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In Vivo Placental Transfer of Fatty Acids Labelled with Stable Isotopes in Gestational Diabetes Mellitus Subjects

Estudio *in vivo* de la Transferencia Placentaria de Ácidos Grasos Marcados con Isótopos Estables en Sujetos con Diabetes Mellitus Gestacional

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A mi yayo y mi yaya

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ABBREVIATIONS

AA	Arachidonic acid
A-FABP	Adipocyte-fatty acid binding protein
AGA	Appropriate for gestational age
ALA	alpha-linolenic acid
APE	Atom percent excess
apoB	Apolipoprotein B
AUC	Area under the curve
B-FABP	Brain-fatty acid binding protein
BHT	Butylated hydroxytoluene
BMI	Body mass index
CE	Cholesterol esters
DHA	Docosahexaenoic acid
EEG	Electroencephalography
EL	Endothelial lipase
EPA	Eicosapentaenoic acid
ERG	Electroretinogram
FA	Fatty acids
FABP	Fatty acid binding protein
FABPpm	Fatty acid binding protein plasma membrane
FAT/CD36	Fatty acid translocase
FATP	Fatty acid transport protein
GC	Gas chromatography
GC-IRMS	Gas chromatography-combustion-isotope ratio mass spectrometry
GDM	Gestational diabetes mellitus
HDL	High density lipoproteins
H-FABP	Heart-fatty acid binding protein
HOMA	Homeostasis model of assessment (for insulin resistance)
HSL	Hormone-sensitive lipase
IDL	Intermediate density lipoproteins
IL	Interleukin
IQ	Intelligence quotient

IUGR	Intrauterine growth restriction
K-ABC	Kaufman assessment battery for children
K-FABP	Keratinocyte-fatty acid binding protein
LA	Linoleic acid
LC-PUFA	Long chain polyunsaturated fatty acids
LD	Lipid droplets
LDL	Low density lipoproteins
LDLR	Low density lipoproteins receptor
LGA	Large for gestational age
L-FABP	Liver-fatty acid binding protein
LPL	Lipoprotein lipase
MDI	Mental development score
MUFA	Monounsaturated fatty acids
MVM	Microvillous plama membrane
NEFA	Non-esterified fatty acids
NOS	Neurological optimality score
OA	Oleic acid
PA	Palmitic acid
PC	Phosphatidylcholine
PDI	Psychomotor development score
PE	Phosphatidylethanolamine
p-FABPpm	Placental plasma membrane fatty acid binding protein
PL	Phospholipids
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
SGA	Small for gestational age
TG	Triglycerides
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor α
VEP	Visual evoked potentials
VLDL	Very low density lipoproteins
VLDLR	Very low density lipoproteins receptor

I. INTRODUCTION

1. GDM: CONCEPT AND WORLD PREVALENCE

Gestational Diabetes Mellitus (GDM) is one of the most common metabolic disorders of pregnancy. This disease is characterized by an abnormal glucose tolerance recognized for the first time during pregnancy due to a decreased insulin sensitivity combined with insufficient insulin secretion (1).

In the last years, the incidence of GDM has been increasing in all countries mainly due to the increased age of pregnant women and the rising rates of obesity in the population. Nowadays, the prevalence of GDM is up to 5% of all pregnancies in the world (2). Unquestionably, there are also large ethnic differences in the prevalence of GDM, thus, depending on the population, the geographic area or the different tests and criteria used for the diagnosis, GDM could affect until 14% of pregnancies (3).

There are different screening tests and criteria for diagnosis between countries, which makes it difficult to compare the prevalence of GDM among different populations. Nowadays, the prevalence in the United States of America (USA) is estimated to be 2.5 to 10.6% depending on the ethnicity, while for the North of Europe (United Kingdom, Netherlands, Sweden and Denmark) the prevalence rates range between 0.6 to 3.6% (4-9).

In Spain, the widest study on the prevalence of GDM was the Multicentre Spanish Study, during 2002 to 2005, including 16 public hospitals with 9270 pregnant women (mainly Spanish women, but there were also some Africans, Asiatic and Caribbean women) included (10). In this study, the new diagnostic criteria of Carpenter and Coustan (C&C) (1982) (11) as well as the classic diagnostic criteria of the National Diabetes Data Group (NDDG) (1979) (12) were applied (Table 1). The results of the Multicentre Spanish Study confirmed the high prevalence of GDM in Spain (8.8%) and it could be even higher according to the new criteria of C&C (up to 11.6%) (10). This high prevalence agrees with recent studies which describe a high prevalence of GDM in Mediterranean women (13).

Table 1. *Oral-glucose-tolerance test criteria of Carpenter & Coustan (11) and National Diabetes Data Group (12) to diagnose gestational diabetes mellitus by screening with an oral load of 100g glucose*

	Carpenter & Coustan Diagnosis Criteria	National Diabetes Data Group Diagnosis Criteria
Fasting measure	95	105
1 hour	180	190
2 hours	155	165
3 hours	140	145

Glycemia values expressed in mg/dl (maximum values above which the screening results are considered abnormal)

2. MATERNAL METABOLIC ADAPTATIONS IN NORMAL PREGNANCY AND IN PREGNANCIES COMPLICATED BY GDM

During gestation, the metabolism of the pregnant women has to undergo changes and adaptations in order to support the nutritional demands to the fetus. These changes are more evident during the last trimester of pregnancy, when the greatest fetal growth takes place and, in consequence, the larger demand of nutrients from the fetus. These adjustments in maternal metabolism are mediated by placental hormones (mainly estrogen and placental lactogen) and by the altered insulin levels as well as changes in insulin sensitivity (14). These adaptations mainly affect the metabolism of carbohydrates and lipid metabolism to ensure a continuous input of energy and nutrients to the fetus. Thus, the mother uses alternative energy sources for carbohydrates and sets aside the glucose to the fetus.

2.1 *Adaptations in carbohydrate metabolism*

Glucose can be obtained in humans by intestinal absorption of dietary glucose after nutrient ingestion, glycogenolysis or gluconeogenesis.

Glucose metabolism is mainly regulated by insulin, a peptide hormone produced in the islets of Langerhans in the β cells of the pancreas. Insulin performs regulatory functions, both directly and indirectly, in most of the tissues in the body, however its main function is to regulate carbohydrate and lipid metabolism in the liver, muscle and adipose tissue.

In normal pregnancies, during the first trimester of gestation, there is an increase in insulin sensitivity compared to prepregnancy status. During this period, both placental lactogen and prolactin promote the secretion of insulin, stimulating the hyperplasia of the β cells islets. From the 20th week until the end of gestation, it is established an insulin resistance condition, with an estimated decrease of 40-60% in insulin activity at the end of pregnancy (15). The mechanisms involved in this insulin resistance condition in late pregnancy are not fully understood, nevertheless, it is known that insulin resistance is mostly mediated by the diabetic effect of placental hormones such as placental lactogen, prolactin, progesterone, growth hormone (GH) and the corticotropin-releasing hormone (CRH). Moreover, this decrease in insulin activity has also been related to increased plasma concentrations of TNF-α, a pro-inflammatory cytokine produced by the immune system cells (16). These changes in insulin production and sensitivity lead to different glucose regulation during pregnancy comparing to the no-gestational condition. Thus, for the period of pregnancy, there is a longer condition of hyperglycaemia and hyperinsulinemia after ingestion in agreement with the insulin resistance condition, which ensures a continuous supply of glucose to the fetus.

In pregnancies complicated by GDM, there is an enhanced insulin resistance respect to normal pregnancies due to several components (17). The first component involved is a pregestational insulin resistance which may be partly inherited and partly acquired. The acquired part, as occurs in type 2 diabetes, is mainly determined by obesity and non-esterified fatty acids (NEFA) concentration, in fact there is a strong negative correlation between NEFA and insulin sensitivity (18). The second component is the gestational insulin resistance which takes place during the second half of

pregnancy; several studies have described that women with GDM have a disturbed function of the β cells in the pancreas, thus, in these women, the insulin resistance during gestation is enhanced by an altered insulin secretion (19). Therefore, GDM results from the failure of pancreatic β cells to compensate the enhanced insulin resistance during the last half of pregnancy (20). A recent study on GDM pregnant women according to prepregnancy body weight have concluded that low insulin secretion capacity is a more important factor for inducing GDM in normal-weight pregnant women whereas insulin resistance is for overweight pregnant women (21).

2.2 *Adjustments in lipid metabolism*

In normal pregnancies, besides the carbohydrate metabolism, lipid metabolism has also to be adapted. During the first trimester of gestation, lipid metabolism is mainly anabolic and in consequence, lipids are accumulated in maternal fat stores leading to weight gain. In this period, there is an enhanced lipoprotein lipase (LPL) activity that results in the hydrolysis of the triglycerides contained in plasma lipoproteins. The products of this hydrolysis are free fatty acids which are captured by adipose tissue. Furthermore, the lipogenesis from glucose is an insulin-sensitive metabolic pathway which is promoted at the beginning of pregnancy due to the hyperinsulinemia condition during this period and may also contribute to the increase in maternal fat depots.

Nevertheless, in the second half of pregnancy, when the greatest fetal growth takes place, the maternal lipid metabolism becomes mainly catabolic with a quick hydrolysis of maternal fat stores. The increased estrogen concentration during the third trimester of gestation activates the production of very low density lipoproteins (VLDL) and increases the lipase activity in the liver. On the other hand, insulin resistance during this period reduces LPL activity in maternal adipose tissue and increases lipolytic activity contributing to the maternal hyperlipidaemia that has been observed in normal pregnancies during the last months of gestation (14; 22). The intensive hydrolysis of fat stores in the adipose tissue is an important source of free fatty acids and glycerol which are moved to the maternal liver to synthesize triglycerides that are released back into

the circulation as part of VLDL. In addition, glycerol may be used to synthesize glucose and the free fatty acids may also result in ketone bodies after its β-oxidation in the liver. After all, maternal hyperlipidaemia at the end of pregnancy is mainly due to an increase in plasma triglycerides concentration and free fatty acids, with smaller rises in phospholipids and cholesterol concentration (14; 23).

There are discrepancies between authors in respect to the lipid alterations and lipid profiles associated with GDM. Most studies have described a higher maternal hypertriglyceridemia during the three trimesters of pregnancy (24), while other authors have found no changes in plasma triglycerides in GDM women compared to control subjects (23; 25). Even more discrepancies have been observed with regard to total cholesterol and lipoprotein concentrations in plasma of GDM women. Increased levels (26) as well as unchanged values (27) of total cholesterol in GDM relative to controls through the first trimester of pregnancy have been described. During the second and third trimester, not only increased or unaltered concentrations of total cholesterol, but also decreased levels in GDM have been found. Both concentrations of HDL and LDL have been also controversial between the different studies; some authors found lower levels of LDL during the last half of pregnancy (24; 26) while others did not find differences between GDM and control subjects (23). Rizzo *et al.* (27) reported an increase in concentrations of small size LDL which is characteristic of insulin resistance situations and has been related to an increased cardiovascular risk (Reviewed by Herrera, 2010) (28).

In summary, available studies comparing lipid profiles in GDM with uncomplicated pregnancies are inconclusive, nevertheless, it seems to be an altered lipid metabolism in GDM that leads to more atherogenic lipid profiles in these women and in consequence, a higher risk of vascular and other metabolic diseases.

3. IMPLICATIONS OF GDM FOR THE MOTHER AND THE OFFSPRING

There are several maternal and fetal risks associated with pregnancies complicated by GDM. Due to the altered glucose levels, women diagnosed with GDM have worse obstetric results than women with a normal pregnancy. Moreover, women with GDM and poor glycemic control have a higher risk to suffer complications during pregnancy in comparison with GDM women with an adequate glycemic control (29). These adverse outcomes affect both the mother and the fetus with several risks and consequences in a short and long term.

3.1 *Maternal hypertensive status during pregnancy (preeclampsia)*

Pregnant women with GDM have a higher risk to develop preeclampsia due in part to the higher insulin resistance (30-32). The higher preeclampsia risk was associated for the first time to the GDM disease by O'Sullivan *et al.* (33); later, it has been confirmed that patients with slight glucose intolerance or a slight GDM have a higher risk of suffering hypertensive alterations during pregnancy (34-35). The higher preeclampsia risk is affected by the length of the pathology (36-37) and by the glucose control degree (30). It is suggested that GDM patients with gestational hypertension or preeclampsia may already have an underlying vascular dysfunction that results in later chronic hypertension and vascular disease (32; 38).

3.2 *Maternal implications after delivery*

More than 90% of women with GDM have normal glucose levels after delivery, but they have a higher risk of GDM in later pregnancies. Older women, women with higher weight gain between pregnancies and multiple birth women have also a higher risk of GDM in later pregnancies. Maternal pregestational and neonatal weight are also positively correlated with the risk of suffering GDM in later pregnancies (39).

GDM women have also a higher risk to develop type 2 diabetes or cardiovascular disease later in life than women with normal glucose levels during pregnancy (40). Bellamy *et al.* reviewed that women with GDM had a higher relative risk of 7.43 of suffering type 2 diabetes; moreover, an observational study described the incidence of type 2 diabetes after GDM with 3.7% at 9 months after delivery, 4.9% at 15 months, 13.1% at 5 years and with 18.9% 9 years after delivery (41). In summary, between 10 to 31% of women diagnosed with type 2 diabetes, suffered previously from gestational diabetes (42). Moreover, different ethnicity also affects the risk of developing type 2 diabetes after GDM pregnancy. Kwak *et al.* (43) have recently reported in an Asian population a prevalence of 12.5% to develop type 2 diabetes after a GDM pregnancy in an early postpartum period of two months. In addition, they also reported a median time to progression to type 2 diabetes of 8 years with an annual incidence estimated to be 6.8% per year after this early postpartum period. Nevertheless, there are a variety of independent risk factors to develop type 2 diabetes after GDM and large interindividual variations of the time to manifestation.

The main anthropometric risk factors associated with developing type 2 diabetes with a previous GDM history are waist circumference and body mass index (BMI) (44-45). Further risk factors are insulin requirements during pregnancy, the diagnosis of GDM very early in pregnancy (before 24 weeks), the presence of islet cells antibodies, fasting hyperglycaemia during pregnancy, a very short period between pregnancies and neonatal hypoglycemia (46-48).

Furthermore, women diagnosed with GDM have a higher risk of developing cardiovascular disorders associated with diabetes such as atherosclerosis, glomerular disruption and pathogenic retinal angiogenesis (32).

3.3 *Fetal macrosomia*

Fetal macrosomia is the most common complication associated with GDM and it occurs when fetal birth weight is above 4500 g, regardless of gestational age (49). In addition to fetal macrosomia, GDM is also related with higher risk of giving birth to

large for gestational age (LGA) babies, defined as a birth weight above the 90th percentile. Although maternal hyperglycemia favours fetal macrosomia (50-52), there are other mechanisms beyond than just maternal hyperglycemia involved in inducing fetal overgrowing. In fact, fetal macrosomia and LGA infants have been related to increased triglyceride concentration in GDM subjects with an adequate glycemic control (53). Moreover, maternal triglyceride levels during the third trimester of gestation seem to be a better predictor of birth weight than maternal glucose levels or BMI in GDM subjects (53-55). The enhanced levels of maternal plasma NEFA associated with GDM pregnancies seem also to be linked to birth weight and fetal growth (56); thus, a disturbed lipid metabolism in this pathology could affect the lipids transfer to the fetus as well as fetal growth and fetal tissues composition. Furthermore, Jansson *et al.* (57) studied the placental signalling and amino acid transport in women with high BMI and without GDM and they proposed that up-regulation of specific placental amino acid transporter isoforms may contribute to fetal overgrowth in obese women. Since obesity and diabetes in pregnancy might have common underlying metabolic disturbances that can result in increased placental nutrient transfer and fetal growth, amino acids could contribute to fetal macrosomia.

It was classically accepted that fetal macrosomia was due to maternal hyperglycemia in addition to an enhanced placental transfer of glucose which led to fetal hyperinsulinemia, according to the Pedersen hypothesis (58-60). Nevertheless, currently the theory of Freinkel (61) is increasingly accepted, which support that glucose, amino acids and lipids all together play a crucial role in the physiopathology development of the fetus.

Despite the metabolic factors described above, there are other preconception- and pregnancy-related risk factors associated with excessive fetal growth. Several authors have reported elevated pre-pregnancy BMI, excessive weight gain during pregnancy and ethnicity to contribute to the prevalence of LGA and macrosomia in women with and without GDM (62-65). Independently, GDM has been significantly associated with increased risk of women giving birth to macrosomic and LGA infants, even among normal-weight women and underweight women (66).

The implications of fetal macrosomia and LGA are an increase in surgical deliveries and fetal obstetric complications like shoulder dystocia, brachial plexus injury and broken clavicle (67-70). Moreover, macrosomia increases the risk of trauma in the labor (71).

3.4 *Intrauterine fetal mortality*

Diabetes before pregnancy (type 1 or type 2 diabetes) has been related with a higher risk of intrauterine fetal mortality (72) with a prevalence of 1 to 2% in women with prepregnancy diabetes. Fetus from GDM women have been reported to have also an increased risk of intrauterine mortality (73-74). This increase in fetal death is more accentuated in GDM women who required insulin treatment than those GDM women with a good glycemic control only with diet and exercise treatment (50; 73; 75). There are many causes for intrauterine fetal mortality during normal gestation, so hyperglycemia in diabetic pregnant women would just be one more.

3.5 *Neonatal morbidity*

After birth, the neonate has a high risk of morbidity, mainly due to the higher risk of suffering hypoglycemia, hyperbilirubinemia, hypocalcemia or respiratory distress syndrome (76-77). These complications are associated with neonates born form mothers with prepregnancy diabetes, although GDM may also induce these complications due to the similar physiopathology with diabetes before pregnancy (78-79).

Most of the fetal metabolic complications are caused by the fetal hyperinsulinemia, which produces hypoglycemia in the neonate during the first hours after delivery without the maternal supply of glucose (80). Moreover, fetal hyperinsulinemia during intrauterine life induces the accumulation of glycogen in fetal liver and lipid accumulation in the adipose tissue (81). This increased fetal metabolism results in higher oxygen consumption that leads to hypoxemia which may produce an increase in the synthesis of erythropoietin that induces polycythemia (82-83). When the red blood cells in excess are removed in the neonate, there is an increased risk of

suffering hyperbilirubinemia (84). Furthermore, hyperinsulinemia also affects the fetal lung maturing process and may cause respiratory distress syndrome in the neonates born from GDM women (85).

3.6 *Fetal programming of obesity*

The concept of intrauterine programming is that a stimulus or insult during a critical or sensitive period of development can have long-term or lifetime effects on the structure, physiology and metabolism of an organism (86). The hypothesis about fetal origins of disease propound that intrauterine programming may critically affect the health in adulthood (87).

Epidemiological studies of Dutch Hunger Winter proposed that the low weight of neonates indicates a poor prenatal nutrition and may have a negative effect in neonatal development with a delayed manifestation later in life with higher risk of major chronic diseases, mainly hypertension, obesity, type 2 diabetes, coronary heart disease and metabolic syndrome (88-95). Nowadays, it is more likely for the fetus to have hyperglycemia or energy excess in the intrauterine environment instead of a poor prenatal nutrition.

An increasing number of studies in rodents show that exposure to maternal obesity/overnutrition during both pregnancy and lactation is associated with the development of obesity in the offspring (96-105). In humans, it is difficult to discern the effect of maternal obesity/overnutrition on the fetal programming of obesity because during childhood and adolescence children are probably under the same dietetic and obesogenic habits than their mothers.

The importance of maternal obesity in the intrauterine environment has reached more relevance subsequent to studies analyzing the perinatal results in children from mothers with morbid obesity, before and after maternal biliopancreatic diversion bariatric surgery which reduced maternal BMI from 48 to 30. Kral *et al.* (106) reported a reduced prevalence of child obesity and overweight after maternal surgery from 60% to

35%, reaching the normal prevalence in the population. With a lower number of subjects, Smith *et al.* (107) observed a reduced fetal weight but also a reduction in fetal macrosomia after maternal surgery. Moreover, Kral *et al.* (106) observed decreased percentages in child overweight and obesity after surgery in children aged 7 and 11 to 18. These results were confirmed by Smith *et al.* (107) in teenagers, with a high reduction in super obesity and an improvement in the cardiometabolic markers during adolescence which are ascribed to an improvement in the intrauterine environment.

Fetus from mothers with GDM are exposed to a hyperglycemic environment that leads to high fetal levels of insulin and fetal lipogenesis, which contribute to the high weight of GDM neonates and the higher risk of infant obesity and type 2 diabetes (108-113). Furthermore, the children born from a GDM pregnancy have a higher risk of developing obesity than their brothers or sisters born from a healthy pregnancy; thus, the higher risk of obesity in the offspring is not only dependent on genetics (111; 114-115). In fact, recent studies in GDM have described maternal pre-pregnancy BMI as the strongest risk factor for childhood obesity and metabolic dysfunction, even in women with well controlled GDM (116). Another study reported that children born LGA exhibit the most severe degrees of obesity and higher prevalence of metabolic syndrome than those born appropriate (AGA) or small for gestational age (SGA) (117). The association between GDM and higher occurrence of giving birth to LGA babies may lead to a higher prevalence of childhood obesity in this pathology. Nevertheless, Pham *et al.* (118) found that childhood obesity was not associated with GDM, while infant obesity was likely more associated with maternal BMI and LGA which were associated with poorly controlled GDM.

3.7 *Neurological implications in the newborn*

Some authors have proposed that some maternal nutritional and metabolic alterations during pregnancy, such as GDM, may affect the fetal development of the neurological system and consequently, the neurological and psychological development of neonates. Rizzo *et al.* described a higher risk of alterations in neonatal development in the offspring of GDM mothers and mothers with pregestational

diabetes at the age of 2-5 years, even when the glucose control was adequate (119). Ornoy *et al*. also reported disturbances in neurological development of neonates at school age from GDM and pregestational diabetes women with delayed manifestations such as motor functions, attention span, hyperactivity disorder, etc; besides, they observed a negative correlation between the degree of maternal glycemic control and the strength of these neurological complications in those children (120).

More recent studies add that maternal metabolic conditions like pregestational type 2 diabetes or GDM could be even considered as a risk factor for autism and delay and impairments in several domains of development, particularly expressive language (121). In agreement with this, Dionne *et al*. observed that the offspring of GDM mothers are two times more at risk of language impairment with an effect of GDM on the expressive language composite and on nonverbal intelligence quotient at the age of 30 months (122), although offspring of educated mothers were less affected. However, Krakowiak *et al.* reported negative scores in language in 2-4 years old children without autism born from GDM or type 2 diabetes mothers, even after adjusting for mother´s educational level (121). Existing prepregnancy diabetes, GDM, and, to a lesser extent, glycosuria have been associated with lower offspring School Entry Assessment scores at age of 4 (an assessment of the four required skills language, reading, writing, and mathematics), lower IQ at the age of 8, and lower General Certificate of Secondary Education results at the age of 16, even when adjusting for offspring sex, maternal age, prepregnancy BMI, smoking in pregnancy, maternal education, and occupational social class (123). Thus, maternal diabetes in pregnancy is consistently associated with lower offspring cognition and educational attainment and the weaker associations with glycosuria suggest a dose-dependent adverse association with IQ.

4. LC-PUFA AND THEIR IMPORTANCE FOR THE FETUS

The critical steps in the development of the human encephalon structure take place during the last trimester of pregnancy and go on until two years after birth (124). This morphogenesis is closely associated with brain function and requires an extra input of long chain polyunsaturated fatty acids (LC-PUFA), especially arachidonic acid (AA) and docosahexaenoic acid (DHA) since brain is a tissue with a high lipid composition, with 60% of the dry weight composed by lipids, of which 40% are LC-PUFA with 10% of arachidonic acid and 15% docosahexaenoic acid (125).

Long chain-polyunsaturated fatty acids are fatty acids composed by 20 or more carbon atoms and include more than one double bond in its chemical structure. LC-PUFA derive from essential fatty acids, which are molecules that cannot be synthesized by humans, thus must be ingested with the diet. The synthesis of LC-PUFA proceeds via enzymatic reactions of desaturation and elongation of the carbon chain (Figure 1). Linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3) are the precursors of AA (20:4n-6) and DHA (22:6 n-3) respectively, by an enzymatic process that mainly occurs in the peroxisomes and endoplasmic reticulum of the hepatocytes (126), although this biochemical reaction may also occur in other human tissues.

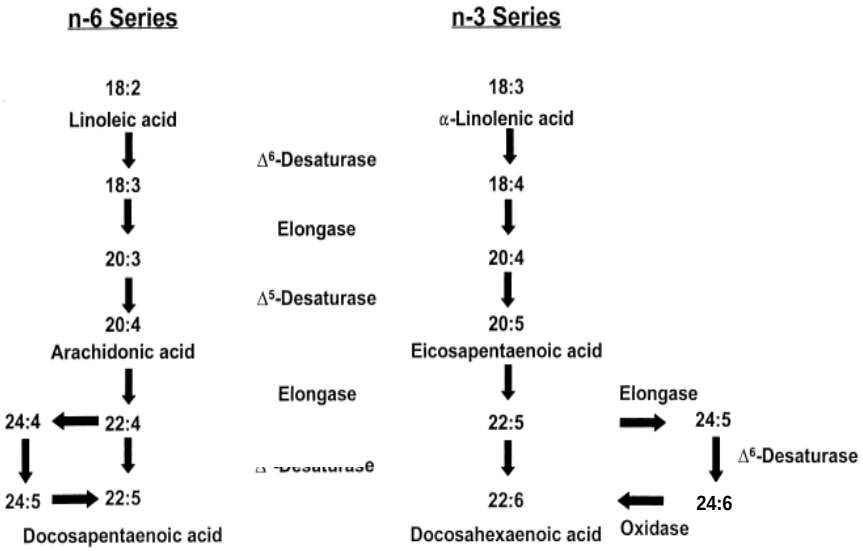


Figure 1. Metabolic pathways of LC-PUFA synthesis from linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3). Lauritzen et al., 2001 (127).

Arachidonic acid is the most important ω-6 LC-PUFA and has a very important role in neonatal development. It is one of the precursors of the eicosanoids which are proinflammatory molecules in a large number of physiological processes such as the blastocyst implantation, the beginning of labor, the hydroelectric regulation in the kidney, platelet aggregation or activation of the immune system. The most important LC-PUFA of the ω-3 family are eicosapentaenoic acid (EPA) and DHA. Both of them have important metabolic and regulatory functions in the organism. EPA is also a precursor of the eicosanoids with antiplatelet and anti-inflammatory functions (128-130) while DHA is involved in the membrane fluidity of the central nervous system and retina with an important role in the neurological function, especially in the early stages of life.

AA and DHA perform most of their functions being esterified in the phospholipids of the cellular lipid bilayer, especially in phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). The phospholipids with high amounts of LC-PUFA provide large fluidity to the membranes due to their high unsaturation degree. The brain is a tissue composed by a large number of membrane structures, thus fluidity of the membranes seems to have a special role in the formation of the nervous system and brain tissue (130-132).

DHA is the LC-PUFA with the greatest distribution in the tissues of the central nervous system which supports the important role of this fatty acid in the neuronal and visual development in the newborns (127; 133). In cell membranes, DHA is one of the principal components of the phospholipids, being the 7.2% of brain composition and mainly distributed in the synaptic membrane of the gray matter; in addition DHA is also found with high abundance in the rod cells, constituting the 19.7 % of retina (133-138).

The most important period in neurological development goes from the third trimester of pregnancy to 18 months of life and it is during this time when more amounts of DHA are required and found in the brain, with a peak at the birth time (139). Clandinin *et al*. estimated a prenatal accumulation of DHA in the human brain around 15-22 mg/week during the last third of pregnancy (140-141) supporting the

importance of this fatty acid in fetal development. Furthermore, other studies described lower levels of DHA in preterm newborn babies due to an early interruption of placental transfer; these lower levels of DHA are correlated with abnormal retinal and cortical functions (142-144).

Some authors have also found a direct relationship between the levels of LC-PUFA in plasma and red blood cells and the visual acuity and the evoked response in lactating babies (145-146). Other authors proposed that poor supply of LC-PUFA during the perinatal period may have consequences for the intelligence and intellectual development of these babies later in life (147). Due to the importance of these fatty acids during pregnancy and fetal development, an especial attention has been focused in adequate intake of LC-PUFA in pregnant women and their supply to the fetus.

Several studies have been focused on the possible beneficial effects of maternal LC-PUFA supplementation during pregnancy on the development of visual and other neural functions in the offspring (Reviewed by Larqué *et al*. (2012) (148). The available results of different studies suggest some association between early DHA status and cognitive function in infancy and early childhood (Table 2). Nevertheless, some authors did not find differences between the treatment and control groups (Table 2). A recent meta-analysis on DHA supplementation during pregnancy concluded that due to the small sample size of most of the studies, the high attrition, the different periods of supplementation and the use of different tests and ages at assessment, there is not enough evidence to support or refute the hypothesis that n-3 LC-PUFA supplementation in pregnancy improves child cognitive or visual development (149). However, this meta-analysis was done considering only one or two studies for each variable under study, which limits very much the results obtained by this statistical meta-analysis. Thus, more studies are needed to get a valuable meta-analysis study on the effect of LC-PUFA supplementation during pregnancy or neurodevelopment.

Table 2. *Randomized controlled trials of n-3 LC-PUFA supplementation during pregnancy on neurodevelopmental function of the offspring*

Study	Participants	Daily Dose	Duration	Primary Functional Outcomes
Stein *et al.* 2012 [150]	452	400 mg DHA	Week 18-22 to delivery	Brainstem auditory-evoked responses at 1 and 3 months or VEP at 3 and 6 months (n.s.)
NUHEAL study Campoy *et al.* 2011 [151] **Escolano-Margarit *et al.* 2011** [152]	270 315	500 mg DHA + 150 mg EPA	Week 20 to delivery	K-ABC test (IQ) at 6.5 years (n.s.) Several functional domains at 4 and 5.5 years (n.s.) NOS score increased with every unit increment in cord blood DHA at 5.5 years
DOMINO study Makrides *et al.* 2010 [153]	2399	0.8g DHA+0.1g EPA	Week 22 to delivery	Language or cognitive skills at 18 months (n.s.) Postpartum maternal depression (n.s.)
Innis and Friesen 2008 [154]	135	400 mg DHA	Week 16 to delivery	More girls in placebo than in the DHA group had visual acuity below average at 2 months
Judge *et al.* 2007 [155]	29	0.214 g DHA	Week 24 to delivery	Problem-solving improved at 9 months. Fagan test of infant intelligence (n.s)
Judge *et al.* 2007 [156]	30			Visual acuity (Teller cards) improved at age 4 months; at age 6 months (n.s)
Tofail *et al.* 2006 [157]	249	1.2g DHA + 1.8g EPA 0.27g ALA + 2.3g LA	Week 25 to delivery	Bayley MDI and PDI at age 10 months (n.s) Associations by multiple regression analyses
Colombo *et al.* 2004 [158]	77	135 mg DHA 35 mg DHA	Weeks 24-28 to delivery	Mental processing improved at 4 and 6 months but not at 8 months. Increase in examining and less distractibility between age 1 and 2y; attentional disengagement (n.s.)
Dunstan *et al.* 2008 [159]	72	2.2g DHA +1.1g EPA	Week 20 to delivery	Eye-hand coordination favored at 2.5 years

Malcolm *et al.* 2003 [160] **Malcolm *et al.* 2003** [161]	100	200 mg fish oil	Week 15 to delivery	Visual acuity (VEP, ERG) (n.s.). Correlations VEP and ERG with infant DHA
Helland *et al.* 2001 [162]	341	1.18g DHA+0.8g EPA	Week 18 to 3 months post-delivery	EEG and Fagan test (n.s.) during the 1st year. Correlation of EEG on day 2 and cord DHA
Helland *et al.* 2003 [142] **(4-year)**	84			IQ at age 4y favoured with DHA. Correlation of IQ with maternal DHA intake
Helland *et al.* 2008 [163] **(7-year)**	142			IQ (n.s.). Significant corrrelation at age 7 with maternal DHA during pregnancy

(n.s.) not significant; EEG, electroencephalography; ERG, electroretinogram; K-ABC, Kaufman assessment battery for children; MDI, mental development score; NOS, neurological optimality score; PDI, psychomotor development score; VEP, visual evoked potentials.

Observational longitudinal studies have also supported a relationship between DHA status and infant development; in Inuits from Arctic Quebec, cord DHA levels were associated with greater visual, cognitive and motor development during the first year of life (164), as well as shorter latencies of VEP in school-age children (165). Further support for the importance of DHA availability in early life comes from the positive associations between maternal fish consumption during pregnancy and cognitive performance of the offspring, observed in large birth cohorts such as ALSPAC (166), VIVA (167) and the Danish National Birth Cohort (168). However, these results should be interpreted with caution, considering the limitations of observational studies which often cannot fully adjust for all confounding variables.

5. PLACENTAL TRANSFER OF FATTY ACIDS

The placental supply of maternal LC-PUFA to the fetus is critical since the ability of both fetus and the human placenta to synthesize LC-PUFA from essential fatty acids by desaturation and elongation reactions is limited (169). The lack of Δ^5- and Δ^6-desaturase activity in the placenta and the physiological immaturity of the fetus to synthesize LC-PUFA (144) make these fatty acids conditionally essential for the fetus and must be provided from the mother by placental transfer. It seems to be an association between DHA levels in maternal plasma, the maternal ingestion of DHA during pregnancy and the development of the cognitive functions of the neonate and later for the child. Thus, an adequate supply of LC-PUFA and especially DHA from the mother to the fetus is of critical importance.

In vitro and perfusion studies demonstrated that placental tissue has a preferential transfer of LC-PUFA to fetal circulation. Using perfused placentas, Haggarty *et al.* found a selective and preferential transfer of DHA (preference order of transfer: DHA>AA>ALA>LA) (170); moreover, Campbell *et al.* developed an *in vitro* study which demonstrated also a preferential DHA uptake by placental cells (DHA>AA>LA>OA) (171).

Larqué *et al.* administered *in vivo* fatty acids labelled with stable isotopes (^{13}Carbon) to healthy pregnant women four hours before a selective caesarean section. The analysis of placental tissue at the time of delivery demonstrates a preferential incorporation of DHA relative to linoleic acid, oleic acid and palmitic acid. The concentration ratio between placenta and maternal plasma was significantly higher for ^{13}C-DHA than for the rest of labelled fatty acids. Nevertheless, this study could not demonstrate a preferential transfer of DHA to fetal circulation which suggests that placental transfer of fatty acids to the fetus needs more than four hours (172). In a subsequent study, Gil-Sánchez *et al.* (173) increased the time between tracer administration and caesarean section up to 12 hours. Under these conditions, they found a significantly higher ratio of ^{13}C-DHA concentrations in cord plasma than in maternal plasma, which was higher than for the other studied fatty acids, supporting

the hypothesis of a selective DHA transfer from the mother to the fetus, and of a preferential accretion of DHA in both placenta and fetus relative to other fatty acids (173).

Placental transfer of LC-PUFA and the optimal supply of these fatty acids to the fetus seem to be disturbed by some pathologies during pregnancy such as gestational diabetes or intrauterine growth restriction (IUGR), with consequences for the fetus that are still unknown.

5.1 *Fatty acid uptake by the human placenta*

In the human placenta, the nutrient exchange occurs in the epithelium called syncytiotrophoblast, it is made of syncytial cells that are polarized as other epithelia by having a brush border or microvillous plasma membrane (MVM), which is bathed in maternal blood and a basal plasma membrane facing the fetal capillary. Maternal to fetal transfer of fatty acids is mediated by the placenta which has been described to take up the fatty acids as NEFA. The maternal plasma NEFA concentration increases during pregnancy generating a gradient of concentration between maternal and fetal circulation that leads to fatty acids transfer across the placenta. Besides NEFA, most fatty acids are esterified in triglycerides, phospholipids and cholesterol esters that are incorporated in plasma lipoproteins (Figure 2). Since placenta takes up NEFA from maternal plasma, esterified fatty acids in the lipoproteins must be hydrolyzed by placental lipases to be available for placental transfer to the fetus.

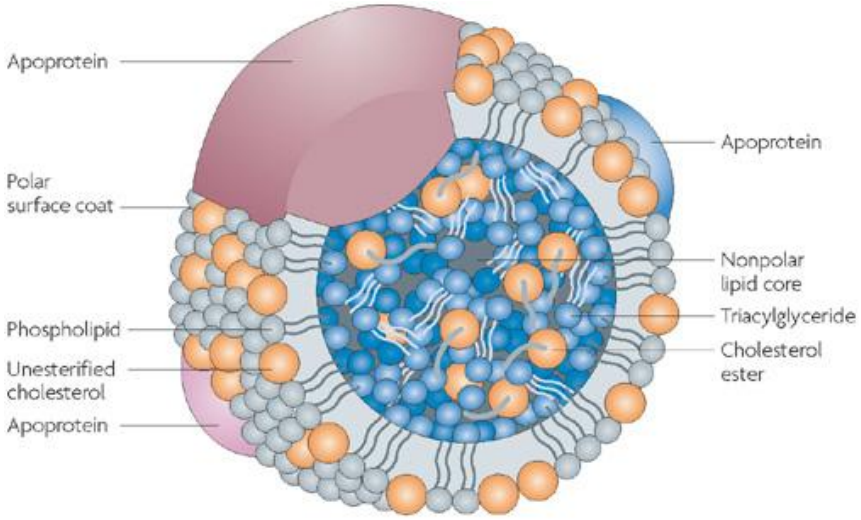


Figure 2. General structure of a lipoprotein. Wasan et al. 2008 (174)

Two lipases have been described in the microvillous membrane: LPL and endothelial lipase (EL) which hydrolyze triglycerides and phospholipids respectively, releasing NEFA for placental accretion (Figure 3) (175). LPL is present in placental microvillous membrane (176) and has been classically described for the placental transfer of fatty acids. This enzyme has triglyceride lipase activity and hydrolyzes the TG present in the nucleus of chylomicrons and VLDL, releasing NEFA for its accretion by the tissues (177-179). EL has been described in the placental endothelium cells and recently, in the membranes of the syncytiotrophoblast (180). EL has A1 phospholipase activity releasing the fatty acid esterified in the position 1 of the phospholipids and has also a minor triglyceride lipase activity (181). The position 1 of the PL is characterized to be esterified by a saturated fatty acid while PUFA are in the position 2; thus, the hydrolysis of PL by EL mainly produces lysophospholipids rich in PUFA which may be an additional placental source of fatty acids. Some studies described that lyso-PC may represent the preferential transfer for DHA into brain tissue comparing with the transfer of DHA in NEFA (182). Moreover, lyso-PC seems to be more efficient than NEFA in releasing DHA to the red blood cells (183). EL is a prevailing enzyme in placental tissue until the end of pregnancy while LPL expression is nearly absent in trophoblasts at the end of gestation. Nevertheless, it has been observed an increase in the expression of both lipases in placental vessels at the end of pregnancy (184).

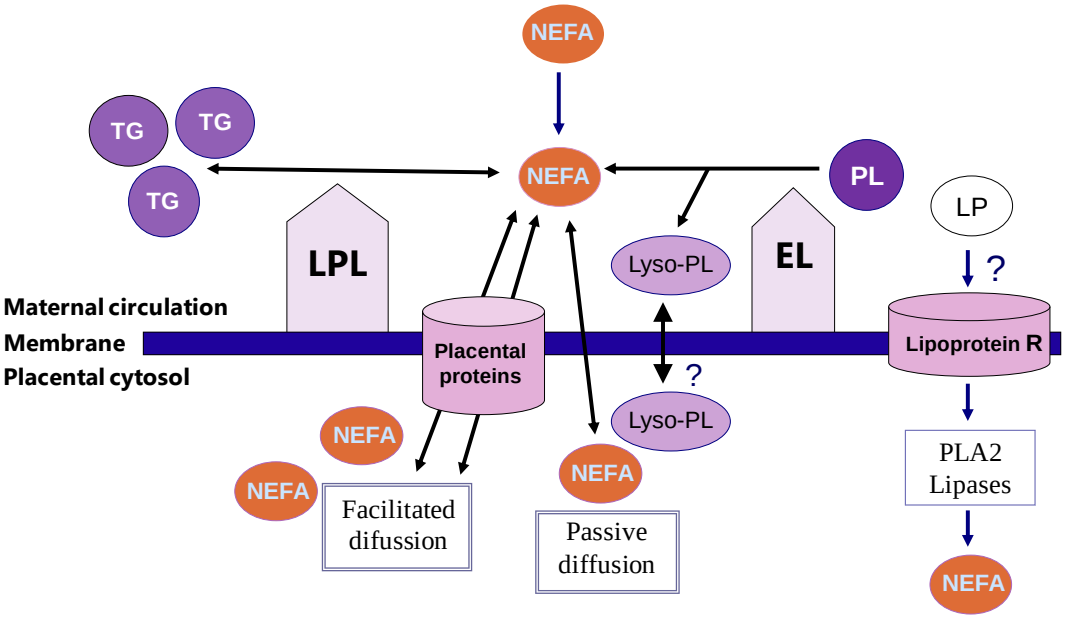


Figure 3. Placental uptake of fatty acids. Gil-Sánchez et al. 2011 (175)

Magnusson *et al.* (185) investigated the placental LPL activity in healthy pregnancies comparing it with pregnant women complicated with IUGR, pregestational diabetes mellitus or GDM. This study demonstrated that placental LPL activity was reduced by 47% in pregnancies complicated with IUGR, while augmented by 39% in pregnant women with pregestational diabetes compared to controls. Lindegaard *et al.* (186) described that placental LPL activity was increased in pregnancies complicated with diabetes associated to fetal overweight. Both studies are in agreement with the hypothesis that alterations in LPL may lead to changes in placental transfer to the fetus and, in consequence, it could modify fetal growth (185).

In GDM, it has been reported not only no differences in the activity of LPL and EL comparing with healthy pregnancies (185; 187), but also increases in EL and even a reduction in placental LPL (188) to counteract excessive placental uptake of fatty acids in this pathology. Thus, available comparisons of the activity of enzymes that release fatty acids from circulating maternal lipids for their placental uptake between GDM and uncomplicated pregnancies are inconclusive.

As regards the regulation of placental lipolysis, Waterman *et al.* (176) identified a hormone-sensitive lipase (HSL) in the cytosol and microvillous membranes of human placenta. Subsequently Lindegaard *et al.* (186) found an increase in HSL and EL mRNA expression in placentas from diabetic women. These alterations in placental lipases of diabetics may be related to the disturbed fatty acids transport from the mother to the fetus in these subjects.

There are other lipases involved in the hydrolysis of esterified fatty acids such as type II phospholipase A2 which is mainly expressed in placenta and has also triglyceride hydrolase activity at a pH 6.0 (different from optimum pH 8.0 of LPL) (189). Moreover, the placenta has receptors for VLDL, HDL and LDL lipoproteins (14; 190); some lipoproteins may join these receptors and suffer endocytosis that would provide fatty acids to the placental tissue after being released by the intracellular lipases. In GDM pregnancies, it has been recently reported an enhanced placental protein expression of the lectin-like oxidized LDL receptor-1 (OLR1) without affecting mRNA expression levels (191) and also an increase of mRNA and protein expression of other lipoprotein receptors as LDLR or VLDLR in both GDM placentas from normal-weight and overweight/obese women (192); nevertheless the relevance of lipoproteins in placental uptake of fatty acids is still unknown.

5.2 *Placental membrane transport proteins*

After the action of the lipases, the esterified fatty acids from the lipoproteins are released as NEFA which are captured by cells through passive transport or by a complex and saturable mechanism mediated by transport proteins (Figure 4). It has been described a large number of proteins involved in fatty acids transport through placental membrane, such as FAT/CD36 (fatty acid translocase), FABPpm (plasma membrane-associated fatty acid-binding protein), p-FABPpm (placental plasma membrane fatty acid-binding protein) and FATP-1 to -6 (fatty acid transport proteins) with Acyl-CoA-synthetase activity (193-194). These proteins are present in placental microvillous membrane and the basal membrane of the syncytiotrophoblast except for p-FABPpm which is only present in placental microvillous membrane (195).

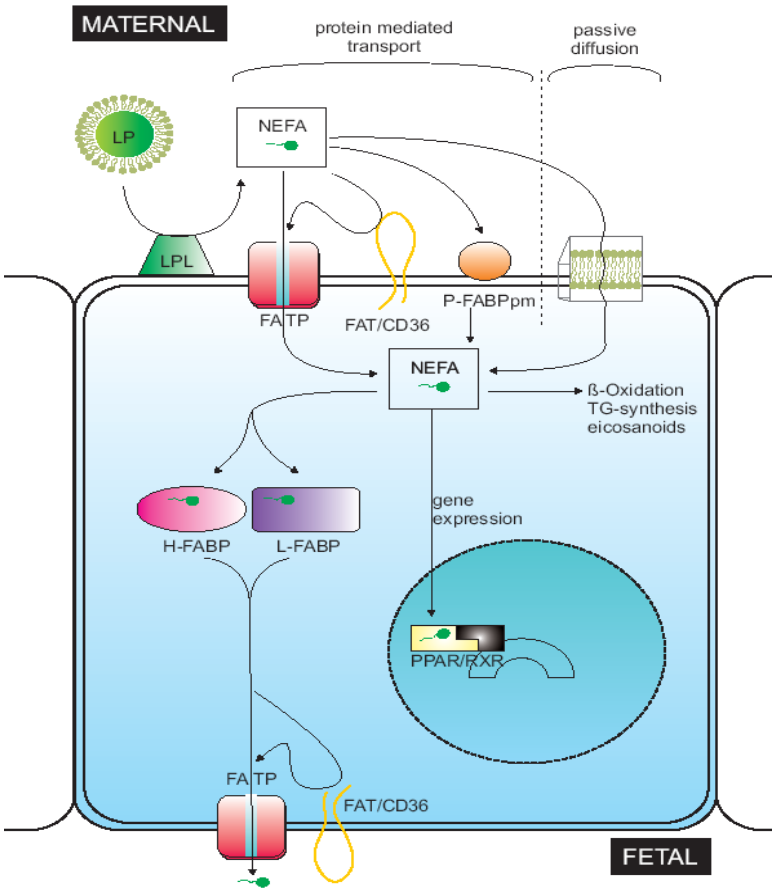


Figure 4. Model of placental fatty acid transport. Hanebutt et al. 2008 (194)

Among the membrane associated fatty acid transport proteins (FATP) expressed in placenta, FATP4 and p-FABPpm seem to be the more involved proteins in the selective raising of DHA by the placenta (195-197). Larqué *et al.* evaluated the association between DHA supplementation and placental expression of FATP in healthy pregnant women. In this study they observed that the placental gene expression of FATP1 and FATP4 was directly correlated with DHA percentages in placental and maternal plasma phospholipids; moreover, placental FATP4 gene expression was significantly correlated with DHA percentages in fetal plasma phospholipids. These results suggest that FATP4 may be involved in the selective placental transfer of DHA and therefore involved in the selective placental transfer of n-3 LC-PUFA (197).

Using BeWo cells as an experimental model of trophoblasts, it was observed a reduced uptake of DHA and AA (64% and 68% respectively) when these cells were incubated with a p-FABPpm antibody; nevertheless, the oleic acid (OA) uptake was only reduced in 32% (171). These results suggest that p-FABPpm is directly involved in placental uptake of DHA and AA; however, p-FABPpm has not been genetically sequenced and has been demonstrated only by Dutta-Roy *et al*.; thus, the function and identification of this protein have to be treated with caution.

To our knowledge, there are not available studies on the expression of FATP in GDM placentas, although Dubé *et al*. studied the mRNA and protein expression of several fatty acid carriers in placentas from obese women. In this study, they found an enhanced FAT/CD36 placental expression and a decreased expression of FATP4 in these subjects compared to that in normal weight women (198). Since Larqué *et al*. suggested that in the placenta, FATP4 in mainly involved in LC-PUFA placental transfer (197), the reduced FATP4 placental expression in obese women could alter their fatty acid uptake.

5.3 *Placental intracellular transport of fatty acids*

Once fatty acids have been transported into the cytosol, they bind to fatty acid binding proteins (FABP). It has been described several FABP in the placenta: FABP1 or L-FABP (Liver FABP), FABP3 or H-FABP (Heart FABP), FABP4 or A-FABP (Adipocyte FABP), FABP5 or K-FABP (Keratinocyte- FABP) and FABP7 or B-FABP (Brain FABP) (reviewed by Cunningham and McDermott 2009) (196). Cytosolic NEFA may also be oxidized into the trophoblast or be esterified and deposited in TG and other lipid fractions inside the placental cell for later hydrolysis (lipolysis) and release of NEFA for their transfer to the fetus (170; 199).

Tobin *et al*. (200) described that the different metabolization of fatty acids into the trophoblast affects the fatty acid transfer to the fetus. They incubated BeWo cells with oleic acid resulting in a higher accumulation of TG in the cytosol and a higher formation of lipid droplets (LD) than incubating with DHA; nevertheless, the relative transfer of DHA comparing with oleic acid was four fold higher for the DHA; thus, the

reduced accumulation of DHA in LD resulted in a higher transfer to the fetus. Moreover, Johnsen *et al*. (201) described that incubating BeWo cells with LC-PUFA (AA, EPA and DHA) modified the gene expression of intracellular acyl-CoA synthetases; this findings suggested that LC-PUFA may have an important regulatory function in the trophoblast fatty acid uptake, mediating the expression of these proteins. These mechanisms may also affect the LC-PUFA biomagnification respect to the rest of fatty acids.

In a recent study, Scifres *et al*. (202) reported an increased expression of FABP4 (also named A-FABP) in placentas of women with pregnancies complicated by obesity and diabetes (either type 2 diabetes or GDM) compared to placentas from nondiabetic obese women or nonobese controls. They also found that inhibition of FABP4 in placental trophoblasts attenuated LD formation and TG accumulation which suggests that FABP4 plays a pivotal role in the uptake and accumulation of lipids in human placentas (202). Furthermore, obese and GDM mothers have elevated pro-inflammatory cytokines and IL-6 has been reported to induce fatty acid accumulation in cultured trophoblast cells; nevertheless, this effect was not mediated by increased expression of LPL or other key genes mediating cellular uptake as FATP-4 or FABP1 (203). Thus, the mechanism by which IL-6 stimulates trophoblasts fatty acid accumulation remains to be established.

5.4 *Fatty acids release to fetal circulation*

The most efficient mechanism to transport lipids from tissues to bloodstream is the formation and secretion of lipoproteins containing apolipoprotein B (apoB). These lipoproteins incorporate large amounts of triglycerides and may transport essential lipids as fat-soluble vitamins and glycolipids (204). Human placenta can synthesize and secrete apoB and one kind of lipoprotein that may transport lipids to the fetus (205); however, the placental secretion of lipoproteins is still controversial. What is accepted for the major part of studies is that the placenta transports fatty acids to the fetal circulation directly by facilitated transport or by lipid transport proteins (FAT/CD36 and FATP) present in the basal membrane of syncytiotrophoblast (194) (Figure 4).

Once fatty acids have been transported to the fetal circulation, they bind to the α-fetoprotein and are captured by fetal liver where fatty acids are esterified into TG and released to the fetal circulation. There are evidences that esterified fatty acids in fetal circulation cannot be re-captured by placental tissue.

6. FATTY ACID STATUS IN GDM PREGNANCIES

6.1 *Maternal fatty acid status in GDM*

In pregnancies complicated with GDM, the concentrations of total fatty acids and the percentages of each individual fatty acid seem to be altered both in maternal plasma and maternal red blood cells. Chen *et al.* (206) described an increase in maternal plasma fatty acid concentration in pregnant women with GDM and healthy pregnant women but with carbohydrate intolerance during pregnancy; moreover, they observed a positive correlation between the hyperglycemia degree and maternal fatty acid levels during the last third of pregnancy. Obesity and overweight during pregnancy observed in these patients also correlate with augmented maternal fatty acid concentrations (206).

In regard to LC-PUFA, different studies showed normal levels of AA and DHA in maternal plasma lipids in GDM women (207), while altered levels of LC-PUFA in maternal erythrocyte membranes of these patients. Min *et al.* demonstrate reduced percentages of AA and DHA in PL of red blood cell membranes in GDM women comparing with control subjects (208); the same results were previously found in subjects with pregestational diabetes (209). These reduced percentages of AA and DHA were more pronounced in obese pregnant women with GDM than in non-obese GDM women and obese pregnant women without GDM (208); this reduction in LC-PUFA levels may be explained by a decreased desaturase activity in diabetic patients. It has been described an impaired Δ-5 and Δ-6 desaturase activity in both human diabetic patients (210) and diabetic animal models (211). Although these desaturases have insulin sensitive activity (210-212), it has not been evidenced their impairment in GDM (213).

Nevertheless, although it has been observed reduced percentages of AA and DHA in erythrocytes membranes of GDM women (208), the plasma levels of these fatty acids have been reported to be unaltered or even higher in GDM women comparing with healthy pregnant women (207-208; 213-214). Particularly for DHA, it has been observed even higher values in maternal plasma triglycerides and phospholipids (213; 215). The discrepancies in the results from these studies may be due to a higher dietary intake or due to the mobilization or synthesis of these fatty acids promoted by insulin. The glycemic control and pregestational BMI seem to be predictors of AA and DHA levels in maternal plasma phospholipids at the end of pregnancy, both in healthy pregnant women and GDM (207).

The disagreement between fatty acid levels in plasma and red blood cell membranes in GDM subjects may suggests an association between the altered glucose metabolism in this pathology and the incorporation of AA and DHA into maternal membrane phospholipids.

In the placental tissue of women diagnosed with GDM it has been described higher levels of AA and DHA than healthy pregnant women, in particular in phospholipids fraction (216). Since placenta lacks desaturase activity, the increased levels of these LC-PUFA cannot be due to *de novo* synthesis by the placenta and are provided from maternal circulation. Thus, it has been suggested that there is an augmented placental uptake of LC-PUFA in GDM pregnancies (216). This could be due to disturbances in placental fatty acid transporters because of GDM, which has been demonstrated in animal models (217-218) and in other tissues than placenta in humans with type 2 diabetes mellitus (219). Moreover, it has been reported an increase in FABP-liver expression in GDM placentas comparing with controls (185) and higher incorporation of AA in placental TG in patients with an insulin dependent GDM (220). It was observed lower levels of AA in TG of placenta than in PL fraction in GDM subjects, which suggests a preferential transfer to the fetus of AA esterified in placental TG fraction in GDM (216).

6.2 *Fetal fatty acid status in GDM*

In neonates born to GDM women it has been reported lower values of DHA and other LC-PUFA, both in cord plasma and erythrocytes PC (215; 221-222). According to these results, Wijendran *et al.* (222) suggested an impaired materno-fetal LC-PUFA transfer during GDM.

The decreased levels of these fatty acids in GDM neonates may be due to a reduced maternal supply, a disturbed materno-fetal transfer, an altered fetal metabolism or a combined effect. Reduced LC-PUFA percentages in cord blood during GDM might occur by augmented *de novo* synthesis of saturated and monounsaturated fatty acids in the fetus from the abundantly available glucose, and hence decreasing proportions of essential fatty acids and their derivatives; thus, the decreased values of LC-PUFA could be explained as a relative shortage respect to other fatty acids, instead of an absolute defect in their concentration (223). Another hypothesis could be a disturbed placental transfer of LC-PUFA, or even higher LC-PUFA accretion by fetal adipose tissue in GDM babies.

Ortega-Senovilla *et al.* studied the venous and arterial lipid profiles in cord blood of GDM neonates and controls (224). The results of this study revealed lower percentages of AA, DHA and other LC-PUFA in arterial cord plasma of GDM than controls but found no differences in venous cord plasma. These differences in cord blood concentrations of LC-PUFA suggests that lower concentrations in neonates of GDM women may be due to a higher fetal metabolization than to a disturbed placental transfer of these fatty acids in GDM.

These lower levels of DHA in the offspring of GDM women might lead to an adverse fetal neurological programming of these children; therefore it is important to discern the reason for the lower levels of DHA in GDM babies in order to identify the best strategy of supplementation with LC-PUFA in the mother, the baby or both.

II. OBJECTIVES

Placenta is a key organ for the fatty acid transfer to the fetus and especially for essential and conditionally essential fatty acids as LC-PUFA. In pregnancies complicated by GDM, lower values of DHA and other LC-PUFA were observed in cord blood of neonates. Nevertheless, is not clear whether the reduced levels of these compounds in fetal blood were due to an impaired placental function or if the hyperglycemia could affect fetal metabolism, modifying the fatty acid profile in the neonate.

A reduction of LC-PUFA levels during fetal neurodevelopment stage might lead to adverse fetal neurological programming of the offspring in GDM. It is important to discern the reason for the lower levels of DHA in GDM babies and to clarify the mechanisms involved in fatty acid placental transfer in this pathology in order to improve LC-PUFA levels in GDM offspring. This information is essential for nutritional policy in order to design appropriate strategies for fatty acid supplementation in the mothers, breast fed babies or both.

The general aim of the present study was to improve the understanding of fatty acid transfer in GDM. We evaluated the *in vivo* placental transfer of fatty acids labelled with stable isotopes consumed by pregnant women with gestational diabetes mellitus or healthy controls 12 hours before caesarean section.

The secondary objectives of this study were:

1. To elucidate the distribution of the labelled fatty acids in the different maternal, placental and fetal compartments in both groups of subjects.
2. To evaluate the rate of fatty acid transfer between the maternal plasma to placental tissue as well as from the placenta to the fetus side in this pathology.
3. To discern if the treatment of GDM with insulin may have additional consequences on LC-PUFA levels in the fetus.

III. MATERIAL AND METHODS

7. SUBJECTS

Subjects undergoing elective caesarean section were recruited at the Gynecology & Obstetrics Service of the Hospital Vírgen de la Arrixaca (Murcia, Spain).

Two groups of subjects were established for the present study:

- Control group: eleven healthy pregnant women
- GDM group: nine pregnant women diagnosed with GDM (6 pregnant women were treated with insulin during pregnancy, while 3 required only dietary treatment)

Participants fulfilled the following inclusion criteria:

- singleton pregnancy
- term delivery
- age from 18 to 40 years
- non smoking women
- consuming omnivorous diet
- fetal Doppler scan within the normal reference range on the day before the caesarean section (225)

The criteria established for the exclusion were:

- reported health problems or pregnancy complications
- smoking women
- consumption of DHA supplements during pregnancy

The study protocol was approved by the local Ethical Committee. Written informed consent was obtained from all participating women after careful explanation of the study.

GDM was diagnosed according to the oral-glucose-tolerance test criteria of O'Sullivan and Mahan (226) between the 24 and 28 weeks of gestation by screening with an oral charge of 50g glucose. A positive screening result (1 hour plasma glucose concentration > 140mg/dL) was followed by a 3-hour oral glucose tolerance test with 100g glucose load and further serum glucose analyses at 1, 2 and 3 hours after glucose intake. The test was considered positive if 2 of the 4 glucose values collected were above the normal range (basal: 105 mg/dL, 1h: 190 mg/dL, 2h: 165 mg/dL and 3h: 145 mg/dL) in accordance with the criteria of the National Diabetes Data Group (NDDG) (12).

The day before the caesarean section maternal weight and height were recorded. In addition, an ecographical control was performed and fetal heart rate, fetal biometry and umbilical artery pulsatility index (PI) and resistance index (RI) were compiled. The results were normalized for gestational age using percentiles, following the criteria of Hadlock *et al.* (227) and those of Acharya *et al.* (228) for the umbilical artery pulsatility index. Weight, height, head circumference and abdominal circumference of neonates was recorded by the paediatricians immediately after birth. Birth weight and length z-score values were calculated according to the data of Carrascosa *et al.* (229).

8. LABELLED FATTY ACIDS ADMINISTRATION

The uniformly ^{13}C labelled fatty acids (Martek Biosciences, Columbia, MD) were given orally to the pregnant women 12 hours before the time of elective caesarean section, since this enables cord blood collection at a predefined time period after tracer application. As the plasma concentrations of palmitic acid (PA), oleic acid (OA) and linoleic acid (LA) are relatively high, 0.5 mg per kg of body weight of the corresponding labelled fatty acids were given. Due to the lower plasma concentration

of DHA, a dose of 0.1 mg/kg DHA (10% ^{13}C enrichment) was adequate. Nevertheless, 5 subjects from the control group received 0.1 mg/kg of ^{13}C-DHA with 99% enrichment; thus the results on ^{13}C-DHA concentrations or enrichments were calculated only for 6 controls versus 9 GDM. Real doses per kg body weight were identical between the groups and consequently any differences were due to different metabolization. The women remained fasted until the time of the caesarean section.

The behaviour of the tracer was the same in the patients receiving ^{13}C-DHA enriched at 10% as in those receiving the ^{13}C-DHA enriched at 99%. The DHA with 99% ^{13}C enrichment gave higher APE values or concentrations than those at 10% enrichment in each compartment, but this did not affect the ratios between compartments; thus, all the subjects from the study were considered for ^{13}FA ratios between compartments.

9. BLOOD AND PLACENTA SAMPLING

Blood samples were collected from the mothers at basal time, before tracer intake (12h before the caesarean section) and also 3 hours before the caesarean section, 2 hours before, 1 hour before, at the time of delivery and 1 hour after the time of caesarean section. Blood samples (8 mL at basal time and 3 mL at the rest of sampling times) were taken by venipuncture and immediately transferred into EDTA-containing tubes. Directly after birth, cord blood from the vein and the artery (2 mL of each one) was collected. Blood cells and plasma were separated within 1 hour by centrifugation at 1200 x g for 3 min and an aliquot of at least 200 μL of plasma was frozen subsequently at –80°C for later analysis.

4 mL from the maternal basal sample (-12h) were analyzed in the Biochemical Laboratory of the Vírgen de la Arrixaca Hospital in order to determine the maternal concentrations of TG, LDL-cholesterol, HDL-cholesterol, total cholesterol and albumin.

The total placenta was weighted immediately after delivery. Samples of 3 g from the placental central cotyledons were cut with a sharp knife. Placenta samples were rinsed with cold isotonic NaCl solution (0.9%) in order to eliminate, as far as possible,

contamination with blood and immediately frozen in liquid nitrogen and stored at –80°C until further analysis. Processing of placenta samples was completed within 10 minutes or less after delivery.

10. FATTY ACID QUANTIFICATION

10.1 *Extraction of plasma fatty acids*

Fatty acids from plasma were extracted using a modification of the Folch method (230). Briefly, 100 µL of internal standard (5mg FA each of pentadecanoic acid, tripentadecanoin, phosphatidylcholine dipentadecanoyl and cholesteryl pentadecanoate dissolved in 50mL methanol:choloroform 1:1) (Sigma, Deisenhofen, Germnay) were added to 250 µL of plasma. Total lipids were extracted into 10 mL chloroform/methanol (2:1) (230) and 2.5 mL of distilled water shaking three times in 15 minutes. Afterwards, the tubes were centrifuged at 1500 × g for 7 minutes at room temperature splitting up three phases in the tubes: a hydroalcoholic one on the top, a chloroformic one at the bottom and the protein phase in the middle. The upper phase was discarded and 2.5 mL of methanol and 2.5 mL of 2% NaCl were added to the remaining two phases in the tubes, shaken three times in 15 minutes and centrifuged again at 1500 × g for 7 minutes at room temperature. Once again three phases were separated and the same process was repeated discarding the upper phase, adding methanol and NaCl, shaking and centrifugating. At this point, the chloroformic phase at the bottom, containing the lipid extract, was taken and dried under reduced pressure at 37ºC for later lipid fraction isolation by thin layer chromatography (TLC).

10.2 *Fatty acids extraction in placental tissue*

Fatty acids from placental tissue were extracted following the method of Klingler *et al.* (231). Approximately 0.3-0.5g of placental tissue with the addition of 250µL of internal standard were homogenized and extracted into 12mL of chloroform/methanol (2:1) plus 5g/L of butylated hydroxytoluene (BHT). The homogenate was incubated at 35ºC for 20 minutes and filtered into new tubes using Whatman nº5 filter paper.

Following this, 4mL of KCl 0.1M were added to the new tubes and centrifugation at 1500 × g for 30 minutes at 10ºC was performed. After 20 minutes at room temperature, two phases were formed in the tubes: the upper watery one was thrown away and the unpolar remaining one was filtered and taken to dryness under reduced pressure at 37ºC for later lipid fraction isolation by TLC.

10.3 *Isolation and methylation of individual lipid fractions by thin layer chromatography*

The dried lipid extract was taken up in 400µL chloroform/methanol (1:1) for application on 20×20cm silica gel plates (Merck, Darmstadt, Germany). Individual lipid fractions were isolated by development of the plates in n-heptane/ diisopropylether/glacial acetic acid (60:40:3, by volume) (232) (Figure 5). Bands corresponding to PL, NEFA, TG and CE were developed under 254 nm ultraviolet light after spray a solution of ethanol:2',7'-dichlorofluorescein (0.2%). Each lipid fraction was identified by its constant retardation factor (Rf) value, described as the ratio of the migration distance of the substance to the migration distance of the solvent font. Lipid fractions of phospholipids, NEFA, triglycerides and cholesterol esters were delimited and scraped carefully.

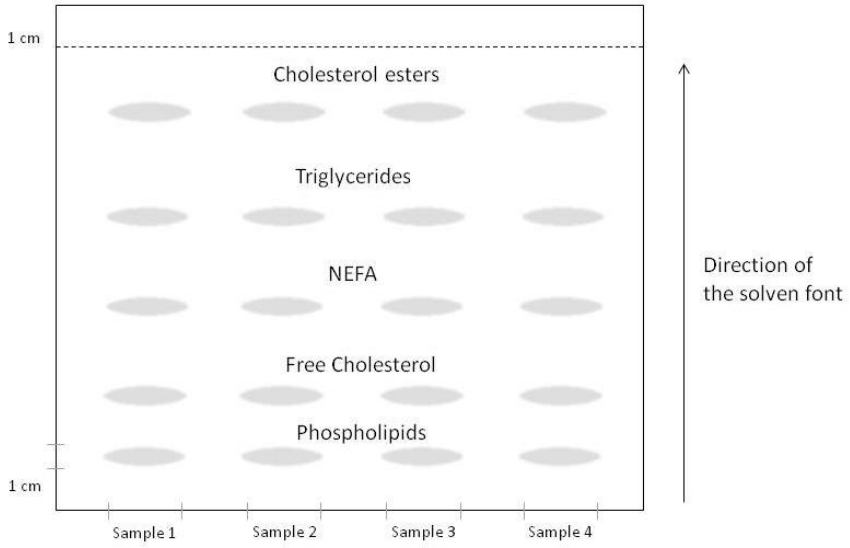


Figure 5. Illustration of a TLC plate with the bands of the different lipid fractions obtained.

From the individual fractions, FA methyl esters were synthesized in 1.5 mL of 3M methanolic HCl (Supelco, Bellfonte, PA, USA) by heating to 85°C for 45 min. After this time, the solution was buffered with sodium sulfat/ sodiumhydrogencarbonate/ sodiumcarbonate (2/2/1). Methyl esters were extracted twice into 1 mL of hexane. The final organic phases were taken to dryness under nitrogen flux, resuspended into 50 µL of hexane containing BHT as antioxidant and stored at -20ºC until gas chromatographic analysis.

10.4 *Chromatographic analysis of fatty acid methyl esters*

Quantification of fatty acids methyl esters was performed by gas chromatography on a Hewlett-Packard 5890 series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany), equipped with a BPX70 column (SGE, Weiterstadt, Germany) with 60 m length and 0.32 mm inner diameter (233).

Helium was used as carrier gas and the analysis conditions with respect to the program of temperatures were set at 130ºC as initial temperature kept during 0.5 min and then heated 3ºC/min until 150ºC, after that heated with a rate of 1.5ºC/min until 180ºC and finally 210ºC were reached by heating at 3ºC/min and it was kept during 23 minutes, falling to initial temperature (130ºC) with a rate of 15ºC/min (Figure 6).

Different fatty acids were identified by the retention time comparison with fatty acids standards supplied by Sigma-Aldrich Chemie (Frankfurt, Germany).

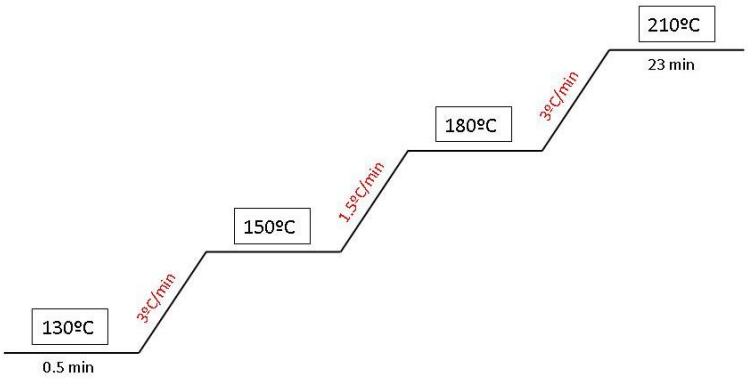


Figure 6: Gas chromatography analysis conditions with respect to the program of temperatures used for fatty acids methyl esters quantification.

11. TRACER ANALYSIS

The ^{13}C-enrichment of individual fatty acid methyl esters was measured by gas chromatography-combustion-isotope ratio mass spectrometry (GC-IRMS) (Hewlett-Packard GC interfaced to a Finningan MAT Delta S mass spectrometer, Bremen, Germany) (233).

To separate the fatty acids, the GC-IRMS was equipped with a DB 23 column (Fissons, Mainz, Germany) with 50 m length and 0.32 mm inner diameter. Temperature program started at 100ºC, rising until 160ºC at 4ºC/min and then reaching the temperature of 195ºC during 28 min with a rate of 2ºC/min (Figure 7). In the oven, at the end of the column, the catalytic combustion took place and the generated CO_2 was headed for the mass spectrometer ion source.

Before and after the peaks of CO_2 generated by the fatty acids, the equipment was calibrated with a CO_2 reference sample with a known isotopic composition.

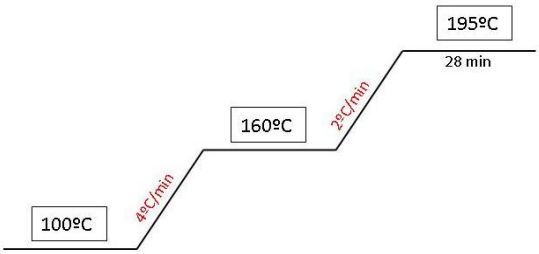


Figure 7: GC-IRMS analysis conditions with respect to the program of temperatures used for measuring the fatty acids methyl esters ^{13}C-enrichment.

11.1 *Expression of results*

The amount of ^{13}C in one substance is expressed as the $\delta^{13}C$ value related to the international standard Pee Dee Belemnite (PDB) (CO_2 obtained from the carbonic shell of the cretaceous mollusc *Belmnitella americana*, from the Pee Dee formation of South Carolina (USA) (234)). $\delta^{13}C$ value is calculated by the formula:

$$\delta^{13}C\ (‰) = [(^{13}C/^{12}C \text{ sample} - {}^{13}C/^{12}C \text{ standard}) / {}^{13}C/{}^{12}C \text{ standard}] * 1000$$

The $\delta^{13}C$ (‰) value was transformed in atom percent (AP) of ^{13}C by the following equation:

$$AP\ (\%) = [100 \times R(0.001\ \delta^{13}C‰ \text{ sample} + 1)] / [1 + R(0.001\ \delta^{13}C‰ \text{ sample} + 1)]$$

R = $^{13}C/^{12}C$ ratio of the PBD international reference (R = 0.0112372).

The AP of the samples after tracer ingestion can be transformed in atom percent excess (APE) by the formula:

$$APE\ (\%) = AP \text{ sample} - AP \text{ basal sample}$$

The ^{13}C atom percent excess (APE) represents the ^{13}C-enrichment (the percentage contribution of tracer ^{13}C to total carbon) (235).

The concentration of the labelled fatty acids (μmol ^{13}C /L or nmol ^{13}C /g) was calculated by multiplying the absolute concentrations of the fatty acids obtained by gas chromatography by their ^{13}C-enrichment (APE values) obtained by GC-IRMS.

From maternal plasma, the area under the tracer concentration curve (AUC; μmol $^{13}C \cdot h/L$) was calculated by integrating the measured tracer concentration until delivery over time according to the trapezoidal rule. AUC of tracer concentration instead of tracer concentration at the time of delivery was chosen because it is considered a better proxy of tracer available for transfer than the concentration at a single time point, even though the limited number of sampling time points possible may not allow describing the true AUC from tracer intake until delivery.

The tracer fatty acids from the mother that go to the fetus was estimated as the tracer concentration ratio in venous cord blood (μmol $^{13}C/L$) relative to the area under the tracer concentration curve (AUC; μmol $^{13}C/L$) in the maternal plasma at delivery.

$$\frac{\text{Venous cord plasma}}{\text{Maternal plasma}} = \frac{\text{fatty acid amount in venous cord plasma (μmol/L)*APE } ^{13}\text{C-cord plasma}}{\text{fatty acid AUC amount in maternal plasma (μmol/L)*APE } ^{13}\text{C-maternal plasma}} \times 100$$

The tracer fatty acid uptake from the placenta was calculated as the tracer concentration ratio in placenta (nmol $^{13}C/g$) relative to the area under the tracer concentration curve (AUC; μmol $^{13}C/L$) in the maternal plasma at delivery. The ratio using concentration gives information on how much ^{13}C-FA from the mother goes to the fetus. In contrast, enrichment ratio indicates how much ^{13}C-FA from the fetus comes from the mother. Both information are complementary but not identical.

$$\frac{\text{Placenta}}{\text{Maternal plasma}} = \frac{\text{fatty acid amount in placenta (nmol/g)*APE } ^{13}\text{C-placenta}}{\text{fatty acid AUC amount in maternal plasma (μmol/L)* APE } ^{13}\text{C-maternal plasma}} \times 100$$

The enrichment ratio of ^{13}C-FA between compartments gives additional information respect to concentration ratio.

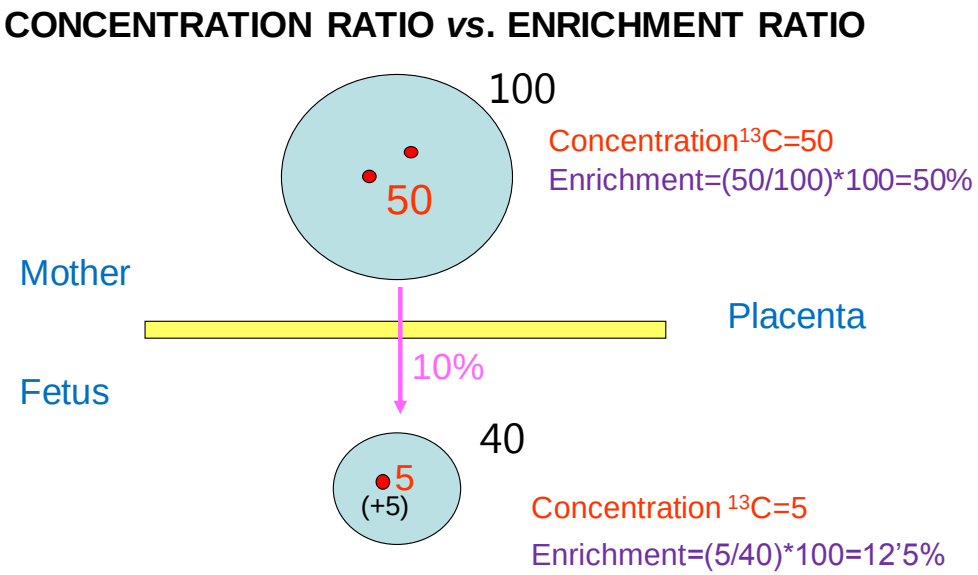
$$\frac{\text{Placenta enrichment}}{\text{Maternal plasma enrichment}} = \frac{\text{APE } ^{13}\text{C-placenta}}{\text{APE } ^{13}\text{C-maternal plasma}} \times 100$$

$$\frac{\text{Venous cord enrichment}}{\text{Maternal plasma enrichment}} = \frac{\text{APE } ^{13}\text{C-venous cord}}{\text{APE } ^{13}\text{C-maternal plasma}} \times 100$$

To try to explain the differences between enrichment or concentration ratios, an example is provided in the Figure 8. For instance, if we have 50 molecules of ^{13}C in a maternal pool of 100 carbon molecules, the ^{13}C-enrichment in the mother would be 50%.

If we estimate a transfer rate to the fetus of for example 10%, that means that 5 molecules of ^{13}C and 5 molecules of ^{12}C will be transferred to the fetal pool of a total size of for example 40 carbon molecules. Thus, we may find in the fetus a ^{13}C-concentration of 5 while the ^{13}C-enrichment in the fetus would be 12.5% (5/40).

If we calculate the concentration ratio (5/50=10%), that means that 10% of the ^{13}C-FA from the mother goes to the fetus; this coincides with the transfer rate for the process suggested initially. On the other hand, if we use the enrichment ratio (12.5/50=25%), that means that 25% of the ^{13}C-FA in the fetus comes from the mother; we can see in the figure that the fetus has received 10 molecules of carbon (5 ^{13}C + 5 ^{12}C) incorporated to a pool of 40 molecules of carbon (10/40=25%) which coincides with the enrichment ratio.



Concentration Ratio = 5/50 =10% (**10% from the mother goes to the fetus**)

Enrichment Ratio = 12.5/50 =25% (**25% from the fetus comes from the mother**)
In fact (5+5)/40=25%

Figure 8. Graphic explanation of the different information obtained if using concentration ratio or enrichment ratio.

12. STATISTICAL ANALYSIS

Results were expressed as mean ± SEM. The comparisons between GDM and control groups were assessed by Student-T test analysis. The ^{13}C-fatty acid concentration in maternal plasma lipid fractions (AUC) was correlated with its total concentration in placenta or cord plasma by Pearson's correlations. The significance level was set to $P < 0.05$. All analyses were performed with the statistical software SPSS, version 15.0 (SPSS, Chicago, IL).

IV. RESULTS

13. DESCRIPTION OF THE SUBJECTS

Anthropometric characteristics of the subjects the day before the caesarean section were reported in Table 3. The length of gestation was significantly shorter in GDM than in controls because of a trend to schedule caesarean sections earlier in GDM to avoid potential complications of infant macrosomia.

Table 3: *Anthropometric characteristics of the subjects*

	Control (n = 11)	GDM (n = 9)	*P*
Age (years)	33.00 ± 1.26	34.50 ± 1.25	0.408
Weight (Kg)	78.03 ± 2.64	84.50 ± 2.24	0.078
Height (cm)	160.90 ± 1.81	163.10 ± 2.77	0.515
BMI (Kg/m^2)	30.12 ± 0.81	31.90 ± 1.07	0.202
Glucose (mg/dl)	70.33 ± 3.29	74.40 ± 4.60	0.484
Gestational Age (weeks)	39.8 ± 0.42	38.19 ± 0.19	**0.004**
Placental Weight (g)	617.00 ± 40.69	648.00 ± 38.20	0.585

Results are expressed as mean ± SEM. BMI, body mass index. Significantly different at $P < 0.05$

Glucose levels were no different between groups at the time of delivery, which could be explained by a well managed glucose control in GDM subjects either by diet treatment or by insulin administration. Moreover, insulin levels at delivery in GDM subjects did not differ between women treated only by diet (n = 3) and those with insulin treatment (n = 6) (17.43 ± 4.64 *vs.* 16.43 ± 4.67 µU/ml respectively) probably because of the low number of subjects; similar results were found for the homeostasis model of assessment (HOMA) for insulin resistance (3.58 ± 1.27 *vs.* 3.05 ± 0.93 respectively).

Ecographical measurements and fetal biometry the day before the caesarean section did not differ between control subjects and GDM with all the results of the fetal and Doppler scanning being according normal range for all the subjects (227-228) (Table 4).

Table 4: *Ecographical measurements and fetal biometry of the subjects the day before the caesarean section.*

	Control (n = 11)	GDM (n = 9)
Biparietal diameter (percentile)	39.27 ± 9.51	59.95 ± 10.50
Head circumference (percentile)	22.33 ± 4.15	39.05 ± 11.47
Abdominal circumference (percentile)	51.65 ± 15.24	56.58 ± 11.91
Femur lenght (percentile)	39.08 ± 10.3	36.54 ± 12.18
Estimated fetal weight (g)	3390 ± 193.59	3368.8 ± 150.4
Umbilical artery pulsatility index (percentile)	39.67 ± 7.75	56.5 ± 10.14

Results are expressed as mean ± SEM. *Significantly different at P <0.05

The anthropometrical measurements of the neonates were not significantly different between GDM and controls (Table 5) probably due the relatively low number of participants. All the newborns were healthy born at term and umbilical pH resulted in a range of values which indicates fetal well-being at birth.

Table 5: *Anthropometric and biochemical characteristics of the neonates*

	Control (n = 11)	GDM (n = 9)	*P*
Birth Weight (g)	3395 ± 149.78	3362 ± 101	0.857
Birth Weight z-score	0.42 ± 0.23	0.88 ± 0.33	0.262
Birth Length (cm)	49.70 ± 0.93	49.50 ± 0.4	0.847
Birth Length z-score	0.05 ± 0.28	0.17 ± 0.27	0.756
Cephalic Circumference (cm)	34.70 ± 0.39	35.00 ± 0.5	0.641
Abdominal Circumference (cm)	34.50 ± 0.96	33.44 ± 0.53	0.363
Cord Artery pH	7.29 ± 0.01	7.26 ± 0.03	0.357
Cord Vein pH	7.35 ± 0.02	7.30 ± 0.03	0.110

Results are expressed as mean ± SEM. Significantly different at P <0.05.

14. FATTY ACID COMPOSITION IN MATERNAL PLASMA, PLACENTA AND CORD PLASMA

Table 6 shows the concentration of total fatty acids in maternal plasma, umbilical vein plasma and placental tissue considering together labelled and unlabelled fatty acids. Fatty acid concentration in maternal plasma was calculated as the mean value of fatty acids in all collected maternal blood samples analyzed by GC. As expected, the concentration of each fatty acid was higher in maternal plasma than in fetal plasma. Moreover, attending to the four fatty acids under following (16:0, 18:1 n-9, 18:2 n-6 and 22:6 n-3), there is a slight trend to higher concentrations in maternal plasma of GDM women respect to control subjects (Table 6), in agreement with the maternal hyperlipidemia attributed to this pathology. It can be also observed a trend to lower DHA concentration in venous cord plasma of the GDM group comparing with controls even with similar levels of this fatty acid in maternal plasma of their mothers (Table 6).

Table 6: *Fatty acid concentration in total lipids of maternal plasma, placental tissue and venous cord plasma*

	Maternal Plasma (mg/L)		Venous Cord Plasma (mg/L)		Placenta (µg/g)	
	Control (n=11)	**GDM (n=9)**	**Control (n=11)**	**GDM (n=9)**	**Control (n=11)**	**GDM (n=9)**
14:0	35.36 ± 3.66	33.11 ± 3.09	7.89 ± 0.66	6.80 ± 0.65	31.40 ± 1.90	23.00 ± 2.25*
14:1 t	0.12 ± 0.04	0.05 ± 0.03	0.18 ± 0.09	0.02 ± 0.01	0.36 ± 0.04	1.70 ± 0.29*
14:1	1.06 ± 0.32	0.24 ± 0.11*	0.25 ± 0.13	0.02 ± 0.02	0.39 ± 0.17	0.51 ± 0.19
15:1	0.30 ± 0.22	1.28 ± 0.18*	0.16 ± 0.12	0.48 ± 0.09	0.98 ± 0.46	3.11 ± 0.30*
16:0	1094.05 ± 50.62	1154.56 ± 67.84	297.80 ± 16.89	291.98 ± 18.92	1865.75 ± 69.58	1778.20 ± 62.10
16:1 t	1.93 ± 0.52	2.19 ± 0.28	0.37 ± 0.19	0.51 ± 0.08	15.84 ± 4.06	20.08 ± 1.02
16:1n-7	69.10 ± 6.20	63.02 ± 5.55	28.89 ± 2.59	34.70 ± 2.87	30.99 ± 2.21	32.42 ± 1.58
17:0	11.39 ± 0.63	11.67 ± 0.64	3.47 ± 0.38	2.34 ± 0.17*	28.06 ± 0.96	23.78 ± 1.37*
18:0	270.40 ± 15.22	248.75 ± 16.69	148.69 ± 16.19	111.26 ± 6.77	962.88 ± 41.78	825.02 ± 37.48*
18:1 t	6.04 ± 0.66	4.39 ± 0.55	0.75 ± 0.09	0.61 ± 0.05	8.94 ± 0.79	9.61 ± 1.06
18:1n-9	893.88 ± 58.67	1096.47 ± 64.08*	155.96 ± 12.18	193.26 ± 18.38	672.77 ± 27.43	662.27 ± 33.80
18:1n-7	66.07 ± 3.11	70.23 ± 3.55	26.58 ± 1.77	30.79 ± 2.06	112.24 ± 6.52	113.85 ± 5.03
18:2 tt	1.26 ± 0.23	1.43 ± 0.24	0.56 ± 0.08	0.39 ± 0.05	4.53 ± 0.42	5.16 ± 0.45
18:2n-6	1167.84 ± 67.59	1299.98 ± 104.69	105.67 ± 7.58	115.30 ± 12.12	774.23 ± 43.31	743.31 ± 36.24
18:3n-6	8.31 ± 0.74	10.1 ± 0.89	2.80 ± 0.34	3.56 ± 0.42	7.57 ± 0.35	8.79 ± 0.53
18:3n-3	11.78 ± 1.07	13.96 ± 1.28	0.44 ± 0.09	0.67 ± 0.11	3.21 ± 0.28	2.58 ± 0.21
20:0	10.75 ± 0.74	10.31 ± 0.78	6.72 ± 0.66	5.44 ± 0.50	23.28 ± 0.87	19.11 ± 0.83*
18:4n-3	0.68 ± 0.15	0.95 ± 0.09	0.20 ± 0.04	0.26 ± 0.04	2.42 ± 0.28	3.29 ± 0.27*
20:1n-9	9.44 ± 0.66	11.96 ± 0.99*	0.67 ± 0.09	1.57 ± 0.18*	13.81 ± 0.54	14.89 ± 0.94
20:2n-6	12.60 ± 0.60	12.28 ± 1.18	2.39 ± 0.13	2.30 ± 0.18	35.98 ± 2.46	33.25 ± 2.25
20:3n-9	3.55 ± 0.38	3.97 ± 0.36	3.84 ± 0.65	5.16 ± 0.61	8.40 ± 0.77	8.28 ± 0.61
20:3n-6	62.25 ± 3.47	63.92 ± 5.74	29.10 ± 1.82	28.65 ± 1.92	329.30 ± 20.82	318.00 ± 12.67
20:4n-6	253.61 ± 15.28	277.56 ± 17.82	134.96 ± 9.60	142.74 ± 10.79	1540.48 ± 69.59	1472.68 ± 81.54
20:3n-3	1.34 ± 0.31	2.1 ± 0.28	0.61 ± 0.16	0.63 ± 0.08	3.68 ± 1.02	14.14 ± 1.52*
22:0	27.22 ± 3.78	20.34 ± 2.13	11.59 ± 1.85	7.86 ± 0.70	99.82 ± 4.59	88.47 ± 5.89
22:1 t	0.96 ± 0.12	1.26 ± 0.19	0.18 ± 0.04	0.07 ± 0.01*	3.06 ± 0.42	6.35 ± 0.64*
20:5n-3	13.24 ± 3.11	15.8 ± 3.07	1.76 ± 0.38	2.08 ± 0.31	10.21 ± 2.52	9.94 ± 1.74
22:2n-6	1.42 ± 0.30	9.34 ± 1.26*	0.44 ± 0.09	1.42 ± 0.19*	4.51 ± 0.32	17.22 ± 0.84*
22:4n-6	8.61 ± 0.47	9.17 ± 0.87	5.89 ± 0.46	6.37 ± 0.65	114.18 ± 4.98	92.18 ± 4.40*
24:0	17.75 ± 0.96	11.3 ± 0.73*	6.47 ± 0.76	8.24 ± 0.80	72.38 ± 5.80	102.32 ± 7.16*
22:5n-6	11.23 ± 0.97	12.78 ± 1.42	9.58 ± 0.72	6.82 ± 0.44*	137.61 ± 10.68	59.74 ± 4.34*
24:1n-9	40.39 ± 2.55	41.36 ± 3.33	14.24 ± 0.81	15.64 ± 1.98	88.97 ± 5.07	82.37 ± 5.05
22:5n-3	11.26 ± 1.20	9.83 ± 1.16	3.35 ± 0.39	1.83 ± 0.21*	45.97 ± 3.44	41.42 ± 3.40
22:6n-3	101.92 ± 8.82	104.1 ± 7.01	40.88 ± 4.38	34.27 ± 2.40	406.78 ± 29.68	335.95 ± 24.10
Total FA	4230.83 ± 171.39	4646.00 ± 274.66	1055.28 ± 65.86	1076.79 ± 75.22	7470.41 ± 287.49	6287.58 ± 743.29
Saturated FA	1466.91 ± 67.50	1490.05 ± 86.90	482.64 ± 33.65	433.92 ± 26.36	3083.58 ± 109.23	2573.92 ± 302.13
MUFA	1083.95 ± 67.77	1300.79 ± 73.08*	228.68 ± 16.29	289.21 ± 24.66*	929.57 ± 38.11	830.35 ± 100.10
Trans FA	10.33 ± 1.35	9.32 ± 0.96	2.04 ± 0.32	1.60 ± 0.17	32.75 ± 4.36	38.61 ± 4.96
PUFA	1669.63 ± 73.03	1845.84 ± 134.99	341.92 ± 20.44	352.06 ± 26.53	3424.51 ± 147.48	2844.69 ± 343.90
LC-PUFA	481.03 ± 22.50	520.85 ± 33.55	232.81 ± 14.48	232.26 ± 15.63	2637.08 ± 122.22	2162.51 ± 262.02
LC-PUFA n-6	349.71 ± 18.04	385.05 ± 25.59	182.37 ± 11.47	188.29 ± 13.39	2162.05 ± 96.18	1793.76 ± 215.78
LC-PUFA n-3	127.77 ± 11.73	131.83 ± 10.77	46.60 ± 4.56	38.81 ±2.79	466.62 ± 32.33	361.30 ± 46.82

Results are expressed as mean ± SEM. FA, fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acids; LC-PUFA, long chain polyunsaturated fatty acids; GDM, gestational diabetes mellitus. *Statistically significant difference between GDM and controls at P <0.05

Respect to the lipid fractions, the concentration of all studied fatty acids in maternal plasma triglycerides tended to be higher in the GDM group compared with those of controls; hence, the maternal hyperlipidemia in GDM was mainly due to the maternal hypertriglyceridemia (Table 7). Furthermore, all fatty acids in the placental tissue were mainly esterified in phospholipids fraction, which represents the major lipid fraction in this tissue (Table 7). The concentrations of the related four fatty acids and even for total fatty acids were significantly reduced in NEFA fraction of GDM comparing with control placentas.

Table 7: *Fatty acid concentration in lipid fractions of maternal plasma, placental tissue and venous cord plasma*

	Maternal Plasma (mg/L)		Venous Cord Plasma(mg/L)		Placenta (µg/g)	
	Control (n=11)	**GDM (n=9)**	**Control (n=11)**	**GDM (n=9)**	**Control (n=11)**	**GDM (n=9)**
Palmitic Acid (16:0)						
PL	529.47 ± 19.45	500.14 ± 24.22	172.12 ± 6.89	172.26 ±10.06	1639.44 ± 67.72	1618.95 ± 59.39
TG	391.03 ± 29.02	476.84 ± 44.44	54.35 ± 9.14	49.45 ± 10.56	52.17 ± 4.67	52.57 ± 3.81
NEFA	50.49 ± 4.29	41.87 ± 3.22	21.06 ± 3.63	12.17 ± 0.81*****	142.67 ± 22.86	83.00 ± 4.60*****
CE	123.05 ± 7.02	135.71 ± 10.90	50.27 ± 3.89	58.10 ± 5.28	31.47 ± 2.49	23.69 ± 2.19*****
Oleic Acid (18:1 n-9)						
PL	155.19 ± 9.47	152.27 ± 6.76	41.79 ± 2.36	47.74 ± 4.62	548.43 ± 25.41	560.98 ± 29.32
TG	493.28 ± 44.14	670.72 ± 55.84*****	47.49 ± 9.60	48.16 ± 7.00	37.45 ± 3.42	43.95 ± 4.73
NEFA	61.26 ± 5.34	62.76 ± 8.01	5.47 ± 1.49	6.47 ± 1.12	53.45 ± 11.52	27.30 ± 3.14
CE	184.14 ± 12.74	210.71 ± 14.98	61.20 ± 4.12	90.89 ± 9.99*****	33.43 ± 3.23	30.04 ± 4.18
Linoleic Acid (18:2 n-6)						
PL	339.45 ± 16.24	341.92 ± 23.17	40.86 ± 2.55	37.91 ± 2.57	622.08 ± 40.89	636.84 ± 32.85
TG	271.01 ± 30.11	318.54 ± 32.89	21.18 ± 3.63	18.61 ± 3.61	25.30 ± 2.27	30.11 ± 3.47
NEFA	29.82 ± 2.16	27.81 ± 2.85	3.65 ± 0.96	4.08 ± 0.39	55.50 ± 11.44	23.57 ± 2.24*****
CE	527.56 ± 35.69	611.71 ± 59.99	39.97 ± 2.83	54.71 ± 7.53	71.35 ± 9.21	52.79 ± 6.29
Docosahexaenoic Acid (22:6 n-3)						
PL	83.47 ± 6.73	79.58 ± 4.08	31.94 ± 3.04	26.42 ± 1.63	361.19 ± 24.32	314.00 ± 22.52
TG	8.96 ± 1.78	13.42 ± 2.64	5.56 ± 1.03	4.16 ± 0.62	15.09 ± 3.10	12.22 ± 1.42
NEFA	1.35 ± 0.27	1.52 ± 0.26	0.63 ± 0.27	0.46 ± 0.06	26.43 ± 6.98	7.41 ± 1.00*****
CE	8.15 ± 0.71	9.57 ± 0.77	2.75 ± 0.34	3.24 ± 0.52	4.06 ± 0.51	2.32 ± 0.37*****
Total Fatty acids						
PL	1699.87 ± 59.58	1660.15 ± 83.80	584.24 ± 27.56	581.97 ± 38.61	6446.91 ± 268.66	6362.12 ± 267.81
TG	1349.06 ± 99.32	1686.48 ± 144	179.28 ± 30.80	160.46 ± 28.12	232.84 ± 23.82	219.50 ± 148.55
NEFA	190.11 ± 14.78	167.90 ± 15.32	59.32 ± 12.43	33.55 ± 2.66	596.45 ± 105.72	259.86 ± 18.26*****
CE	1020.38 ± 62.76	1131.47 ± 95.67	232.44 ± 15.82	300.81 ± 30.04	194.21 ± 19.21	144.72 ± 16.01

Results are expressed as mean ± SEM. PL, phospholipids; TG, triglycerides; NEFA, non-esterified fatty acids; CE, cholesterol esters. *Statistically significant differences between GDM and controls at P <0.05

The percentages of each fatty acid respect the total amount of fatty acids are usually used to evaluate the fatty acid profile of a sample as well as to compare the behaviour of the different fatty acids. Table 8 shows the percentage values of the four selected fatty acids in maternal plasma, placenta and umbilical vein plasma in both groups of subjects. The percentage of oleic acid was significantly higher in GDM than in controls in maternal plasma, placenta and venous cord plasma, while for the rest of fatty acids no differences were found.

Table 8: *Fatty acid percentages in total lipids of maternal plasma, placental tissue and venous cord plasma of the four studied fatty acids*

	Maternal Plasma (%)		Venous Cord Plasma(%)		Placenta (%)	
	Control (n=11)	**GDM (n=9)**	**Control (n=11)**	**GDM (n=9)**	**Control (n=11)**	**GDM (n=9)**
Palmitic Acid (16:0)	25.83 ± 0.36	24.90 ± 0.43	28.33 ± 0.47	27.25 ± 0.57	25.01 ± 0.27	25.52 ± 0.26
Oleic Acid (18:1 n-9)	21.02 ± 0.82	23.66 ± 0.88*****	14.76 ± 0.57	17.67 ± 0.66*****	9.02 ± 0.20	9.51 ± 0.39
Linoleic Acid (18:2 n-6)	27.68 ± 1.28	27.73 ± 0.65	10.06 ± 0.47	10.53 ± 0.58	10.37 ± 0.44	10.62 ± 0.18
Docosahexaenoic Acid (22:6 n-3)	2.40 ± 0.17	2.28 ± 0.14	3.81 ± 0.28	3.19 ± 0.10	5.40 ± 0.24	4.76 ± 0.19

Results are expressed as mean ± SEM. *Statistically significant difference between GDM and controls at P <0.05

15. LABELLED FATTY ACIDS IN MATERNAL PLASMA

At the time of delivery (0h), ^{13}C-DHA concentration was significantly lower in total lipids of maternal plasma in GDM than in controls, while for the other ^{13}C-FA no differences were found (Figure 9). Moreover, when we analyzed the clearance or area under the curve (AUC) in maternal plasma, ^{13}C-DHA AUC tended to be reduced in GDM mothers (P = 0.057), while ^{13}C-PA and ^{13}C-OA had significantly higher AUC in GDM than in controls (and ^{13}C-LA followed also the same trend) corresponding to lower maternal plasma clearance of these non-LC-PUFA (Table 9).

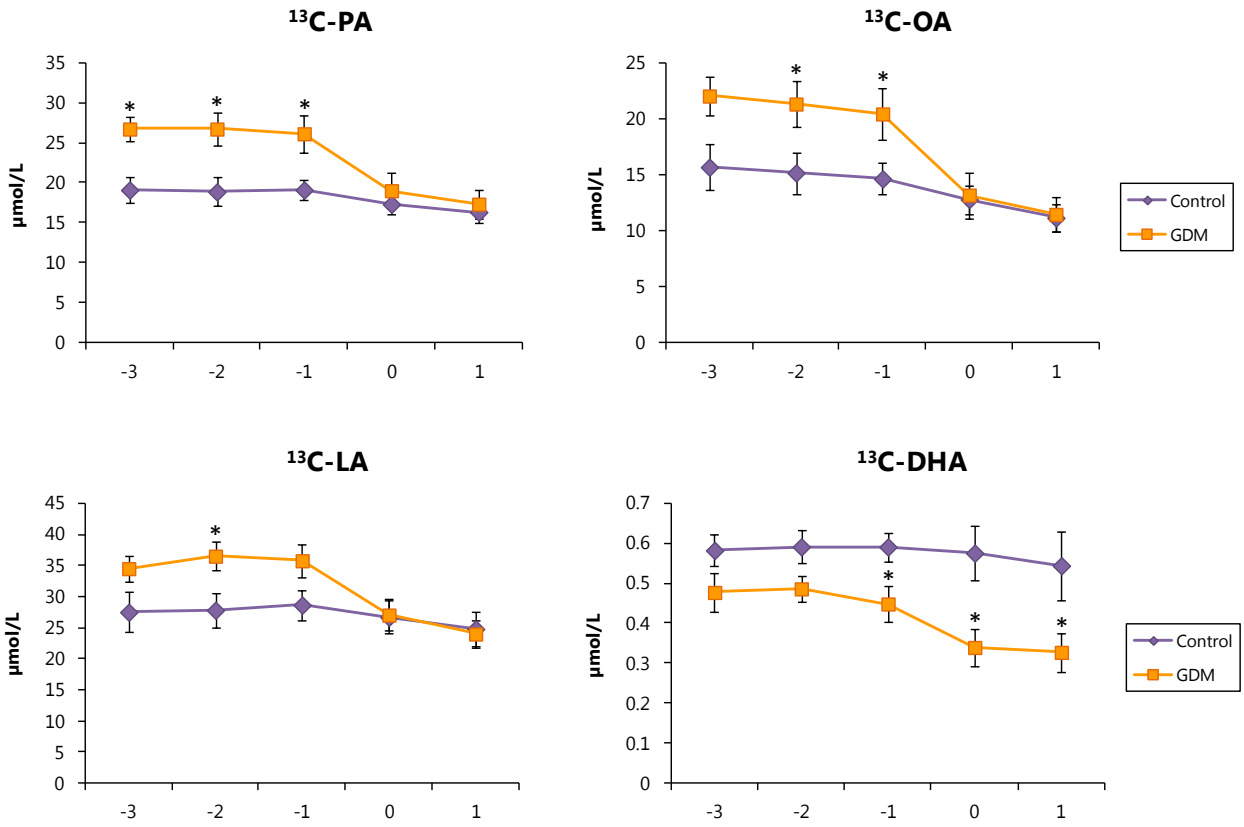


Figure 9. *Time course of ^{13}C-FA concentrations in maternal plasma total lipids during the time of study.* Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Time point 0 indicates the time of caesarean section. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Significant differences T-test with $P<0.05$.

Table 9: *Labelled fatty acids AUC concentration in total lipids of maternal plasma*

	Maternal Plasma AUC (µmol ·h/L)		
	Control	**GDM**	***P***
^{13}C-PA	142.07 ± 11.77	195.99 ± 12.88	**0.006**
^{13}C-OA	114.58 ± 13.94	158.56 ± 13.37	**0.038**
^{13}C-LA	207.26 ± 22.68	258.34 ± 16.14	0.096
^{13}C-DHA	4.38 ± 0.27	3.49 ± 0.33	0.057

Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9.

As commented in the material and methods, the concentration of the labelled fatty acids was calculated by multiplying the absolute concentrations of the unlabelled fatty acids by their ^{13}C enrichment values (APE values). Since in gestational diabetes there is a hyperlipidemia that could affect unlabelled fatty acid concentration respect to controls, we also include the ^{13}C enrichment in each compartment in both groups of subjects. We found similar enrichments for ^{13}C-PA, ^{13}C-OA, and ^{13}C-LA between GDM and controls (Table 10) but again, significantly lower ^{13}C enrichments for ^{13}C-DHA in maternal plasma. The lower ^{13}C-DHA concentration in maternal plasma was mainly due to its lower enrichment values in GDM subjects (Table 10), while the higher ^{13}C-FA concentration of non-LC-PUFA in GDM was mainly due to the maternal hyperlipidemia in these subjects.

Table 10: *^{13}C-enrichment of fatty acids (tracer/tracee ratio) in total lipids of each compartment*

		Maternal Plasma (Mean)	Placental Tissue	Venous Cord Plasma	Artery Cord Plasma
^{13}C-Palmitic acid	Control (n=11)	0.022 ± 0.002	0.006 ± 0.0004	0.003 ± 0.0002	0.003 ± 0.0002
	GDM (n=9)	0.026 ± 0.002	0.008 ± 0.0004*****	0.004 ± 0.0002	0.004 ± 0.0002*****
^{13}C-Oleic acid	Control (n=11)	0.021 ± 0.002	0.009 ± 0.0008	0.004 ± 0.0004	0.004 ± 0.0003
	GDM (n=9)	0.02 ± 0.002	0.01 ± 0.0008	0.005 ± 0.0003	0.005 ± 0.0003
^{13}C-Linoleic acid	Control (n=11)	0.029 ± 0.003	0.029 ± 0.0034	0.011 ± 0.0018	0.01 ± 0.0017
	GDM (n=9)	0.031 ± 0.003	0.033 ± 0.0026	0.012 ± 0.001	0.011 ± 0.001
^{13}C-Docosahexaenoic acid	Control (n=6)	0.019 ± 0.005	0.017 ± 0.005	0.004 ± 0.0005	0.004 ± 0.0006
	GDM (n=9)	0.005 ± 0.0003*****	0.003 ± 0.0003*	0.002 ± 0.0005*****	0.002 ± 0.0005*****

Results are expressed as mean ± SEM. *Statistically significant difference between groups at P <0.05

Concerning maternal plasma lipid fractions, the concentration of labelled non-LC-PUFA was again higher in maternal plasma triglycerides of GDM respect to controls, being the differences statistically significant for ^{13}C-PA and ^{13}C-OA, confirming that the maternal hyperlipidemia in GDM was mainly a hypertriglyceridemia (Figure 10). ^{13}C-DHA concentration tended to be lower in maternal plasma phospholipids and higher in triglycerides of GDM (Figure 10) which could influence its placental uptake. In fact, ^{13}C-DHA concentration tended to be lower in all maternal plasma lipid fractions of GDM subjects except in triglycerides, although the differences were not statistically significant (Figure 10). Moreover, all the ^{13}C-FA analyzed were less incorporated into NEFA in maternal plasma of GDM hence its AUC tended towards lower levels in GDM than controls (Figure 10).

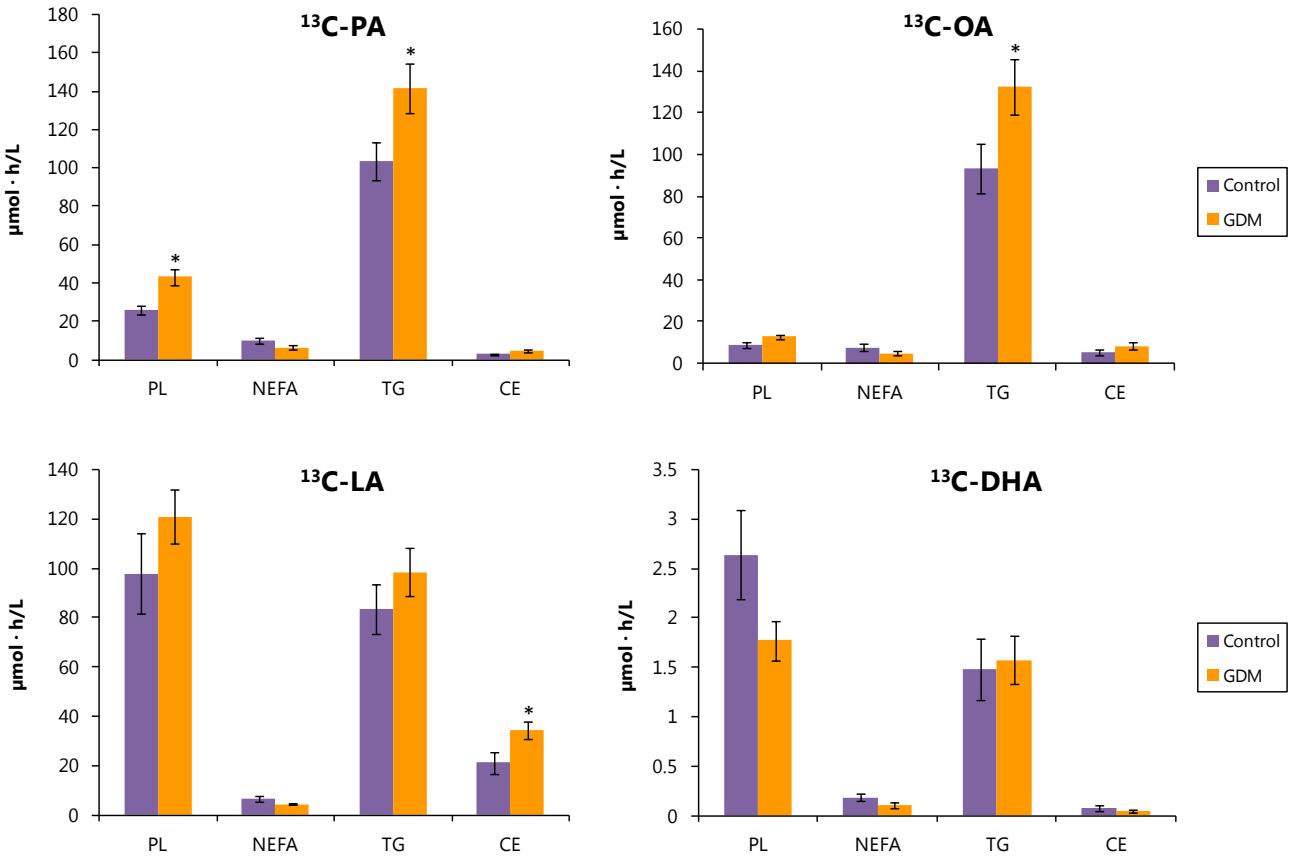


Figure 10: *Labelled fatty acids AUC concentration within the lipid fractions of maternal plasma*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid; PL, phospholipids; TG, triglycerides; NEFA, non-esterified fatty acids; CE, cholesterol esters. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at P <0.05

As expected, ^{13}C-FA enrichment in maternal lipid fractions followed the same pattern than ^{13}C-FA concentration, except to for maternal plasma triglycerides, which were mainly affected by GDM; ^{13}C-FA enrichment in maternal triglycerides tended to be lower for all studied fatty acids in the GDM group compared with the healthy group (Figure 11) but the higher concentrations of TG bound fatty acids related to the higher hypertriglyceridemia in GDM explains the higher concentration of non-LC-PUFA ^{13}C-FA in maternal plasma triglycerides (Figure 10).

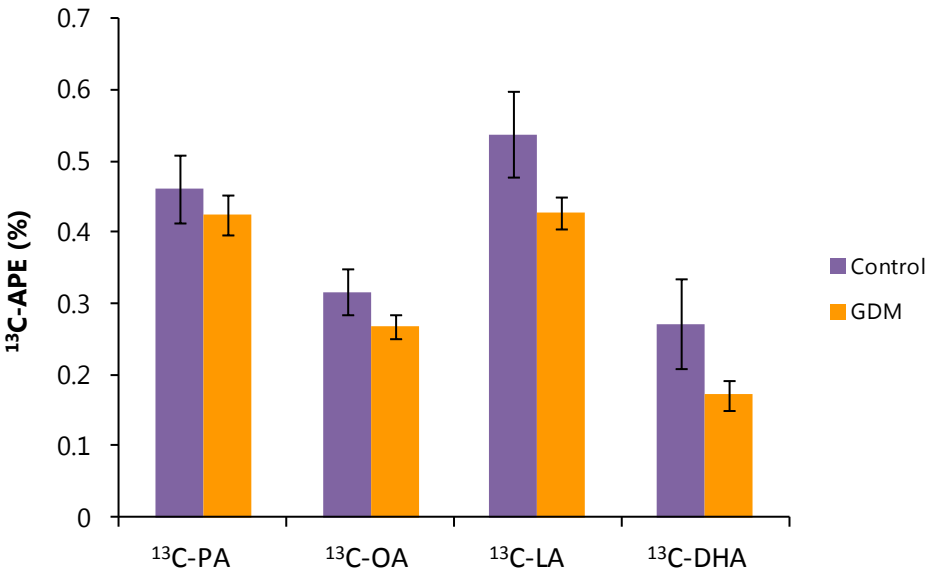


Figure 11: *^{13}C-enrichment of fatty acids (AUC) within the lipid fraction of triglycerides of maternal plasma*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at P <0.05

16. LABELLED FATTY ACIDS IN PLACENTAL TISSUE

In total lipids of placental tissue, we found significantly lower concentration of ^{13}C-DHA in GDM than in controls (Figure 12), and also significantly lower enrichment values (Table 10). For all other ^{13}C-FA, no significant differences were observed between groups, except for ^{13}C-PA which both concentration and enrichment were significantly higher in GDM (Figure 12 and Table 10).

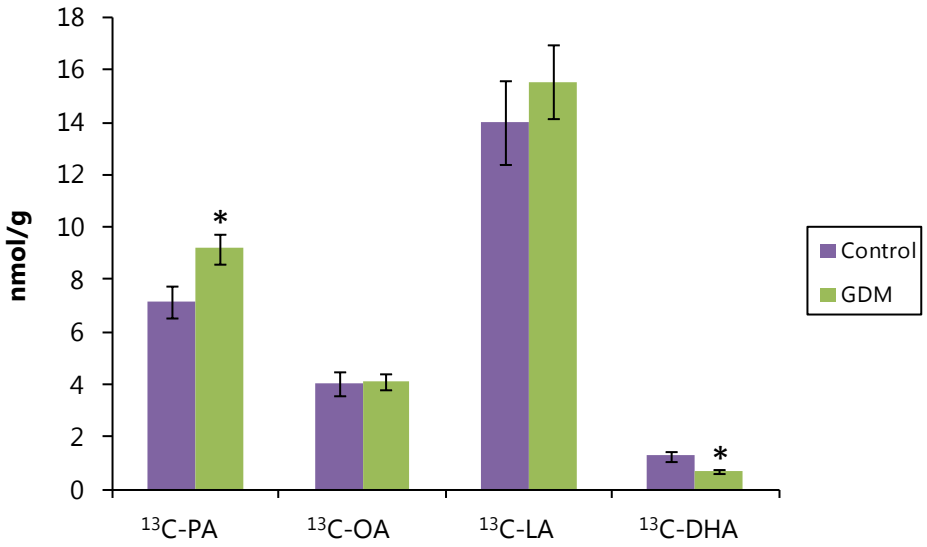


Figure 12. *Labelled fatty acids concentration in total lipids of placental tissue at the time of delivery*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at P <0.05

As expected, ^{13}C-FA were mostly found in the phospholipids of the placenta since this lipid fraction constitutes the bulk of lipids in placental tissue (80-90%). In agreement with maternal plasma, all the ^{13}C-FA analyzed tended towards lower concentration in placental NEFA in GDM than controls (Figure 13) indicating a hard reduction in all ^{13}C-FA levels in this lipid fraction of placental tissue in GDM women.

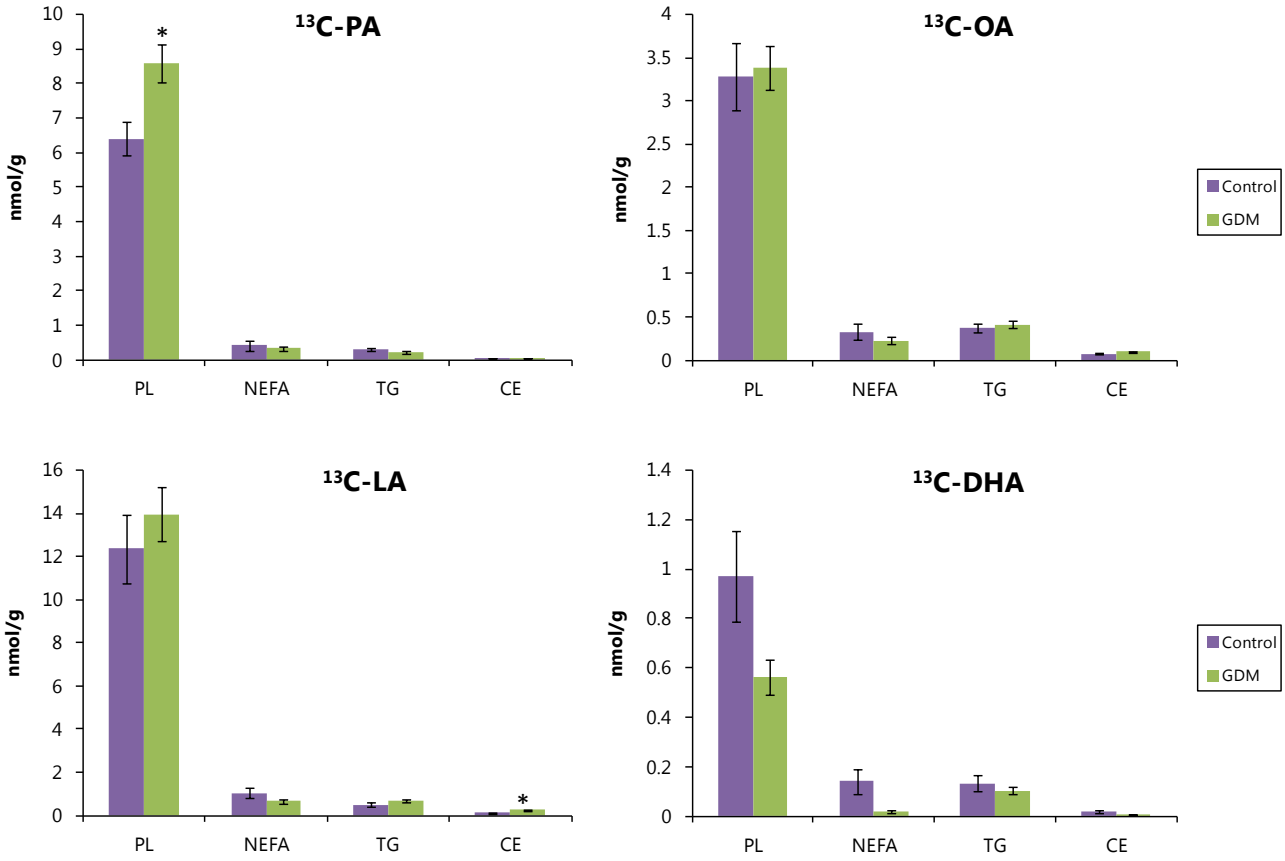


Figure 13: *Labelled fatty acids concentration within the lipid fractions of placenta*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid; PL, phospholipids; TG, triglycerides; NEFA, non-esterified fatty acids; CE, cholesterol esters. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at P <0.05

It is important to note that the enrichment values of non-LC-PUFA and specially ^{13}C-OA and ^{13}C-LA, were significantly higher in placental NEFA in GDM, but the low amount of unlabelled NEFA within the placenta in GDM made finally that these differences disappear (Figure 14). It is known that placentas of GDM store lipid droplets and thus NEFA in GDM could be esterified in triglycerides and incorporated to the lipid droplets of the placenta. As regards to ^{13}C-DHA, enrichments values tended also to be higher in triglycerides of GDM placenta like for the other fatty acids (Figure 14) in agreement with higher lipid droplets accumulation.

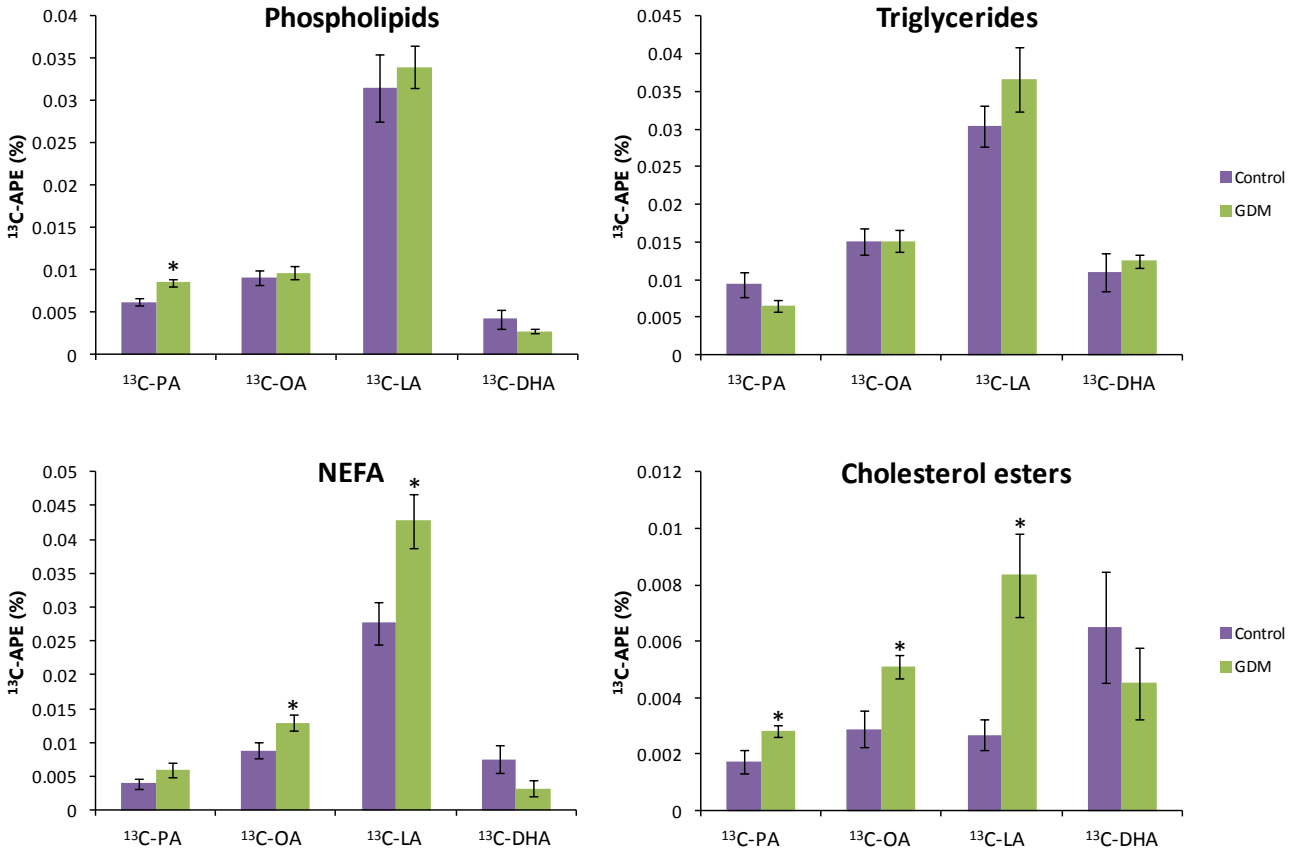


Figure 14: *^{13}C-enrichment of fatty acids within the lipid fractions of placental tissue*. Results are expressed as mean ± SEM. NEFA, non-esterified fatty acids; ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at P <0.05

17. LABELLED FATTY ACIDS IN CORD PLASMA

In total lipids of both venous cord blood (Figure 15) and arterial cord blood (Figure 16), we found lower concentration of ^{13}C-DHA in GDM than in controls, being statistically significant in venous cord plasma. For the rest of ^{13}C-FA, their concentrations tended to be higher in GDM, with a significant difference for ^{13}C-OA in both cord vessels (Figure 15, 16). The same pattern was observed for enrichments values in cord blood (Table 10) since there was not hyperlipidemia in venous or arterial fetal blood (results not shown).

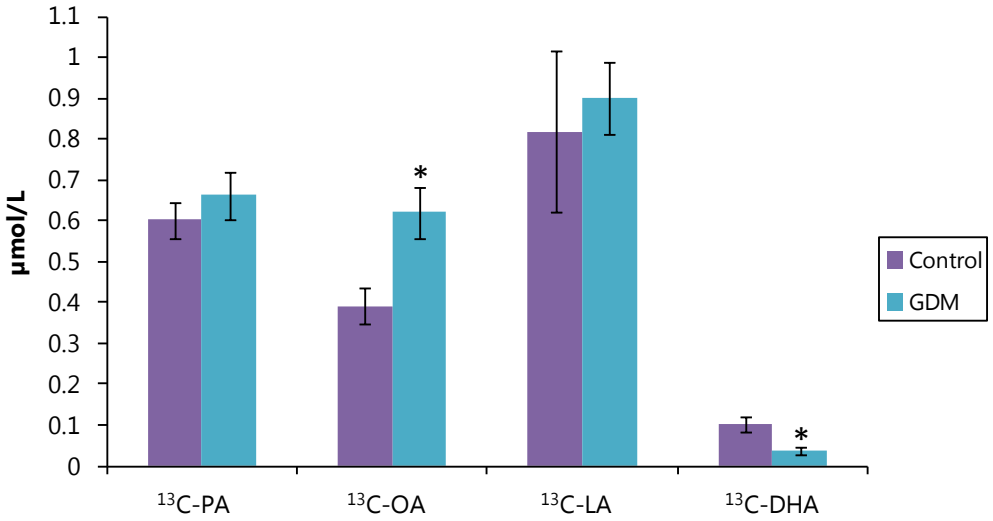


Figure 15. *Labelled fatty acids concentration in total lipids of venous cord plasma at the time of delivery*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at *P* <0.05

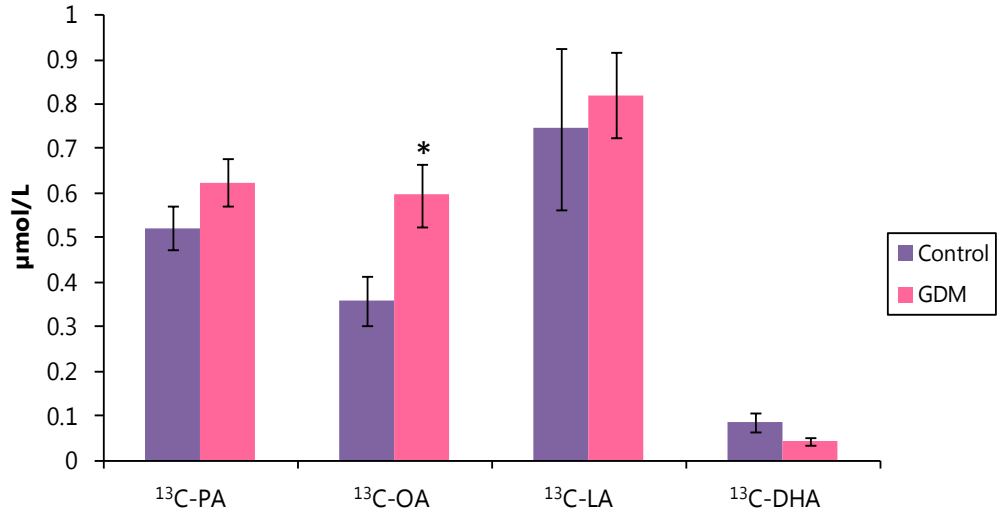


Figure 16. *Labelled fatty acids concentration in total lipids of arterial cord plasma at the time of delivery*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at *P* <0.05

In both venous and arterial cord plasma, ^{13}C-DHA tended to lower concentrations in all lipid fractions of GDM comparing with controls (Figure 17), showing significant differences in venous cord phospholipids (Figure 17) and arterial cord triglycerides (results not shown). It is interesting to see that, while ^{13}C-DHA decreased in cord phospholipids in GDM, the other polyunsaturated fatty acid analyzed in this study (^{13}C-LA) increased significantly in cord plasma of GDM (both in artery and vein). The lipid fraction of cholesterol esters showed the highest increase in ^{13}C-PA and ^{13}C-OA in cord plasma of the GDM group.

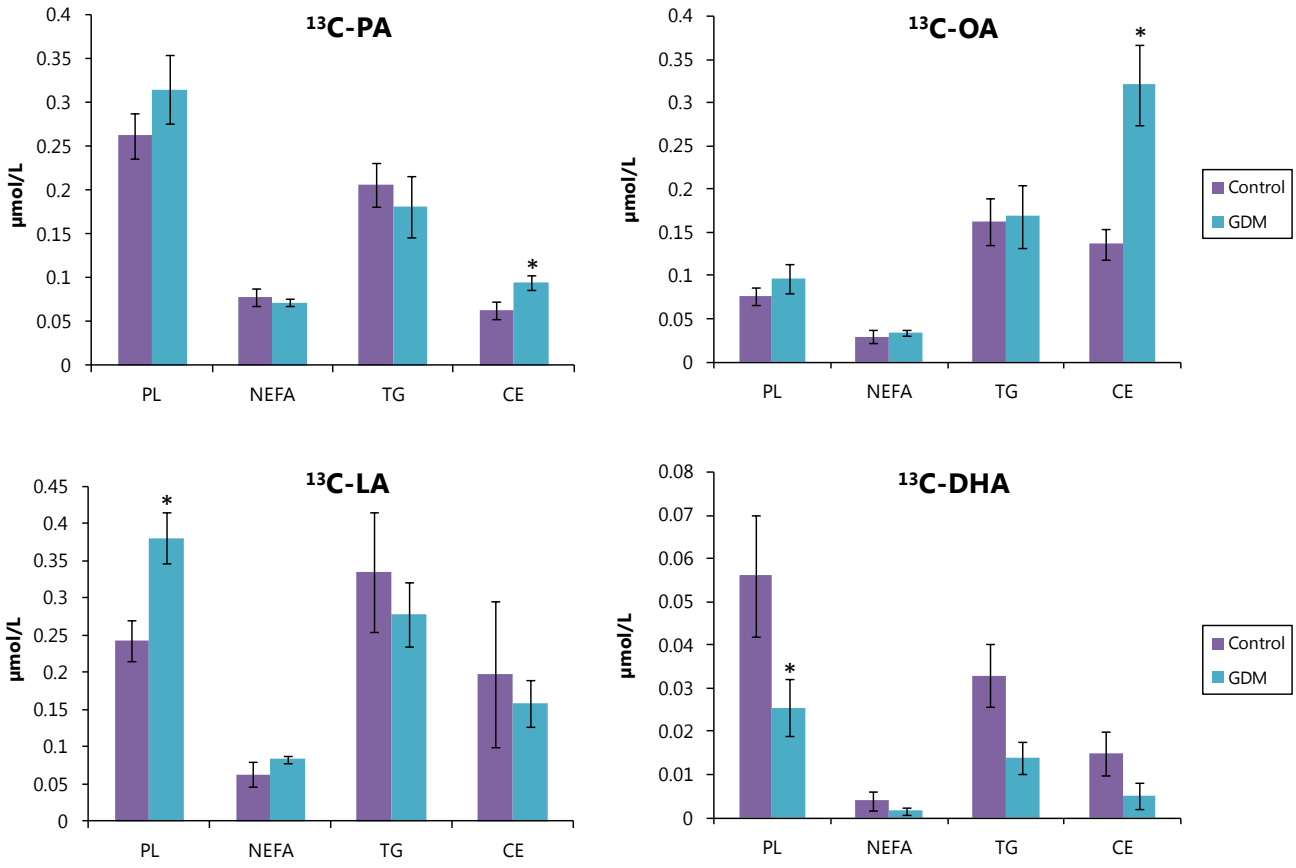


Figure 17: *Labelled fatty acids concentration within the lipid fractions of venous cord plasma*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid; PL, phospholipids; TG, triglycerides; NEFA, non-esterified fatty acids; CE, cholesterol esters. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at P <0.05

17.1 *Artery-venous differences*

As an estimation of fetal tissue fatty acid accretion, we compared ^{13}C-FA concentration in total lipids of umbilical artery and umbilical vein at birth, but no differences were observed in controls or in GDM patients (Figure 18). Thus, the distribution of labelled fatty acids in the lipid fractions of arterial and venous cord blood was very similar.

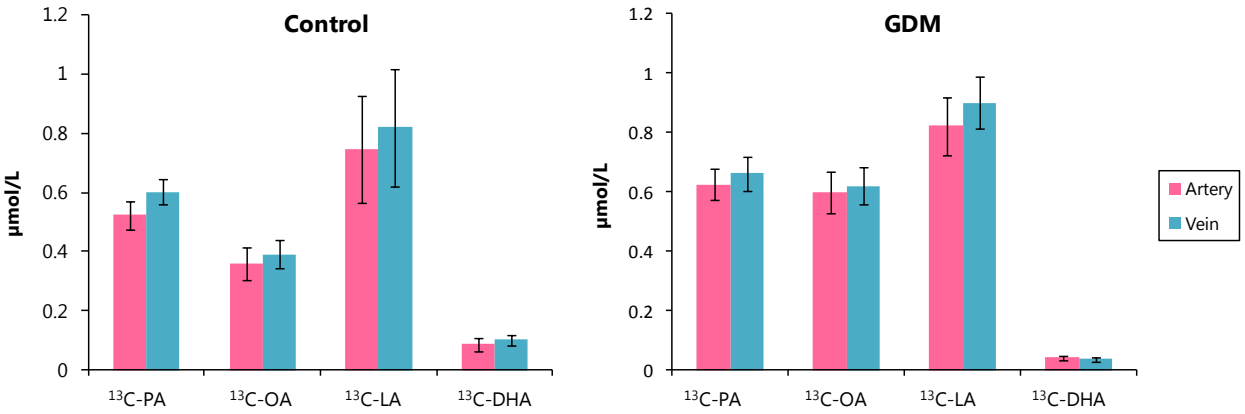


Figure 18: *Labelled fatty acids concentration in total lipids of arterial and venous cord plasma at the time of delivery in both control and GDM groups*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at P <0.05

Moreover, the umbilical artery and umbilical vein difference did not show any statistically significant difference (Figure 19). Maybe the time between the tracer intake and the caesarean section in the present study (12 h) was not enough to detect differences in the fatty acid uptake by fetal tissues.

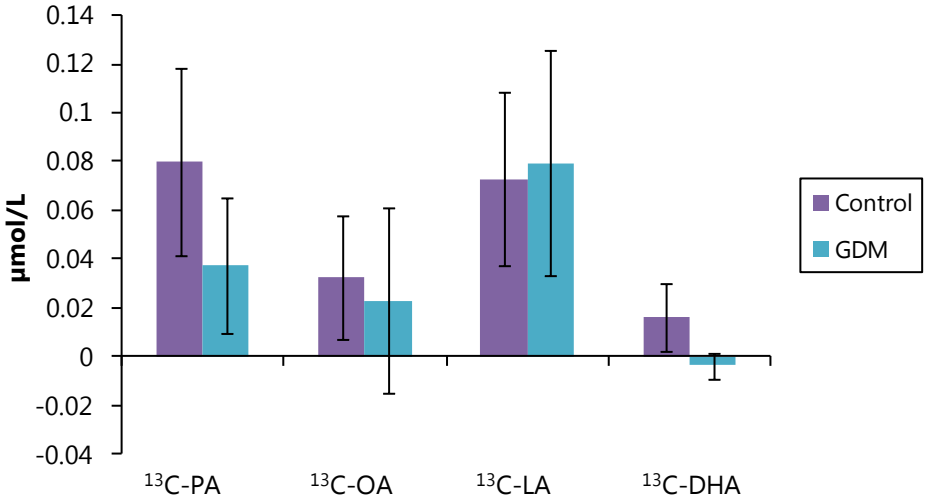


Figure 19: *Difference between labelled fatty acids concentration in total lipids of arterial and venous cord plasma at the time of delivery*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at P <0.05

18. PLACENTAL UPTAKE RATIO OF ^{13}C-FATTY ACIDS

To estimate the ratio of placental uptake of the different ^{13}C-FA, we calculated the ratio between the ^{13}C-FA concentration in placenta and the maternal plasma AUC concentration which tended towards lower ^{13}C-DHA accretion in GDM subjects (Figure 20). If we consider the overall control subjects (n = 11) to calculate this ratio (within each subject the dose of ^{13}C-DHA consumed by the mothers affected equally both compartments) GDM showed a statistically significant lower ratio of ^{13}C-DHA in placental tissue to maternal AUC than controls (P = 0.021). Thus, the placental uptake of ^{13}C-DHA was reduced in GDM subjects.

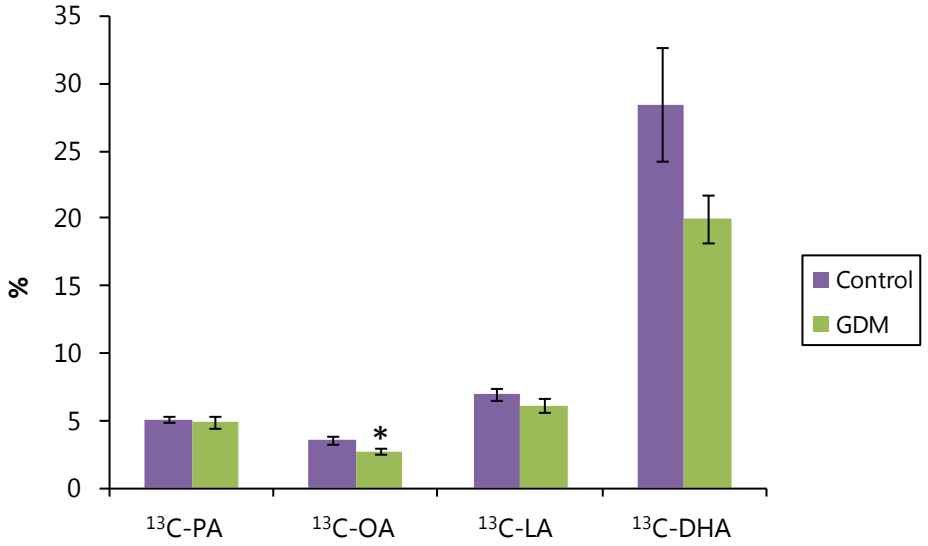


Figure 20: *Ratio (%) between placental concentration and maternal plasma AUC of ^{13}C-fatty acids*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at P <0.05

Similar results were obtained if the ratios from enrichment values were calculated (Figure 21). The ratio of ^{13}C-enrichment between compartments indicated how much tracer fatty acid in the second compartment comes from the former (see pag. 36-38 materials and methods). Thus not only less ^{13}C-DHA from the mother goes to the placenta (Figure 20), but in tendency less ^{13}C-DHA in the placenta comes from the mother in GDM (Figure 21).

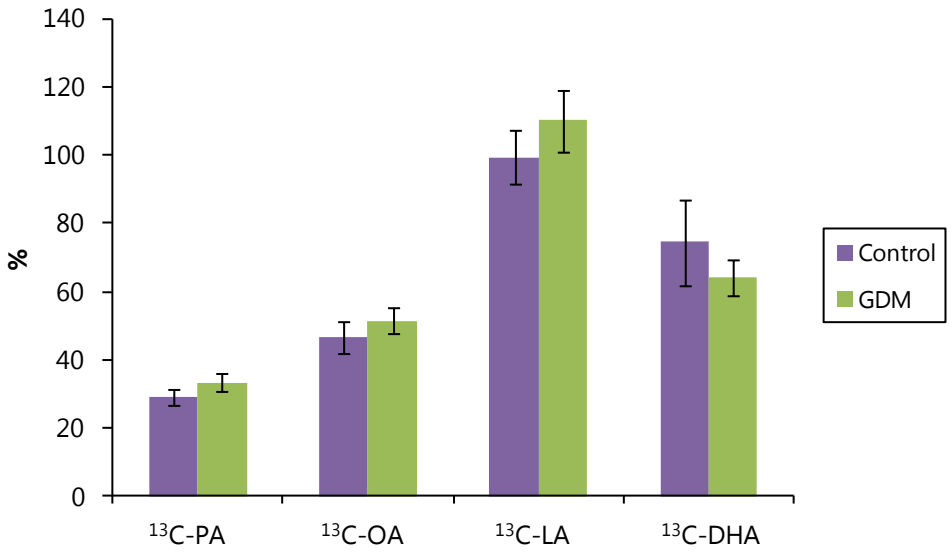


Figure 21: *Ratio (%) between placental and maternal plasma ^{13}C-enrichment values*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at P <0.05

19. MATERNAL-FETAL ^{13}C-FATTY ACID TRANSFER RATIO

Similarly, the ratio between the ^{13}C-DHA in venous cord blood and the AUC maternal plasma was significantly lower in GDM than in controls independently if using concentration (Figure 22) or enrichment values (P = 0.187, Figure 23), while for the other tracer fatty acids the ratios were not different between the groups. These results indicate that lower ^{13}C-DHA from the mother goes to the fetus (Figure 22) and that lower ^{13}C-DHA from the fetus comes from the mother (Figure 23).

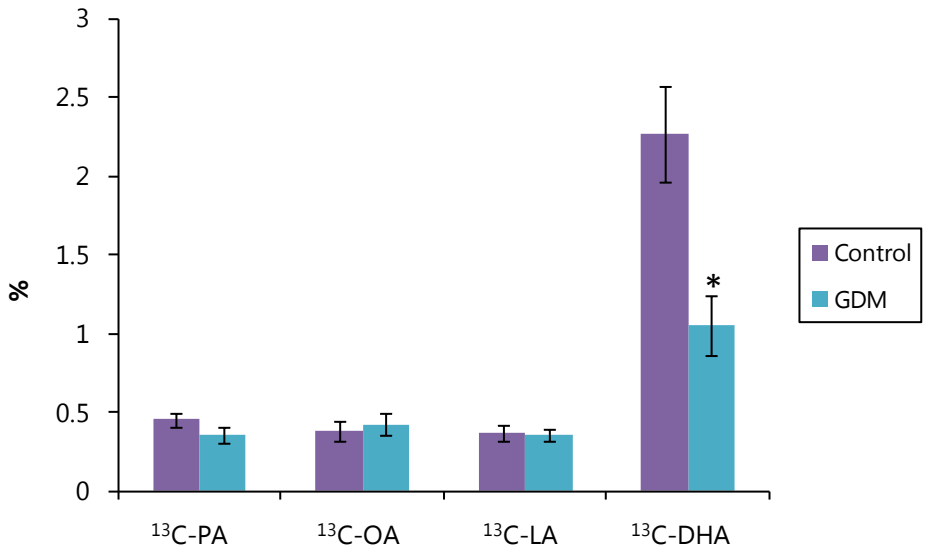


Figure 22: *Ratio (%) between venous cord plasma concentration and maternal plasma AUC of ^{13}C-fatty acids*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at *P* <0.05

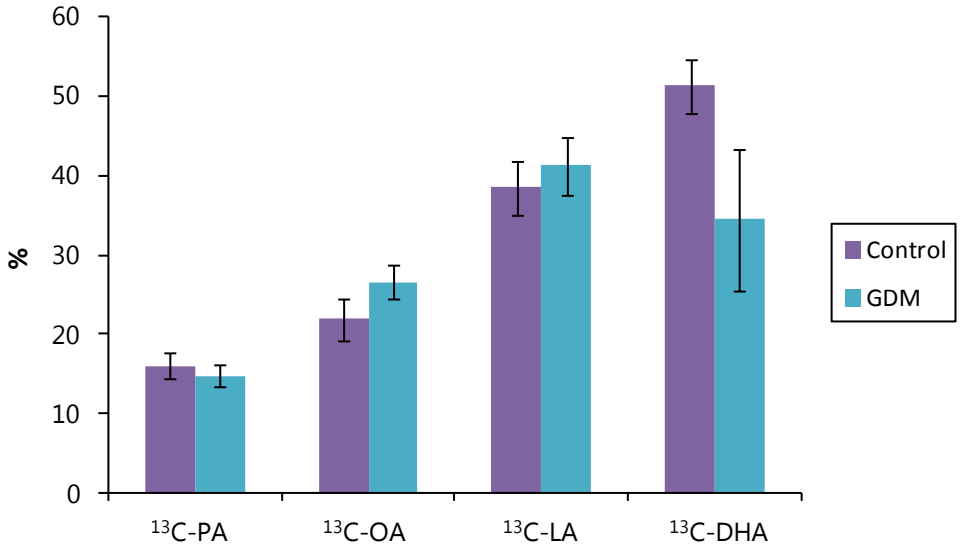


Figure 23: *Ratio (%) between venous cord plasma and maternal plasma ^{13}C-enrichment values*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. Controls, n = 11 (except for ^{13}C-DHA in which n = 6); GDM, n = 9. *Statistically significant difference between groups at *P* <0.05

Thus, in agreement with the lower ^{13}C-DHA placental uptake in GDM, our results indicate that the fetus of GDM receives lower ^{13}C-DHA from maternal plasma than controls.

20. GDM TREATED WITH DIET *VERSUS* GDM TREATED WITH INSULIN

Although only 6 GDM subjects received insulin treatment while 3 GDM only dietary treatment, we attempted to see if there were differences between these two groups. It was surprising that despite no differences were found in the placental uptake between the groups (Figure 24 A), the ratio of ^{13}C-DHA concentration in cord respect to maternal plasma was significantly lower in GDM subjects treated with insulin respect to those treated with diet (Figure 24 B). Thus, fetus from GDM women treated with insulin have lower ^{13}C-DHA in cord blood because of another mechanisms apart from placental uptake, probably higher fat accretion by fetal adipose tissue.

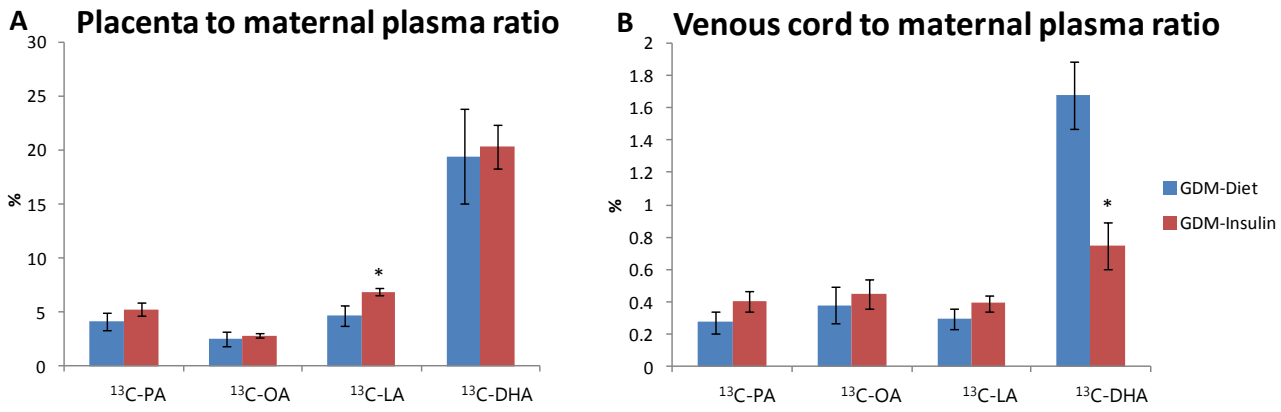


Figure 24. *Ratio (%) between placental concentration and maternal plasma AUC and between venous cord plasma concentration and maternal plasma AUC of ^{13}C-fatty acids in GDM subjects*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. GDM-Diet, n = 3; GDM-Insulin, n = 6. *Statistically significant difference between groups at P <0.05

Anyhow, both GDM groups (insulin or diet) tended to lower uptake and transfer of ^{13}C-DHA respect to control subjects without GDM (Table 11).

Table 11. *Ratio (%) between compartments of ^{13}C-fatty acids concentration in GDM under different clinical treatments respect to controls.*

Placenta/Maternal plasma AUC	Control (n=11)	GDM-Diet (n=3)	GDM-Insulin (n=6)	*P**	*P†*
^{13}C-Palmitic acid	5.10 ± 0.25	4.13 ± 0.78	5.26 ± 0.59	0.147	0.770
^{13}C-Oleic acid	3.59 ± 0.28	2.50 ± 0.65	2.83 ± 0.25	0.114	0.099
^{13}C-Linoleic acid	6.96 ± 0.44	4.64 ± 0.91	6.84 ± 0.35	**0.033**	0.862
^{13}C-Docosahexaenoic acid	28.43 ± 4.23	19.41 ± 4.42	20.31 ± 1.97	0.228	0.125
Venous Cord/ Maternal plasma AUC	**Control (n=11)**	**GDM-Diet (n=3)**	**GDM-Insulin (n=6)**	***P****	***P†***
^{13}C-Palmitic acid	0.45 ± 0.05	0.27 ± 0.06	0.40 ± 0.06	0.087	0.537
^{13}C-Oleic acid	0.38 ± 0.06	0.38 ± 0.11	0.45 ± 0.09	0.977	0.565
^{13}C-Linoleic acid	0.37 ± 0.05	0.29 ± 0.06	0.39 ± 0.05	0.453	0.764
^{13}C-Docosahexaenoic acid	2.27 ± 0.31	1.68 ± 0.21	0.75 ± 0.14	0.155	**0.003**

Results are expressed as mean ± SEM. *P**, *P* values between control group and GDM-Diet; *P†*, *P* values between control group and GDM-Insulin. Statistically significant difference between groups at $P < 0.05$

The differences in fetal transfer ratio of ^{13}C-DHA in GDM according to maternal treatment (Figure 24) resulted in lower ^{13}C-DHA concentration in total lipids of venous cord plasma in neonates of GDM women treated with insulin respect to those from mothers treated only with diet (Figure 25). However, the concentration of ^{13}C-DHA in maternal plasma or placental tissue was similar between the groups (Figure 26).

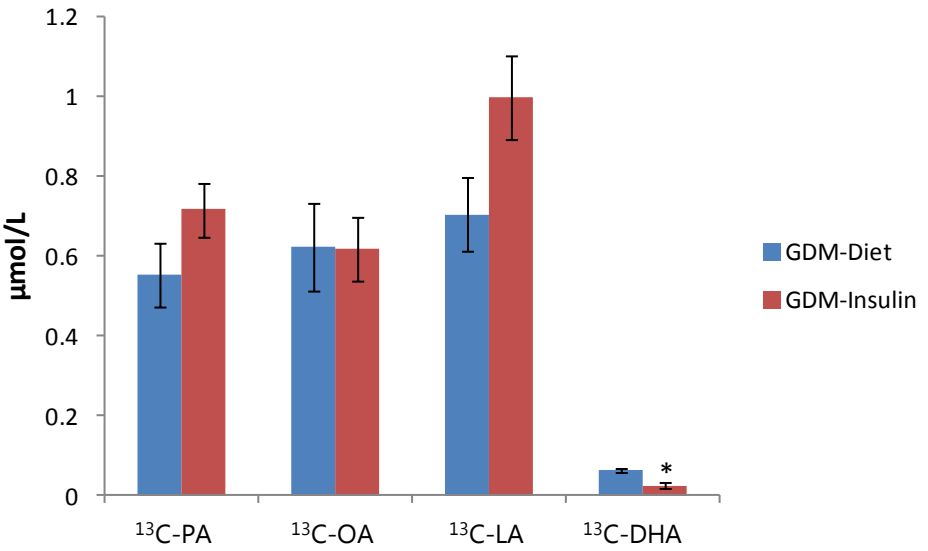


Figure 25. *Labelled fatty acids concentration in total lipids of venous cord plasma at the time of delivery in GDM subjects*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. GDM-Diet, n = 3; GDM-Insulin, n = 6. *Statistically significant difference between groups at *P* <0.05

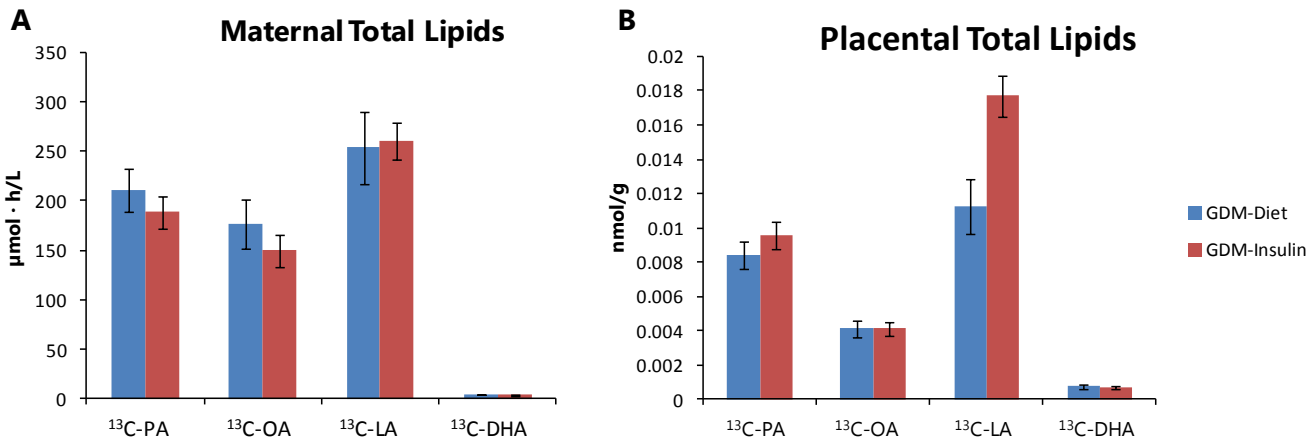


Figure 26. *Labelled fatty acids concentration in total lipids of maternal plasma (AUC) (A) and placental tissue at the time of delivery (B) in GDM subjects*. Results are expressed as mean ± SEM. ^{13}C-PA, ^{13}C-Palmitic acid; ^{13}C-OA, ^{13}C-Oleic acid; ^{13}C-LA, ^{13}C-Linoleic acid; ^{13}C-DHA, ^{13}C-Docosahexaenoic acid. GDM-Diet, n = 3; GDM-Insulin, n = 6. *Statistically significant difference between groups at *P* <0.05

There were no changes in the umbilical vein-artery difference between both groups of GDM subjects treated with diet or insulin (results not shown) maybe because of the commented limitation on the time schedule of the study. The differences in ^{13}C-DHA concentration of neonates from GDM could be due to higher fetal insulin levels in offspring of GDM women who required insulin treatment which could contribute additionally to reduce LC-PUFA levels in cord blood.

V. DISCUSSION

21. EFFECTS OF GDM ON FATTY ACID PROFILE

Many adaptations of maternal lipid metabolism are taking place throughout gestation which could have major consequences for fetal growth. In lipid metabolism, the role of lipoproteins is essential, since they constitute the fundamental transport of lipids in the blood circulation. Chylomicrons are lipoproteins which are synthesized in the gut and they transport the lipids from the diet, mainly triglycerides. On the other hand, VLDL are TG-rich lipoproteins synthesized in the liver either *de novo* or from the fatty acids derived from adipose tissue lipolysis.

The main destinies of TG-rich lipoproteins are peripheral tissues with high lipoprotein lipase activity like adipose tissue or muscle. This enzyme hydrolyzes triglycerides circulating in plasma in both chylomicrons and VLDL, which are converted into chylomicron remnant particles and intermediate-density lipoproteins (IDL) respectively and the hydrolytic products, NEFA and glycerol, are partially taken up by the subjacent tissue (236). The IDL can be uptaken by the liver to be recycled or can be turned into low-density lipoproteins (LDL) which are mainly made up by phospholipids and esterified cholesterol; thus LDL are the major transporters of cholesterol to the peripheral tissues. On the other hand, high-density lipoproteins (HDL) adhere PL and mainly non-esterified cholesterol; in this way, HDL clear the cholesterol up from peripheral tissues transporting it to the liver or other tissues where it can be used to synthesize different compounds as steroid hormones in the adrenal glands or ovary. In this manner, HDL redistribute cholesterol among the different tissues and facilitate the cholesterol return from peripheral tissues to the liver giving rise to an antiatherogenic effect of the HDL.

During late pregnancy, fat uptake by adipose tissue decreases because of an enhanced lipolytic activity and a decreased lipoprotein lipase activity of this tissue. These changes result in a accelerated net breakdown of fat depots, which contribute to the development of maternal hyperlipidemia, coinciding with the phase of maximal fetal growth (14). This condition of maternal hyperlipidemia during late gestation

mainly corresponds to plasma rises in triglycerides, with smaller rises in phospholipids and cholesterol (23). The greatest increase in plasma triglycerides corresponds to VLDL, which results from enhanced production by the liver and decreased removal from the circulation as consequence of reduced adipose tissue lipoprotein lipase activity (237-238). The insulin-resistant condition occurs by increasing placental lactogen and rising plasma estrogen levels during late pregnancy which contributes to both enhanced lipolytic activity of adipose tissue and the decreased lipoprotein lipase activity; in addition, the progressive increase in plasma estrogen levels during gestation (239-240) also actively contributes to maternal hypertriglyceridemia by enhancing liver production of VLDL (241-242).

Since the underlying pathophysiology of GDM is a function of a pronounced peripheral resistance to insulin, those pathways become further enhanced in this condition, being responsible for the increase in plasma NEFA and triglycerides seen in women with GDM (23; 243). In diabetic pregnancy, exaggerated hypertriglyceridaemia has been found in the first (26), second (24; 26) and third (24; 26; 244) trimesters of gestation compared to normal pregnancy. Thus, it has been reported an enhanced concentration of triglycerides in pregnant women with GDM. Despite the hypertriglyceridaemia classically reported in GDM subjects, some authors did not find differences in triglycerides concentration in GDM with respect to healthy pregnant women (23; 25; 27). An adequate glycemic control and treatment of the pathology either by diet or insulin and also the small size of some studies may explain the discordant findings.

In the present study, our subjects tended to maternal hyperlipidemia with higher total plasma fatty acid concentration in GDM compared to control women (Table 6), and a trend to higher fatty acid concentration in plasma triglycerides in GDM women than in controls, with the differences very close to significance (P = 0.065) (Table 7). Moreover, in venous cord blood there were no differences between groups in total fatty acid concentration in plasma triglycerides that even tended towards lower concentration in the GDM group. This could indicate an impaired lipid transfer from the

mother to the fetus in GDM or it could represent an enhanced triglycerides uptake and lipid deposition by the fetal adipose tissue.

With regard to total NEFA concentration, we found surprisingly a trend to lower levels in maternal plasma and venous cord blood in women with GDM comparing to normal pregnancies (Table 7). Some authors reported an increased plasma NEFA concentration in GDM women respect to controls whereas others did not find any change (25; 198; 245). To better discern the behaviour of the fatty acids, we decided not to work only with classical percentage values or fatty acid profile, but using labelled fatty acids administered orally to the pregnant women to study their distribution among compartments.

22. EFFECTS ON LABELLED FATTY ACIDS METABOLISM

Using fatty acids labelled with stable isotopes in pregnant women offers an opportunity for evaluating *in vivo* the process of the fatty acids placental transfer. In the present study we administered four fatty acids labelled with stable isotopes, ^{13}C-PA, ^{13}C-OA, ^{13}C-LA and ^{13}C-DHA, to healthy pregnant women and women with GDM with the aim to elucidate and understand whether an impaired placental transfer of fatty acids occurs in this pathology.

It is difficult to identify a time for caesarean section in which the steady state of the four ^{13}C-FA was reached in all blood lipid fractions, since each lipid fraction has different kinetics of appearance and disappearance of fatty acids. The chosen time for caesarean section should also take into consideration the process of uptake and release of ^{13}C-FA from placenta to the umbilical cord. Larqué *et al.* (172) previously administered labelled fatty acids to pregnant women 4h before a caesarean section. In that study, the incorporation of tracer into maternal plasma phospholipids and cholesterol esters was hardly detected after 4h, thus the evaluation of the maternal-fetal fatty acid transfer required longer time of study. More recently, Gil-Sánchez *et al.*

(173) increased the time between tracer administration and caesarean section to 12 hours. With this study, it was clearly demonstrated tracer enrichment in all maternal plasma lipid fractions at the time of caesarean section, 12 hours after oral fatty acid tracer application. Thus, we followed this methodological approach to assess human placental fatty acids transfer in GDM.

As the clinical setting of a scheduled caesarean section early in the morning does not allow frequent maternal blood sampling during the 12 h period and placenta and cord blood only can be sampled at the time of delivery, we had some limitations on data analysis and interpretation. Since no samples could be collected at night previous to a surgery, to avoid disturbance of the patient, the kinetics of fatty acids in the maternal plasma lipid pool could only be determined by AUC for individual fractions and fatty acids with data obtained close to delivery. Moreover, for the data analysis we had to assume that turnover does not differ between the studied fatty acids and, that the total area under the tracer concentration curves was proportional to the area calculated from the available data. With respect to tracer appearance on the fetal side only the concentration at one time point is available, and so no kinetic data are obtained. As a consequence, we must focus data analysis on the tracer distribution observed at the time of delivery.

In the present study, women with GDM showed significantly lower ^{13}C-DHA concentrations or enrichments in total lipids of maternal plasma, placenta and venous cord plasma than healthy pregnant women. Furthermore, the ratio between ^{13}C-DHA concentration in both placenta and cord blood respect to maternal plasma AUC was significantly lower in GDM than in controls, which indicates both lower materno-placental transfer and lower materno-fetal transfer of DHA in GDM. As transfer related ratios were not different between groups for the other studied fatty acids, this indicates an effect of GDM specific for DHA which might influence the neurological development of the neonates.

The timing of the maternal blood sampling (beginning 9 hours after ingestion) is well past the expected peak in chylomicron triglycerides concentration, explaining why the concentrations at -3h, -2h and -1h were relatively stable (Figure 9). At that point in time, most of the labelled fatty acids have been hydrolyzed by lipoprotein lipase and the majority of tracer taken up by adipose tissue. However, a significant amount remained in triglycerides (probably chylomicron remants for the most part) and phospholipids, with smaller amounts in NEFA (derived from incomplete uptake of fatty acids in tissues after lipoprotein lipase hydrolysis) and cholesterol esters. The labelled material in phospholipids presumably represents cycling of fatty acids through hepatic pools.

There was a different behaviour between the ^{13}C-DHA and the rest of labelled fatty acids. We reported higher AUC concentration of the ^{13}C-non-LC-PUFA in total lipids of GDM maternal plasma (Figure 8 and Table 9). This could be due to the lower clearance of maternal plasma triglycerides in gestational diabetes that contributes to the hyperlipidemia in these subjects. In type 2 diabetes, it was reported that insulin resistance causes high postprandial levels of triglyceride rich lipoproteins and their prolonged residence in the circulation (246). The maternal hypertriglyceridemia resulted in higher AUC concentration of ^{13}C-non LC-PUFA in maternal plasma total lipids and in maternal plasma triglycerides despite similar enrichment values among groups. Moreover, labelled non-LC-PUFA concentration in maternal plasma phospholipids and cholesterol esters was also enhanced in GDM, as well as their enrichments. All of them contribute to the higher labelled non-LC-PUFA in GDM maternal plasma.

In contrast, for ^{13}C-DHA, we found significantly lower tracer concentration in maternal plasma of GDM (Figure 9 and Table 9). The smaller AUC of ^{13}C-DHA concentration in total lipids of GDM compared to controls (P = 0.057) could be due to different effects of GDM on the metabolism of triglycerides and phospholipids. DHA is a fatty acid which is mainly incorporated into phospholipids, while PA and OA go into maternal plasma triglycerides (173; 247). A different incorporation of DHA in maternal plasma lipid fractions could affect the plasma clearance of ^{13}C-DHA and its placental

uptake in this pathology. We found lower ^{13}C-DHA concentration in all maternal plasma lipid fractions in GDM except for maternal triglycerides; ^{13}C-DHA incorporation in triglycerides was enhanced while its incorporation in phospholipids decreased (Figure 10). Wijendran *et al.* (207) reported significantly higher percentages and concentrations of DHA in maternal plasma phospholipids in women with GDM compared to controls. Nevertheless, in this study, women with GDM had a significantly higher dietary intake of EPA and DHA than the control subjects. It is well-known that dietary intake of LC-PUFA correlates with plasma LC-PUFA levels, thus higher DHA intake could explain the higher percentages observed in this subjects. Furthermore, Thomas *et al.* (213) described significantly higher percentages of DHA in plasma triglycerides of GDM women while no differences in plasma phospholipids.

In contrast to plasma, other authors observed significantly lower percentages of arachidonic acid and DHA in red blood cells phospholipids of GDM women (222), particularly in choline phospholipids (208). Thomas *et al.* (213) suggested a potential failure to incorporate LC-PUFA into the red cell phospholipids in addition to a defect in placental transport. The higher ^{13}C concentrations and ^{13}C enrichments for PA and OA in maternal plasma phospholipids of GDM women, could disturb DHA incorporation into this lipid fraction and hence, its placental uptake. Our results using labelled fatty acids support a disturbed incorporation of DHA into maternal plasma phospholipids and into further maternal lipid fractions in GDM but not in plasma triglycerides, while for the other labelled fatty acids this effect was not observed. Erythrocytes could provide LC-PUFA by flip-flop exchange to syncitiotrophoblast membrane (248); nevertheless, we did not measure labelled fatty acids in phospholipids of red blood cells, but only in phospholipids of total blood.

GDM has been associated with enhanced maternal plasma NEFA content (249), because of the higher insulin resistance and increased rate of lipolysis of adipose tissue (28); nevertheless, Radaelli (188) and Schaefer-Graf (245) studies did not detect such an increase of maternal plasma NEFA. In the current study we have observed a trend towards lower appearance of all studied ^{13}C-FA in plasma NEFA in the GDM group and

also lower enrichment (Figures 10, 11). The lower enrichment of ^{13}C-FA in NEFA could indicate that most of the NEFA analyzed in this study were provided by lipolysis from adipose tissue where ^{13}C-FA would be too highly diluted in GDM to cause measurable ^{13}C enrichment in NEFA after re-liberation by lipolysis.

Placental tissue may uptake NEFA or fatty acids released from lipoproteins by placental lipases. Available comparisons of the activity of enzymes that release fatty acids from circulating maternal lipids for placental uptake (lipoprotein lipase and endothelial lipase) between GDM and uncomplicated pregnancies are inconclusive. Magnusson *et al.* (185) studied the lipoprotein lipase activity in placentas of GDM women and found no differences compared with controls. Nevertheless, Dubé *et al.* (198) have reported an increased placental lipoprotein lipase activity in GDM women while Radaelli *et al.* (188) described a reduction in placental lipoprotein lipase to counteract excessive placental uptake of fatty acids in GDM. Thus, the results on placental LPL, that mainly hydrolizes serum triglycerides are controversial. In contrast, Radaelli *et al.* (188) reported an enhancement of endothelial lipase in GDM which is an enzyme that mainly releases fatty acids from serum phosppholipids. This result is in part in agreement with the higher endothelial lipase activity observed in placentas from obese women with GDM compared to controls, although no differences were found with placentas from lean women with GDM (187). If the concentration of ^{13}C-DHA in maternal plasma phospholipids is smaller in GDM, we could speculate about a high relevance of endothelial lipase for DHA release and transfer in these subjects. As less DHA is available in maternal plasma of GDM this leads to a lower transfer of DHA from the mother to the placenta in GDM. ^{13}C-DHA in placental total lipids was significantly correlated with its concentration in each maternal plasma lipid fractions in control subjects (^{13}C-DHA maternal plasma PL r = 0.697, P = 0.017; TG r = 0.724, P = 0.012; NEFA r = 0.922, P = 0.001; CE r = 0.643, P = 0.045). In contrast, in GDM subjects there were no significant correlations, which points towards other factors than availability in maternal plasma, which limit placental DHA uptake.

The results of the present study showed a significantly lower concentration of ^{13}C-DHA in placental total lipids of women with GDM compared to control subjects but not for the other studied fatty acids. The decrease of ^{13}C-DHA concentrations in the placenta of GDM tended to occur in all placental lipid fractions, although the differences were not statistically significant. Shafrir *et al.* (250) investigated the materno-fetal fat transport in rats made diabetic by injection of streptozotocin and administered ^{14}C-OA intragastrically; the diabetic rats showed significantly higher radioactivity 24h later in placenta than control rats. The treatment of GDM subjects during pregnancy with diet or insulin seems to ameliorate theses differences. An increase in total lipids of human placenta has been reported in GDM women (216; 251), but we could not detect such increase, maybe due to a limitation in the number of subjects. Furthermore, Bitsanis *et al.* (216) described a higher accumulation of DHA in placental phospholipids, but no differences of DHA in placental triglycerides of women with GDM. Since, phospholipids contribute about 80-90% to total placental lipids (231), Bitsanis *et al.* hypothesized that LC-PUFA were taken up by the GDM placenta and retained after esterification into phosphoglycerides instead of being transferred to the fetus (216). We found a significant increase of ^{13}C-PA in phospholipids fraction in the placenta of GDM, and a trend to higher ^{13}C-LA levels but no trend for ^{13}C-DHA. Thus, our results do not support the concept that LC-PUFA uptake by the placenta is increased by GDM.

Similarly to maternal plasma, the concentration of the labelled non-LC-PUFA tended to be higher in placenta lipid fractions with esterified fatty acids (triglycerides, phospholipids and cholesterol esters) but not in placental NEFA. In fact, the ^{13}C-enrichment of non-LC-PUFA in placental NEFA of GDM was higher than in controls, but the amount of NEFA in the placenta of GDM was smaller. Histological studies have classically reported more intensive esterification of fatty acids into lipids in GDM placentas, in agreement with higher accumulation of lipid droplets (188), which leads to a smaller NEFA pool in GDM placenta with fast turnover. This is in agreement with our observation of a low concentration and high enrichment. Moreover, a consistent activation of the expression of several genes involved in placental lipid biosynthesis

pathways was found in GDM (188). Recently, Visiedo *et al.* (252) reported by *in vitro* studies with diabetic and healthy placental explants that hyperglycemia reduces fatty acid oxidation and increases triglycerides accumulation in human placenta.

The storage of ^{13}C-DHA or other ^{13}C-FA in triglycerides of placental lipid droplets could reduce its rate of transfer from the mother to the fetus. It is interesting to highlight that, in contrast to the rest of lipid fractions, ^{13}C-DHA tended to higher enrichment in triglycerides of GDM placentas although without statistical differences. However, finally the unlabelled DHA level in triglycerides from GDM placentas was not higher than in controls, resulting in practically no differences in ^{13}C-DHA concentrations in placental triglycerides. Maybe a methodological problem could be arising in the triglycerides quantification in the placenta, due to the large amount of blood inside placental tissue. Considering that the triglycerides content in placental tissue is low (the major lipid fraction in placenta is phospholipids) and due to the high level of triglycerides in maternal blood of GDM, it is no clear how much the triglycerides that we analyzed were influenced by maternal blood contamination. It could be of interest to analyze these labelled fatty acids specifically in placental lipid droplets to test this hypothesis.

^{13}C-DHA concentration was significantly lower in both AUC of maternal plasma and placental total lipids but GDM subjects showed also a lower ratio of ^{13}C-DHA concentration in placenta to maternal plasma than controls indicating lower ^{13}C-DHA uptake by the placental tissue in GDM. This also agrees with lower ratio of ^{13}C-DHA enrichments in placenta to maternal plasma, indicating that a lower proportion of the placental DHA is maternally derived. Thus, not only the levels of ^{13}C-DHA in maternal circulation were lower, but also its uptake by the placenta was reduced by GDM. Nevertheless, as previously reported for the healthy subjects (173), in GDM women we also found a preferential placental uptake of DHA relative to the other studied fatty acids.

The concentration ratio indicates how much tracer fatty acid from the mother goes to the placenta, while the enrichment ratio indicates how much tracer fatty acid in the placenta comes from the mother. It is interesting to note that for essential fatty acids like linoleic acid, the 100% of enrichment ratio indicates that practically all ^{13}C-LA in placenta comes from the maternal plasma (Figure 21) in agreement with the behaviour of an essential fatty acid. For conditionally essential fatty acids like DHA, the enrichment ratio in placenta respect to maternal plasma was about 70% (Figure 21), thus, most of it comes also from maternal plasma, although, this is a measurement performed at only one time point (delivery time).

We have also found lower ^{13}C-DHA in venous cord blood in GDM, not only in total lipids but also in phospholipids, which are mainly synthesized by the fetal liver from the available NEFA. Our results are in agreement with observational studies in neonates born to mothers with GDM who had decreased percentages of DHA, both in cord plasma (215; 221) and cord red blood cells (215; 222). The reduced ratio of ^{13}C-DHA concentrations between fetal and maternal circulation is consistent with lower absolute materno-fetal transfer of ^{13}C-DHA in GDM subjects. The lower ratio of ^{13}C-DHA using ^{13}C enrichment values also confirms that lower ^{13}C-DHA in the fetus are maternally derived in GDM. Our results clearly demonstrate an impaired materno-fetal function in GDM pregnancies for the transfer of LC-PUFA. In contrast, non-LC-PUFA ^{13}C concentrations tended to be higher in the offspring of GDM women, thus it seems that transfer of non-LC-PUFA is not affected by GDM in a relevant way. This result is in agreement with similar fetal to maternal plasma concentration ratio for non-LC-PUFA between controls and GDM (Figure 22). Moreover, the ratio of fetal to maternal enrichments also supports a trend to higher materno-fetal transfer of non-LC-PUFA in GDM offspring, indicating that higher non-LC-PUFA from venous cord in GDM was maternally derived (Figure 23).

It was interesting to note differences between GDM treated with diet or insulin in the transfer ratio of ^{13}C-DHA from the mother to the fetus (Figure 24). Despite we had a limited number of subjects from each group (GDM-Diet n=3 and GDM-Insulin

n=6), placental uptake of labelled fatty acids was similar between groups (Figure 24A) while materno-fetal transfer of ^{13}C-DHA was significantly lower in GDM treated with insulin (Figure 24B). Moreover, we found lower ^{13}C-DHA concentration in venous cord blood of GDM-Insulin (Figure 25). Fetal insulin levels in these children could enhance the fatty acid accretion by fetal adipose tissue, contributing also to the reduction of the ^{13}C-DHA in cord blood. Thus, in both GDM groups, placental function is similarly affected but in those GDM subjects treated with insulin there is an enhanced fetal fat accretion that reduces DHA availability in fetal circulation.

Schaefer-Graf *et al.* demonstrated in a population of well-controlled GDM pregnancies that both maternal triglycerides and NEFA levels correlated with fetal growth during pregnancy and with neonatal anthropometric measurements, with the best correlation found with neonatal fat mass (55). They highlighted the role of maternal lipids as strong determinants of fetal growth in pregnant women with GDM. Nevertheless, in a further study, they did not observe such correlation of maternal lipids and fetal anthropometric measurements in control subjects without GDM (245). Cord blood triglycerides or cord NEFA were both positively correlated to neonatal anthropometrics in control pregnancies, while, in GDM they observed a negative correlation of triglycerides *versus* fetal growth (245) supporting a higher accretion of fatty acids by fetal peripheral tissues as adipose tissue in GDM offspring.

Maternal hyperglycemia during pregnancy may lead to an increase in fetal insulin levels to counteract the excess of glucose provided from the mother. The augmented levels of fetal insulin might cause effects on fetal lipid metabolism, increasing fat depots by enhancing LPL activity; in addition, fetal insulin levels have been shown to correlate well with the fetal abdominal circumference (253). Thus, a more severe GDM with higher hyperglycemia as occurs in subjects treated with insulin could result in an additional alteration in fetal lipid metabolism with higher fat uptake by fetal adipose tissue. Moreover, Ortega-Senovilla *et al.* (224) found lower percentages of arachidonic acid, DHA and total n-6 or n-3 polyunsaturated fatty acids in umbilical arterial but not in venous plasma of neonates of GDM than those from controls. On the fetal side, an

altered handling or metabolism of fatty acids in neonates of mothers with GDM could also contribute to the lower proportion of LC-PUFA in the plasma of neonates of GDM mothers, apart from the disturbed placental function. We did not find differences in the umbilical artery and vein difference between GDM subjects treated with diet or insulin, probably because 12h was not enough time to detect differences in the fatty acid uptake by fetal tissues or because the major differences occur in NEFA and the ^{13}C determination in NEFA is less precise than in other lipid fractions due to the small NEFA concentrations in cord blood.

Reduced DHA levels in the perinatal period might affect brain and retinal development in the offspring resulting in decreased visual function and altered learning, behaviour, and neurotransmitter metabolism. It could lead to a situation like in preterm infants who display lower levels of DHA than term infants due to the loss of intrauterine supply during the last months of pregnancy (254). Studies on DHA supplementation involving preterm infants revealed consistent benefits for visual acuity following DHA supplementation, at least over the first six months of life (255-260) and other authors have also found positive results on outcomes related to cognitive and motor development in preterm infants (141; 261). Our results highlight the potential benefit of a supplementation in GDM mothers or in their offspring to avoid the risk of deficits.

VI. CONCLUSIONS

1. The concentrations of labelled long-chain polyunsaturated fatty acids (LC-PUFA) were significantly lower in maternal, placental and fetal compartments in gestational diabetes mellitus (GDM) compared to controls, reflecting differences in the docosahexaenoic acid (DHA) metabolism between healthy mothers and mothers with GDM.

2. The concentration of the studied non-LC-PUFA fatty acids (^{13}C-palmitic acid, ^{13}C-oleic acid and ^{13}C-linoleic acid) was higher in maternal plasma total lipids of GDM women compared with controls due to the hypertrygliceridemia in GDM subjects.

3. The ^{13}C-docosahexaenoic acid concentration tended to be lower in all maternal plasma lipid fraction of GDM women than in controls except for maternal plasma triglycerides, pointing to a disturbed incorporation of DHA into maternal plasma phospholipids which could affect its placental uptake.

4. While the placental transfer of the other ^{13}C-FA was not significantly affected, there was a disturbed placental uptake of DHA in GDM as indicated by both lower ratio of ^{13}C-DHA in placenta respect to maternal plasma and lower ^{13}C-DHA concentration in placental tissue. This could affect the fetal supply of this important fatty acid.

5. The lower ^{13}C-DHA in cord blood in addition with the lower ratio of ^{13}C-docosahexaenoic acid in umbilical vein plasma respect to maternal plasma was consistent with an impaired materno-fetal transfer of DHA in pregnancies complicated by GDM, which was not observed for the rest of fatty acids.

6. Insulin treatment in GDM mothers may have an additional role on the lower ^{13}C-DHA in cord blood respect to GDM treated with only diet. Both GDM groups have similar placental uptake ratio, but different materno-fetal DHA ratio; apart from placental uptake, probably higher fat accretion from fetal adipose tissue could explain these differences between GDM subjects.

GENERAL CONSIDERATION

Our research *in vivo* shows in humans that DHA transfer to the fetus seems to be reduced in pregnancies complicated by GDM compared to controls. The impaired supply of DHA in this pathology emphasizes the interest for the supplementation of these neonates with DHA to prevent potential adverse effects on neurodevelopmental programming of these children.

VII. SUMMARY IN SPANISH/ RESUMEN EN ESPAÑOL

La diabetes mellitus gestacional (GDM) es la alteración metabólica más asociada a la gestación, alcanzando una prevalencia de hasta el 12% y aumentando la incidencia de esta patología paralelamente a las tasas de obesidad. La diabetes gestacional se caracteriza por un descenso pronunciado en la sensibilidad a la insulina y una secreción insuficiente de la misma, provocando una tolerancia anormal a la glucosa durante el embarazo que produce hiperglucemia tanto en la madre como en el feto. Diversos estudios sugieren que la GDM es un importante factor de riesgo para el feto, presentándose macrosomía y con frecuencia daños fetales asociados (distress fetal, distocia de hombros, aumento de incidencia de partos distócicos y mediante cesárea, dislipemia...). Además, las mujeres con historia de GDM y su descendencia tienen un mayor riesgo de desarrollar hipertensión arterial y diabetes mellitus tipo II en la edad adulta.

Durante el proceso normal del embarazo, se producen cambios en la madre tanto en el metabolismo de la glucosa como en el metabolismo lipídico. La deposición de lípidos tiene lugar durante el último trimestre de gestación. Los ácidos grasos esenciales (ácido linoleico (18:2 n-6) y ácido α-linolénico (18:3 n-3)) y aquellos ácidos grasos que no pueden ser sintetizados en suficiente extensión por el feto (ácidos grasos poliinsaturados de cadena larga (LC-PUFA)), han de ser transferidos por la madre a través de la placenta. Las nuevas membranas en formación y el tejido nervioso son estructuras muy ricas en lípidos, conteniendo principalmente LC-PUFA, especialmente ácido docosahexanoico (DHA).

En los niños de madres diabéticas, se ha encontrado una reducción de los niveles de DHA y LC-PUFA en plasma y fosfatidil colina de eritrocitos. El hecho de padecer diabetes durante la gestación se ha relacionado con la afectación del desarrollo intelectual y del comportamiento del recién nacido; relacionando de forma inversa la cetoacidosis materna y los niveles de ácidos grasos libres con el coeficiente intelectual de los niños a la edad de dos años. Además, la diabetes pregestacional y la GDM parecen tener un efecto adverso en la capacidad de atención y la función motora de los niños en edad escolar, aunque parece no afectar a las habilidades cognitivas. Estos

efectos se han correlacionado de forma negativa con el grado del control de la glucemia materna. Esta situación de deficiencia de LC-PUFA en los recién nacidos de madres diabéticas podría ser en parte responsable del retraso de maduración cerebral de estos niños comparados con recién nacidos de madres sanas. Sin embargo, no está claro si la reducción en los niveles de estos ácidos grasos se debe a una alteración de la función placentaria por la GDM o si el metabolismo fetal estaría afectado por la hiperglucemia, modificando así el perfil de ácidos grasos en los recién nacidos; por lo tanto, la causa de los bajos porcentajes de LC-PUFA en estos niños no se conoce aún.

La placenta es un órgano clave en el transporte de ácidos grasos de la madre al feto, especialmente para los ácidos grasos esenciales y condicionalmente esenciales como son los LC-PUFA. La transferencia placentaria de ácidos grasos es un proceso complejo en el cual están involucradas un gran número de proteínas transportadoras de ácidos grasos (plasma membrane fatty-acid binding protein (FABPpm/GOT2), fatty acid translocase (FAT/CD36), fatty acid transport proteins (FATP), and fatty acid binding proteins (FABP)). Es importante conocer la razón de los bajos niveles de DHA en los niños de mujeres con GDM y clarificar los mecanismos placentarios implicados en la transferencia de estos ácidos grasos en esta patología para poder mejorar los niveles de LC-PUFA en los recién nacidos de madres con GDM. Esta información es esencial para diseñar estrategias de suplementación adecuadas para las madres con diabetes gestacional durante el embarazo, así como para los recién nacidos lactantes e incluso ambos.

El objectivo general de esta tesis doctoral fue mejorar el conocimiento sobre la transferencia de ácidos grasos de la madre al feto en embarazadas con diabetes mellitus gestacional, evaluando *in vivo* la transferencia placentaria de ácidos grasos marcados con isótopos estables.

Para el estudio *in vivo* de la transferencia placentaria, se reclutaron once mujeres embarazadas sanas y 9 mujeres embarazadas diagnosticadas con diabetes mellitus gestacional (3 de ellas fueron tratadas sólo con dieta y 6 recibieron tratamiento adicional con insulina). 12 horas antes de ser sometidas a una cesárea electiva por dificultades obstétricas para el parto, los sujetos recibieron 4 ácidos grasos, ácido palmítico (PA), ácido oleico (OA), ácido linoleico (LA) y DHA, marcados con ^{13}C (isótopo estable del carbono). Se tomaron muestras de sangre de la madre 12h antes del parto (momento previo a la administración de los ácidos grasos marcados y correspondiente al momento basal), así como 3 horas antes del parto, 2 horas antes, una hora antes, en el momento del parto y una hora después del parto. Se tomaron también muestras de tejido placentario y de sangre venosa del cordón umbilical en el momento del parto. Las muestras fueron almacenadas a -80ºC y posteriormente se llevó a cabo la extracción de los ácidos grasos y su cuantificación mediante cromatografía gaseosa, así como el enriquecimiento en ^{13}C mediante cromatografía gaseosa acoplada a un espectrómetro de masas de isótopo ratio.

Los principales resultados de la presente tesis doctoral muestran una mayor concentración de los ácidos grasos marcados en los lípidos totales del plasma materno en las mujeres con GDM con la excepción del DHA, cuya concentración es menor en estas mujeres que en los sujetos controles. La mayor concentración del PA, OA y LA en los lípidos totales del plasma materno en las mujeres con diabetes gestacional podría indicar un menor aclaramiento de estos ácidos grasos en esta patología, contribuyendo a la dislipemia en estos sujetos. De hecho, la resistencia a la insulina en la diabetes mellitus se asocia con altos niveles de lipoproteínas ricas en triglicéridos y una mayor permanencia de las mismas en la circulación materna. Sin embargo, la menor concentración de DHA observada en el plasma materno en las mujeres con diabetes gestacional podría indicar una metabolización diferente del DHA, ya que es un ácido graso que se esterifica principalmente en fosfolípidos, lo cual podría incluso causar un mayor aclaramiento plasmático en estos sujetos.

Al analizar la concentración de los ácidos grasos marcados en las distintas fracciones lipídicas del plasma materno, el DHA tendió a concentraciones menores en todas las fracciones lipídicas en las mujeres con GDM, especialmente en la fracción de fosfolípidos. Para el resto de ácidos grasos, las concentraciones tendieron a ser mayores en todas las fracciones lipídicas del plasma materno de las mujeres diabéticas con respecto a las mujeres sanas. No obstante, en la fracción de ácidos grasos libres, las concentraciones de todos los ácidos grasos marcados tendieron a ser menores en las mujeres con diabetes gestacional; lo cual se debería a que los ácidos grasos marcados provienen de la lipolisis del tejido adiposo por lo que se diluirían en el gran pool de ácidos grasos libres de estas mujeres, resultando en valores más bajos en este grupo de pacientes.

Tanto en los lípidos totales del tejido placentario como en sangre venosa de cordón, se observaron concentraciones significativamente menores de DHA marcado en las embarazadas con diabetes gestacional respecto a las controles, mientras que para los otros ácidos grasos no se observaron diferencias entre los grupos, e incluso tendieron a mayores valores en sangre venosa de cordón en el grupo de GDM. La menor concentración de DHA en el tejido placentario no apoya la hipótesis de una mayor captación y acumulación de LC-PUFA por la placenta en mujeres con diabetes gestacional. Sin embargo, los menores niveles de DHA observados en sangre venosa de cordón en hijos de mujeres con GDM sí coinciden con los resultados en porcentajes obtenidos en estudios observacionales, e indican un descenso real en los niveles de DHA en el plasma de estos recién nacidos.

La tasa de transferencia de ácidos grasos de la madre a la placenta se calculó mediante la relación de cada ácido graso marcado en el tejido placentario con respecto a su concentración en el plasma materno; esta realción mostró una menor captación placentaria de DHA en mujeres con GDM con respecto a mujeres sanas. Asimismo, al calcular la tasa de transferencia de la madre al feto como la relación entre la concentración de cada ácido graso marcado en sangre venosa de cordón y su

respectiva concentración en plasma materno, se observó una menor transferencia absoluta de DHA al feto en sujetos con diabetes gestacional con respecto a controles.

Finalmente, se analizaron los datos del grupo de GDM separando los sujetos según el tratamiento recibido (sólo dieta o insulina). A pesar del bajo número de sujetos se observó una tasa de transferencia de DHA marcado de la madre al feto significativamente menor en mujeres con GDM tratadas con insulina respecto a aquellas tratadas sólo con dieta. No obstante, la tasa de transferencia de DHA de la madre a la placenta fue similar en ambos grupos de mujeres con GDM. Por lo tanto, los recién nacidos de mujeres con GDM tratadas con insulina parecen tener menos DHA en plasma debido a otros mecanismos implicados, aparte de la captación placentaria, probablemente una mayor captación por el tejido adiposo fetal.

CONCLUSIONES

1. La concentración de los ácidos grasos poliinsaturados de cadena larga (LC-PUFA) marcados con ^{13}C fue significativamente menor en plasma materno, placenta y plasma fetal en sujetos con diabetes mellitus gestacional (GDM) con respecto a controles, reflejando diferencias en el metabolismo del ácido docosahexaenoico (DHA) entre mujeres sanas y mujeres con GDM.

2. La concentración de los ácidos grasos no poliinsaturados de cadena larga estudiados (^{13}C-ácido palmítico, ^{13}C-ácido oleico y ^{13}C-ácido linoleico) fue mayor en los lípidos totales del plasma materno en mujeres con GDM comparado con controles debido a la hipertrigliceridemia que tiene lugar en los sujetos con GDM.

3. La concentración del ^{13}C-DHA tendió a ser menor en todas las fracciones lipídicas del plasma materno de mujeres con GDM respecto a las controles excepto en los triglicéridos del plasma materno, indicando una alteración en la incorporación del DHA en la fracción de fosfolípidos del plasma materno, lo cual, podría afectar a su captación placentaria.

4. Mientras que la transferencia placentaria del resto de ácidos grasos marcados no se vio afectada significativamente, sí se observó una alteración en la captación placentaria del DHA en los casos con GDM, indicado por la menor relación de ^{13}C-DHA de la placenta respecto a la madre y la menor concentración de ^{13}C-DHA en el tejido placentario. Lo cual, podría afectar al suministro fetal de este importante ácido graso.

5. La menor concentración de ^{13}C-DHA en sangre de cordón así como la menor relación de ^{13}C-DHA en plasma de vena umbilical con respecto al plasma materno es consistente con una transferencia materno-fetal de DHA alterada en embarazos complicados con GDM, que no se observó para el resto de ácidos grasos estudiados.

6. El tratamiento con insulina en mujeres con GDM podría tener un papel adicional en los bajos niveles de ^{13}C-DHA en sangre de cordón respecto a aquellos sujetos tratados sólo con dieta. Ambos grupos de mujeres con GDM tuvieron una tasa similar de captación placentaria pero diferencias en la tasa de transferencia materno-fetal para el DHA; aparte de la captación placentaria, probablemente una mayor captación de grasa por el tejido adiposo fetal podría explicar estas diferencias entre sujetos con GDM.

CONSIDERACIÓN GENERAL

Nuestra investigación *in vivo* mostró en humanos que la transferencia de DHA al feto parece estar reducida en embarazos complicados con GDM comparado con controles. La reducción en el suministro de DHA en esta patología resalta el interés de una suplementación con DHA en estos recién nacidos para prevenir los posibles efectos adversos en la programación del desarrollo neurológico de estos niños.

VIII. SUMMARY

Gestational Diabetes Mellitus (GDM) is one of the most common metabolic disorders of pregnancy, affecting up to 12% of all pregnancies. This disorder is characterized by an abnormal glucose tolerance diagnosed for the first time during pregnancy due to a decreased insulin sensitivity combined with insufficient insulin secretion. The increased insulin resistance induces alterations in lipid metabolism that lead to dyslipidemia in GDM women. Moreover, women with a history of GDM have a higher risk of developing type 2 diabetes mellitus later in life. Maternal diabetes during pregnancy might affect behavioural and intellectual development of the offspring; pregestational and gestational diabetes mellitus were found to adversely affect attention span and motor functions of offspring at school age. Since long-chain polyunsaturated fatty acids (LC-PUFA), especially docosahexaenoic acid (DHA), are of critical importance for the fetal development of the central nervous system, adverse effects of GDM on their transfer would be of major relevance.

The placental supply of maternal LC-PUFA to the fetus is critical since the ability of both fetus and the human placenta to synthesize LC-PUFA from essential fatty acids by desaturation and elongation is limited. In pregnancies complicated by GDM, some studies have shown normal levels of LC-PUFA in maternal plasma lipids, and even higher values for DHA in maternal triglycerides and phospholipids. In contrast, lower values of DHA and other LC-PUFA were observed in cord blood of GDM neonates. According to these results, it has been suggested an impaired materno-fetal LC-PUFA transfer during GDM that might lead to adverse fetal neurological programming of the offspring. Reduced LC-PUFA percentages in cord blood during GDM might result from augmented *de novo* synthesis of saturated and monounsaturated fatty acids in the fetus from the abundantly available glucose, and hence decreasing proportions of essential fatty acids and their derivatives. Another hypothesis could be a disturbed placental LC-PUFA transfer, or even higher LC-PUFA accretion by fetal adipose tissue in GDM babies. It is important to discern the reason for the lower levels of DHA in GDM babies in order to identify the best strategy of supplementation with LC-PUFA in the mother, the baby or both.

The general aim of the present study was to improve the understanding of fatty acid transfer in GDM, evaluating the *in vivo* placental transfer of fatty acids labelled with stable isotopes.

Eleven healthy pregnant women and nine pregnant women diagnosed with GDM (3 were treated only with diet and 6 also required insulin treatment) were recruited at the Virgen de la Arrixaca Hospital (Murcia, Spain). All subjects received 12 hours before an elective caesarean section, 4 fatty acids labelled with the stable isotope ^{13}C, palmític acid (PA), oleic acid (OA), linoleic acid (LA) and DHA. Maternal blood samples were collected 12 hours before the caesarean section (corresponding to basal time), and subsequently 3 hours, 2 hours, 1 hour before delivery, at the time of delivery and 1 hour afterward. Placental tissue and venous cord blood were also collected at delivery. Samples were stored at-80ºC until further analysis. Fatty acids were quantified by gas chromatography (GC) and ^{13}C-enrichments by GC-isotope ratio mass spectrometry.

In the present study, women with GDM showed significantly lower ^{13}C-DHA concentrations in total lipids of maternal plasma, placenta and venous cord plasma than healthy pregnant women. Furthermore, the ratio between ^{13}C-DHA concentration in both placenta and cord blood and maternal plasma AUC was lower in GDM than in controls, which indicates both lower materno-placental transfer and lower materno-fetal transfer of DHA in GDM. As transfer related ratios were not different between groups for the other studied fatty acids this indicates an effect of GDM specific for DHA.

At the timing of the maternal blood sampling (beginning 9 hours after ingestion) most of the lipids containing stable isotopes tracers have been hydrolyzed by lipoprotein lipase (LPL) and the majority of tracer has been taken up by adipose tissue. However, significant amounts remained in triglycerides (probably chylomicron remants for the most part) and phospholipids, with smaller amounts in non-esterified fatty acids (NEFA) (derived from incomplete tissue uptake of fatty acids after LPL hydrolysis) and cholesterol esters. The higher AUC of concentrations of the non-LC-PUFA in total lipids

and triglycerides of GDM maternal plasma could be due to the lower clearance of maternal plasma triglycerides in gestational diabetes that contributes to the hyperlipidemia in these subjects. In type 2 diabetes mellitus insulin resistance causes high postprandial levels of triglyceride rich lipoproteins and their prolonged residence in the circulation. The hyperlipidemia could affect the rate of fatty acids transfer across the placenta in GDM subjects respect to controls. However the smaller AUC of ^{13}C-DHA concentration in total lipids of GDM compared to controls (P = 0.057) might indicate different effects of GDM on the metabolism of triglycerides and phospholipids, which might even cause faster plasma clearance of ^{13}C-DHA.

We found a trend to reduced ^{13}C-DHA incorporation into maternal phospholipids (P = 0.068) respect to maternal triglycerides. Some authors have reported increased percentages of DHA in maternal plasma phospholipids and triglycerides in women with GDM; in contrast to plasma, red cells of the GDM women had significantly lower levels of arachidonic acid and DHA, particularly in choline phospholipids. It has been suggested a potential failure to incorporate LC-PUFA into the red cell phospholipids in addition to a defect in placental transport. Our results using stable isotopes tracers support a disturbed incorporation of DHA into maternal plasma phospholipids and into further maternal lipid fractions in GDM but not in plasma triglycerides, while for the other labelled fatty acids this effect was not observed.

GDM has been associated with higher maternal plasma NEFA content, because of the higher insulin resistance and increased rate of lipolysis of adipose tissue; nevertheless, some studies did not detect such an increase of maternal plasma NEFA. In the current study we have observed a trend towards lower appearance of all studied ^{13}C-FA in plasma NEFA in the GDM group and also lower enrichment. This could indicate that most of the NEFA analyzed in this study were provided by lipolysis from adipose tissue where ^{13}C-FA would be too highly diluted in GDM to cause measurable ^{13}C enrichment in NEFA after re-liberation by lipolysis.

The results of the present study showed a significantly lower concentration of ^{13}C-DHA in placental total lipids of women with GDM compared to control subjects but not for the other fatty acids. The decrease of ^{13}C-DHA concentrations in the placenta of GDM tended to occur in all placental lipid fractions, although the differences were not statistically significant. Rats made diabetic by injection of streptozocin, that received intragastrically ^{14}C-OA had significantly higher radioactivity 24h later in placenta than control rats; the treatment of GDM subjects during pregnancy with diet or insulin seems to ameliorate theses differences. An increase in total lipids of human placenta in GDM, and higher accumulation of DHA in placental phospholipids, but no differences of DHA in placental triglycerides was reported. Phospholipids contribute about 80-90% to total placental lipids and we found a significant increase of ^{13}C-PA in placental phospholipids, but no trend for ^{13}C-DHA. Thus, our results do not support the concept that LC-PUFA uptake by the placenta is increased by GDM. There is more intensive esterification of fatty acids into lipids in GDM placentas, in agreement with higher accumulation of lipid droplets, which leads to a smaller NEFA pool in GDM placenta with fast turnover. Moreover, a consistent activation of the expression of several genes involved in placental lipid biosynthesis pathways was found in GDM. Recently, it has been reported by *in vitro* studies in diabetic and healthy placental explants that hyperglycemia reduces fatty acid oxidation and increases triglycerides accumulation in human placenta.

GDM subjects showed a lower ratio of ^{13}C-DHA concentration in placenta to maternal plasma than controls. This agrees with lower ratio of ^{13}C-DHA enrichment in placenta to maternal plasma indicating that lower proportion of the placental DHA is maternally derived. Thus, not only the levels of ^{13}C-DHA in maternal circulation were lower, but also its uptake by the placenta was reduced by GDM. Nevertheless, as previously reported for the healthy subjects, there was a preferential placental uptake of DHA relative to the other studied fatty acids in GDM. Available comparisons of the activity of enzymes that release fatty acids from circulating maternal lipids for placental uptake (lipoprotein lipase and endothelial lipase) between GDM and uncomplicated pregnancies are inconclusive. No differences, increased endothelial lipase but not LPL

activity and even a reduction in placental LPL to counteract excessive placental uptake of fatty acids in GDM have been reported. If the concentration of ^{13}C-DHA in maternal plasma phospholipids is smaller in GDM, we could speculate about a high relevance of endothelial lipase for DHA release and transfer in these subjects. As less DHA is available in maternal plasma of GDM this leads to a lower transfer of DHA from the mother to the placenta in GDM. It has been reported an increased expression of phospholipid transfer protein (PLTP) in the endothelial cells of the fetal placental surface of GDM subjects, although no differences were found in fetal plasma. PLTP has a role in plasma lipoprotein metabolism and enhances transfer and exchange of phospholipids between HDL particles but its role in LC-PUFA fetal metabolism in GDM is uncertain.

We confirm an impaired LC-PUFA transfer from the mother to venous cord blood in GDM. Moreover, we found lower ^{13}C-DHA in cord blood phospholipids, which are mainly synthesized by the fetal liver from the available NEFA received from the mother. Other studies reported that neonates born to mothers with GDM had decreased percentages of DHA, both in plasma and red blood cells. The reduced ratio of ^{13}C-DHA concentrations between fetal and maternal circulation is consistent with lower absolute transfer of ^{13}C-DHA to venous cord plasma in GDM subjects; moreover when this ratio was calculated from ^{13}C enrichment values, lower proportion of the fetal DHA seems to be maternally derived. In contrast, non-LC-PUFA ^{13}C concentrations in the offspring of GDM women were similar to controls, and even higher for ^{13}C-OA, thus it seems that transfer of non-LC-PUFA is not affected by GDM in a relevant way, which agrees with similar fetal to maternal plasma concentration ratio for non-LC-PUFA between controls and GDM. This is new information that can only be carried out by isotope labelling.

GDM is associated with higher fetal fat mass that could be related to higher incorporation of circulating fatty acids into fetal adipose tissue. It was interesting to note that placental uptake of ^{13}CDHA was similar between GDM treated with diet or insulin, while materno-fetal transfer ratio of ^{13}C-DHA was significantly lower in GDM treated with insulin *vs*. only diet. Moreover, ^{13}C-DHA concentration in venous cord

blood was significantly reduced in GDM treated with insulin *vs*. GDM treated with diet. Thus, in addition to disturbed placental function by GDM, fetal insulin on GDM subjects with more severe hyperglycemia could enhance fatty acid accretion by fetal adipose tissue, contributing also to the reduction of ^{13}C-DHA in cord blood. Our results are in accordance with the negative correlation between cord triglycerides and fetal growth in GDM offspring but not in healthy controls previously reported. Moreover, other authors found lower percentages of arachidonic acid and DHA in umbilical arterial but not in venous plasma of neonates of GDM than those from controls pointing toward an altered handling or metabolism of these fatty acids in neonates of GDM mothers. In our study, we did not find changes in the umbilical artery and vein difference between GDM subjects treated with diet or insulin, or between GDM *vs.* controls, probably because 12h was not enough time to detect differences in the fatty acid uptake by fetal tissues or because the major differences occur in NEFA and the ^{13}C determination in NEFA is less precise than in other lipid fractions due to the small NEFA concentrations in cord blood. Thus, with this tracer approach we cannot exclude enhanced accumulation of ^{13}C-DHA in fetal adipose tissue by GDM as reason for the lower fetal to maternal ^{13}C-DHA ratio. It would be interesting to evaluate LC-PUFA supplementation to neonates of GDM mothers with respect to neurodevelopmental and other outcomes.

In conclusion, the concentrations of labelled LC-PUFA were significantly lower in maternal, placental and fetal compartments in GDM compared to controls, reflecting differences in the DHA metabolism between healthy mothers and mothers with GDM. Placental LC-PUFA uptake is impaired in GDM and in combination with enhanced fetal fat accretion in more severe GDM under insulin treatment can well explain the reduced DHA levels in cord blood. More studies are needed to elucidate the benefit for LC-PUFA supplementation during this time of development and to establish the best strategy to supplement the mother, the fetus or both.

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