and IIB fibres.

Materials and methods

Animals and nutritional manipulations

This study is conformed with Good Publishing Practice in Physiology (Persson & Henriksson 2011). Twelve virgin female Wistar rats weighing 200–220 g were obtained from Charles River (France) and placed under a 12-h light/dark cycle (lights on at 7 AM),

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with food and water *ad libitum* for at least 8 days before any experimental manipulation. They were then mated to 3-month-old males of the same strain in a proportion of two females for one male. After confirmation of mating by the visualization of spermatozoa in a vaginal smear, dams were housed individually and allocated at random to one or two groups (six females per group) to be fed either a control (170 g protein kg^{-1}) or an isocaloric low-protein diet (80 g protein kg^{-1}) until the end of gestation. Diets were prepared in the Department of Nutrition of the Federal University of Pernambuco (Brazil), and their composition has been described previously (Toscano *et al.* 2008). One day after birth, litter size was adjusted to eight pups per dam maintaining a 1: 1 male/female ratio as close as possible. At weaning (21 days), only male animals remained for the study, and all control (C) and protein-restricted (PR) pups were fed standard chow. All experimental analyses were performed in a group of 4-month-old animals constituted by one male offspring selected at random from each control and protein-restricted litter.

Measurement of body weight and spontaneous food intake

The weight of each pup was recorded on the day after birth and every 3–4 days thereafter. At 35 and 60 days of age, each pup was housed individually during 5 days and its daily food consumption determined by the difference between the amount of food provided at the onset of the light cycle and the amount of food remaining 24 h later. Body and food weights were recorded to 0.01 g.

Oral glucose tolerance test and metabolite determinations

After overnight fasting, animals were given an oral dose of 1 g kg^{-1} of glucose. A drop of blood was taken immediately before the administration of glucose and 20, 40, 80 and 120 min after by severing the tip of the tail. Blood glucose was determined with a blood glucose monitor (Accu-Check® Active, Roche Diagnostics). Plasma from trunk blood collected at the time of killing was assayed for triglycerides (kit PAP 150, BioMérieux, Craponne, France), glucose (kit PAP 1200, BioMérieux), cholesterol (Cholesterol RTU, Biomerieux) and free fatty acids (NEFA-HR2, Wako, Richmond, VA, USA).

Killing and muscle sampling

Animals were anaesthetized with an intra-peritoneal injection of sodium pentobarbital (50 mg kg^{-1}). For histochemistry analysis, the soleus and EDL muscles from one limb were carefully dissected and immediately immersed in isopentane cooled by liquid nitrogen and kept at −80 °C until analysis. The same muscles from the contralateral limb were frozen in liquid nitrogen and kept at −80 °C until assayed for enzyme activity and gene expression. After muscle dissection, animals were killed by cervical dislocation. All animals were killed between 8 and 9 o'clock in the morning under *ad libitum* feeding conditions.

Histochemical staining

Serial cross sections (10 μm) were cut with the use of a cryostat and stained for myosin adenosine triphosphatase (mATPase) or NADH-tetrazolium reductase (NADH-TR) as described elsewhere (Marquez *et al.* 2006). Muscle fibres were classified into types I and II based on the presence (type I) or absence (type II) of staining for ATPase after acid pre-incubation at pH 4.3. Using a different set of muscle samples, fibres were further classified as type IIa or IIb using as criteria differences in staining intensity for ATPase after acid pre-incubation at pH 4.55. After NADH-TR stain, fibres were differentiated into oxidative (stained in bleu) and non-oxidative (non-stained). The number and phenotype of the fibres were determined by computerized image analysis with the use of a light microscope (Olympus Optical U-CMAD-2, Tokyo, Japan; 10x objective lens) and the Mesurim PRO 3.2 software developed by Jean-François Madre (http://pedagogie.ac-amiens.fr/svt/info/logiciels/Mesurim2/Telecharge.htm). At least 1 500 fibres in fields that were equally distributed over the sample were counted to determine the fibre-type composition of the muscle samples.

Enzymatic assays

Muscle samples were minced and homogenized in 1 : 25 volumes (wt/vol) of 50 mM Tris buffer (pH 7.4) containing 1 mM EDTA, using a glass-Teflon Porter homogenizer. Homogenates were then centrifuged at 10 000 × *g* at 4 °C for 10 min. Aliquots (10 μL) of the resulting supernatant were used for the enzymatic assays. Proteins were determined by the method of Lowry using the *DC*™ Protein Assay from Biorad (Marnes-la-Coquette, France).

Hexokinase (HK, EC 2.7.1.1) activity was determined as described by Regmi *et al.* (2008) with the following modifications: reaction cocktail contained 8.3 mM glycylglycine buffer (pH 8.5), 17 mM ATP, 0.001% cresol red, 14 mM magnesium chloride, 27 mM glucose, and the absorbance of the solution

was measured at 560 nm at 25 °C. β-Hydroxyacyl-CoA-dehydrogenase (β-HAD EC1.1.1.35), and citrate synthase (CS, EC 4.1.3.7) were assayed as described by Bass *et al.* (1969) with some modifications. Briefly, the reaction mix for β-HAD contained 93 mM potassium phosphate buffer (pH 7.3), 0.09 mM acetoacetyl-CoA and 0.1 mM β-NADH. For CS assay, the reaction mix contained 46 mM Tris (pH 8.0), 6.7 mM malic acid, 1.7 mM β-NAD, 0.13 mM acetyl-CoA and 56 units of malate dehydrogenase. Both enzymatic activities were measured at 340 nm using a Varioskan 4 3.00.7 spectrophotometer (Thermo Scientific) at 37 °C.

Real-time quantitative reverse transcription PCR experiments

Total RNA from soleus and EDL was extracted 5 using the Trizol reagent (Invitrogen) and treated with 6 DNAse (RQ1 DNase Promega) for 45 min at 37 °C. Afterwards, 1 μg of purified RNA was reverse transcribed using M-MLV Reversed Transcriptase (Promega) in a total volume of 25 μL, and the resulting cDNA was diluted 40-fold in DNAse- and RNAse-free water. Thereafter, 5 μL of each cDNA diluted sample was used as template for PCR amplification using SYBR Green (Qiagen, Courtaboeuf, France) as fluorogenic intercalating dye and the iCycler iQ Real-Time PCR detection system instrument from Bio-Rad Laboratories (Hercules, CA, USA). PCR parameters were as follows: an initial denaturation step of 5 min at 95 °C followed by 40 cycles of 30 s at 95 °C and 30 s at 60 °C. The relative expression levels were calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001) and 18S RNA as housekeeping gene. The primers used for the amplification are listed in Table 1. The applicability of the C_T method was first validated by determining how the amplification efficiencies of the different transcripts including 18S varied with template dilution. These preliminary experiments showed that the PCR gave a single product of the expected size for all the genes, as determined by melting curve analysis and agarose gel electrophoresis (data not shown), and that all of them were amplified with the same efficiency.

Data analysis

Experimental results are expressed as means ± SEM. Statistical differences between the groups were assessed by unpaired Student's *t*-test or two-way ANOVA followed by Bonferroni's test. Histochemical data were log-transformed before application of the two-way ANOVA test to approximate a normal distribution. Statistical significance was set at $P < 0.05$.

Results

Body weight, food intake and metabolic determinations

There were no differences between control and protein-restricted dams neither in the number of pups per dam (C = 15 ± 0.88 pups per litter; PR = 15 ± 0.97 pups per litter) nor in the sex ratio of born pups (C = 52 ± 3.5% of male pups; PR = 47 ± 6.9% of male pups. However, pups born to protein-restricted dams weighed significantly less than controls at birth as determined by Student's *t*-test (C = 6.34 ± 0.13 g, $n = 22$ vs. PR = 5.98 ± 0.09 g, $n = 31$, $P < 0.05$), and this difference in body weight increased during lactation. Actually, at weaning, PR pups weighed in average 57% less than controls (C = 44.53 ± 0.46 g, $n = 22$ vs. PR = 19.43 ± 0.66 g, $n = 31$ $P < 0.0001$, Fig. 1).

The differences in body weight between the two groups persisted until the end of the experiment and cannot be attributed to impaired food intake. Actually, at 35 days of age, PR rats exhibited a higher consumption of food than their control counterparts (daily food intake C = 56.7 ± 2.3 kcal per 100 g of body weight, $n = 6$ vs. PR = 64.6 ± 1.1 kcal per 100 g of body weight, $n = 6$, $P < 0.05$), but from 60 days onwards both groups consumed daily the same amount of food (C = 29.4 ± 0.7 kcal per 100 g of body weight, $n = 6$ vs. PR = 29.0 ± 0.7 kcal per 100 g of body weight, $n = 6$). At killing, 120 days, PR rats exhibited reduced 7 visceral fat (C = 30.42 ± 3.14 g; $n = 6$; PR = 12.99 ± 1.1G g, $n = 6$, $P < 0.001$) and lower plasmatic levels of triglycerides. In contrast, we observed no differences between C and PR rats in the concentration of glucose, free fatty acids or cholesterol in plasma under *ad libitum* feeding conditions. Likewise, control and PR rats exhibited similar fasting glucose concentrations (Control = 6.1 ± 0.40 mM, $n = 6$; PR = 5.2 ± 0.30 mM, $n = 6$; $P > 0.05$). Moreover, both C and PR animals showed an identical evolution of the plasmatic concentration of glucose after an oral glucose challenge, and the area under the glucose curve was the same in both groups (Fig. 1).

Fibre-type profile

As expected, the number of type I fibres, identified by ATPase staining at pH 4.3, was significantly higher in soleus than in EDL of both control and PR rats (Fig. 2c,d). Conversely, EDL contained a higher density of type II fibres. Protein restriction during pregnancy and lactation had no effect on the number of type I cells neither in soleus nor in EDL. However, a small, albeit statistically significant, reduction in the percentage of type I fibres in relation to the total

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Table 1 Sequences of primers used for the real-time RT-PCR analysis

Gene	Symbol	Forward	Reverse	Gene Bank
Hexokinase II	HKII	GCGGTGCTGTGGCGAATC	AGCCTCCTCACTGCCTTATGG	NM_012735.2
Glucose Transporter type 4	Glut4	CAGCACTTTAGCCCTCTCTTCC	CCACAGCCTAGCCACAACAC	M25482.1
Carbohydrate response element binding protein	ChREBP	GTACTGTTCCCTGCCTGCTCTC	CCCTCTGTGACTGCCCTTGTG	FN432819.1
Carnitine palmitoyltransferase 1a	CPT1a	TGCCTGCCAGTTCCATTAAGC	GTCTCACTCCTCTTGCCAACAG	NM_031559.2
Medium-chain acyl coenzyme A dehydrogenase	MCAD	GAGGCTACAAGGTCCTGAGAAGTG	TCTGCTGCTCCGTCAACTCG	NM_016986.2
Hydroxyacyl-Coenzyme A dehydrogenase	β-HAD	CTCCATGTCCTCCTGTTCCTCTGC	CAGCCCGCCGCCGATGAC	NM_057186.1
Pyruvate Dehydrogenase Kinase, Isozyme 4	PDK4	GGTGGCGGTGTTCCTCTGAG	TGAATTGTCCATCACAGGCGTTG	NM_053551.1
Citrate Synthase	CS	CTCTCTCTCCGATCGCTTCCC	AGGACGAGGCAGGATGAGTTCTTG	NM_130755.1
Cytochrome c oxidase subunit IV isoform 1	COX IV	GGCAGCAGTGGCAGAATGTTG	GAAGGCACACCGAAGTAGAAATGG	NM_017202.1
Uncoupling protein 3	UCP3	CCGTTAAGCCTTCAGCCTTCC	CGAGAGTCCATCCTGTCCTTCC	NM_013167.2
Peroxisome proliferator-activated receptor alpha	PPARα	CACGATGCTGTCCTCCTTGATG	ATGATGTCGCAGAATGGCTTCC	NM_013196.1
Peroxisome proliferator-activated receptor delta	PPARδ	ACTCTCCTTTCTTCCTGCCTGTG	TGTGCTGCTGCTCCTTCTGG	NM_013141.2
Peroxisome proliferator-activated receptor gamma, co-activator 1	PGC1α	ACACCGCACACATCGCAATTC	TTCGTCCCTCTTGAGCCTTTCG	NM_031347.1
Myosin Heavy chain 7	MHCI	ACAGAGGAAGACAGGAAGAACCTAC	GGGCTTCACAGGCATCCTTAG	NM_017240.1
Protein kinase AMP-activated alpha 1	AMPKα1	TTGCGTGTGCGAAGGAAGAAC	CCAAATCAGGGACTGCTACTCCA	NM_019142.1
Protein kinase AMP-activated alpha 2	AMPKα2	GATGATGAGGTGGTGGAGCAGAC	CACTGTCTGGCTCTCTCACTGC	NM_023991.1
Ribosomal 18S	18S	GATGCGGCGGCGTTATTC	CTCCTGGTGGTGCCCTTCC	M11188.1

LOW RESOLUTION FIG

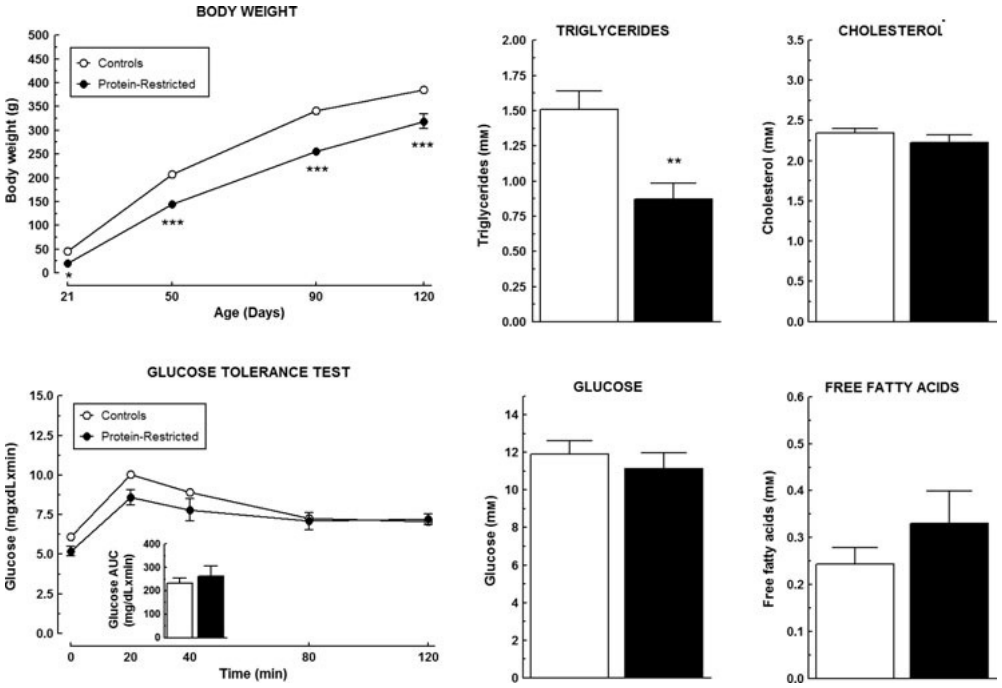

Figure 1 Body weight and metabolic determinations in plasma of adult rats born to protein-restricted dams during gestation and lactation. Animals were killed under *ad libitum* feeding conditions within the first hour after the beginning of the light cycle. Data were analysed by student's *t*-test or two-way ANOVA (glucose tolerance test and body weight evolution). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. White bars correspond to control animals and black bars to malnourished rats. 10

number of fibres was found in soleus but not in EDL of PR rats ($C = 95.90 \pm 1.32\%$, $n = 6$ vs. PR = $88.93 \pm 2.85\%$, $n = 5$; $P < 0.05$). In addition, adult animals born to protein-restricted dams exhibited a threefold increase in the number ($C = 7.56 \pm 2.16$ fibres mm^{-2}, $n = 6$ vs. PR = 22.34 fibres mm^{-2}, $n = 5$, $P < 0.05$) and proportion ($C = 4.10 \pm 1.32\%$, $n = 6$ vs. LP = $11.07 \pm 2.85\%$, $n = 5$; $P < 0.05$) of type II fibres in soleus but not in EDL (Fig. 2c,d). Further phenotyping of type II fibres by ATPase staining at pH 4.55 revealed that the numbers of both type IIa and type IIb fibres were increased by early protein restriction (Fig. 2g). ATPase staining at pH 4.55 also confirmed that although PR rats possesses the same density of type I fibres than controls in both soleus and EDL, the percentage of type I fibres in relation to the total number of fibres is reduced in the soleus of malnourished rats ($C = 95.61 \pm 1.26\%$, $n = 6$ vs. PR = $87.64 \pm 3.26\%$, $n = 5$, $P < 0.05$).

Histochemical staining with NADH-tetrazolium reductase, a marker of mitochondrial activity, revealed an increase in the number (Fig. 3) and proportion ($C = 2.31 \pm 0.04\%$, $n = 6$ vs. PR = $3.71 \pm 0.05\%$, $n = 6$, $P < 0.001$) of non-oxidative (non-stained) fibres in the soleus muscle of PR rats without any change in the density of oxidative (stained) fibres. In contrast, we observed a reduction in the total number of non-oxidative fibres in EDL of PR rats in relation to control animals (Fig. 3).

Enzymatic activity

To determine to what extend the structural changes induced by early protein restriction on skeletal muscle could be affecting its metabolic properties, we evaluated the activity of hexokinase (HK), β-hydroxyacyl-CoA dehydrogenase (β-HAD) and citrate synthase (CS). Hexokinase is the rate-limiting enzyme of glycolysis, β-HAD regulates the oxidation of long-chain saturated fatty acids within the mitochondria, and CS plays a key regulatory role in the tricarboxylic acid cycle by catalysing the condensation of oxaloacetate and acetyl-coA to citrate.

Under our experimental conditions, HK activity was significantly reduced in muscle homogenates from

COLOR

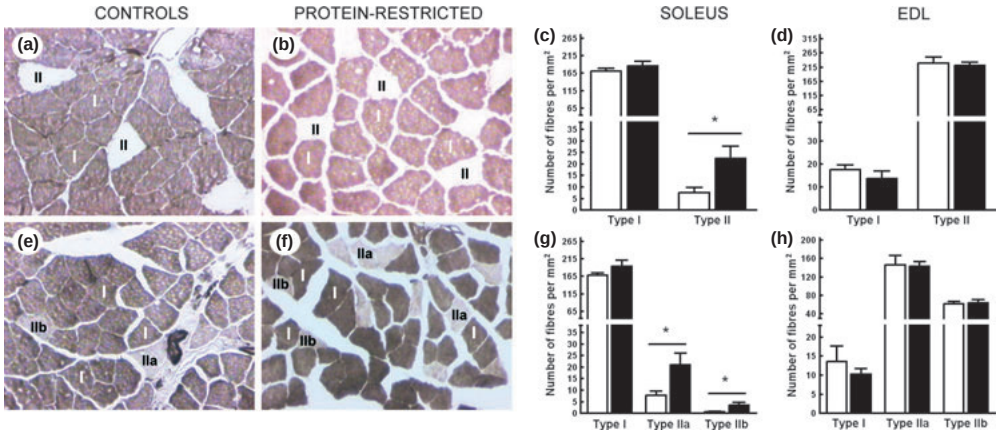

Figure 2 Effect of early protein restriction on the number and phenotype of muscle fibres in soleus and EDL as determined by ATPase staining after pre-incubation at pH 4.3; (a), (b), (c) and (d), or pH 4.55; (e), (f), (g) and (h). Numbers within the sections indicate the type of fibre. *$P < 0.05$ as determined by two-way ANOVA followed by Bonferroni multiple comparison test using mother diet and muscle type as independent factors. White bars correspond to control animals and black bars to malnourished rats.

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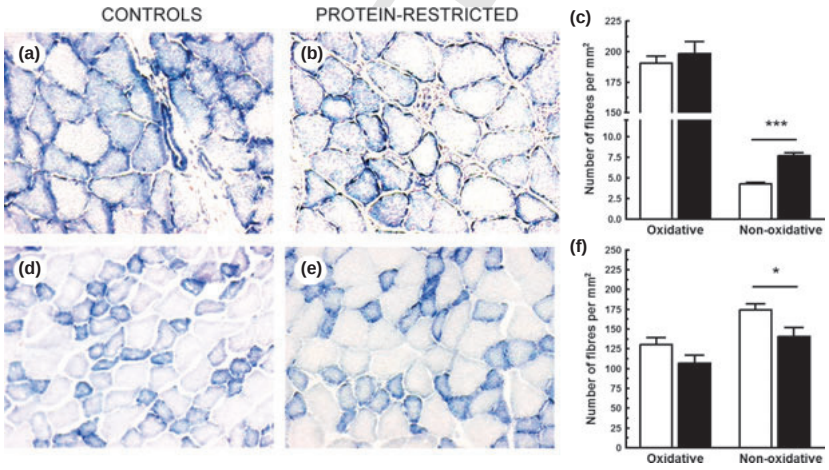

Figure 3 Effect of early protein restriction on the number of oxidative and non-oxidative muscle fibres in soleus; (a), (b), (c) and EDL; (d), (e), (f) as determined by NADH-TR staining. Oxidative fibres appear stained in bleu, whereas non-oxidative fibres are non-stained. *$P < 0.05$; ***$P < 0.001$ as determined by two-way ANOVA followed by Bonferroni multiple comparison test using mother diet and muscle type as independent factors with $n = 6$ animals per experimental group. White bars correspond to control animals and black bars to malnourished rats.

protein-restricted rats as compared with their control counterparts. However, these changes were observed exclusively in soleus (Fig. 4). We observed no differences between control and PR animals in the basal activities of β-HAD and CS neither in soleus nor in EDL (Fig. 4). Nonetheless, the activity of β-HAD in proportion to that of CS was decreased in PR animals (Fig. 5), indicating that the rate of fatty acid oxidation was actually reduced by maternal protein restriction. Again, these effects were observed in soleus but not in the EDL. The proportionality between the activity of HK and CS was also reduced (Fig. 5) further confirming that the soleus muscle of PR rats has decreased glycolytic capacity.

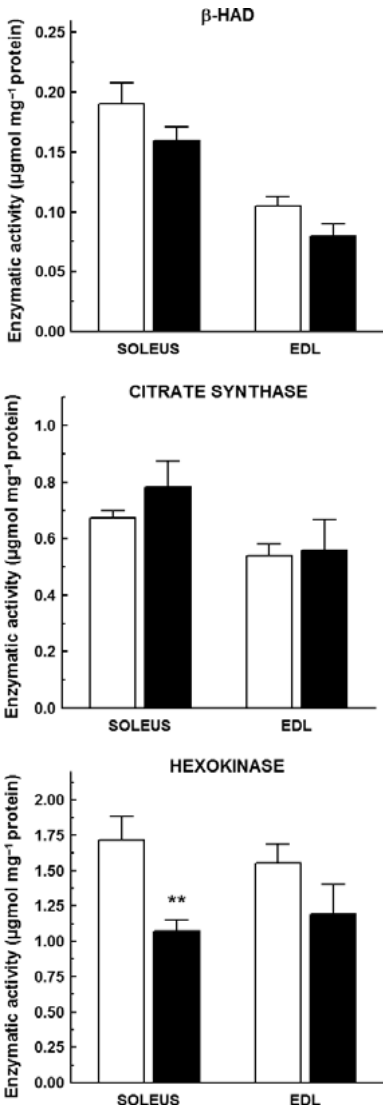

Figure 4 Enzymatic activity in skeletal muscles from adult offspring born to control (white bars) or from protein-restricted dams (black bars) during gestation and lactation. $^{**}P < 0.001$ as determined by two-way ANOVA followed by Bonferroni multiple comparison test using mother diet and muscle type as independent factors with $n = 6$ animals per experimental group.

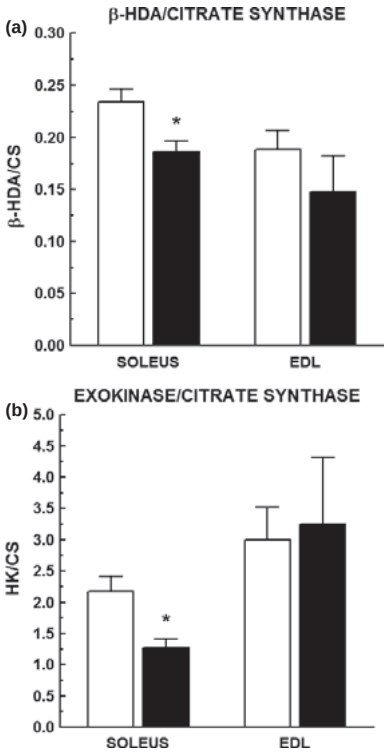

Figure 5 Rate of fatty acid oxidation (a) and glycolysis (b) in skeletal muscle from adult rats born to control (white bars) or protein-restricted (black bars) dams during gestation and lactation. The enzymatic activities of β-hydroxylacyl dehydrogenase (β-HAD) and hexokinase (HK) are expressed in proportion to the activity of citrate synthase (CS). All enzymatic activities were determined in parallel in the same muscle samples. $^{*}P < 0.05$ with $n = 6$ animals per group.

Gene expression

To further characterize the effects induced by metabolic programming on the metabolic properties of skeletal muscle, we analysed by quantitative RT-PCR the expression levels of several genes regulating glucose metabolism (ChREBP, HKII, Glut4), fatty acid oxidation (CPT1a, MCAD, PDK4, β-HAD) and mitochondrial function (CS,COXIV,UCP3). The transcript levels of genes involved in the transcriptional regulation of metabolism (PPARα, PPARδ, PGC1α), fibre-type composition (MHCI) and nutrient sensing (AMPKα1, AMPKα2) were also examined.

In soleus, no differences between control and PR rats were observed in the transcript levels of the genes regulating glucose metabolism that we analysed (Fig. 6). In contrast, the expression of CPT1a and UCP3 increased approx. 2,5-fold in skeletal muscle from the offspring of malnourished dams compared with controls ($P < 0.05$), but there were no differences in expression of the other genes involved in β-oxidation or mitochondrial function (Fig. 6). Concerning the transcriptional regulation of metabolism, PGC1α

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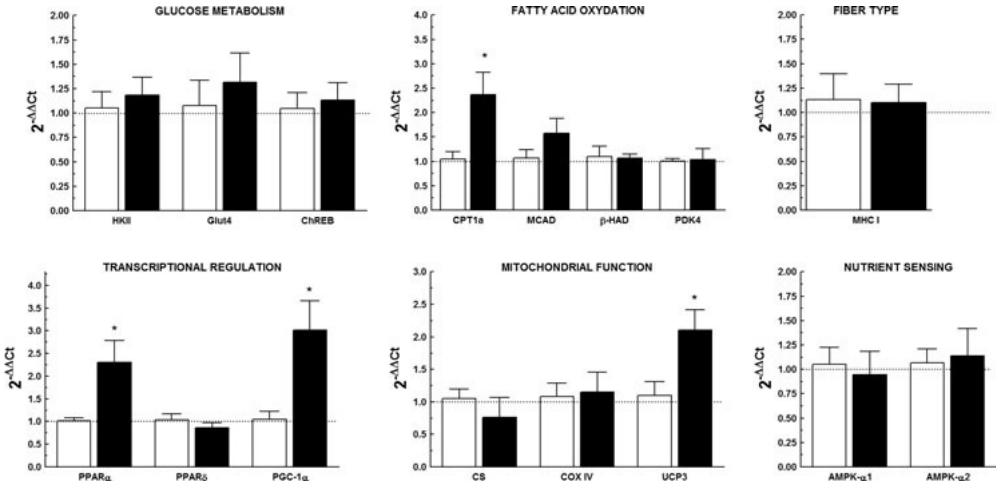

Figure 6 Messenger RNA expression levels of several genes regulating energy metabolism and mitochondrial function in soleus muscle from adult rats born to control (white bars) or from protein-restricted (black bars) dams during gestation and lactation. $^*P < 0.05$ with $n = 5$ animals per group.

and PPARα were up-regulated in PR rats. Finally, control and PR exhibited similar expression levels of the genes encoding the two isoforms of the nutrient sensor AMPK and the myosin heavy chain isform 1 (Fig. 6).

The transcriptional profile of EDL was also affected by early protein restriction but in a different way than in soleus. Among the genes regulating glucose metabolism, the expression of ChREBP was increased (Fig. 7). The levels of the glucose transporter Glut4 mRNA transcripts tended also to increase in PR rats in relation to controls, but this difference was not statistically different. Concerning the genes involved in fatty acid oxidation, the expression of MCAD was reduced, whereas that of the nuclear receptors PPARα and PPARδ was increased (Fig. 7). We found no change in the expression levels of the genes regulating mitochondrial function, nutrient sensing or fibre-type composition.

Discussion

Insulin resistance of skeletal muscle is a hallmark of obesity and type 2 diabetes. Given the tight relationship between early malnutrition and the risk of developing these metabolic disorders in later life (Fernandez-Twinn & Ozanne 2010, Wiedmeier *et al.* 2011), here we examined the long-term consequences of protein restriction during pregnancy and lactation on the structural and metabolic properties of skeletal muscle. Our results clearly indicate that skeletal muscle is metabolically programmed in a fibre-type-specific manner. Actually, adult rats born to protein-restricted dams exhibited enhanced density of type II fibres along with decreased rate of fatty acid oxidation and glycolysis in soleus but not in EDL. Maternal malnutrition induced also a different gene expression profile in soleus and EDL. Altogether, our results are indicative of altered mitochondrial function and oxidative capacity of soleus skeletal muscle in PR rats as compared with controls.

Our observation that protein restriction during pregnancy and lactation alters the structural properties of skeletal muscle depending on muscle type is consistent with previous reports in the literature showing that poor maternal nutrition results in fibre-type-specific deficiencies in the skeletal muscle of the offspring. Thus, whereas in the soleus muscle of female rats born to protein-restricted dams, the number of both type I and type II fibres was decreased, in the gastrocnemius of the same animals, only the number of type I fibres was reduced (Mallinson *et al.* 2007). Similarly, maternal nutrient restriction in the sheep produced a global reduction in the number of myofibres in the triceps brachii, but not the soleus, of the offspring (Costello *et al.* 2008, Zhu *et al.* 2006). Nevertheless, in contrast to these studies in which a decrease in the density of type I myofibres following maternal malnutrition was systematically found, we observed an increase in the number of type II fibres without any modification in the density of type I fibres. These differences might be explained by the severity and timing of the nutritional insult and the age of the animals at which the histochemical analysis was performed. In fact, although (Mallinson *et al.*

POOR QUALITY FIG

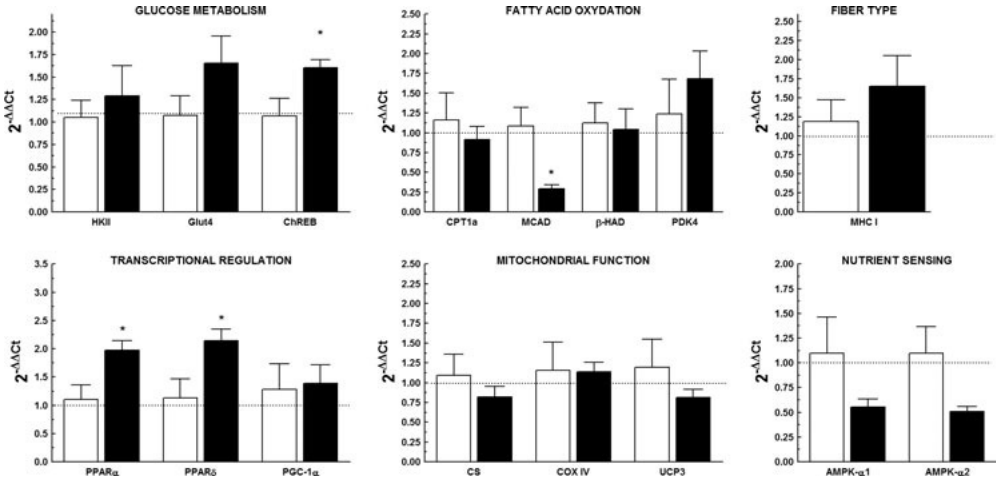

Figure 7 Messenger RNA expression levels of several genes regulating energy metabolism and mitochondrial function in EDL from adult rats born to control (white bars) or from protein-restricted (black bars) dams during gestation and lactation. *$P < 0.05$ with $n = 5$ animals per group. 14

(2007) used also protein restriction of the dam as nutritional insult, in their study the dams were subjected to malnutrition for a maximum of 1 week during pregnancy and the skeletal muscle from the offspring was analysed at 4 weeks of age.

In rodents, hindlimb skeletal muscle fibres are formed during the second half of gestation from progenitor cells originating in the somites. These myogenic precursor cells proliferate and fuse to form new multinucleated myofibres through two overlapping waves of differentiation (Kelly & Rubinstein 1980, Condon *et al.* 1990). The first wave takes place between embryonic days 15 and 17 (E15-Z17) and generates slow muscle fibres, whereas the second one is initiated around E17, continues into early post-natal life and generates fast muscle fibres. The final fibre profile proper to each skeletal muscle is nevertheless established during the first weeks after birth by the disappearance of type I fibres from fast muscles and a progressive transformation of type II into type I fibres in slow-twitch muscles (Lyons *et al.* 1984, Whalen *et al.* 1984). In the present study, we found no differences between control and PR rats in the total number of muscle fibres neither in soleus nor in EDL. This observation indicates that the genetic programme determining the primary differentiation of muscle cells is not affected by early protein restriction. In contrast, the number of type II fibres is soleus was significantly increased in PR rats as compared with controls suggesting an impairment of the transition from embryonic/neonatal to adult phenotype. This latter process is regulated in a muscle-type-dependent manner by neuronal signals and the thyroid hormone. Actually, denervation of the motor unit does not alter the neonatal to adult type II fibre transition in EDL (Gambke *et al.* 1983). Yet, the same experimental manipulation reduces significantly the proportion of type I fibres in soleus (Gambke *et al.* 1983). Similarly, pharmacologically induced hyperthyroidism induces a precious expression of myosin heavy chain IIB, a marker of fast-glycolytic fibres, in soleus and gastrocnemius but not in the levator ani, the bulbocavernosus complex or the masseter muscles (d'Albis *et al.* 1990, Russell *et al.* 1988). Interestingly, foetal growth restriction in humans (Radetti *et al.* 2004) as well as protein restriction during early development in rats (Lisboa *et al.* 2008) has been associated with enhanced circulating levels of thyroid hormones. Based on these observations, it is tempting to speculate that the increased number of type II fibres in the soleus muscle of PR rats is due to enhanced thyroid hormone signalling. However, the involvement of other factors cannot be excluded. In this respect, it is interesting to note that inhibition of calcineurin signalling in adult rats increases the proportion of type II vs. type I fibres in soleus (Chin *et al.* 1998). Further studies are warranted to determine the molecular mechanisms underlying the structural alterations of skeletal muscle associated with metabolic programming.

Differences in fibre-type composition have been associated with obesity and insulin resistance both in animal models and humans. Indeed, obese and diabetic individuals exhibit reduced amounts of type I (oxidative) fibres concomitantly with a enhanced

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number of type IIb (glycolytic) fibres (Hickey *et al.* 1995, Nyholm *et al.* 1997, MÅrin *et al.* 1994, Tanner *et al.* 2002). Similarly, a reduced percentage of type IIa fibres, which posses mixed oxidative/glycolytic properties, has been reported in skeletal muscle from Zucker (Adachi *et al.* 2007) or from Otsuka Long-Evans Tokushima Fatty (OLETF) rats (Yasuda *et al.* 2001), two animal models of type 2 diabetes mellitus. Moreover, skeletal muscles from diabetic and obese patients exhibit reduced hexokinase activity under basal conditions and impaired hexokinase mRNA expression in response to an insulin load (Pendergrass *et al.* 1998). Here, we observed that the soleus of PR rats possesses a higher density of glycolytic fibres, as revealed by both ATPase and NADH-TR staining, as well as reduced HK activity. Interestingly, however, these structural and metabolic alterations in muscle were not associated with glucose intolerance or an obese phenotype. Indeed, at the moment of killing, offspring from malnourished dams had lower body weight and lower visceral fat and exhibited the same quantitative and temporal decrements in the plasmatic levels of glucose during the glucose tolerance test than controls.

It is also interesting to note that the animals used in our study were killed in the fed state. Under these conditions, skeletal muscle should present a high rate of glucose uptake and oxidation. However, PR rats exhibited reduced HK activity and decreased rate of glycolysis in soleus in spite of presenting the same plasmatic levels of glucose than controls. Reduced use of glucose as fuel under normal (*ad libitum*) provision of nutrients in PR rats might suggest that developmental programming is associated with metabolic inflexibility of skeletal muscle. This phenomenon, defined as the impaired capacity of the organism to increase fatty acid oxidation upon increased lipids availability and to use glucose as the main energy source after a meal, has also been linked with the development of obesity and insulin resistance (Corpeleijn *et al.* 2009, Storlien *et al.* 2004).

Obesity and insulin resistance have also been associated with increased circulating levels of lipids (free fatty acids and triglycerides), as a result of impaired mitochondrial oxidation of long-chain fatty acids (LCFA). It is hypothesized that when the excess of lipids overcomes the oxidative capacity of mitochondria, they are transported in the form of fatty acid anions inside the mitochondrial matrix where their accumulation is potentially toxic because they are highly susceptible to peroxidation and, consequently, can lead to the exacerbated production of free radicals (Schrauwen *et al.* 2010, Hirabara *et al.* 2007). In addition, the accumulation of lipids within the mitochondrial matrix can trigger the activation of protein kinase C (PKC) and other protein kinases which inhibit insulin signalling (Samuel *et al.* 2010). Accumulation of fatty acids into the mitochondria is prevented by uncoupling oxidative phosphorylation from ATP production (mitochondrial uncoupling). In skeletal muscle, this function is performed by the mitochondrial uncoupling protein type 3 (UCP3) that transports fatty acid anions from the mitochondrial matrix to the cytosol to limit their peroxidation. Notably, fatty acids activate the expression of UCP3 thus establishing a feedback loop to prevent excessive production of free radicals and mitochondrial damage (Schrauwen *et al.* 2010, Hirabara *et al.* 2007, Son *et al.* 2001).

In the present study, the levels of mRNA encoding carnitine palmitoyltransferase 1 (*CPT1*), which drives the transport of long-chain fatty acids into the mitochondrial matrix (Bonnefont *et al.* 2004), were increased in soleus from PR rats. This result suggests that early protein restriction promotes the oxidation of fat by muscle. Surprisingly, however, we did not detect any difference between control and PR in the activity or gene expression levels of β-HAD. Control and protein-restricted rats exhibited also similar transcript levels of medium-chain acyl coenzyme A dehydrogenase (*MCAD*), another key enzyme of β-oxidation. These results indicate that the increased translocation of fatty acids into the mitochondria does not result in enhanced fatty acid oxidation or in increased production of ATP. In line with this idea, the activity of CS, a marker of mitochondrial oxidative phosphorylation capacity, was not affected in PR rats. In fact, the rate of fat oxidation in soleus muscle is actually reduced by early protein restriction as indicated by the decreased capacity of fatty acid oxidation to supply substrates for mitochondrial phosphorylation (β-HAD/CS ratio). We detected also enhanced levels of UCP3 transcripts, a gene which is up-regulated when fatty acid supply to the mitochondria exceeds its capacity to oxidize fatty acids (Schrauwen *et al.* 2001)

Altogether, these observations favour a scenario in which protein restriction during pregnancy and lactation stimulates the use of lipids as fuel in soleus muscle by promoting the incorporation of LCFA into the mitochondria through the enhanced expression of CPT1. Yet, the concomitant impairment of fatty acid β-oxidation induced by this nutritional insult leads to the accumulation of intermediates of fatty acid metabolism within the mitochondrial matrix. This in turn inhibits hexokinase activity (Thompson & Cooney 2000) and activates the expression of UCP3 which results in the outward translocation of fatty acids from the mitochondrial matrix. Hence, early protein restriction seems to induce a metabolic paradox in skeletal muscle in the sense that it enhances the

transfer of fatty acids to the mitochondrial matrix, but this increased translocation does not result in increased energy production.

In conclusion, we observed altered fibre-type composition, gene expression profile and enzymatic capacities in skeletal muscle from adult (4 months) and offspring born to protein malnourished rats. These alterations are muscle dependent, correspond to a state of energy deficiency that would result from the combined impairment of fatty acid oxidation and glycolysis and are present in animals which no show yet any sign of obesity or glucose intolerance. This latter observation suggests that the alterations in muscle structure and enzymatic activity induced by protein restriction during pregnancy and lactation might have a causative role in the development of obesity and related metabolic disorders later in life.

Conflict of interest

The authors have nothing to declare.

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References

Adachi, T., Kikuchi, N., Yasuda, K., Anahara, R., Gu, N., Matsunaga, T., Yamamura, T., Mori, C., Tsujimoto, G., Tsuda, K. & Ishihara, A. 2007. Fibre type distribution and gene expression levels of both succinate dehydrogenase and peroxisome proliferator-activated receptor-gamma coactivator-1alpha of fibres in the soleus muscle of Zucker diabetic fatty rats. *Exp Physiol* **92**, 449–455.

d'Albis, A., Chanoine, C., Janmot, C., Mira, J.-C. & Couteaux, R. 1990. Muscle-specific response to thyroid hormone of myosin isoform transitions during rat postnatal development. *Eur J Biochem* **193**, 155–161.

Bass, A., Brdiczka, D., Eyer, P., Hofer, S. & Pette, D. 1969. Metabolic differentiation of distinct muscle types at the level of enzymatic organization. *Eur J Biochem/FEBS* **10**, 198–206.

Bayol, S., Jones, D., Goldspink, G. & Stickland, N.C. 2004. The influence of undernutrition during gestation on skeletal muscle cellularity and on the expression of genes that control muscle growth. *Br J Nutr* **91**, 331–339.

Bayol, S.A., Simbi, B.H. & Stickland, N.C. 2005. A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning. *J Physiol* **567**, 951–961.

Bonnefont, J.-P., Djouadi, F., Prip-Buus, C., Gobin, S., Munnich, A. & Bastin, J. 2004. Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. *Mol Aspects Med* **25**, 495–520.

Brooke, M.H. & Kaiser, K.K. 1970. Three myosin adenosine triphosphatase systems: the nature of their pH lability and sulfhydryl dependence. *J Histochem Cytochem* **18**, 670–672.

Chin, E.R., Olson, E.N., Richardson, J.A., Yang, Q., Humphries, C., Shelton, J.M., Wu, H., Zhu, W., Bassel-Duby, R. & Williams, R.S. 1998. A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev* **12**, 2499–2509.

Cianfarani, S., Agostoni, C., Bedogni, G., Berni Canani, R., Brambilla, P., Nobili, V. & Pietrobelli, A. 2012. Effect of intrauterine growth retardation on liver and long-term metabolic risk. *Int J Obes* ??????, ?????–??????. 9

Condon, K., Silberstein, L., Blau, H.M. & Thompson, W.J. 1990. Development of muscle fiber types in the prenatal rat hindlimb. *Develop Biol* **138**, 256–274.

Corpeleijn, E., Saris, W.H.M. & Blaak, E.E. 2009. Metabolic flexibility in the development of insulin resistance and type 2 diabetes: effects of lifestyle. *Obes Rev* **10**, 178–193.

Costello, P.M., Rowlerson, A., Astaman, N.A., Anthony, F.E.W., Sayer, A.A., Cooper, C., Hanson, M.A. & Green, L.R. 2008. Peri-implantation and late gestation maternal undernutrition differentially affect fetal sheep skeletal muscle development. *J Physiol* **586**, 2371–2379.

Fahey, A.J., Brameld, J.M., Parr, T. & Buttery, P.J. 2005. The effect of maternal undernutrition before muscle differentiation on the muscle fiber development of the newborn lamb. *J Anim Sci* **83**, 2564–2571.

Fernandez-Twinn, D.S. & Ozanne, S.E. 2010. Early life nutrition and metabolic programming. *Ann N Y Acad Sci* **1212**, 78–96.

Gambke, B., Lyons, G.E., Haselgrove, J., Kelly, A.M. & Rubinstein, N.A. 1983. Thyroidal and neural control of myosin transitions during development of rat fast and slow muscles. *FEBS Lett* **156**, 335–339.

Grace, C.E., Kim, S.-J. & Rogers, J.M. 2011. Maternal influences on epigenetic programming of the developing hypothalamic-pituitary-adrenal axis. *Birth Defects Res A* **91**, 797–805.

Hickey, M.S., Carey, J.O., Azevedo, J.L., Houmard, J.A., Pories, W.J., Israel, R.G. & Dohm, G.L. 1995. Skeletal muscle fiber composition is related to adiposity and in vitro glucose transport rate in humans. *Am J Physiol – Endocrinol Metab* **268**, E453–E457.

Hirabara, S.M., Silveira, L.R., Abdulkader, F., Carvalho, C.R.O., Procopio, J. & Curi, R. 2007. Time-dependent effects of fatty acids on skeletal muscle metabolism. *J Cell Physiol* **210**, 7–15.

Jensen, C.B., Martin-Gronert, M.S., Storgaard, H., Madsbad, S., Vaag, A. & Ozanne, S.E. 2008. Altered PI3-Kinase/Akt signalling in skeletal muscle of young men with low birth weight. *PLoS ONE* **3**, e3738.

Kelly, A.M. & Rubinstein, N.A. 1980. Why are fetal muscles slow? *Nature* **288**, 266–269.

Lisboa, P.C., Fagundes, A.T.S., Denolato, A.T.A., Oliveira, E., Bonomo, I.T., Alves, S.B., Curty, F.H., Passos, M.C.F.

& Moura, E.G. 2008. Neonatal low-protein diet changes deiodinase activities and pituitary TSH response to TRH in adult rats. *Exp Biol Med* **233**, 57–63.

Livak, K.J. & Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–408.

Lyons, G.E., Haselgrove, J., Kelly, A.M. & Rubinstein, N.A. 1984. Myosin transitions in developing fast and slow muscles of the rat hindlimb. *Differentiation* **25**, 168–175.

Mallinson, J.E., Sculley, D.V., Craigon, J., Plant, R., Langley-Evans, S.C. & Brameld, J.M. 2007. Fetal exposure to a maternal low-protein diet during mid-gestation results in muscle-specific effects on fibre type composition in young rats. *Brit J Nut* **98**, 292–299.

MÅrin, P., Andersson, B., Krotkiewski, M. & Björntorp, P. 1994. Muscle fiber composition and capillary density in women and men with NIDDM. *Diabetes Care* **17**, 382–386.

Marquez, J., Sweazea, K.L. & Braun, E.J. 2006. Skeletal muscle fiber composition of the English sparrow (Passer domesticus). *Comp Biochem Physiol B Biochem Mol Biol* **143**, 126–131.

Muhlhausler, B.S., Duffield, J.A., Ozanne, S.E., Pilgrim, C., Turner, N., Morrison, J.L. & McMillen, I.C. 2009. The transition from fetal growth restriction to accelerated postnatal growth: a potential role for insulin signalling in skeletal muscle. *J Physiol* **587**, 4199–4211.

Nordby, D.J., Field, R.A., Riley, M.L. & Kercher, C.J. 1987. Effects of maternal undernutrition during early pregnancy on growth, muscle cellularity, fiber type and Carcass Composition in Lambs. *J Anim Sci* **64**, 1419–1427.

Nyholm, B., Qu, Z., Kaal, A., Pedersen, S.B., Gravholt, C.H., Andersen, J.L., Saltin, B. & Schmitz, O. 1997. Evidence of an increased number of type IIb muscle fibers in insulin-resistant first-degree relatives of patients with NIDDM. *Diabetes* **46**, 1822–1828.

Ozanne, S.E., Wang, C.L., Coleman, N. & Smith, G.D. 1996. Altered muscle insulin sensitivity in the male offspring of protein-malnourished rats. *Am J Physiol – Endocrinol Metab* **271**, E1128–E1134.

Ozanne, S., Olsen, G., Hansen, L., Tingey, K., Nave, B., Wang, C., Hartil, K., Petry, C., Buckley, A. & Mosthaf-Seedorf, L. 2003. Early growth restriction leads to down regulation of protein kinase C zeta and insulin resistance in skeletal muscle. *J Endocrinol* **177**, 235–241.

Ozanne, S., Jensen, C., Tingey, K., Storgaard, H., Madsbad, S. & Vaag, A. 2005. Low birthweight is associated with specific changes in muscle insulin-signalling protein expression. *Diabetologia* **48**, 547–552.

Pendergrass, M., Koval, J., Vogt, C., Yki-Jarvinen, H., Iozzo, P., Pipek, R., Ardehali, H., Printz, R., Granner, D., DeFronzo, R.A. & Mandarino, L.J. 1998. Insulin-induced hexokinase II expression is reduced in obesity and NIDDM. *Diabetes* **47**, 387–394.

Persson, P.B. & Henriksson, J. 2011. Editorial. *Acta Physiol* **203**, 403–407.

Punkt, K., Naupert, A. & Asmussen, G. 2004. Differentiation of rat skeletal muscle fibres during development and ageing. *Acta Histochem* **106**, 145–154.

Radetti, G., Renzullo, L., Gottardi, E., D'Addato, G. & Messner, H. 2004. Altered thyroid and adrenal function in children born at term and preterm, small for gestational age. *J Clin Endocrinol Metab* **89**, 6320–6324.

Regmi, P.R., Dixon, W.T. & Oba, M. 2008. Effects of ammonia load on glucose metabolism by isolated ovine duodenal mucosa. *J Anim Sci* **86**, 2321–2327.

Reusens, B., Theys, N., Dumortier, O., Goosse, K. & Remacle, C. 2011. Maternal malnutrition programs the endocrine pancreas in progeny. *Am J Clin Nut* **94**, 1824S–1829S.

Russell, S.D., Cambon, N., Nadal-Ginard, B. & Whalen, R.G. 1988. Thyroid hormone induces a nerve-independent precocious expression of fast myosin heavy chain mRNA in rat hindlimb skeletal muscle. *J Biol Chem* **263**, 6370–6374.

Samuel, V.T., Petersen, K.F. & Shulman, G.I. 2010. Lipid-induced insulin resistance: unravelling the mechanism. *The Lancet* **375**, 2267–2277.

Schiaffino, S. & Reggiani, C. 2011. Fiber types in mammalian skeletal muscles. *Physiol Rev* **91**, 1447–1531.

Schrauwen, P., Saris, W.H.M. & Hesselink, M.K.C. 2001. An alternative function for human uncoupling protein 3: protection of mitochondria against accumulation of nonesterified fatty acids inside the mitochondrial matrix. *FASEB J* **15**, 2497–2502.

Schrauwen, P., Schrauwen-Hinderling, V., Hoeks, J. & Hesselink, M.K. 2010. Mitochondrial dysfunction and lipotoxicity. *Biochim Biophys Acta* **1801**, 266–271.

Selak, M.A., Storey, B.T., Peterside, I. & Simmons, R.A. 2003. Impaired oxidative phosphorylation in skeletal muscle of intrauterine growth-retarded rats. *Am J Physiol – Endocrinol Metab* **285**, E130–E137.

Shelley, P., Martin-Gronert, M.S., Rowlerson, A., Poston, L., Heales, S.J.R., Hargreaves, I.P., McConnell, J.M., Ozanne, S.E. & Fernandez-Twinn, D.S. 2009. Altered skeletal muscle insulin signaling and mitochondrial complex II-III linked activity in adult offspring of obese mice. *Am J Physiol – Regulat, Int Comp Physiol* **297**, R675–R681.

Son, C., Hosoda, K., Matsuda, J., Fujikura, J., Yonemitsu, S., Iwakura, H., Masuzaki, H., Ogawa, Y., Hayashi, T., Itoh, H., Nishimura, H., Inoue, G., Yoshimasa, Y., Yamori, Y. & Nakao, K. 2001. Up-Regulation of Uncoupling Protein 3 Gene Expression by Fatty Acids and Agonists for PPARs in L6 Myotubes. *Endocrinology* **142**, 4189–4194.

Storlien, L., Oakes, N.D. & Kelley, D.E. 2004. Metabolic flexibility. *Proc Nutr Soc* **63**, 363–368.

Tanner, C.J., Barakat, H.A., Dohm, G.L., Pories, W.J., MacDonald, K.G., Cunningham, P.R., Swanson, M.S. & Houmard, J.A. 2002. Muscle fiber type is associated with obesity and weight loss. *Am J Physiol – Endocrinol Metab* **282**, E1191–6.

Thompson, A.L. & Cooney, G.J. 2000. Acyl-CoA inhibition of hexokinase in rat and human skeletal muscle is a potential mechanism of lipid-induced insulin resistance. *Diabetes* **49**, 1761–1765.

Toscano, A.E., Manhaes-de-Castro, R. & Canon, F. 2008. Effect of a low-protein diet during pregnancy on skeletal muscle mechanical properties of offspring rats. *Nutrition* **24**, 270–8.

Whalen, R.G., Johnstone, D., Bryers, P.S., Butler-Browne, G.S., Ecob, M.S. & Jaros, E. 1984. A developmentally regulated disappearance of slow myosin in fast-type muscles of the mouse. *FEBS Lett* **177**, 51–56.

Wiedmeier, J.E., Joss-Moore, L.A., Lane, R.H. & Neu, J. 2011. Early postnatal nutrition and programming of the preterm neonate. *Nutr Rev* **69**, 76–82.

Yan, X., Huang, Y., Zhao, J.-X., Long, N.M., Uthlaut, A.B., Zhu, M.-J., Ford, S.P., Nathanielsz, P.W. & Du, M. 2011. Maternal obesity-impaired insulin signaling in sheep and induced lipid accumulation and fibrosis in skeletal muscle of offspring. *Biol Reprod* **85**, 172–178.

Yasuda, K., Ishihara, A., Adachi, T., Shihara, N., Seino, Y. & Tsuda, K. 2001. Growth-related changes in skeletal muscle fiber type and insulin resistance in diabetic Otsuka Long-Evans Tokushima fatty rats. *Acta Histochemica et Cytochemica* **34**, 371–382.

Zhu, M.J., Ford, S.P., Means, W.J., Hess, B.W., Nathanielsz, P.W. & Du, M. 2006. Maternal nutrient restriction affects properties of skeletal muscle in offspring. *J Physiol* **575**, 241–250.

Artigo 2

Obesity

Nutritional programming impairs metabolic flexibility of skeletal muscle before any sign of obesity or glucose intolerance

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Nutritional programming impairs metabolic flexibility of skeletal muscle before any sign of obesity or glucose intolerance

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Running head: Programming skeletal muscle metabolic flexibility

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What is already known about this subject?

- Obesity and diabetes are associated with impaired metabolic flexibility of skeletal muscle but whether this is a cause or a consequence of these pathological conditions is unknown.
- Malnutrition during early life is a predisposing factor for the development of obesity and related metabolic diseases in adulthood.
- Skeletal muscle from the offspring born to protein-restricted dams exhibit initially better insulin sensitivity as compared with control skeletal muscle but become insulin resistant in the elderly.

What does this study add?

- This study shows that the metabolic flexibility of skeletal muscle is impaired in the adult offspring born to protein-restricted dams adding to the detrimental effects of malnutrition during early life.
- Young adult malnourished animals exhibit impaired transcriptional responses of skeletal muscle to fasting but show a normal metabolic state indicating that impaired metabolic flexibility precedes and may underpin the development of obesity and insulin resistance induced by maternal protein-restriction.
- Early protein-restriction affected differentially the responses of soleus and Extensor Digitorum Longus muscles to fasting indicating that metabolic inflexibility of skeletal muscle is fiber-type specific.

Abstract

Impaired metabolic flexibility in skeletal muscle has been associated with obesity and type 2 diabetes but it is not known if it is a cause or a consequence of these pathological entities. Inasmuch as a poor nutritional environment during early life is a predisposing factor for the development of metabolic diseases in adulthood, here we search to determine whether maternal malnutrition alters the metabolic flexibility of offspring skeletal muscle. The transcriptional responses of soleus and Extensor Digitorum Longus (EDL) muscles to fasting were evaluated in skeletal muscle from adult rats born to dams fed a control (17% protein) or a low-protein (8% protein, PR) diet throughout pregnancy and lactation. PR rats exhibited the same metabolic profile and glucose tolerance as control animals along with decreased body weight and reduced levels of plasmatic triglycerides. In the fed state, PR rats showed enhanced expression levels of key regulatory genes of mitochondrial metabolism. However, in response to fasting PR rats showed impaired expression of several genes implicated in the regulation of mitochondrial function that increase the capacity for fat oxidation. These results indicate that metabolic inflexibility precedes and may contribute to the development of metabolic disorders associated with early malnutrition.

Keywords: Metabolic flexibility, skeletal muscle, metabolic programming, maternal malnutrition.

Introduction

Skeletal muscle has a remarkable ability to adapt its metabolism in response to caloric intake and the energy demands of the organism. Actually, both food deprivation and feeding a high-fat diet, favor the use of lipids by skeletal muscle as energy source at the expense of glucose, a phenomenon known as metabolic flexibility. Impaired metabolic flexibility has been associated with obesity and insulin resistance (1, 2), but the exact mechanisms linking metabolic inflexibility to these pathological conditions are not known. We also ignore if the lack of adaptation to metabolic demands is a cause or a consequence of obesity or diabetes.

It is now widely accepted that malnutrition during early life is an important predisposing factor for the development of obesity and metabolic diseases. Indeed, based on epidemiological studies demonstrating that people born at low birth weight are at high risk of developing the metabolic syndrome in adulthood, Hales and Barker (3) proposed that in a developing organism exposed to nutrient deficiency there is a selective distribution of nutrients to preserve brain growth at the expense of other organs. This results in the "programming" of energy metabolism in order to survive under conditions of food restriction. However, when the adverse nutritional environment disappears, the physiological incompatibility between the “thrifty” metabolic programming and the new nutritional conditions generates obesity, impaired glucose tolerance, hypertension and atherosclerosis (3).

Experimental studies in different animal species have provided major support for the hypothesis proposed by Hales and Barker and have led to the identification of several cellular and physiological adaptive responses as potential underlying mechanisms of metabolic programming including alterations in skeletal muscle. Notably, it has been shown that reduced nutrient supply during the last three days of gestation in the rat leads to decreased ATP production and decreased glucose transport and glycogen biosynthesis in red muscle (4).

It has also been shown that the offspring born to dams fed a low protein diet during gestation and lactation, exhibit initially better glucose tolerance and enhanced insulin sensitivity in soleus muscle (5), but become insulin resistant in the elderly (6, 7). In addition to these functional alterations, decreased (8), increased (9) or no change (10) in the number of type II fibres have been documented in rat following perinatal undernutrition.

The overall objective of this study was to define the consequences of protein restriction during gestation and lactation on the metabolic flexibility of skeletal muscle at adulthood. Our working hypothesis is that nutrient restriction during the critical period of perinatal development impairs the ability of skeletal muscle to adapt to the use of energy substrates (lipids, glucose) in response to the nutritional and energy demands of the body.

Materials and Methods

Animals and experimental manipulations

We used a typical model of metabolic programming which has been described in detail elsewhere (11). In brief, virgin female Wistar rats weighing 200–220 g were obtained from Charles River (France) and mated to 3-month-old males of the same strain. After confirmation of mating, pregnant rats were housed individually and fed *ad libitum* during gestation and lactation either a control (170 g protein / Kg) or an isocaloric low-protein (LP) diet (80 g protein / Kg). The diets were kindly provided by the Department of Nutrition of the Federal University of Pernambuco (Brazil) and their composition has been described previously (9). One day after delivery, litter size was adjusted at random to eight pups per dam maintaining an equal sex ratio. At weaning (21 days), female pups were discarded from the study and all male pups from the control (C) and Protein-restricted (PR) groups were fed standard chow until the end of the experiment. The body weight of the pups was recorded every three days during lactation and every week after weaning.

Sacrifice and muscle sampling

At 4 months of age, animals were sacrificed under *ad libitum* feeding conditions or after a fasting period of 48-hours. Soleus and the Extensor Digitorum Longus (EDL) muscles were carefully dissected under sodium pentobarbital anaesthesia (50 mg/kg), immediately frozen in liquid nitrogen and kept at -80°C until analysis. Animals were then sacrificed by cervical dislocation. All animals were sacrificed within the first hour after the beginning of the light cycle to avoid the bias induced by the circadian variations in gene expression.

Hormone and metabolite determinations

Insulin resistance was evaluated 15 days before sacrifice by the glycemic response to an oral glucose tolerance test after an overnight fast of 16h. Plasma from trunk blood collected at the time of sacrifice was assayed for insulin and leptin using assay kits from Millipore (Billerica, MA). Enzymatic diagnostic kits from BioMérieux (Craponne, France), were used to quantify the plasmatic levels of triglycerides, glucose and cholesterol. Free fatty acids were evaluated using an assay kit from Wako (Richmond, VA).

Real-time quantitative -PCR experiments

PCR experiments were performed in reversed-transcribed RNA extracts using SYBR Green (Qiagen, Courtaboeuf, France) as fluorogenic intercalating dye and the iCycler iQ Real-Time PCR detection system instrument from Bio-Rad Laboratories (Hercules, CA, USA). The relative expression levels were calculated using the comparative $2^{-\Delta\Delta Ct}$ method (12) and 18S RNA as housekeeping gene. Primers were designed using the information contained in the public GeneBank database of the National Center for Biotechnology Information (NCBI), and their sequences are presented in Table 1.

Western Blot

Muscles samples were homogenized in 1:8 volumes (wt/vol) of extraction buffer containing: 50 mM HEPES (pH 7.6), 50 mM KCl, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5 mM β-glycerophosphate, 1% NP-40, 1 mM Na_3VO_4 and a cocktail of protease inhibitors (Sigma, P2714). Homogenates were then centrifuged at 10000x *g* at 4°C for 10 min. The resulting supernatants were used for Western Blot analysis. In brief, 100 µg of protein extracts were separated with a 10% SDS-PAGE gel and transferred onto PVDF membranes which were incubated overnight at 4°C with a primary antibody against total AMPK or with a rabbit polyclonal antibody recognising the phosphorylated form of AMPK at Thr172. Both antibodies were used at a dilution of 1:1000 and were purchased from Cell Signaling (Danvers, MA, USA). To control for protein loading, the same blots were incubated with an anti-α-tubulin antibody (Santa Cruz Biotechnology, INC. Dallas, Texas, U.S.A.) diluted at 1:100. Afterwards, membranes were incubated for 1 h at room temperature with goat anti-Rabbit peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Immunoblotted proteins were visualized with the UptiLight™ HRP blot substrate (Interchim, Montluçon, France) and analyzed using the G:BOX Chemi XL system (Syngene, Cambridge, UK).

Data Analysis

Experimental results are expressed as means ± SEM. Statistical differences between the groups in plasma metabolite concentrations were assessed by two-way ANOVA followed by Bonferroni's test using maternal diet (control or protein-restricted), and nutritional status at sacrifice (Ad libitum or fasted), as independent factors. Differences in gene expression were assessed by unpaired Student's t-test and by two-way ANOVA. Statistical significance was set at $p<0.05$.

Results

Phenotypic characteristics of the pups and metabolite variations in plasma in response to fasting

There were no differences in body weight at birth between control and protein-restricted pups (C = 5.21±0.18g, n =12; PR = 4.86±0.21g, n=12). However, the offspring born to, and nursed by, protein-restricted dams gained significantly less weight during lactation such that at the end of this period they weighed in average 58% less than their control counterparts (C = 43.38g ± 0.46, n=12; PR = 19.50g ± 0.66, n =12, $p<0.0001$, Figure 1A). During the first two months after weaning, PR pups exhibited enhanced body weight gain (Figure 1B). However, in spite of this catch-up growth they continued to weigh less than controls until the end of the experiment (Figure 1A). In addition, PR pups exhibited less abdominal fat both in absolute terms (C = 18.88 ± 2.01g, n = 12; PR = 9.17.± 0.76 g, n = 9, $p<0.001$) and in proportion to their body weight (C = 8.26 ± 0.70 %, n = 12; PR = 4.68 ± 0.34 %, n = 9, $p< 0.001$).

Control and PR rats displayed the same glucose tolerance as indicated by the identical area under the curve of the glucose tolerance test (C = 332.60 ± 30.65 mg/dLxmin, n = 6; PR = 322.30.65 ± 30.28 mg/dLxmin, n=6), and the same temporal decrease in plasmatic glucose after the glucose load (data not shown).With the exception of a reduced concentration of triglycerides and insulin, the metabolite profile in plasma under *ad libitum* feeding conditions of control and PR adult rats was the same (Figure 2). Both groups exhibited also similar variations in plasma metabolite concentrations in response to a 48-hour fast. Of notice, however, fasting reduced the plasmatic content of insulin in control but not in PR rats (Figure 2).

Gene expression profile under ad libitum feeding conditions

We detected higher mRNA expression levels for several genes involved in the regulation of fatty oxidation in the soleus, but not the EDL, of PR animals sacrificed in the fed state as compared with their control counterparts. Thus, the mRNA levels of carnitine palmitoyltransferase 1a (CPT1a), were upregulated in PR rats (Table 2). CPT1a is the key enzyme in the carnitine-dependent transport of fatty acids across the mitochondrial inner membrane. Moreover, the expression of the nuclear receptors PPARα as well as that of the co-regulator of transcription PGC1α, which in a coordinated fashion regulate positively the expression of several genes playing a key role in fatty acid oxidation, was increased by more than two-fold by perinatal protein-restriction (Table 2). We also detected increased expression of uncoupling protein 3 (UCP3), which acts to export non sterified fatty acids (NEFA), from the mitochondrial matrix when fatty acid supply exceeds oxidation (13, 14), and of the α2 isoform of AMP-activated protein kinase (AMPK). This kinase is activated by energy depletion and regulates a wide number of cellular process that synergistically inhibit protein and lipid biosynthesis and stimulate β-oxidation, glucose uptake and glycolysis (15). In contrast, protein-restriction decreased the expression of Pyruvate Dehydrogenase Kinase, Isozyme 4 (PDK4), which negatively regulates glucose metabolism through the inhibition of the pyruvate dehydrogenase complex (Table 2). In contrast to these multiple gene expression changes in soleus, the only transcriptional change detected in EDL was an increased expression of mRNA levels encoding PPARδ nuclear receptors.

Genes expression changes in response to fasting

We observed both divergent and similar mRNA transcription changes in response to fasting between control and PR rats that differed also from one muscle type to the other. Thus, while fasting increased the expression of CPT1a in soleus from control animals, no change in

mRNA levels were detected in fasted PR animals in relation to ad libitum fed malnourished rats (Figure 3). Moreover, fasting decreased the expression of medium chain acyl coenzyme A dehydrogenase (MCAD) and beta-Hydroxyacyl-Coenzyme A dehydrogenase (β-HAD) in protein-restricted but not in control animals (Figure 3). These two enzymes catalyze several steps of the beta-oxidation of long chain fatty acids by the mitochondria. In addition, although fasting enhanced the expression of uncoupling protein 3 in both groups of animals, PR rats exhibited an attenuated response as compared with controls (Figure 3). Finally, the expression of PDK4 and AMPK was not affected by fasting neither in control nor in PR rats (Figure 3). Concerning the genes involved in the transcriptional regulation of metabolism, their expression, as that of Myosin Heavy chain 7 (MHCI), which defines the formation of type I (oxidative) fibers, was decreased by fasting both in control and PR rats (Figure 3).

With regard to the transcriptional changes in EDL, our analysis revealed that fasting increased the expression of CPT1a and UCP3 in both groups of animals (Figure 4). However, fasted PR animals, but not fasted control rats, exhibited increased expression of PDK4 and decreased mRNA levels encoding PPARα and PPARδ in relation to their *ad libitum* fed counterparts (Figure 4). Moreover, MCAD was down regulated by fasting in controls but up regulated in malnourished animals. Lastly, food deprivation for 48-hours did not alter the mRNA expression levels of β-HAD, PGC1α, AMPK and MHCI neither in control nor in PR rats (Figure 4).

Effect of maternal protein restriction on the activation of AMPK

The nutrient sensor AMPK is considered to play a central role in the metabolic adaptation to fasting through the regulation of multiple metabolic enzymes and transcription factors that stimulate glucose uptake and fatty acid oxidation. We were therefore surprised to observe no changes in AMPK gene expression in the soleus of fasted animals. To clearly determine if AMPK was activated under our experimental conditions, we quantified by Western blot the

phosphorylated (activated), form of AMPK at Thr172. As illustrated in Figure 5, PR rats exhibited enhanced phosphorylation levels of AMPK in the fed state in soleus but not EDL. Moreover, whereas fasting induced a significant activation of AMPK in the soleus of control animals, we did not detect any change of AMPK phosphorylation in response to fasting in PR rats (Figure 5A). Fasting increased also the phosphorylation levels of AMPK in the EDL from control and PR animals though in control animals this change did not reach statistical significance (Figure 5B).

Discussion

While reduced energy expenditure and increased consumption of highly caloric foods are important factors contributing to the current epidemic of obesity, many epidemiological and experimental studies have demonstrated that an unbalanced provision of nutrients during perinatal development increases the risk of developing obesity and the metabolic syndrome in adulthood (16). Several mechanisms have been proposed to account for this enhanced disease risk including altered organ structure (17, 18), elevated glucocorticoids and endocrine sensitivity (19, 20), impaired mitochondrial function (21, 22) and altered feeding behaviour (23, 24). Here we show that the metabolic flexibility of skeletal muscle is impaired in the adult offspring born to dams fed a low protein diet during gestation and lactation adding to the detrimental effects of malnutrition during early life.

To determine the effects of early malnutrition on the metabolic flexibility of skeletal muscle, we examined the expression of several genes involved in lipid metabolism and mitochondrial function in soleus, which relies mainly on lipids as energy substrate, and EDL, a fast-twitch glycolytic muscle. Under *ad libitum* feeding conditions, PR rats exhibited, increased AMPK gene expression and phosphorylation in soleus along with enhanced mRNA levels of several genes encoding for key proteins favouring fatty acid oxidation including CPT-1a, PGC-1α, UCP3 and PPARα. In contrast, the only statistically significant

modification observed in EDL was an increase in PPARδ mRNA levels indicating that, in addition to have divergent metabolic properties, these two muscles are affected differentially by metabolic programming.

Increased β-Oxidation (25) and over-expression of CPT-1 (26) in skeletal muscle cells have been shown to improve insulin resistance. Our gene expression data, along with the observation that PR rats exhibited also reduced body fat and decreased plasmatic levels of triglycerides, therefore suggest that early protein restriction induces a long-lasting metabolic state favouring lipid catabolism and insulin function. This observation seems to be at odds with the thrifty phenotype hypothesis stating that a poor nutritional environment during early life increases the risk of developing the metabolic syndrome at adulthood (27). However, enhanced capacity for fat oxidation in muscle is not in itself predictive of a healthy phenotype. Actually, transgenic mice in which increased fatty acid oxidation was induced by muscle-specific over-expression of PPARα or PGC-1α are more susceptible to diet-induced obesity and diabetes (28). Moreover, high-fat feeding increases the lipid-oxidation capacity of skeletal muscle through the up-regulation of several key genes regulating mitochondrial biogenesis (29, 30). On the basis of these results, it has been suggested that incomplete fatty acid oxidation and accumulation of beta-oxidative intermediates resulting from the mismatch between lipid overload and energy needs is the primary biochemical defect underlying insulin resistance (1, 31). In this respect, it is interesting to note that *ad libitum* fed PR rats exhibited enhanced expression of CPT-1a and UCP3 without any modification of the mRNA levels encoding MCAD or β-HAD. This transcriptional profile is indicative of an enhanced flux of lipids into the mitochondria without a concomitant increase of fatty acid oxidation. This would lead to increased export of fatty acid anions outside the mitochondrial matrix which might result in the accumulation of lipid accumulation in skeletal muscle.

Fasting is a nutritional challenge that leads to rapid metabolic adaptations of skeletal muscle which result in the shift from glucose to fatty acid oxidation as source of fuel. This phenomenon involves a complex mechanism of transcriptional regulation which includes the nuclear receptors PPARα and PPARδ, the co-transcriptional regulator PGC-1α and the nutrient sensors SIRT1 and AMPK (32, 33, 34). These two latter factors, which are activated under physiological conditions that require a large amount of energy such as nutritional restriction or exercise, induce PGC-1α expression and directly enhance its activity through deacetylation (SIRT1), or phosphorylation (AMPK) (35). The expression of PGC-1α can also be enhanced via the nuclear receptor PPARδ (36) and a calcium-dependent signaling pathway involving the activation of the transcription factor CREB (37). Once activated, PGC-1α facilitates the expression of several genes involved in fatty acid oxidation and mitochondrial function by forming a complex with the nuclear receptors PPARα (38) and, simultaneously, regulates the reprogramming of fast-twitch, glycolytic fibers (type II), into slow-twitch, oxidative fibers (type I), through co-activation of myosin Enhancer Factor 2 (39).

The transcriptional responses of skeletal muscle to fasting are time-dependent. Thus, PPARδ and PGC-1α are initially up-regulated by food deprivation but after a 48h fast their expression decrease below control levels (40). In agreement with these results, we observed that a 48h starvation period reduced the expression of these two transcription factors as well as that of PPARα in soleus from both control and PR rats. However, we found a clear difference between control and PR rats in the gene expression profile of CPT-1a, MCAD and β-HAD in response to fasting. Actually, the mRNA levels of the former gene increased by starvation in control but not in PR rats. Moreover, whereas fasted PR rats exhibited reduced expression of MCAD and β-HAD in relation to their *ad libitum* fed counterparts, starvation induced no change in the expression of these genes in control animals. Similarly, the activation of

AMPK, as measured by the phosphorylation of its alpha subunit on Thr-172, was increased by fasting in control but not in PR rats. Altogether, these gene expression changes are indicative of impaired fatty acid uptake and oxidation in response to fasting in soleus from PR rats. Furthermore, as expected from their divergent metabolic properties, soleus and EDL reacted differently to food deprivation and their metabolic flexibility was differentially affected by early protein restriction. Thus, while starvation down regulated the expression of the transcriptional regulators of lipid metabolism (PPARα, PPARδ, PGC-1α) in soleus from both control and PR rats, in EDL these changes were observed only in early malnourished rats. In addition, in sharp contrast to its effects in soleus, fasting induced a gene expression profile indicative of enhanced fatty acid β-oxidation and reduced glycolysis in EDL from PR rats. Collectively, these results show that fasting alters in a very fine and selective manner the metabolic properties of skeletal muscle

Kelly et al., were the first to propose that metabolic inflexibility of skeletal muscle, defined as decreased fatty acid oxidation under fasting conditions or reduced capacity to switch from lipid oxidation to glucose uptake and catabolism during the transition from fasting to feeding, is associated with obesity and diabetes and predicts the severity of insulin resistance (1). Further studies have corroborated that skeletal muscle from obese or diabetic patients exhibit decreased fatty acid oxidation during fasting or exercise as well as reduced capacity to shift between lipid and glucose oxidation under insulin-stimulating conditions (41, 42). However, it has not been determined if metabolic inflexibility is an additional manifestation of obesity and diabetes or if it is an underlying cause of these metabolic disorders. Here we show that 4 moths-old rats born to protein-restricted dams, exhibit an altered gene expression pattern in skeletal muscle in response to fasting. The fact that, at this age, these animals do not show yet any sign of obesity or glucose intolerance, suggests that metabolic inflexibility might be the cause and not the consequence of obesity and diabetes.

Interestingly, early protein-restriction affected differentially the responses of soleus and EDL to fasting further indicating that metabolic inflexibility of skeletal muscle might be fiber-type specific.

In summary, the herein presented results indicate that adult offspring born to protein-restricted dams exhibit under *ad libitum* feeding conditions a gene expression pattern in skeletal muscle favouring the transport of long chain fatty acids into the mitochondrial matrix without a concomitant increase of fatty acid oxidation. Moreover, the transcriptional network regulating the metabolic switch from carbohydrate to fat oxidation during fasting was clearly altered in PR animals which exhibit otherwise a normal metabolic state. This observation indicates that impaired metabolic flexibility precedes and may underpin the development of obesity and insulin resistance induced by maternal protein-restriction. Given that the deleterious effects of early malnutrition develop with age, further studies are warranted to determine how the herein reported impaired metabolic plasticity of skeletal muscle might contribute to insulin resistance and obesity.

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References

1. Kelley DE, Goodpaster B, Wing RR, Simoneau J-A. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *American Journal of Physiology - Endocrinology And Metabolism* 1999;277: E1130-E1141.

2. Storlien L, Oakes ND, Kelley DE. Metabolic flexibility. *The Proceedings of the Nutrition Society* 2004;63: 363-368.

3. Hales CN, Barker DJP. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* 1992;35: 595-601.

4. Selak MA, Storey BT, Peterside I, Simmons RA. Impaired oxidative phosphorylation in skeletal muscle of intrauterine growth-retarded rats. *American Journal of Physiology - Endocrinology And Metabolism* 2003;285: E130-E137.

5. Ozanne SE, Wang CL, Coleman N, Smith GD. Altered muscle insulin sensitivity in the male offspring of protein-malnourished rats. *American Journal of Physiology - Endocrinology And Metabolism* 1996;271: E1128-E1134.

6. Ozanne S, Olsen G, Hansen L, Tingey K, Nave B, Wang C, *et al.* Early growth restriction leads to down regulation of protein kinase C zeta and insulin resistance in skeletal muscle. *Journal of Endocrinology* 2003;177: 235-241.

7. Ozanne S, Jensen C, Tingey K, Storgaard H, Madsbad S, Vaag A. Low birthweight is associated with specific changes in muscle insulin-signalling protein expression. *Diabetologia* 2005;48: 547-552.

8. Mallinson JE, Sculley DV, Craigon J, Plant R, Langley-Evans SC, Brameld JM. Fetal exposure to a maternal low-protein diet during mid-gestation results in muscle-specific effects on fibre type composition in young rats. *The British journal of nutrition* 2007;98: 292-299.

9. Toscano AE, Manhaes-de-Castro R, Canon F. Effect of a low-protein diet during pregnancy on skeletal muscle mechanical properties of offspring rats. *Nutrition* 2008;24: 270-278.

10. Bayol S, Jones D, Goldspink G, Stickland NC. The influence of undernutrition during gestation on skeletal muscle cellularity and on the expression of genes that control muscle growth. *British Journal of Nutrition* 2004;91: 331-339.

11. Orozco-Solis R, Lopes de Souza S, Barbosa Matos RJ, Grit I, Le Bloch J, Nguyen P, *et al.* Perinatal undernutrition-induced obesity is independent of the developmental programming of feeding. *Physiology & behavior* 2009;96: 481-492.

12. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25: 402-408.

13. Abe T, Mujahid A, Sato K, Akiba Y, Toyomizu M. Possible role of avian uncoupling protein in down-regulating mitochondrial superoxide production in skeletal muscle of fasted chickens. *FEBS letters* 2006;580: 4815-4822.

14. Himms-Hagen J, Harper ME. Physiological role of UCP3 may be export of fatty acids from mitochondria when fatty acid oxidation predominates: an hypothesis. *Experimental biology and medicine* 2001;226: 78-84.

15. Lage R, Dieguez C, Vidal-Puig A, Lopez M. AMPK: a metabolic gauge regulating whole-body energy homeostasis. *Trends Mol Med* 2008;14: 539-549.

16. Ozanne SE, Constancia M. Mechanisms of disease: the developmental origins of disease and the role of the epigenotype. *Nature clinical practice Endocrinology & metabolism* 2007;3: 539-546.

17. Merezak S, Reusens B, Renard A, Goosse K, Kalbe L, Ahn MT, *et al.* Effect of maternal low-protein diet and taurine on the vulnerability of adult Wistar rat islets to cytokines. *Diabetologia* 2004;47: 669-675.

18. Vehaskari VM, Aviles DH, Manning J. Prenatal programming of adult hypertension in the rat. *Kidney Int* 2001;59: 238-245.

19. Cottrell EC, Holmes MC, Livingstone DE, Kenyon CJ, Seckl JR. Reconciling the nutritional and glucocorticoid hypotheses of fetal programming. *The FASEB Journal* 2012;26: 1866-1874.

20. Fowden AL, Forhead AJ. Hormones as epigenetic signals in developmental programming. *Experimental Physiology* 2009;94: 607-625.

21. Reusens B, Theys N, Remacle C. Alteration of mitochondrial function in adult rat offspring of malnourished dams. *World J Diabetes* 2011;15: 149-157.

22. Leduc L, Levy E, Bouity-Voubou M, Delvin E. Fetal programming of atherosclerosis: Possible role of the mitochondria. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 2010;149: 127-130.

23. Vickers MH, Breier BH, Cutfield WS, Hofman PL, Gluckman PD. Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. *American Journal of Physiology - Endocrinology And Metabolism* 2000;279: E83-E87.

24. Bellinger L, Langley-Evans SC. Fetal programming of appetite by exposure to a maternal low-protein diet in the rat. *Clin Sci* 2005;109: 413-420.

25. Perdomo G, Commerford SR, Richard A-MT, Adams SH, Corkey BE, O'Doherty RM, *et al.* Increased β-Oxidation in Muscle Cells Enhances Insulin-stimulated Glucose Metabolism and Protects against Fatty Acid-induced Insulin Resistance Despite Intramyocellular Lipid Accumulation. *Journal of Biological Chemistry* 2004;279: 27177-27186.

26. Bruce CR, Hoy AJ, Turner N, Watt MJ, Allen TL, Carpenter K, *et al.* Overexpression of Carnitine Palmitoyltransferase-1 in Skeletal Muscle Is Sufficient to Enhance Fatty Acid Oxidation and Improve High-Fat Diet–Induced Insulin Resistance. *Diabetes* 2009;58: 550-558.

27. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* 1992;35: 595-601.

28. Choi CS, Befroy DE, Codella R, Kim S, Reznick RM, Hwang Y-J, *et al.* Paradoxical effects of increased expression of PGC-1α on muscle mitochondrial function and insulin-stimulated muscle glucose metabolism. *Proceedings of the National Academy of Sciences* 2008;105: 19926-19931.

29. Hancock CR, Han D-H, Chen M, Terada S, Yasuda T, Wright DC, *et al.* High-fat diets cause insulin resistance despite an increase in muscle mitochondria. *Proceedings of the National Academy of Sciences* 2008;105: 7815-7820.

30. Turner N, Bruce CR, Beale SM, Hoehn KL, So T, Rolph MS, *et al.* Excess Lipid Availability Increases Mitochondrial Fatty Acid Oxidative Capacity in Muscle: Evidence Against a Role for Reduced Fatty Acid Oxidation in Lipid-Induced Insulin Resistance in Rodents. *Diabetes* 2007;56: 2085-2092.

31. Muoio Deborah M, Neufer PD. Lipid-Induced Mitochondrial Stress and Insulin Action in Muscle. *Cell Metabolism* 2012;15: 595-605.

32. Bassel-Duby R, Olson EN. Signaling Pathways in Skeletal Muscle Remodeling. *Annual Review of Biochemistry* 2006;75: 19-37.

33. Jørgensen SB, Richter EA, Wojtaszewski JFP. Role of AMPK in skeletal muscle metabolic regulation and adaptation in relation to exercise. *The Journal of Physiology* 2006;574: 17-31.

34. Rodgers JT, Lerin C, Gerhart-Hines Z, Puigserver P. Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways. *FEBS letters* 2008;582: 46-53.

35. Cantó C, Auwerx J. PGC-1[alpha], SIRT1 and AMPK, an energy sensing network that controls energy expenditure. *Current Opinion in Lipidology* 2009;20: 98-105 110.1097/MOL.1090b1013e328328d328320a328324.

36. Schuler M, Ali F, Chambon C, Duteil D, Bornert J-M, Tardivel A, *et al.* PGC1α expression is controlled in skeletal muscles by PPARβ, whose ablation results in fiber-type switching, obesity, and type 2 diabetes. *Cell Metabolism* 2006;4: 407-414.

37. Chowanadisai W, Bauerly KA, Tchaparian E, Wong A, Cortopassi GA, Rucker RB. Pyrroloquinoline Quinone Stimulates Mitochondrial Biogenesis through cAMP Response Element-binding Protein Phosphorylation and Increased PGC-1α Expression. *Journal of Biological Chemistry* 2010;285: 142-152.

38. Vega RB, Huss JM, Kelly DP. The Coactivator PGC-1 Cooperates with Peroxisome Proliferator-Activated Receptor α in Transcriptional Control of Nuclear Genes Encoding Mitochondrial Fatty Acid Oxidation Enzymes. *Molecular and Cellular Biology* 2000;20: 1868-1876.

39. Lin J, Wu H, Tarr PT, Zhang C-Y, Wu Z, Boss O, *et al.* Transcriptional co-activator PGC-1[alpha] drives the formation of slow-twitch muscle fibres. *Nature* 2002;418: 797-801.

40. de Lange P, Farina P, Moreno M, Ragni M, Lombardi A, Silvestri E, *et al.* Sequential changes in the signal transduction responses of skeletal muscle following food deprivation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2006;20: 2579-2581.

41. Blaak EE, van Aggel-Leijssen DP, Wagenmakers AJ, Saris WH, van Baak MA. Impaired oxidation of plasma-derived fatty acids in type 2 diabetic subjects during moderate-intensity exercise. *Diabetes* 2000;49: 2102-2107.

42. Thyfault JP, Rector RS, Noland RC. Metabolic inflexibility in skeletal muscle: a prelude to the cardiometabolic syndrome? *Journal of the cardiometabolic syndrome* 2006;1: 184-189.

Figure legends

Figure 1 Evolution of body weight (A) and body weight gain (B) of male rats born to dams fed a control (17% protein) or a low-protein (8% protein) diet through gestation and lactation. At weaning, all animals went to standard chow. Data in (B) correspond to the percentage in body weight gain between two recording periods (i.e. 30 and 45 days). Mean ± SEM. ***p<0.0001 vs. Control group (Student's t–test).

Figure 2 Metabolite determinations in plasma from adult rats born to dams fed a control (17% protein) or a low-protein (8% protein) diet through gestation and lactation. Animals were sacrificed under *ad libitum* feeding conditions within the first hour after the beginning of the light cycle or after a fast of 48 h. Data were analysed by two-way ANOVA using maternal diet (control or protein-restricted), and nutritional status at sacrifice (Ad libitum or fasted), as independent factors followed by Bonferroni's post-test. *p<0.05; **p< 0.01; ***p<0.001; $p^{§§§}$ compared to *ad libitum* fed control animals.

Figure 3 Gene expression changes in soleus from control and protein-restricted rats in response to fasting. Histograms illustrate the variations in gene expression in relation to those observed under *ad libitum* feeding conditions using the expression in fed animals for each one of the groups as calibrator. *p<0.05; **p< 0.01; ***p<0.001.

Figure 4 Gene expression changes in EDL from control and protein-restricted rats in response to fasting. Histograms illustrate the variations in gene expression in relation to those observed under *ad libitum* feeding conditions using the expression in fed animals for each one of the groups as calibrator. *p<0.05; **p< 0.01; ***p<0.001.

Figure 5 Changes in AMPK activity in response to fasting in soleus (A) and EDL (B) skeletal muscles in adult rats born to control or protein-restricted dams. Each bar corresponds to the ratio of the phosphorylated versus the non phosphorylated levels of AMPK determined by

Western Blot analysis. White bars correspond to data from animals sacrificed under *ad libitum* feeding conditions and grey bars to data from animals sacrificed after a fast of 48 h. *$p < 0.05$; **$p < 0.01$; $^{§}p < 0.05$ in relation to *ad libitum* fed control animals (two way ANOVA followed by Bonferroni's test).

Table 1. Sequences of primers used for the real time RT-PCR analysis.

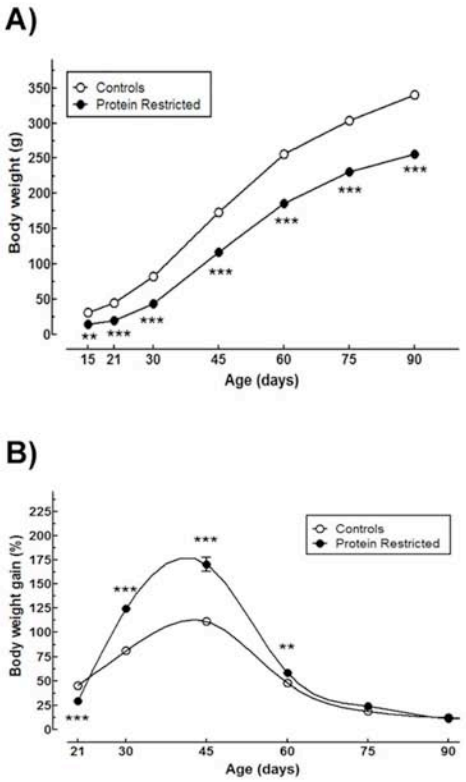
Gene	Symbol	Forward	Reverse	Gene Bank
Carnitine palmitoyltransferase 1a	CPT1a	TGCCTGCCAGTTCCATTAAGC	GTCTCACTCCTCTTGCCAACAG	NM_031559.2
Medium chain acyl coenzyme A dehydrogenase	MCAD	GAGGCTACAAGGTCCTGAGAAGTG	TCTGCTGCTCCGTCAACTCG	NM_016986.2
Hydroxyacyl-Coenzyme A dehydrogenase	β-HAD	CTCCATGTCCTCCTCTTCCTCTGC	CAGCCCGCCGCCGATGAC	NM_057186.1
Pyruvate Dehydrogenase Kinase, Isozyme 4	PDK4	GGTGGCGGTGTTCCTCTGAG	TGAATTGTCCATCACAGGCGTTG	NM_053551.1
Uncoupling protein 3	UCP3	CCGTTAAGCCTTCAGCCTTCC	CGAGAGTCCATCCTGTCCTTCC	NM_013167.2
Peroxisome proliferator-activated receptor alpha	PPARα	CACGATGCTGTCCTCCTTGATG	ATGATGTCGCAGAATGGCTTCC	NM_013196.1
Peroxisome proliferator-activated receptor delta	PPARδ	ACTCTCCTTTCTTCCTGCCTGTG	TGTGCTGCTGCTCCTTCTGG	NM_013141.2
Peroxisome proliferator-activated receptor gamma, co-activator 1alpha	PGC-1α	ACACCGCACACATCGCAATTC	TTCGTCCCTCTTGAGCCTTTCG	NM_031347.1
Myosin Heavy chain 7	MHCI	ACAGAGGAAGACAGGAAGAACCTAC	GGGCTTCACAGGCATCCTTAG	NM_017240.1
Protein kinase AMP-activated alpha 2	AMPKα2	GATGATGAGGTGGTGGAGCAGAC	CACTGTCTGGCTCTCTCACTGC	NM_023991.1
Ribosomal 18S	18S	GATGCGGCGGCGTTATTC	CTCCTGGTGGTGCCCTTCC	M11188.1

Table 2. Gene expression under *ad libitum* feeding conditions in skeletal muscle from adult offspring born to dams fed a control or a low protein diet during gestation and lactation.

Gene	Soleus		EDL	
	Controls	PR	Controls	PR
CPT1a	1.04± 0.14	2.36 ± 0.46*	1.16±0.34	0.94±0.13
MCAD	1.06±0.16	2.22±0.49*	1.08±0.23	0.37±0.09*
β-HAD	0.97±0.19	0.95±0.12	0.98±0.25	1.04±0.26
UCP3	1.10±0.21	2.10±0.30*	1.08±0.23	1.38±0.37
PDK4	1.00±0.05	0.82±0.04*	1.13±0.32	0.34±0.02*
PGC-1α	1.04±0.17	3.42±0.61*	1.27±0.45	2.24.72±0.51
PPARα	1.01±0.07	2.30±0.48*	1.10±0.26	2.05±0.11*
PPARδ	1.03±0.13	0.86±0.10	1.07±0.23	1.99±0.19*
AMPKα2	1.05±0.14	2.98±0.89*	1.06±0.22	0.49±0.04*
MHCI	0.91±0.18	1.10±0.18	1.04±0.15	0.99±0.29

Values correspond to gene expression levels calculated by the formula $2^{-\Delta\Delta CT}$ where C_T corresponds to the amplification cycle at which the fluorescence exceeds 10 times the standard deviation of the baseline.*$p < 0.05$ in relation to the expression in control animals.

Da Silva Aragão et al., Figure 1



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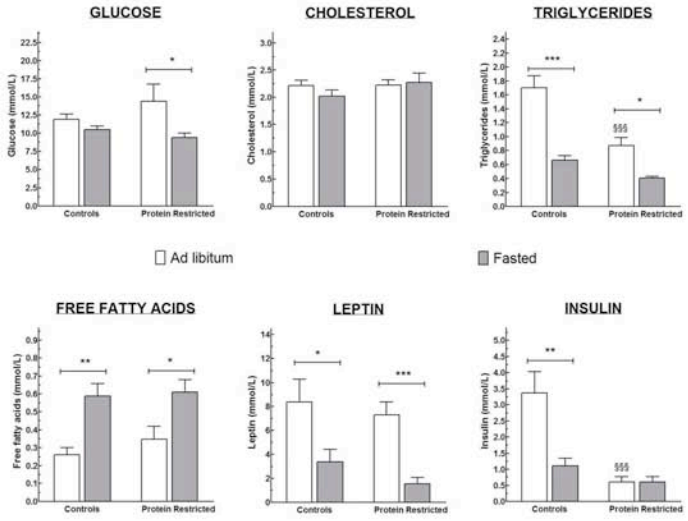
Da Silva Aragão et al., Figure 2



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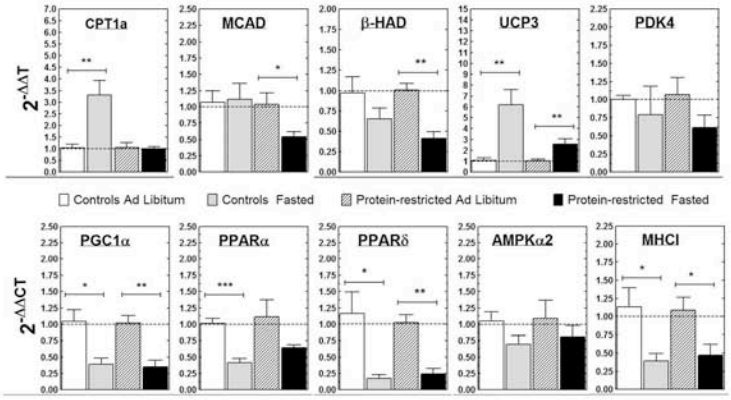
Da Silva Aragão et al., Figure 3



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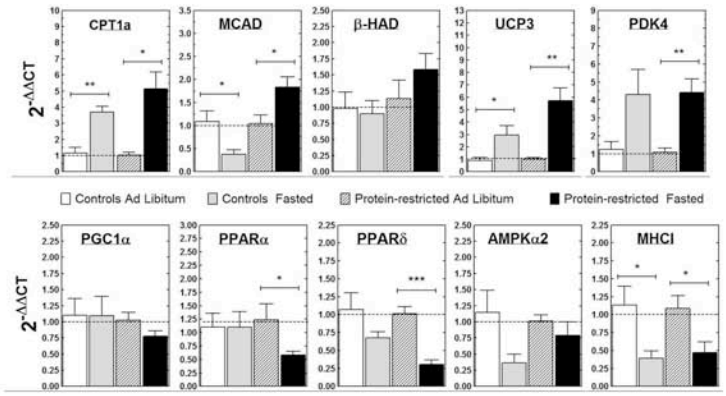
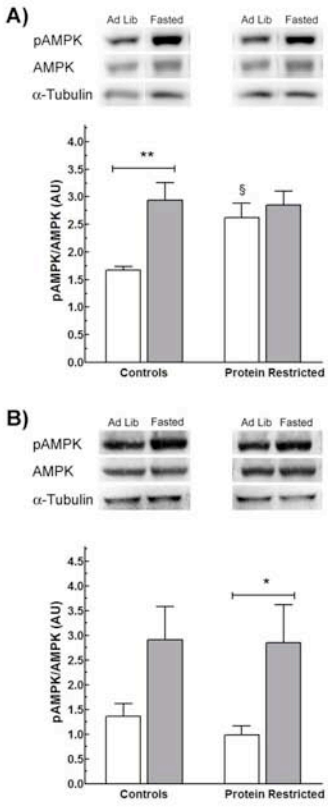
Da Silva Aragão et al., Figure 4



152x93mm (300 x 300 DPI)

Da Silva Aragão et al., Figure 5



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CONSIDERAÇÕES FINAIS

6 Considerações Finais

Desnutrição proteica materna durante a gestação e lactação já está estabelecida como um modelo experimental para induzir a programação metabólica (OZANNE *et al.*, 1996a; OZANNE *et al.*, 1996b; OZANNE *et al.*, 1998; OZANNE, 1999; OZANNE *et al.*, 2003a; OZANNE *et al.*, 2004; TOSCANO, MANHAES-DE-CASTRO e CANON, 2008; LEANDRO *et al.*, 2012). Em geral, considera-se que esta programação torna o organismo mais propenso a desenvolver doenças metabólicas na vida adulta (OZANNE *et al.*, 2003a).

O primeiro artigo desta tese teve como objetivo analisar os efeitos a longo prazo da desnutrição perinatal sobre as propriedades metabólicas e fenotípica do músculo esquelético. Dois músculos de diferentes características metabólicas e de composição de fibra foram estudados. No segundo artigo, foram estudados as alterações no metabolismo, da prole de mães desnutridas durante a gestação e lactação, face a um desafio energético, jejum por 48 horas.

A desnutrição proteica maternal perinatal induziu a redução do peso corporal da prole, mesmo após um período de recuperação nutricional, quando os animais são submetidos a uma ração controle após o desmame. Esta observação já é conhecida na literatura (TOSCANO, MANHAES-DE-CASTRO e CANON, 2008; LEANDRO *et al.*, 2012), mas parece ser dependente do tempo em que o animal foi submetido à desnutrição e também o tempo no qual a recuperação nutricional foi induzida (COUPE *et al.*, 2009; COUPE *et al.*, 2012).

Os animais que foram utilizados nos dois artigos ainda não apresentavam a síndrome metabólica no momento do sacrifício. Além disso, estes animais apresentam menor percentagem de massa gorda, redução nos triglicerídeos, e não eram intolerantes à glucose quando submetidos ao teste oral de tolerância à glicose. Ozanne e colegas (OZANNE *et al.*, 1996b) também observou que os filhotes de mães desnutridas apresentavam melhor resposta à glicose e sensibilidade à insulina quando jovens. No entanto, quando os animais estão com 15 meses de vida ele apresentam-se francamente diabéticos (PETRY *et al.*, 2001; OZANNE *et al.*, 2003a). No caso do nosso artigo, as alterações metabólicas e fenotípicas foram observadas mesmos sem indicador importante da síndrome metabólica (SM). Dessa forma, propomos que essas alterações antecedem ao aparecimento da SM, podendo ser utilizados como prováveis indicadores de maior predisposição à SM.

O fenótipo muscular foi modificado pela desnutrição materna perinatal. No músculo sóleo, foi observado aumento no número de fibras por áreas das fibras IIA e intermediárias, da mesma forma, observou-se aumento no número de fibras não-oxidativas. Tanner e colegas (2002) mostraram que existe uma associação entre o percentil do tipo de fibra e a obesidade. Os indivíduos obesos têm menor proporção de fibras do tipo I e maior proporção de fibras do tipo IIB, e há forte correlação positiva entre o IMC e a proporção de fibras IIB (TANNER *et al.*, 2002a). Redução percentual de fibras oxidativas caracterizadas pela succinato desidrogenase (SDH) foi observada em seres humanos obesos e com diabetes de tipo II (HE, WATKINS e KELLEY, 2001). Ademais ratos Zucker obesos e diabéticos também apresentam redução da proporção de fibras oxidativas (ADACHI *et al.*, 2007). Em pessoas obesas que foram submetidas a um programa de perda de peso, houve correlação entre perda de peso e aumento percentual no tipo de fibra I (TANNER *et al.*, 2002a). Em nosso estudo, pudemos observar diferenças entre os percentuais de fibra, mesmo em animais que ainda não apresentam SM. Dessa forma, observamos que um dos mecanismo pelo qual a desnutrição perinatal pode programar a prole, é pela alteração do fenótipo muscular.

Em animais, foi observado que a desnutrição materna reduz a densidade de fibras esqueléticas (BAYOL *et al.*, 2004; YATES *et al.*, 2012), além de redução nas fibras secundárias da prole ainda em período precoce (ZHU *et al.*, 2004; QUIGLEY *et al.*, 2005). Desta forma, a desnutrição materna pode afetar a proliferação e diferenciação muscular (BAYOL *et al.*, 2004; QUIGLEY *et al.*, 2005). Desnutrição proteica somente na gestação aumentou a percentagem de fibra tipo IIA no sóleo, e reduziu a percentagem de fibras tipo IIA e aumentou de tipos IIB no EDL, na prole de 3 meses (TOSCANO, MANHAES-DE-CASTRO e CANON, 2008). Porém, outro estudo com o músculo tibial anterior, não foram observadas alterações pela desnutrição (OZANNE *et al.*, 1996b). Essas diferenças entre os estudos dos efeitos da restrição de nutriente materno e a repercussão sobre o fenótipo muscular pode ser explicada pelo tipo de insulto aplicado, a duração do insulto e principalmente o tipo muscular estudado, visto que os diferentes grupos musculares podem ser diferentemente afetados por insultos externos. Neste trabalho observamos que o fenótipo muscular é diferencialmente afetado pela restrição proteica materna. Esta influência diferenciada também pode refletir na importância da desordem fisiológica que será observada. O músculo sóleo tem função postural, ou seja, está constantemente sendo exigido de seu trabalho na manutenção da postura tanto em bípedes quanto em quadrúpedes. Além de que os músculos oxidativos são os maiores responsáveis pela captação da glicose pelo sistema

muscular, sendo associados ao aparecimento da resistência à insulina (BASS *et al.*, 1969; HENRIKSEN *et al.*, 1990; KONG *et al.*, 1994; PATTI *et al.*, 2003).

Uma outra questão a discutir é que as mudanças observadas em nossos estudos foram mais fortemente presentes no músculo sóleo, rico em fibras oxidativas. Este músculo tem o seu desenvolvimento no início da miogênese período embrionário / fetal (BARBET *et al.*, 1991; BARBET, THORNELL e BUTLER-BROWNE, 1991; WIGMORE e DUNGLISON, 1998; MALTIN *et al.*, 2001; TE e REGGIANI, 2002). Segundo Bedi e colaboradores (1982) o período gestacional e lactacional é o mais importante para o desenvolvimento muscular.

Portanto, essas duas questões abordadas nos levam a crer que o sóleo é mais susceptível à programação metabólica pela desnutrição durante o período perinatal e, também mais propenso alterações ao longo da vida, pois ele apresenta um maior nível de atividade do que a EDL (RABOL *et al.*, 2010; SCHIAFFINO e REGGIANI, 2011). Entretanto, esses efeitos são dependentes da natureza do insulto, da extensão e do tempo ao qual foi induzido, podendo afetar o desenvolvimento de fibras primária e secundárias ou somente uma dessas gerações (MALTIN *et al.*, 2001).

A atividade enzimática de três enzimas chaves de diferentes vias metabólica também foi estudada, são elas HK, β-HAD e CS. A HK , que é uma enzima chave para a glicólise, apresentou sua atividade reduzida no músculo sóleo dos animais do grupo restrito em proteína. No entanto, não foi observada alteração na atividade de β-HAD ou CS. Da mesma forma, não houve diminuição na expressão de HKII. Observou-se também que a relação de HK/CS e β-HAD/CS também estavam reduzidas, indicando que houve tanto diminuição da capacidade glicolítica, como redução da oxidação de ácidos graxos.

Em concordância com os nossos achados, pacientes obesos e diabéticos submetidos a doses suprafisiológicas de insulina apresentam expressão e atividade reduzida da HKII (PENDERGRASS *et al.*, 1998). É importante ressaltar que estes animais foram sacrificados num estado alimentado, o que indicaria um maior circulação de insulina e maior captação da glicose. Entretanto, o que se observou foi a redução da atividade da HK nos animais do grupo desnutrido, indicando que eles podem já apresentar problemas para a manutenção da glicose no citoplasma e sua entrada na via glicolítica. Além de serem metabolicamente menos flexíveis entre a alternância da utilização de ácidos graxos ou glicose como fonte energética (STORLIEN, OAKES e KELLEY, 2004; CORPELEIJN, SARIS e BLAAK, 2009), segundo

os pressupostos do ciclo de Randle (RANDLE *et al.*, 1963). Esta diminuição na fosforilação da glicose devida à presença de AG foi comprovada pela diminuição na quantidade de G-6-P e da formação de glicogênio na célula durante infusão continua de AG (RODEN *et al.*, 1996; RODEN, 2004).

Interessantemente, outro estudo de nosso grupo verificou que estes animais, mesmo em estado alimentado apresentavam menor nível de ativação de Akt (Guzman-Quevedo, em preparação). Indicando uma redução na via de cascata de sinalização de insulina, o que pode também a ajudar a explicar o fato de termos observado redução da atividade de HK, porém sem redução da expressão dos genes da HKII e do Glut4.

Também foram analisados, no primeiro artigo, a expressão de vários genes relacionados ao metabolismo de glicose e de ácidos graxos, ciclo de Krebs, fenotipagem muscular, fatores de transcrição e sensores nutricionais. Da mesma forma que nas primeiras análises, o músculo sóleo de animais do grupo desnutrido apresentaram maiores alterações quando comparados com o grupo controle do que o músculo EDL.

Não houve diferenças na expressão dos genes estudados que estavam envolvidos com o metabolismo de glicose, nem fenotipagem, nem sensores nutricionais, no sóleo. As alterações foram observadas em relação aos genes que de alguma forma estão envolvidos no metabolismo de ácidos graxos. Observou-se aumento de na expressão de CPT1, que está envolvido na entrada, principalmente, dos ácidos graxos de cadeia longa (LCFA, do inglês Long Chain Fatty Acids) (BONNEFONT *et al.*, 2004). Por si só, este resultado nos levaria a crer que há aumento da β-oxidação, porém, como foi observado anteriormente, a atividade da β-HAD não foi alterada. Nem tão pouco foram alteradas as expressões de MCAD e β-HAD.

Entretanto, foi observado aumento a expressão de UCP3. Esta proteína apresenta várias funções, além da termogênese, atuando como desacopladora da produção de ATP, ela também atua reduzindo a produção de ROS, expulsando da mitocôndria ânions de AG que possam vim facilmente a serem peroxidados levando à dano celular (SCHRAUWEN *et al.*, 2010; VAN DEN BERG *et al.*, 2011). Estas ações da UCP3 tendem a aumentar a capacidade oxidativa da célula evitando o dano pelo excesso de AG na mitocôndria, por isso que essa proteína é mais expressa em fibras do tipo II e em indivíduos sedentários, pois estes tem naturalmente menor capacidade oxidativa (RUSSELL *et al.*, 2003a; RUSSELL *et al.*, 2003b).

Analisando os dados em conjunto, podemos observar que a desnutrição proteica perinatal induz aumento da entrada de AG na mitocôndria, porém sem aumentar a capacidade oxidativa das células musculares, o que leva, provavelmente, ao acúmulo de AG na matriz mitocondrial, com concomitante redução da atividade de glicólise, e como mecanismo de defesa, aumento da expressão de UCP3. Porém, está proteção mitocondrial promovida pela UCP3 pode apresentar um limite de atuação, visto que foram observadas que disfunções mitocondriais podem estar associada à obesidade e resistência à insulina (BOUDINA *et al.*, 2012). Ademais tem sido proposto que a disfunção mitocondrial possa estar relacionada ao início ou ao desenvolvimento da SM (PETERSEN e SHULMAN, 2002).

Corroborando com nossos achados, prole que foi programada para ser resistente à insulina apresenta desregulação do metabolismo de AG, possivelmente devido a defeito na atividade de fosforilação oxidativa (BEFROY *et al.*, 2007). Embora outros estudos tenham mostrado que resistência à insulina pode acontecer sem disfunção mitocondrial (BONNARD *et al.*, 2008; DE FEYTER *et al.*, 2008). Foi proposto que a disfunção mitocondrial pode aumentar os derivados de AG e ROS, sendo que os dois podem prejudicar a sinalização à insulina (MARTINS *et al.*, 2012). Uma revisão da literatura foi observado que, na maioria dos estudos, animais obesos apresentam aumento da β -oxidação, mas esta se dá de forma incompleta aumentando a possibilidade de prejudicar a sensibilidade à insulina (ZHANG *et al.*, 2010).

Interessantemente, nós observamos aumento na expressão de PGC1α, um conhecido co-ativador transcricional de vários receptores nucleares e de fatores de transcrição (KNUTTI e KRALLI, 2001) e também de com influência em diversas funções em relação ao metabolismo energético, incluindo indução de biogênese mitocondrial, regulação do metabolismo de glicose e oxidação de ácidos graxos (FINCK e KELLY, 2006).

A superexpressão muscular de PGC1α resulta em aumento de fibras tipo I nos músculos vasto lateral e plantar, além de maior resistência à fadiga e maior expressão de proteínas envolvidas na oxidação fosforilativa (LIN *et al.*, 2002b; MIURA *et al.*, 2003; MIURA *et al.*, 2007), além de aumentar a sensibilidade e a sinalização de insulina em animais obesos (BENTON *et al.*, 2006; BENTON *et al.*, 2010). Entretanto, entretanto a sobre-expressão em animais normais diminui a expressão de Glut4 e prejudica o controle glicêmico após teste de resistência à insulina (MIURA *et al.*, 2003).

Ratos que que apresentaram retardo no crescimento uterino apresentam alteração diferenciadas no níveis de PGC1α, observando-se redução no sóleo (LANE *et al.*, 2003) e aumento no EDL (LANE *et al.*, 2003) e no quadríceps (MUHLHAUSLER *et al.*, 2009). Contudo, convém esclarecer que esses resultados foram observados ainda na prole em desenvolvimento (3ª semana pós-natal) (LANE *et al.*, 2003; MUHLHAUSLER *et al.*, 2009). Em nosso estudo, o aumento da expressão de PGC1α foi acompanhado do aumento da expressão de alguns de seus genes-alvos CPT1 e UCP3. Ademais, esses animais também apresentaram redução nos níveis de acetilação (Guzman-Quevedo, em preparação). A desacetilação de PGC1α é promovida pela Sirt1, tanto *in vitro* e *in vivo* (NEMOTO, FERGUSSON e FINKEL, 2005), além de ser necessário para a desacetilação de PGC1α durante o jejum prologado, aumentando a sua atividade (GERHART-HINES *et al.*, 2007; RODGERS *et al.*, 2008a).

PGC1α também pode ser regulado por fosforilação, mas ao contrário da acetilação, quando fosforilado PGC1α tende a aumentar sua atividade e sua estabilidade (FERNANDEZ-MARCOS e AUWERX, 2011). AMPK, MAPK p38, e Akt são proteínas quinases que podem fosforilar PGC1α, entretanto a fosforilação por Akt tem o efeito inverso na atividade de PGC1α, reduzindo-a (FERNANDEZ-MARCOS e AUWERX, 2011). Trabalho de nosso grupo observou que a atividade de AMPK está aumentada no músculo sóleo de animais cujas mães foram restritas em proteínas durante o período perinatal (Guzman-Quevedo, submetido). Este achado além de corroborar com os dados de menor taxa de oxidação de glicose e AG, ainda suporta os achados de maior expressão de PGC1α. Visto que AMPK é capaz de induzir aumento na transcrição e na atividade de PGC1α (JAGER *et al.*, 2007). Corroborando com estes achados, nós também observamos redução na atividade de Akt, que é conhecida por inibir a atividade de PGC1α (LI *et al.*, 2007).

Tomados em conjunto, os resultados do primeiro artigo aqui apresentado e os resultados de outros artigos produzidos pelo grupo, indicam que o músculo sóleo de animais submetidos à restrição proteica durante a gestação e lactação apresenta-se em um estado de baixa utilização de energia, tendo em vista que esses animais foram sacrificados em estado alimentados. Observamos, redução da atividade de HK e da taxa HK/CS, e aumento na expressão de genes relacionados ao metabolismo de lipídios. Entretanto, nesta última observação, não podemos sugerir que este lipídio esteja sendo utilizado até sua completa oxidação, visto que não houve alteração da atividade da β-HAD, nem da taxa β-HAD/CS,

nem alteração na expressão de genes do metabolismo intermediário de AG. Ademais, nossos outros trabalhos corroboram com os resultados ao passo que foi observado aumento da atividade de AMPK (Guzman-Quevedo, submetido) e redução da atividade de Akt (Guzman-Quevedo, em preparação).

Resistência à insulina e diabetes tipo 2 tem sido associada com habilidade prejudicada de apresentar mudança entre o metabolismo de lipídios e de glicose (THYFAULT, RECTOR e NOLAND, 2006). Objetivando estudar se a desnutrição proteica materna perinatal seria capaz de alterar a plasticidade metabólica muscular, a prole foi submetida a um desafio energético com restrição alimentar por 48 horas. Foram comparados os animais controles e desnutridos submetidos ao jejum com os seus respectivos pares sacrificados em condição alimentada. Estudou-se o perfil plasmático e o perfil de expressão de genes relacionados ao metabolismo de AG, ciclo de Krebs, fenótipo muscular e sensores nutricionais. Além da expressão proteica de PGC1α e nível de ativação de AMPK.

Como esperado os resultados divergiram entre os dois tipos musculares estudados. Mais uma vez mostrando que o músculo sóleo (mais oxidativo) é mais afetado pela desnutrição materna perinatal. Além disso, o grupo desnutrido apresentou resposta prejudicada ao jejum quando comparados ao grupo controle.

Inicialmente observamos que os animais desnutridos não apresentaram aumento nos AGL após 48 horas de privação alimentar. É sabido que durante o jejum prolongado, os AG tornam-se a principal fonte de energia para o músculo (DE LANGE *et al.*, 2006). Aumento da utilização de lipídios como fonte de combustível muscular durante o jejum exige aumento na expressão dos genes envolvidos no metabolismo de AG, na fosforilação oxidativa e biogênese mitocondrial (WANG *et al.*, 2004b; DE LANGE *et al.*, 2007; RODGERS *et al.*, 2008a). A inabilidade em aumentar os nível plasmáticos de AGL, mesmo tendo apresentado uma maior percentagem de perda de peso, pode prejudicar a resposta ao jejum, visto que os AGL agem como sinalizadores do estado energético e assim induzir as modificações necessárias para a manutenção do suprimento de energia (DE LANGE *et al.*, 2006; DE LANGE *et al.*, 2007).

Privação alimentar por 2 dias é capaz de induzir alterações na expressão de 700 genes (HAKVOORT *et al.*, 2011). Em nosso estudo, no músculo sóleo, o jejum induziu aumento da expressão de CPT1a e UCP3 , além de redução na expressão de PGC1α, PPARα, PPARδ e MyHC I, nos animais do grupo controle. O aumento na expressão de CPT1 é importante pois

possibilita os AG a serem transportados para a mitocôndria e serem completamente oxidados (DE LANGE *et al.*, 2006; FRIER, JACOBS e WRIGHT, 2011), sendo um das respostas esperada após 48h de jejum (SAMEC *et al.*, 2002; DE LANGE *et al.*, 2006). Entretanto o grupo desnutrido não apresentou alteração na expressão de CPT1, além de ter apresentado menor expressão de β-HAD. Isto pode indicar que este animais já consumiram a maior da sua fonte de AG e já estariam utilizando outras fontes energéticas, como corpos cetônicos ou aminoácidos (CAHILL, 2006). Entretanto, estes animais já tem a expressão de CPT1 aumentada nos níveis basais, indicando que talvez haja um limite para aumentar a sua expressão no músculo.

O aumento da expressão de UCP3 é um achado comum em resposta ao aumento oxidação de AG (SAMEC *et al.*, 2002; TUNSTALL *et al.*, 2002; THOMPSON e KIM, 2004; ABE *et al.*, 2006; DE LANGE *et al.*, 2006). Este aumento tem por fim evitar a lipotoxicidade causada pelo aumento da disponibilidade de AG (ABE *et al.*, 2006; SCHRAUWEN *et al.*, 2010). Seja por expulsar os ânions de AG da mitocôndria (HIMMS-HAGEN e HARPER, 2001) ou por diminuir a produção de ERO (BEVILACQUA *et al.*, 2004; ABE *et al.*, 2006). Apesar de não ter apresentado aumento dos AGL, os animais do grupo desnutrido ao menos aumentaram a expressão de UCP3, o que pode indicar que os mecanismos de defesa celular ainda estejam atuantes, apesar do nível de expressão de UCP3 já está aumentado nesses animais no estado basal.

Foi observado que a expressão de PGC1α encontrava-se reduzida em ambos o grupo. Este achado corrobora com outros dados da literatura que mostram que PGC1α é um dos primeiros genes a aumentar sua expressão, parecendo orquestrar a expressão de outros RNA (DE LANGE *et al.*, 2006). Entretanto durante o jejum prolongado sua expressão volta aos níveis basais ou está reduzida (DE LANGE *et al.*, 2006). Corroborando com esse achado, a expressão de PPARδ e MyHCI também foi reduzida com o jejum. Ademais, a expressão de PPARα também esteve reduzida no grupo controle. Sabe-se que há uma sobreposição entre as funções dos PPAR α e δ em relação ao metabolismo lipídico (MUOIO *et al.*, 2002a).

No grupo controle, no EDL, observamos apenas aumento na expressão de CPT1a e redução de MCAD. Apesar de parecer contraditório, convém lembrar que este é um músculo de ativação esporádica, o que pode indicar que sua participação na resposta metabólica geral seja menor que um músculo de ativação tônica, como o sóleo. Nos animais desnutridos, também houve aumento da expressão de CPT1a, além de aumento na expressão de UCP3 e

MCAD, indicando que este músculo pode estar tentando compensar o prejuízo da inflexibilidade metabólica do músculo sóleo em resposta ao jejum.

Considerando os achados dos segundo artigo, podemos inferir que a prole de mães submetidas à desnutrição proteica durante a gestação e lactação apresenta redução da sua capacidade de responder à uma demanda energética, como a privação alimentar. Entretanto, esta alteração é dependente do tipo muscular estudado.

Como conclusões gerais da tese, podemos afirmar que a desnutrição proteica materna induz alterações no metabolismo de AG da prole, o que pode a longo prazo levar à danos celulares que vão propiciar o desenvolvimento da SM. Além disso, os insultos perinatais reduzem a flexibilidade metabólica muscular. Ademais de ambos os resultados serem dependentes do tipo de fibra muscular.

PERSPECTIVAS

7 Perspectivas

- Estudos de alterações epigenéticas induzidas pela desnutrição materna no músculo esquelético;

- Estudos em culturas primárias de células satélites musculares, obtidas de animais cujas mães foram desnutridas no período perinatal, do perfil metabólico;

- Estudos *in vitro* com manipulação de insulina, lipídios, agonistas de enzimas ou moléculas que têm influência sobre o metabolismo para avaliar a resposta dos músculos isolados obtidos a partir de animais de mães desnutridas durante a gestação e lactação .

REFERÊNCIAS BIBLIOGRÁFICAS

8 Referências Bibliográficas / Référence bibliographique

AAGAARD-TILLERY, K. M. et al. Developmental origins of disease and determinants of chromatin structure: maternal diet modifies the primate fetal epigenome. **J Mol Endocrinol,** v. 41, n. 2, p. 91-102, Aug 2008.

ABE, T. et al. Possible role of avian uncoupling protein in down-regulating mitochondrial superoxide production in skeletal muscle of fasted chickens. **FEBS letters,** v. 580, n. 20, p. 4815-4822, Sep 4 2006.

ABERNETHY, P. J.; THAYER, R.; TAYLOR, A. W. Acute and chronic responses of skeletal muscle to endurance and sprint exercise. A review. **Sports medicine,** v. 10, n. 6, p. 365-389, Dec 1990.

ADACHI, T. et al. Fibre type distribution and gene expression levels of both succinate dehydrogenase and peroxisome proliferator-activated receptor-gamma coactivator-1alpha of fibres in the soleus muscle of Zucker diabetic fatty rats. **Experimental physiology,** v. 92, n. 2, p. 449-455, Mar 2007.

AGBULUT, O. et al. Myosin heavy chain isoforms in postnatal muscle development of mice. **Biology of the cell / under the auspices of the European Cell Biology Organization,** v. 95, n. 6, p. 399-406, Sep 2003.

ALBALA, C. et al. Nutrition transition in Latin America: the case of Chile. **Nutrition reviews,** v. 59, n. 6, p. 170-176, Jun 2001.

ALBERTI, K. G.; ZIMMET, P.; SHAW, J. International Diabetes Federation: a consensus on Type 2 diabetes prevention. **Diabetic medicine : a journal of the British Diabetic Association,** v. 24, n. 5, p. 451-463, May 2007.

ALBERTI, K. G.; ZIMMET, P. Z. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. **Diabetic medicine : a journal of the British Diabetic Association,** v. 15, n. 7, p. 539-553, Jul 1998.

ALLEN, D. L. et al. Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse. **Journal of applied physiology,** v. 90, n. 5, p. 1900-1908, May 2001.

ALMOND, K. et al. The influence of maternal protein nutrition on offspring development and metabolism: the role of glucocorticoids. **Proceedings of the Nutrition Society,** v. 71, n. 01, p. 198-203, 2012.

ARMITAGE, J. A. et al. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? **The Journal of physiology,** v. 561, n. Pt 2, p. 355-377, Dec 1 2004.

BAAR, K. Epigenetic control of skeletal muscle fibre type. **Acta physiologica,** v. 199, n. 4, p. 477-487, Aug 2010.

BAAR, K. et al. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology,** v. 16, n. 14, p. 1879-1886, Dec 2002.

BAIRD, J. et al. Testing the fetal origins hypothesis in twins: the Birmingham twin study. **Diabetologia,** v. 44, n. 1, p. 33-39, Jan 2001.

BALKAU, B. et al. Frequency of the WHO metabolic syndrome in European cohorts, and an alternative definition of an insulin resistance syndrome. **Diabetes & metabolism,** v. 28, n. 5, p. 364-376, Nov 2002a.

BARBET, J. P. et al. [Quantification of the diameter of muscular fibres in the course of the development of the quadriceps]. **Bulletin de l'Association des anatomistes,** v. 75, n. 230, p. 25-29, Sep 1991.

BARBET, J. P.; THORNELL, L. E.; BUTLER-BROWNE, G. S. Immunocytochemical characterisation of two generations of fibers during the development of the human quadriceps muscle. **Mechanisms of development,** v. 35, n. 1, p. 3-11, Aug 1991.

BARKER, D. J. The developmental origins of adult disease. **European journal of epidemiology,** v. 18, n. 8, p. 733-736, 2003.

BARKER, D. J. et al. Weight in infancy and death from ischaemic heart disease. **Lancet,** v. 2, n. 8663, p. 577-580, Sep 9 1989.

BARRES, R.; ZIERATH, J. R. DNA methylation in metabolic disorders. **The American journal of clinical nutrition,** v. 93, n. 4, p. 897S-900, Apr 2011.

BARSH, G. S.; FAROOQI, I. S.; O'RAHILLY, S. Genetics of body-weight regulation. **Nature,** v. 404, n. 6778, p. 644-651, 2000.

BASS, A. et al. Metabolic differentiation of distinct muscle types at the level of enzymatic organization. **European journal of biochemistry / FEBS,** v. 10, n. 2, p. 198-206, Sep 1969.

BASSEL-DUBY, R.; OLSON, E. N. Signaling Pathways in Skeletal Muscle Remodeling. **Annual Review of Biochemistry,** v. 75, n. 1, p. 19-37, 2006a.

BATESON, P. et al. Developmental plasticity and human health. **Nature,** v. 430, n. 6998, p. 419-421, Jul 22 2004.

BATESON, P.; GLUCKMAN, P. Plasticity and robustness in development and evolution. **International journal of epidemiology,** v. 41, n. 1, p. 219-223, Feb 2012.

BATISTA FILHO, M.; RISSIN, A. [Nutritional transition in Brazil: geographic and temporal trends]. **Cadernos de saude publica / Ministerio da Saude, Fundacao Oswaldo Cruz, Escola Nacional de Saude Publica,** v. 19 Suppl 1, p. S181-191, 2003.

BAYOL, S. et al. The influence of undernutrition during gestation on skeletal muscle cellularity and on the expression of genes that control muscle growth. **The British journal of nutrition,** v. 91, n. 3, p. 331-339, Mar 2004.

BAYOL, S. P. A.; SIMBI, B. H.; STICKLAND, N. C. A maternal cafeteria diet during gestation and lactation promotes adiposity and impairs skeletal muscle development and metabolism in rat offspring at weaning. **The Journal of Physiology,** v. 567, n. 3, p. 951-961, September 15, 2005 2005.

BEAUCHAMP, J. R. et al. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. **The Journal of cell biology,** v. 151, n. 6, p. 1221-1234, Dec 11 2000.

BEDI, K. S. et al. Early life undernutrition in rats. 1. Quantitative histology of skeletal muscles from underfed young and refed adult animals. **The British journal of nutrition,** v. 47, n. 3, p. 417-431, May 1982.

BEFROY, D. E. et al. Impaired mitochondrial substrate oxidation in muscle of insulin-resistant offspring of type 2 diabetic patients. **Diabetes,** v. 56, n. 5, p. 1376-1381, May 2007.

BELLINGER, L.; LANGLEY-EVANS, S. C. Fetal programming of appetite by exposure to a maternal low-protein diet in the rat. **Clinical science,** v. 109, n. 4, p. 413-420, Oct 2005.

BENTON, C. R. et al. Inverse relationship between PGC-1alpha protein expression and triacylglycerol accumulation in rodent skeletal muscle. **Journal of applied physiology,** v. 100, n. 2, p. 377-383, Feb 2006.

BENTON, C. R. et al. Increased levels of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) improve lipid utilisation, insulin signalling and glucose transport in skeletal muscle of lean and insulin-resistant obese Zucker rats. **Diabetologia,** v. 53, n. 9, p. 2008-2019, 2010.

BERCHTOLD, M. W.; BRINKMEIER, H.; MUNTENER, M. Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. **Physiological reviews,** v. 80, n. 3, p. 1215-1265, Jul 2000.

BERLEZE, K. J. et al. Gestational and postnatal low protein diet alters insulin sensitivity in female rats. **Experimental biology and medicine,** v. 234, n. 12, p. 1437-1444, Dec 2009.

BERTRAM, C. E.; HANSON, M. A. Animal models and programming of the metabolic syndrome. **Br Med Bull,** v. 60, p. 103-121, 2001a.

BEVILACQUA, L. et al. Effects of short- and medium-term calorie restriction on muscle mitochondrial proton leak and reactive oxygen species production. **American journal of physiology. Endocrinology and metabolism,** v. 286, n. 5, p. E852-861, May 2004.

BIKMAN, B. T. et al. Lipid-induced insulin resistance is prevented in lean and obese myotubes by AICAR treatment. **American journal of physiology. Regulatory, integrative and comparative physiology,** v. 298, n. 6, p. R1692-1699, Jun 2010.

BIRD, A. Perceptions of epigenetics. **Nature,** v. 447, n. 7143, p. 396-398, May 24 2007.

BIRESSI, S.; MOLINARO, M.; COSSU, G. Cellular heterogeneity during vertebrate skeletal muscle development. **Developmental biology,** v. 308, n. 2, p. 281-293, Aug 15 2007.

BJORNHOLM, M. et al. Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation. **Diabetes,** v. 46, n. 3, p. 524-527, Mar 1997.

BLAAK, E. E. et al. Impaired oxidation of plasma-derived fatty acids in type 2 diabetic subjects during moderate-intensity exercise. **Diabetes,** v. 49, n. 12, p. 2102-2107, Dec 2000.

BO, S. et al. The metabolic syndrome in twins: a consequence of low birth weight or of being a twin? **Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association,** v. 109, n. 3, p. 135-140, 2001.

BODEN, G. Obesity, insulin resistance and free fatty acids. **Current opinion in endocrinology, diabetes, and obesity,** v. 18, n. 2, p. 139-143, Apr 2011.

BONNARD, C. et al. Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. **The Journal of clinical investigation,** v. 118, n. 2, p. 789-800, Feb 2008.

BONNEFONT, J. P. et al. Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. **Molecular aspects of medicine,** v. 25, n. 5-6, p. 495-520, Oct-Dec 2004.

BONOMO, I. T. et al. Prolactin inhibition in dams during lactation programs for overweight and leptin resistance in adult offspring. **The Journal of endocrinology,** v. 192, n. 2, p. 339-344, Feb 2007.

BONOW, R. O. Primary prevention of cardiovascular disease: a call to action. **Circulation,** v. 106, n. 25, p. 3140-3141, Dec 17 2002.

BORRELLI, E. et al. Decoding the epigenetic language of neuronal plasticity. **Neuron,** v. 60, n. 6, p. 961-974, Dec 26 2008.

BOUDINA, S. et al. Early mitochondrial adaptations in skeletal muscle to diet-induced obesity are strain dependent and determine oxidative stress and energy expenditure but not insulin sensitivity. **Endocrinology,** v. 153, n. 6, p. 2677-2688, Jun 2012.

BRAMELD, J. M. The influence of undernutrition on skeletal muscle development. **The British journal of nutrition,** v. 91, n. 3, p. 327-328, Mar 2004.

BRANDÃO, A. P. et al. I Diretriz brasileira de diagnóstico e tratamento da síndrome metabólica. . **Arquivos brasileiros de cardiologia,** v. 84, n. suppl 1, p. 3-28, 2005.

BRENNER, B. M.; CHERTOW, G. M. Congenital oligonephropathy: an inborn cause of adult hypertension and progressive renal injury? **Current opinion in nephrology and hypertension,** v. 2, n. 5, p. 691-695, Sep 1993.

BRETON, C. et al. Maternal prenatal undernutrition alters the response of POMC neurons to energy status variation in adult male rat offspring. **American journal of physiology. Endocrinology and metabolism,** v. 296, n. 3, p. E462-472, Mar 2009.

BRONS, C. et al. Effects of high-fat overfeeding on mitochondrial function, glucose and fat metabolism, and adipokine levels in low-birth-weight subjects. **American journal of physiology. Endocrinology and metabolism,** v. 302, n. 1, p. E43-51, Jan 1 2012.

BRONS, C. et al. Deoxyribonucleic acid methylation and gene expression of PPARGC1A in human muscle is influenced by high-fat overfeeding in a birth-weight-dependent manner. **The Journal of clinical endocrinology and metabolism,** v. 95, n. 6, p. 3048-3056, Jun 2010.

BROOKE, M. H.; KAISER, K. K. Three "myosin adenosine triphosphatase" systems: the nature of their pH lability and sulfhydryl dependence. **The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society,** v. 18, n. 9, p. 670-672, Sep 1970.

BRUNMAIR, B. et al. Activation of PPAR-δ in isolated rat skeletal muscle switches fuel preference from glucose to fatty acids. **Diabetologia,** v. 49, n. 11, p. 2713-2722, 2006.

BUCKINGHAM, M. Skeletal muscle formation in vertebrates. **Current opinion in genetics & development,** v. 11, n. 4, p. 440-448, Aug 2001.

_____. Skeletal muscle progenitor cells and the role of Pax genes. **Comptes rendus biologies,** v. 330, n. 6-7, p. 530-533, Jun-Jul 2007.

BUCKINGHAM, M.; MONTARRAS, D. Skeletal muscle stem cells. **Current opinion in genetics & development,** v. 18, n. 4, p. 330-336, Aug 2008.

BUCKLEY, A. J. et al. Altered body composition and metabolism in the male offspring of high fat-fed rats. **Metabolism: clinical and experimental,** v. 54, n. 4, p. 500-507, Apr 2005.

BULLER, A. J.; ECCLES, J. C.; ECCLES, R. M. Interactions between motoneurones and muscles in respect of the characteristic speeds of their responses. **The Journal of physiology,** v. 150, p. 417-439, Feb 1960.

BURDGE, G. et al. Dietary protein restriction of pregnant rats in the F0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F1 and F2 generations. **Br J Nutr.,** v. 97, n. 3, p. 435-439, 2007

BURDGE, G. C. et al. Effect of reduced maternal protein consumption during pregnancy in the rat on plasma lipid concentrations and expression of peroxisomal proliferator–activated receptors in the liver and adipose tissue of the offspring. **Nutrition Research,** v. 24, n. 8, p. 639-646, 2004.

BURDGE, G. C. et al. Dietary protein restriction of pregnant rats in the F0 generation induces altered methylation of hepatic gene promoters in the adult male offspring in the F1 and F2 generations. **The British journal of nutrition,** v. 97, n. 3, p. 435-439, Mar 2007.

BURNS, S. P. et al. Gluconeogenesis, glucose handling, and structural changes in livers of the adult offspring of rats partially deprived of protein during pregnancy and lactation. **The Journal of clinical investigation,** v. 100, n. 7, p. 1768-1774, Oct 1 1997.

BUSCHER, D.; IZPISUA BELMONTE, J. C. Muscle development during vertebrate limb outgrowth. **Cell and tissue research,** v. 296, n. 1, p. 131-139, Apr 1999.

CAHILL, G. F., JR. Fuel metabolism in starvation. **Annual review of nutrition,** v. 26, p. 1-22, 2006.

CAMM, E. J. et al. Prenatal hypoxia independent of undernutrition promotes molecular markers of insulin resistance in adult offspring. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology,** v. 25, n. 1, p. 420-427, Jan 2011.

CANTO, C.; AUWERX, J. PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. **Current opinion in lipidology,** v. 20, n. 2, p. 98-105, Apr 2009.

CARONE, B. R. et al. Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. **Cell,** v. 143, n. 7, p. 1084-1096, Dec 23 2010.

CHAKKALAKAL, J. V. et al. Stimulation of calcineurin signaling attenuates the dystrophic pathology in mdx mice. **Human molecular genetics,** v. 13, n. 4, p. 379-388, Feb 15 2004.

CHAKRAVARTHY, M. V.; BOOTH, F. W. Eating, exercise, and "thrifty" genotypes: connecting the dots toward an evolutionary understanding of modern chronic diseases. **J Appl Physiol,** v. 96, n. 1, p. 3-10, January 1, 2004 2004.

CHAMSON-REIG, A. et al. Exposure of the pregnant rat to low protein diet causes impaired glucose homeostasis in the young adult offspring by different mechanisms in males and females. **Experimental biology and medicine,** v. 234, n. 12, p. 1425-1436, Dec 2009.

CHEMELLO, F. et al. Microgenomic analysis in skeletal muscle: expression signatures of individual fast and slow myofibers. **PloS one,** v. 6, n. 2, p. e16807, 2011.

CHEN, J. H. et al. Maternal protein restriction affects postnatal growth and the expression of key proteins involved in lifespan regulation in mice. **PloS one,** v. 4, n. 3, p. e4950, 2009.

CHERIF, H. et al. A protein-restricted diet during pregnancy alters in vitro insulin secretion from islets of fetal Wistar rats. **The Journal of nutrition,** v. 131, n. 5, p. 1555-1559, May 2001.

CHONG, S.; WHITELAW, E. Epigenetic germline inheritance. **Current opinion in genetics & development,** v. 14, n. 6, p. 692-696, Dec 2004a.

CHOWANADISAI, W. et al. Pyrroloquinoline quinone stimulates mitochondrial biogenesis through cAMP response element-binding protein phosphorylation and increased PGC-1alpha expression. **The Journal of biological chemistry,** v. 285, n. 1, p. 142-152, Jan 1 2010.

CINTRA IDE, P. et al. Evolution of body mass index in two historical series of adolescents. **Jornal de pediatria,** v. 83, n. 2, p. 157-162, Mar-Apr 2007.

CIVITARESE, A. E. et al. Role of adiponectin in human skeletal muscle bioenergetics. **Cell metabolism,** v. 4, n. 1, p. 75-87, Jul 2006.

CLARET, M. et al. AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. **The Journal of Clinical Investigation,** v. 117, n. 8, p. 2325-2336, 2007.

CLINE, G. W. et al. Impaired glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes. **The New England journal of medicine,** v. 341, n. 4, p. 240-246, Jul 22 1999.

COOPER, R. N. et al. In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. **Journal of cell science,** v. 112 (Pt 17), p. 2895-2901, Sep 1999.

CORPELEIJN, E.; SARIS, W. H.; BLAAK, E. E. Metabolic flexibility in the development of insulin resistance and type 2 diabetes: effects of lifestyle. **Obesity reviews : an official journal of the International Association for the Study of Obesity,** v. 10, n. 2, p. 178-193, Mar 2009.

COSTELLO, P. M. et al. Peri-implantation and late gestation maternal undernutrition differentially affect fetal sheep skeletal muscle development. **The Journal of Physiology,** v. 586, n. 9, p. 2371-2379, May 2008 2008.

COUPE, B. et al. The timing of "catch-up growth" affects metabolism and appetite regulation in male rats born with intrauterine growth restriction. **American journal of physiology. Regulatory, integrative and comparative physiology,** v. 297, n. 3, p. R813-824, Sep 2009.

COUPE, B. et al. Postnatal growth after intrauterine growth restriction alters central leptin signal and energy homeostasis. **PloS one,** v. 7, n. 1, p. e30616, 2012.

CUEVAS, A.; ALVAREZ, V.; CARRASCO, F. Epidemic of metabolic syndrome in Latin America. **Current opinion in endocrinology, diabetes, and obesity,** v. 18, n. 2, p. 134-138, Apr 2011.

CUEVAS, A.; ALVAREZ, V.; OLIVOS, C. The emerging obesity problem in Latin America. **Expert review of cardiovascular therapy,** v. 7, n. 3, p. 281-288, Mar 2009.

DAVIS, T. A.; FIOROTTO, M. L. Regulation of muscle growth in neonates. **Current opinion in clinical nutrition and metabolic care,** v. 12, n. 1, p. 78-85, Jan 2009.

DAVIS, T. A. et al. Enhanced response of muscle protein synthesis and plasma insulin to food intake in suckled rats. **The American journal of physiology,** v. 265, n. 2 Pt 2, p. R334-340, Aug 1993.

DE FEYTER, H. M. et al. Increased intramyocellular lipid content but normal skeletal muscle mitochondrial oxidative capacity throughout the pathogenesis of type 2 diabetes. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology,** v. 22, n. 11, p. 3947-3955, Nov 2008.

DE LANGE, P. et al. Sequential changes in the signal transduction responses of skeletal muscle following food deprivation. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology,** v. 20, n. 14, p. 2579-2581, Dec 2006.

DE LANGE, P. et al. Fuel economy in food-deprived skeletal muscle: signaling pathways and regulatory mechanisms. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology,** v. 21, n. 13, p. 3431-3441, Nov 2007.

DE MOURA, E. G. et al. Malnutrition during lactation changes growth hormone mRNA expression in offspring at weaning and in adulthood. **The Journal of nutritional biochemistry,** v. 18, n. 2, p. 134-139, Feb 2007.

DE OLIVEIRA, E. P. et al. Dietary factors associated with metabolic syndrome in Brazilian adults. **Nutrition journal,** v. 11, p. 13, 2012.

DEFRONZO, R. A. Pathogenesis of type 2 (non-insulin dependent) diabetes mellitus: a balanced overview. **Diabetologia,** v. 35, n. 4, p. 389-397, Apr 1992.

DEL PRATO, S. et al. Characterization of cellular defects of insulin action in type 2 (non-insulin-dependent) diabetes mellitus. **The Journal of clinical investigation,** v. 91, n. 2, p. 484-494, Feb 1993.

DESAI, M. et al. Organ-selective growth in the offspring of protein-restricted mothers. **The British journal of nutrition,** v. 76, n. 4, p. 591-603, Oct 1996.

DESAI, M.; HALES, C. N. Role of fetal and infant growth in programming metabolism in later life. **Biological reviews of the Cambridge Philosophical Society,** v. 72, n. 2, p. 329-348, May 1997.

DOBBING, J. The effect of undernutrition on myelination in the central nervous system. **Biologia neonatorum. Neo-natal studies,** v. 9, n. 1, p. 132-147, 1965.

DOLINOY, D. C. et al. Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. **Environ Health Perspect,** v. 114, n. 4, p. 567-572, Apr 2006.

DOLINSKY, V. W. et al. Continued postnatal administration of resveratrol prevents diet-induced metabolic syndrome in rat offspring born growth restricted. **Diabetes,** v. 60, n. 9, p. 2274-2284, Sep 2011.

DUTRA, E. S. et al. Metabolic syndrome in central Brazil: prevalence and correlates in the adult population. **Diabetology & metabolic syndrome,** v. 4, n. 1, p. 20, May 14 2012.

ECKEL, R. H.; GRUNDY, S. M.; ZIMMET, P. Z. The metabolic syndrome. **The Lancet,** v. 365, n. 9468, p. 1415-1428, 2005/4/22/ 2005.

EGGER, G. et al. Epigenetics in human disease and prospects for epigenetic therapy. **Nature,** v. 429, n. 6990, p. 457-463, May 27 2004.

ERHUMA, A. et al. Prenatal exposure to a low-protein diet programs disordered regulation of lipid metabolism in the aging rat. **American journal of physiology. Endocrinology and metabolism,** v. 292, n. 6, p. E1702-1714, Jun 2007.

ERIKSSON, J. et al. Obesity from cradle to grave. **Int J Obes Relat Metab Disord,** v. 27, n. 6, p. 722-727, Jun 2003a.

ERIKSSON, J. et al. Size at birth, childhood growth and obesity in adult life. **International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity,** v. 25, n. 5, p. 735-740, May 2001a.

ERIKSSON, J. G. et al. Early growth and coronary heart disease in later life: longitudinal study. **BMJ,** v. 322, n. 7292, p. 949-953, Apr 21 2001b.

_____. Early adiposity rebound in childhood and risk of Type 2 diabetes in adult life. **Diabetologia,** v. 46, n. 2, p. 190-194, Feb 2003b.

ERIKSSON, J. G. et al. Pathways of infant and childhood growth that lead to type 2 diabetes. **Diabetes care,** v. 26, n. 11, p. 3006-3010, Nov 2003c.

ESCHER, P. et al. Rat PPARs: quantitative analysis in adult rat tissues and regulation in fasting and refeeding. **Endocrinology,** v. 142, n. 10, p. 4195-4202, Oct 2001.

FAGUNDES, A. T. S. et al. Maternal low-protein diet during lactation programmes body composition and glucose homeostasis in the adult rat offspring. **British Journal of Nutrition,** v. 98, n. 05, 2007.

FERNANDEZ-MARCOS, P. J.; AUWERX, J. Regulation of PGC-1alpha, a nodal regulator of mitochondrial biogenesis. **The American journal of clinical nutrition,** v. 93, n. 4, p. 884S-890, Apr 2011.

FIDALGO, M. et al. Programmed changes in the adult rat offspring caused by maternal protein restriction during gestation and lactation are attenuated by maternal moderate-low physical training. **The British journal of nutrition**, p. 1-8, May 1 2012.

FINCK, B. N.; KELLY, D. P. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. **The Journal of clinical investigation,** v. 116, n. 3, p. 615-622, Mar 2006.

FINLEY, L. W. et al. Skeletal muscle transcriptional coactivator PGC-1alpha mediates mitochondrial, but not metabolic, changes during calorie restriction. **Proceedings of the National Academy of Sciences of the United States of America,** v. 109, n. 8, p. 2931-2936, Feb 21 2012.

FIOROTTO, M. L.; DAVIS, T. A. Food intake alters muscle protein gain with little effect on Na(+)-K(+)-ATPase and myosin isoforms in suckled rats. **The American journal of physiology,** v. 272, n. 5 Pt 2, p. R1461-1471, May 1997.

FORRESTER, T. Historic and early life origins of hypertension in Africans. **The Journal of nutrition,** v. 134, n. 1, p. 211-216, Jan 2004.

FRAGA, M. F. et al. Epigenetic differences arise during the lifetime of monozygotic twins. **Proceedings of the National Academy of Sciences of the United States of America,** v. 102, n. 30, p. 10604-10609, Jul 26 2005.

FRIER, B. C.; JACOBS, R. L.; WRIGHT, D. C. Interactions between the consumption of a high-fat diet and fasting in the regulation of fatty acid oxidation enzyme gene expression: an evaluation of potential mechanisms. **American journal of physiology. Regulatory, integrative and comparative physiology,** v. 300, n. 2, p. R212-221, Feb 2011.

FU, Q. et al. Growth retardation alters the epigenetic characteristics of hepatic dual specificity phosphatase 5. **FASEB J,** v. 20, n. 12, p. 2127-2129, Oct 2006.

FU, Q. et al. Uteroplacental insufficiency induces site-specific changes in histone H3 covalent modifications and affects DNA-histone H3 positioning in day 0 IUGR rat liver. **Physiol Genomics,** v. 20, n. 1, p. 108-116, Dec 15 2004.

FUKS, F. et al. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. **Nat Genet,** v. 24, n. 1, p. 88-91, Jan 2000.

FUKS, F. et al. Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. **EMBO J,** v. 20, n. 10, p. 2536-2544, May 15 2001.

FUKS, F. et al. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. **J Biol Chem,** v. 278, n. 6, p. 4035-4040, Feb 7 2003.

GARDNER, D. S. et al. Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. **American journal of physiology. Regulatory, integrative and comparative physiology,** v. 289, n. 4, p. R947-954, Oct 2005.

GASTER, M. et al. GLUT4 is reduced in slow muscle fibers of type 2 diabetic patients: is insulin resistance in type 2 diabetes a slow, type 1 fiber disease? **Diabetes,** v. 50, n. 6, p. 1324-1329, Jun 2001.

GE, Y.; CHEN, J. MicroRNAs in skeletal myogenesis. **Cell cycle,** v. 10, n. 3, p. 441-448, 2011.

GERHART-HINES, Z. et al. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. **The EMBO journal,** v. 26, n. 7, p. 1913-1923, Apr 4 2007.

GERRITS, M. F. et al. Distinct skeletal muscle fiber characteristics and gene expression in diet-sensitive versus diet-resistant obesity. **Journal of lipid research,** v. 51, n. 8, p. 2394-2404, Aug 2010.

GHOSHAL, K. et al. A folate- and methyl-deficient diet alters the expression of DNA methyltransferases and methyl CpG binding proteins involved in epigenetic gene silencing in livers of F344 rats. **The Journal of nutrition,** v. 136, n. 6, p. 1522-1527, Jun 2006.

GIBSON, M. C.; SCHULTZ, E. The distribution of satellite cells and their relationship to specific fiber types in soleus and extensor digitorum longus muscles. **The Anatomical record,** v. 202, n. 3, p. 329-337, Mar 1982.

GLUCKMAN, P. D. et al. The fetal, neonatal, and infant environments-the long-term consequences for disease risk. **Early human development,** v. 81, n. 1, p. 51-59, Jan 2005a.

GLUCKMAN, P. D.; HANSON, M. A. The developmental origins of the metabolic syndrome. **Trends in endocrinology and metabolism: TEM,** v. 15, n. 4, p. 183-187, May-Jun 2004a.

_____. Living with the past: evolution, development, and patterns of disease. **Science,** v. 305, n. 5691, p. 1733-1736, Sep 17 2004b.

GLUCKMAN, P. D.; HANSON, M. A.; PINAL, C. The developmental origins of adult disease. **Matern Child Nutr,** v. 1, n. 3, p. 130-141, Jul 2005.

GLUCKMAN, P. D. et al. Environmental influences during development and their later consequences for health and disease: implications for the interpretation of empirical studies. **Proceedings. Biological sciences / The Royal Society,** v. 272, n. 1564, p. 671-677, Apr 7 2005b.

GOLLNICK, P. D. et al. Muscular enlargement and number of fibers in skeletal muscles of rats. **Journal of applied physiology: respiratory, environmental and exercise physiology,** v. 50, n. 5, p. 936-943, May 1981.

GOODMAN, C. A. et al. Muscle fiber type-dependent differences in the regulation of protein synthesis. **PloS one,** v. 7, n. 5, p. e37890, 2012.

GRIMALDI, P. A. Roles of PPARdelta in the control of muscle development and metabolism. **Biochemical Society transactions,** v. 31, n. Pt 6, p. 1130-1132, Dec 2003.

GRONNER, M. F. et al. Prevalence of metabolic syndrome and its association with educational inequalities among Brazilian adults: a population-based study. **Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al.],** v. 44, n. 7, p. 713-719, Jul 2011.

GRUNDY, S. M. Metabolic syndrome pandemic. **Arteriosclerosis, thrombosis, and vascular biology,** v. 28, n. 4, p. 629-636, Apr 2008.

HAKVOORT, T. B. et al. Interorgan coordination of the murine adaptive response to fasting. **The Journal of biological chemistry,** v. 286, n. 18, p. 16332-16343, May 6 2011.

HALES, C. N.; BARKER, D. J. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. **Diabetologia,** v. 35, n. 7, p. 595-601, Jul 1992.

_____. The thrifty phenotype hypothesis. **British medical bulletin,** v. 60, p. 5-20, 2001a.

HALES, C. N. et al. Fetal and infant growth and impaired glucose tolerance at age 64. **BMJ,** v. 303, n. 6809, p. 1019-1022, Oct 26 1991.

HALES, C. N.; BARKER, D. J. P. The thrifty phenotype hypothesis: Type 2 diabetes. **Br Med Bull,** v. 60, n. 1, p. 5-20, November 1, 2001 2001b.

HAMALAINEN, N.; PETTE, D. The histochemical profiles of fast fiber types IIB, IID, and IIA in skeletal muscles of mouse, rat, and rabbit. **The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society,** v. 41, n. 5, p. 733-743, May 1993.

HANDSCHIN, C. et al. Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1alpha muscle-specific knock-out animals. **The Journal of biological chemistry,** v. 282, n. 41, p. 30014-30021, Oct 12 2007.

HARRIDGE, S. D. Plasticity of human skeletal muscle: gene expression to in vivo function. **Experimental physiology,** v. 92, n. 5, p. 783-797, Sep 2007.

HAWKE, T. J.; GARRY, D. J. Myogenic satellite cells: physiology to molecular biology. **Journal of applied physiology,** v. 91, n. 2, p. 534-551, Aug 2001.

HE, J.; WATKINS, S.; KELLEY, D. E. Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. **Diabetes,** v. 50, n. 4, p. 817-823, Apr 2001.

HEIJMANS, B. T. et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. **Proceedings of the National Academy of Sciences of the United States of America,** v. 105, n. 44, p. 17046-17049, Nov 4 2008.

HENRIKSEN, E. J. et al. Glucose transporter protein content and glucose transport capacity in rat skeletal muscles. **The American journal of physiology,** v. 259, n. 4 Pt 1, p. E593-598, Oct 1990.

HENRY, R. R. et al. Glycogen synthase activity is reduced in cultured skeletal muscle cells of non-insulin-dependent diabetes mellitus subjects. Biochemical and molecular mechanisms. **The Journal of clinical investigation,** v. 98, n. 5, p. 1231-1236, Sep 1 1996.

HICKEY, M. S. et al. Skeletal muscle fiber composition is related to adiposity and in vitro glucose transport rate in humans. **The American journal of physiology,** v. 268, n. 3 Pt 1, p. E453-457, Mar 1995a.

HICKEY, M. S. et al. The insulin action-fiber type relationship in humans is muscle group specific. **Am J Physiol Endocrinol Metab,** v. 269, n. 1, p. E150-154, July 1, 1995 1995b.

HIMMS-HAGEN, J.; HARPER, M. E. Physiological role of UCP3 may be export of fatty acids from mitochondria when fatty acid oxidation predominates: an hypothesis. **Experimental biology and medicine,** v. 226, n. 2, p. 78-84, Feb 2001.

HIRABARA, S. M. et al. Acute effect of fatty acids on metabolism and mitochondrial coupling in skeletal muscle. **Biochimica et biophysica acta,** v. 1757, n. 1, p. 57-66, Jan 2006a.

HO, K. W. et al. Postnatal muscle fibre histochemistry in the rat. **Journal of embryology and experimental morphology,** v. 76, p. 37-49, Aug 1983.

HOLEMANS, K.; AERTS, L.; VAN ASSCHE, F. A. Fetal growth restriction and consequences for the offspring in animal models. **J Soc Gynecol Investig,** v. 10, n. 7, p. 392-399, Oct 2003.

HOWALD, H. et al. Influences of endurance training on the ultrastructural composition of the different muscle fiber types in humans. **Pflugers Archiv : European journal of physiology,** v. 403, n. 4, p. 369-376, Apr 1985.

HUXLEY, R.; NEIL, A.; COLLINS, R. Unravelling the fetal origins hypothesis: is there really an inverse association between birthweight and subsequent blood pressure? **The Lancet,** v. 360, n. 9334, p. 659-665, 2002.

ICHINOSEKI-SEKINE, N. et al. Fiber-type specific expression of alpha-actinin isoforms in rat skeletal muscle. **Biochemical and biophysical research communications,** v. 419, n. 2, p. 401-404, Mar 9 2012.

INGELFINGER, J. R. Pathogenesis of perinatal programming. **Curr Opin Nephrol Hypertens,** v. 13, n. 4, p. 459-464, Jul 2004.

JABLONKA, E.; LAMB, M. J. The changing concept of epigenetics. **Ann N Y Acad Sci,** v. 981, p. 82-96, Dec 2002.

JADDOE, V. W.; WITTEMAN, J. C. Hypotheses on the fetal origins of adult diseases: contributions of epidemiological studies. **European journal of epidemiology,** v. 21, n. 2, p. 91-102, 2006.

JAGER, S. et al. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. **Proceedings of the National Academy of Sciences of the United States of America,** v. 104, n. 29, p. 12017-12022, Jul 17 2007.

JANSEN, J. K.; FLADBY, T. The perinatal reorganization of the innervation of skeletal muscle in mammals. **Progress in neurobiology,** v. 34, n. 1, p. 39-90, 1990.

JENUWEIN, T.; ALLIS, C. D. Translating the histone code. **Science,** v. 293, n. 5532, p. 1074-1080, Aug 10 2001.

JEUKENDRUP, A. E. Regulation of fat metabolism in skeletal muscle. **Annals of the New York Academy of Sciences,** v. 967, p. 217-235, Jun 2002.

JONES, P. L. et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. **Nat Genet,** v. 19, n. 2, p. 187-191, Jun 1998.

JORGENSEN, S. B.; RICHTER, E. A.; WOJTASZEWSKI, J. R. F. P. Role of AMPK in skeletal muscle metabolic regulation and adaptation in relation to exercise. **The Journal of Physiology,** v. 574, n. 1, p. 17-31, July 1, 2006 2006.

JORNAYVAZ, F. R. et al. Metabolism of oral glucose in children born small for gestational age: evidence for an impaired whole body glucose oxidation. **Metabolism: clinical and experimental,** v. 53, n. 7, p. 847-851, Jul 2004.

JUNIEN, C. et al. [Nutritional epigenomics of metabolic syndrome]. **Med Sci (Paris),** v. 21, n. 4, p. 396-404, Apr 2005.

KARDON, G.; CAMPBELL, J. K.; TABIN, C. J. Local extrinsic signals determine muscle and endothelial cell fate and patterning in the vertebrate limb. **Developmental cell,** v. 3, n. 4, p. 533-545, Oct 2002.

KASSAR-DUCHOSSOY, L. et al. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. **Nature,** v. 431, n. 7007, p. 466-471, Sep 23 2004.

KE, X. et al. Nonresponsiveness of cerebral p53-MDM2 functional circuit in newborn rat pups rendered IUGR via uteroplacental insufficiency. **Am J Physiol Regul Integr Comp Physiol,** v. 288, n. 4, p. R1038-1045, Apr 2005.

KELLEY, D. E. et al. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. **The American journal of physiology,** v. 277, n. 6 Pt 1, p. E1130-1141, Dec 1999.

KELLEY, D. E. et al. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. **Diabetes,** v. 51, n. 10, p. 2944-2950, Oct 2002.

KIBERSTIS, P. A. A Surfeit of Suspects. **Science,** v. 307, n. 5708, p. 369-, January 21, 2005 2005.

KIM, J. Y. et al. Evidence of a malonyl-CoA-insensitive carnitine palmitoyltransferase I activity in red skeletal muscle. **American journal of physiology. Endocrinology and metabolism,** v. 282, n. 5, p. E1014-1022, May 2002.

KNUTTI, D.; KRALLI, A. PGC-1, a versatile coactivator. **Trends in endocrinology and metabolism: TEM,** v. 12, n. 8, p. 360-365, Oct 2001.

KONG, X. et al. Glucose transporters in single skeletal muscle fibers. Relationship to hexokinase and regulation by contractile activity. **The Journal of biological chemistry,** v. 269, n. 17, p. 12963-12967, Apr 29 1994.

KOVES, T. R. et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. **Cell metabolism,** v. 7, n. 1, p. 45-56, Jan 2008.

LAGORD, C. et al. Differential myogenicity of satellite cells isolated from extensor digitorum longus (EDL) and soleus rat muscles revealed in vitro. **Cell and tissue research,** v. 291, n. 3, p. 455-468, Mar 1998.

LANE, R. H. Increased Hepatic Peroxisome Proliferator-Activated Receptor- Coactivator-1 Gene Expression in a Rat Model of Intrauterine Growth Retardation and Subsequent Insulin Resistance. **Endocrinology,** v. 143, n. 7, p. 2486-2490, 2002.

LANE, R. H. et al. IUGR alters postnatal rat skeletal muscle peroxisome proliferator-activated receptor-gamma coactivator-1 gene expression in a fiber specific manner. **Pediatric research,** v. 53, n. 6, p. 994-1000, Jun 2003.

LANGFORT, J. et al. Expression of hormone-sensitive lipase and its regulation by adrenaline in skeletal muscle. **The Biochemical journal,** v. 340 (Pt 2), p. 459-465, Jun 1 1999.

LANGLEY-EVANS, S. C. Developmental programming of health and disease. **The Proceedings of the Nutrition Society,** v. 65, n. 1, p. 97-105, Feb 2006.

LEANDRO, C. G. et al. Moderate physical training attenuates muscle-specific effects on fibre type composition in adult rats submitted to a perinatal maternal low-protein diet. **European journal of nutrition,** v. 51, n. 7, p. 807-815, Oct 2012.

LEE, C. H.; OLSON, P.; EVANS, R. M. Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. **Endocrinology,** v. 144, n. 6, p. 2201-2207, Jun 2003.

LEE, W. J. et al. AMPK activation increases fatty acid oxidation in skeletal muscle by activating PPAR[alpha] and PGC-1. **Biochemical and Biophysical Research Communications,** v. 340, n. 1, p. 291-295, 2006.

LEITAO, M. P.; MARTINS, I. S. [Prevalence and factors associated with metabolic syndrome in users of primary healthcare units in Sao Paulo--SP, Brazil]. **Revista da Associacao Medica Brasileira,** v. 58, n. 1, p. 60-69, Jan-Feb 2012.

LEMON, B.; TJIAN, R. Orchestrated response: a symphony of transcription factors for gene control. **Genes & Development,** v. 14, n. 20, p. 2551-2569, October 15, 2000 2000.

LERIN, C. et al. GCN5 acetyltransferase complex controls glucose metabolism through transcriptional repression of PGC-1alpha. **Cell metabolism,** v. 3, n. 6, p. 429-438, Jun 2006.

LEVIN, B. E. The obesity epidemic: metabolic imprinting on genetically susceptible neural circuits. **Obes Res,** v. 8, n. 4, p. 342-347, Jul 2000.

LEVY-COSTA, R. B. et al. [Household food availability in Brazil: distribution and trends (1974-2003)]. **Revista de saude publica,** v. 39, n. 4, p. 530-540, Aug 2005.

LI, J. B.; GOLDBERG, A. L. Effects of food deprivation on protein synthesis and degradation in rat skeletal muscles. **The American journal of physiology,** v. 231, n. 2, p. 441-448, Aug 1976.

LI, X. et al. Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1alpha transcription coactivator. **Nature,** v. 447, n. 7147, p. 1012-1016, Jun 21 2007.

LIANG, H. et al. Whole body overexpression of PGC-1alpha has opposite effects on hepatic and muscle insulin sensitivity. **American journal of physiology. Endocrinology and metabolism,** v. 296, n. 4, p. E945-954, Apr 2009.

LILLIOJA, S. et al. Skeletal muscle capillary density and fiber type are possible determinants of in vivo insulin resistance in man. **The Journal of Clinical Investigation,** v. 80, n. 2, p. 415-424, 1987.

LILLYCROP, K. A.; BURDGE, G. C. Epigenetic changes in early life and future risk of obesity. **International journal of obesity,** v. 35, n. 1, p. 72-83, Jan 2011.

LILLYCROP, K. A. et al. Dietary Protein Restriction of Pregnant Rats Induces and Folic Acid Supplementation Prevents Epigenetic Modification of Hepatic Gene Expression in the Offspring. **J. Nutr.,** v. 135, n. 6, p. 1382-1386, June 1, 2005 2005a.

LIN, J. et al. Transcriptional co-activator PGC-1[alpha] drives the formation of slow-twitch muscle fibres. **Nature,** v. 418, n. 6899, p. 797-801, 2002a.
LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. **Methods,** v. 25, n. 4, p. 402-408, Dec 2001.

LOPES DE SOUZA, S. et al. Perinatal protein restriction reduces the inhibitory action of serotonin on food intake. **The European journal of neuroscience,** v. 27, n. 6, p. 1400-1408, Mar 2008.

LOWRY, C. V. et al. Enzyme patterns in single human muscle fibers. **The Journal of biological chemistry,** v. 253, n. 22, p. 8269-8277, Nov 25 1978.

LUCAS, A. Programming by early nutrition in man. **Ciba Foundation symposium,** v. 156, p. 38-50; discussion 50-35, 1991.

LUCAS, A.; FEWTRELL, M. S.; COLE, T. J. Fetal origins of adult disease-the hypothesis revisited. **BMJ,** v. 319, n. 7204, p. 245-249, Jul 24 1999.

MACLENNAN, N. K. et al. Uteroplacental insufficiency alters DNA methylation, one-carbon metabolism, and histone acetylation in IUGR rats. **Physiol Genomics,** v. 18, n. 1, p. 43-50, Jun 17 2004.

MALLINSON, J. E. et al. Fetal exposure to a maternal low-protein diet during mid-gestation results in muscle-specific effects on fibre type composition in young rats. **British Journal of Nutrition,** v. 98, n. 02, p. 292-299, 2007.

MALTIN, C. A. et al. Impact of manipulations of myogenesis in utero on the performance of adult skeletal muscle. **Reproduction,** v. 122, n. 3, p. 359-374, Sep 2001.

MARCHAND, M. C.; LANGLEY-EVANS, S. C. Intrauterine programming of nephron number: the fetal flaw revisited. **Journal of nephrology,** v. 14, n. 5, p. 327-331, Sep-Oct 2001.

MARCHEVA, B. et al. Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. **Nature,** v. 466, n. 7306, p. 627-631, Jul 29 2010.

MARQUEZ, J.; SWEAZEA, K. L.; BRAUN, E. J. Skeletal muscle fiber composition of the English sparrow (Passer domesticus). **Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology,** v. 143, n. 1, p. 126-131, Jan 2006.

MARTINS, A. R. et al. Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function. **Lipids in health and disease,** v. 11, p. 30, 2012.

MATSUDA, G. The light chains of muscle myosin: its structure, function, and evolution. **Advances in biophysics,** v. 16, p. 185-218, 1983.

MATSUMOTO, A. et al. Cell size and oxidative enzyme activity of rat biceps brachii and triceps brachii muscles. **The journal of physiological sciences : JPS,** v. 57, n. 5, p. 311-316, Oct 2007.

MATTHEWS, A. G. et al. RAG2 PHD finger couples histone H3 lysine 4 trimethylation with V(D)J recombination. **Nature,** v. 450, n. 7172, p. 1106-1110, Dec 13 2007.

MAURO, A. Satellite cell of skeletal muscle fibers. **The Journal of biophysical and biochemical cytology,** v. 9, p. 493-495, Feb 1961.

MENSINK, M. et al. Improved skeletal muscle oxidative enzyme activity and restoration of PGC-1 alpha and PPAR beta/delta gene expression upon rosiglitazone treatment in obese patients with type 2 diabetes mellitus. **International journal of obesity,** v. 31, n. 8, p. 1302-1310, Aug 2007.

MISRA, A.; KHURANA, L. Obesity and the Metabolic Syndrome in Developing Countries. **J Clin Endocrinol Metab,** v. 93, n. 11_Supplement_1, p. s9-30, November 1, 2008 2008.

MIURA, S. et al. Overexpression of peroxisome proliferator-activated receptor gamma coactivator-1alpha down-regulates GLUT4 mRNA in skeletal muscles. **The Journal of biological chemistry,** v. 278, n. 33, p. 31385-31390, Aug 15 2003.

MIURA, S. et al. An increase in murine skeletal muscle peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) mRNA in response to exercise is mediated by beta-adrenergic receptor activation. **Endocrinology,** v. 148, n. 7, p. 3441-3448, Jul 2007.

MOORE, T. Genetic conflict, genomic imprinting and establishment of the epigenotype in relation to growth. **Reproduction,** v. 122, n. 2, p. 185-193, Aug 2001.

MORGANE, P. J. et al. Prenatal malnutrition and development of the brain. **Neuroscience and biobehavioral reviews,** v. 17, n. 1, p. 91-128, Spring 1993.

MORISON, I. M.; REEVE, A. E. A catalogue of imprinted genes and parent-of-origin effects in humans and animals. **Human molecular genetics,** v. 7, n. 10, p. 1599-1609, 1998.

MORRIS, T. J. et al. Transcriptional profiling of rats subjected to gestational undernourishment: implications for the developmental variations in metabolic traits. **PloS one,** v. 4, n. 9, p. e7271, 2009.

MORTENSEN, O. H. et al. A maternal low protein diet has pronounced effects on mitochondrial gene expression in offspring liver and skeletal muscle; protective effect of taurine. **Journal of biomedical science,** v. 17 Suppl 1, p. S38, 2010.

MOY, F. M.; BULGIBA, A. The modified NCEP ATP III criteria maybe better than the IDF criteria in diagnosing Metabolic Syndrome among Malays in Kuala Lumpur. **BMC public health,** v. 10, p. 678, 2010.

MUHLHAUSLER, B. S. et al. The transition from fetal growth restriction to accelerated postnatal growth: a potential role for insulin signalling in skeletal muscle. **The Journal of physiology,** v. 587, n. Pt 17, p. 4199-4211, Sep 1 2009.

MUOIO, D. M.; KOVES, T. R. Skeletal muscle adaptation to fatty acid depends on coordinated actions of the PPARs and PGC1 alpha: implications for metabolic disease. **Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme,** v. 32, n. 5, p. 874-883, Oct 2007.

MUOIO, D. M. et al. Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) alpha knock-out mice. Evidence for compensatory regulation by PPAR delta. **The Journal of biological chemistry,** v. 277, n. 29, p. 26089-26097, Jul 19 2002a.

MUOIO, D. M. et al. Peroxisome proliferator-activated receptor-alpha regulates fatty acid utilization in primary human skeletal muscle cells. **Diabetes,** v. 51, n. 4, p. 901-909, Apr 2002b.

NAN, X. et al. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. **Nature,** v. 393, n. 6683, p. 386-389, May 28 1998.

NEMOTO, S.; FERGUSSON, M. M.; FINKEL, T. SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1{alpha}. **The Journal of biological chemistry,** v. 280, n. 16, p. 16456-16460, Apr 22 2005.

NG, S. F. et al. Chronic high-fat diet in fathers programs beta-cell dysfunction in female rat offspring. **Nature,** v. 467, n. 7318, p. 963-966, Oct 21 2010.

NYHOLM, B. et al. Evidence of an increased number of type IIb muscle fibers in insulin-resistant first-degree relatives of patients with NIDDM. **Diabetes,** v. 46, n. 11, p. 1822-1828, Nov 1997.

NYIRENDA, M. J. et al. Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and

causes glucose intolerance in adult offspring. **The Journal of clinical investigation,** v. 101, n. 10, p. 2174-2181, May 15 1998.

OKEN, E.; GILLMAN, M. W. Fetal origins of obesity. **Obes Res,** v. 11, n. 4, p. 496-506, Apr 2003.

OROZCO-SOLIS, R. et al. Perinatal undernutrition-induced obesity is independent of the developmental programming of feeding. **Physiology & behavior,** v. 96, n. 3, p. 481-492, Mar 2 2009.

OROZCO-SÓLIS, R. et al. Perinatal undernutrition-induced obesity is independent of the developmental programming of feeding. **Physiology & Behavior,** v. 96, n. 3, p. 481-492, 2009.

OROZCO-SOLIS, R. et al. Nutritional programming in the rat is linked to long-lasting changes in nutrient sensing and energy homeostasis in the hypothalamus. **PloS one,** v. 5, n. 10, p. e13537, 2010.

OROZCO-SOLIS, R. et al. Perinatal nutrient restriction induces long-lasting alterations in the circadian expression pattern of genes regulating food intake and energy metabolism. **International journal of obesity,** v. 35, n. 7, p. 990-1000, Jul 2011.

OZANNE, S. E. Programming of hepatic and peripheral tissue insulin sensitivity by maternal protein restriction. **Biochemical Society transactions,** v. 27, n. 2, p. 94-97, Feb 1999.

OZANNE, S. E.; HALES, C. N. The long-term consequences of intra-uterine protein malnutrition for glucose metabolism. **The Proceedings of the Nutrition Society,** v. 58, n. 3, p. 615-619, Aug 1999.

OZANNE, S. E.; HALES, C. N. Early programming of glucose-insulin metabolism. **Trends in Endocrinology and Metabolism,** v. 13, n. 9, p. 368-373, 2002.

OZANNE, S. E.; HALES, C. N. Lifespan: catch-up growth and obesity in male mice. **Nature,** v. 427, n. 6973, p. 411-412, Jan 29 2004.

OZANNE, S. E. et al. Low birthweight is associated with specific changes in muscle insulin-signalling protein expression. **Diabetologia,** v. 48, n. 3, p. 547-552, 2005.

OZANNE, S. E. et al. Early programming of weight gain in mice prevents the induction of obesity by a highly palatable diet. **Clinical science,** v. 106, n. 2, p. 141-145, Feb 2004.

OZANNE, S. E. et al. Maternal low protein diet in rats programmes fatty acid desaturase activities in the offspring. **Diabetologia,** v. 41, n. 11, p. 1337-1342, Nov 1998.

OZANNE, S. E. et al. Early growth restriction leads to down regulation of protein kinase C zeta and insulin resistance in skeletal muscle. **The Journal of endocrinology,** v. 177, n. 2, p. 235-241, May 2003a.

OZANNE, S. E. et al. Altered regulation of hepatic glucose output in the male offspring of protein-malnourished rat dams. **The American journal of physiology,** v. 270, n. 4 Pt 1, p. E559-564, Apr 1996a.

OZANNE, S. E. et al. Altered muscle insulin sensitivity in the male offspring of protein-malnourished rats. **The American journal of physiology,** v. 271, n. 6 Pt 1, p. E1128-1134, Dec 1996b.

OZANNE, S. E. et al. Dissection of the metabolic actions of insulin in adipocytes from early growth-retarded male rats. **The Journal of endocrinology,** v. 162, n. 2, p. 313-319, Aug 1999.

PAGEL-LANGENICKEL, I. et al. The role of mitochondria in the pathophysiology of skeletal muscle insulin resistance. **Endocrine reviews,** v. 31, n. 1, p. 25-51, Feb 2010.

PARSONS, T. J.; POWER, C.; MANOR, O. Fetal and early life growth and body mass index from birth to early adulthood in 1958 British cohort: longitudinal study. **BMJ,** v. 323, n. 7325, p. 1331-1335, 2001.

PASSOS, M. C. F.; RAMOS, C. F.; MOURA, E. G. Short and long term effects of malnutrition in rats during lactation on the body weight of offspring. **Nutrition Research,** v. 20, n. 11, p. 1603-1612, 2000.

PATTI, M. E. et al. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. **Proceedings of the**

National Academy of Sciences of the United States of America, v. 100, n. 14, p. 8466-8471, Jul 8 2003.

PENDERGRASS, M. et al. Insulin-induced hexokinase II expression is reduced in obesity and NIDDM. **Diabetes,** v. 47, n. 3, p. 387-394, Mar 1998.

PERDIGUERO, E. et al. Epigenetic regulation of myogenesis. **Epigenetics : official journal of the DNA Methylation Society,** v. 4, n. 8, p. 541-550, Nov 16 2009.

PETER, J. B. et al. Metabolic profiles of three fiber types of skeletal muscle in guinea pigs and rabbits. **Biochemistry,** v. 11, n. 14, p. 2627-2633, Jul 4 1972.

PETERS, S. J. et al. Human skeletal muscle PDH kinase activity and isoform expression during a 3-day high-fat/low-carbohydrate diet. **American journal of physiology. Endocrinology and metabolism,** v. 281, n. 6, p. E1151-1158, Dec 2001.

PETERSEN, K. F.; SHULMAN, G. I. Pathogenesis of skeletal muscle insulin resistance in type 2 diabetes mellitus. **The American journal of cardiology,** v. 90, n. 5A, p. 11G-18G, Sep 5 2002.

PETRIK, J. et al. A low protein diet alters the balance of islet cell replication and apoptosis in the fetal and neonatal rat and is associated with a reduced pancreatic expression of insulin-like growth factor-II. **Endocrinology,** v. 140, n. 10, p. 4861-4873, Oct 1999.

PETRY CJ, D. M., PAWLAK DB, OZANNE SE, HALES CN. Diabetes in old male offspring of rat dams fed a reduced protein diet. **Int J Exp Diabetes Res.,** v. 2, n. 2, p. 139-143, 2001.

PETRY, C. J. et al. Diabetes in old male offspring of rat dams fed a reduced protein diet. **International journal of experimental diabetes research,** v. 2, n. 2, p. 139-143, 2001.

PETTE, D.; STARON, R. S. Myosin isoforms, muscle fiber types, and transitions. **Microscopy research and technique,** v. 50, n. 6, p. 500-509, Sep 15 2000.

_____. Transitions of muscle fiber phenotypic profiles. **Histochemistry and cell biology,** v. 115, n. 5, p. 359-372, May 2001.

PHAM, T. D. et al. Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney. **Am J Physiol Regul Integr Comp Physiol,** v. 285, n. 5, p. R962-970, Nov 2003.

PIEROBON-BORMIOLI, S. et al. "Fast" isomyosins and fiber types in mammalian skeletal muscle. **Journal of Histochemistry & Cytochemistry,** v. 29, n. 10, p. 1179-1188, 1981.

PIETER DE LANGE et al. Fuel economy in food-deprived skeletal muscle: signaling pathways and regulatory mechanisms. **FASEB J.,** v. 21, n. 13, p. 3431-3441, November 1, 2007 2007.

PIMENTA, A. M.; GAZZINELLI, A.; VELASQUEZ-MELENDEZ, G. [Prevalence of metabolic syndrome and its associated factors in a rural area of Minas Gerais State (MG, Brazil)]. **Ciencia & saude coletiva,** v. 16, n. 7, p. 3297-3306, Jul 2011.

PLAGEMANN, A. et al. Hypothalamic proopiomelanocortin promoter methylation becomes altered by early overfeeding: an epigenetic model of obesity and the metabolic syndrome. **The Journal of physiology,** v. 587, n. Pt 20, p. 4963-4976, Oct 15 2009.

POPKIN, B. M. The shift in stages of the nutrition transition in the developing world differs from past experiences! **Public Health Nutr,** v. 5, n. 1A, p. 205-214, Feb 2002.

POTTHOFF, M. J.; OLSON, E. N.; BASSEL-DUBY, R. Skeletal muscle remodeling. **Current opinion in rheumatology,** v. 19, n. 6, p. 542-549, Nov 2007.

POWER, C.; JEFFERIS, B. J. Fetal environment and subsequent obesity: a study of maternal smoking. **Int J Epidemiol,** v. 31, n. 2, p. 413-419, Apr 2002.

PRENTICE, A. M. The emerging epidemic of obesity in developing countries. **Int J Epidemiol,** v. 35, n. 1, p. 93-99, Feb 2006.

PRESCOD, A. L.; HALLIDAY, W. C.; TAYLOR, C. G. Protein deficiency, but not zinc deficiency, reduces recovery of type 1 and type 2 muscle fibre diameters in the gastrocnemius muscle of growing rats. **The British journal of nutrition,** v. 106, n. 5, p. 675-682, Sep 2011.

PRIOR, L. J. et al. Undernutrition during suckling in rats elevates plasma adiponectin and its receptor in skeletal muscle regardless of diet composition: a protective effect? **International journal of obesity,** v. 32, n. 10, p. 1585-1594, Oct 2008.

PUNKT, K.; NAUPERT, A.; ASMUSSEN, G. Differentiation of rat skeletal muscle fibres during development and ageing. **Acta histochemica,** v. 106, n. 2, p. 145-154, 2004.

PUTMAN, C. T. et al. AMPK activation increases uncoupling protein-3 expression and mitochondrial enzyme activities in rat muscle without fibre type transitions. **The Journal of physiology,** v. 551, n. Pt 1, p. 169-178, Aug 15 2003.

QUIGLEY, S. P. et al. Myogenesis in sheep is altered by maternal feed intake during the peri-conception period. **Animal reproduction science,** v. 87, n. 3-4, p. 241-251, Jul 2005.

RABOL, R. et al. Regional anatomic differences in skeletal muscle mitochondrial respiration in type 2 diabetes and obesity. **The Journal of clinical endocrinology and metabolism,** v. 95, n. 2, p. 857-863, Feb 2010.

RANDLE, P. J. et al. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. **Lancet,** v. 1, n. 7285, p. 785-789, Apr 13 1963.

RAVELLI, A. C. J. et al. Glucose tolerance in adults after prenatal exposure to famine. **The Lancet,** v. 351, n. 9097, p. 173-177, 1998.

REEVES, P. G.; NIELSEN, F. H.; FAHEY, G. C., JR. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. **The Journal of nutrition,** v. 123, n. 11, p. 1939-1951, Nov 1993.

REGMI, P. R.; DIXON, W. T.; OBA, M. Effects of ammonia load on glucose metabolism by isolated ovine duodenal mucosa. **Journal of animal science,** v. 86, n. 9, p. 2321-2327, Sep 2008.

REIK, W.; DEAN, W.; WALTER, J. Epigenetic reprogramming in mammalian development. **Science,** v. 293, n. 5532, p. 1089-1093, Aug 10 2001.

REMACLE, C. et al. Intrauterine programming of the endocrine pancreas. **Diabetes Obes Metab,** v. 9 Suppl 2, p. 196-209, Nov 2007.

REUSENS, B.; REMACLE, C. Intergenerational effect of an adverse intrauterine environment on perturbation of glucose metabolism. **Twin Res,** v. 4, n. 5, p. 406-411, Oct 2001.

REUSENS, B.; THEYS, N.; REMACLE, C. Alteration of mitochondrial function in adult rat offspring of malnourished dams. **World journal of diabetes,** v. 2, n. 9, p. 149-157, Sep 15 2011.

REZNICK, R. M.; SHULMAN, G. I. The role of AMP-activated protein kinase in mitochondrial biogenesis. **The Journal of Physiology,** v. 574, n. 1, p. 33-39, July 1, 2006 2006.

ROBERTSON, K. D. DNA methylation and human disease. **Nat Rev Genet,** v. 6, n. 8, p. 597-610, Aug 2005.

ROBERTSON, K. D. et al. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. **Nat Genet,** v. 25, n. 3, p. 338-342, Jul 2000.

ROCKL, K. S. et al. Skeletal muscle adaptation to exercise training: AMP-activated protein kinase mediates muscle fiber type shift. **Diabetes,** v. 56, n. 8, p. 2062-2069, Aug 2007a.

RODEN, M. How Free Fatty Acids Inhibit Glucose Utilization in Human Skeletal Muscle. **News in Physiological Sciences,** v. 19, n. 3, p. 92-96, 2004.

RODEN, M. et al. Mechanism of free fatty acid-induced insulin resistance in humans. **The Journal of clinical investigation,** v. 97, n. 12, p. 2859-2865, Jun 15 1996.

RODGERS, J. T. et al. Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways. **FEBS letters,** v. 582, n. 1, p. 46-53, Jan 9 2008a.

ROGERS, I. The influence of birthweight and intrauterine environment on adiposity and fat distribution in later life. **Int J Obes Relat Metab Disord,** v. 27, n. 7, p. 755-777, Jul 2003.

ROUNTREE, M. R.; BACHMAN, K. E.; BAYLIN, S. B. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. **Nat Genet,** v. 25, n. 3, p. 269-277, Jul 2000.

RUSSELL, A. P. et al. UCP3 protein regulation in human skeletal muscle fibre types I, IIa and IIx is dependent on exercise intensity. **The Journal of physiology,** v. 550, n. Pt 3, p. 855-861, Aug 1 2003a.

SALAROLI, L. B. et al. [Prevalence of metabolic syndrome in population-based study, Vitoria, ES-Brazil]. **Arquivos brasileiros de endocrinologia e metabologia,** v. 51, n. 7, p. 1143-1152, Oct 2007.

SAMEC, S. et al. Skeletal muscle heterogeneity in fasting-induced upregulation of genes encoding UCP2, UCP3, PPARgamma and key enzymes of lipid oxidation. **Pflugers Archiv : European journal of physiology,** v. 445, n. 1, p. 80-86, Oct 2002.

SANDRI, M. et al. Muscle Fiber Type-Dependent Differences in the Regulation of Protein Synthesis. **PloS one,** v. 7, n. 5, p. e37890, 2012.

SCHIAFFINO, S.; HANZLIKOVA, V.; PIEROBON, S. Relations between structure and function in rat skeletal muscle fibers. **The Journal of cell biology,** v. 47, n. 1, p. 107-119, Oct 1970.

SCHIAFFINO, S.; REGGIANI, C. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. **Physiological reviews,** v. 76, n. 2, p. 371-423, Apr 1996.

_____. Fiber types in mammalian skeletal muscles. **Physiological reviews,** v. 91, n. 4, p. 1447-1531, Oct 2011.

SCHRAUWEN, P.; HESSELINK, M. K. Oxidative capacity, lipotoxicity, and mitochondrial damage in type 2 diabetes. **Diabetes,** v. 53, n. 6, p. 1412-1417, Jun 2004a.

_____. The role of uncoupling protein 3 in fatty acid metabolism: protection against lipotoxicity? **The Proceedings of the Nutrition Society,** v. 63, n. 2, p. 287-292, May 2004b.

SCHRAUWEN, P. et al. Uncoupling Protein 3 Content Is Decreased in Skeletal Muscle of Patients With Type 2 Diabetes. **Diabetes,** v. 50, n. 12, p. 2870-2873, December 2001 2001.

SCHRAUWEN, P. et al. Mitochondrial dysfunction and lipotoxicity. **Biochimica et biophysica acta,** v. 1801, n. 3, p. 266-271, Mar 2010.

SCHULER, M. et al. PGC1[alpha] expression is controlled in skeletal muscles by PPAR[beta], whose ablation results in fiber-type switching, obesity, and type 2 diabetes. **Cell Metabolism,** v. 4, n. 5, p. 407-414, 2006a.

SEALE, P.; RUDNICKI, M. A. A new look at the origin, function, and "stem-cell" status of muscle satellite cells. **Developmental biology,** v. 218, n. 2, p. 115-124, Feb 15 2000.

SELAK, M. A. et al. Impaired oxidative phosphorylation in skeletal muscle of intrauterine growth-retarded rats. **Am J Physiol Endocrinol Metab,** v. 285, n. 1, p. E130-137, Jul 2003.

SHELLEY, P. et al. Altered skeletal muscle insulin signaling and mitochondrial complex II-III linked activity in adult offspring of obese mice. **American journal of physiology. Regulatory, integrative and comparative physiology,** v. 297, n. 3, p. R675-681, Sep 2009.

SHI, X. et al. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. **Nature,** v. 442, n. 7098, p. 96-99, Jul 6 2006.

SILVA, K. F.; PRATA, A.; CUNHA, D. F. Frequency of metabolic syndrome and the food intake patterns in adults living in a rural area of Brazil. **Revista da Sociedade Brasileira de Medicina Tropical,** v. 44, n. 4, p. 425-429, Jul-Aug 2011.

SILVADO, C. E.; WERNECK, L. C. Alterations in the gastrocnemius muscle of undernourished suckling rats. **Muscle & nerve,** v. 34, n. 1, p. 72-77, Jul 2006.

SILVEIRA, L. R. et al. Updating the effects of fatty acids on skeletal muscle. **Journal of cellular physiology,** v. 217, n. 1, p. 1-12, Oct 2008a.

SIMS, R. J., 3RD et al. Recognition of trimethylated histone H3 lysine 4 facilitates the recruitment of transcription postinitiation factors and pre-mRNA splicing. **Mol Cell,** v. 28, n. 4, p. 665-676, Nov 30 2007.

SINGHAL, A.; LUCAS, A. Early origins of cardiovascular disease: is there a unifying hypothesis? **The Lancet,** v. 363, n. 9421, p. 1642-1645, 2004.

SMITH, F. M.; GARFIELD, A. S.; WARD, A. Regulation of growth and metabolism by imprinted genes. **Cytogenetic and genome research,** v. 113, n. 1-4, p. 279-291, 2006.

SNOW, M. H. The effects of aging on satellite cells in skeletal muscles of mice and rats. **Cell and tissue research,** v. 185, n. 3, p. 399-408, Dec 19 1977.

SONG, X. M. et al. Muscle fiber type specificity in insulin signal transduction. **The American journal of physiology,** v. 277, n. 6 Pt 2, p. R1690-1696, Dec 1999.

STABELINI NETO, A. et al. Physical activity, cardiorespiratory fitness, and metabolic syndrome in adolescents: a cross-sectional study. **BMC public health,** v. 11, p. 674, 2011.

STARON, R. S. et al. Fiber type composition of four hindlimb muscles of adult Fisher 344 rats. **Histochemistry and Cell Biology,** v. 111, n. 2, p. 117-123, 1999.

STEWART, P. M.; KROZOWSKI, Z. S. 11 beta-Hydroxysteroid dehydrogenase. **Vitamins and hormones,** v. 57, p. 249-324, 1999.

STOPPANI, J. et al. AMP-activated protein kinase activates transcription of the UCP3 and HKII genes in rat skeletal muscle. **American journal of physiology. Endocrinology and metabolism,** v. 283, n. 6, p. E1239-1248, Dec 2002.

STORLIEN, L.; OAKES, N. D.; KELLEY, D. E. Metabolic flexibility. **The Proceedings of the Nutrition Society,** v. 63, n. 2, p. 363-368, May 2004.

TANNER, C. J. et al. Muscle fiber type is associated with obesity and weight loss. **American journal of physiology. Endocrinology and metabolism,** v. 282, n. 6, p. E1191-1196, Jun 2002a.

TANNER, C. J. et al. Muscle fiber type is associated with obesity and weight loss. **Am J Physiol Endocrinol Metab,** v. 282, n. 6, p. E1191-1196, June 1, 2002 2002b.

TE, K. G.; REGGIANI, C. Skeletal muscle fibre type specification during embryonic development. **Journal of muscle research and cell motility,** v. 23, n. 1, p. 65-69, 2002.

THEYS, N. et al. Maternal malnutrition programs pancreatic islet mitochondrial dysfunction in the adult offspring. **The Journal of nutritional biochemistry,** v. 22, n. 10, p. 985-994, Oct 2011.

THEYS, N. et al. Maternal low-protein diet alters pancreatic islet mitochondrial function in a sex-specific manner in the adult rat. **American journal of physiology. Regulatory, integrative and comparative physiology,** v. 297, n. 5, p. R1516-1525, Nov 2009a.

THEYS, N. et al. Early low protein diet aggravates unbalance between antioxidant enzymes leading to islet dysfunction. **PloS one,** v. 4, n. 7, p. e6110, 2009b.

THOMPSON, A. L.; COONEY, G. J. Acyl-CoA inhibition of hexokinase in rat and human skeletal muscle is a potential mechanism of lipid-induced insulin resistance. **Diabetes,** v. 49, n. 11, p. 1761-1765, Nov 2000.

THOMPSON, M. P.; KIM, D. Links between fatty acids and expression of UCP2 and UCP3 mRNAs. **FEBS letters,** v. 568, n. 1-3, p. 4-9, Jun 18 2004.

THOMPSON, N. M. et al. Prenatal and postnatal pathways to obesity: different underlying mechanisms, different metabolic outcomes. **Endocrinology,** v. 148, n. 5, p. 2345-2354, May 2007.

THOMPSON, R. F. et al. Experimental intrauterine growth restriction induces alterations in DNA methylation and gene expression in pancreatic islets of rats. **The Journal of biological chemistry,** v. 285, n. 20, p. 15111-15118, May 14 2010.

THYFAULT, J. P.; RECTOR, R. S.; NOLAND, R. C. Metabolic inflexibility in skeletal muscle: a prelude to the cardiometabolic syndrome? **Journal of the cardiometabolic syndrome,** v. 1, n. 3, p. 184-189, Summer 2006.

TOSCANO, A. E.; MANHAES-DE-CASTRO, R.; CANON, F. Effect of a low-protein diet during pregnancy on skeletal muscle mechanical properties of offspring rats. **Nutrition,** v. 24, n. 3, p. 270-278, Mar 2008.

TOSCHKE, A. M. et al. Childhood obesity is associated with maternal smoking in pregnancy. **Eur J Pediatr,** v. 161, n. 8, p. 445-448, Aug 2002.

TOSH, D. N. et al. Epigenetics of programmed obesity: alteration in IUGR rat hepatic IGF1 mRNA expression and histone structure in rapid vs. delayed postnatal catch-up growth. **American journal of physiology. Gastrointestinal and liver physiology,** v. 299, n. 5, p. G1023-1029, Nov 2010.

TREVENZOLI, I. H. et al. Neonatal hyperleptinaemia programmes adrenal medullary function in adult rats: effects on cardiovascular parameters. **The Journal of physiology,** v. 580, n. Pt. 2, p. 629-637, Apr 15 2007.

TUNSTALL, R. J. et al. Fasting activates the gene expression of UCP3 independent of genes necessary for lipid transport and oxidation in skeletal muscle. **Biochemical and biophysical research communications,** v. 294, n. 2, p. 301-308, Jun 7 2002.

TURNER, B. M. Cellular memory and the histone code. **Cell,** v. 111, n. 3, p. 285-291, Nov 1 2002.

VAAG, A. A. et al. The thrifty phenotype hypothesis revisited. **Diabetologia,** v. 55, n. 8, p. 2085-2088, 2012.

VAN DEN BERG, S. A. et al. Skeletal muscle mitochondrial uncoupling, adaptive thermogenesis and energy expenditure. **Current opinion in clinical nutrition and metabolic care,** v. 14, n. 3, p. 243-249, May 2011.

VAN SPEYBROECK, L. From epigenesis to epigenetics: the case of C. H. Waddington. **Annals of the New York Academy of Sciences,** v. 981, p. 61-81, Dec 2002.

VAN STRATEN, E. M. et al. Sex-dependent programming of glucose and fatty acid metabolism in mouse offspring by maternal protein restriction. **Gender medicine,** v. 9, n. 3, p. 166-179 e113, Jun 2012.

VEGA, R. B.; HUSS, J. M.; KELLY, D. P. The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. **Molecular and cellular biology,** v. 20, n. 5, p. 1868-1876, Mar 2000.

VELASQUEZ-MELENDEZ, G. et al. Prevalence of metabolic syndrome in a rural area of Brazil. **Sao Paulo medical journal = Revista paulista de medicina,** v. 125, n. 3, p. 155-162, May 3 2007.

VICKERS, M. H. et al. Sedentary behavior during postnatal life is determined by the prenatal environment and exacerbated by postnatal hypercaloric nutrition. **American journal of physiology. Regulatory, integrative and comparative physiology,** v. 285, n. 1, p. R271-273, Jul 2003.

VOLLESTAD, N. K.; VAAGE, O.; HERMANSEN, L. Muscle glycogen depletion patterns in type I and subgroups of type II fibres during prolonged severe exercise in man. **Acta physiologica Scandinavica,** v. 122, n. 4, p. 433-441, Dec 1984.

WANG, Y.-X. et al. Regulation of Muscle Fiber Type and Running Endurance by PPARÎ´. **PLoS Biol,** v. 2, n. 10, p. e294, 2004a.

WANG, Y. X. et al. Regulation of muscle fiber type and running endurance by PPARdelta. **PLoS biology,** v. 2, n. 10, p. e294, Oct 2004b.

WATERLAND, R. A. Do maternal methyl supplements in mice affect DNA methylation of offspring? **The Journal of nutrition,** v. 133, n. 1, p. 238; author reply 239, Jan 2003.

WATERLAND, R. A.; MICHELS, K. B. **Epigenetic Epidemiology of the Developmental Origins Hypothesis**. 27**:** 363-388 p. 2007.

WELLS, J. C. The thrifty phenotype hypothesis: thrifty offspring or thrifty mother? **Journal of theoretical biology,** v. 221, n. 1, p. 143-161, Mar 7 2003.

_____. The thrifty phenotype as an adaptive maternal effect. **Biological reviews of the Cambridge Philosophical Society,** v. 82, n. 1, p. 143-172, Feb 2007.

WEST-EBERHARD, M. J. Developmental plasticity and the origin of species differences. **Proceedings of the National Academy of Sciences of the United States of America,** v. 102 Suppl 1, p. 6543-6549, May 3 2005.

WHITE, P. et al. Developmental expression analysis of thyroid hormone receptor isoforms reveals new insights into their essential functions in cardiac and skeletal muscles. **FASEB journal : official publication of the Federation of American Societies for Experimental Biology,** v. 15, n. 8, p. 1367-1376, Jun 2001.

WIGMORE, P. M.; DUNGLISON, G. F. The generation of fiber diversity during myogenesis. **The International journal of developmental biology,** v. 42, n. 2, p. 117-125, Mar 1998.

WOLFF, G. L. et al. Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice. **FASEB J,** v. 12, n. 11, p. 949-957, Aug 1998.

YADA, E.; YAMANOUCHI, K.; NISHIHARA, M. Adipogenic potential of satellite cells from distinct skeletal muscle origins in the rat. **The Journal of veterinary medical science / the Japanese Society of Veterinary Science,** v. 68, n. 5, p. 479-486, May 2006.

YATES, D. T. et al. Developmental programming in response to intrauterine growth restriction impairs myoblast function and skeletal muscle metabolism. **Journal of pregnancy,** v. 2012, p. 631038, 2012.

ZHANG, L. et al. Role of fatty acid uptake and fatty acid beta-oxidation in mediating insulin resistance in heart and skeletal muscle. **Biochimica et biophysica acta,** v. 1801, n. 1, p. 1-22, Jan 2010.

ZHU, M. J. et al. Maternal nutrient restriction affects properties of skeletal muscle in offspring. **The Journal of Physiology,** v. 575, n. 1, p. 241-250, August 15, 2006 2006.

ZHU, M. J. et al. Effect of maternal nutrient restriction in sheep on the development of fetal skeletal muscle. **Biology of reproduction,** v. 71, n. 6, p. 1968-1973, Dec 2004.

ZIERATH, J. R.; HAWLEY, J. A. Skeletal Muscle Fiber Type: Influence on Contractile and Metabolic Properties. **PLoS Biol,** v. 2, n. 10, p. e348, 2004.

ZIMMET, P.; SHAW, J.; ALBERTI, K. G. Preventing Type 2 diabetes and the dysmetabolic syndrome in the real world: a realistic view. **Diabet Med,** v. 20, n. 9, p. 693-702, Sep 2003.

ZINKHAN, E. K. et al. Maternal Hyperglycemia Disrupts Histone 3 Lysine 36 Trimethylation of the IGF-1 Gene. **Journal of nutrition and metabolism,** v. 2012, p. 930364, 2012.

ANEXOS / ANNEXES

Anexo / Annexe A

Subject: APH-2012-10-0255.R1 Differential developmental programming by early protein restriction of rat skeletal muscle according to its fibre type composition
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Re: Differential developmental programming by early protein restriction of rat skeletal muscle according to its fibre type composition

Dear Miss Francisco Bolaños-Jiménez,
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Sincerely

Anexo/ Annexe B

Obesity

Nutritional programming impairs metabolic flexibility of skeletal muscle before any sign of obesity or glucose intolerance

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Anexo / Annexe C

OPEN ACCESS Freely available online

PLOS ONE

Long-Lasting Effect of Perinatal Exposure to L-tryptophan on Circadian Clock of Primary Cell Lines Established from Male Offspring Born from Mothers Fed on Dietary Protein Restriction

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Abstract

Background & Aims: Maternal undernutrition programs metabolic adaptations which are ultimately detrimental to adult. L-tryptophan supplementation was given to manipulate the long-term sequelae of early-life programming by undernutrition and explore whether cultured cells retain circadian clock dysregulation.

Methods: Male rat pups from mothers fed on low protein (8%, LP) or control (18%, CP) diet were given, one hour before light off, an oral bolus of L-tryptophan (125 mg/kg) between Day-12 and Day-21 of age. Body weight, food intake, blood glucose along with the capacity of colonization of primary cells from biopsies were measured during the young (45–55 days) and adult (110–130 days) phases. Circadian clock oscillations were re-induced by a serum shock over 30 hours on near-confluent cell monolayers to follow PERIOD1 and CLOCK proteins by Fluorescent Linked ImmunoSorbent Assay (FLISA) and period1 and bmal1 mRNA by RT-PCR. Cell survival in amino acid-free conditions were used to measure circadian expression of MAP-LC3B, MAP-LC3B-FP and Survivin.

Results: Tryptophan supplementation did not alter body weight gain nor feeding pattern. By three-way ANOVA of blood glucose, sampling time was found significant during all phases. A significant interaction between daily bolus (Tryptophan, saline) and diets (LP, CP) were found during young ($p = 0.0291$) and adult ($p = 0.0285$) phases. In adult phase, the capacity of colonization at seeding of primary cells was twice lower for LP rats. By three-way ANOVA of PERIOD1 perinuclear/nuclear immunoreactivity during young phase, we found a significant effect of diets ($p = 0.049$), daily bolus ($p < 0.0001$) and synchronizer hours ($p = 0.0002$). All factors were significantly interacting ($p = 0.0148$). MAP-LC3B, MAP-LC3B-FP and Survivin were altered according to diets in young phase.

Conclusions: Sequelae of early-life undernutrition and the effects of L-tryptophan supplementation can be monitored non-invasively by circadian sampling of blood D-glucose and on the expression of PERIOD1 protein in established primary cell lines.

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Introduction

Early-life stressors such as maternal undernutrition, overnutrition, hypercholesterolemia, corticosteroid therapy, uteroplacental insufficiency, or hypoxia program metabolic adaptations that initially favor survival but are ultimately detrimental to adult health. In laboratory rodents, low-protein diet during gestation and lactation has been known to reduce the life expectancy of offspring [1]. The maternal protein restriction (5–8% as compared to 18–20% in normal diet) in the rat model of In Utero Protein Restriction is one of the most extensively explored model. The low-protein fed mothers give birth to growth-restricted offspring [2], [3], and when suckled by their mothers maintained on the same low-protein diet, they remain permanently growth-restricted, despite being weaned on a normal diet [4]. Also, early-life undernutrition is associated with higher blood tryptophan levels [5], brain serotonin [6] and impairment of the serotonergic control

of feeding in female adult rats [7]. Recently, we have shown that circadian clock of the hypothalamus is altered in young rats subsequently to perinatal undernutrition [8], however there is no proof that this dysregulation exists in other tissues as well. In rodents, the emergence of circadian clock outputs occur during the first 2 or 3 weeks after birth [9]. The pre and postnatal developments of the molecular clockwork in the rat liver proceed gradually with clock transcript oscillations well-organized after 30 days of life [10]. Early rhythm is entrained by the rhythm in breast feeding and care of the newborns [11]. Apparently, before weaning, peripheral clocks' setting by the feeding regime may prevail upon entrainment by the suprachiasmatic nuclei. Some potentially entraining substrates, like melatonin which derives from L-tryptophan, may be delivered in milk [11]. From human studies, we also know that the circadian rhythm of tryptophan in breast milk affects the rhythms of 6-sulfatoxymelatonin and sleep in newborn [12], [13] and that infant formulas supplemented in L-tryptophan during the night can alter the expression of genes in cerebellum of nursing rat neonates [14]. It has been found that acute supplementation with tryptophan show transitory increase of melatonin plasma levels [15] as well as alteration in insulin secretion [16].

Several interventions (dietary or pharmacological) to reduce the long-term sequelae of early-life programming effects of several stressors have been used in animal models. The administration of folic acid with a low-protein diet during pregnancy prevents the altered phenotype and epigenotype in rat offspring [17], and administration of a diet rich in methyl donors prevents the transgenerational increase in obesity in agouti yellow mice [18]. Some works underline that the timing of such interventions can be crucial. For instance, neonatal leptin treatment which reverses the programming effects of prenatal undernutrition can be reversed with leptin treatment between Day-3 and Day-13 [19]. Here we apply L-tryptophan supplementation from Day-12 of age because Coupé et al [20] have identified extensive changes in gene expression of neurodevelopmental process related to cell differentiation and cytoskeleton organization, in the hypothalamus of rat pups born from low protein-fed mothers. As shown on adult rats [21], a daily bolus of L-tryptophan during 7 days enhances serotonin levels over a 24 hour period, and produces an advance in the peak of serotonin in both plasma and different brain regions. Long-term influence of a daily bolus can be studied on the feeding pattern, growth curves as well as on plasma D-glucose which has been described to follow a circadian rhythm during the development of obesity in rats [22]. Restricted feeding by providing a single meal at the same time each day is changing the daily profiles of PERIOD1 and PERIOD2 protein expression in brain nucleus of rats [23]. To determine whether these alterations can be measured on somatic cells accessible by non-invasive means, we have chosen to establish primary cultures of rat tail. Somatic cells like fibroblasts can be synchronized by a serum shock to re-induce clock gene expression [24] and they are believed to harbor a complete set of clock genes, retaining a function similar to the one observed in the subject [25], [26]. Moreover, primary cultured cells are easily amenable to survival under amino acid-free conditions to follow the microtubule-associated-protein light chain 3b (MAP-LC3B) which is currently the only molecular marker available for following the autophagosome in cells [27], [28], [29].

In this paper we have demonstrated a long-lasting effect of perinatal exposure to L-tryptophan on the blood D-glucose profile of male rats during the young and adult phases. On established primary cell lines, the expression of PERIOD1 protein after serum shock synchronization were different between L-tryptophan and undernourished saline groups with their controls. However, the capacity of colonization at seeding was left unchanged suggesting that developmental metabolic programming related to longevity was not reversed by our tryptophan supplementation.

Materials and Methods

Studies on rats were realized according to the rules of the Nantes animal experimental unit (in compliance with the European Communities Directive of 24 November 1986 (86/609/EEC) and the Principles of laboratory animal care (NIH publication no. 85–23, revised 1985)). The protocol was approved by the « Comité d'éthique pour l'expérimentation animale, Pays de la Loire, France » under number 06 (March 20th, 2011; CEEA.2010.38). Animals were euthanized by carbon dioxide exposure.

Diet, Animal Care and Experimental Design

Ten virgin female Wistar rats were used at the beginning of study (8 weeks, weighing 200–224 g purchased from Janvier, Rennes, France). On arrival, the rats were housed either under a photoperiod 12 h light/dark cycle (lights on at 08:00 to 20:00 h) or a 12 h dark/light (period from 20:00 to 08:00 h) reversed cycle. All handling during the dark period was done under dim red light (<2 lux), in a temperature-controlled (21±1°C) and air conditioned housing room (relative humidity: 60±10%). Animals were kept undisturbed for 2 weeks for adaptation. After confirmation of mating by visualization of spermatozoa in vaginal smears, the dams were housed individually and randomly assigned as low-protein (LP) and control (CP) groups. During all experiments, the animals were maintained with diet and water *ad libitum*. The composition of diets is shown in **Table 1**. Isocaloric (18 g% protein) or low-protein (8 g% of protein) diets were offered during gestation and lactation. Timing of delivery, litter size and pup weight were recorded at birth.

The litters were homogenized for 5–6 males: 3–2 females. The sex was judged according to whether the ano-genital distance was less (female) or greater than (male) around 2.5 mm. The daily records of weight and food (7 h–9 h) allowed to calculate body weight gain, food intake and energy efficiency of dams, energy and protein intake during gestation and lactation. At Day-21, the offspring were weaned and received commercial diet for rodents (Standard diet AO4 16% protein; 4% fibers; 5% minerals and vitamins; 12% humidity and 60% glucides; 2.9 Kcal/g°) until the end of experiments.

Beside neurophysiological consideration [20], we had chosen Day-12 as the first day allowing pup's oral gavage in Wistar rat; this first day may be different with other rat strains. From Day-12 to Day-21, every pup was administered one hour before lights off with a single bolus either of L-tryptophan (125 mg/kg; BioUltra, #93659, Sigma, France) or similar volume of vehicle saline solution (NaCl) by gavage needle. Experimental groups were abbreviated either low protein tryptophan (LPT) and low-protein saline (LPS) or control tryptophan (CTT); control saline (CTS);. Prior to bolus administration, pups were weighed to calculate the exact injection volume to be administered.

Measurement of Food Intake

Pups aged 28–30 days were housed individually in metabolic cages (Charles River). Standard laboratory chow (SAFE, Augy, France), presented in powder form, was available *ad libitum* from a hopper recessed in to the front wall of the cage eliminating fouling of the food with urine and feces. Access to food was restricted to a horizontal slot in the hopper that allowed the rat to eat but not to remove the food. A hollow in the front portion of the

Table 1. Composition of low protein (8 g% protein) and control (18 g% protein) diets.

	Diets (g/kg)			Diets (g%)	
Ingredient	**Low protein**	**Control**	**Nutrient**	**Low protein**	**Control**
Casein (80%)	100	220			
Cornstarch (88%)	610	510	Protein	8.0	18.0
Sucrose	120	100	Carbohydrates	65.6	54.8
Soybean oil	70	70	Lipid	7.0	7.0
Fiber	50	50	Cellulose	5.0	5.0
Mineral Mix (AIN-93G)	35	35	Mineral Mix (AIN-93G)	3.5	3.5
Vitamin Mix (AIN-93G)	10	10	Vitamin Mix (AIN-93G)	1.0	1.0
L-methionin	2.5	3.0	L-methionin	0.2	0.3
Choline bitartrate	2.5	2.5	Choline bitartrate	0.25	0.25
Tert-butylhydroquinon (TBHQ) mg	8	14	Tert-butylhydroquinon (TBHQ) mg	0.8	1.4
Energetic value				357.4	352.6
% energy by protein				9.0	20
% energy by carbohydrate				73	62
% energy by lipid				18	18

The diets were isoenergetic and isolipidic. The quantity of reduced protein was replaced by carbohydrates.
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hopper retained any food spilled out from the hopper. Water was dispensed from a bottle fixed to the front wall. After a habituation period of 7 days, during which the animals attained a stable pattern of feeding, food intake was monitored every 4 h for the next 3 consecutive days.

Total Blood D-glucose

Rats aged 40–50 days and 110–120 days were sampled at the tail of one blood drop to determine the total blood glucose by Accu Chek® Active (Roche-Diagnostics GmbH, Mannheim, Germany).

Biocollection of Primary Cell Lines

Rats aged between 45–55 days and 110–130 days, were aseptically sampled at the tip of the tail (1 mg tissue) at the end of the blood sampling cycle. The primary cells established between 45–55 days will be referred as « young » or "50" days and the cells established between 110–130 days will be referred as "adult" or « 120 days ». The young phase was chosen because it corresponds to the "hyperphagic" phase of undernourished rat pups described by all authors in the field [30]. The adult phase was chosen according to the average mating age of male rat (between 56 and 70 days) and well before the onset of obesity which is described to occur at 17 months [31]. The biopsy was briefly exposed to Javelle water, rinsed twice in a large volume of phosphate-buffered saline solution before exposure to Trypsin-EDTA during 15 min at 37°C. Cellular aggregates were mechanically disaggregated by vigorous pipetting (20 times). Cellular suspensions were layered on top of a cushion of 3 ml DMEM +20% fetal calf serum and centrifuged at 1,300×g for 3 min at room temperature. The cellular pellet was resuspended in 10 ml DMEM medium +10% fetal calf serum, amphotericin-B (1/1000) and gentamicin (100 µg/50 ml) and inoculated in 25 cm^2 flask (Nunc®) in an humidified incubator (37°C, 5% CO_2). Within three to five days, colonies of active cells were seen. In flask inoculated with fast-growing cells, confluency was reached within a week. Cells were resuspended by trypsinization and used to prepare a cryotube (in 95% fetal calf serum with 5% DMSO, stored in a Nalgen box before being kept permanently at −70°C), and to inoculate tissue culture dishes (LabTek, P-96 or 25 cm^2 flask).

Capacity of Colonization at Seeding, Adhesion and Phenotypes of Primary Cells

Freshly trypsinized primary cells from rat tail were inoculated on conventional tissue culture plastics (25 cm2 flask, Nunc®). A week later, primary cultures with actively growing cellular colonies were enumerated to calculate the capacity of colonization at seeding, and subcultured to establish cell lines. On Cytoo chambers (Starter kit; [32], suspension of 60,000 cells from 4 representative fibroblast cell lines were inoculated and left to attach 20 min before changing the medium and allowing over-night adhesion at 37°C, 5% CO2. Cells were fixed and immunolabeled for tryptophan-hydroxylase [33] and Hoechst 33258.

Time-series Experimental Design

Rats were housed in two separate chambers and blood sampled every 4 h between Zeitgeber time (ZT) 0 to 12 and 12 to 24 h. The values obtained on both groups at ZT-12 were not statistically different. Established primary cells were cultured in various vessels (25 cm^2 flask, Nunc, P-96 or LabTek) to obtain time-series of cellular monolayers for immunodetection or RT-PCR every 6 h over 30 hours.

Selection of Biomarkers, Source and Specificity of Primary Antibodies

Period1 is an immediate response gene involved in the quick resetting of the circadian clock (Rabbit polyclonal antibody, Santa-Cruz sc-25362). We targeted CLOCK and PERIOD1, both involved in circadian rhythms. The transcription factor CLOCK was detected by antibodies from Santa-Cruz, sc-25361. CLOCK harbors a Histone-acetyl-transferase activity, and histone acetylation is thought to play a key role in the effects of early nutrition on gene expression, possibly mediating the long-term effects of early

nutrition (nutritional imprinting). Anti-tryptophan-hydroxylase (TPH, Santa-Cruz, sc-30079) antibody was used as tryptophan-hydroxylase is the first and rate-limiting enzyme in the biosynthesis of serotonin. *In situ* detection of microtubule-associated protein light chain 3b (MAP-LC3B) by primary antibodies (Santa-Cruz (sc-28266) has been recommended when this protein constitutive of the autophagosome is overexpressed during progressive autophagy [34]. Rabbit polyclonal anti-survivin (Santa-Cruz, sc-10811) antibody was used to characterize non-apoptotic status of our cells, as survivin is a member of the inhibitors-of-apoptosis protein (IAP) family. Antibodies were used in serial dilution in confocal microscopy according to manufacturers' requirements.

Immunocytochemistry and Fluorescent Linked ImmunoSorbent Assay (FLISA)

Fixed cell monolayers were rehydrated by overnight incubation in phosphate buffered saline solution without Ca++ and Mg++ (PBS0). Incubations of primary antibodies were carried out overnight at 4°C in PBS0 containing 0.2% bovine serum albumin (weight/volume; fraction V, Eurotech). After 3 washing cycles with PBS0, cell preparations were incubated with Hoechst 33258 and secondary antibodies (Goat-anti-rabbit-Alexa-568, Molecular Probes) during one hour at 37°C in PBS0 containing 0.2% bovine serum albumin. After 3 washing cycles with PBS0, cellular preparations were mounted into ProLong Gold (In Vitrogen) and visualized under Leica videomicroscope (x 40 magnification) or Zeiss apotome microscope (x 63 magnification). For quantification, preparations were observed under a Leica fluorescent video microscope (x 40 magnification) with Metamorph software. The intensity of labeling by the primary – secondary antibodies complex was normalized by the total surface of the cellular body at the best plane of acquisition by densitometry with ImageJ 1.42. software as described [35].

Microscopic Detection of Transduced LC3B-FP during Autophagosome Formation

Fluorescent Protein fused to MAP-LC3B transduced on primary cells with a baculovirus vector is also a well accepted approach to monitor autophagy whereby the appearance of fluorescent puncta are indicative of the recruitment of MAP-LC3B to the forming autophagosomes [27], [28], [29]. Autophagosome formation was detected utilizing the Premo Autophagy Sensors (LC3B-FP) BacMam 2.0 kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Prem Autophagy Sensors (LC3B-FP) BacMam 2.0 kit allows transduction of cultured mammalian cells with an MAP-LC3B-fluorescent protein chimera (LC3B-FP), and a negative control fluorescent LC3B protein containing a mutation that renders the protein unable to be processed to form MAP-LC3B-II-FP (LC3B(G120A)-FP. Transduction occurs via an insect Baculovirus vector containing a mammalian promoter. Transduced cells were cultured and treated in the same way as described above. Incorporation of MAP-LC3B-II-FP in cytosolic vacuole was vizualized with a Leica fluorescent videomicroscope (x 40 magnification) or an Apotome Zeiss Microscope (x 63 magnification). Induction of vacuolization in cells was visualized by phase contrast microscopy at 100× magnification.

Quantitative RT-PCR Experiments

We designed forward and reverse primers with Beacon Designer or PerlPrimer [36] software; the specificities were assigned independently on line with the Blast software.

Total RNA was extracted from tryptophan and saline treated fibroblasts collected at 0, 6, 12, 18, 24, and 30 h after a serum shock using the Trizol reagent (Invitrogen, Cergy Pontoise, France), treated with DNAse (RNAse free) for 30 min at 37 °C (Promega). The quality was checked on agarose gels and the quantity determined using a NanoVue^{TM} Plus SpectrophotometerGE Healthcare at 260 and 280 nm. Afterwards, 1 µg of purified RNA was reversed-transcribed using the reverse transcription system (Promega) according to the manufacturer's instructions. Real-time PCR was performed to measure relative mRNA expression in a Bio-Rad iCycler iQ system using the iQ SYBRGreen Supermix PCR kit (Bio-Rad Laboratories) and specific primers. PCR reactions (15 µL) were assayed in triplicate on a 96-well heat-sealed PCR plate (Thermo Scientific). Each reaction contained 7,5 µL SYBR green Supermix, 1,5 µL of forward and reverse primers, and 5 µL of cDNA (1:40 dilution). PCR parameters were: an initial denaturation step of 5 min at 95 °C followed by 45 cycles of 30 s at 95 °C and 30 s at 60 °C. The primers used for the amplification [8] are: *Bmal1* forward 5′CAATGCGATGTCCCGGAAGTTAGA3′, reverse 5′TCCCTCGGTCACATCCCTGAGAAT3′; *Period1* forward 5′TTCGGAGCAGGCAGGTGTC3′, reverse 5′GGCAGGCGAGATGGTGTAGTAG3′ and *18S* forward 5′GATGCGGCGGCGTTATTC3′, reverse 5′CTCCTGGTGGTGCCCTTCC3′ as housekeeping gene. Relative expression levels of *Bmal1* and *Period1 mRNAs* was calculated using the comparative ΔC_T method [37]. Absolute cycle number at threshold for 18S was unchanged by tryptophan treatment.

Data and Statistical Analyses

On data obtained from immunofluorescence imaging, normality of distribution of the intensity of a specific labeling was tested on 30 cells at a 5% level according to Kolmogorov test. Concerning, FLISA values, experimental results are expressed as means +/− s.e.m. With n = four-six cell monolayers per time point. Each cell monolayer was from a different rat.

To test for the presence of circadian rhythms, time series data were first analyzed by three-ways ANOVA using the on line available R package [38] based on classic statistics [39]. All tests were two-tailed and the significance level was set at 5% level. There is no consensus to analyze rhythms, however we have used the methodology of Exploratory Data Analysis (http://www.itl.nist.gov/div898/handbook/index.htm) and TSA Cosinor software (Expertsoft technologies, Esvres, France).

Results

Effects of Protein Restriction during Gestation and Lactation on Body Weight, Food Intake and Energy of Dams

Body weight and body weight gain of LP and CP dams were not different both at the beginning and at the end of experimental observation (**Table 2**). Furthermore we did not observe any alteration in the duration of gestation, the sex ratio (LP = 6.0±1.4; 5.4±1.5; males and females respectively, CP = 6.6±0.5) nor on the litters weight. However, the litters size of LP group was significantly lower than the controls (**Table 2**, $p = 0.021$).

The protein intake (g/day) of LP dams was approximately 44% lower than the CP (LP = 1.6±0.13; CP = 3.7±0.4). No difference between the mean body weight of rat pups at birth was found. This fact can be related to the equivalence of food or protein intakes of all mothers throughout gestation (**Table 3**). However in agreement with previous studies, alterations in food intake and body weight were observed during lactation. During lactation, the

Table 2. Gestational performance of dams fed on low-protein or control diet.

Parameters	Groups		P*
	Low- protein (n = 5)	Control (n = 5)	
Body weight initial (g)	228.8±7.0	234.8±20.13	0.543
Body weight gain (g)	133.9±52.1	164.0±22.8	0.272
Body weight end (g)	362.7±48.9	398.8±34.7	0.215
Duration of gestation	21.0±0.6	20.8±0,4	0.568
Litters size	11.2±1.6*	13.6±0,9	**0.021**
Litters weight (g) at 1 day of life	57.4±3.9	52.7±7.9	0.102

*Unpaired Student's t-test. Overall results are means ±SD, *<0.05.
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mean food intake (g) of LP dams was of 59% of intake of CP dams (LP = 79.8±9.1; CP = 136.0±15.9). This reduction in food intake induced a deficit in energy at the end of the period around 50% (LP = 1756.5±298.2, CP = 3467.5±608.3). The protein intake of LP dams was approximately 75% of the one of CP dams.

The evolution of body weight, food intake and energy of LP dams **(Table 2)** showed a global deficiency in nutrients and calories, since LP dams ingest 30% less carbohydrate (LP = 52.3±6.0; CP = 74.5±8.7 g) and 40% less lipids (LP = 5.6±0.6; CP = 9.5±1.1 g) than CP dams.

The protein intake (g/100 g/day) of LP dams was approximately 44% lower than of CP dams **(Table 3**). The total food intake of LP dams was half of CP dams (LP = 487.9±82.8; CP = 963.2±169.0 g, P<0.01). This reduction in food intake induced a deficit in energy at the end of the period around 50% (LP = 1756.5±298.2, CP = 3467.5±608.3, P<0.001).

At the end of lactation, the dams fed on low protein has 24% less body weight than control dams (LP = 232,7±44.2; CP = 305.7±19.9). The variation of body weight recorded during the lactation period confirms this trend (LP = −22.64±4.6 g and CP = 4.45±3.88 g).

Body Weight, Body Growth and Food Intake of Male Offspring

Body weight gain of low- protein and Control groups were divergent at day-7 independently of tryptophan supplementation (**Figure 1 A**). At weaning, the pups of LP dams were 47% lighter than the control ones (LPT = 27.5±1.4; LPS = 28.3±1.4; CTT = 53.9±3.4; CTS = 53.1±1.3) indicating growth retardation. The daily body weight gain was maximum at the 1st post natal week and decreased thereafter (**Figure 1B**). The daily body weight gain was never higher after post weaning (4th and 5th week) with all groups from low-protein diet.

A daily bolus of L-tryptophan between D-12 and D-21 did not alter the catch-up growth of offspring from weaning to final growth (around ×530% with LP and ×370% with CP). Our results confirm that the post-weaning period (22–40th days of life) is crucial in the compensation of body weight gain of pups suffering of growth retardation. These data are consistent with the temporary hyperphagy previously demonstrated [40]. However the body weight of low protein group (saline or tryptophan) in our experiment remained lower until the end of observations (**Figure 1C**) but the groups did not differ in visceral fat (**Figure 1D**).

The daily food intake of rat pups measured between Day-39 and Day-42 showed that the absolute consumption of the LP group is lower than control group, irrespectively of a supplementation by L-tryptophan **(Figure S1)**. In addition, the relative food intake was higher in the LP group during dark cycle, but it was similar during light cycle (**Figure 2**).

Taken together these observations demonstrate that a daily supplementation of tryptophan between Day-12 and Day-21 did not alter up to 140 days, body weight, body weight gain, and feeding pattern of low-protein or control groups.

Total Blood Glucose Profiles

The total blood glucose profiles were strictly different between the young and adult phases (Note that the range of D-glucose values are between 130 to 170 mg/dL for young (**Figure 3A**) and 105–140 mg/dL for adult (**Figure 3B**) phases). A significant effect of the sampling time was found for the groups of young and adult rats, p = 0.0007 and p<0.0001, respectively by 3-way ANOVA. The absence of interaction between sampling time and other factors indicates that all groups had a representative profile with the maxima of all series at 16 h. By applying Cosinor analysis, we found that the maximum at 16 h was representative of a rhythm for the group of rats fed as control and receiving daily bolus of L-tryptophan (Fourier analysis, autospectral plot and spectral density analysis gave a maximum at 16.7 h). A striking observation was the reversion between CT and CS profiles according to the phases. The CT profile (filled circle in A and B) showed the highest values in the young rats and only low values in the adult rats. The CS profile (white circle in A and B) showed low values in the young rats and the highest values in the adult rats (except at 4 hours). The profiles of undernourished rats remained between the CS and CT profiles.

A significant interaction between daily bolus (L-tryptophan or saline) and diets (Low protein versus Control) were found for the young rats (p = 0.0291 by 3-way ANOVA; **Figure 3C**) and for the adult rats (p = 0.0285; **Figure 3D**). These data show that the metabolic status of low protein as well as of control rats was profoundly altered by the daily bolus of L-tryptophan. By comparing **figures 3C and D**, interactions between factors were reversed indicating that we had selected 2 strictly different phases. By following food intake during 4 consecutive days of observations, we found that only LPT rats had a significantly different profile from others (**Figure 4**).

Observations from **figures 3A and 4** demonstrate that the daily bolus of tryptophan had an effect on the phenotype of rat pups from the low-protein fed mothers during the young phase.

In order to explore the interaction between perinatal L-tryptophan supplementation and perinatal undernutrition of rat pups, we chose to select primary cells from the tip of the tail of every rat. The general aim was to design functional assays with living cells which may be sampled in rats and in humans.

Capacity of Primary Cells to Adhere and Colonize Plastics at Seeding and Diversity of Phenotypes Selected from the Biopsies

During the young phase, the capacity of colonization of primary cells at seeding was of 100%. All cellular preparations successfully prepared gave colonies of actively growing cells colonizing plastics within one week (**Figure 5**). However, we

Table 3. Weekly evolution of body weight gain, food intake, and energy of dams fed on low-protein or control diet during gestation and lactation.

Gestation (weeks)							
Variables	**Low protein Group**			**Control Group**			
	1[a]	**2[a]**	**3[a]**	**1[a]**	**2[a]**	**3[a]**	**P**
Weight gain (%)	12.1	10.9	22.4	13.7	13.7	26.7	<0.001
	±3.5[a]	±6.6[a]	±11.4[b]	±3.6[a]	±2.4[a]	±1.1[b]	
Daily Food intake (g)	24.7	20.2	21.6	18.8±3.0	21.1	23.8	
	±7.6	±4.7	±6.6		±3.5	±1.7	>0.05
Food intake relative (g/100 g)	7.9	7.5	6.2	7.5	7.3	6.6	
	±0.5[a]	±1.5[a]	±1.3[b]	±0.8[a]	±0.8[a]	±0,2[b]	= 0.001
Daily Energy relative (kcal/100 g)	28.4	27.5	24.8	26.6±2.7[a]	26.7	24.2	
	±1.9 [a]	±4.3 [a]	±4.2 [b]		±2.9[a]	±1.9[b]	= 0.035
Protein relative (g/100 g)	0.6±0.05[b]	0.7±0.04[b]	0.6	1.3	1.3	1.2	>0.05
			±0.07[b]	±0.13 [a]	±0.14[a]	±0.10 [a]	
Lactation (weeks)							
Variables	**Low protein Group**			**Control Group**			
	1[a]	**2[a]**	**3[a]**	**1[a]**	**2[a]**	**3[a]**	**P**
Weight variation (g/%)	−12.7	−5.2	−3.6	1.6	−1.1	−1.2	<0.001
	±6.5[b]	±1.8[bc]	±6.8[bc]	±4.3[a]	±3.5[a]	±3.2[a]	
Food intake (g)	26.4[d]	23.3[d]	30.0[e]	37.2 [a b]	46.1 [b c]	52.7[c]	<0.001
	±6.7	[e] ±4.0	±8.3	±12.4	±3.9	±3.7	
Food intake relative (g/100 g)	6.7	10.4	13.5	10. 8	14.9	17.2	
	±1.8[d]	±2.3[e]	±2.2[f]	±1.4[a]	±0.9[b]	±1.4[c]	<0.001
Energy relative (kcal/100 g)	23.9	37.1	48.3	39.0	53.7	63.5	
	±7.2[d]	±8.6[e]	±18.1[f]	±4.5[a]	±3.3[b]	±4.1[c]	<0.001
Protein relative (g/100 g)	0.5[d]	0.8[e]	1.5[f]	1.9[a]	2.60[b]	3.10[c]	<0.001
	(±0.1)	(±0.2)	(±1.0)	±0.2	(±0.2)	(±0.2)	

Two-way ANOVA followed by Bonferronis test. Data correspond to the mean ±DP of each experimental group. Different letters in same line indicate statistically significance.

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have found that the speed of adhesion was slower with cell preparations of the LP rats. To test the homogeneity of cellular preparation and the capacity to adhere to a substratum, we have seeded established cell lines of LP and CP groups onto Cytoo chamber starter kit. The Cytoo kit proposes different preset forms onto plastic substratum pre-coated with fibronectin. After adhesion according to manufacturer's requirements, cells were fixed, and nuclei were revealed by Hoechst staining along with an immunostaining for the expression of tryptophan-hydroxylase. The diversity of cell phenotypes was identical between established cell lines as well as the expression of tryptophan-hydroxylase (**Figure S2**). However the number of cells able to adhere to fibronectin substratum from the low protein groups (receiving a daily bolus of L-tryptophan or of saline solution) was lower (percentage of sites with a cell: 23.6% ±7.6% than for the cells isolated from rats of control group (percentage of sites with a cell: 65.9% ±4.9%) confirming observation at the onset of primary cultures. Primary cells collected from adult rats were significantly less able to colonize plastics after one week in culture than corresponding cells from young rats (**Figure 5**).

Together these data indicate that rats derived from LP dams were less prone to give rise to primary cell cultures after the young phase, irrespectively of a daily bolus of L-tryptophan. In the following experiments, we have chosen to focus on the fastest growing cell lines derived from each group of rats. Cell monolayers were studied either after synchronization to follow the circadian expression of tryptophan-hydroxylase, PERIOD1, CLOCK or under conditions of starvation to follow the circadian expression of

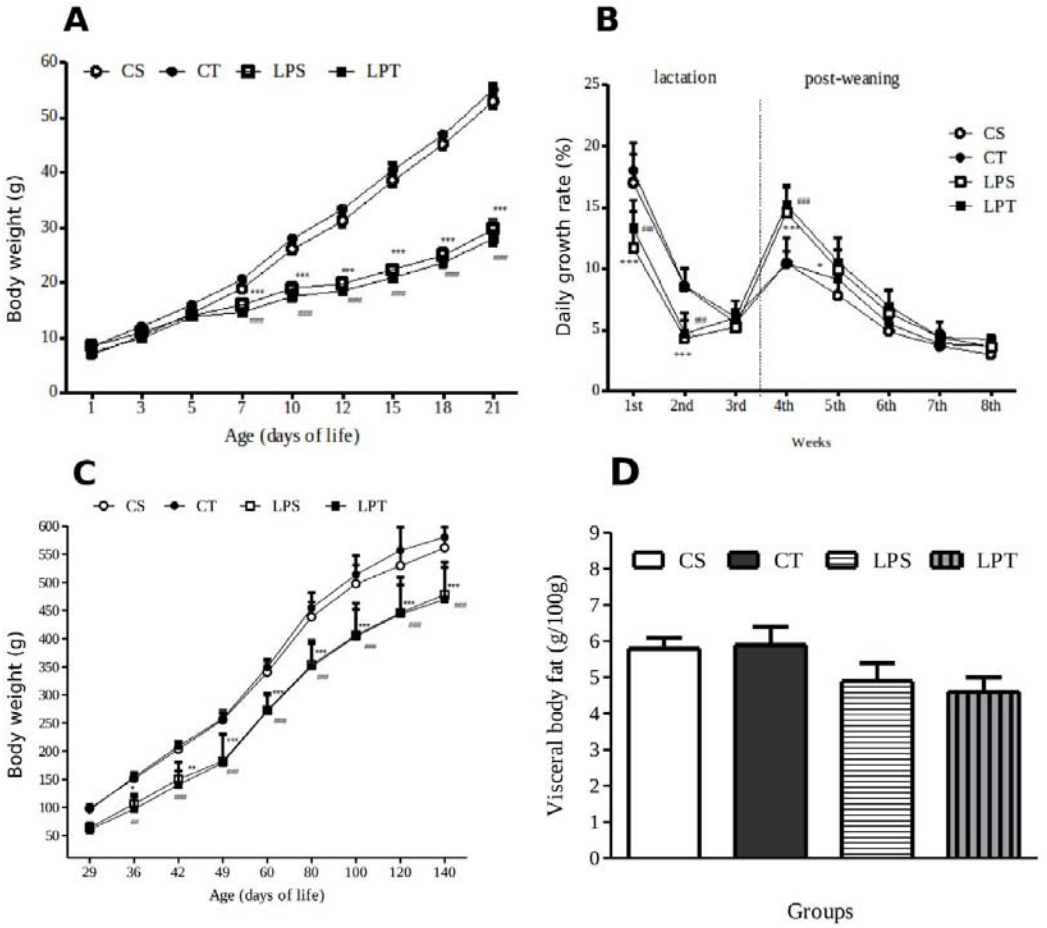

Figure 1. Growth of offspring and amount of visceral fat at sacrifice. Evolution of body weight (A) and daily growth rate (B) of offspring receiving a daily bolus of tryptophan or of saline solution from Day-12 to 21 of age. Evolution of body weight of offspring after weaning (C) and visceral fat at day 140 (D). Four groups of rat pups are shown referred as LPS = Low-protein saline (n = 9); LPT = low-protein tryptophan (n = 9). Body weight s' gain of low protein and control groups were divergent at day-7 and remained so independently of tryptophan supplementation. Data are expressed as means and ±SEM. *P<0.05; **P<0.01; ***P<0.001 by two-way ANOVA followed by Bonferroni test. (*LPS vs CS and #LPT vs CT). The body weight of offspring (n = 42) after weaning until 140-old age remained lower until the end of experiment (C) with similar visceral fat (g/100 g) at sacrifice (D). Data are expressed as means ±SEM. CS = control saline (n = 12); CT = control tryptophan (n = 12); LPS = Low-protein saline (n = 9); LPT = low-protein tryptophan (n = 9). *P<0.05; **P<0.01; ***P<0.001 by RM two-way ANOVA followed by Bonferroni test (*LPS vs CS and #LPT vs CT.).
doi:10.1371/journal.pone.0056231.g001

autophagic (LC3-B, Survivin) biomarkers and of PERIOD1 proteins.

Expressions of Tryptophan-hydroxylase and CLOCK Proteins Over 30 Hours after a Serum Shock

We did not find any difference over time in the expression of tryptophan-hydroxylase as well as of CLOCK proteins by primary cultured cells of all experimental groups (unshown results).

Expression of PERIOD1 Protein Over 30 Hours after a Serum Shock

Synchronization by fetal calf serum could be checked by the localization of PERIOD1 proteins in nuclear or perinuclear sites for all examined cells at 6 h (**Figure 6 A**). Cycling expression of PERIOD1 proteins were shown by the clear-cut nuclear labeling obtained at 6 and 30 h (**Figure 6 A**). By confocal image analysis made on the best plane of nuclear PERIOD1 labeling, we have shown that the profiles of PERIOD1 nuclear localization were different between all groups. A range of 31 to 74 nuclei were measured for the intensity of PERIOD1 from at least 3 cell lines

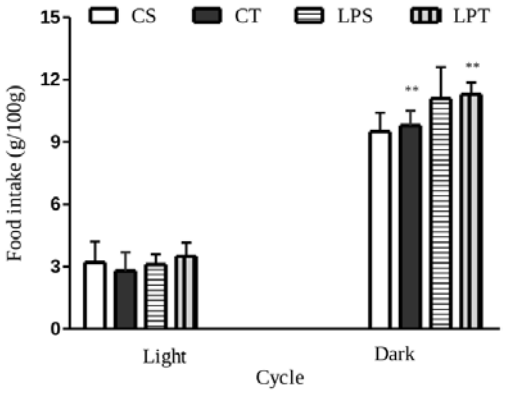

Figure 2. Relative food intake of pups between day-39 and day-42, from dams fed on low-protein or control diet during perinatal period. Rat pups received a daily bolus of L-tryptophan or Saline from day-12 to day-21. On rat pups from mothers fed on low protein diet, means of food intake measured in dark cycle were significantly different from control, irrespectively to L-tryptophan supplementation (Low-Protein Tryptophan (LPT, n = 9 pups); Low protein Saline (LPS, n = 9 pups); Control Tryptophan (CT, n = 12 pups), Control Saline (CS as white) *P<0.05 ***P<0.0001 by one way ANOVA followed by Bonferroni test. Data are expressed as means ±SEM (*LPS vs CS and #LPT vs CT.).
doi:10.1371/journal.pone.0056231.g002

per group (**Figure 6 B**). By 3 Way ANOVA, we found a significant effect of diets ($p = 0.049$), of daily of bolus of L-tryptophan ($p<0.0001$) and of Zeitgeber hours ($p = 0.0002$). All factors were significantly interacting ($p = 0.0148$) indicating that PERIOD1 profiles of LPT, LPS and CT were strictly different from CS profile. In parallel we have performed by FLISA the analysis of the total intensity of PERIOD1 labeling and we did not obtain any difference in the profiles of PERIOD1 immunolabeling when integrating cytoplasmic and nuclear labeling (**Figure S3**).

Evolution of Period1 Transcript Over 30 Hours after a Serum Shock

As shown **Figure S4**, the profiles of period1 mRNA as well as of bmal1 mRNA of primary cells collected from control-fed rats were not different from the one obtained with control-fed rats supplemented with L-tryptophan.

Expression of Autophagic Biomarkers (MAP-LC3B and Survivin) Over 30 Hours of Starvation

Near-confluent cell monolayers were rinsed and exposed to phosphate-buffered saline solution supplemented with 1% fetal cal serum following Chiou et al (2011) [41]. Time series were realized to follow the level of expression of MAP-LC3B and Survivin over 2, 6, 12, 18, 24 and 30 h by using FLISA (**Figure S5**). Expression of MAP-LC3B and Survivin did not differ according to the daily bolus of L-tryptophan. Significant differences were found between cell lines isolated from rats with LP dams and from rats with control dams on the density of cells (**Figure S5**) and on the total intensity of immunolabeling (**Figure 7 A**) during the young phase. The expression of Survivin was following a similar pattern during the young phase (**Figure 7 A**). However the profiles of MAP-LC3B and Survivin obtained on primary cells established during the adult phase were different (**Figure 7 B**).

In parallel experiments, we have used a baculovirus vector to study the expression of MAP-LC3B-FP in the autophagosome corresponding to the form II of MAP-LC3B. Primary cells were found to be easily infected by baculovirus construction in the range of 80–90% positive cells per culture. Representative cell lines of each group were submitted to starvation during 4 hours before recording the typical localization of LC3B in autophagosomes (**Figure S6**) or submitted to one hour serum shock followed by 4 hours starvation (unshown results). We have found that the expression of red fluorescent punta were qualitatively higher in cell lines isolated from control-fed rats than in undernourished rats (**Figure S6 C and D**) according to our quantification realized by FLISA (**Figure 7**). However, we cannot confirm the later points as the fluorescence of our baculovirus construction was quenched after 4 hours in culture by repetitive recording of fluorescence. Under starving conditions, we did not show any difference in the level of PERIOD1 immunoreactivity between these cell lines.

Discussion

L-tryptophan supplementation was given to manipulate the long-term sequelae of early-life programming by undernutrition. We found that the effects of this dietary intervention can be monitored non invasively by circadian sampling of blood D-glucose. Expressions of PERIOD1 protein by synchronized primary cell lines established from young rats were altered according to diet and L-tryptophan supplementation. However the capacity of colonization at seeding and the adhesion potentials of primary cells were clearly altered in rats born from mothers fed on dietary protein restriction, irrespectively of the supplementation with L-tryptophan.

The body weight means of low-protein groups (tryptophan or saline) in our experiment remain lower than control groups until the end of the observation period (**Figure 1C**), but, the weight of visceral fat of undernourished offspring was similar to control group at the end of experiment (**Figure 1D**). L-tryptophan supplementation between D-12 and D-21 did not alter the catch-up growth nor the absolute food consumption of male offspring from weaning to the end of growth period ($22–50^{th}$ days of life). The daily food intake of rat pups measured between Day 39 and Day-42 showed that the food consumption of the LP group is lower than the CP group irrespectively of a supplementation by L-tryptophan (**Figure S1**). These observations on nursing rats with offspring are similar to results obtained in lactating sows [16]. In addition, the relative food intake was higher for the LP group during the night cycle but the relative intakes of both groups were similar during the light cycle (**Figure 2**). These data are consistent with the temporary hyperphagy that we previously demonstrated in undernourished rat pups [40].

A daily bolus of L-tryptophan between D-12 and D-21 did alter the level of D-glucose both in LPT and CPT groups (**Figure 3**). Our D-glucose profiles displayed a maximum around 16 h Zeitgeber time like the profiles obtained on plasma D-glucose titration [22]. However a significant circadian rhythm of D-glucose oscillation was only obtained with the control-fed group receiving a perinatal bolus of L-tryptophan. Results shown on **Figures 3 and 5** suggest that there are a long-term effect of tryptophan supplementation on rats enduring perinatal undernutrition as well as on control-fed rats. As shown on **Figure 4**, we found a significant difference on the cycle of food intake during 4 consecutive days of observations on rats exposed to perinatal undernutrition and receiving a daily bolus of L-tryptophan. These results are suggesting that our supplementation triggered discrete phenotypic alterations. We think that our results indicate that milk

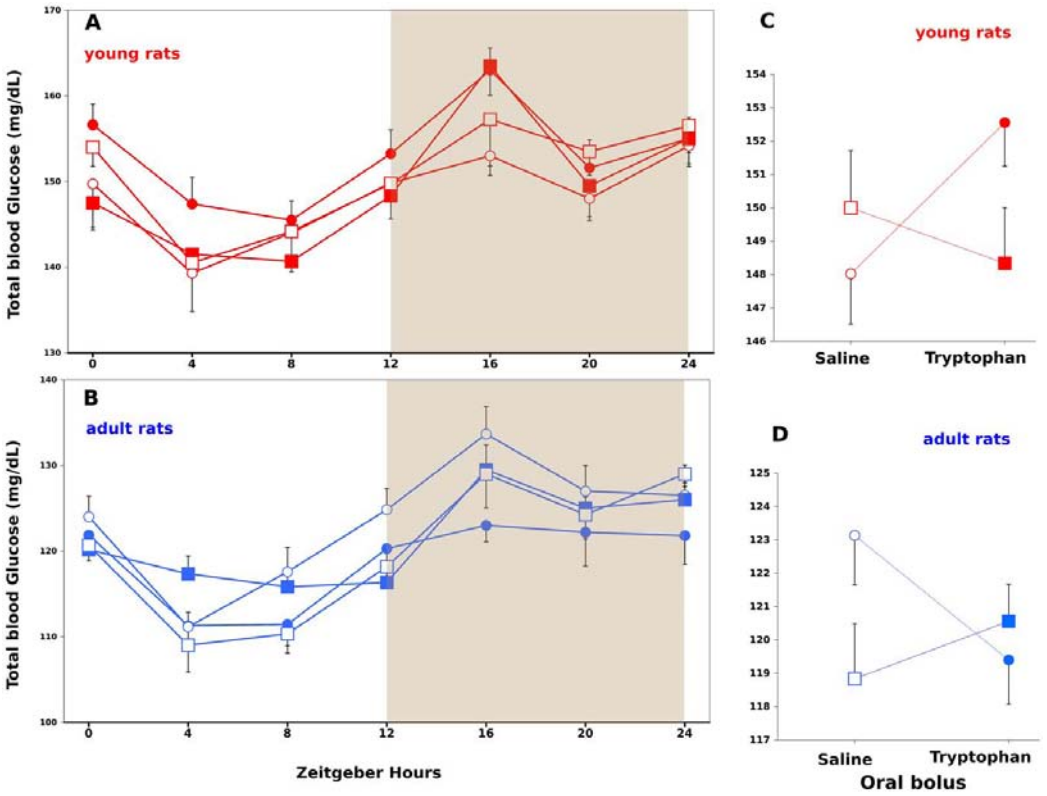

Figure 3. Evolution of total blood glucose over 24 hours of rats sampled during young (red) and adult (blue) phases. Rats were from mothers fed on Low protein diet (square) with bolus of L-tryptophan (filled, LPT) or without (white, LPS) and Control diet (circle) with bolus of L-tryptophan (filled, CT) or without (white, CS). Data are expressed as means ±SEM. By three-way ANOVA we found a significant effect of the Zeitgeber (Hours) on both time series ($p<0.001$; A & B) and a significant interaction between Diet (Low protein, Control) and daily bolus (L-tryptophan or saline) for young phase ($p=0.0291$; C) and for the adult phase ($p=0.0285$; D). Note that interactions between factors shown on C and D are reversed. By applying Cosinor analysis, we found that the maximum at 16 h was representative of a rhythm for the group of rats fed as control and receiving daily bolus of L-tryptophan (Fourier analysis, autospectral plot and spectral density analysis gave a maximum at 16.7 h for CT series on A).
doi:10.1371/journal.pone.0056231.g003

formulas designed to improve sleep-wake cycles of babies have to be tested on rat models under several conditions of feeding to check for global phenotypic consequences. Beside oral gavage, L-tryptophan supplementation has to be tested from birth in formulated milk by using gastrostomized rat pups [42] or with subcutaneous or intraperitonal injections. In any case, subsequently to our experiment, testing formula fortified with L-tryptophan on cerebellum gene expression of nursing rat neonates [14] is clearly insufficient.

To explore whether our supplementation with L-tryptophan interacted with the influence of perinatal undernutrition on male somatic cells, we have selected primary cells from the tip of the tail of every rat. We have tested the capacity of these primary cells to adhere and colonize plastics (**Figure 5**) and the diversity of phenotypes selected from the biopsies (**Figure S2**). Together these data indicate that adult rats derived from LP dams were less prone to give rise to primary cell cultures, irrespectively of a daily bolus of L-tryptophan. This influence of maternal undernutrition on rat pups is in line with previous works on mice reporting that SIRT1 expression was reduced and many insulin-related signaling molecules were altered [43] explaining a reduction in longevity. Tryptophan supplementation has clearly the potential to alter clock-related dysregulation but it is not sufficient to revert the reduction in longevity related to perinatal undernutrition.

A daily bolus of L-tryptophan had a profound effect on the profiles of PERIOD1 protein expression for both diets (**Figure 6A and 6B**). Our microscopic approach is taking advantage of confocal imaging to trace the distribution of PERIOD1 in the different cellular compartments. The re-induction of PERIOD1 protein expression in our primary cells observed between 6 and 18 h was similar to the PERIOD1 reactivity described in rat brain region between 6 and 13 h [**23**]. By focusing on the perinuclear and nuclear localization of PERIOD1, we have been able to appreciate the level of synchronization of our cells (PERIOD1 can be detected during transit at the perinuclear and nuclear membranes locations) as well as the total nuclear intensity of expression according to previously described methods [23], [35]. A daily bolus of L-tryptophan had a profound effect on the profiles of

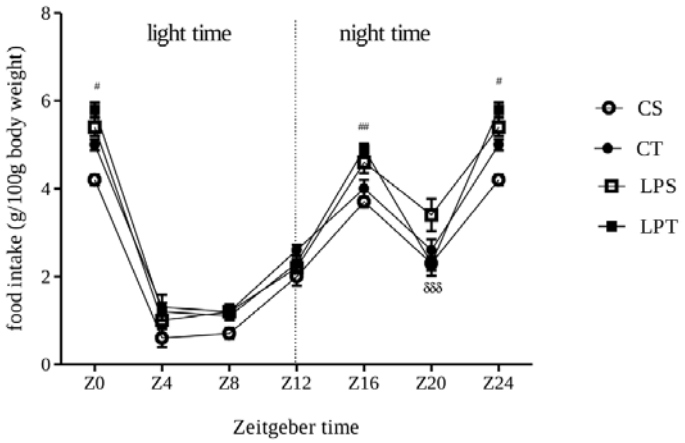

Figure 4. Daily percentages of food intake of rats during 4 days of observation of consumption every 4 h. The litter mate (n = 38) derived from dams fed control or low protein diet during perinatal period. The pups received L-tryptophan or saline (125 mg/kg body weight) between Day-12 and 21. Only litter mates LPT (45–55 old age) showed difference on the cycle of food intake during 4 consecutive days of observations. Data (g/100 g body weight) are expressed as means ±SEM. *#p<0.05 by two-way ANOVA followed by Bonferroni test.; *LPS X CT and CS; #LPT X LPS.
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PERIOD1 protein expression for both diets. These results are in line with our previous work indicating that perinatal undernutrition alters the circadian expression of period1 mRNA of hypothalamus of young rats [8].

The environmental synchronizers are integrated by response elements located in the promoter region of period genes that drive the central oscillator complex. The period genes are also members of the immediate early gene family because cells like human normal fibroblasts exposed to cycloheximide, an inhibitor of transcription, retain a response toward stressful conditions characterized by a dramatic increase in PERIOD proteins [***44***]. As shown on **Figure S4**, the expression profiles of period1 and bmal1 mRNA by cells collected from control-fed rats were not different from the ones obtained with control-fed rats supplemented with L-tryptophan suggesting that these alterations may be at the protein level, further works with cycloheximide are needed to clarify this point. The promoters of period1 and 2 genes (but not of period3) contain a cAMP-responsive element (called CRE) that binds to CREB proteins. These CRE sites are integrating the cAMP response to a wide category of synchronizers (like serotonin, glutamate, calcium ions, and light) as well as the response to a second wide category of synchronizers (like growth factors, hormones, and cytokines) acting through the extracellular signal regulated kinase leading to the mitogen-activated kinase pathways, independently of the CLOCK: BMAL1 activity [45]. We have used a serum shock to re-induce clock machinery; experiments are scheduled to explore which specific pathways are dysregulated by using molecular compounds like dexamethasone, Forskolin, dibutyryryl cAMP, phorbol-12-myristate, calcimycin, epidermal growth factor, insulin, or fibroblast growth factor.

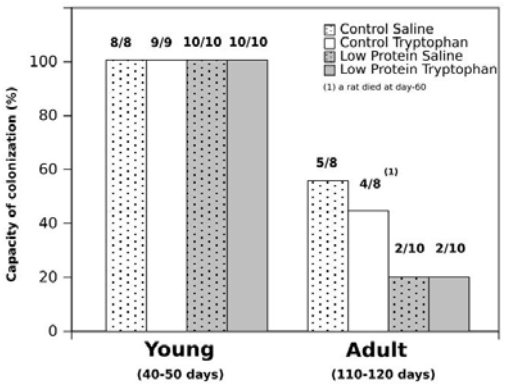

Figure 5. Adhesion and colonization of primary cells on conventional plastics according to age. Cells were isolated by trypsinization from tail biopsies within 7 days of culture. Identical capacity to rise primary cultures were found for rats whatever their mother's diet and perinatal treatment during the young phase. A significant loss in the capacity of colonization (p<0.05) was found for cellular preparations obtained from undernourished adult rats whatever the perinatal treatment (with or without a daily bolus of L-tryptophan).
doi:10.1371/journal.pone.0056231.g005

The expression of autophagic biomarkers (MAP-LC3B and Survivin) over 30 hours of starvation (**Figure 7**, and for monolayer microscopic observations **Figure S5**) were suggesting that a daily bolus of L-tryptophan did not alter the autophagic machinery of primary cells but that the phenotypes derived during the hyperphagic phase from rats enduring a perinatal malnutrition had deeply altered autophagic machinery. Similar cellular phenotypes obtained during the prediabetic phase (110–130 days) did not show similar deregulation indicating that the alteration of autophagic machinery was only transient. Reversibility of molecular alterations induced in living cells by early-life nutritional stress is a major drawback to the long-term monitoring of the sequelae of early-life programming effects, especially by non-invasive means. The lower lifespan of rats whose dams fed low-protein diet during perinatal period had been shown in earlier studies [46] but the

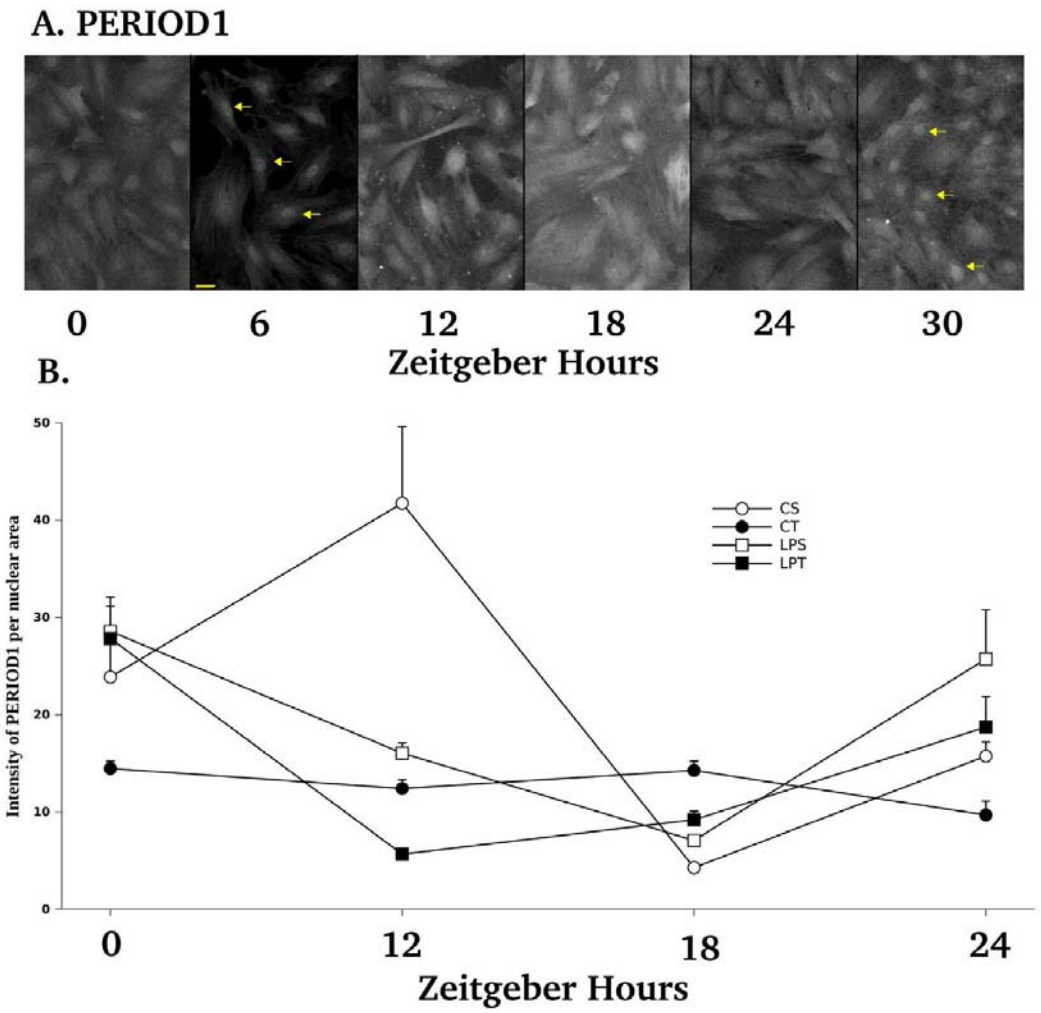

Figure 6. Immunodection of PERIOD1 on primary cell monolayers from young rats over 30 hours after a 2 h serum shock according to diets and tryptophan supplementation. A. Expression of PERIOD1 was found localized in the nucleus (yellow arrows) at 6 and 30 h after serum shock. Consistent observations of the nuclear localization of PERIOD1 at 6 h were indicative of correct cell synchronization by the serum shock. Re-occurence of nuclear staining at 30 h showed PERIOD1 cycling. The yellow bar at bottom of 6H plane stands for 10 μm. B. Quantification of nuclear PERIOD1 staining on confocal images of cellular monolayers by using the Hoechst staining to delineate nuclei area and to integrate PERIOD1 staining. By three-way ANOVA, we found a significant effect of diets ($p = 0.0490$), of daily bolus of L-tryptophan ($p < 0.0001$) and of Hours ($p = 0.0002$). All factors were significantly interacting ($p = 0.0148$). Data are expressed as means ±SEM. A range of 31 to 74 nuclei were measured for the intensity of PERIOD1 from at least 3 cell lines per group.
doi:10.1371/journal.pone.0056231.g006

underlying mechanisms remain unclear [47]. The potential factors have been investigated as oxidative injury in key tissues [48] and telomere shortening [49], [50]. During the young phase, high blood glucose levels were indeed observed at ZT-0 in control tryptophan and control saline groups but could not be related to food intake. However, during the adult phase, we have found a shift in profiles (at ZT-16 the maxima were lower for both groups). These results indicate that there are intimate interactions between the clockwork and the cellular metabolism. In the future, we could realize epigenetic profiling of each cell line to dissect the molecular cascades altered relatively to the original rat diet. Another avenue of research is to establish primary cell lines of embryos from Low-protein or control-fed mothers to check for some difference at the onset of period1 gene regulation by CLOCK:BMAL1 activity in relation to autophagy.

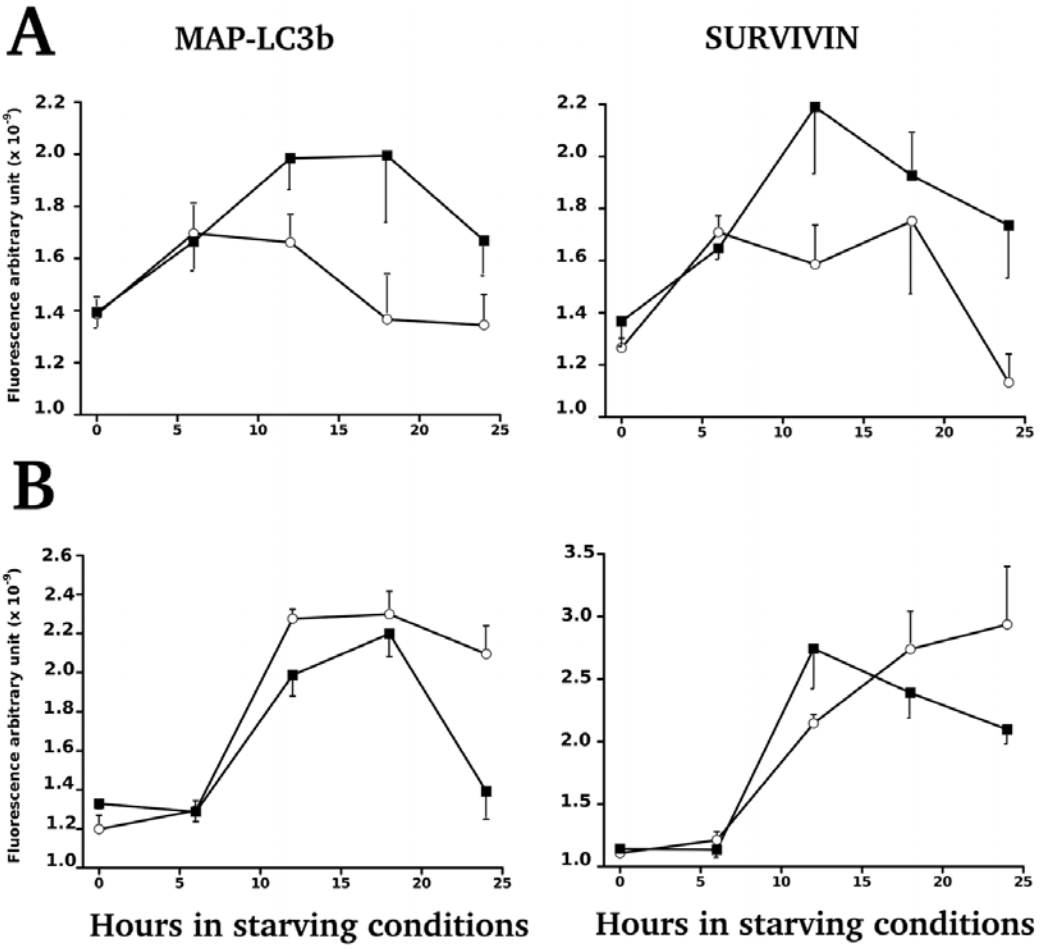

Figure 7. Expression of MAP-LC3B and Survivin by primary cells submitted to starvation. A. Primary cell cultures established during the young phase from rats fed either a Low protein (black square) or control diet (white circle). By two-way ANOVA, we found a significant effect of diet ($p = 0.0005$) and of duration of starvation ($p = 0.0343$) for MAP LC3B, even if Survivin expressions were following a similar trend they were not statistically significant. B. Primary cell cultures established from adult rats, fed either a Low protein (black square) or control diet (white circle). Data are expressed as means ±SEM. By two-way ANOVA, we found a significant effect of diet ($p = 0.0173$) and of duration of starvation ($p<0.0001$) for MAP LC3B with a significant interaction of both factors ($p = 0.038$). Only the interaction of both factors for Survivin was significant ($p<0.0001$).
doi:10.1371/journal.pone.0056231.g007

In conclusion, our results demonstrate that the young phase is characterized by transient behavior and metabolic variations which can be traced at the molecular level on living cells. The general aim was to design functional assays with living cells which may be sampled in long term experiments under similar conditions as ours or with humans by non-invasive means like skin fibroblasts [26], hair follicles [51], urinary cells [52] or exfoliated cells of gastric [35], or nasal [53] epitheliums. The availability of 50 primary cell lines retaining nutritional stress-related alterations in PERIOD1 expression open the way to design functional assays on living cells on the dynamic of the circadian epigenome [54] like determining if the profile of H3K9/K14 histone acetylation [55] in fibroblasts is comparable to the one found in neurons.

Supporting Information

Figure S1 Daily food intake of pups between day-39 and day-42, from dams fed on low-protein or control diet during perinatal period. Rat pups received a daily bolus of L-tryptophan or Saline from day-12 to day-21. On rat pups from mothers fed on low protein diet, daily food intake was significantly different from control, irrespectively to L-tryptophan supplementation (Low-Protein Tryptophan (LPT, n = 9 pups); Low protein Saline (LPS, n = 9 pups); Control Tryptophan (CT, n = 12 pups), Control Saline (CS as white) *$P<0.05$ ***$P<0.0001$ by one way ANOVA followed by Bonferroni test. Data are expressed as means ±SEM (*LPS vs CS and #LPT vs CT.).

(TIF)

Figure S2 Morphology of representative primary cells, all expressing tryptophan hydroxylase (red) on Cytoo well preset with a wide array of forms. Similar diversity of phenotypes (A to E) was found on primary cells isolated from rat submitted to perinatal undernutrition or not. Supplementation with a bolus of L-tryptophan did not alter the phenotypes.
(TIF)

Figure S3 Total intensity of PERIOD1 proteins expressed by primary cells of young (A) and adult (B) rats. Data are expressed as means ±SEM. Note that we did not find any difference between these cell lines (n = 4 to 6 cell lines per point).
(TIF)

Figure S4 Evolution of mRNA expression of period1 (circle) and bmal1 (triangle) after a serum shock of cell lines obtained from at least 3 rats of the control group receiving a daily bolus of L-tryptophan (closed symbol) or a saline solution (open circle). Expression of circadian clock Per1 (A) and BMAL1 (B) transcripts in primary cultures from tryptophan (black square) and saline (white circle) rat offspring from mother fed on control diet. The transcript levels at 6 h intervals were measured by quantititative PCR and synchronized to time 0 h by fetal calf serum. Graphs represent the relative transcriptional level of genes averaged over at least 4 independent samples isolated by offspring of 45 d old derived from dams fed control diet and supplemented or not wit L-tryptophan early 12 d old at 21 d old. Each point corresponds to the means ±S.E.M. expression levels of 4–6 cells by groups (two-way ANOVA followed by Bonferroni test).
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Figure S5 Expression of MAP LC3B protein in representative cultures of primary cells from rats with perinatal undernutrition or control-fed. Autophagosomes were clearly labeled after 6 under starving conditions (yellow arrows). Note that the density of cells are equivalent up to 12 h, thereafter the density of cells isolated from the rat with perinatal undernutrition is higher than the density of control. All cultures were made of surviving cells at 30 h and were not used for quantification.
(TIF)

Figure S6 Expression of the chimeric LC3B-FP protein after infection of primary cells by a baculovirus construction and 4-hour starvation. Cells were isolated during the hyperphagic period from undernourished rats receiving daily bolus of L-tryptophan (A) or saline solution (B) and from control-fed rats receiving a daily bolus of L-tryptophan (C) or saline solution (D). Note that the number of autophagosomes labeled in red (yellow arrowheads) are equivalent between infected cells.
(TIF)

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Author Contributions

Conceived and designed the experiments: EN OG ND RA GP FB BK. Performed the experiments: EN OG ND RA BK. Analyzed the data: EN BK. Contributed reagents/materials/analysis tools: EN SL RM BK. Wrote the paper: EN BK.

References

1. Zamenhof S, van Marthens E (1982) Effects of prenatal and chronic undernutrition on aging and survival in rats. J Nutr 112(5): 972–7.
2. Snoeck A, Remacle C, Reusens B, Hoet JJ (1990) Effect of a low protein diet during pregnancy on the fetal rat endocrine pancreas. Biology of the Neonate 57 (2): 107–118.
3. Fernandez-Twinn DS, Wayman A, Ekizoglou S, Martin MS, Hales CN, et al. (2005) Maternal protein restriction leads to hyperinsulinaemia and reduced insulin-signaling protein expression in 21-mo-old female rat offspring. American Journal of Physiology Regulatory Integrative and Comparative Physiology 288(2): R368–R373.
4. Desai M, Crowther NJ, Lucas A, Hales CN (1996) Organ-selective growth in the offspring of protein-restricted mothers. British Journal of Nutrition 76(4): 591–603.
5. Manjarrez G, Contreras JL, Chagoya G, Biochem E, HernBndez-RJ (1998) Free tryptophan as an indicator of brain serotonin synthesis in infants. Pediatric Neurology 18(1): 57–62.
6. Huether G, Thömke F, Lothar A (1992) Administration of tryptophan-enriched diets to pregnant rats retards the development of the serotonergic system in their offspring. Developmental Brain Research 68(2): 175–181.
7. Pôrto LCJ, Sardinha FLC, Telles MM, Guimarães RB, Albuquerque KT, et al. (2009) Impairment of the serotonergic control of feeding in adult female rats exposed to intra-uterine malnutrition. British Journal of Nutrition 101: 1255–1261.
8. Orozco-Sólis R, Matos RJ, Lopes de Souza S, Grit I, Kaeffer B, et al. (2011) Perinatal nutrient restriction induces long-lasting alterations in the circadian expression pattern of genes regulating food intake and energy metabolism. International Journal of Obesity 35: 990–1000.
9. Vallone D, Lahiri K, Dickmeis T, Foulkes NS (2007) Start the Clock! Circadian Rhythms and Development Developmental Dynamics 236: 142–155.
10. Sládek M, Jindráková Z, Bendová Z, Sumová A (2007) Postnatal ontogenesis of the circadian clock within the rat liver. Am J Physiol Regul Integr Comp Physiol 292: R1224–R1229.
11. Sumova A, Bendova Z, Sladek M, El-Hennamy R, Laurinova K, et al. (2006) Setting the biological time in central and peripheral clocks during ontogenesis FEBS Letters 580: 2836–2842.
12. Cubero J, Valero V, Sanchez J, Rivero M, Parvez H, et al. (2005) The circadian rhythm of tryptophan in breast milk affects the rhythms of 6-sulfatoxymelatonin and sleep in newborn. Neuroendocrinol Lett 26(6): 657–661.
13. Cubero J, Narciso D, Terron P, Rial R, Esteban S, et al. (2007) Chrononutrition applied to formula milks to consolidate infants' sleep/wake cycle. Neuroendocrinol Lett 28(4): 360–366.
14. Puigjaner J, Fabrega J, de Diego I, Subirada F, Durany O, et al. (2007) Two circadian infant formulas produce differential cerebellum gene expression in lactating rat neonates. Genes Nutr 2: 129–131; doi 10.1007/s12263-007-0037-2.
15. Esteban S, Nicolaus C, Garmundi A, Rial RV, Rodríguez AB, et al. (2004) Effect of orally administered L-tryptophan on serotonin, melatonin, and the innate immune response in the rat. Mol Cell Biochem 267(1–2): 39–46.
16. Mosnier E, Le Floc'h N, Etienne M, Ramaekers P, Sève B et al. (2010) Low feed intake of lactating primiparous sows is associated with high insulin with dietary tryptophan resistance during the peri partum period and is not modified through supplementation. J Anim Sci 88(2): 612–25.
17. Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC (2005) Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. Journal of Nutrition 135(6): 1382–1386.
18. Waterland RA, Travisano M, Tahiliani KG, Rached MT, Mirza S (2008) Methyl donor supplementation prevents transgenerational amplification of obesity. International Journal of Obesity 32(9): 1373–1379.
19. Vickers MH, Gluckman PD, Coveny AH, Hofman PL, Cutfield WS, et al. (2005) Neonatal leptin treatment reverses developmental programming. Endocrinology 146(10): 4211–4216.
20. Coupé B, Amarger V, Grit I, Benani A, Parnet P (2010) Nutritional programming affects hypothalamic organization and early response to leptin. Endocrinology 151: 702–713.
21. Mateos SS, Sanchez CL, Paredes SD, Barriga C, Rodriguez AB (2009) Circadian levels of serotonin in plasma and brain after oral administration of tryptophan in rats. Basic Clin Pharmacol Toxicol 104(1): 52–59.
22. Sukumaran S, Xue B, Jusko WJ, Dubois DC, Almon RR (2010) Circadian variations in gene expression in rat abdominal adipose tissue and relationship to physiology. Physiol Genomics 42A: 141–52.

23. Verwey M, Amir S (2011) Nucleus-specific effects of meal duration on daily profiles of PERIOD1 and PERIOD2 protein expression in rats housed under restricted feeding. Neuroscience 192: 304–311.
24. Balsalobre A, Damiola F, Schibler U (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell 93: 929–937.
25. Yagita K, Tamanini F, van der Horst G, Okamura H (2001) Molecular mechanisms of the biological clock in cultured fibroblasts. Science 292: 278–281.
26. Brown SA, Fleury-Olela F, Nagoshi E, Hauser C, Juge C, et al. (2005) The period length of fibroblast circadian gene expression varies widely among human individuals. PLoS Biol 3(10): e338. doi:10.1371/journal.pbio.0030338.
27. Pera MF, Trounson AO (2004) Human embryonic stem cells: prospects for development. Development 131: 5515–5525.
28. Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, et al. (2000) A ubiquitin-like system mediates protein lipidation. Nature 408: 488–492.
29. Tra T, Gong L, Kao L-P, Li X-L, Grandela C, et al. (2011) Autophagy in Human Embryonic Stem Cells. PLoS ONE 6(11): e27485.
30. Portha B, Chavey A, Movassat J (2011) Early-Life Origins of Type 2 Diabetes: Fetal Programming of the Beta-Cell Mass. Exp Diabetes Res. 2011;2011:105076. doi: 10.1155/2011/105076.
31. Petry CJ, Dorling MW, Pawlak DB, Ozanne SE (2001) Diabetes in old male offspring of rat dams fed a reduced protein diet. International Journal of Experimental Diabetes Research 2(2) : 139–143.
32. Théry M, Racine V, Piel M, Pepin A, Dimitrov A, et al. (2006) Anisotropy of cell adhesive microenvironment governs cell internal organization and orientation of polarity. Proc Natl Acad Sci U S A 103(52): 19771–6.
33. Slominski A, Pisarchik A, Johansson O, Jing C, Semak I, et al. (2003) Tryptophan hydroxylase expression in human skin cells. Biochimica et Biophysica Acta 1639: 80–86.
34. Martinet W, De Meyer GR, Andries L, Herman AG, Kockx MM (2006) Detection of autophagy in tissue by standard immunohistochemistry: possibilities and limitations. Autophagy 2: 55–57.
35. Kaeffer B, Legrand A, Moyon T, Frondas-Chauty A, Billard H, et al. (2011) Non-invasive exploration of neonatal gastric epithelium by using exfoliated epithelial cells. PLoS One. 6(10): e25562.
36. Marshall OJ (2004) PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. Bioinformatics 20: 2471–2472.
37. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–8.
38. R Development Core Team (2008) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3–900051–07–0, URL http://www.R-project.org.
39. Chambers JM, Hastie TJ (1992) Statistical Models in S_, Wadsworth & Brooks/Cole.
40. Orozco-Sólis R, Matos RJB, Guzmán-Quevedo O, Lopes de Souza S, Bihouée A, et al. (2010) Nutritional programming in the rat is linked to long-lasting changes in nutrient sensing and energy homeostasis in the hypothalamus. PLoS One. 2010 Oct 21;5(10): e13537. doi: 10.1371/journal.pone.0013537.
41. Chiou S-K, Moon WS, Jones MK, Tarnawski AS (2003) Survivin expression in the stomach: implications for mucosal integrity and protection. Biochem Biophys Res Commun 305: 374–379.
42. Delamaire E, Parnet P, Coupé B, Hoebler C, Blat S, et al. (2012) Long term metabolic impact of high protein neonatal feeding: A preliminary study in male rat pups born with a low birth weight. Clin Nutr. 2012 Oct;31(5): 741–8. doi: 10.1016/j.clnu.2012.02.005.
43. Chen J-H, Martin-Gronert MS, Tarry-Adkins J, Ozanne SE (2009) Maternal protein restriction affects postnatal growth and the expression of key proteins involved in lifespan regulation in mice. Plos ONE 4(3): e4950. Doic10.1371/journal.pone.0004950.
44. Miyazaki K, Nagase T, Mesaki M, Narukawe J, Ohara O, et al. (2004) Phosphorylation of clock protein PER1 regulates its circadian degradation in human normal fibroblasts. Biochemical Journal 380(Pt 1): 95–103.
45. Travnickova-Bendova Z, Cermakian N, Reppert SM, Sassone-Corsi P (2002) Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. Proc. Natl. Acad. Sci. USA 99(11): 7728–7733.
46. Ozanne SE, Hales CN (2004) Lifespan: catch-up growth and obesity in male mice. Nature 427: 411–412.
47. Martin-Gronert MS, Ozanne SE (2012) Mechanisms underlying the developmental origins of disease. Reviews in Endocrine & Metabolic Disorders 13(2) : 85–92, DOI: 10.1007/s11154-012-9210-z.
48. Langley-Evans SC, Sculley D (2006) The association between birthweight and longevity in the rat is complex and modulated by maternal protein intake during fetal life FEBS Letters 580: 4150–4153.
49. Jennings BJ, Ozanne SE, Hales CN (2000) Nutrition, oxidative damage, telomere shortening, and cellular senescence: individual or connected agents of aging? Mol Genet Metab 71: 32–42.
50. Tarry-Adkins JL, Chen JH, Smith NS, Jones RH, Cherif H, et al. (2009) Poor maternal nutrition followed by accelerated postnatal growth leads to telomere shortening and increased markers of cell senescence in rat islets FASEB J 23: 1521–1528.
51. Akashi M, Soma H, Yamamoto T, Tsugitomi A, Yamashita S, et al. (2010) Noninvasive method for assessing the human circadian clock using hair follicle cells. Proc. Natl. Acad. Sci. USA 107 : 15643–15648.
52. Hernández-Zavala A, Valenzuela OL, Matous T, Drobná Z, Ddina J, et al. (2008) Speciation of Arsenic in Exfoliated Urinary Bladder Epithelial Cells from Individuals Exposed to Arsenic in Drinking Water. Environ Health Perspect 116: 1656–1660.
53. van Meegen MA, Terheggen-Lagro SW, van der Ent CK, Beekman JM (2011) CFTR Expression Analysis in Human Nasal Epithelial Cells by Flow Cytometry. PLoS ONE 6(12): e27658. doi:10.1371/journal.pone.0027658.
54. Masri S, Sassone-Corsi P (2010) Plasticity and specificity of the circadian epigenome. Nature Neuroscience 13(11) : 1324–1329.
55. Cheung P, Tanner KG, Cheung WL, Sassone-Corsi P, Denu JM, et al. (2000) Synergistic Coupling of Histone H3 Phosphorylation and Acetylation in Response to Epidermal Growth Factor Stimulation. Molecular Cell 5 : 905–915.

Anexo / Annexe D

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Automatic system for analysis of locomotor activity in rodents—A reproducibility study

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ABSTRACT

Automatic analysis of locomotion in studies of behavior and development is of great importance because it eliminates the subjective influence of evaluators on the study. This study aimed to develop and test the reproducibility of a system for automated analysis of locomotor activity in rats. For this study, 15 male Wistar were evaluated at P8, P14, P17, P21, P30 and P60. A monitoring system was developed that consisted of an open field of 1 m in diameter with a black surface, an infrared digital camera and a video capture card. The animals were filmed for 2 min as they moved freely in the field. The images were sent to a computer connected to the camera. Afterwards, the videos were analyzed using software developed using MATLAB® (mathematical software). The software was able to recognize the pixels constituting the image and extract the following parameters: distance traveled, average speed, average potency, time immobile, number of stops, time spent in different areas of the field and time immobile/number of stops. All data were exported for further analysis. The system was able to effectively extract the desired parameters. Thus, it was possible to observe developmental changes in the patterns of movement of the animals. We also discuss similarities and differences between this system and previously described systems.

1. Introduction

The acquisition of locomotion is an important part of the proper development of animals (Barros et al., 2006). Motor development follows a pre-determined series of events that is conserved among different species (Muir, 2000). Many different methods can be used for the study of locomotion (Dunne et al., 2007; Noldus et al., 2001; Bailoo et al., 2010), and one widely used apparatus is the open field apparatus (Noldus et al., 2001; Prut and Belzung, 2003; Barros et al., 2006; Basso et al., 2006; Bellinger et al., 2006; Dunne et al., 2007). This apparatus was initially designed to assist in the study of emotionality in rodents (Hall, 1934). However, over the years, its use has been expanded, and its specific characteristics have been adjusted accordingly (Noldus et al., 2001; Prut and Belzung, 2003). Ratings of behavior displayed in the open field can be made through observational analysis and can be automatic (or semi-automatic) (Barros et al., 2006). In the automatic (or semi-automatic) evaluations, different techniques may be used to acquire information from the open field, such as the use of infrared photocells (Bellinger et al., 2006) or the capturing of images (Sams-Dodd, 1995; Noldus et al., 2001; Dunne et al., 2007). Studies using image analysis usually are of higher cost than observational studies but suffer less influence from the researcher during data processing, as they often use algorithms that analyze all the data in the same way (Noldus et al., 2001; Kulikov et al., 2008). Image analysis also allows for the possibility of acquiring more accurate data, such as tracking the animal, the average speed and the time or frequency at which it presents specific behaviors (Noldus et al., 2001; Dunne et al., 2007). Meanwhile, this kind of analysis must fit the experimental conditions of each laboratory. This is why there are some differences between the analysis systems that are currently available. Most of the existing video tracking systems cannot track an animal under low illumination and use visible light (Spink et al., 2001; Dunne et al., 2007), which can interfere with the animal's behavior. Application of infrared illumination seems to be promising. This study aimed to develop and test the reproducibility of a system capable

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of evaluating the behavioral and biomechanical properties of locomotor activity in freely moving rodents based on analysis of digital images captured under infrared light.

2. Materials and methods

2.1. Animals

For this study, 15 male Wistar rats (from eight litters) of the Animal Nutrition Department, Federal University of Pernambuco (UFPE), were used. The animals were kept at a room temperature of 22 ± 2 °C and in a light-dark cycle of 12 h/12 h (lights on at 9 pm). Twenty-four hours after birth (P1), the animals were selected and assigned to groups of eight pups per dam. Females were used to complete the number of pups per dam, but they were discarded from the analysis. Weaning occurred at 21 days postnatal (P21), and after weaning, each cage contained a maximum of four animals. These were housed in polypropylene cages (46 cm × 34 cm × 20 cm). Water and standard diet (chow – Purina, Brazil) were offered freely. All procedures were approved by the Ethics Committee on Animal Experimentation (ECAE) of the Federal University of Pernambuco and follow the recommendations of the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.2. Supported monitoring

The monitoring apparatus consisted of a circular open field (⌀ 1 m), bounded by walls 30 cm high. The inner surfaces were painted black, and a soft surface of EVA (ethyl vinyl acetate), also black, was placed in the bottom of the apparatus to facilitate the movement of the animal and provide a greater contrast between the animal and the surface (Fig. 1A). A digital camera was mounted on the ceiling of the room (VTR® 6638 – CCTV System) and aligned with the vertical line through the center of the open field; the camera filmed the animal while it moved freely. Its distance from the place of attachment to the ground of the field was 2.40 m. The camera was attached to the computer through a video acquisition board. Its rate of image capture was set to 30 frames per second, and each image was 240 pixels high and 320 pixels wide. It features 420 lines of resolution and a speed between 1/60 and 1/100 frames per second. The images were captured in a dark room using an infrared camera. Infrared LEDs were mounted on the camera, and when activated, they illuminated the open field and allowed for imaging of rodents in the dark. The camera image is saved for further processing in the mpeg file type using a program developed in the Delphi programming language. It is possible to record images with lighting as low as 0.1 lux. The room where the experiments were performed had light less than 1 lux. The use of an infrared camera is important because it allows experimentation with animals during the dark phase of the cycle. The video compression was performed during recording in MPEG. There is no histogram of brightness because we recorded in the dark and the image capture was performed using the infrared. The storage capacity of the video depends on the computer's hard disk, but the system splits the video into files of 15 min each. Afterwards, the videos were divided into frames for analysis (Fig. 1B). Using Paint® software, we created a mask to isolate the image of the animal in the field of objects surrounding it (Fig. 1C and D).

2.3. Systems analysis

We developed a software platform in MATLAB® for analysis of images taken from each video. Through an interface, the evaluator enters the data to register the animal and the information used for the analysis of frames (mass, number of frames, and time between frames).

Due to the difference in color between the open field and the animal, the program can use the recognized intensity of pixels to establish all the points that make up the image of the animal. The binary code analysis system analyzed images using a thresholding process. A fixed value in pixels of the threshold recognized by the program was prepared for each age because the animals show differences in their coat color tone due to growth. The value of each limit was inserted by the examiner using a scale with specific values for each age, thus avoiding errors in the recognition of the animal. The program determines the midpoint in the image of the animal and uses this to represent the animal's center of mass. From that

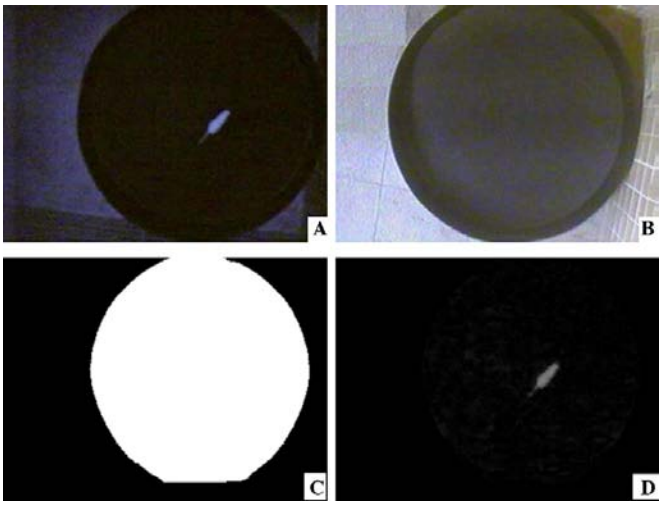

Fig. 1. (A) A view of the open field with the light on. (B) A view of open field with the light off. (C) A mask created with Paint® software. (D) The final image (C on B) used for analysis.

point, the *xy* coordinates of the animal were determined in each frame. Once the positions of the animal in each of the frames was determined, it was possible to reconstruct its trajectory, and by adding information about the animal's mass, the number of frames and time between each frame, the following could be established.

- Distance traveled (m): the sum of all displacements performed by the animal. The animal was considered to be moving when it ran a distance of over 50% of its length in a maximum of three frames. These values were chosen to prevent the system from considering small rotational movements as displacement. Only translational movements that were able to move the center of mass were considered displacement. The program featured an internal converter from pixels to meters, where the scale of conversion was added by the evaluator.
- Average speed (m/s): the ratio of total displacement by the time the animal remained in motion. Formula: $\Delta S/\Delta T$, where ΔS corresponds to the total displacement in meters and ΔT the total analysis time minus the time of immobility in seconds.
- Average potency (mW): potency produced during the period of displacement. The amount of kinetic energy dissipated by a body in motion over the travel time was calculated. Formula: $mV^2/2\Delta T$, where m is the mass of the animal in grams, V is the average speed in meters/seconds, and ΔT the total analysis time minus downtime in seconds.
- Time immobile (s): total time the animal remained standing in the open field.
- Number of stops: total number of stops made in the field.
- Time immobile/number of stops (s): relationship between the time immobile and the total number of stops.
- Length of stay in the area (s): the open field was divided virtually into three areas (central, intermediate, and peripheral, with radii of 0.165, 0.33 and 0.50 m, respectively). This parameter gives the total time the animals were within these areas. The division into three areas of the arena was the largest possible value of divisions that prevented older (and longer) animals from being erroneously positioned in two areas by the analysis system.

These calculations are made assuming that the animal physically moving is a point object endowed with a certain mass and performing random motion. The movements that do not shift the centroid of the image of the animal, such as simple head movements, are not considered in the calculation because we only consider those movements that shift the position of the animal. Thus, it is assumed that any potential energy is converted into muscle kinetic energy associated with the actual motion of the centroid of the image of the animal, which is quite appropriate because we are only interested in monitoring the locomotive activity of the animal.

2.4. Reproducibility study

The animals were evaluated at P8, P14, P17, P21, P30 and P60. These dates in the neonatal period were chosen because they represent milestones in the development of locomotion (Westerga and Gramsbergen, 1990). Evaluations were performed during the dark phase of the circadian cycle (between 12:00 pm and 2:00 pm) so that they occurred during the period when the animals are normally most active. Each animal was placed individually in the center of the open field and recorded for 2 min while it moved freely. The test duration was chosen based on a pilot experiment, when it was observed that animals reduce their locomotion after that period, and also with the intention to not accustom the animals to the apparatus and to allow the animals to cover the entire field. When the animals were exchanged, the field was cleaned with sodium hypochlorite and water, and the EVA was changed to eliminate odors that could affect the behavior of the next animal.

2.5. Statistical analyses

The data were statistically analyzed using SigmaStat (v. 3.1). The Kolmogorov–Smirnov test was performed to determine if the data were normally distributed. To assess the reproducibility of the data, each film was analyzed by two different evaluators. Reliability was determined using the Pearson correlation coefficient for each age and evaluated parameter.

To study development, we performed a one-way ANOVA for repeated measurements followed by the Tukey test for the parameter distance, average speed, average potency, time immobile and number of stops. For the time immobile/number of stops, we used Friedman's test followed by Dunn's test, and for the time spent in each of the areas, we used two-way ANOVA (age × area) for repeated measures followed by the Tukey test. A p value <0.05 was considered significant. The results are expressed as the mean ± standard error of the mean (SEM).

3. Results

In the analysis of data reproducibility, there was significant correlation between different evaluators for all ages and parameters analyzed ($r > 0.813$, $p < 0.00001$). Thus, for the developmental study, we present the mean of values from both evaluators.

The changes observed in the weight of the rats closely matched the normal course of weight gain for the Wistar strain (Fig. 2). We observed a progressive increase in the distance traveled ($F_{5,\,107} = 107{,}86$) with age until the 21st postnatal day ($p < 0.001$) (Fig. 3A). There was no difference between P21 and P30. We observed a decrease in distance between P30 and P60 ($p = 0.031$) (Fig. 3A). The average speed ($F_{5,\,107} = 364{,}66$) showed a progressive increase with age, with significant differences between all ages tested ($p < 0.001$) (Fig. 3B). A slight increase in the average potency produced ($F_{5,\,107} = 481{,}81$) was observed in early life, with differences only between P21 and P30, and P30 and P60 ($p < 0.001$) (Fig. 3C). The time the animal remained standing in the field ($F_{5,\,107} = 90{,}45$) declined by P17, and there were differences between P8 and P14, and P14 and P17 ($p < 0.001$) (Fig. 3D). There was an increase in immobility time at P60 compared to P30 ($p < 0.001$) (Fig. 3D). The number of stops in the open field ($F_{5,\,107} = 35{,}49$) showed an increase between P8 and P14 ($p < 0.001$), followed by a decline between P14 and P17 ($p < 0.001$) (Fig. 3E). After 60 days, another increase in the number of stops was observed ($p < 0.001$) (Fig. 3E). The average time spent by the animal at each stop decreased between P8 and P14 ($p < 0.001$), remaining constant in the other age groups (Fig. 3F).

One can observe the influence of age on the time spent in different areas of the open field ($F_{5,\,251} = 8.49$, $p < 0.001$) and the study area ($F_{2,\,251} = 217.28$, $p < 0.001$) and the interaction of age × area ($F_{10,\,251} = 89.70$, $p < 0.001$). There was a decrease in the time spent in the central area for P17, with differences between P8 and P14, and P14 and P17 ($p < 0.001$) (Fig. 4). The residence time in the intermediate area increased between P8 and P14 ($p < 0.001$) and decreased by P17 compared to P14 ($p < 0.001$) (Fig. 4). In the periphery, there was an increased duration of residence time until P17 ($p < 0.001$), followed by reduction at P21 ($p < 0.001$) (Fig. 4). When analyzed according to age, the time that the animals remained in the central area was higher compared to the two other areas in P8 ($p < 0.001$) (Fig. 4). At P14, the time in the peripheral areas differed only in the intermediate area, becoming greater ($p < 0.001$) (Fig. 4). In all other ages studied, the animals spent longer in the peripheral area ($p < 0.001$), with no difference between the time spent in the central and intermediate areas (Fig. 4).

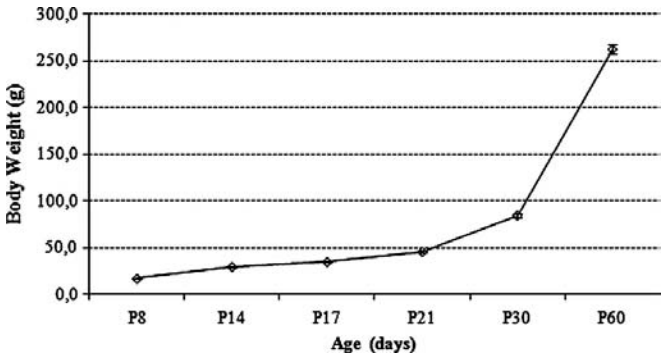

Fig. 2. The body weight of normal rats. Values are the mean ± SEM.

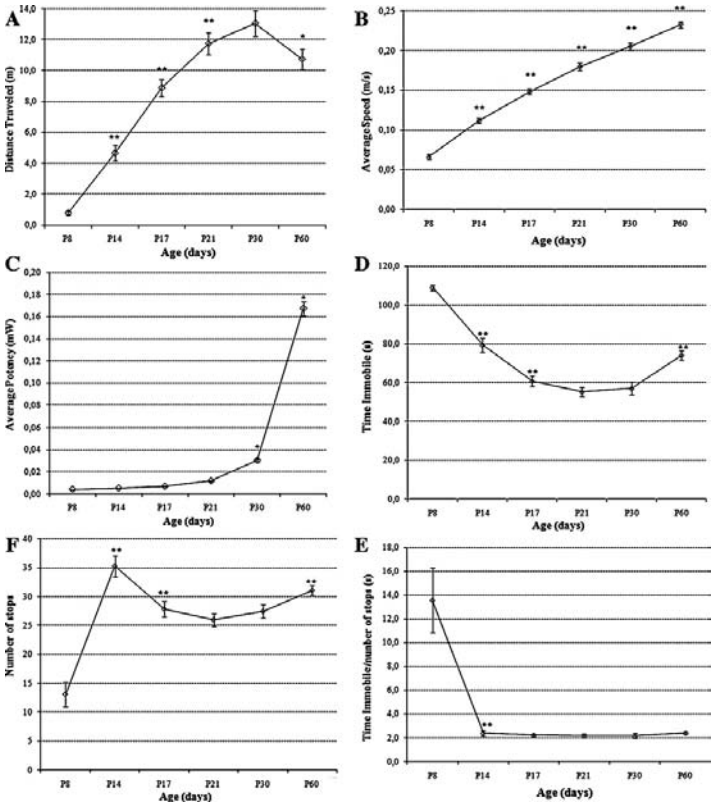

Fig. 3. The development of locomotor activity. Normal rats ($n = 15$) were evaluated in an open field at the ages specified for 2 min. A, distance traveled (m), B, average speed (m/s); C, average potency (mW); D, time immobile (s); E, number of stops; F, time immobile/number of stops (s). Values are the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.001$, vs. earlier age using a one way ANOVA for repeated measures with Tukey's post-hoc test (A, B, C, D and E) or Friedman test and Dunn's post-hoc test (F).

4. Discussion

We present an automatic analysis of locomotion for rodents. This system was able to recognize the animal, even under low illumination, and analyze all the desired parameters using the same algorithms for all tests. The evaluators were responsible for creating the mask to isolate the open field, entering information into the system about the animal and the process of converting the video frames without interfering in the algorithm's values. To test the system, we analyzed the development of locomotion in normal rats and the reproducibility of the results between two independent evaluators. The results show that the system is able to extract

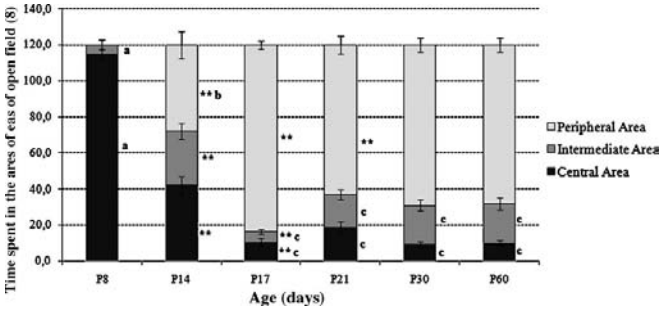

Fig. 4. Development and the time spent in the areas of the open field. Normal rats ($n = 15$) were evaluated in the open field at the ages specified for 2 min. Values are the mean ± SEM. $^{**}p < 0.001$, vs. the same area at an earlier age. a, b, c $p < 0.001$, vs. a different area at the same age (a, central, b, intermediate area, c, peripheral area). Two-way ANOVA for repeated measures with the Tukey post-hoc test. $F_{age} = 8.49$, $p < 0.001$; $F_{area} = 217.28$, $p < 0.001$; $F_{area \times age} = 89.70$, $p < 0.001$.

the video information on the movement of animals and that these data are reproducible; a high agreement between the results for different evaluators was observed, as expected because they only have influence on area delimitation. Although the period recorded (2 min) is not the most commonly used time (Prut and Belzung, 2003) to analyze open field behavior and locomotion, we were able to detect differences between the locomotor patterns among days. Furthermore, this period was long enough for the animals (14 postnatal days or older) to cover the range of the whole arena, thus allowing us to be sure that the analysis system was capable to track the animal in all positions of the field.

New systems of locomotion analysis have been developed. Some systems use analysis and present video images to extract the parameters of the study (Sams-Dodd, 1995; Noldus et al., 2001; Bailoo et al., 2010). Some require that images recorded initially in analog mode be scanned for further analysis (Noldus et al., 2001; Dunne et al., 2007). This often causes loss of video quality, with the appearance of noise that can interfere with the extraction of data (Noldus et al., 2001). Therefore, our acquisitions were performed by a video camera directly in digital mode with no analog storage. Although some authors have captured images directly in digital form, they still fail to perform the test in a dark environment (Dunne et al., 2007; Kulikov et al., 2008). In these studies, they used or a dim light or indirect illumination. Bailoo et al. (2010) provided lighting conditions from 563 to 1743 lux by fluorescents lights, while Dunne et al. (2007) supplied 60 lux in the center of arena. However, these lighting intensities could influence animal behavior. Some authors have already suggested that tracking animals in darkness using infrared light could minimize the influence of lights on behavior (Lind et al., 2005). Spink et al. (2001) stated that illumination should be indirect and even. In our work, we were able to assess animal behavior even in an environment with very low illumination, which allows us to perform the tests during the dark phase of the cycle.

Altman and Sudarshan (1975) performed a comparative analysis of the qualitative aspects of the locomotion of laboratory rats (*Rattus norvegicus*) subjected to the open field test at ages from P1 to P21. Despite observed differences in the course of development, they could not establish values that could be compared to other studies. Westerga and Gramsbergen (1990) studied the development of locomotion in rats between P10 and P20. Unlike the first study, these authors used analysis of pictures of animals walking on a corridor for the study of locomotion. They were able to perform qualitative analysis and quantitatively evaluate the kinematics of walking. However, they could not assess the behavioral differences presented by the animals during development. Our work, as well as those cited, was able to show the changes in locomotion during development. We observed lower activity early (P8), which stabilized by P21, and the subsequent development of behavioral issues and improved physical performance. Unlike the work of Westerga and Gramsbergen (1990), our study allowed us to verify the overall performance of the animal through the parameters of distance, speed and potency. Our results are in accordance with the literature on increased exploration activity and interaction with the environment between weaning and the 30th postnatal day (Bâ and Seri, 1995).

In our system, we present two types of approaches to locomotion: biomechanics and behavior. The first was expressed by parameters such as distance traveled, average speed and average potency. In these, we seek to express the mechanical movement capacity of the animal. By studying the animal at different stages of life and comparing the findings with literature data, we observed that the animal's ability to generate movement is linked to the level of maturation, activation and coordination of neural structures (sensory and motor) (Vinay et al., 2002) and mainly to muscle activation (Gramsbergen, 1998). For behavioral analysis, we analyzed the time and number of stops and their relationship in addition to the spatial distribution of the time the animal spent in the open field. The possibility of extracting behavioral and biomechanical parameters in the same test is useful because it allows us a broader vision of locomotion because behavior is influenced by the ability to generate movement and vice versa (Prut and Belzung, 2003). For example, by studying the time spent in the different areas, we observed that the time spent by P8 animals in the peripheral area was very small compared to other ages. However, rather than representing a less active animal (Prut and Belzung, 2003), this represents a failure of these rats to move to the outer areas, due to the immaturity of their muscle control and neural maturation (Gramsbergen, 1998; Vinay et al., 2002). As development continued, the animals were able to explore larger areas in the open field and demonstrate a more coordinated pattern of locomotion due to increasing maturation of descending pathways of the central nervous system (for review, Jamon, 2006). In addition, some drugs that act on emotional behavior can alter the movement of animals without acting directly on the muscular system (Sams-Dodd, 1995; Siemiatkowski et al., 2000). In the study of potency, we are able to compare animals with different weights and observe if they are capable of dissipating energy similarly to animals from a standard situation. Thus, it is possible to study locomotion proportionally to the weight of animals.

The analysis system was shown to be a useful tool for the study of locomotion, with a low cost of implementation. The system has many benefits: it is automatic, requires little time for analysis, the results are not influenced by the researcher, and the data are highly

reproducible and reliable. Moreover, it is able to reveal changes in locomotor activity and can be used in many different studies on the subject.

Conflict of interest

The authors declare no conflict of interest.

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References

Altman J, Sudarshan K. Postnatal development of locomotion in the laboratory rat. Anim Behav 1975;23:896–920.
Bâ A, Seri BV. Psychomotor functions in developing rats: ontogenetic approach to structure–function relationships. Neurosci Biobehav Rev 1995;19(3):413–25.
Bailoo JD, Bohlen MO, Wahlsten D. The precision of video and photocell tracking systems and the elimination of tracking errors with infrared backlighting. J Neurosci Methods 2010;188:45–52.
Barros KMFT, Manhães-De-Castro R, Sousa SL, Matos RJB, Deiró TCBJ, Cabral-Filho JE, et al. A regional model (Northeast Brazil) of induced malnutrition delays ontogeny of reflexes and locomotor activity in rats. Nutrit Neurosci 2006;9:99–104.
Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. J Neurotrauma 2006;23(5):635–59.
Bellinger L, Sculley DV, Langley-Evans SC. Exposure to undernutrition in fetal life determines fat distribution, locomotor activity and food intake in ageing rats. Int J Obes (Lond) 2006;30(5):729–38.
Dunne F, O'Halloran A, Kelly JP. Development of a home cage locomotor tracking system capable of detecting the stimulant and sedative properties of drugs in rats. Prog Neuropsychopharmacol Biol Psychiatry 2007;31(7):1456–63.
Gramsbergen A. Posture and locomotion in the rat: independent or interdependent development? Neurosci Biobehav Rev 1998;22:547–53.
Hall CS. Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. J Comp Psychol 1934;18:385–403.
Jamon M. The early development of motor control in neonate rat. C R Palevol 2006;5:657–66.
Kulikov AV, Tikhonova MA, Kulikov VA. Automated measurement of spatial preference in the open field test with transmitted lighting. J Neurosci Methods 2008;170:345–51.
Lind NM, Vinther M, Hemmingsen RP, Hansen AK. Validation of a digital video tracking system for recording pig locomotor behaviour. J Neurosci Methods 2005;143(2):123–32.
Muir GD. Early ontogeny of locomotor behaviour: a comparison between altricial and precocial animals. Brain Res Bull 2000;53(5):719–26.
Noldus LPJJ, Spink AJ, Tegelenbosch RAJ. EthoVision: a versatile video tracking system for automation of behavioral experiments. Behav Res Met Instr Comp 2001;33(3):398–414.
Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 2003;463:3–33.
Sams-Dodd F. Automation of the social interaction test by a video-tracking system: behavioural effects of repeated phencyclidine treatment. J Neurosci Methods 1995;59:157–67.
Siemiatkowski M, Sienkiewicz-Jarosz H, Członkowska AI, Bidziński A, Płaźnik A. Effects of buspirone, diazepam, and zolpidem on open field behavior, and brain [3H]muscimol binding after buspirone pretreatment. Pharmacol Biochem Behav 2000;66(3):645–51.
Spink AJ, Tegelenbosch RA, Buma MO, Noldus LP. The EthoVision video tracking system—a tool for behavioral phenotyping of transgenic mice. Physiol Behav 2001;73(5):731–44.
Vinay L, Brocard F, Clarac F, Norreel J, Pearlstein E, Pflieger JF. Development of posture and locomotion: an interplay of endogenously generated activities and neurotrophic actions by descending pathways. Brain Res Rev 2002;40:118–29.
Westerga J, Gramsbergen A. The development of locomotion in the rat. Dev Brain Res 1990;57:163–74.