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Angiopoietin-like protein 8 (ANGPTL8) in pregnancy: A brown adipose tissue-derived endocrine factor with a potential role in fetal growth.

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Short running title: ANGPTL8 in pregnancy, BAT and fetal growth

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Abbreviations

ANGPTL8: Angiopoietin-like protein 8

GDM: Gestational diabetes mellitus

WAT: White adipose tissue

BAT: Brown adipose tissue

OGTT: Oral glucose tolerance test

BMI: Body mass index

LGA: Large for gestational age

AGA: Adequate for gestational age

SGA: Small for gestational age

SSF: Sum of three skinfolds

HOMA-IR: Insulin Resistance Homeostatic Model Assessment

HOMA-B: Beta cell function – Homeostatic Model Assessement

hWA: human white adipocytes

hBA: human brown adipocytes

mBA: murine brown adipocytes

hASCS: Adipose derived stem cells

UCP1: Uncoupling protein 1

ABSTRACT

Angiopoietin-like protein 8 (ANGPTL8), a protein implicated in lipid and glucose homeostasis, is present only in mammals, suggesting that it is involved in processes unique to these vertebrates such as pregnancy and homeothermy. We explored the role of ANGPTL8 in maternal-fetal crosstalk and its relationship with newborn adiposity. In a longitudinal analysis of healthy pregnant women, ANGPTL8 levels decreased progressively during pregnancy although remained higher than levels in the postpartum period. In a cross-sectional observational study of women with or without gestational diabetes (GDM), and their offspring, ANGPTL8 levels were higher in venous cord blood than in maternal blood, and were significantly lower in GDM patients than in healthy women. Infants small for gestational age and with low fat mass had the highest ANGPTL8 cord blood levels. Studies *in vitro* revealed that ANGPTL8 was secreted by brown adipocytes and its expression was increased in experimental models of white-to-brown fat conversion. Additionally, ANGPTL8 induced the expression of markers of brown adipocytes. The high levels of ANGPTL8 found in fetal life together with its relationship with newborn adiposity and brown adipose tissue point to ANGPTL8 as a potential new player in the modulation of the thermogenic machinery during the fetal-neonatal transition.

INTRODUCTION

Angiopoietin-like protein 8 (ANGPTL8)[1], also known as lipasin[2], betatrophin[3] and RIFL[4] (refeeding induced in fat and liver), is a novel but atypical member of the angiopoietin-like protein family that is implicated in lipid and glucose homeostasis *via* its ability to inhibit lipoprotein lipase activity[1] and to induce pancreatic β-cell proliferation in insulin resistance states[3]. In humans, the highest expression levels of *ANGPTL8* are found in the liver[2] and this organ seems to be the main source of circulating ANGPTL8, although other potential sources have been proposed[4,5]. Circulating ANGPTL8 concentrations are increased in obesity and type 2 diabetes[6–9], however this finding is contentious[10–12]. Intriguingly, while *ANGPTL8* expression has been detected in mammals, neither the transcript nor its polypeptide homologs are present in other vertebrate species, suggesting that ANGPTL8 may be implicated in physiologic functions that are unique to mammals such as homeothermy, pregnancy and lactation[13].

Pregnancy is characterized by increased insulin secretion and marked insulin resistance. This physiologic adaptation facilitates a constant net flow of glucose (and other nutrients) to the growing fetus. The morphological basis for this hyperinsulinism is pronounced β-cell hypertrophy and hyperplasia, and hyperactivity of individual β-cells[14]. In murine models, liver *ANGPTL8* expression increases as pregnancy progresses[3], but data on ANGPTL8 regulation in human pregnancy is scarce and inconclusive[15–17]. Circulating ANGPTL8 concentrations are reported to be higher in gestational diabetes mellitus (GDM) than in normal pregnancy[15–17], but there is no consensus on ANGPTL8 concentrations between pregnancy and postpartum[15,16] and nothing is known about the evolution of ANGPTL8 concentrations during pregnancy.

ANGPTL8 is also present in fetal life and higher ANGPTL8 levels have been found

in umbilical cord blood than in maternal serum[16], suggesting a role in fetal growth and development. In the fetus, total adipose tissue mass increases through late gestation and comprises a mixture of white and brown adipocytes. Fetal nutrient supply during late gestation determines white adipose tissue (WAT) and brown adipose tissue (BAT) depots. BAT is essential for postnatal adaptation to temperature and the onset of non-shivering thermogenesis after birth and, in humans, comparatively greater amounts of BAT are present in the newborn than in other mammals[18]. ANGPTL8 is expressed in both WAT and BAT depots[1,4], and its regulation depends on nutritional and environmental factors[1,2,4,19]. Specifically, *ANGPTL8* mRNA is up-regulated during white and brown adipocyte differentiation[4], suppressed by starving and agents that induce lipolysis[1,2], and induced by insulin[4], a cold environment[19] and feeding[1,2]. Since pregnancy and homeothermy are situations unique to mammals, we hypothesized that ANGPTL8 may be involved in mother-fetus crosstalk, to maintain metabolic homeostasis during pregnancy, and may be an inductor of adipose tissue browning. We evaluated ANGPTL8 concentrations during normal pregnancy and explored the relationship between maternal and cord blood ANGPTL8 with newborn adiposity in normal pregnancy and GDM. We also evaluated circulating levels and BAT expression of ANGPTL8 during pregnancy in mice. Finally, we assessed the ability of ANGPTL8 to act as an endocrine factor secreted by brown adipocytes, among others, which might facilitate white-to-brown fat conversion.

RESEARCH DESIGN AND METHODS

Study population

The study was undertaken at the Hospital Universitari de Tarragona Joan XXIII and was carried out according to the code of ethics of the World Medical Association

(Declaration of Helsinki). Participants were women with singleton term births with no major birth defects, with an accurate gestational age confirmed by an ultrasound examination before 20 weeks of gestation and a gestational age at delivery of 37 weeks or more. The study protocol was reviewed and approved by the research ethics board of the center and all participants provided written informed consent before inclusion. To assess the effects of pregnancy on ANGPTL8 concentrations and its relationship with insulin secretion, 15 pregnant women were enrolled in the first prenatal visit, before the 12th week of pregnancy, and followed up until the end of pregnancy (cohort 1). A blood sample was collected in each trimester of pregnancy and stored at -80^{o} C until analysis. None of the women included in the longitudinal study had diabetes or any other chronic disease.

To analyze the relationship of ANGPTL8 to GDM, whether it was present in fetal life and its relationship with fetal development, we included 46 GDM and 37 control pregnant women, matched for age and body mass index, enrolled in a prospective pre-birth cohort, and their children (cohort 2). The participants were recruited at the time of the antenatal oral glucose tolerance test (OGTT) before the 30th week of pregnancy, and were monitored from the time of inclusion until delivery. All participants underwent a 3 h, 100 g OGTT and those with 2 or more values above the threshold proposed by the *National Diabetes Data Group* were considered GDM. Those with values below the threshold were classified as control. Umbilical cord blood was obtained at the time of delivery and a complete anthropometric evaluation was performed in 64 neonates (33 born to control women and 31 born to GDM women). Fifthteen control and 29 GDM women were re-evaluated 12-20 weeks after pregnancy. The following three exclusion criteria were applied before 36 weeks of pregnancy: identified fetal anomalies, inflammatory diseases or preeclampsia.

Clinical and demographic data

Upon inclusion, demographic and historical information of participants was collected using an interviewer-administered questionnaire focused on personal medical and obstetrical history, and also information regarding the current pregnancy with particular attention to risk factors for GDM. Maternal anthropometry was recorded as follows: height, pre-pregnancy weight and weight at the end of pregnancy in all pregnancies. In the longitudinal study, weight was also recorded at each evaluation, in the first, second and third trimester of pregnancy. Pre-pregnancy BMI was calculated using the formula: pre-pregnancy weight (kg)/(height (m))^2. The same procedure was used to calculate BMI in first, second and third trimester and postpartum evaluations. Increased BMI was calculated by the formula BMI gain = final BMI – pre-pregnancy BMI.

Neonatal length and weight were determined in all participants within 48 hours post-delivery using a measuring board to the nearest 0.1 cm and a calibrated scale to the nearest 10 g. Infant size was defined according to gestational age and sex population specific growth charts[20] as large for gestational age (LGA) if the infants were >90th percentile birth weight at each gestational age for fetal sex, and small for gestational age (SGA) when they were <10th percentile. Those with birth weights ≥10th and ≤90th percentiles were considered adequate for gestational age (AGA). Tricipital, subscapular and suprailiac skinfold thickness were measured within the first 48 hours of life. The sum of the three skinfolds (SSF) was used as a surrogate marker of neonatal fat mass[21]. SSF showed a normal distribution without significant differences between infants born to GDM and those born to control mothers. The cutoff points used to establish the three subgroups were the 25th percentile (10.84 mm) and the 75th percentile (12.46 mm). Subgroup 1 included pregnant women and their infants with

SSF below 10.84 mm, subgroup 2 included those with SSF between 10.84 and 12.46 mm and subgroup 3 included those with neonatal SSF above 12.46 mm.

Laboratory analysis

Maternal blood samples were obtained after an overnight fast and during pregnancy at the time of OGTT. Cord blood samples were obtained from the umbilical vein at delivery. Serum was immediately separated by centrifugation and frozen at -80º C until laboratory determinations. Circulating ANGPTL8 levels in human samples were determined using a commercially available ELISA kit (Wuhan Eiaab Science, Wuhan, China; catalog No. E11644h), with an intra-assay coefficient of variation (CV) <6.5%, inter-assay CV < 9.2% and spike average recovery of 102%. ELISA validation was performed by western blotting and a good correlation between both methods was observed (Supplementary Figure 1). In mouse samples, ANGPTL8 levels were determined using the Mouse ANGPTL8 ELISA Kit (Aviscera Bioscience, Santa Clara, CA.; catalog No. SK00528-16), with an intra-CV 4-6% and inter-CV 8-12%. To avoid the influence of interassay variations between maternal and fetal concentrations, ANGPTL8 levels in the same maternal-fetal pair were assayed in the same experiment. Serum fasting glucose, insulin, triglycerides, total cholesterol and high-density lipoprotein were determined by standard enzymatic methods. Insulin resistance and β-cell function were estimated using homeostatic model assessment (HOMA) IR and HOMA-β as described [22].

***In vitro* cell culture**

The Simpson-Golabi-Behmel Syndrome (SGBS) preadipocyte cell line, provided by Dr Wabitsch (University of Ulm, Germany), was used as a cellular model of human subcutaneous white adipocytes (hWA) and was differentiated as described[23]. PAZ6 cells, kindly provided by Dr. Tarik Issad (Institut Cochin, France), were used as a

cellular model of human brown adipocytes (hBA)[24]. An immortalized brown preadipocyte cell line[25] was used as a cellular model of murine brown adipocytes (mBA). To analyze ANGPTL8 secretion, cell culture medium was collected from the last two days of differentiation (between 10-14 days). Medium was concentrated using Amicom ultra columns (Millipore, Bilerica, MA) prior to western blotting. Adipose-derived stem cells (hASCs) were isolated from the subcutaneous adipose tissue of lean patients and differentiated following a published protocol[26]. During differentiation, hASCs cells were treated every second day with ANGPTL8 (20 ng/mL; Aviscera Biosciences, Santa Clara, CA) or FGF21 (100 nM; PeproTech, Rocky Hill, NJ).

Animal studies

Chronic AMPK activation *in vivo* was used as an induction model of browning[25]. Eight-week-old mice were treated with AICAR (0.5 mg/g; intra-peritoneal) three times weekly for two weeks. Mice in the control group received an equivalent volume of vehicle. At the end of the treatment period, animals were sacrificed and adipose tissue was harvested. The study was approved by the local ethics committee and all procedures were performed in accordance with the Federation of European Laboratory Animal Science Association.

For studies in pregnant mice and fetuses, C57/Bl6 female mice were mated overnight with male mice. The male was removed from the cage the next morning. When pregnant, the night after mating was considered day 0. At day 18, pregnant mice and age-matched non-pregnant female mice (controls, n=5-6 animals per group) were sacrificed by decapitation and blood was obtained for plasma preparation. Fetuses were removed by caesarean section. Interscapular BAT from pregnant mice, female controls and fetuses was dissected and frozen for subsequent analysis.

Gene expression analysis

Total RNA was extracted from adipose cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Two micrograms of RNA was retrotranscribed with random primers using the Reverse Transcription System (Applied Biosystems, Foster City, CA). Quantitative gene expression was analyzed using the TaqMan Gene Expression Assay (Applied Biosystems) on a 7900HT fast real-time PCR system. The following genes were evaluated: *ADRB3* (Hs 00609046_m1), *COX4I1* (Hs00971639_m1), *COX7A1* (Hs03045102_g1), *CPT1a* (Hs00912671_m1), *CTBP1* (Hs00972284_m1), *ELOVL3* (Hs00537016_m1), *FOXC2* (Hs00270951_s1), *PPARGC1A* (Hs01016719_m1), *PPARGC1B* (Hs00991677_m1), *PRDM16* (Hs00922674_m1), *ANGPTL8* (Mm01175863_g1), *B2M* (Mm00437762_m1), *TMEM26* (Hs00415619_m1), *TBX1* (Hs00271949_m1), *UCP1* (Hs00218820_m1 and Mm01244861_m1), and *18S* (Hs03928985_g1).

Western blot analysis

Equal amounts of protein were subjected to SDS-PAGE, transferred to immobilon membranes and blocked[27]. Immunoreactive bands were visualized using SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford, IL) and images were captured using the VersaDoc Imaging System and Quantity One software (Bio-Rad, Hercules, CA). The following antibodies were used: ANGPTL8 (Aviscera Biosciences, catalog No. SAB3501080), UCP1 (Santa Cruz Biotechnology, Santa Cruz, CA; catalog No. sc-6528) and GAPDH (Sigma-Aldrich, St. Louis, MO; catalog No. GW22763). For all the experiments, antibody dilutions were 1/1000 and incubation was carried out at 4º C overnight.

Statistical analysis

Data were analyzed with SPSS software version 17.0 (IBM, Armonk, NY). The 1-sample Kolmogorov-Smirnov test was performed to verify the normal distribution of the quantitative variables. Normally distributed data are expressed as mean±SD, whereas variables with a skewed distribution are represented as the median [Q25-Q75]. Categorical variables are reported as number (percentages) and chi-square test was used to analyze differences between groups. Student's t-test and the paired t-test were used to compare the mean values of continuous variables normally distributed between independent groups. Mann-Whitney-U and Wilconxon tests were used for variables with skewed distributions. One-way ANOVA, Kruskal Wallis test and the repeated measures ANOVA with post-hoc analysis were used to test differences between more than two variables as required. The Pearson correlation coefficient was used to assess univariate relationships. Variables with skewed distribution were log-transformed before analysis. A two-sided *P* value <0.05 was considered statistically significant.

RESULTS

Circulating levels of ANGPTL8 are regulated during pregnancy

We first measured ANGPTL8 levels from the first to third trimester in a longitudinal cohort of pregnant women. Table 1 summarizes clinical and metabolic data of this group (cohort 1). Serum ANGPTL8 levels progressively decreased across pregnancy (Figure 1A), while BMI, HOMA-IR index, total cholesterol, triglycerides and insulin concentrations increased. ANGPTL8 levels in each trimester were positively correlated between each other (data not shown), but no other association was observed.

We next measured maternal, cord blood and postpartum ANGPTL8 concentrations in a larger cohort of GDM and control women and their offspring to assess differences according to the maternal glucose tolerance status, and to investigate possible implications for fetal growth (cohort 2). Mother and child clinical metabolic data are presented in Table 2. The HOMA-IR index and triglycerides concentrations were higher in the GDM group than in the control group (P=0.019 and P=0.006, respectively). No differences were observed in pregnancy or postpartum maternal ANGPTL8 levels between both groups, whereas cord blood ANGPTL8 levels were lower in GDM women than in control women (P=0.033). GDM women gained less weight at the end of pregnancy, mainly because of a lower weight increase in the third trimester.

In the whole group, ANGPTL8 concentrations were significantly lower in maternal serum than in cord blood (2887.47±1195.62 vs. 3593.74±628.12 pg/mL; P=0.003) (Figure 1B); however, this difference disappeared when the groups where considered separately (Figure 1C). ANGPTL8 concentrations were significantly lower in postpartum than in pregnancy (2221.98±1143.00 vs. 321.52±175.39 pg/mL; P<0.001) when the whole group was analyzed, and remained significant when the groups were considered separately (Figure 1B and 1C, respectively). This increase in ANGPTL8 concentrations associated with pregnancy was also observed in mice (Supplementary Figure 2). Similar cord blood ANGPTL8 concentrations were observed according to offspring sex (3089.57±978.65 in males vs. 3093.97±783.97 pg/mL in females; P=NS). Maternal ANGPTL8 and cord blood ANGPTL8 levels were positively correlated (r=0.426; P<0.001) (Figure 1D), but no other significant associations were detected between ANGPTL8 levels and other maternal or neonatal parameters.

Cord blood ANGPTL8 and fetal growth

To investigate whether cord blood ANGPTL8 levels associated with fetal growth, we compared cord blood concentrations according to birth weight (SGA, AGA and LGA). ANGPTL8 concentrations were significantly different among the three groups (SGA: 3128±1074.02, AGA: 2251.80±1017.64 and LGA: 2906.35±1240.25 pg/mL; *P*=0.020) (Figure 1E). To assess whether this distribution was related to differences in the amount of fat mass, we analyzed neonates according to the sum of skinfolds. We observed a U-shaped curve, with the highest values in the first and third tertile, and the lowest in the second tertile. (1^{st} tertile: 3305.03±840.70, 2^{nd} tertile: 2670.27±883.55 and 3^{rd} tertile: 3039.76±764.59 pg/mL; *P*=0.047) (Figure 1F). These differences remained after adjusting for GDM diagnosis. No differences in ANGPTL8 concentrations were observed according to the type of birth (vaginal, scheduled or unplanned cesarean).

ANGPTL8 is secreted by brown adipocytes *in vitro* and is related to a browning phenotype in mice and humans

Given the high levels of cord blood ANGPTL8 and its relationship with neonatal fat mass, we next examined the potential effect of this protein on adipose tissue metabolism. Furthermore, considering the relevance of BAT in fetal life and in pregnancy together with the high levels of ANGPTL8 observed in both settings, we also explored whether this protein may be involved in the regulation of BAT and browning.

Analysis of several adipocyte cell lines revealed the presence of ANGPTL8 in the culture medium as determined by western blotting of concentrated supernatants (Figure 2A), suggesting a process of secretion. Moreover, higher amounts of

ANGPTL8 were found in supernatants from human and murine brown adipocytes (hBA and mBA) than in the white adipose cell line (hWA) (Figure 2A). To investigate the relationship between ANGPTL8 and browning of WAT *in vivo*, we used prolonged AICAR-induced AMPK activation as a browning stimulus in mice[24]. Consistent with previous findings observed in gonadal fat[28], an increase in protein expression of the BAT marker Uncoupling protein 1 (UCP1) was detected in subcutaneous WAT of mice following pharmacological activation of AMPK (Figure 2B). Notably, this increase in browning correlated with a significant increase in ANGPTL8 protein levels (Figure 2B). Alternative browning agents, such as FGF21[29,30] and irisin [31,32], also increased ANGPTL8 levels of *in vitro* differentiated hASCs (Figure 2C). A unique browning model in adulthood is pheochromocytoma, as this adrenal tumor has an excess of catecholamine secretion that induces BAT markers in peritumoral adipose tissue. Remarkably, a significant increase of ANGPTL8 levels in visceral adipose tissue of pheochromocytoma patients was also found (Figure 2D), confirming that expression of ANGPTL8 is particularly high in inducible beige/brite adipose tissue[33]. Finally, chronic ANGPTL8 exposure during hASC differentiation *in vitro* induced a BAT expression profile as illustrated by a significant increase in the mRNA levels of brown adipocyte markers such as UCP1, TMEM26, TBX1 and β3-AR (Figure 2D). Overall, our results point to ANGPTL8 as a new factor released by both classic and inducible BAT, with a significant browning effect on precursor cells derived from human WAT.

DISCUSSION

Pregnancy is associated with changes in maternal metabolic homeostasis that induce modifications in the fetal and maternal hormonal milieu. In the present study, we

show that serum ANGPTL8 levels fluctuate dramatically during this period, with peak levels found during the first trimester and thereafter decreasing as pregnancy progresses. Furthermore, we confirm that ANGPTL8 is present during fetal life with high circulating levels in cord blood, and exhibits a U-shaped relationship with neonatal fat mass. Interestingly, we also show for the first time that ANGPTL8 is secreted by brown adipocytes, suggesting that BAT may be an alternative source of the peptide. Intriguingly, ANGPTL8 also induces a browning effect on WAT.

Few studies have explored the regulation of ANGPTL8 during pregnancy and, to the best of our knowledge, changes in ANGPTL8 concentrations along pregnancy have not previously been investigated. We found that during normal pregnancy, maternal circulating ANGPTL8 levels are approximately ten-fold-higher than in the non-pregnant state, and decrease progressively from the first to the third trimester. Our results are in accord with recent reports from Ebert et al.[15] and Trebotic et al.[17], who observed increased ANGPTL8 concentrations in pregnancy. Contrastingly, Wawrusiewicz-Kurylonek et al.[16] failed to find differences in ANGPTL8 concentrations during pregnancy, although in this study pregnancy blood samples were obtained at a later timepoint, close to the peripartum period. The role of ANGPTL8 in glucose homeostasis, as a promoting factor for β-cell proliferation in insulin resistance states[3], has been recently questioned[34,35]. In line with this controversy and in agreement with other reports[15,16], we were unable to demonstrate a relationship between insulin secretion markers and ANGPTL8 concentrations in a physiological environment of marked insulin resistance. Indeed, we found that as maternal weight, insulin concentrations and HOMA-IR index increased, ANGPTL8 concentrations decreased but not in a synchronous manner because no inverse relationship was found. Supporting this observation, similar ANGPTL8

concentrations were found in GDM and control women during pregnancy, unlinking this protein to glucose metabolism in pregnancy. These data conflict with previous reports that describe increased ANGPTL8 levels in GDM women[15,16], which could be attributed to differences in the sampling times or to the ELISA used[36]. However, no clear relationship with insulin sensitivity or insulin-secretion variables were found in these studies and only an inverse correlation with C-peptide was described, both in normal and in GDM pregnant women. These data reinforce the lack of association between ANGPTL8 and glucose metabolism during pregnancy. Outwith pregnancy, data for type 2 diabetes and obesity has also yielded equivocal results[8,10,11,37], and assay-dependent variability because of differences in proteolytic degradation of ANGPTL8 have been proposed[36]. Full length ANGPTL8 is cleaved at a proprotein convertase consensus site, releasing the N-terminal domain. The ELISA kit used in the present study (EIABB) detects the N terminus and measures full-length ANGPTL8 concentrations. By contrast, other assays such as those from Phoenix Pharmaceuticals detect an epitope from the C-terminal domain, measuring all ANGPTL8 species including full-length and C-terminal fragments. In pregnancy, a recent report showed that ANGPTL8 concentrations measured by two methods, despite correlating significantly with one another, yielded contrasting results[17]. Moreover, given that ANGPTL8 expression is regulated by cold exposure[19], fasting and feeding[1,2], and that pregnancy is characterized by changes in the regulation of postprandrial and postabsortive glucose and lipid metabolism, differences in diet composition and sampling time might also explain these discrepancies.

We show that ANGPTL8 is present during fetal life and its concentrations are much higher in umbilical cord blood than in maternal serum. Some authors have suggested a role for ANGPTL8 in β-cell proliferation during fetal life[16]; however, the lack of a

relationship of ANGPTL8 with insulin-secretory markers together with the recent doubts raised concerning its capacity to induce β-cell expansion[34,35], lead us to propose a potential involvement of ANGPTL8 in fetal growth and development. *ANGPTL8* is known to be highly expressed in the liver[3], WAT and BAT[4], and to a lesser extent in placenta[16]. *ANGPTL8* expression is induced by environmental and nutritional factors[1,2,19], and also by stimuli known to induce browning[31]. Indeed, we demonstrate for the first time that BAT *ANGPTL8* expression in both pregnant mice and fetuses follows the same pattern as *UCP1* (Supplementary Figure 3). Thus, a down-regulation of *ANGPTL8* is detected in BAT of pregnant mice, in line with the decrease in the thermogenic activity of BAT during pregnancy[38,39]. Conversely, higher expression levels of *ANGPTL8* were detected in BAT from fetuses at term than in adult mice.

Until now, no data have been published on BAT as an alternative source of ANGPTL8. We show here that murine and human brown adipocyte cell lines express this protein. The protein characteristics of ANGPTL8 suggest that it might function as a secreted factor since it lacks features indicative of enzymatic activity[3,5]; however, until now, no study had reported ANGPTL8 expression in adipocyte cultures. Additionally, we also show that ANGPTL8 has a browning effect on WAT and induces brownish characteristics in differentiated hASCs as demonstrated by an increase in UCP1 expression. Moreover, and in a manner similar to that proposed for irisin[31], we found that FGF21, a well-known brown fat thermogenic effector in murine models[30], induces ANGPTL8 and UCP1 expression in hASCs. Collectively, our results imply a possible role for ANGPTL8 in regulating browning.

BAT is visible in fetuses from around mid gestation, when fetal adipose tissue first appears. BAT mass increases through late gestation[40] to a maximun just before birth, providing the newborn with protection against cold exposure. This process requires the orchestration of several endocrine stimulatory factors which act to maximize both the amount and the thermogenic potential of UCP1[41]. However, *UCP1* gene expression up to term is susceptible to changes in the maternal nutritional and endocrine environment, which can result in either an increase in the total amount of fat and/or a change in UCP1 abundance and therefore BAT. It is proposed that reduced maternal food intake through late gestation in normal pregnancies is accompanied by a decrease in fetal WAT and normal amounts of BAT[41]. On the other hand, increased maternal nutrient supply to the fetus induces an increase in the amount of BAT and WAT adipose tissue depots, although changes due to GDM remain unknown[41].

Interestingly, in our cohort, cord blood ANGPTL8 concentrations were higher in SGA newborns and also in those with the lowest amount of fat mass, and this association was independent of the maternal glucose tolerance status. ANGPTL8 concentrations were lowest in AGA and in those in the second tertile of fat mass. Considering that BAT can secrete ANGPTL8, which can then stimulate browning, we are tempted to speculate that in fetuses with low amounts of fat mass, and therefore low protective insulator capacity, the high ANGPTL8 concentrations observed are a compensatory mechanism to a condition of low BAT mass. ANGPTL8 concentrations exhibited a trend to be higher in newborns with the highest amount of fat mass and LGA. Considering that emerging evidence suggests that the association between birth weight and metabolic disorders may not be linear[42], the U-shaped relationship

observed in our study with cord blood ANGPTL8 concentrations point to a potetnial role for ANGPTL8 in the modulation of energy metabolism later in life.

In contrast to a recent report[16], we observed lower cord blood ANGPTL8 concentrations in offspring of GDM mothers than in those born to control mothers, despite the finding that newborns had similar birth weights and neonatal adiposity. In our cohort, BMI gain was lower in GDM than in control women, resulting from a reduction in food intake that may be accompanied by a reduction in the nutrient supply to the fetus. It is well known that treated GDM is associated with deceleration of fetal growth velocity and normalization of birth weight and fetal adiposity[43,44]. In animal models, a reduction in food consumption in the final months of gestation is accompanied by a reduction in WAT rather than in brown adipocyte number[41]. Considering this and the fact that the reduced amount of fat present in the newborn has a greater capacity to retain UCP1, indicative of a protective mechanism against cold exposure after birth, we believe that offspring of GDM women might have more efficient BAT and therefore the compensatory increase in ANGPTL8 is not needed.

We are aware that this work has some limitations. The observational design of the study does not allow for causal inference between the analyzed variables and so we cannot establish a direct relationship between cord blood ANGPTL8 and brown adipose homeostasis in the setting of pregnancy. Nevertheless, in support of our hypothesis two recent reports have proposed that cord blood irisin, a myokine involved in browning, is also a marker of the BAT depot in early life, suggesting that in low birth weight infants it might be a biomarker for the appearance of insulin resistance in later life[45,46].

In summary, we show that maternal ANGPTL8 concentrations are increased in pregnancy and in fetal life and, in the latter situation, seem to be regulated by nutritional factors. We propose that ANGPTL8 might function to activate and expand the thermogenic machinery during the fetal-neonatal transition, thereby providing a robust defense against hypothermia.

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REFERENCES

1. Quagliarini F, Wang Y, Kozlitina J, et al. Atypical angiopoietin-like protein that regulates ANGPTL3. *Proc Natl Acad Sci U S A*. 2012;109(48):19751-19756. doi:10.1073/pnas.1217552109.

2. Zhang R. Lipasin, a novel nutritionally-regulated liver-enriched factor that regulates serum triglyceride levels. *Biochem Biophys Res Commun*. 2012;424(4):786-792. doi:10.1016/j.bbrc.2012.07.038.

3. Yi P, Park J-S, Melton DA. Betatrophin: a hormone that controls pancreatic β cell proliferation. *Cell*. 2013;153(4):747-758. doi:10.1016/j.cell.2013.04.008.

4. Ren G, Kim JY, Smas CM. Identification of RIFL, a novel adipocyte-enriched insulin target gene with a role in lipid metabolism. *Am J Physiol Endocrinol Metab*. 2012;303(3):E334-E351. doi:10.1152/ajpendo.00084.2012.

5. Stephens JM. RIFL aims to be a new player in lipid metabolism. *Am J Physiol Endocrinol Metab*. 2012;303(3):E332-E333. doi:10.1152/ajpendo.00169.2012.

6. Espes D, Martinell M, Carlsson P-O. Increased circulating betatrophin concentrations in patients with type 2 diabetes. *Int J Endocrinol*. 2014;2014:323407. doi:10.1155/2014/323407.

7. Hu H, Sun W, Yu S, et al. Increased circulating levels of betatrophin in newly diagnosed type 2 diabetic patients. *Diabetes Care*. 2014;37(10):2718-2722. doi:10.2337/dc14-0602.

8. Chen X, Lu P, He W, et al. Circulating betatrophin levels are increased in patients with type 2 diabetes and associated with insulin resistance. *J Clin Endocrinol Metab*. 2015;100(1):E96-E100. doi:10.1210/jc.2014-2300.

9. Ebert T, Kralisch S, Hoffmann A, et al. Circulating angiopoietin-like protein 8 is independently associated with fasting plasma glucose and type 2 diabetes mellitus. *J Clin Endocrinol Metab*. 2014;99(12):E2510-E2517. doi:10.1210/jc.2013-4349.

10. Gómez-Ambrosi J, Pascual E, Catalán V, et al. Circulating betatrophin concentrations are decreased in human obesity and type 2 diabetes. *J Clin Endocrinol Metab*. 2014;99(10):E2004-E2009. doi:10.1210/jc.2014-1568.

11. Fenzl A, Itariu BK, Kosi L, et al. Circulating betatrophin correlates with atherogenic lipid profiles but not with glucose and insulin levels in insulin-resistant individuals. *Diabetologia*. 2014;57(6):1204-1208. doi:10.1007/s00125-014-3208-x.

12. Barja-Fernández S, Folgueira C, Seoane LM, et al. Circulating betatrophin levels are increased in anorexia and decreased in morbid obese women. *J Clin Endocrinol Metab*. 2015:JC20151595. doi:10.1210/JC.2015-1595.

13. Tseng Y-H, Yeh Y-H, Chen W-J, Lin K-H. Emerging regulation and function of betatrophin. *Int J Mol Sci*. 2014;15(12):23640-23657. doi:10.3390/ijms151223640.

14. Guelinckx I, Devlieger R, Beckers K, Vansant G. Maternal obesity: pregnancy complications, gestational weight gain and nutrition. *Obes Rev*. 2008;9(2):140-150. doi:10.1111/j.1467-789X.2007.00464.x.

15. Ebert T, Kralisch S, Wurst U, et al. Betatrophin levels are increased in women with gestational diabetes mellitus compared to healthy pregnant controls. *Eur J Endocrinol*. 2015;173(1):1-7. doi:10.1530/EJE-14-0815.

16. Wawrusiewicz-Kurylonek N, Telejko B, Kuzmicki M, et al. Increased Maternal and Cord Blood Betatrophin in Gestational Diabetes. *PLoS One*. 2015;10(6):e0131171. doi:10.1371/journal.pone.0131171.

17. Trebotic LK, Klimek P, Thomas A, et al. Circulating Betatrophin Is Strongly Increased in Pregnancy and Gestational Diabetes Mellitus. *PLoS One*. 2015;10(9):e0136701. doi:10.1371/journal.pone.0136701.

18. Symonds ME. Brown adipose tissue growth and development. *Scientifica (Cairo)*. 2013;2013:305763. doi:10.1155/2013/305763.

19. Fu Z, Yao F, Abou-Samra AB, Zhang R. Lipasin, thermoregulated in brown fat, is a novel but atypical member of the angiopoietin-like protein family. *Biochem Biophys Res Commun*. 2013;430(3):1126-1131. doi:10.1016/j.bbrc.2012.12.025.

20. Direcció General de Salut Pública. Generalitat de Catalunya. *Corbes de Referència de Pes, Perímetre Cranial I Longitud En Néixer de Nounats D ' Embarassos Únics , de Bessons I de Trigèmins a Catalunya*.; 2008. http://canalsalut.gencat.cat/web/.content/home_canal_salut/professionals/temes_de_salut/salut_maternoinfantil/documentacio/arxius/corbesdef3.pdf.

21. Schmelzle HR, Fusch C. Body fat in neonates and young infants: validation of skinfold thickness versus dual-energy X-ray absorptiometry. *Am J Clin Nutr*. 2002;76(5):1096-1100. http://www.ncbi.nlm.nih.gov/pubmed/12399284.

22. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28:412-419.

23. Ceperuelo-Mallafré V, Duran X, Pachón G, et al. Disruption of GIP/GIPR axis in human adipose tissue is linked to obesity and insulin resistance. *J Clin Endocrinol Metab*. 2014;99(5):E908-E919. doi:10.1210/jc.2013-3350.

24. Kazantzis M, Takahashi V, Hinkle J, et al. PAZ6 cells constitute a representative model for human brown pre-adipocytes. *Front Endocrinol (Lausanne)*. 2012;3:13. doi:10.3389/fendo.2012.00013.

25. Vila-Bedmar R, Lorenzo M, Fernández-Veledo S. Adenosine 5'-monophosphate-activated protein kinase-mammalian target of rapamycin cross talk regulates brown adipocyte differentiation. *Endocrinology*. 2010;151(3):980-992. doi:10.1210/en.2009-0810.

26. Pachón-Peña G, Yu G, Tucker A, et al. Stromal stem cells from adipose tissue and bone marrow of age-matched female donors display distinct immunophenotypic profiles. *J Cell Physiol*. 2011;226(3):843-851. doi:10.1002/jcp.22408.

27. Fernández-Veledo S, Nieto-Vazquez I, Rondinone CM, Lorenzo M. Liver X receptor agonists ameliorate TNFalpha-induced insulin resistance in murine brown adipocytes by downregulating protein tyrosine phosphatase-1B gene expression. *Diabetologia*. 2006;49(12):3038-3048. doi:10.1007/s00125-006-0472-4.

28. Vila-Bedmar R, Garcia-Guerra L, Nieto-Vazquez I, et al. GRK2 contribution to the regulation of energy expenditure and brown fat function. *FASEB J*. 2012;26(8):3503-3514. doi:10.1096/fj.11-202267.

29. Fisher M, Kleiner S, Douris N, et al. FGF21 regulates PGC-1a and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev*. 2012;26:271-281. doi:10.1101/gad.177857.111.

30. Hondares E, Rosell M, Gonzalez FJ, et al. Hepatic FGF21 expression is induced at birth via PPARα in response to milk intake and contributes to thermogenic activation of neonatal brown fat. *Cell Metab*. 2010;11(3):206-212. doi:10.1016/j.cmet.2010.02.001.

31. Zhang Y, Li R, Meng Y, et al. Irisin stimulates browning of white adipocytes through mitogen-activated protein kinase p38 MAP kinase and ERK MAP kinase signaling. *Diabetes*. 2014;63(2):514-525. doi:10.2337/db13-1106.

32. Sanchis-Gomar F, Perez-Quilis C. The p38-PGC-1α-irisin-betatrophin axis: Exploring new pathways in insulin resistance. *Adipocyte*. 2014;3(1):67-68. doi:10.4161/adip.27370.

33. Hondares E, Gallego-Escuredo JM, Flachs P, et al. Fibroblast growth factor-21 is expressed in neonatal and pheochromocytoma-induced adult human brown adipose tissue. *Metabolism*. 2014;63(3):312-317. doi:10.1016/j.metabol.2013.11.014.

34. Gusarova V, Alexa CA, Na E, et al. ANGPTL8/betatrophin does not control pancreatic beta cell expansion. *Cell*. 2014;159(3):691-696. doi:10.1016/j.cell.2014.09.027.

35. Cox AR, Lam CJ, Bonnyman CW, Chavez J, Rios JS, Kushner JA. Angiopoietin-like protein 8 (ANGPTL8)/betatrophin overexpression does not increase beta cell proliferation in mice. *Diabetologia*. 2015;58(7):1523-1531. doi:10.1007/s00125-015-3590-z.

36. Fu Z, Abou-Samra AB, Zhang R. An explanation for recent discrepancies in levels of human circulating betatrophin. *Diabetologia*. 2014;57(10):2232-2234. doi:10.1007/s00125-014-3346-1.

37. Fu Z, Berhane F, Fite A, Seyoum B, Abou-Samra AB, Zhang R. Elevated circulating lipasin/betatrophin in human type 2 diabetes and obesity. *Sci Rep*. 2014;4:5013. doi:10.1038/srep05013.

38. Martin I, Giralt M, Viñas O, Iglesias R, Mampel T, Villarroya F. Adaptative decrease in expression of the mRNA for uncoupling protein and subunit II of cytochrome c oxidase in rat brown adipose tissue during pregnancy and lactation. *Biochem J*. 1989;263(3):965-968.

39. Martínez de Morentin PB, Lage R, González-García I, et al. Pregnancy induces resistance to the anorectic effect of hypothalamic malonyl-CoA and the thermogenic effect of hypothalamic AMPK inhibition in female rats. *Endocrinology*. 2015;156(3):947-960. doi:10.1210/en.2014-1611.

40. Clarke L, Bryant MJ, Lomax MA, Symonds ME. Maternal manipulation of brown adipose tissue and liver development in the ovine fetus during late gestation. *Br J Nutr*. 1997;77(6):871-883.

41. Symonds ME, Pope M, Sharkey D, Budge H. Adipose tissue and fetal programming. *Diabetologia*. 2012;55:1597-1606.

42. Curhan GC, Willett WC, Rimm EB, Spiegelman D, Ascherio AL, Stampfer MJ. Birth Weight and Adult Hypertension, Diabetes Mellitus, and Obesity in US Men. *Circulation*. 1996;94(12):3246-3250. doi:10.1161/01.CIR.94.12.3246.

43. Au CP, Raynes-Greenow CH, Turner RM, Carberry AE, Jeffery HE. Body composition is normal in term infants born to mothers with well-controlled gestational diabetes mellitus. *Diabetes Care*. 2013;36(3):562-564. doi:10.2337/dc12-1557.

44. Bahado-Singh RO, Mele L, Landon MB, et al. Fetal male gender and the benefits of treatment of mild gestational diabetes mellitus. *Am J Obs Gynecol*. 2012;206(5):422.e1-e422.e5.

45. Baka S, Malamitsi-Puchner A, Boutsikou T, et al. Cord blood irisin at the extremes of fetal growth. *Metabolism*. 2015;64:1515-1520. doi:10.1016/j.metabol.2015.07.020.

46. Joung KE, Park K-H, Filippiaos A, Dincer F, Christou H, Mantzoros CS. Cord Blood Irisin Levels are Positively Correlated with Birth Weight in Newborn Infants. *Metabolism*. 2015;64:1507-1514. doi:10.1016/j.metabol.2015.07.019.

Table 1. ANGPTL8 concentrations, clinical and metabolic parameters of the population studied during pregnancy

	1st trimester Evaluation	**2nd trimester Evaluation**	**3rd trimester Evaluation**	***P* value**
Gestational week (weeks)	10.09±1.38	25.64±1.63	33.27±1.56	-
BMI (kg/m^2)*†‡	24.91.00±4.77	26.97±4.46	28.12±4.55	<0.001
Glucose (mg/dL)*	83.67±5.73	78.07±7.00	81.04±6,78	0.003
Insulin (mcUI/mL)†‡	6.83[4.80-11.37]	9.34[7.62-10.67]	11.94[7.01-16.09]	0.023
HOMA-IR†‡	1.37[1.07-2.25]	1.78[1.42-2.19]	2.56[1.33-3.47]	0.036
HOMA-β *†	126.00[70.38-227.70]	213.90[152.16-552.15]	244.40[184.35-297.56]	0.002
Total Cholesterol (mg/dL)*†‡	173.24±27.83	236.98±27.98	264.42±33.05	<0.001
HDL Cholesterol (mg/dL)*	61.28±8.07	70.06±12.27	69.96±13.99	0.014
Triglycerides (mg/dL)*†‡	75.27±36.28	161.18±62.16	201.27±74.12	<0.001
ANGPTL8 (pg/mL)	3540.15±2782.54	3247.54±2374.89	2191.16±1274.57	0.031

*. $P<0.05$ between 1st trimester and 2nd trimester;
†. $P<0.05$ between 1st trimester and 3rd trimester.
‡. $P<0.05$ between 2nd trimester and 3rd trimester

Table 2. Clinical and metabolic characteristics of the mother and infants pairs

	Control (N=37)	**GDM (N=46)**	***P* value**
Maternal Characteristics in Pregnancy			
Age (years)	31.49±5.44	32.11±4.92	NS
Gestational week (weeks)	27.24±1.30	27.55±1.89	NS
Prepregnancy BMI (kg/m^2)	24.75±4.14	25.66±4.89	NS
ΔBMI (kg/m^2)	4.80±1.76	3.57±2.08	0.005
ΔBMI in third trimester (kg/m^2)	2.13±1.18	0.80±1.12	<0.001
Fasting Glucose (mg/dL)	80.30±7.24	85.38±11.03	0.018
Total Cholesterol (mg/dL)	254.64±36.52	254.06±41.63	NS
HDL Cholesterol (mg/dL)	76.19±13.48	71.50±13.98	NS
Triglycerides (mg/dL)	159.92±39.55	193.06±65.98	0.006
Fasting Insulin (mUI/L)	7.39 (5.46-11.20)	9.86 (6.01-14.72)	0.033
HOMA-IR	1.48 (1.05-2.24)	2.08 (1.39-3.37)	0.019
HOMA-β Pregnancy	184.99 (127.69-249.75)	157.15 (128.73-279.14)	NS
mANGPTL8 (pg/mL)	2941.28±1128.46	2667.30±1198.11	NS
Newborn Characteristics[a]			
Gestational week delivery (week)	39.32±1.56	39.09±1.23	NS
Male sex, n (%)	17 (45.95%)	27 (57.45%)	NS
Birth weight (g)	3320.65±530.08	3283.40±434.19	NS
Birth weight Z-Score	0.48±0.92	0.36±0.91	NS
Sum Skinfolds (mm)	12.37±2.67	11.35± 1.79	NS
Cord blood Insulin (mUI/L)	4.37 (2.66-8.48)	4.89 (3.17-11.05)	NS
Cord blood ANGPTL8 (pg/mL)	3325.02±617.44	2883.26±1043.79	0.033
Postpartum[b]			
Postpartum Evaluation (months)	5.04±7.54	3.90±5.63	NS
BMI (kg/m^2)	25.27±4.94	26.25±5.24	NS
Glucose (mg/dL)	91.927±7.42	91.93±9.40	NS
Insulin (mUI/L)	6.17[5.34-10.48]	5.73[4.98-10.79]	NS
Total Cholesterol (mg/dL)	204.41±33.35	211.41±31.89	NS
HDL Cholesterol (mg/dL)	69.03±14.20	62.82±15.03	NS
Triglycerides (mg/dL)	76.07±34.04	81.46±34.09	NS
HOMA-IR	1.45 (1.08-2.12)	1.35 (1.06-2.47)	NS
HOMA-β	87.87 (72.33-138.00)	82.92 (167.38-110.91)	NS
ANGPTL8 (pg/mL)	394.10±245.97	294.98±129.30	NS

a. Newborn evaluation: 33 born to control women and 31 born to GDM women.

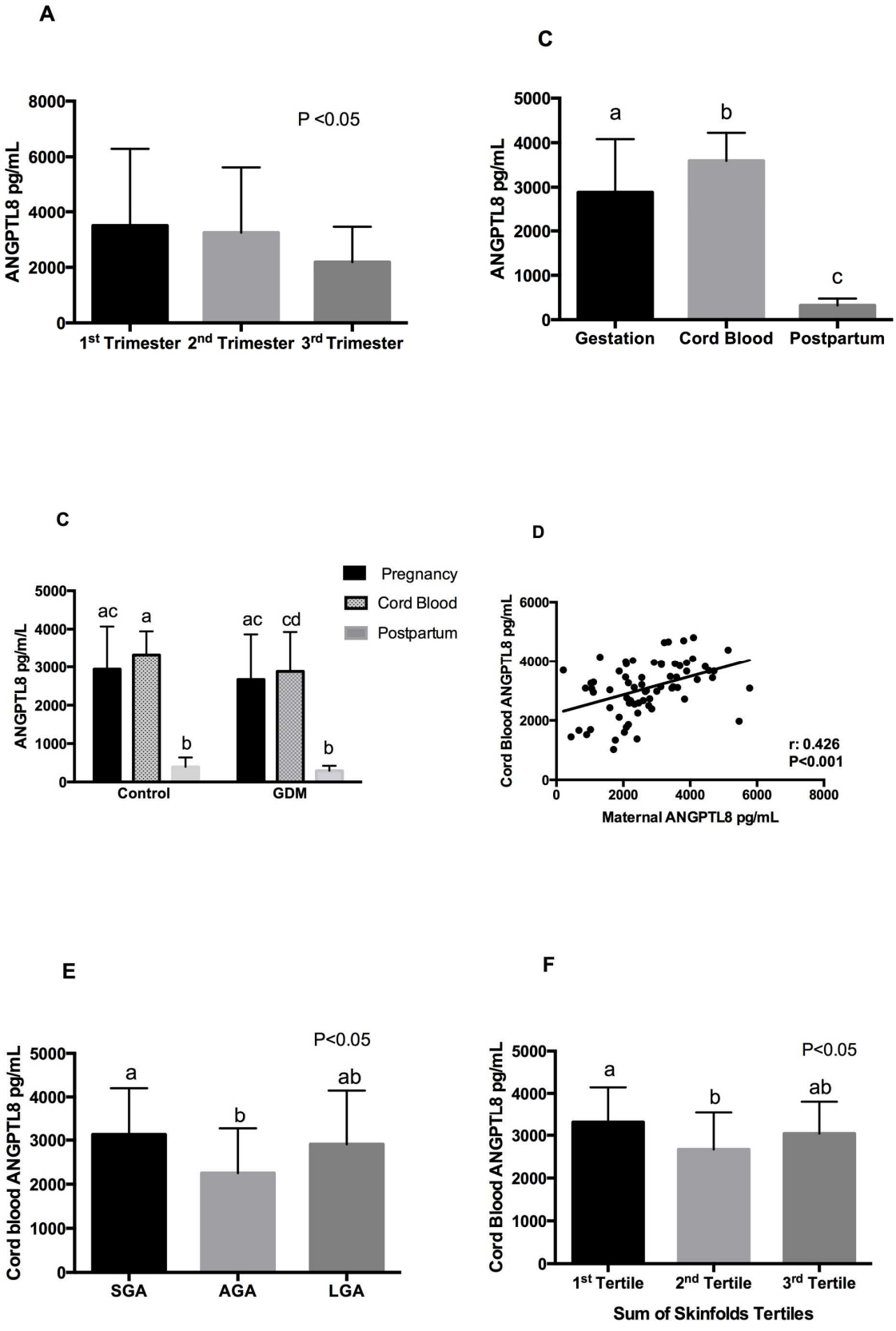
b. Postpartum evaluation: 15 control and 29 GDM women.

FIGURE LEGENDS

Figure 1. ANGPTL8 concentrations in pregnancy, postpartum and cord blood. **(A)** Maternal serum ANGPTL8 concentrations in each trimester of normal pregnancy were compared with repeated measures ANOVA (P=0.011) **(B)** Paired t-test between ANGPTL8 concentrations in gestation and in cord blood, between ANGPTL8 in gestation and postpartum, and between ANGPTL8 concentrations in cord blood and postpartum in the whole group and **(C)** in the control and GDM group analyzed separately. **(D)** The Pearson correlation between maternal (27 weeks of pregnancy) and cord blood ANGPTL8 concentrations **(E)** Cord blood ANGPTL8 concentrations in newborns according to birth weight and **(F)** to sum of skinfolds tertiles analyzed by ANOVA (P<0.05 for both). For all graphics, groups with different letters are significantly different (P<0.05).

Figure 2. ANGPTL8 is secreted by brown adipocytes and stimulates browning. **(A)** Brown adipocytes secrete ANGPTL8. Cells were differentiated as described and conditioned medium was collected after 12 days of differentiation. Secreted ANGPTL8 was analyzed by western blotting of concentrated medium obtained from murine and human brown adipocytes (mBA and hBA, respectively) and human white adipocytes (hWA). Ponceau S staining was used to verify equal loading of proteins from the conditioned media. A representative experiment is shown together with densitometric analysis (n=3). **(B)** Prolonged AICAR-induced AMPK activation *in vivo* increases ANGPTL8 protein levels in inguinal adipose tissue. UCP1 expression was used as a positive control of browning. GAPDH was used as a loading control. **(C)** Browning of hASCs is related to increased ANGPTL8 expression. hASCs were treated with FGF21 (100 nM) or Irisin (10 nM) every second day during

differentiation and mRNA ANGPTL8 levels were analyzed by qPCR. Gene expression of ribosomal 18s was used for normalization. Mean±SEM. *, $P<0.05$, Student´s t-test. **(D)** *ANGPTL8* is increased in pheocromocytoma-induced adult human brown adipose tissue. The expression of *UCP1* and *ANGPTL8* were determined by qPCR in visceral AT from healthy and pheochromocytoma subjects. Gene expression of *PPIA* was used for normalization. Mean±SEM. *, $P<0.05$, Student´s t-test. **(E)** ANGPTL8 induces *UCP1* expression in hASCs. Cells were treated with ANGPTL8 (20 ng/mL) every second day during differentiation and mRNA levels of several browning markers were studied. Gene expression of ribosomal 18s was used for normalization. Mean ± SEM. *, $P<0.05$. Sudent´s t-test.


A
ANGPTL8 pg/mL
8000
6000
4000
2000
0
P <0.05
1st Trimester
2nd Trimester
3rd Trimester
C
ANGPTL8 pg/mL
5000
4000
3000
2000
1000
0
a
b
c
Gestation
Cord Blood
Postpartum
C
ANGPTL8 pg/mL
5000
4000
3000
2000
1000
0
ac
a
b
ac
cd
b
Control
GDM
Pregnancy
Cord Blood
Postpartum
D
Cord Blood ANGPTL8 pg/mL
6000
4000
2000
0
0
2000
4000
6000
8000
Maternal ANGPTL8 pg/mL
r: 0.426
P<0.001
E
Cord blood ANGPTL8 pg/mL
5000
4000
3000
2000
1000
0
P<0.05
a
b
ab
SGA
AGA
LGA
F
Cord Blood ANGPTL8 pg/mL
5000
4000
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P<0.05
a
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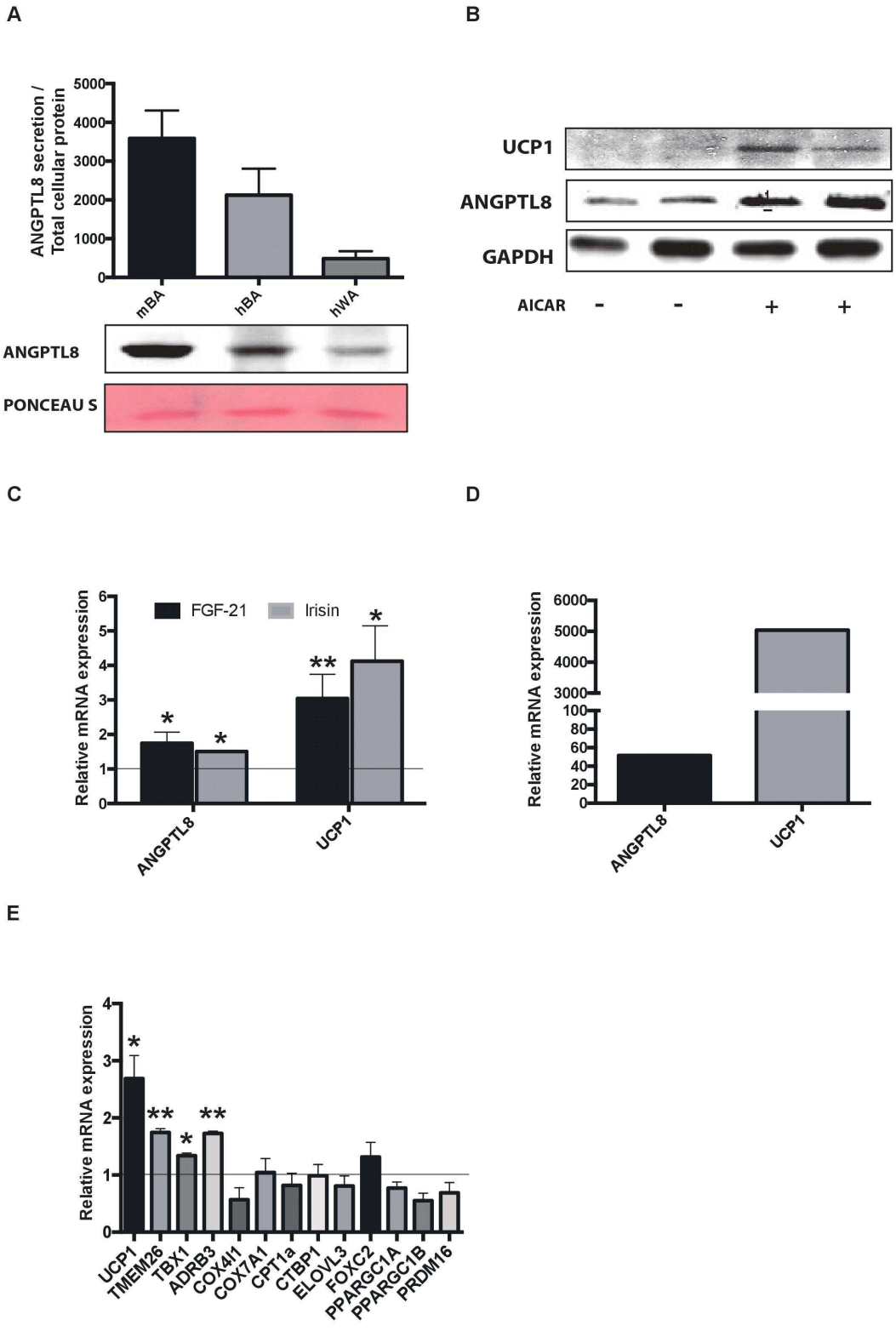
1st Tertile
2nd Tertile
3rd Tertile
Sum of Skinfolds Tertiles

Figure 2


A
ANGPTL8 secretion / Total cellular protein
5000
4000
3000
2000
1000
0
mBA
hBA
hWA
ANGPTL8
PONCEAU S
B
UCP1
ANGPTL8
GAPDH
AICAR - - + +
C
Relative mRNA expression
FGF-21
Irisin
ANGPTL8
UCP1
D
Relative mRNA expression
6000
5000
4000
3000
100
80
60
40
20
0
ANGPTL8
UCP1
E
Relative mRNA expression
UCP1
TMEM26
TBX1
ADRB3
COX4I1
COX7A1
CPT1a
CTBP1
ELOVL3
FOXC2
PPARGC1A
PPARGC1B
PRDM16

Brief Commentary

Background

ANGPTL8, a protein implicated in lipid and glucose homeostasis, is present only in mammals, suggesting a role in processes unique to these vertebrates such as pregnancy and homeothermy.

Translational Significance:

We have studied ANGPTL8 levels in both maternal and cord blood during normal pregnancy and gestational diabetes, and its potential relationship with newborn adiposity. Additionally we have explored the connection between ANGPTL8 and brown fat using *in vivo* and *in vitro* experimental models of white-to-brown fat conversion (browning). Our study suggests that ANGPTL8 might be involved in fetal growth by acting on the thermogenic machinery during the fetal-neonatal transition.