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Association of placental nutrient sensing pathways with birth weight

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Abstract

Birth weight (BW) is an important indicator for newborn health. Both high and low BW is associated with increased risks for adult metabolic diseases. AMPK (AMP-activated protein kinase), mTOR (mechanistic target of rapamycin), and insulin/IGF1 (insulin-like growth factor 1) pathways may function as placental sensors of maternal hormonal and nutritional status. However, the physiological role of these pathways in placenta has not been completely elucidated. To evaluate expression and activation of AMPK, mTOR, and insulin/IGF1 pathways and its association with placental weight (PW), BW, and maternal hormonal and metabolic status, we performed a cross-sectional study in placentas from non-obese mothers with SGA ($n=17$), AGA ($n=19$) and LGA ($n=10$) newborns. We analyzed placental expression of total and phosphorylated key proteins from the AMPK, mTOR and insulin/IGF1 pathways. Maternal and cord blood hormones were determined by ELISA. AMPK and LKB1 activation correlated negatively with PW and BW, cord leptin, and pregestational BMI. Placental SIRT1 inversely correlated with BW, cord leptin, neonatal HOMA-IR, and maternal IGF1. PGC1α correlated negatively with PW and BW. Phosphorylated mTOR positively correlated with maternal glucose, PW and BW. IGF1R was lower in SGA. No changes in p-IGF1R, INSRb, total AKT or p-AKT were found, and pPDK1 was lower in SGA and LGA. These results suggest that placental AMPK, insulin/IGF1, and mTOR pathways may influence fetal growth, perhaps regulating placental physiology, even in metabolically healthy pregnancies. Our study highlights these nutrient sensing pathways as potential molecular mechanisms modulating placental adaptations and, thus, long-term metabolic health.

Reproduction (2020) **160** 455–468

Introduction

Birth weight is an important indicator of postnatal health and survival as well as for metabolic health during adult life. Several studies have suggested that events during fetal development may program metabolic outcomes in adult life, leading to the field of developmental origins of health and disease (DOHaD) (Suzuki 2018). Extremes of birth weight increase the risk of adult metabolic diseases. Being born small for gestational age (SGA) has been related to adult metabolic syndrome, obesity, type 2 diabetes mellitus, cardiovascular and renal diseases (Kruger & Levitt 2017). Nearly 40% of individuals born large for gestational age (LGA) present obesity when adults (Mingrone *et al*. 2008). Furthermore, LGA newborns have an increased risk for metabolic, cardiovascular and neoplasic diseases (Monasta *et al*. 2010).

Intrauterine growth and birth weight establishment are influenced by a wide variety of signals, such as hormones and nutrients. These are integrated by the placenta which, in turn, modulates fetal nutrient and oxygen availability. Among these signals, nutrient-sensing pathways, such as AMPK (AMP-activated protein kinase), mTOR (mechanistic Target of Rapamycin), and Insulin/IGF1 (insulin-like growth factor 1), have been suggested to play a crucial role in fetal and placental physiology (Sferruzzi-Perri *et al*. 2017, Gupta & Jansson 2019).

AMPK is an evolutionarily conserved serine/threonine kinase which activates energy-producing metabolic pathways in response to low intracellular energetic status, oxidative stress, and hypoxia (Hardie *et al*. 2012), all conditions associated with alterations in placental function and intrauterine growth restriction (Aljunaidy *et al*. 2017). It has been suggested that AMPK

ISSN 1470–1626 (paper) 1741–7899 (online)
https://doi.org/10.1530/REP-20-0186
Online version via https://rep.bioscientifica.com

modulates fetal growth through placental development (Egawa *et al*. 2008), cellular metabolism (Landau *et al*. 2019) and nutrient transport (Jones *et al*. 2010, Ma *et al*. 2011, Landau *et al*. 2019); this is evidenced by studies in cell lines (Egawa *et al*. 2008), primary cultures (Jones *et al*. 2010, Landau *et al*. 2019), and animal models (Ma *et al*. 2011, Kavitha *et al*. 2014). Studies associating maternal nutrient status with placental AMPK have been performed, mostly in animal models, with discordant results (Ma *et al*. 2011, Rosario *et al*. 2011, Kavitha *et al*. 2014).

AMPK is phosphorylated by tumour suppressor kinase LKB1 (liver kinase B1), which regulates cell proliferation and stress responses. Lkb1$^{-/-}$ mice exhibit defective placental development (Ylikorkala *et al*. 2001). Nevertheless, LKB1 expression and roles in the human placenta has scarcely been studied (Skeffington *et al*. 2016).

After phosphorylation by LKB1, AMPK activates the metabolic sensor SIRT1 (sirtuin-1) by increasing NAD^+ levels, resulting in deacetylation and modulation of downstream proteins, such as the coactivator 1α of PPARγ (PGC1α). Along with metabolism, SIRT1 influences cell proliferation, apoptosis and autophagy (Cantó & Auwerx 2009). SIRT1 is expressed in both human and mouse placenta (Lappas *et al*. 2011, Arul Nambi Rajan *et al*. 2018) and has been suggested to play an important role in embryogenesis, fetal growth and placental development. SIRT1-null mice have small placentas and fetal growth restriction, with concomitant fetal lethality (Arul Nambi Rajan *et al*. 2018).

The mTOR pathway modulates energy generation and nutrient use, by forming two protein complexes: mTORC1 and mTORC2. While mTORC1 mediates macromolecule biosynthesis, autophagy, proliferation, metabolism, and growth, mTORC2 regulates cell survival, cytoskeletal organisation, and metabolism. mTORC1 responds to oxygen, amino acids, energy levels, growth factors, hormones, and stress. mTORC2 responds to growth factors through the PI3K/AKT pathway (Saxton & Sabatini 2017) and acute energetic stress through AMPK pathway (Kazyken *et al*. 2019). Moreover, it has been recently shown that AMPK is a mTORC2 positive modulator, which may explain paradoxical effects of AMPK seen in tumorigenesis (Kazyken *et al*. 2019).

mTOR is expressed in trophoblasts, being regulated by nutrients (i.e. glucose, fatty acids and folate) and hormones such as IGFs, corticosterone and adiponectin (Gupta & Jansson 2019). In cultured primary human trophoblasts, mTORC1 regulates mitochondrial oxidative phosphorylation (Rosario *et al*. 2019), aminoacid transport (Gupta & Jansson 2019), and folate uptake (Silva *et al*. 2017). In pregnancies complicated with IUGR, placental mTOR activation is reduced (Roos *et al*. 2007), correlating positively with birth weight in LGA babies from obese (Jansson *et al*. 2013) and gestational diabetes (GDM) mothers (Shang & Wen 2018).

The IGF axis (IGF1, IGF2 and IGF binding proteins: IGFBPs) modulates cell differentiation and growth by mediating growth hormone's action (Harrela *et al*. 1996) and plays an important role in fetal growth regulation. IGF1, IGFBP1 and IGFBP3 levels in umbilical cord blood are correlated to birth weight (Giudice *et al*. 1995). Maternal production of IGFs is stimulated by hormonal signals, and the IGF axis promotes placental growth and function by modulating the trophoblast invasion and amino acid transport (Sferruzzi-Perri *et al*. 2017). IGFs exert their actions through IGF receptor 1 (IGF1R) and insulin receptor (INSR). IGFs activate PI3K/AKT and MAPK pathways (Sferruzzi-Perri *et al*. 2017). Some studies have addressed placental expression of IGF1R/INSR and PI3K/AKT pathway in SGA (Iniguez *et al*. 2010), LGA (Iniguez *et al*. 2010), IUGR (Laviola *et al*. 2005, Street *et al*. 2011) and GDM (Shang & Wen 2018). AMPK activation increases IRS1 (Insulin receptor substrate 1) phosphorylation, leading to a crosstalk between insulin/IGF1 and AMPK pathways (Hardie *et al*. 2012). Accordingly, AMPK downregulation, together with IGF1 signaling increase, has been observed in placentas of LGA newborns from obese (Jansson *et al*. 2013) and GDM (Shang & Wen 2018) mothers.

Given the importance of nutrient-sensing pathways for placental function and potentially for birth weight establishment, we hypothesise that AMPK, mTOR, and insulin/IGF1 pathways in placenta are differentially activated in idiopathic SGA and LGA from non-obese mothers with normal pregnancy. We also investigated whether maternal hormonal and metabolic environments could be associated with the expression and activation of these pathways and how these pathways are regulated and interrelated to the placenta at both ends of altered birth weight.

Materials and methods

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the Mexican Regulation of the General Health Law regarding Health Research and with the Helsinki Declaration of 1975, as revised in 2008, and have been approved by the Institutional Bioethics Committee of the University of Guanajuato (CIBIUG-P14-2016), the Research Committee of the General Hospital of Leon (16042012) and the Local Research and Ethics Committee in health Research from UMAE-48 IMSS (R-2012-1002-51).

Design and study population

A cross-sectional observational study was carried out in women without obesity with a singleton pregnancy, with no previous metabolic or associated disease. Subjects included were part of a previously studied cohort, recruited from public

health centres from Leon, Guanajuato, Mexico, described elsewhere (Gonzalez-Dominguez *et al*. 2016). Informed consent was signed before entering the study. Ethics and research committees from the hospitals where the participants were recruited, as well as the ethics committee of the Institute of Medical Research at University of Guanajuato, approved the study. All procedures were done according to the Mexican General Health Laws and the Declaration of Helsinki.

Pregnant women between 18 and 35 years of age were included, with full-term newborns of either sex from vaginal delivery or non-elective caesarean section (indicated by previous caesarean section or pelvic insufficiency), without signs of perinatal asphyxia or acute fetal distress. The presence of pregestational obesity (BMI > 29.9 kg/m^2), preeclampsia, diabetes, antiphospholipid syndrome, connective tissue diseases, chronic infection, previous IUGR diagnosis by Doppler ultrasound and alcohol consumption or smoking habit during the current pregnancy were taken as the exclusion criteria. Gestational age was determined by a neonatologist using the Capurro method (Capurro *et al*. 1978), following standardised procedures from the health centres. Newborns were classified according to their birth weight as SGA (small for gestational age, weight in the lowest 10th percentile, $n=17$), AGA (appropriate for gestational age, weight between 10th and 90th percentiles, $n=19$), and LGA (large for gestational age, weight above 90th percentile, $n=10$), using validated tables for the Mexican population (Jurado 1971, Flores-Huerta & Martínez-Salgado 2012). Clinical and anthropometrical data from mothers and newborns were obtained via direct interview and clinical records. No maternal or neonatal complications were observed during the study.

Sampling methods

Maternal blood samples were drawn at the time the mother entered labor ward (latent labor phase), within 3–8 h from the last meal. Cord blood from the umbilical artery was collected immediately after delivery. A 5 × 5 cm piece of placental tissue, including fetal and maternal sides, was cut halfway between the cord insertion and the placental border immediately after delivery, frozen in dry ice and stored at −70 °C until use. Maternal and cord blood serum was obtained by centrifugation at 4°C within 1 h after collection and stored at −20 °C until used.

Biochemical and hormonal assays

Glucose, triglycerides, and cholesterol concentrations (total, HDL, LDL and VLDL) in both maternal and neonatal serum were measured by colorimetric enzymatic assays (Spinreact) following manufacturer's instructions. HbA1c was measured from the maternal plasma by chromatography with Labona Check equipment and reagents. Insulin concentration in maternal and neonatal serum was measured using an ultra-sensitive ELISA kit (ALPCO). HOMA IR index was calculated using the following formula: Fasting insulin (µU/mL) × fasting glucose (mmol/L)/22.5. IGF1, IGF2, IGFBP1 and IGFBP3 serum concentrations were measured using ELISA kits (ALPCO). Total adiponectin in maternal serum was measured by ELISA (MyBioSource). Leptin in maternal and umbilical cord blood was measured using the human Leptin immunoassay (Quantikine, R&D Systems) ELISA kit. Maternal serum Acyl (MyBioSource) and Desacylghrelin (SPI-BIO) were measured by ELISA following the manufacturer's instructions.

Total and phosphorylated protein expression

Pieces of approximately 150 mg of tissue, cut transversally from each placental sample, were washed with 300 µL of cold PBS 1 and homogenised using a PT 12000 E Polytron handheld homogenizer at maximum speed for 20 s in cold lysis buffer (100 mM Tris-HCl, 1% NP-40, 1 mM de EDTA, 250 mM sucrose, 150 mM de NaCl, 100 mM NaPPi, 100 mM NaF, 10 mM Sodium orthovanadate, and protease inhibitors (mini complete ROCHE), left 15 s in ice, and homogenized again for 20 s. Tissue extracts were incubated for 2 h at 4 °C, centrifuged at 10,000 ***g*** for 15 min at 4 °C, and the supernatant collected. Protein was quantified via the Lowry assay, resolved by electrophoresis in 10% SDS – PAGE gels for 40 – 65 kDa proteins (AMPK, LKB1, AKT and PDK1) and 7.5% SDS – PAGE gels for 90 – 140 kDa proteins (INSRb, IGFR1, SIRT1 and PGC1a), and electrotransferred to nitrocellulose membranes (Hybond-C Super, Amersham Pharmacia Biotech). After blocking for 1 h in 5% BSA (for phosphorylated proteins) or 5% skimmed milk (for non-phoshporylated proteins) in TBS-0.1% Tween 20 (20 mM Tris-Base, 150 mM NaCl and 0.1% Tween 20), the membranes were incubated overnight at 4 °C with the corresponding primary antibodies. Primary antibodies were purchased from Cell Signaling Technology and used as follows: 1:2500 AMPKa, 1:2500 p-AMPKa (Thr172), 1:2000 LKB1, 1:2000 p-LKB1 (Ser482), 1:2500 SIRT1, 1:1000 PGC1a, 1:1000 mTOR, 1:1000 p-mTOR (Ser2448), 1:1000 IGF1Rb, 1:1000 p-IGF1Rb (Tyr980), 1:1000 INSRb, 1:1000 p-PDK1 (Ser241), 1:1000 AKT, 1:1000 p-AKT (Thr308) and 1:1000 p-AKT (Ser273). Secondary antibodies from Abcam were used in 1:80,000–1:55,000 dilution for 2 h at 4 °C. Bands were detected using chemiluminescence with Luminol ECL Plus Western Blotting Detection System (Amersham, GE Healthcare). Normalisation was done with alpha-tubulin detection (1:8000, Sigma), using secondary anti-mouse IgG-HRP antibody (1:4000, Sigma). Band intensities were quantified by densitometry in a Chemidoc Molecular Imager®, using Image-Lab software 3.0 (BioRad). Equal random samples per group were run on the same gel, and simultaneous assays were performed for all samples, performing triplicate immunodetections for each sample.

Statistical analysis

Data were analysed with the software Statview V.4.5 (Abacus Concepts, Berkeley, CA, USA). Clinical data are presented as mean ± S.D. when normally distributed or otherwise as median with interquartile ranges. Differences between SGA, AGA and LGA groups were assessed using ANOVA or Kruskal–Wallis and Mann–Whitney *U*-test. Association was analysed using Spearman's correlation coefficients. Significance was stated as P value ≤0.05.

Table 1 Clinical and anthropometric characteristics of mothers of SGA, AGA and LGA newborns. Data are presented as mean ± S.D. or as median (quartile range).

	SGA mothers ($n=17$)	**AGA mothers** ($n=19$)	**LGA mothers** ($n=10$)	***P*-value**
Age (years)	25 (21–28.5)	21 (20–28.5)	27.5 (23.8–31.3)	0.23
Pregestational weight (kg)	52.2 ± 8.0	56.1 ± 8.4	63.2 ± 9.0*+	**0.01**
Height (m)	1.55 ± 0.05	1.54 ± 0.04	1.56 ± 0.05	0.38
Pregestational BMI (kg/m^2)	21.7 ± 3.5	23.5 ± 3.5	25.4 ± 2.8+	**0.03**
<18.5 kg/m^2	3	1	0	0.07
18.5–24.9 kg/m^2	11	11	3	
25–29.9 kg/m^2	3	7	7	
Gestational weight gain (kg)	11.8 ± 2.7	13.4 ± 4.5	13.8 ± 4.5	0.38

*$P<0.05$ compared to control AGA.
+$P<0.05$ compared to SGA.

Results

Anthropometric, clinical, and biochemical data

Clinical and anthropometric data of mothers (Table 1) and their newborns (Table 2) in each of the three groups are shown. Pregestational weight was higher in the LGA group compared to AGA and SGA, while pregestational BMI in LGA was only different from SGA. Height, gestational weight gain and maternal age were similar for the three groups (Table 1). As expected, birth weight, anthropometric parameters of newborns, and placental weight of SGA and LGA were significantly different from the AGA group (Table 2).

None of the mothers from the three groups had previously known metabolic alterations, and no biochemical nor hormonal measurements were different between them. Maternal adiponectin was significantly higher for SGA mothers as compared to AGA (Table 3), but did not correlate with pregestational BMI ($r=0.104$, $P=0.509$). Maternal leptin did not correlate with pregestational BMI ($r=-0.003$, $P=0.987$), although it was associated with gestational weight gain ($r=0.353$, $P=0.028$).

Biochemical and hormonal parameters in the newborns were similar between the three groups. Cord blood insulin, HOMA-IR, IGF1 and IGFBP3 were lower in SGA and higher in LGA when compared to AGA (Table 3). Birth weight was correlated with placental weight ($r=0.89$, $P<0.001$), pregestational BMI ($r=0.52$, $P<0.001$), gestational age ($r=0.44$, $P=0.003$), as well as with maternal glucose, triglycerides, and HDL, but not with maternal weight gain or any other maternal metabolic or hormonal measurements (Supplementary Table1, see section on supplementary materials given at the end of this article).

Placental AMPK and LKB1 protein expression and activation

AMPK protein is phosphorylated in Thr-172 in response to the increase in the AMP/ATP ratio. We evaluated the total AMPK and phosphorylated (p-AMPK) protein expression in placenta (Fig. 1). AMPK expression in the LGA group was 30% lower compared to SGA (Fig. 1A and B). p-AMPK was two-fold higher in SGA group and 1.6-fold lower in LGA group as compared to AGA. Also, p-AMPK of the SGA group was five-fold higher compared to LGA (Fig. 1A and C).

The main kinase for AMPK activation, LKB1, and phosphorylated LKB1 (p-LKB1) placental protein expression was different between groups (Fig. 2). Total LKB1 protein expression in LGA was 37% lower compared to SGA (Fig. 2A and B). p-LKB1 was significantly higher in SGA and lower in LGA, compared

Table 2 Clinical and anthropometric characteristics of SGA, AGA and LGA newborns. Data are presented as mean ± S.D. or as median (25%/75% quartile range).

	SGA ($n=17$)	**AGA** ($n=19$)	**LGA** ($n=10$)	***P*-value**
Baby's gender (male/female)	8/9	11/8	7/3	0.50
Delivery method (vaginal/C-section)	9/8	13/6	6/4	0.64
Gestational age (weeks)	38.8 ± 1.2	38.3 ± 1.2	39.4 ± 1.1*	0.06
Placental weight (g)	400.8 ± 62.9*	608.2 ± 108.9	730.1 ± 80.3*+	**<0.001**
Birth weight (g)	2314 (2137.5/2407.5)*	3265 (2910/3325)	3915 (3787.5/4102.5)*+	**<0.001**
Birth lenght (cm)	45.7 ± 2.55*	50.9 ± 2.04	52.8 ± 2.10*+	**<0.001**
Birth weight z-score	−2.1 (−2.58/−1.7)*	−0.11 (−0.73/0.24)*	1.52 (1.23/1.93)*	**<0.001**
Ponderal index	2.48 (1.88/2.79)	2.38 (2.23/2.54)	2.71 (2.53/2.88)	**0.038**
Head circumference (cm)	32.2 ± 1.6*	34.6 ± 1.4	36.4 ± 1.6*+	**<0.001**
Thoracic circumference (cm)	30 (29–31)*	34 (32–34)	35 (34.87–37)+	**<0.001**
Abdominal circumference (cm)	27.2 ± 1.8*	30.7 ± 1.7	34.1 ± 2.2*+	**<0.001**

*$P<0.05$ compared to control AGA.
+$P<0.05$ compared to SGA.

Table 3 Metabolic and hormonal status of mothers and SGA, AGA and LGA newborns. Data are presented as mean ± S.D. or as median (quartile range).

	SGA ($n=17$)	**AGA** ($n=19$)	**LGA** ($n=10$)	***P*-value**
Maternal metabolic and hormonal status				
Blood glucose (mg/dL)	73.82 ± 13.47	82.43 ± 10.76	75.64 ± 13.75	0.11
HbA1C (%)	5.1 (4.7–5.8)	5.6 (5.3–5.8)	5.9(5.4–6.0)	0.08
Triglycerides (mg/dL)	175 (154.8–207.6)	228.2 (202.2–245.9)	216.6 (159.5–302.1)	0.07
Total cholesterol (mg/dL)	201.1 ± 43.9	209.6 ± 29.4	198.3 ± 41.4	0.69
HDL (mg/dL)	65.75 ± 8.96	65.28 ± 7.94	60.58 ± 6.21	0.24
LDL (mg/dL)	96.62 ± 43.00	97.48 ± 28.89	92.83 ± 34.33	0.99
Insulin (uU/L)	9.66 (5.34–26.26)	10.18 (6.09–13.84)	19.40 (7.73–30.90)	0.32
IGF1 (ng/mL)	171.82(120.51–216.01)	171.22(154.09–249.91)	225.82(116.12–295.92)	0.51
HOMA-IR	1.54 (0.94–5.22)	2.19 (1.12–3.21)	3.59 (1.622–6.04)	0.41
Leptin (pg/mL)	23,196.44 ± 10,653.66	24,976.64 ± 16,704.06	22,416.10 ± 10,461.18	0.87
Adiponectin	6.17 ± 0.88*	5.20 ± 1.38	6.11 ± 1.74	0.07
Desacylghrelin (ng/mL)	3.72 (2.61–8.06)	3.72 (1.46–7.75)	3.9 (2.13–10.30)	0.91
Acylghrelin (ng/mL)	3.56 (2.56–18.66)	3.48 (2.75–4.89)	3.62 (3.19–5.76)	0.91
Neonatal metabolic and hormonal status (cord blood)				
Triglycerides (mg/dL)	48.5 (35.5–69.7)	58.0 (54.0–63.3)	49.0 (45.0–58.83)	0.25
Total cholesterol (mg/dL)	73.0 (61.35–86.6)	83.7 (75.1–95.0)	76.5 (70.3–79.43)	0.13
HDL (mg/dL)	42.63 ± 8.30	47.05 ± 7.35	42.21 ± 10.45	0.21
LDL (mg/dL)	21.75 ± 13.93	25.40 ± 9.35	23.22 ± 11.76	0.65
Glucose (mg/dL)	63.9 (47.5–85.2)	77.0 (65.4–89.8)	61.8 (56.7–70.5)	0.07
Insulin (μU/L)	1.62 (0.87–2.98)*	3.39 (2.46–4.75)	4.05 (3.48–5.29)$^{+}$	**<0.001**
HOMA-IR	0.25 (0.17–0.35)*	0.55 (0.45–0.90)	0.64 (0.48–1.01)$^{+}$	**<0.001**
IGF1 (ng/mL)	14.18 (7.47–35.26)*	32.20 (24.92–44.46)	40.82 (29.61–53.38)$^{+}$	**0.003**
IGF2	262.89 ± 37.32	281.04 ± 65.06	267.42 ± 50.42	0.60
IGFBP3	1539.65 ± 342.63*	1850.15 ± 427.91	1741.31 ± 367.03	0.09
IGFBP1	90.54 ± 56.16	76.31 ± 42.34	53.0 ± 47.81	0.17
Leptin (pg/mL)	8827.6 ± 4915.7	13218.2 ± 6069.4	26037.4 ± 9550.5^{+}*	**<0.001**

*$P<0.05$ compared to control AGA.
$^{+}P<0.05$ compared to SGA.

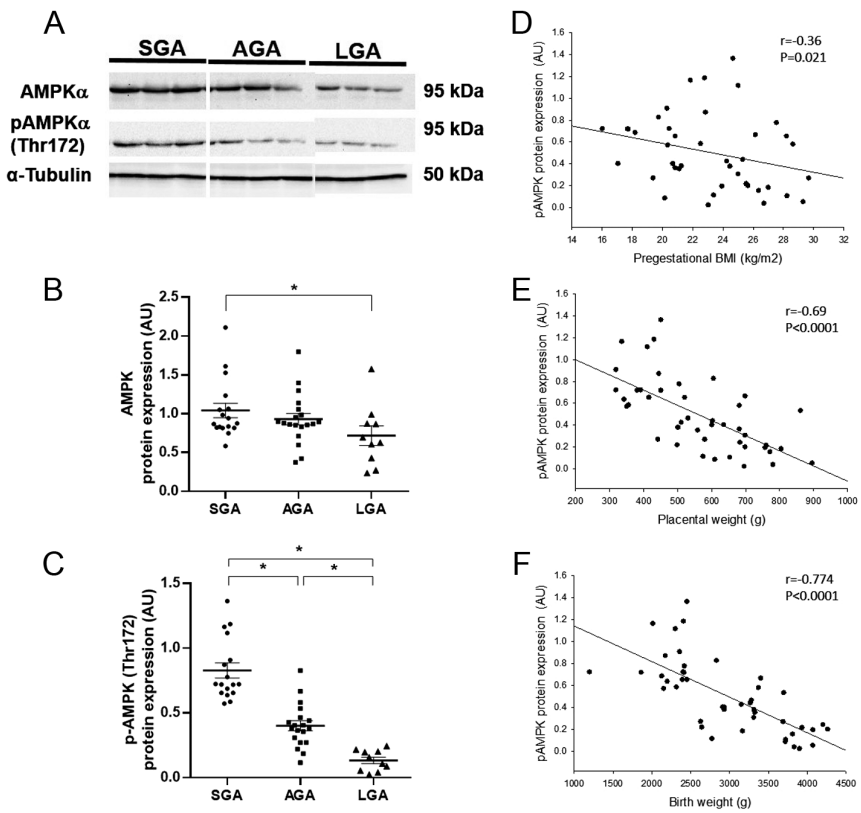

Figure 1 AMPK and p-AMPK protein expression in placentas of SGA, AGA and LGA newborns. Representative Western Blot of AMPK and p-AMPK in placentas of SGA ($n=17$), AGA ($n=19$) and LGA ($n=10$) newborns (A). Bands presented correspond to the same membrane. Quantification of AMPK (B) and p-AMPK (C) protein expression in placenta by immunodetection. Data are mean ± S.E. *Denotes significant difference ($P<0.05$) between groups. Correlation between placental expression of p-AMPK with pregestational weight (D), placental weight (E) and birth weight (F).

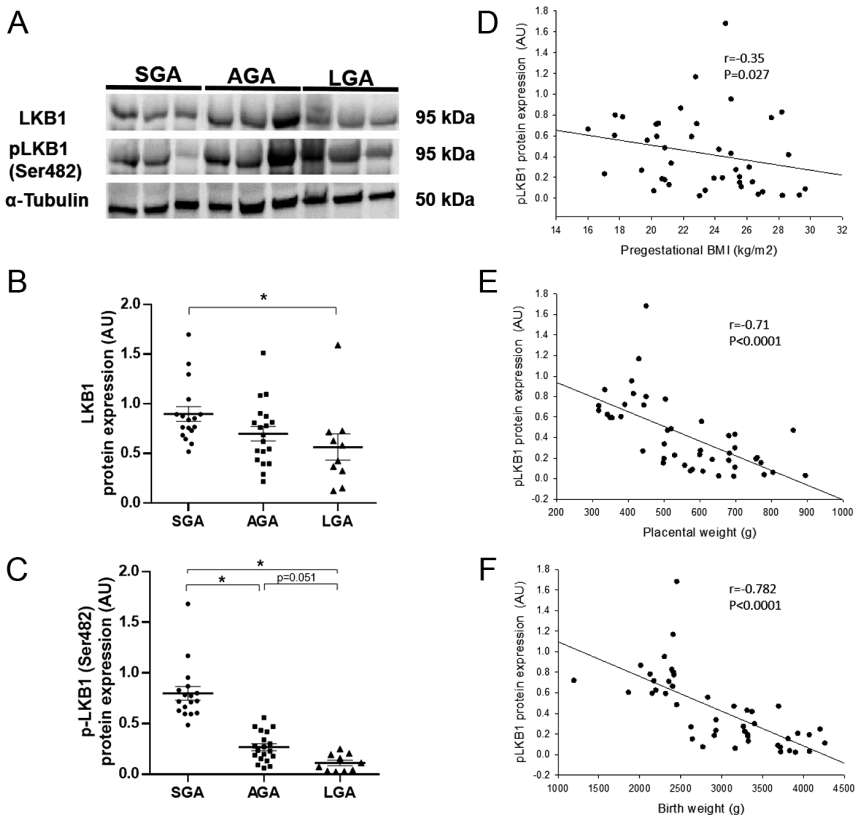

Figure 2 LKB1 and p-LKB1 protein expression in placentas of SGA, AGA and LGA newborns. Representative Western Blot of LKB1 and p-LKB1 in placentas of SGA ($n=17$), AGA ($n=19$) and LGA ($n=10$) newborns (A). Bands presented correspond to the same membrane. Quantification of LKB1 (B) and p-LKB1 (C) protein expression in placenta by immunodetection. Data are mean ± S.E. *Denotes significant difference ($P<0.05$) between groups. Correlation between placental expression of p-LKB1 with pregestational weight (D), placental weight (E) and birth weight (F).

to AGA (3.12- vs 0.54-fold of AGA, $P<0.0000$ expression respectively) (Fig. 2A and C). Total AMPK correlated both with total and p-LKB1 ($r=0.715$ and $r=0.482$ respectively, $P<0.0001$), while p-AMPK was highly correlated with p-LKB1 ($r=0.933$, $P<0.0001$). Both p-AMPK and p-LKB1 correlated negatively with placental weight (Figs 1E and 2E), birth weight (Figs 1F and 2F), neonatal anthropometry variables and cord blood leptin (Supplementary Table 2). Total LKB1 expression also correlated with weight, length, and thoracic perimeter at birth. Total and phosphorylated forms of AMPK and LKB1 were also inversely correlated with cord blood insulin and neonate HOMA-IR (Supplementary Table 2).

Interestingly, both phosphorylated proteins correlated negatively with pregestational weight and BMI (Figs 1D and 2D) but not with pregnancy weight gain, maternal IGF1, glucose, lipid profile, or HbA1c (Supplementary Table 3). In the multivariate analysis, p-AMPK predicted birth weight independently of placental weight, pregestational BMI, maternal glucose, maternal triglycerides, and gestational age ($t=-2.5$, $P=0.018$).

Placental SIRT1 and PGC1a protein expression

Placental expression of SIRT1 was lower in LGA when compared to SGA (Fig. 3A and B). SIRT1 correlated directly with both p-AMPK and p-LKB1 ($r=295$, $P=0.046$ and $r=0.297$, $P=0.045$ respectively). SIRT1 expression correlated with birth length ($r=-0.3$, $P<0.05$), birth weight ($r=-0.418$ and $P=0.004$) (Fig. 3C) and placental IGF1R ($r=-0.292$, $P=0.048$) but not with placental weight. SIRT1 also correlated with cord blood leptin and neonatal HOMA-IR (Supplementary Table 2). Circulating IGF1 was the only maternal variable associated with SIRT1 (Fig. 3D).

PGC1a, an effector of AMPK and SIRT1 activation, was elevated in SGA and decreased in LGA compared to AGA placentas ($P<0.001$) (Fig. 4A and B). This expression was directly correlated with total and p-AMPK ($r=0.333$ and $r=0.721$), total and p-LKB1 ($r=0.509$ and $r=0.693$), and SIRT1 ($r=0.32$, $P<0.05$ for all). We found a correlation of placental PGC1a with placental weight (Fig. 4D), birth weight (Fig. 4E), as well as birth length, thoracic, and abdominal perimeters (Supplementary Table 2). Neonatal leptin, insulin and HOMA-IR were correlated with PGC1a as well. Interestingly, IGF1R expression was negatively correlated with PGC1a ($r=-0.297$, $P=0.045$) (Fig. 4C), but not with the rest of the insulin/IGF1 pathway proteins or any maternal parameters (Supplementary Table 3).

Placental mTOR protein expression and activation

Placental expression of total mTOR was lower in SGA as compared to AGA and LGA (Fig. 5A and B).

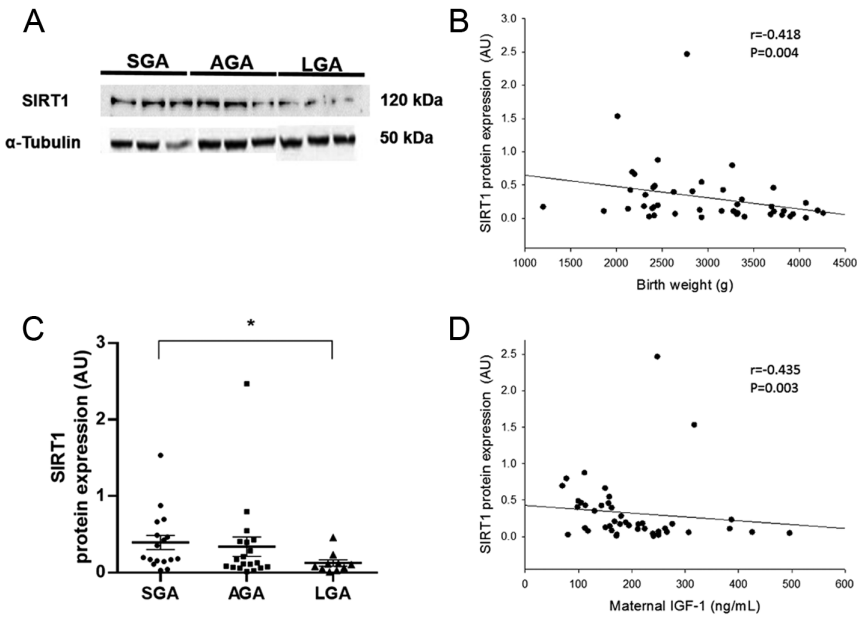

Figure 3 SIRT1 protein expression in placentas of SGA, AGA and LGA newborns. Representative Western Blot of SIRT1 in placentas of SGA ($n=17$), AGA ($n=19$) and LGA ($n=10$) newborns (A). Bands presented correspond to the same membrane. Quantification of SIRT1 (B) protein expression in placenta by immunodetection. Data are mean ± S.E. *Denotes significant difference ($P<0.05$) between groups. Correlation between placental expression of SIRT1 with birth weight (C) and maternal IGF1 (D).

Phosphorylated mTOR (p-mTOR) was 40% lower in SGA compared to AGA and LGA (Fig. 5A and C). Both total ($r=0.39$, $P=0.02$) and p-mTOR (Fig. 5D) were positively associated with birth weight, as well as with other neonatal variables such as cephalic perimeter, thoracic perimeter, abdominal perimeter, and cord blood IGF1 (Supplementary Table 2). Additionally, p-mTOR correlated with placental weight (Fig. 5E) and birth length (Supplementary Table 2). After adjusting for gestational age, pregestational BMI, maternal glucose, and placental weight, only total mTOR protein, but not p-mTOR, remained significant for predicting birth weight (t=2.35, $P=0.025$).

p-mTOR was associated with placental AMPK total expression ($r=-0.33$, $P=0.04$) and activation ($r=-0.5$, $P<0.0001$), as well as with total ($r=-0.37$, $P=0.02$) and p-LKB1 ($r=-0.55$, $P<0.0001$). Regarding maternal variables, only blood glucose was positively correlated with p-mTOR (Fig. 5F), but not with total mTOR expression (Supplementary Table 3).

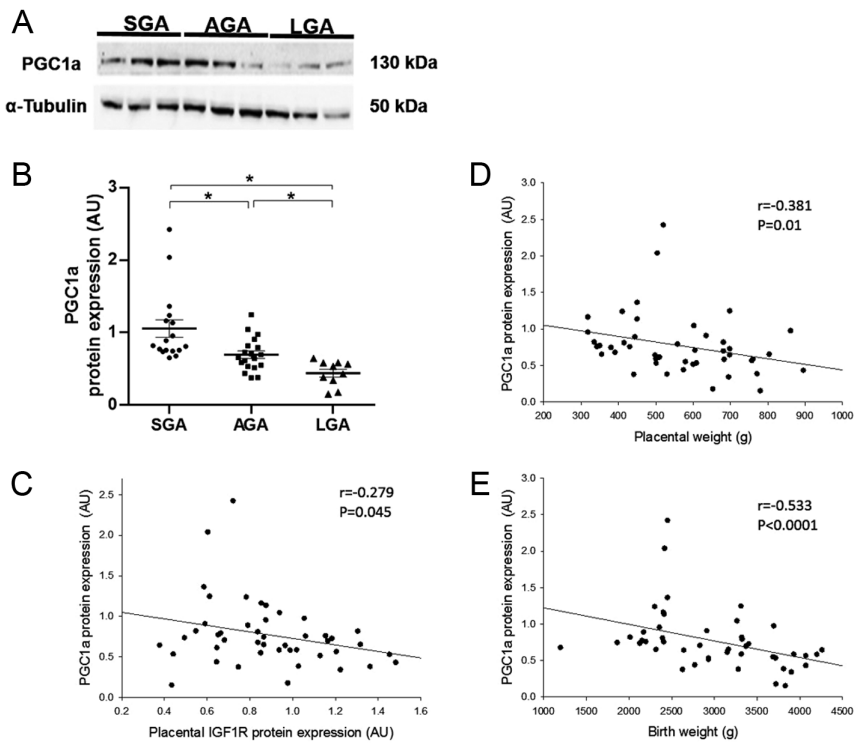

Figure 4 PGC1a protein expression in placentas of SGA, AGA and LGA newborns. Representative Western Blot of PGC1a in placentas of SGA ($n=17$), AGA ($n=19$) and LGA ($n=10$) newborns (A). Bands presented correspond to the same membrane. Quantification of PGC1a (B) protein expression in placenta by immunodetection. Data are mean ± S.E. *Denotes significant difference ($P<0.05$) between groups. Correlation between placental expression of PGC1a with placental IGF1R (C), placental weight (D) and birth weight (E).

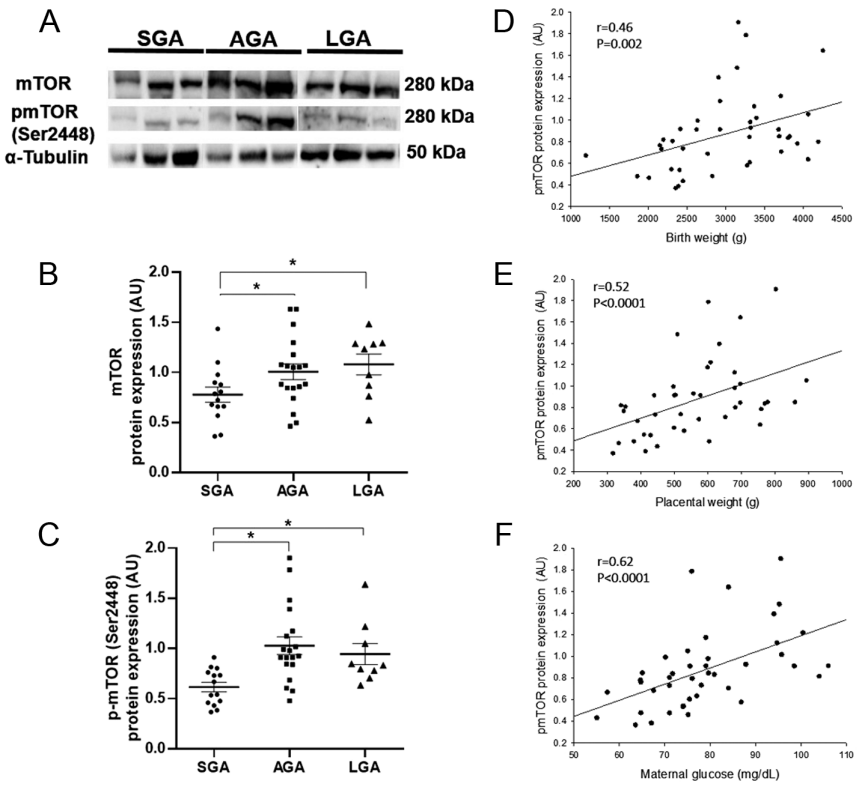

Figure 5 mTOR and p-mTOR protein expression in placentas of SGA, AGA and LGA newborns. Representative Western Blot of mTOR and p-mTOR in placentas of SGA ($n=14$), AGA ($n=19$) and LGA ($n=10$) newborns (A). Bands presented correspond to the same membrane. Quantification of mTOR (B) and p-mTOR (C) protein expression in placenta by immunodetection. Data are mean ± S.E. *Denotes significant difference ($P<0.05$) between groups. Correlation between placental expression of p-mTOR with birth weight (D), placental weight (E) and maternal glucose (F).

Placental insulin/IGF1 pathway protein expression and activation

Placental IGF1R expression was found to be 20% lower in SGA compared to control AGA ($P=0.034$) (Fig. 6A and B) and correlated positively with birth length ($r=0.33$, $P=0.02$) and marginally with birth weight (Fig. 6C) but not placental weight (Supplementary Table 2) or maternal variables (Supplementary Table 3). No changes in the kinase docking-associated phosphorylation p-IGF1R–Tyr980 were found (Fig. 6D and E). p-IGF1R correlated with length at birth ($r=0.298$, $P=0.048$) (Fig. 6F) and maternal weight gain ($r=0.379$, $P=0.016$) (Fig. 6I). INSRb protein expression was not modified between the groups (Fig. 6G and H).

Phosphorylation of PDK1, the main kinase for Thr308 AKT activation, decreased 40% in SGA ($P=0.017$) and 50% in LGA ($P=0.008$) groups compared to AGA (Fig. 7A and B). No differences in total AKT expression or its phosphorylated forms in SGA and LGA were found (Fig. 7C, D, E and F). However, p-AKT-Thr308 showed a positive correlation with maternal glucose ($r=0.403$, $P=0.005$) and leptin ($r=0.336$, $P=0.02$), as well as with p-mTOR ($r=0.31$, $P=0.05$). Total and pAKT-Ser473 positively correlated with INSRb expression ($r=0.493$, $P<0.001$ and $r=0.301$, $P=0.041$, respectively), but not IGF1R. Total AKT, but not its phosphorylated forms, was inversely correlated to weight gain during pregnancy ($r=-0.374$, $P=0.017$).

Effects of sex and maternal BMI on placental protein expression

We analysed whether differential placental protein expression was different by neonate's sex or pregestational BMI, independent of birth weight classification. None of the proteins differed between females and males overall. Only in the SGA group, the expression of AMPK, LKB1, and their phosphorylated forms was higher in females than in males (Supplementary Table 4). Protein expression was not different between normal and overweight pregestational BMIs in any birth weight group (P values for interaction: AMPK $P=0.834$, p-AMPK $P=0.373$, LKB1 $P=0.49$, p-LKB1 $P=0.831$, SIRT1 $P=0.919$, PGC1a $P=0.756$, mTOR $P=0.416$ and p-mTOR $P=0.475$).

Discussion

The physiological role of nutrient-sensing pathways in the placenta has not been completely elucidated. In the present study, we aimed to analyse protein expression and activity of AMPK, mTOR and insulin/IGF1 pathways in placental tissue of non-obese mothers with normal pregnancy, in order to look for possible interactions within these pathways and maternal endocrine or metabolic status involved in placental growth and function.

We found placental AMPK protein expression in SGA newborns was increased compared to LGA group.

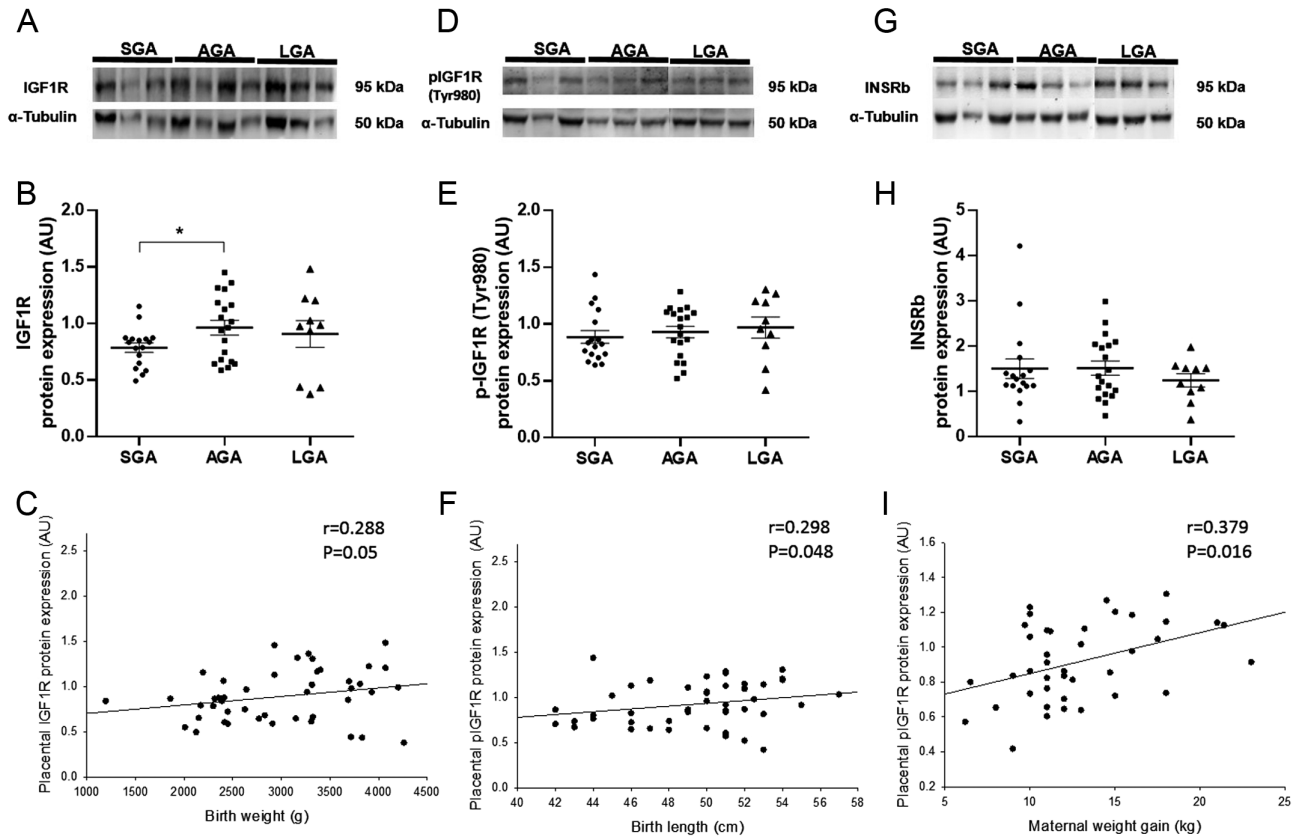

Figure 6 IGF1R, p-IGF1R and INSRb protein expression in placentas of SGA, AGA and LGA newborns. Representative Western Blot of IGF1R (A), p-IGF1R (D) and INSRb (G) in placentas of SGA ($n=17$), AGA ($n=19$) and LGA ($n=10$) newborns. Bands presented correspond to the same membrane. Quantification of IGF1R (B), p-IGF1R (E) and INSRb (H) protein expression in placenta by immunodetection. Data are mean ± S.E. *Denotes significant difference ($P<0.05$) between groups. Correlation between placental expression of IGF1R with birth weight (C). Correlation between placental expression of p-IGF1R with birth length (F) and maternal weight gain (I).

Accordingly, p-AMPK was higher in SGA and lower in LGA compared to AGA, which may reflect higher and lower placental AMP/ATP ratios, respectively. AMPK activation requires LKB1 phosphorylation (Hardie *et al*. 2012). Placental LKB1 was higher in SGA compared to LGA, while p-LKB1 decreased with higher birth weight. Accordingly, p-LKB1 and p-AMPK were strongly correlated, suggesting that AMPK phosphorylation is carried out by LKB1 in the placenta. This is consistent with AMPK function in most tissues (Huang *et al*. 2008) and a previous study, the only one measuring LKB1 expression in human placenta so far, in which LKB1 activates AMPK in human and mouse placentas (Skeffington *et al*. 2016).

Placental AMPK is modulated by maternal nutrient status, oxygen conditions, and hormones. In sheep, 50% caloric restriction in pregnancy increases AMPK activity (Ma *et al*. 2011), and *LKB1* and *AMPK* mRNA is increased in placentas of mothers living at high altitudes (hypoxic conditions) (Skeffington *et al*. 2016). On the contrary, placental p-AMPK decreases in rats under a high-fat diet (Gaccioli *et al*. 2013). Lower placental AMPK protein, p-AMPK, (Jansson *et al*. 2013) and mRNA (Martino *et al*. 2016) are associated with pregestational obesity in women, together with higher maternal leptin concentrations, while *AMPK* mRNA is similar in overweight mothers, as compared to normal BMI (Martino *et al*. 2016). Adiponectin activates AMPK in placenta cell lines (Benaitreau *et al*. 2009) and primary trophoblasts (Jones *et al*. 2010). Ghrelin activates AMPK in several tissues (Kola *et al*. 2005) and its receptor GHSR1 (growth hormone secretagogue receptor 1) is expressed in human placenta (Gonzalez-Dominguez *et al*. 2016); however, ghrelin's role in placental function is unclear. Neither maternal adiponectin, leptin nor ghrelin values in the present study differed by pregestational BMI, similar to previous studies (Martino *et al*. 2016, Stefaniak *et al*. 2019). Interestingly, LKB1 and AMPK expression and activation were higher in SGA females, in agreement with a study where androgens inhibited and oestrogens stimulated LKB1/AMPK activation in 3T3-L1 adipocytes (McInnes *et al*. 2012).

Although it is widely known that AMPK exerts metabolic effects through SIRT1 activation in other tissues (Cantó & Auwerx 2009), the modulation of SIRT1 in placenta is still controversial and has not been

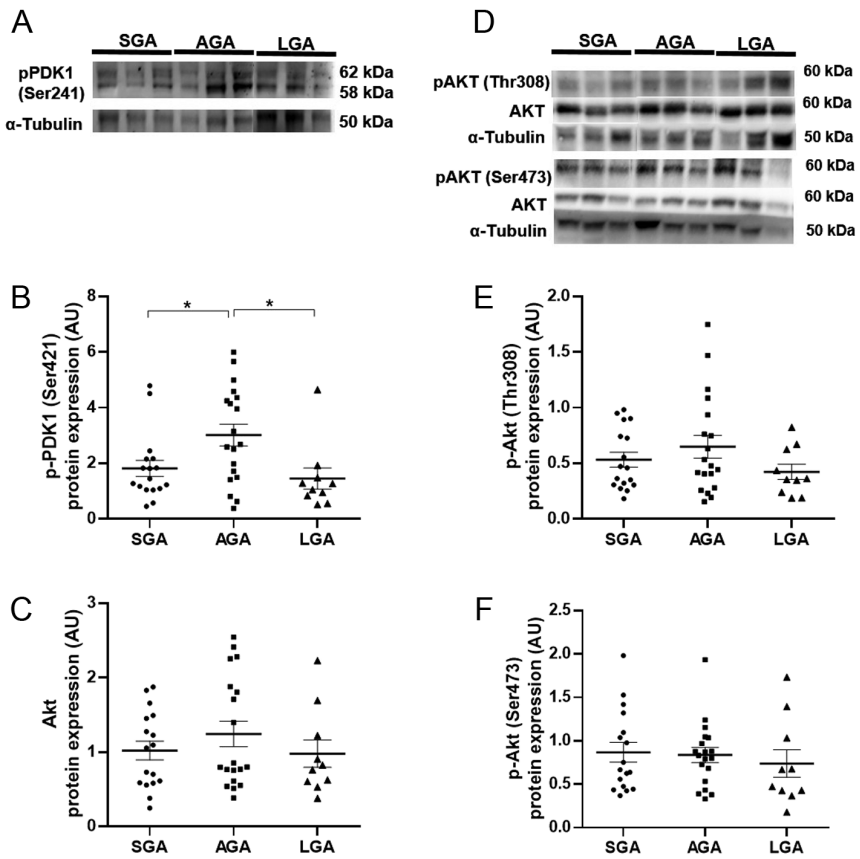

Figure 7 p-PDK1, AKT and pAKT protein expression in placentas of SGA, AGA and LGA newborns. Representative Western Blot of p-PDK1 (A), AKT and p-AKT (D) in placentas of SGA ($n=17$), AGA ($n=19$) and LGA ($n=10$) newborns. Bands presented correspond to the same membrane. Quantification of p-PDK1 (B), AKT (C) and p-AKT (E and F) protein expression in placenta by immunodetection. Data are mean ± S.E. *Denotes significant difference ($P<0.05$) between groups.

previously studied concomitantly with AMPK activation. Placentas from obese animal models have shown either reduced SIRT1 protein (Qiao *et al*. 2015) or no changes in *SIRT1* mRNA (Borengasser *et al*. 2014). In contrast, *SIRT1* mRNA was higher in placentas from overweight/obese women compared to normal BMI ones (Martino *et al*. 2016). In the present study, placental SIRT1 was reduced in LGA and negatively correlated with birth weight. It is plausible that placental SIRT1 mRNA and protein are subject to distinct regulation in conditions such as oxidative stress, which may modulate placental nutrient uptake (Lappas *et al*. 2012). As suggested by the correlation found with p-AMPK, IGF1R and maternal IGF1 with SIRT1, our findings point to an interaction of AMPK and IGF with the SIRT1 pathway in human placenta, as it has been seen in other tissues (Tran *et al*. 2014, Kang *et al*. 2016).

AMPK and SIRT1 converge in activating PGC-1α (Cantó & Auwerx 2009). We found higher placental PGC1a in SGA compared to AGA and LGA groups. Studies in animal models with pregestational obesity showed that placental *PGC1a* mRNA was not modified (Borengasser *et al*. 2014), while its protein expression was increased (Leiros *et al*. 2018). In contrast, in our study PGC1a expression is inversely correlated with placental and fetal growth. *PGC1a* mRNA is inversely associated with maternal glycemia (Wang *et al*. 2018) and PGC1a decreased in placental BeWo cells exposed to high glucose (Jiang *et al*. 2017). GDM placentas have increased *PGC1a* gene promoter methylation (Xie *et al*. 2015, Wang *et al*. 2018) and reduced mRNA expression (Wang *et al*. 2018), but not protein expression (Xie *et al*. 2015). In contrast, we did not find a correlation of PGC1a with maternal glucose or HbA1c. The positive correlation between p-AMPK and SIRT1 found in our study suggests that human placental PGC1a expression may depend on energy/nutrient sensing either directly from AMPK or through AMPK–SIRT1.

The role of the AMPK pathway in placental function is yet to be elucidated. While in most tissues its activation leads to increased metabolic energy production and enhanced mitochondrial function (Hardie *et al*. 2012), intriguing results have been found in placenta. Pharmacological activation of placental AMPK in explants from normal pregnancies decreased fatty acid oxidation, glucose metabolism, and mitochondrial function, as well as polyunsaturated fatty acid and glucose uptake, but a normal uptake of amino acids, saturated and monosaturated fatty acids (Landau *et al*. 2019). As we found an inverse association of AMPK activation with neonatal and placental weight independently of maternal variables, it is plausible that the AMPK pathway modulates placental growth and metabolism with differential results in SGA and LGA outcomes. Our results pose new questions regarding the use of supplements or pharmacological agents

targeting LKB-AMPK-SIRT1, which have been suggested as therapeutic alternatives during pregnancy, such as resveratrol (Yao *et al*. 2015, Lan *et al*. 2017) or metformin (Garcia-Contreras *et al*. 2019), and their potential effects in placental function and birth weight establishment.

mTOR is a nutrient-sensing pathway targeted by AMPK, which is prone to hormonal modulation by leptin, insulin/IGF1, and adiponectin in the placenta (Kelly *et al*. 2020). We found that total mTOR protein expression, together with its phosphorylation, were lower in placentas of SGA newborns and correlated positively with birth weight. Obese women with LGA (Jansson *et al*. 2013) and GDM women with macrosomic newborns (Shang & Wen 2018) have increased mTORC1 and mTORC2 and decreased AMPK placental activation. In our study, mTOR phosphorylation was similar between AGA and LGA, perhaps since mothers did not present obesity, altered hormonal status, or a prior pathological condition. Nevertheless, as expected, mTOR phosphorylation was negatively associated with AMPK and LKB activation. Of note, placental and birth weights were strongly associated with p-AMPK, pregestational weight and total mTOR, but not phosphorylated mTOR. Moreover, no maternal variables other than glucose correlated with mTOR phosphorylation, which is in line with downregulation of mTORC1 by glucose deprivation in primary trophoblast cultures, with no change in AMPK expression or activation (Roos *et al*. 2009). Our results suggest that AMPK-mediated placental growth and function may involve mTOR-dependent and mTOR-independent pathways, as seen in other tissues (Stevanovic *et al*. 2013).

IGFs modulate placental growth and efficiency (Sferruzzi-Perri *et al*. 2017). Similar to previous reports in IUGR (Laviola *et al*. 2005), we did not observe changes in INSRb in the SGA or LGA groups as compared to the AGA, while placental expression of the IGF1R receptor was lower in SGA. This supports the concept that INSRb and IGF1R exert different functions in the placenta (Sferruzzi-Perri *et al*. 2017). Placental IGF1R expression is contradictory, finding both an increase (Iniguez *et al*. 2010) and decrease (Laviola *et al*. 2005) in IUGR and no changes in *IGF1R* mRNA in mothers with different pregestational BMIs (Martino *et al*. 2016). Additionally, *IGF1R* mutations are associated with IUGR (Klammt *et al*. 2011), and the downregulation of *Igf1r* in trophoblast leads to AMPK activation (Louden *et al*. 2008). In our study, correlation of IGF1R and p-IGF1R to neonatal length supports the role of placental IGF1R pathway in fetal growth (Agrogiannis *et al*. 2014). According to the IGF1R decrease in SGA, phosphorylation of p-PDK1, the kinase that phosphorylates AKT–Thr308 for its activation (Bayascas 2010), was decreased in the SGA group as compared to AGA group. Intriguingly, p-PDK1 was also lower in LGA, while no differences were found in any AKT phosphorylation site. Paradoxical results in AKT protein expression and its activation have been shown in IUGR placentas, where p-INSRb was found in contrast to the decreased AKT expression and undetectable pAKT (Street *et al*. 2011).

AKT crosstalk with mTORC pathway has been largely reported. The phosphorylation of AKT in Ser473 depends on the interactions with mTORC2, while phosphorylation in AKT in Thr308 activates mTORC1 downstream (Saxton & Sabatini 2017). In this line, p-AKT–Thr308 is positively correlated with mTOR phosphorylation in our study, consistent with the role of insulin/IGF1 signaling in placental mTOR modulation (Gupta & Jansson 2019). However, we did not evaluate mTOR downstream effector molecules, which would have been useful in discerning the roles of mTORC1 and mTORC2 in SGA and LGA in non-obese pregnancy.

One of the main limitations of our study is the small number of samples included. As such, it is plausible that we have missed some of the differential effects on placental protein expression. The use of whole placenta samples instead of fetal and maternal sides separately is another potential limitation. In this regard, mTOR activation responds in the same direction in both trophoblasts and decidua in IUGR (Gupta & Jansson 2019). IGF1R protein expression is similar for both sides (Iniguez *et al*. 2010), while SIRT1 is more abundant in trophoblasts compared to decidual cells (Lappas *et al*. 2011). Notwithstanding these limitations, the AMPK and mTOR expression changes we found are in accordance with previous studies in animal models (Ma *et al*. 2011, Rosario *et al*. 2011, Gaccioli *et al*. 2013) and in human placenta of similar sample sizes (Roos *et al*. 2007, Jansson *et al*. 2013, Martino *et al*. 2016, Shang & Wen 2018).

To our knowledge, this is the first study evaluating placental nutrient-sensing pathways at the protein level in SGA and LGA newborns of idiopathic origin in non-obese mothers. Notably, exploring whether maternal metabolic and endocrine variables at normal levels are related to nutrient sensing modulation is a strength of the present study. While we did not assess detailed maternal nutritional status, maternal weight gain and hormone concentrations were similar between our groups. Our findings suggest that the changes found in placental nutrient-sensing pathways may not be due to maternal nutritional status at the macronutrient level (Martino *et al*. 2016), but perhaps subtle nutritional differences of specific nutrients (Silva *et al*. 2017). Additionally, variations in placental blood flow may affect both oxygen status and nutritional availability, rendering differential AMPK (Skeffington *et al*. 2016) and mTOR activation (Gupta & Jansson 2019).

In conclusion, our results suggest that the LKB1-AMPK-SIRT1–PGC1a pathway, together with mTOR and IGF1R expression, is associated with fetal growth in normal pregnancy, perhaps through regulation of placental physiology. LKB1–AMPK activation is associated with pregestational BMI, highlighting the importance of

pre-conception maternal status in placental programming. Our results shed light on possible molecular mechanisms linking the maternal environment to fetal outcomes, which may be mediated by placental adaptations. More studies in this line are required to clarify the physiological role of these pathways in the placenta, to aid in the establishment of potential therapeutic targets for improving fetal development and, consequently, preventing metabolic diseases in later life.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/REP_200186.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Basic Science CONACYT (CB-2013-222563) and University of Guanajuato CIIC 2016-2017 (1089/2016).

Author contribution statement

MLLVM and GBS conceived and designed the study. MLLVM, KAMT, MIGD, JAGS and ACF generated, collected and assembled the data. MLLVM, GBS, HMGZ, JMMH, and LDB analyzed and interpreted the data, drafted, and critically revised the article. All authors approved the final version to be published.

Acknowledgements

MIGD was recipient of a postdoctoral Fellowship Scholarship from CONACYT (CVU: 22283). KAMT was recipient of a Master's Scholarship from CONACYT (CVU: 743088). JAGS was recipient of a Master's Scholarship from CONACYT (CVU: 819241).

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Received 11 February 2020
First decision 26 February 2020
Revised manuscript received 29 May 2020
Accepted 25 June 2020