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# “Metabolic syndrome (MetS) and pregnancy”

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## Introduction

The increasing prevalence of overweight and obesity among women of childbearing age, is a growing public health problem in the world.[1] The nutritional status represents one of the most important factor that determines individual wellbeing, in particolar maternal- fetal health, during and after pregnancy. [2] Obese women compared to normal-weight have a higher risk of having reduced sensitivity to insulin. The combination of obesity and reduced insulin sensitivity, increases the long-term risk of developing the metabolic syndrome. The number of pre-obese women (25> BMI <30) and obese (BMI> 30) who become pregnant is definitely on the rise [3] . The state of nutrition is one of the most important determinant the welfare of the individual, especially of the maternal-fetal development; often even the flexibility of the metabolic response of the pregnant woman is able to correct for the imbalance and nutritional pregravid / or metabolic alterations induced by lifestyle; therefore, negative events can be correlated with being overweight.. For metabolic disorders evolved during a normal pregnancy, particularly showing a 60% reduction in insulin sensitivity, these women have a higher risk of metabolic disregulation and complications and adverse fetal outcomes, including preeclampsia, gestational diabetes, increased incidence of cesarean section, macrosomia and fetal death [4]. The metabolic

syndrome is a combination of cardiometabolic risk determinants such as obesity, insulin resistance, glucose intolerance, dyslipidemia, non-alcoholic fatty liver, hypertension. Pregnancy in these women may be seen as a metabolic stress test for the future risk of metabolic syndrome.

The prevalence of the metabolic syndrome is increasing worldwide as a result of obesity, with a significant impact on the incidence of cardiovascular disease and type 2 diabetes. [5]

**Definition and epidemiology of obesity**

Obesity is a chronic multifactorial etiology characterized by excessive body weight for accumulation of adipose tissue. Recently, the WHO (World Health Organization) has set new criteria for classifying obesity based on BMI (Body Mass Index), obtainable from the weight / height squared ( kg /m2) : as the upper limit of normal was set a BMI value of 24.9 Kg/m2 while were defined obesity I, II and III, respectively, including the values of BMI between 25 and 29.9Kg/m2, between 30 and 39.9Kg/m2 and more than 40Kg/m2.

Being overweight or obese, increases the risk of morbidity and mortality caused by the onset of other diseases such as diabetes mellitus type 2, hypertension, heart disease and several forms of cancer [6]. Obesity is the

most common nutritional disorder in the western world and its prevalence is on the increase even in countries in development[7]. Today there is a lot of concern about their body weight, in fact recent estimates indicate a prevalence of obesity in 15% of the European adult population, and in many regions is increasing. The most worrying data come from the U.S., where about half the population is overweight and about one-fourth net is obese. More specifically, recent data indicate that 22.5% of the population is obese versus 14.5% in 1980 [7-8]. Experts have called the explosion a veritable epidemic of obesity that has touched all demographic groups even children.

In Italy, the estimates derived from the first two quarters of the ISTAT survey (1999/2000), whereas the WHO classification [9] in the four classes: underweight (BMI <18.5), normal weight (BMI between 18.5 -24.99), overweight (BMI 25 to 29.99) and obesity (BMI ≥ 30) (4), indicates that the majority of adults, 53.8% is in a state of normal weight, well an adult three turns out to be overweight (33.4%), 9.1% is obese, and the remaining 3.6% are underweight. *(Tab.1)*

| | |
|---|---|
| BMI < 18.5 Kg/m2 | Underweight |
| BMI 18.5-24.9 Kg/m2 | Normal |
| BMI 25-29.9 Kg/m2 | Overweight |
| BMI >30 Kg/m2 | Obese |

**Tab 1. BMI score (WHO Classification).**

**Pathogenesis of obesity**

An understanding of the causes and ways through which obesity is spreading so rapidly, is the subject of increasing attention from reserchers and experts, who agree in emphasizing the existence of a strong interrelationship between genetic , physiological, metabolic, behavioral, and psychosocial factors.

Obesity is therefore the resultant of the variable combination of two elements: genetic susceptibility, ie of a genetic predisposition of the individual to become obese, and the presence of environmental factors (eg: easy access to high-energy foods, sedentary lifestyle).

It is believed that the genetic component impinges for the 30 - 40% and the environmental / behavioral for the remaining 60-70% (10)

In the years , research conducted to improve the understanding of the development of obesity have led ever more to consider genetic factors as key players in the onset of human Obesity stimulating numerous studies to

evaluate the role of family factors. There are many studies emphasize the high incidence of Obesity in children of obese parents: the numbers ranging from an incidence of around 40% if one parent is obese, over 70% if they are both.

However, it's important be taken into account of environmental factors. It is recognized that some genotypes thrive in a specific environment and less in another; and conversely a certain environment will exalt some manifestations and it will inhibit other. (11)

Obesity is widely recognized as a chronic disease, which can have a significant impact on collective health. It is the main factor responsible for non-insulin dependent diabetes mellitus and coronary heart disease, but also increases the risk of biliary disorders and certain cancers, and play a role in musculoskeletal disorders and in the respiratory diseases. Despite these evidence, the prevalence of obesity is growing, so that experts believe that obesity should be considered one of the greatest public health issue of our time.(12)

**Metabolic Syndrome**

Metabolic syndrome also known as **metabolic syndrome X**, **cardiometabolic syndrome**, **syndrome X**, **insulin resistance syndrome**, **Reaven's syndrome** is a disorder of energy utilization and storage, diagnosed by a co-occurrence of three out of five of the following medical conditions: abdominal (central) obesity, elevated blood pressure, elevated fasting plasma glucose, high serum triglycerides, and low high-density cholesterol (HDL) levels. Metabolic syndrome increases the risk of developing cardiovascular disease, particularly heart failure, and diabetes. Some studies have shown the prevalence in the USA to be an estimated 34% of the adult population, and the prevalence increases with age.

Therefore, the syndrome has been renamed "plurimetabolic", including the association of insulin resistance, central obesity, type 2 diabetes mellitus, dyslipidemia and hypertension. Many epidemiological studies have evaluated the various aspects of the metabolic syndrome evaluating them individually, and only some of them have considered a "cluster" of risk factors. [13]

The metabolic syndrome, according to the definition proposed by the World Health Organization (WHO) and then modified in part by the European Group for the Study of Insulin Resistance (EGIR), is characterized by the following risk factors:

**Insulin- resistance** defined by the presence of hyperinsulinemia and by high levels of glucose in fasting blood of at least 110 mg / dL and by the presence of at least two of the following criteria:

**abdominal obesity** defined on the basis of two definitions ***:***

- according to the original definition of the WHO, as waist-hip ratio> 0.90 or BMI≥ 30kg/m2;

- according to EGIR, as waist-hip ratio ≥ 94cm;

**dyslipidemia** with serum triglycerides ≥ 150 mg / dL (≥ 1.70 mmol / L) or HDL cholesterol <35 mg / dL (<0.90 mmol / L)

**hypertension** that is based on two definitions:

- according to the original definition of the WHO, with pressure values ≥ 160/90mmHg;

- according to the modification by EGIR, with pressure values of 140/90mmHg;

The clinical significance of the metabolic syndrome becomes particularly important when you consider that the set of metabolic disorders typical of this syndrome seems to play an essential role in the genesis of cardiovascular disease and diabetes mellitus type 2. In patients with metabolic syndrome there is a high mortality from ischemic heart disease. [(14)]

## Obesity and pregnancy

Obesity in pregnancy (pregravid body mass index ≥ 30) has been linked to several adverse pregnancy outcomes, including spontaneous abortion, preeclampsia, gestational diabetes, fetal macrosomia, cesarean delivery, and many complications post–cesarean section. Intrapartum and postpartum management of obese gravidas requires multidisciplinary consultations between obstetricians, anesthesiologists, nurses, and pediatricians in order to improve the pregnancy outcomes of the mother and neonate. [15]

The assessment of BMI in the preconception period allows the identification of pregnants will present an additional risk related to changes in metabolic parameters. The abnormalities of nutrition if not corrected have the ability to compromise the maternal-fetal health short-and long-term.

Maternal nutritional deficiencies are most commonly associated with preterm low, placental and neonatal low birth weight as confirmed by recent studies;[16] controversial are those relating to the risk of fetal and neonatal death.

A prominent study carried out between 89 and '96 has evaluated the association between pregravid BMI and obstetric outcome. In the study carried out on a total sample of 24. 505 singleton pregnancies was not significantly increased the incidence of stillbirth and neonatal death in

women with low BMI compared to obese women, who had twice the risk compared to the rest of the sample examined. [17]

It was also demonstrated that obesity and overweight is associated with an increased risk of gestational diabetes, pre-eclampsia, hypertension, eclampsia, macrosomia and fetal morbidity, preterm delivery, and cesarean section compared to normal weight pregnant women. [17-18]

Among the fetal complications appear to be increased in the obese population neural tube defects (NTDs) [19] and minor malformations such as cleft lip and palate, clubfoot, and cardiac defects.

Still controversial results regarding the incidence of complicated deliveries from shoulder dystocia and brachial plexus injuries.

Analyzing the most recent data in the literature is how many of these do not show an increased frequency of these complications in pregnant women with high BMI contrary to what proved to be older studies.

This failure to increase should be in relation with the highest percentage of caesarean sections performed in pregnancies complicated; this percentage increases with increasing weight of the pregnant woman.

However, there are studies which emphasize the idea that obstetric complications occur more frequently in infants born to mothers with obesity and / or diabetes; these conditions favoring the increase in fetal weight > 90 ° percentile. [20] In obese patients there are higher anesthetic complications

(difficult intubations, complicated and long procedures). Epidemiological studies have also found a direct correlation between high maternal weight , the weight of the child at birth and in adulthood demonstrating how pregravidi inadequate nutritional status of the mother may be a pathogenic factor responsible for the onset of obesity in future generations.

An appropriate weight gain during pregnancy can ensure the well-being of the mother and fetus, influencing the structure metabolic maternal-fetal unit.

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Abstract
**Obesity pregnancy and maternal-fetal outcomes.**
*Quaglia F. et al.*

*Introduction**:***

The prevalence of obesity in pregnant women is approximately 20-30%. This is an increasingly growing health problem. Obesity during pregnancy is considered a state high-risk because there is a correlation between pre-gestational BMI and adverse maternal and fetal outcomes,as gestational hypertension, gestational diabetes, the failure of induction of labour, preterm birth, intrauterine fetal death, first caesarean section, fetal growth restriction and macrosomia.

*Material and Methods*.

The main objective of the study was to correlate pre-gestational weight, measured by the body mass index (BMI), and gestational weight gain with NICU admission and fetal or neonatal death.

In our retrospective study, we included 35 singleton pregnancies of women with a BMI> 30 followed in our High-Risk pregnancy department between 2008 and 2013.

For each pregnant with single fetus were collected data about age, anthropometric measurements necessary for calculating the BMI,

information about obstetric anamnesis, complications and any medications taken during pregnancy. We collected also data on maternal diseases in particular diabetes, hypertension, preeclampsia. For the neonatal outcome were collected data on: sex, weight, Apgar scores at 1 and 5 minutes and admission at NICU. We compared pregestational BMI and gestational weight gain in cases with NICU admission versus controls; we also compared pregestational BMI and gestational weight gain in women with fetal or neonatal death and controls. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) Statistics v. 19 (IBM Inc., Armonk, New York, USA).

*Results*

In our population 12 women had babies admitted at NICU and 4 women had fetal or neonatal death. No statistical difference was observed in mean pregestational BMI between women with or without NICU admission (37.45; SD 6.74 vs 40.17; SD 8.07; p=0.37) and in women with or without fetal or neonatal death (35.85; SD 4.16 vs 39.93; SD 8.02; p=0.33). A statistical difference was observed in mean gestational weight gain weight between women with or without NICU admission (14.87; SD 16.67 vs 6.02; SD 7.33; $p<0.05$) and in women with or without fetal or neonatal death (23.00; SD 17.58 vs 6.12; SD 7.93; $p<0.05$).

*Conclusion*

Our results show that even if pregestational weight has been showed to be associated with adverse fetal and neonatal outcomes, gestational weight gain maybe is more important as risk factor for the developing of adverse outcomes in pregnancies complicated by obesity. Thus a strict medical control on maternal nutrition and weight gain should be done in pregnancies complicated by obesity.

*Submission will be in process.*

**The role of epigenetic in obese pregnant women.**

In the past fifty years, the prevalence of obesity in the world has increased at an alarming rate . In addition, recent data indicate that 30% of American women of reproductive age are obese and 8% achieved a degree of severe obesity , it strives to clarify the harmful effects of obesity in pregnancy. [(1)] Different critical periods during childhood appear to influence the subsequent development of obesity: the prenatal period, the stabilization of adiposity between 5 and 7 years old, and puberty. [(2)]

The prenatal period, ie from conception to birth is a period of rapid growth, replication and cell differentiation, and maturation of cellular and organ systems.

During pregnancy, the mother, placenta and fetus interact to modulate fetal growth. The fetal placenta ensures homeostasis playing a wide range of physiological functions. The alterations that modify one or more aspects of placental structure and function may have long-term repercussions on the health of the individual. [(3)]

For example, the placental weight and birth weight (correlated) may predispose to adult diseases such as obesity, type 2 diabetes and hypertension . In addition, changes in the structure placenta (placenta thicker change the barrier and increases the placental vascular resistance) are directly involved in the fetal programming of cardiovascular disease.[(3)]

The placenta may act as a sensor of nutrients, such as may change the availability of nutrients and hormones to the feto-placental tissues in relation to environmental conditions in order to maximize fetal growth, it also supports the pregnancy in order to ensure the delivery of a newborn able to live. [4]

The intrauterine growth retardation induced in animal models in different ways (including hyper-and hypo-nutrition, isocaloric modification of the content of macro-and micro-nutrients in the diet, maternal stress, hypoxemia, glucocorticoid administration, and restriction of blood flow uterine) causes functional postnatal abnormalities . Finally, obesity has been demonstrated an intense inflammatory response in the placenta and that, by changing the environment in which the fetus develops, it could have consequences in the short-and long-term effects on his health.

These observations suggest that adult diseases may originate in utero as a result of the development of intrauterine conditions that induce optimal permanent changes in the morphology and function of tissue, a process called "fetal programming or intrauterine". [4-6]

The fetal programming implies the plasticity of development, so as to allow the fetus to respond to environmental influences present or planned, with adaptive mechanisms (ie changing its metabolism and growth). The process by which an individual in development work permanent changes

advantageous for the future life, is known as "response presumed adaptation". Such fetal response may be inappropriate if the post-natal conditions are different from those expected, and this discrepancy can cause a disease in the adult. [7] Since the placenta is the active interface between the maternal and fetal blood, alterations in the levels of nutrients, cytokines, hormones, and reduced utero placental flow, must be transmitted through the placenta to the fetus in order to influence.

The mechanism that binds an intrauterine environment unfavorable to the disease of the adult is still in the study, however, studies in animal models have shown that the epigenetic regulation (eg. DNA methylation) of fetal genes in metabolic pathways key is an important mechanism of fetal programming. [6]

In humans, have been also identified non-coding RNA imprinted genes, although their role has not yet been clarified is hypothesized to play an important role in some pathological conditions (eg, cancer, and Prader-Willi syndrome, a disease associated with the latter obesity). [8]

In addition, altered expression of miRNAs (non-coding RNA) (compared to control placentas) was detected in human placentas of women with pre-eclampsia, suggesting that the down-regulation of genes potential targets can contribute to this pathology. [9-10] In conclusion, there is evidence to show

that the epigenetic regulation of fetal genes could be an important mechanism that mediates the fetal programming of obesity.

In view of the following findings, we therefore assessed in placentas and amniotic mesenchymal stromal cells (hAMSCs) isolated at birth from placentas of obese pregnant women, the presence of epigenetic mechanisms of gene regulation (DNA methylation, miRNAs) different from those observed pregnant women in non-obese and that may represent risk factors for obesity in the neonate.

**miRNA-mediated gene regulation is different in placental tissues from obese and not obese pregnant women at delivery.**

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Epidemiological studies indicated that human adult diseases can be originated in uterus, as a result of changes in development during suboptimal intrauterine conditions that could alter the structure and function of the tissues. This process is called "fetal or intrauterine programming". Experimental data indicate that the epigenetic regulation of fetal genes could be an important mechanism of the fetal programming of obesity. The aim of this study was to evaluate, in the placentas from obese and not obese pregnant women at delivery, the presence of a different miRNA-mediated regulation of the gene transcription. We recruited 5 not obese (BMI<30 $kg/m^2$) and 10 obese (BMI>30 $kg/m^2$) pregnant women, to the term of the pregnancy. Total RNA was purified from sections of placentas collected during the Caesarean delivery. A panel of 365 human miRNAs was evaluated by the TaqMan Array Human MicroRNA Panel v1.0 (Applied Biosystems) system. By the TargetScan program we selected the target

genes of the miRNAs differently expressed in obese and not obese pregnant women, while the Gene Ontology program was used to identify the biological processes in which such genes were predicted to be involved. The results evidenced that: 1) 78% of the miRNAs studied were expressed in the placental tissue; 2) 48% of the miRNAs showed a different expression in obese and not obese placentas; 3) gene targets of the overexpressed miRNAs belonged to the TGFβ- and insulin-signalling pathways.

## Introduction

Obesity in pregnancy is increasing worldwide to almost epidemic proportions in developed countries and it is associated with increased risk of adverse outcomes for both mother and child.[1] Maternal obesity and alteration in postnatal nutrition are associated in the offspring with increased risks for obesity, and for obese associated diseases such as hypertension and type 2 diabetes.[2] The process by which the structure and function of tissues could be altered permanently by insults acting during intrauterine development is called "intrauterine or fetal programming".[3] The in utero environment can substantially modify the fetal genome expression and exert stimulatory or inhibitory effects on fetal growth and adiposity.[4] The mechanisms, whereby maternal obesity and nutrient excess in utero, in part increased risk for future metabolic disease, are poorly understood but likely include changes in fetal

nutrient supply in combination with genetic and epigenetic mechanisms.[4] Epigenetics investigates mechanisms of inheritance which are not associated with changes in the gene sequence. The major epigenetic processes are DNA methylation, hystone modifications and microRNAs (miRNAs).[5-7] MiRNAs are a class of endogenous non-coding RNA molecules, 18-25 nucleotides long that exert a tissue-specific regulation in a variety of biological and pathological processes such as adipocyte differentiation, insulin resistance, appetite regulation, diabetes and cancer.[8] Altered miRNAs expression in human placenta has been involved in preeclampsia or small for gestational age (SGA) newborns.[9,10] Further, the potential role of maternal serum miRNAs as marker for fetal congenital heart defects has also been described.[11] It follows that the study of miRNA-regulated pathways during pregnancy both in normal weight and obese women could highlight patogenetic mechanisms underlying this metabolic disorder and address therapeutic approaches to prevent obesity insurgence in newborns. Our aim was to characterize the miRNA expression profile of amnion from obese and non-obese pregnant women at delivery in order to define a miRNA-signature associated with obesity and to evidentiate metabolic pathway potentially deregulated by this mechanisms. We focused on the amnion because alterations in this fetal membrane could have important consequences for the growth and intrauterine programming of the fetus.

## Materials and Methods

*Patients*

Fifteen Caucasian pregnant women were enrolled in this study. The women were divided into two groups based on body mass index (BMI) before pregnancy: non-obese pregnant women <25Kg/$m^2$ (n=5) and obese pregnant women >30Kg/$m^2$ (n=10). Clinical and anamnestic mother's data were collected before the delivery. Cancer, viral infection and drug abuse were exclusion criteria. Maternal blood was obtained immediately before delivery and was centrifuged at 2500 rpm for 15 minutes, then the serum sample was stored at -80 °C until using. A cord blood sample was collected at delivery and within 1h from the collection cord plasma and mononuclear cells (MNC) were obtained by Ficoll density gradient centrifugation. Cord plasma and MNCs were immediately recovered and cryopreserved at -20°C and in liquid nitrogen, respectively.

Human term placentas were collected from all pregnant women. The samples were obtained after caesarean section and were immediately processed. Firstly, the maternal decidua was removed, and then the amnion was manually separated from chorion and washed in phosphate-buffered saline (PBS, Sigma-Aldrich, St Louis, USA). Finally, the amnion was mechanically minced into small pieces and immediately cryopreserved in liquid nitrogen.[12]

The MNC isolated from cord blood and visceral and subcutaneous adipose tissues collected from all pregnant women were not used for this study.

Newborns general characteristics, that are birth weight, length, head circumference, Apgar score (Appearance, Pulse, Grimace, Activity, Respiration) were registered immediately at birth. Each pregnant women provided written informed consent for herself and her newborn to the study and the research protocol was approved by the Ethics Committee of our Faculty of Medicine.

*Laboratory investigations*

Main biochemical parameters, leptin and adiponectin were measured respectively by routine laboratory methods and Luminex xMAP Technology on a BioRad Multiplex Suspension Array System (Bio-Rad, Hemel Hempstead, Herts), according to the manufacturer's instructions both in mother's serum and in cord plasma.

*RNA isolation*

Total RNA (including miRNAs) was purified from amnion using the mirVana$^{TM}$ miRNA isolation kit (Ambion) and its concentration was evaluated by NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The RNA isolated from amnion of non-obese pregnant women was pooled to set up a control pool RNA.

*MiRNA expression profile*

TaqMan low density arrays (TLDA), micro fluidic cards were used to detect and quantify mature miRNAs according to manufacturer's instructions. Each TLDA Human MicroRNA Panel v1.0 card contained 365 preloaded human miRNA targets and two endogenous controls (small nuclear RNAs: RNU48 and RNU44). TLDAs were prepared in two-steps. In the first step, 640 ng of total RNA were reverse transcribed in eight multiplex reverse transcriptase (RT) reaction pools using stem loop RT primers specific for mature miRNA species. Then, each of the resulting eight cDNA pools was diluted, mixed with TaqMan Universal PCR master mix, and loaded into one of the eight fill ports on the TLDA microfluidic card. The card was centrifuged for 2 min at 1200 rpm to distribute samples to the multiple wells of the fill ports and sealed to prevent well-to-well contamination.

Finally, the cards were processed on an 7900 HT Real-Time PCR System (Applied Biosystems). The miRNA expression values were normalized to RNU48 (endogenous control), and relative expression values were obtained using the ΔΔCT method (Relative Quantification, $RQ=2^{-\Delta\Delta CT}$) with Sequence Detection System (SDS) v2.3 and RQ Manager 1.2 software (Applied Biosystems). To further normalize our miRNA data and to minimize interindividual variability, we considered differently expressed the

miRNAs whose mean RQ levels were <0.5 (down-expressed) or >2.0 (up-expressed) in at least 7/10 obese pregnant women vs the control pool.

*Bioinformatic analysis*

Biological targets of miRNAs differently expressed in obese pregnant women vs the control pool were predicted using the TargetScan Release 6.2 algorithm (http://www.targetscan.org). This algorithm assigns a "total context score" for each predicted target. Target genes with an "total context score" <-0.30 were further analyzed using the KEGG database (http://www.genome.ad.jp/kegg/) to identify the pathways involving the target genes of miRNAs. By this latter program we selected the biological pathways that contained at least two predicted genes to be miRNA-altered in obese samples with a statistically significant probability ($P<0.001$).

*Statistical analysis*

All variables were expressed as mean ± standard error of the mean (SEM). Student "t" test was used to compare group means and P values <0.05 were considered significant. Statistical analyses were carried out by using the PASW Statistics (Ver.18; SPSS Inc. Headquarters, Chicago, Ill).

**Results**

Higher values of leptin (*P*-value=0.01) and L/A ratio (*P*-value =0.001) were measured in obese vs non-obese pregnant women. Instead, the adiponectin serum levels were lower, even if not at statistically significant level. The weight gain and the total cholesterol levels were significantly lower in obese than in non-obese pregnant women (*P*-value=0.008 and *P*-value 0.016). In addition, among parameters evaluated in newborns we found that only the cord plasma adiponectin was statistically different with lower levels in the newborns from obese mothers than those from non-obese mothers (*P*-value=0.004).

Alarge percentage of the tested miRNAs about 74% (271/365) was expressed in amnion, whereas 26% (94/365) was not. Most of the expressed miRNAs (90% 243/271) did not differ between obese women and controls and were not further investigated. Conversely. 4.8% of miRNAs (13/271) were higher (Fig 1a) and 4.4% (12/271)were lower (Fig.1b) in obese women than in controls.

Interestingly, 7/271 miRNAs (miR-422b, miR-219, miR-575,miR-523, miR-579, miR-618 and miR-659) were obesity-specific, being expressed only in amnion from obese women.

Bioinformatics showed that miRNAs had a significant probability ($P<0.01$) of deregulating several genes and metabolic pathways (Figures 1c, d and 2).

The expression levels of several miRNAs were further validated by quantitative real-time polymerase chain reaction and, except for slight differences because of the two different methodologies, they were in close agreement with those obtained with TaqMan array (mean RQ values in obese vs control: miR-422b¼6.4;miR-23b¼1.19; miR-338¼1.25; miR-139¼0.68; miR-449b¼0.9).

## Discussion

The present study is the first to evaluate the miRNAs profiling in human amnion of obese pregnant women. Previous reports revealed that altered miRNA expression profile in placenta may have important implications for several placental injuries such as preeclampsia, hypoxia, fetal growth restriction, preterm labor and choriocarcinoma.[9,10,13] No data are yet available in literature regarding differential expression of miRNAs in placentas of obese women, so our aim has been to characterize the miRNA expression profile of amnion from obese and non-obese pregnant women at delivery. Furthermore, in our study population, we also evaluated some biochemical parameters and the leptin and adiponectin levels. In Table 1 are reported the clinical and biochemical characteristics of obese and non-obese women, and of their respective children.

In According to Gestational Weight Gain (GWG) guidelines US Institute of Medicine (IOM) published on 2009, we found an higher weight gain in non-obese women than obese during pregnancy.[14] In our obese group we found the increased leptin and decreased adiponectin levels, normally associated with elevated body mass index.[15] Leptin is an adipokine mainly produced by adipose tissue, its major biological role is regulation of energy balance, regulating food intake, glucose uptake, insulin secretion and action. Leptin is also secreted by placenta and so its concentrations increases during pregnancy.[16] In fact, an increase of four fold of leptin levels is measured in our non-obese pregnant women than non-obese women without pregnancy (these latter were previously analyzed in another our study). Instead, in our obese pregnant women we observed an increase of about seven fold than obese women without pregnancy (these latter were previously analyzed in another our study), probably due to additive effect of obesity and pregnancy. Our results are in agreement with previous studies, that showed a higher serum leptin concentrations in overweight pregnant women when compared to normal weight.[17] As previously reported, it is possible that exposure to high or low concentrations of leptin, during critical periods of development of the neural network that regulates appetite may be have longer term consequences for this network and for the regulation of energy balance after birth.[18]

Adiponectin is another adipokine produced by adipose tissue that acts as an antidiabetic, anti-inflammatory and antiaterogenic hormone. Previous reports showed no differences in adiponectin levels between non pregnant and pregnant women, suggesting that the placenta might not be the primary source of circulating adiponectin in pregnant women and that adipocytes play a major role in adiponectin production.[19] This finding supports our lower adiponectin levels observed in our obese than non-obese pregnant women. We also observed a significantly lower levels of adiponectin in cord plasma of newborns from obese than non-obese mothers, agreeing with a previous report in which the authors suggested that, due to lower circulating anti-inflammatory cytokines, fetuses from obese women may be less able to control the inflammatory process and affecting the optimal fetal development.[20] Furthermore, we found that the obese pregnant women showed a lower levels of total cholesterol than non-obese women. These findings are previously reported by Varhatian A. et al. that observed a different lipid profile during pregnancy in obese than normal weight women. In particular, these authors found in the third-trimester of pregnancy lower total and LDL cholesterol levels in overweight or obese than normal-weight women, probably due to metabolic deregulation associated with maternal obesity that could affect the fetus predisposing it to metabolic diseases.[21]

It is now recognized that epigenetic modifications of the fetal genome contribute to disease development in adults (7). Here, we show that miRNAs regulate gene expression in the amnion during obesity. In particular, we found that 25 miRNAs are differently expressed in obese with respect to control women, and have identified seven obesity-specific miRNAs.

Our differently expressed placental miRNAs include miRNAs previously associated with endometriosis (miR-196b, let-7d and miR-107), preeclampsia (miR-181 and let-7d),(9-10) cancer(miR-422b) or identified in sera from normal pregnancies (miR-23b,miR-372 and miR-519e). The predicted target genes of ourmiRNAs belong to pathways potentially important for placental development and/or functions. In fact, the neurotrophin pathway was regulated by three obesity-specific miRNAs (miR-422b, miR-659 and miR-575), six miRNAs upexpressed (miR-196b, miR-96, miR-338-3p, miR-372, miR-492 and miR-107) and four miRNAsdownexpressed (miR-591, miR-139, miR-624 and miR-554).

The brain-derived neurotrophic factor was recently implicated in placental development and fetal growth in mice and its placental expression was increased in intrauterine growth restriction and decreased in maternal type 1 diabetes. Obesity-specific miRNAs (miR-422b, miR-659 and miR-219) also downregulated the mammalian target of rapamycin (mTOR) and the insulin

signaling pathways. mTOR is highly expressed in syncytiotrophoblasts of the human placenta and exists as two complexes with distinct regulation and function based on its association with accessory proteins: raptor in Complex 1 (mTORC1) and rictor in Complex 2 (mTORC2). (Fig.2)

Some of these signaling pathways have been demonstrated to have important roles in placental development and functions. For example, TGF-□ signaling exerts several modulatory effects on trophoblast cells such as inhibition of proliferation and invasiveness, stimulation of differentiation.[22] An altered expression of TGF-□1 was found in fetal growth restriction pre-eclamptic placentae.[23]

In humans, Wnts and GnRH signaling promote decidualization, endometrial function and trophoblast differentiation. Changes in Wnt signaling components were noticed in cancers of reproductive tissues, in endometriosis and in gestational diseases.[24, 25]

Previous reports showed that also members of MAPKs family as ERK1/2, were expressed in the placenta, until the 12$^{th}$ week of gestation, suggesting a predominant role during early pregnancy;[26] furthermore, its activation was found elevated also during the early stages of adipogenesis.[27]

Another study indicated that ERK was increased in placentas from patients with pre-eclampsia.[28] Further investigations demonstrated an altered placental expression of insulin signaling components in insulin controlled

GDM (gestational diabetes mellitus) in comparison with normal pregnant controls in both obese and non-obese cohorts, changes were also detected in normal obese pregnant women than normal non-obese.[29] Placental expression of several insulin signaling components were also reduced in pregnancies complicated by IUGR (intrauterine growth restriction).[30]

The cytoskeleton plays a crucial role in basic cellular processes, such as mitosis, growth, motility, aging, smooth muscle contraction, and apoptosis. The reorganization of the cytoskeleton is involved in decidualization,[31] and so an altered regulation of actin cytoskeleton signaling was observed in preeclamptic placentae.[32]

Regulation of epithelial organization, structure, and subsequent function is modulated by two forms of junctional complexes, tight junctions (TJs) and adherens junctions. Amniotic TJs may be regulated by several factors in the amniotic fluid and they may be involved in normal or abnormal (oligohydramnios and polyhydramnios) volume changes of the amniotic fluid.[33] Has been found a decreased expression of some tight junction proteins in preclamptic human term placentae.[34]

The adherens junction has a considerable effect on endothelial integrity and has been implicated in the stability of junctions, angiogenesis and intracellular signaling, therefore they are important in understanding how the

placental endothelial barrier is regulated during pregnancy in health and in disease.[35]

The intracellular cytoskeleton is connected to the extracellular matrix by the focal adhesion complexes that transmit force at cell adhesion site and serve as signaling centers from which numerous intracellular pathways can regulate cell growth, proliferation, survival, gene expression, development, tissue repair, migration, and invasion.[36,37] Previous studies in rodent myometrium have demonstrated that focal adhesion signaling is activated at late pregnancy.[36]

Recent evidence indicated that in the development of the feto-placental unit may also be implicated the neurotrophins. In mice, in fact, it has been shown that the brain-derived neurotrophic factor (BDNF) exerts autocrine/paracrine roles in the placenta, potentiates both placental development and fetal growth from mid gestation to late gestational stages. The placental BDNF/TrkB signaling system is modulated in pregnancies with fetal growth perturbations, in fact, in IUGR the placental BDNF expression was increased while its expression was decreased in maternal type 1 diabetes.[38]

An altered expression of some proteins involved in selective proteolysis via the ubiquitin-proteasome system was revealed in placenta from IUGR and preeclamptic pregnant women.[39]

Furthermore, among dysregulated pathways in our patients, we also found some associated with cardiovascular diseases. An increased susceptibility to myocardial injury in offspring from obese dams has been previously described.[40]

More recent studies suggested that obesity also results in cognitive impairment and the mechanisms involved include hyperglycemia, hyperinsulinemia and vascular damage to the central nervous system. [41] In particular, leptin regulates hippocampal plasticity by converting short term potentiation in long term potentiation, a functional enhancement that may contribute to leptin's ability to improve hippocampal-dependent behavioral performance.[42] Hypertriglyceridemia, observed in obesity, can impair the transport of leptin across the blood-brain barrier causing the cognitive disturbances in diet-induced obesity.[41]

In conclusion, the results of the present study reveal, for the first time, an altered miRNA expression in human amnion from obese women. This suggests that the differently expressed miRNAs could deregulate the pathways above mentioned and have therefore potential effects on fetal development, increasing the risks of metabolic diseases in adulthood. Further studies are needed to corroborate our hypotheses and to clarify the molecular mechanisms involved in fetal programming of obesity. It would be interesting to monitor the children measuring anthropometrics and

biochemical parameters, to evidence risk factors predisposing to obesity in children from obese mothers.

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SHORT COMMUNICATION

# Characterization and predicted role of the microRNA expression profile in amnion from obese pregnant women

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Maternal obesity and nutrient excess *in utero* increase the risk of future metabolic diseases. The mechanisms underlying this process are poorly understood, but probably include genetic, epigenetic alterations and changes in fetal nutrient supply. We have studied the microRNA (miRNA) expression profile in amnion from obese and control women at delivery to investigate if a specific miRNA signature is associated with obesity. The expression profile of 365 human miRNAs was evaluated with the TaqMan Array in amnion from 10 obese and 5 control (prepregnancy body mass index (BMI) $>30$ and $<25\,kg\,m^{-2}$, respectively) women at delivery. Target genes and miRNA-regulated pathways were predicted by bioinformatics. Anthropometric and biochemical parameters were also measured in mothers and newborns. Seven miRNAs were expressed only in obese women (miR-422b, miR-219, miR-575, miR-523, miR-579, miR-618 and miR-659), whereas 13 miRNAs were expressed at a higher level and 12 miRNAs at a lower level in obese women than in controls. MicroRNAs significantly downregulated the neurotrophin, cancer/ErbB, mammalian target of rapamycin, insulin, adipocytokine, actin cytoskeleton and mitogen-activated protein kinase signaling pathways. In conclusion, we show that the miRNA profile is altered in amnion during obesity and hypothesize that this could affect pathways important for placental growth and function, thereby contributing to an increase in the newborn's risk of future metabolic diseases.

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**Keywords:** pregnancy; miRNA; pathway

## INTRODUCTION

Maternal obesity and nutrient excess *in utero* increase the risk of future metabolic diseases.[1] The mechanisms underlying this process are poorly understood, but probably include genetic, epigenetic alterations and changes in fetal nutrient supply.[1] Placenta, and particularly amnion, is the *in utero* environment that could modify fetal growth and adiposity by exerting stimulatory or inhibitory effects on fetal genome expression.[1] Examples of epigenetic mechanisms are DNA methylation, histone modifications and microRNAs (miRNAs),[1] the latter being a class of endogenous non-coding RNA molecules (18–25 nucleotides long) that exert tissue-specific regulation in a variety of biological and pathological processes.[2] The expression of placental miRNAs is altered during pregnancy in such pathological conditions as preeclampsia, small-for-gestational-age newborns[3] and fetal congenital heart defects,[4] but their role in obesity has not yet been investigated. We previously reported that miRNAs were implicated in the deregulation of metabolic pathways in subcutaneous and visceral adipose tissues in obese adults.[5,6] The aim of this study was to analyze the miRNA expression profile in amnion from obese and control women at delivery to determine if a specific miRNA signature could be associated with obesity.

## MATERIALS AND METHODS

### Patients and samples collection

Fifteen Caucasian pregnant women (5 controls and 10 obese subjects of a similar age (mean/s.e.m.: 29/1.3 and 31.1/1.0 years, $P=$ NS, respectively) and prepregnancy body mass index (BMI) (mean/s.e.m.: 22.8/1.4 and 38.7/1.4 $kg\,m^{-2}$, respectively)) were consecutively enrolled at the Dipartimento Neuroscienze/Scienze Riproduttive/Odontostomatologiche (University of Naples Federico II). The exclusion criteria were neoplasia, viral infection, diabetes and drug assumption. One maternal blood sample and one cord blood sample were collected at delivery. Sera and cord plasma were obtained and stored at $-80\,^{\circ}C$ until required for the measurement of biochemical parameters and leptin/adiponectin concentrations by routine methods and immunoassay (Bio-Rad, Hemel Hempstead, Herts, UK), respectively.

The term placentas, collected at delivery by cesarean section, were processed immediately. The maternal decidua was removed, the amnion was first manually separated from the chorion, washed in phosphate-buffered saline (Sigma-Aldrich, St Louis, MO, USA), mechanically minced into small pieces and immediately cryopreserved in liquid nitrogen. The newborn's weight, length, head circumference and Appearance-Pulse-Grimace-Activity-Respiration score (Apgar 1 and 5 min) were recorded at birth. All patients and controls gave their informed consent to the study and both parents gave consent for their newborns. The study was performed according to the Helsinki II Declaration and was approved by the Ethics Committee of our Faculty.

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### RNA isolation

Total RNA (including miRNAs) was purified from amnion (mirVana miRNA Isolation Kit; Ambion, Austin, TX, USA) and quantified by NanoDrop ND-1000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

### MiRNAs detection

We studied the miRNA profiles by the TaqMan low-density arrays human MicroRNA Panel v.1.0 (Applied Biosystems, Foster City, CA, USA) that contains 365 preloaded miRNAs and two endogenous controls (RNU48 and RNU44). We used 640 ng of total RNA for reverse transcription. Reverse transcription–polymerase chain reaction was performed using the 7900 HT Real-Time PCR System (Applied Biosystems). The miRNA expression values were normalized to RNU48 and to RNA isolated from controls as calibrator; relative expression values were obtained using the ΔΔCt method (relative quantification, $RQ = 2^{-\Delta\Delta Ct}$) by sequence detection system v.2.3 and RQ Manager 1.2 software (Applied Biosystems). To normalize our miRNA data further and to minimize interindividual variability, we considered the miRNAs whose mean RQ levels were $<0.5$ (downexpressed) or $>2.0$ (upexpressed) in 70% of the obese vs control women to be expressed differently. The expression levels of selected miRNAs (miR-422b, miR-23b, miR-338, miR-449b and miR-139) were also validated by single TaqMan miRNA assay (Applied Biosystems) in accordance with the manufacturer's instructions on the 7900 HT Real-Time PCR System.

### Bioinformatic analysis

TargetScan Release 6.2 algorithm (http://www.targetscan.org) and the KEGG database (http://www.genome.ad.jp/kegg/) were used to predict biological targets of miRNAs and to select biological pathways, respectively.

### Statistical analysis

All variables were expressed as mean and s.e.m. Student's *t*-test was used to compare groups and *P*-values $<0.05$ were considered statistically significant. Statistical analyses were carried out with the PASW Statistics (v. 18; SPSS Headquarters, Chicago, IL, USA).

## RESULTS

Obese and control women differed in weight gain (mean/s.e.m.: 7.6/1.0 and 15.0/2.6 kg) and total cholesterol levels (mean/s.e.m.: 5.8/0.4 and 7.3/0.2 mmol $l^{-1}$), which were significantly lower ($P = 0.008$ and 0.016) in obese women than in controls. In addition, levels of triglycerides (mean/s.e.m.: 3.1/0.2 and 2.4/0.3 mmol $l^{-1}$, $P = 0.048$) and leptin (mean/s.e.m.: 47.7/10.4 and 12.4/3.9 ng $ml^{-1}$, $P = 0.010$) were significantly higher in obese women than in controls.

Newborns of obese and control women showed similar birth weight (mean/s.e.m.: 3.268/0.374 and 3.340/0.190 kg), length (mean/s.e.m.: 50.4/1.1 and 50.4/0.7 cm), head circumference (mean/s.e.m.: 33.9/0.7 and 34.5/0.7 cm) and Apgar scores (mean/s.e.m. (1–5 min): 7.3/0.4–8.5/0.2 and 8.0/0.3–8.8/0.2), respectively. Cord plasma adiponectin levels were significantly lower in newborns of obese women than in newborns of controls (mean/s.e.m.: 47.0/7.7 and 107.9/18.9 μg $ml^{-1}$, respectively, $P = 0.004$), whereas cord plasma leptin levels did not differ between the two groups.

A large percentage of the tested miRNAs, about 74% (271/365), was expressed in amnion, whereas 26% (94/365) was not. Most of the expressed miRNAs (90%, 243/271) did not differ between obese women and controls and were not further investigated. Conversely, 4.8% of miRNAs (13/271) were higher (Figure 1a) and 4.4% (12/271) were lower (Figure 1b) in obese women than in controls. Interestingly, 7/271 miRNAs (miR-422b, miR-219, miR-575, miR-523, miR-579, miR-618 and miR-659) were obesity-specific, being expressed only in amnion from obese women. Bioinformatics showed that miRNAs had a significant probability ($P<0.01$) of deregulating several genes and metabolic pathways (Figures 1c, d and 2).

The expression levels of several miRNAs were further validated by quantitative real-time polymerase chain reaction and, except for slight differences because of the two different methodologies,

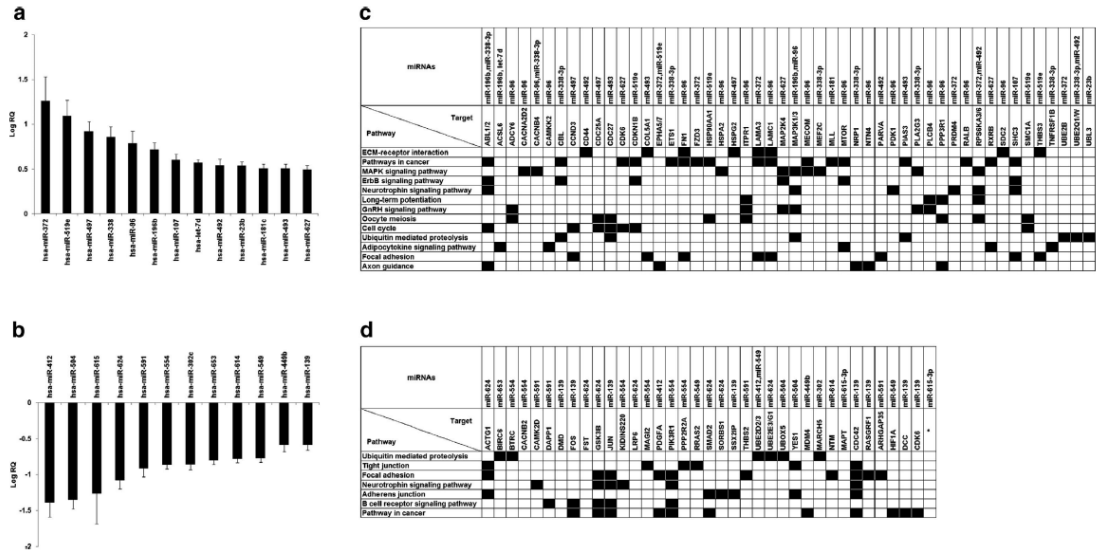


**Figure 1.** Differently expressed miRNAs in amnion from obese and control women. Panels (**a**) and (**b**) depict miRNAs upexpressed (13/271) and downexpressed (12/271), respectively. miRNA expression levels are shown as Log RQ mean and s.e.m. (The miRNA expression values were first normalized to RNU48, after which, the relative quantification was calculated as: $RQ = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{obese}\ (miRNA) - Ct_{obese}\ (RNU48)) - (Ct_{calibrator}\ (miRNA) - Ct_{calibrator}\ (RNU48))$). Panels (**c**) and (**d**) show metabolic pathways (black squares) and genes targeted by miRNAs ($P<0.01$) upexpressed and downexpressed, respectively. *Pathways significantly regulated by target genes of miR-615-3p were not reported by TargetScan.

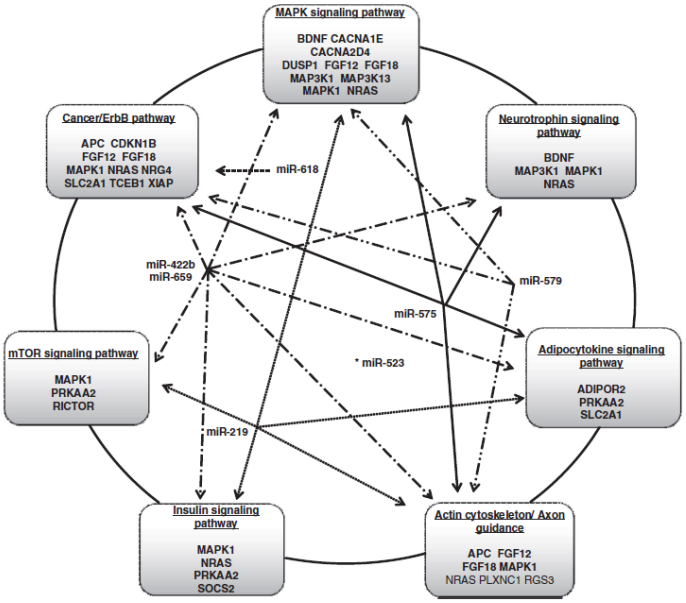


**Figure 2.** Pathways and genes predicted by bioinformatics to be regulated by obesity-specific miRNAs in the amnion. In boxes are reported the metabolic pathways and genes targeted by obesity-specific miRNAs (miR-422b, miR-219, miR-575, miR-579, miR-618 and miR-659) ($P<0.01$). The arrows connect miRNAs to target genes in each pathway. *Conserved target genes of miR-523 were not predicted by TargetScan.

they were in close agreement with those obtained with TaqMan array (mean RQ values in obese vs control: miR-422b = 6.4; miR-23b = 1.19; miR-338 = 1.25; miR-139 = 0.68; miR-449b = 0.9).

## DISCUSSION

It is now recognized that epigenetic modifications of the fetal genome contribute to disease development in adults.[7] Here, we show that miRNAs regulate gene expression in the amnion during obesity. In particular, we found that 25 miRNAs are differently expressed in obese with respect to control women, and have identified seven obesity-specific miRNAs.

Our differently expressed placental miRNAs include miRNAs previously associated with endometriosis (miR-196b, let-7d and miR-107),[8] preeclampsia (miR-181 and let-7d),[9,10] cancer (miR-422b)[11] or identified in sera from normal pregnancies (miR-23b, miR-372 and miR-519e).[12,13] The predicted target genes of our miRNAs belong to pathways potentially important for placental development and/or functions. In fact, the neurotrophin pathway was regulated by three obesity-specific miRNAs (miR-422b, miR-659 and miR-575), six miRNAs upexpressed (miR-196b, miR-96, miR-338-3p, miR-372, miR-492 and miR-107) and four miRNAs downexpressed (miR-591, miR-139, miR-624 and miR-554). The brain-derived neurotrophic factor was recently implicated in placental development and fetal growth in mice[14] and its placental expression was increased in intrauterine growth restriction and decreased in maternal type 1 diabetes.[14]

Obesity-specific miRNAs (miR-422b, miR-659 and miR-219) also downregulated the mammalian target of rapamycin (mTOR) and the insulin signaling pathways. mTOR is highly expressed in syncytiotrophoblasts of the human placenta and exists as two complexes with distinct regulation and function based on its association with accessory proteins: raptor in Complex 1 (mTORC1) and rictor in Complex 2 (mTORC2).[15] A multitude of upstream regulators of mTORC1, including insulin, cellular energy and oxygen levels, could affect placental size and modulate fetal growth by regulating placental nutrient transporter function.[15] Conversely, mTORC2 is involved in lipid metabolism; in fact, in *C. elegans* loss of rictor function causes developmental delay, reduced size and reproduction, and increased fat accumulation.[16] Here, we highlight a new potential mechanism based on miRNA downregulation of mTOR that could impact on fetal growth during obesity. Mammalian mTORC2 controls the actin cytoskeleton organization, as shown by the finding that its knockdown prevents actin polymerization.[17] The cytoskeleton has a crucial role in basic cellular processes and in decidualization,[18] and the downregulation of placenta actin cytoskeleton signaling was observed in preeclampsia.[19] In our study, the actin cytoskeleton pathway was predicted to be downregulated by obesity-specific miR-422b, miR-659, miR-575 and miR-579, which indicates that the placental structural organization and function are also altered in human obesity.

We also found that adiponectin levels were higher in umbilical cord plasma than in the mother's serum, and lower in obese than in control samples, in agreement with a previous report.[20] Hypoadiponectinemia might have a role in placental development and in pregnancy-related complications,[20] and also through p38 mitogen-activated protein kinase signaling activation. In this study, both the adipocytokine and mitogen-activated protein kinase signaling pathways were downregulated by obesity-specific miR-422b, miR-659, miR-579, miR-575 and miR-219, which supports the concept that epigenetic regulation of placental adiponectin and of its signaling could be involved in the pathophysiology of obesity during pregnancy.

In conclusion, we show that the miRNA profile is altered in amnion during obesity and hypothesize that this could affect pathways important for placental growth and function, thereby contributing to an increase in the newborn's risk of future metabolic diseases.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# High Aminopeptidase N/CD13 Levels Characterize Human Amniotic Mesenchymal Stem Cells and Drive Their Increased Adipogenic Potential in Obese Women

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Maternal obesity is associated to increased fetal risk of obesity and other metabolic diseases. Human amniotic mesenchymal stem cells (hA-MSCs) have not been characterized in obese women. The aim of this study was to isolate and compare hA-MSC immunophenotypes from obese (Ob-) and normal weight control (Co-) women, to identify alterations possibly predisposing the fetus to obesity. We enrolled 16 Ob- and 7 Co-women at delivery (mean/SEM prepregnancy body mass index: 40.3/1.8 and 22.4/1.0 kg/m$^2$, respectively), and 32 not pregnant women. hA-MSCs were phenotyped by flow cytometry; several maternal and newborn clinical and biochemical parameters were also measured. The expression of membrane antigen CD13 was higher on Ob-hA-MSCs than on Co-hA-MSCs ($P<0.005$). Also, serum levels of CD13 at delivery were higher in Ob- versus Co-pregnant women and correlated with CD13 antigen expression on Ob-hA-MSCs ($r^2=0.84$, $P<0.0001$). Adipogenesis induction experiments revealed that Ob-hA-MSCs had a higher adipogenic potential than Co-hA-MSCs as witnessed by higher peroxisome proliferator-activated receptor gamma and aP2 mRNA levels ($P<0.05$ and $P<0.05$, respectively), at postinduction day 14 associated with increased CD13 mRNA levels from baseline to day 4 postinduction ($P<0.05$). Adipogenesis was similar in the two sets of hA-MSCs after CD13 silencing, whereas it was increased in Co-hA-MSCs after CD13 overexpression. CD13 expression was high also in Ob-h-MSCs from umbilical cords or visceral adipose tissue of not pregnant women. In conclusion, antigen CD13, by influencing the adipogenic potential of hA-MSCs, could be an in utero risk factor for obesity. Our data strengthen the hypothesis that high levels of serum and MSC CD13 are obesity markers.

## Introduction

The increase in the incidence of obesity in pregnant women in the last two decades has paralleled that observed in the general population [1–3]. Although maternal fat stores increase in all pregnant women, irrespective of prepregnancy weight [4], the storage capacity of subcutaneous adipose tissue is impaired, and fat predominantly accumulates in the visceral adipose tissue (VAT) in obeses [5]. VAT is an important risk factor for metabolic imbalance in human subjects, also during pregnancy [6–8]. In fact, maternal obesity is related to offspring obesity [9], and there is an increased risk of adverse outcomes for both mother and child [10–13]. Moreover, the risk of childhood obesity was quadrupled if the mother was obese before pregnancy [14], which suggests that the in utero environment is obesogenic. In mammals, the placenta is the main interface between the fetus and the mother; it regulates intrauterine development and modulates adaptive responses to suboptimal in utero conditions [15,16].

Placenta is also an important source of stem/progenitor cells [17–19]. In particular, human amniotic mesenchymal stem cells (hA-MSCs) have been shown to differentiate into cell types of mesenchymal origin such as chondrocytes, adipocytes, and osteocytes [20–22]. The phenotype of hA-MSCs from normal pregnant women has been characterized and found to differ in terms of cytokine expression from that of pregnant women affected by pre-eclampsia [23].

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Thus far, little is known about hA-MSCs from obese women.

The aim of this study was to characterize hA-MSCs from term placenta of obese (Ob-) women and to test their adipogenic potential with respect to that of normal weight control (Co-) women. We also measured several maternal and newborn clinical and biochemical parameters, and looked for correlations between these parameters with the hA-MSC immunophenotype. We found that the Ob-hA-MSC immunophenotype was characterized by increased expression levels of the CD13 surface antigen that correlated with maternal CD13 serum levels. Adipogenesis was higher in Ob-hA-MSCs than in Co-hA-MSCs, and returned to the control value after CD13 silencing. On the other hand, CD13 overexpression increased the adipogenic potential of Co-hA-MSCs. Our findings suggest that CD13 could contribute to obesity programming in the fetus and indicate that maternal serum CD13 is an obesity risk marker.

## Materials and Methods

### *Patients and controls*

Sixteen Ob- (age range: 26–39 years) and seven Co-pregnant women, (age range: 26–38 years), prepregnancy body mass index (BMI) (mean/SEM) 40.3/1.8 and 22.4/1.0 $kg/m^2$, respectively, and thirty-two not pregnant women (16 obese and 16 normal weight, BMI >30 $kg/m^2$ and <25 $kg/m^2$, respectively), were recruited at the Dipartimento di Neuroscienze e Scienze Riproduttive ed Odontostomatologiche, University of Naples "Federico II." The clinical, personal, and family history of the 23 women was recorded during a medical interview conducted by an expert upon hospitalization. Data relative to each pregnancy follow-up and delivery were also recorded. The general characteristics of the newborn and clinical data (birth weight, length, head circumference, Apgar score) were recorded at birth.

### *Sample collection*

Two fasting peripheral blood samples were collected in the morning from not pregnant women and from Ob- and Co-pregnant women, immediately before delivery. One sample was used for DNA extraction, whereas the other was centrifuged at 2,500 rpm for 15 min and serum was stored at −80°C until further processing. At delivery, placentas were collected by the C-section from each enrolled woman and immediately processed. Bioptic samples of VAT were also collected from not pregnant obese and control women during obstetric surgery (ovarian cysts). All patients and controls gave their informed consent to the study and both parents gave consent for their newborns. The study was performed according to the Helsinki II Declaration and was approved by the Ethics Committee of our Faculty.

### *Biochemical evaluations*

The main serum biochemical parameters were evaluated by routine assays. Leptin and adiponectin were measured in maternal serum with Luminex xMAP Technology on a BioRad Multiplex Suspension Array System (Bio-Rad), according to the manufacturer's instructions. The ratio leptin/adiponectin (L/A) was also calculated.

### *Aminopeptidase N/CD13 ELISA assay*

Aminopeptidase N (APN)/CD13 serum levels were measured by ELISA (Life Science). Briefly, the microtiter plate was precoated with a specific anti-CD13 antibody. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for CD13. Next, avidin conjugated to horseradish peroxidase was added to each microplate well and incubated for 15 min at room temperature. A TMB substrate solution (3,3′,5,5′-tetramethylbenzidine) was then added to each well. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm. The amount of CD13 in each sample was determined by comparing the absorbance of the sample to a standard curve.

### *Cell isolation from placenta tissue*

Placentas were collected and immediately processed, according to Parolini et al. [24]. After removal of the maternal decidua, the amnion was manually separated from the chorion and extensively washed 5 times in 40 mL of phosphate-buffered saline (PBS) containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 µg/mL amphotericin B (all from Sigma-Aldrich), after which, it was mechanically minced into small pieces [24]. Amnion fragments were digested overnight at 4°C in the ACCUMAX® reagent (Innovative Cell Technology), a combination of DNase, protease, and collagenolytic enzymes [25], containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 µg/mL amphotericin B. The next day, digestion enzymes were inactivated with a complete culture medium constituted by the low-glucose DMEM (Sigma-Aldrich) supplemented with 10% of heat-inactivated bovine serum (FBS), 1% of nonessential amino acids, and 2% of ultraglutamine (all from Lonza). After centrifugation at 300 *g* for 10 min, cell pellets and digested tissue fragments were seeded in a cell culture dish (BD Falcon) in the complete culture medium and incubated at 37°C in 5% $CO_2$. One week later, digested tissue pieces were removed from the dish and discarded, and isolated cells formed distinct fibroblast colony-forming units. When the colonies reached 70% confluence, they were washed with PBS and detached with trypsin/EDTA (Sigma-Aldrich), counted, and reseeded in the complete medium for expansion at a concentration of about 5,000/$cm^2$ [24].

### *Cell preparation*

hA-MSCs were expanded for several passages. The absence of mycoplasma contamination was assessed as described previously [26]. The population doubling level was calculated for each subcultivation with the following equation: population doubling = $[\log_{10}(N_H) - \log_{10}(N_I)]/\log_{10}(2)$, where $N_I$ is the cell inoculum number and $N_H$ is the cell harvest number [27]. The increase in population doubling was added to the population doubling levels of the previous passages to yield the cumulative population doubling level. When 70%–80% confluent cultures reached about 4 population doublings, they were detached with trypsin/EDTA, resuspended in PBS with 10% FBS, and processed for flow cytometry, DNA, and RNA extraction. Cellular viability was

TABLE 1. SURFACE IMMUNOPHENOTYPIC PROFILE INVESTIGATED IN HUMAN AMNIOTIC MESENCHYMAL STEM CELLS BY FLOW CYTOMETRY

| *Fluorochrome* | *CD antigen* | *Other names* | *Molecular weight (kDa)* | *Cell expression* | *Function* |
|---|---|---|---|---|---|
| FITC | CD9 | Tspan-29 | 24–26 | Platelets, pre-B cells, activated T cells | Adhesion, migration, platelet activation |
| APC | CD10 | CALLA | 100 | B/T precursors, stromal cells | Endopeptidase |
| PE | CD13 | APN | 150 | Granulocytes, monocytes and their precursors, endothelial cells, epithelial cells, mesenchymal stem cells | Metalloproteinase |
| PE | CD14 | LPS-R | 53–55 | Monocytes, macrophages | Receptor for LPS/LPB complex |
| APC | CD15 | Lewis X | _ | Granulocyte, monocyte, epithelial cells | Cell adhesion |
| PE | CD16 | FCγRIIIa | 50–65 | Neutrophils, NK, macrophages | Low affinity with FCγ receptor, mediates phagocytosis |
| APC | CD19 | Bgp95 | 95 | B cells, not on plasma cells | Signal transduction |
| FITC | CD26 | DPP IV | 110 | Mature thymocytes, T, B, NK cells | Exoprotease, costimulation |
| APC | CD28 | Tp44 | 44 | Most T cells, thymocytes, NK and plasma cells | Costimulation |
| APC | CD29 | VLA β1-chain | 130 | T, B, granulocytes, monocytes, fibroblasts, endothelial cells, NKs, platelet | Adhesion activation, embryogenesis, and development |
| FITC | CD31 | ECAM-1 | 130–140 | Monocytes, platelets, granulocytes, and endothelial cells | Cell adhesion |
| APC | CD33 | My9 | 67 | Monocytes, granulocytes, mastocytes, and myeloid progenitors | Cell adhesion |
| APC | CD34 | My10 | 105–120 | Hematopoietic stem cells and progenitors, endothelial cells | Cell adhesion |
| APC | CD36 | Platelet GPIV | 85 | Platelets, monocytes, macrophages, endothelial cells, erythroid precursors | Adhesion and phagocytosis |
| FITC | CD40 | Bp50 | 48 | Monocytes, macrophages, B cells, endothelial cells, fibroblasts, keratinocytes | Costimulation to B cells, growth, differentiation, and isotype switching |
| APC | CD44 | H-CAM | 90 | Leukocytes, erythrocytes, and epithelial cells | Rolling, homing, and aggregation |
| Per Cp | CD45 | LCA | 180–220 | Hematopoietic cells, except erythrocytes and platelets | Critical for T and B cell receptor-mediated activation |
| FITC | CD47 | IAP I | 50–55 | Hematopoietic, epithelial, endothelial, and brain mesenchymal cells | Adhesion |
| FITC | CD49d | VLA-4 | 150 | B cells, T cells, monocytes, eosinophils, basophils, NKs, dendritic cells | Adhesion, migration, homing, activation |
| APC | CD54 | ICAM-1 | 80–114 | Epithelial and endothelial cells monocytes. Low on resting lymphocytes, upregulate on activated | T cell activation |
| PE | CD56 | NCAM | 175–220 | Neural, tumors, embryonic tissue, NK | Homophilic and heterophilic adhesion |
| PE | CD58 | LFA-3 | 40–70 | Leucocytes, erythrocytes, epithelial endothelial cells, and fibroblasts | Costimulation |
| FITC | CD71 | Transferrin recepor | 95 | Reticulocytes, erythroid precursor | Controls iron intake during cell proliferation |
| APC | CD81 | TAPA-1 | 26 | B and T cells, monocytes, endothelial cells | Signal transduction |

(*continued*)

TABLE 1. (CONTINUED)

| Fluorochrome | CD antigen | Other names | Molecular weight (kDa) | Cell expression | Function |
|---|---|---|---|---|---|
| FITC | CD90 | Thy-1 | 25–35 | Hematopoietic stem cells, neurons, mesenchymal stem cells | Inhibition of hematopoietic stem cells and neuron differentiation |
| PE | CD99 | MIC2 | 32 | Leucocyte, NK, monocytes, endothelial and epithelial cells | Leucocyte migration, T cell activation, cell adhesion |
| PE | CD105 | Endoglin | 90 | Endothelial and mesenchymal stem cells, erythroid precursors, monocytes | Angiogenesis, modulates cellular response to TGFβ1 |
| PE | CD117 | c-kit | 145 | Hematopoietic stem cells and progenitors | Crucial for hematopoietic stem cells |
| PE | CD133 | Prominin-1 | 120 | Hematopoietic stem cell, endothelial, epithelial, and neural precursors | Unknown function, stem cell marker |
| PE | CD151 | PETA-3 | 32 | Endothelial and epithelial cells, megakaryocytes, platelets | Adhesion |
| PE | CD166 | ALCAM | 100–105 | Neurons, activated T cells, epithelial cells, mesenchymal stem cells | Adhesion, T cell activation |
| PE | CD200 | OX-2 | 33 | B cells, activated T cells, thymocytes, neurons, endothelium | Downregulatory signal for myeloid cell functions |
| FITC | CD243 | MDR-1 | 170 | Stem cells, multidrug-resistant tumors | Influences the uptake, distribution, elimination of drugs |
| APC | CD271 | NGFR | 75 | Neurons, stromal and dendritic follicular cells | Low affinity for NGF receptor |
| APC | CD324 | E-cadherin | 120 | Epithelial, keratinocytes, platelet | Adhesion, growth, differentiation |
| APC | CD338 | ABCG-2 | 72 | Hematopoietic stem cells, liver, kidney, intestine, side population of stem cells | Absorption and excretion of xenobiotics |
| FITC | HLA-ABC | Class I MHC | 46 | All nucleated cells and platelets | Antigen presentation |
| FITC | HLA-DR | Class II MHC | 30 | B cells, monocytes, myeloid progenitors, activated T and dendritic cells | Antigen presentation |

assessed by both Trypan blue dye exclusion and the analysis of light scatter proprieties in flow cytometry, and it was never lower than 90%.

Using the above cell isolation and preparation procedures, h-MSCs were also isolated from the umbilical cord (hUC-MSCs) of one obese and one control pregnant woman.

### *Isolation of hVAT-MSCs*

Briefly, VAT bioptic samples were washed with PBS containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 μg/mL amphotericin B (all from Sigma-Aldrich), minced into small pieces, and digested with 1.5 mg/mL collagenase type I (GIBCO) at 37°C. The digestion enzymes were inactivated with FBS. After centrifugation at 1500 *g* for 5 min, cell pellets and digested tissue fragments were washed and seeded in a cell culture dish (BD Falcon) in the complete culture medium and incubated at 37°C in 5% $CO_2$. When the colonies reached 60%–70% confluence, they were washed with PBS and detached with trypsin/EDTA (Sigma-Aldrich), counted, and reseeded in the complete medium for expansion at a concentration of about 5,000/$cm^2$ [28].

### *DNA typing*

The fetal origin of both amnion and hA-MSCs was verified by DNA typing. Genomic DNA was extracted from the mother's peripheral blood, from amnion samples, and from hA-MSCs using the Nucleon BACC2 extraction kit (Illustra DNA Extraction Kit BACC2; GE Healthcare). The DNA concentration was evaluated using the NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies). Genomic DNA (1 ng) was amplified in a final volume of 25 μL using the AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems). The AmpFlSTR Identifiler PCR Amplification Kit is a short tandem repeat (STR) multiplex assay that amplifies 15 repeat loci and the Amelogenin gender determining marker in a single polymerase chain reaction (PCR) amplification using a primer set labeled with four fluorescent molecules. The amplification was performed with the GeneAmp PCR System 9700 (Applied Biosystems) instrument. PCR products were then analyzed by capillary electrophoresis on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems) together with an allelic ladder, which contained all the most common alleles for the analyzed loci

that were present in Caucasian populations and both a negative- and a positive-quality control sample. Typically, 1μL of each sample was diluted in 18.7μL of deionized formamide; each sample was supplemented with 0.3 μL of an internal size standard (LIZ 500 Applied Biosystems) labeled with an additional fluorophore. The samples were denatured at 95°C for 4 min, and then placed in the auto sampler tray (maximum of 96 samples) on the ABI Prism 3130 for automatic injection in the capillaries. The data were analyzed by Gene Mapper Software (Applied Biosystems).

### *Immunophenotyping of h-MSCs by flow cytometry*

We analyzed the expression of 38 hematopoietic, mesenchymal, endothelial, epithelial, and no-lineage membrane antigens on the surface of hA-MSCs, hUC-MSCs, and hVAT-MSCs by four-color flow cytometry (Table 1). The antibody cocktails contained in each tube are detailed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/scd). All monoclonal antibodies (MoAbs) were from Becton Dickinson (San Jose) except anti-CD338-APC, which was from R&D (Minneapolis), anti-CD-133-PE and anti-CD271-APC MoAbs, which were from Milenyi Biotec (Bergisch Gladbach). For all antibody staining experiments, at least $1 \times 10^5$ hA-MSCs isolated from each placenta sample were incubated at 4°C for 20 min with the appropriate amount of MoAbs, washed twice with PBS, and finally analyzed with an unmodified Becton-Dickinson FACSCanto II flow cytometer (Becton-Dickinson), that was set up according to published guidelines [29]. For each sample, the respective control was prepared to determine the level of background cellular autofluorescence without antibody staining.

CaliBRITE beads (Becton-Dickinson, catalog no. 340486) were used as quality controls across the study as described elsewhere [30,31], according to the manufacturer's instructions. Daily control of CaliBRITE intensity showed no change in instrument sensitivity throughout the study. The relative voltage range for each detector was assessed *una tantum* using the eight-peak technology (Rainbow Calibration Particles, Becton-Dickinson, catalog no. 559123) at the beginning of the study.

Compensation was set in the FACS-DiVa (Becton-Dickinson) software, and compensated samples were analyzed. Samples were acquired immediately after staining using the FACSCanto II instrument, and at least 10,000 events were recorded for each monoclonal combination. Levels of CD antigen expression were displayed as median fluorescence intensity (MFI). The FACS-DiVa software (Becton-Dickinson) was used for cytometric analysis.

### *Differentiation potential toward the adipogenic lineage*

hA-MSCs and hVAT-MSCs were cultured in the low-glucose DMEM (Sigma-Aldrich) supplemented with 10% of FBS, 2% of ultraglutamine, and 1% of nonessential amino acids at 37°C in 5% $CO_2$ (all from Lonza). The cells were passaged twice before the addition of the differentiation medium composed of the DMEM with the addition of 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxhantine, 200 μM indomethacin, and 10 μg/mL insulin. Media were changed every 2 days and cells were either stained or collected for RNA extraction.

### *CD13 RNA interference and overexpression*

hA-MSCs plated at a density of 5,000 cells/cm$^2$ were transfected using 20 μL Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen) with 8 μg short hairpin RNAs (shRNAs) expressing plasmids (Open Biosystem) or with an 8 μg pCMV-Sport 6 Vector (Invitrogen), to silence or to overexpress CD13 mRNA, respectively. Transfected cells were induced to differentiate toward the adipogenic lineage up to 4 days.

### *Effect of interferon-γ on the expression of CD13 on the surface of h-MSCs*

The expression of CD13 on the surface of Co- and of Ob-h-MSCs isolated from the amnion, umbilical cord, and VAT was measured after exposure of cells to 0.8 and 12.5 ng/mL interferon (IFN)-γ at 37°C for 24 h, using untreated Co- and Ob-h-MSCs as controls. At the end of incubation, the cells were

Table 2. Clinical and Biochemical Characteristics of Obese and Normal Weight Control Pregnant Women at Delivery and Their Newborns

**A**

| *Mother's parameters* | *Ob-pregnant women (n=16)* | *Co-pregnant women (n=7)* |
|---|---|---|
| Age (years) | 32.6 (0.9) | 30.7 (1.5) |
| Weight (kg)[a] | 110.1 (5.4) | 65.2 (3.6) |
| Height (m) | 163.3 (1.6) | 169.0 (1.7) |
| BMI prepregnancy (kg/m$^2$)[a] | 40.3 (1.8) | 22.4 (1.0) |
| Weight gain in pregnancy[b] | 8.4 (1.3) | 14.3 (1.8) |
| Systolic blood pressure (mmHg) | 124.3 (2.7) | 117.1 (5.1) |
| Diastolic blood pressure (mmHg)[c] | 82.5 (2.2) | 74.2 (2.0) |
| Frequency cardiac | 79.6 (1.7) | 79.0 (3.7) |
| Gestational age | 38.4 (0.3) | 38.7 (0.2) |
| Glucose (mmol/L) | 4.3 (0.1) | 4.0 (0.3) |
| Total cholesterol (mmol/L) | 6.9 (0.4) | 7.3 (0.1) |
| Triglycerides (mmol/L) | 2.8 (0.2) | 2.3 (0.3) |
| AST (U/L) | 15[d] (12.2–26.5[d]) | 14.8 (0.7) |
| ALT (U/L) | 13[d] (9.2–17.7[d]) | 12.1 (1.1) |
| ALP (U/L) | 124.2 (11.1) | 115.0 (12.6) |
| GGT (U/L) | 11.0 (1.7) | 8.8 (1.5) |
| Leptin (L) (ng/mL)[a] | 38.5 (2.2) | 15.2 (3.3) |
| Adiponectin (A) (μg/mL) | 6.0 (0.7) | 7.5 (1.4) |
| L/A[a] | 7.7 (0.6) | 2.6 (0.5) |

**B**

| *Newborn features* | *Ob-newborns (n=16)* | *Co-newborns (n=7)* |
|---|---|---|
| Birth weight (kg) | 3162 (0.1) | 3401 (0.1) |
| Length (cm) | 49.6 (0.7) | 50.8 (0.7) |
| Head circumference (cm) | 34.0 (0.4) | 34.8 (0.3) |
| Apgar 1′ | 7.0[d] (7.0–8.0[d]) | 7.8 (0.2) |
| Apgar 5′ | 9.0[d] (8.5–9.0[d]) | 8.7 (0.1) |

Data are expressed as mean (SEM) (parametric distributions). Statistically significant difference at the Student *t* test.
[a]$P<0.0001$.
[b]$P<0.05$.
[c]$P<0.05$.
[d]Median value and 25th–75th percentiles (nonparametric distributions).

harvested by trypsin, washed in PBS, counted, and adjusted to the same concentrations of $1\times10^5$ h-MSCs. Subsequently, their immunophenotype was examined by flow cytometry.

### Adipocyte staining

After 14 days of differentiation, the adipocyte cultures were stained for lipid droplets, which are an index of differentiation. The cells were washed in PBS and fixed in 10% formalin for 1 h. Then, they were washed in PBS and the lipids were stained for 15 min with Oil Red O prepared by mixing vigorously three parts of a stock solution (0.5% Oil Red O in 98% isopropanol) with two parts of water, and then eliminating undissolved particles with a 0.4-μm filter. Cells were then washed with water and the number of adipocytes was evaluated with a microscope. Relative lipid levels were assessed

Table 3. Immunophenotyping of Human Amniotic Mesenchymal Stem Cells Isolated from Obese and Control Pregnant Women

| | | *Ob-hA-MSCs* | | *Co-hA-MSCs* | | |
|---|---|---|---|---|---|---|
| *Fluorochrome* | *Antigen* | *MFI* | *25th-75th Percentiles* | *MFI* | *25th-75th Percentiles* | *P value* |
| Not expressed antigens | | | | | | |
| FITC | CD31 | 364.0 | 278.3–511.3 | 306.5 | 286.8–368.0 | 0.3254 |
| | CD40 | 444.0 | 336.5–568.3 | 388.0 | 357.0–457.8 | 0.4824 |
| | CD243 | 363.0 | 292.3–528.3 | 307.5 | 274.0–378.3 | 0.2061 |
| | HLA-DR | 355.0 | 283.8–530.0 | 297.0 | 272.5–374.8 | 0.2415 |
| | NC | 325.0 | 250.0–516.3 | 279.0 | 218.8–418.8 | 0.4260 |
| PE | CD14 | 166.5 | 125.5–197.8 | 134.5 | 124.5–157.8 | 0.2815 |
| | CD16 | 36.0 | 11.5–71.7 | 65.5 | 50.5–72.0 | 0.2407 |
| | CD117 | 142.5 | 109.5–189.3 | 121.0 | 116.0–176.8 | 0.6065 |
| | CD133 | 95.5 | 80.5–112.8 | 87.5 | 76.50–110.5 | 0.5423 |
| | NC | 115.5 | 91.75–181.5 | 102.5 | 93.25–135.0 | 0.5427 |
| APC | CD15 | 122.0 | 91.0–283.5 | 122.5 | 80.0–162.5 | 0.6065 |
| | CD36 | 241.5 | 194.0–388.5 | 200.5 | 145.5–267.3 | 0.2417 |
| | CD271 | 208.0 | 127.0–296.5 | 170.0 | 127.5–233.3 | 0.6065 |
| | CD338 | 200.5 | 106.5–373.0 | 107.5 | 101.8–146.8 | 0.1223 |
| | CD19 | 192.0 | 154.0–241.3 | 144.0 | 120.3–182.2 | 0.1012 |
| | CD28 | 91.0 | 5.2–228.5 | 85.5 | 0–122.0 | 0.1722 |
| | CD33 | 147.5 | 10.0–189.5 | 105.5 | 90.0–132.3 | 0.6734 |
| | CD34 | 186.5 | 105.0–241.5 | 133.0 | 17.5–206.0 | 0.4250 |
| | NC | 169.0 | 99.5–244.8 | 92.0 | 64.0–204.0 | 0.2061 |
| Per Cp | CD45 | 215.5 | 133.8–251.3 | 167.0 | 146.0–208.0 | 0.6734 |
| | NC | 305.0 | 252.0–483.3 | 288.5 | 261.3–348.8 | 0.7431 |
| Expressed antigens | | | | | | |
| FITC | CD9 | 3,538.0 | 2,172.0–6,871.0 | 2,156.0 | 1,743.0–3,495.0 | 0.2417 |
| | CD26 | 1,287.0 | 651.8–3,235.0 | 1,308.0 | 742.3–1,920.0 | 0.9626 |
| | CD47 | 1,339.0 | 980.3–2,312.0 | 1,287.0 | 1,106.0–1,344.0 | 0.4824 |
| | CD49d | 1,185.0 | 946.8–1,393.0 | 941.0 | 708.3–1,140.0 | 0.2061 |
| | CD71 | 1,271.0 | 796.5–2,147.0 | 1,093.0 | 897.0–1,538.0 | 0.7431 |
| | CD90 | 37,140.0 | 22,740.0–52,690.0 | 36,210.0 | 21,640.0–50,260.0 | 0.8149 |
| | CD324 | 517.0 | 463.0–551.0 | 436.0 | 375.0–545.0 | 0.3027 |
| | HLA-ABC | 9,363.0 | 4,033.0–14,180.0 | 5,424.0 | 3,987.0–6,539.0 | 0.1223 |
| | NC | 325.0 | 250.0–516.3 | 279.0 | 218.8–418.8 | 0.4260 |
| PE | CD13 | 9,802.0 | 6,786.0–17,130.0 | 3,950.0 | 3,634.0–4,961.0 | 0.0043[a] |
| | CD56 | 496.0 | 293.8–711.0 | 528.0 | 151.0–1,048.0 | 0.9626 |
| | CD58 | 2,432.0 | 1,723.0–2,792.0 | 2,009.0 | 1,798.0–2,459.0 | 0.5427 |
| | CD99 | 405.0 | 296.5–586.3 | 467.5 | 360.5–651.0 | 0.3736 |
| | CD105 | 652.0 | 507.0–1,329.0 | 790.0 | 746.0–847.8 | 0.6734 |
| | CD151 | 16,010.0 | 10,970.0–21,430.0 | 19,410.0 | 11,090.0–23,690.0 | 0.4260 |
| | CD166 | 5,215.0 | 3,551.0–7,382.0 | 4,634.0 | 3,962.0–5,608.0 | 0.6734 |
| | CD200 | 722.5 | 205.8–1,699.0 | 1,137.0 | 631.5–1,444.0 | 0.3736 |
| | NC | 115.5 | 91.7–181.5 | 102.5 | 93.2–135.0 | 0.5427 |
| APC | CD10 | 1,247.0 | 999.3–2,319.0 | 1,890.0 | 1,122.0–3,031.0 | 0.3736 |
| | CD29 | 45,150.0 | 25,130.0–54,610.0 | 24,240.0 | 17,660.0–40,000.0 | 0.0832 |
| | CD44 | 11,440.0 | 8,186.0–16,290.0 | 7,259.0 | 6,613.0–9,753.0 | 0.0678 |
| | CD54 | 9,910.0 | 5,404.0–14,260.0 | 10,660.0 | 9,486.0–24,670.0 | 0.3736 |
| | CD81 | 31,240.0 | 19,110.0–55,050.0 | 38,890.0 | 24,500.0–44,640.0 | 0.8149 |
| | NC | 169.0 | 99.5–244.8 | 92.0 | 64.0–204.0 | 0.2061 |

[a]Significant *P* value at the Mann–Whitney test.
hA-MSC, human amniotic mesenchymal stem cells; MFI, median fluorescence intensity.

by redissolving the Oil Red O present in stained cells in 98% isopropanol, and then determining absorbance at 550 nm.

### RNA isolation

Total RNA was purified from hA-MSCs isolated from term placentas of Co- and of Ob-pregnant women using the mirVana™ miRNA isolation kit (Ambion) and its concentration was evaluated with the NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies).

### Quantitative real-time PCR of mRNAs

Real-time quantitative PCR (qRT-PCR) was carried out on the Applied Biosystems 7900HT Sequence Detection system (Applied Biosystems). cDNAs were synthesized from 2 μg of total RNA using hexamer random primers and M-MuLV Reverse Transcriptase (New England BioLabs). The PCR was performed in a 20 μL final volume containing cDNA, 1×SYBR Green PCR mix, and 10 μM of each specific primer. Supplementary Table S2 lists the oligonucleotide primers used for PCR of selected genes: peroxisome proliferator-activated receptor gamma (PPARγ), CD13, protein homologous to myelin P2 (aP2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR conditions for reverse transcription were stage 1: 50°C, 2 min; stage 2: 95°C, 10 min; stage 3: 95°C, 15 s; 60°C, 1 min/40 cycles; and stage 4: 95°C, 15 s; 60°C, 1 min. Levels of target genes were quantified using specific oligonucleotide primers and normalized for GAPDH expression.

### Statistical analysis

The parameters investigated were expressed as mean and standard error of the mean (SEM) (parametric distributions) or as the median value and 25th and 75th percentiles (nonparametric distributions). The Student's *t* and Mann–Whitney tests were used to compare parametric and nonparametric data, respectively. *P* values <0.05 were considered statistically significant. Correlation analysis was performed with the SPSS package for Windows (ver. 18; SPSS, Inc.).

## Results

The clinical and biochemical characteristics of the mothers and their newborns are reported in Table 2 (A and B, respectively). Weight gain was lower ($P<0.05$) and diastolic blood pressure was higher ($P<0.05$) in Ob- than in Co-pregnant women. Both the leptin concentration ($P<0.0001$) and the L/A ratio ($P<0.0001$) were higher in Ob- than in Co-pregnant women at delivery. Biometric characteristics did not differ significantly between Ob- and Co-newborns.

### Isolation of hA-MSCs

We isolated hA-MSCs from the mesenchymal layer of amniotic membranes obtained from our Ob- and Co-pregnant women at delivery. The fetal origin of all isolated hA-MSCs was confirmed by STR typing of DNA of the mother and of the hA-MSCs. Mycoplasma contamination of cultures was checked and excluded (data not shown). All isolated hA-MSCs were characterized by a high proliferation potential and collected after four population doublings. Morphologically, cultured Ob- and Co-hA-MSCs showed a similar fibroblastic-like morphology after four population doublings (Supplementary Fig. S1).

### Immunophenotyping of h-MSCs

The antigenic mosaic displayed by Ob- and Co-hA-MSCs is shown in Table 3. Seventeen of the 38 antigens investigated were not expressed on the surface of hA-MSCs (hematopoietic antigens: CD14, CD15, CD16, CD19, CD28, CD33, CD34, CD45, and CD117; the endothelial marker PECAM-1/CD31; and no-lineage markers: thrombospondin receptor/CD36, Bp50/CD40, Prominin-1/CD133, MDR-1/CD243, NGFR/CD271, ABCG-2/CD338, and HLA-DR). Both Ob- and Co-hA-MSCs were positive for the following mesenchymal markers: CD9, CD10, CD13, CD26, CD29, CD44, CD47, CD49d, CD54, CD56, CD58, CD71, CD81, CD90, CD99, CD105, CD151, CD166, CD200, and HLA-ABC. A very weak positivity for the epithelial antigen E-cadherin/CD324 was

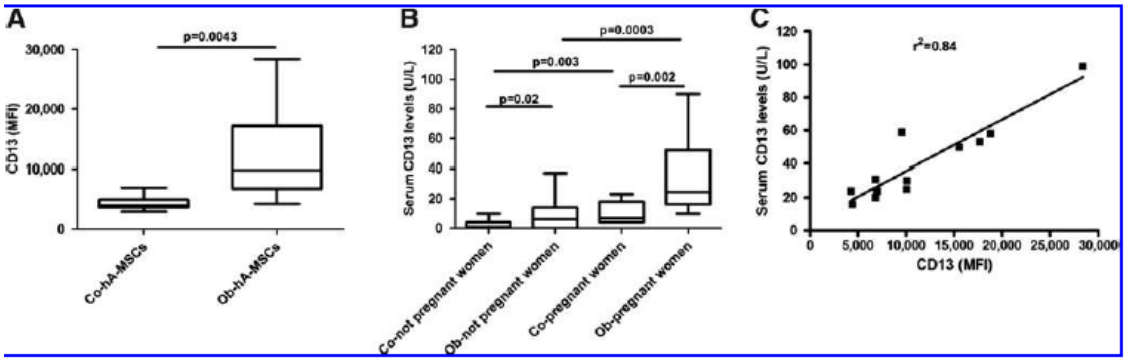


**FIG. 1.** Expression of CD13 antigen in control (Co-) and obese (Ob-) pregnant women. **(A)** Ob-Human amniotic mesenchymal stem cells (hA-MSCs) expressed significantly higher amounts (at Mann–Whitney test) of CD13 surface antigen compared with Co-hA-MSCs ($P<0.005$); **(B)** serum levels of CD13 were significantly higher both in Ob- than in Co-not pregnant women ($P<0.05$) and in Ob- than in Co-women at delivery ($P<0.005$); **(C)** serum CD13 levels were correlated with CD13 surface expression levels in Ob-pregnant women ($r^2=0,84$; $P<0.0001$). The box plots provide a vertical view of the data expressed as median, 25th percentile, 75th percentile, and extreme values.

also observed. Interestingly, CD13 expression was significantly higher in Ob-hA-MSCs than in Co-hA-MSCs, that is, MFI: 9,802.0 and 3,950.0, respectively ($P<0.005$) (Table 3 and Fig. 1A). The immunophenotype characterization confirmed the mesenchymal origin and the higher CD13 expression in hVAT-MSCs and hUC-MSCs from Ob- than from Co-women (hVAT-MSCs - MFI: 8,200.0 vs. 1,100.0 and hUC-MSCs - MFI: 4,965.0 vs. 3,155.0, respectively).

### *APN/CD13 serum levels*

We first measured baseline serum levels of CD13 in a small group of not pregnant obese and normal weight women and found significantly higher values in the obese subset (medians: 6.00 and 1.00 U/L, $P<0.05$, respectively) (Fig. 1B). The serum levels of CD13 were also significantly higher in Ob- than in Co-pregnant women at delivery (medians: 24.00 and 7 U/L, $P<0.005$, respectively), (Fig. 1B). CD13 levels were significantly higher in Ob- and Co-pregnant women than in not pregnant Ob- and Co-women: four ($P<0.0005$) and seven times ($P<0.005$), respectively. Furthermore, in Ob-pregnant women, serum CD13 levels were significantly correlated to the levels of CD13 on the surface of hA-MSCs ($r^2=0.84$; $P<0.0001$) (Fig. 1C).

### *CD13 h-MSC expression and adipogenic differentiation*

To investigate whether CD13 is involved in adipogenesis, we cultured Ob- and Co-hA-MSCs for 14 days in the adipogenic induction medium. At the end of incubation, the adipogenic potential, as measured by PPARγ and aP2 mRNA levels, was higher in Ob- than in Co-hA-MSCs. In fact, as shown in Fig. 2A and B, the mean RQs at day 14 were 0.04 and 0.02, respectively, for PPARγ ($P<0.05$), and 0.02 and 0.01, respectively, for aP2 ($P<0.05$). The same results were obtained with Oil Red staining; in fact, staining was more intense in Ob- than in Co-hA-MSCs at day 14 of differentiation [Abs (550 nm) = 0.6 and 0.4, $P<0.05$, respectively] (Fig. 2C). During adipogenesis, CD13 mRNA levels remained higher in Ob- than in Co-hA-MSCs. CD13 silencing by shRNA in Ob-hA-MSCs resulted in a switch-off of CD13 mRNA expression, as evaluated by RT-PCR (Fig. 3A), and at the same time, the adipogenic potential of these cells did not differ from that observed in Co-hA-MSCs, as shown by similar PPARγ mRNA levels measured in silenced Ob-hA-MSCs and in Co-hA-MSCs ($P=0.71$) (Fig. 3B). In agreement to CD13 involvement in adipogenesis, we overexpressed CD13 in Co-hA-MSCs (mRNA CD13 mean RQ = 7.23) and observed at day 4 of differentiation that PPARγ mRNA levels were higher in treated (mean RQ = 0.015) than in untreated (mean RQ = 0.001) Co-hA-MSCs. The adipogenic potential at day 14 was also higher in Ob- than in Co-hVAT-MSCs isolated from not pregnant women [aP2: RQs were 0.050 and 0.036; Oil Red O Abs (550 nm): 0.559 and 0.437, respectively].

### *Upregulation of CD13 h-MSC expression by IFN-γ*

We next evaluated if CD13 expression could be upregulated in h-MSCs by IFN-γ as occurs in murine cellular models [32]. To this aim, we treated the Co- and Ob-hA-MSCs with 0.8 ng/mL or 12.5 ng/mL IFN-γ for 24 h. We found that CD13 expression was significantly higher on membranes of

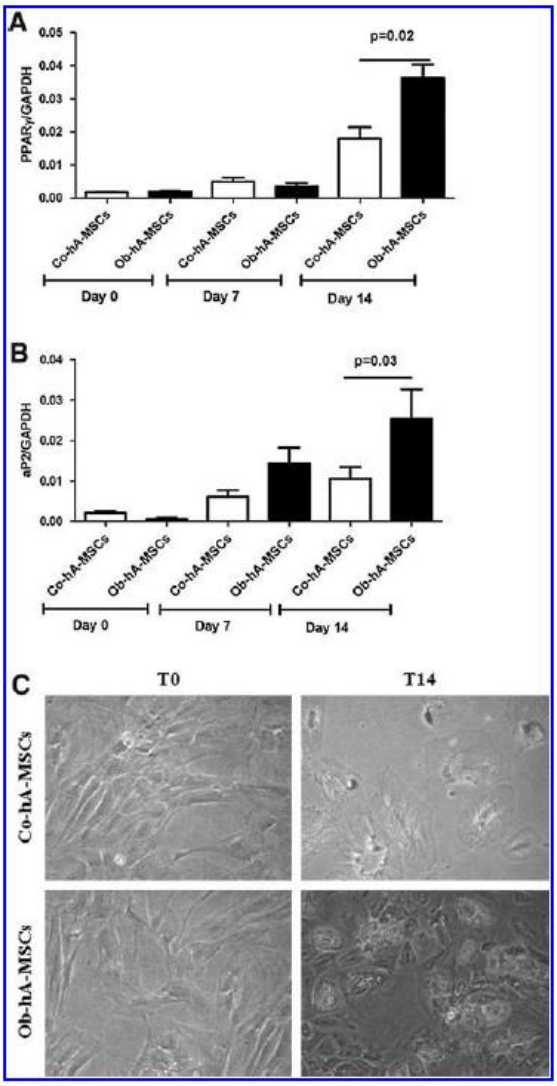


**FIG. 2.** Adipogenic potential in Ob-hA-MSCs and in Co-hA-MSCs. The statistically significant higher mRNA expression levels of PPARγ ($P<0.05$) **(A)** and of aP2 ($P<0.05$) **(B)** measured 14 days after the adipogenic induction, indicated increased adipogenesis in Ob- versus Co-hA-MSCs. **(C)** The higher adipogenesis in Ob- than in Co-hA-MSCs was also confirmed by Oil Red staining [Abs (550 nm) = 0.6 and 0.4, $P<0.05$, respectively].

Co-hA-MSCs treated with 12.5 ng/mL IFN-γ ($P<0.05$) than in untreated cells, whereas there was a slight, not significant, increase in treated Ob-hA-MSCs (Supplementary Fig. S2) versus the untreated counterpart cells. In addition, IFN-γ treatment (12.5 ng/mL at 37°C for 24 h) induced the increase of CD13 membrane expression in hVAT-MSCs (Ob- and Co-MSCs: 39% and 8%, respectively), and in Co-hUC-MSCs (4%) versus the untreated counterpart cells, but not in Ob-hUC-MSCs. Our results suggest that high levels of INF-γ drive

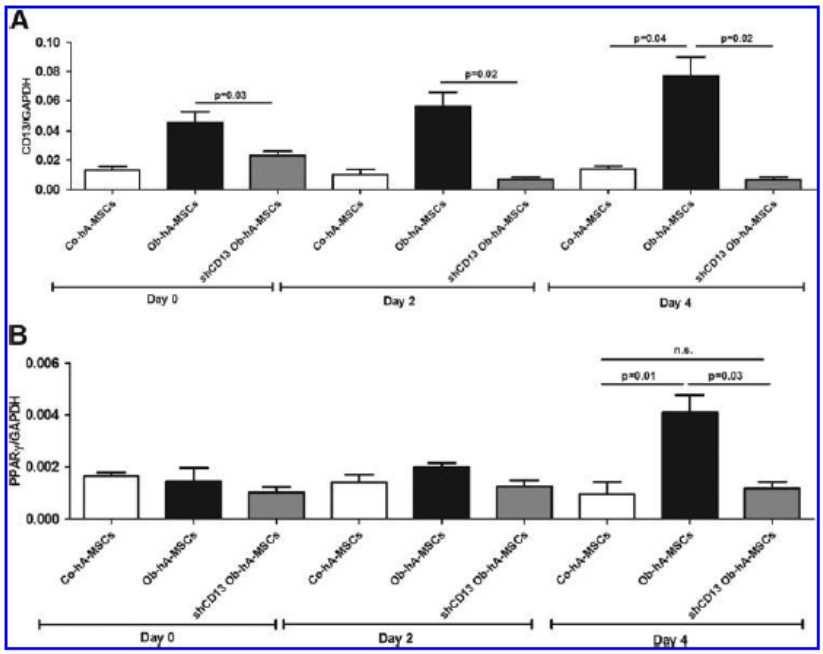


**FIG. 3.** Role of CD13 in adipogenesis. **(A)** mRNA expression levels of CD13 were significantly higher in Ob- than in Co-hA-MSCs at day 0 ($P<0.05$), day 2 ($P<0.05$), and day 4 ($P<0.05$) when cultured with the adipogenic medium. CD13 mRNA expression was switched-off in Ob-hA-MSCs after CD13 silencing with shRNA. **(B)** At day 4 of adipogenic induction, PPARγ mRNA expression levels that were significantly higher in Ob-hA-MSCs than in Co-hA-MSCs ($P<0.02$), decreased to the levels detected in Co-hA-MSCs after CD13 silencing ($P=0.71$), which indicates that CD13 enhances adipogenesis in hA-MSCs. n.s.: not statistically significant difference.

the upregulation of CD13 expression in Co-h-MSCs, irrespective of their source and of pregnancy, whereas its effect on Ob-h-MSC CD13 expression during obesity is ambiguous.

## Discussion

Human amniotic membrane is a readily available source of abundant fetal MSCs that are free from ethical concerns [33]. hA-MSCs isolated from normal weight healthy women at delivery have been characterized [24,34,35], but, to our knowledge, the features of hA-MSCs from obese women are largely unknown. In this study, we used flow cytometry to characterize hA-MSCs isolated at delivery from two groups of women: prepregnancy normal weight and prepregnancy severely obese women. The immunophenotypic characterization confirmed the mesenchymal origin of the isolated cells [36]. In particular, the distribution of CD56 was in agreement with the placental origin of the isolated hA-MSCs. In fact, this marker is absent from bone marrow [34] and from adipose tissue-derived mesenchymal stem cells [37]. Similarly, the endothelial marker PECAM-1/CD31, and the hematopoietic antigens CD14, CD15, CD16, CD19, CD28, CD33, CD34, CD45, and CD117 were absent from isolated Ob- and Co-hA-MSCs. Staining for the E-cadherin/CD324 epithelial antigen was very weak in our Ob- and Co-hA-MSC preparations; the coexpression of epithelial, although at a low intensity, and mesenchymal markers on our hA-MSCs was in agreement with previous findings [38,39]. Overall, our results are similar to those reported by Parolini et al. [24] and/or Roubelakis [35] regarding the expressed (CD49d, CD90, HLA-ABC, CD13, CD56, CD105, CD166, CD10, CD29, CD44, and CD54) and not expressed (PECAM-1/CD31, HLA-DR, CD14, Prominin-1/CD133, NGFR/CD271, CD34, and CD45) membrane-bound antigens in hA-MSCs. We found that the Ob-hA-MSC immunophenotype is characterized by a significantly higher expression of the APN/CD13 antigen with respect to the Co-hA-MSC phenotype. Besides amnion, CD13 was overexpressed in h-MSCs isolated from the umbilical cord in obese women and in those isolated from VAT in not pregnant obese women.

Type II metalloprotease APN/CD13 (EC. 3.4.11.2) is a heavily glycosylated membrane-bound protein (~960aa, ~150 kDa) that is encoded by the human ANPEP gene located on chromosome 15 (q25-q26) [40]. This protein exists also in a soluble form. APN/CD13 is a ubiquitous enzyme present in a wide variety of human organs, tissues, and cell types, including the placenta, human umbilical vein endothelial cells, monocytes, lymphocytes T, hypothalamus, and epithelial intestinal cells [41]. It has various mechanisms of action: enzymatic cleavage of peptides, endocytosis, and signal transduction [42]. APN/CD13 is involved in inflammation, cellular differentiation and proliferation, apoptosis, cell adhesion, and motility [42]. Dysregulated expression of membrane and/or soluble forms of APN/CD13 has been observed in many diseases [43], but until now, it has never been associated with obesity. Here we provide the first demonstration that the CD13 antigen is increased on hA-MSCs during obesity and could play a role in adipogenesis. In fact, we first detected a higher adipogenic potential in Ob- than in Co-hA-MSCs after 14 days of adipogenic differentiation, and then observed that the adipogenic potential of Ob-hA-MSCs was comparable to that of Co-hA-MSCs after CD13 silencing. Conversely, the adipogenic potential increased in Co-hA-MSCs after CD13 overexpression. Furthermore, we provide evidence that INFγ upregulated CD13 expression in Co-hA-MSCs.

Intriguingly, in Ob-pregnant women, APN/CD13 serum levels at delivery were higher than in Co-pregnant women and correlated with CD13 surface Ob-hA-MSC expression ($r^2 = 0.84$, $P < 0.0001$), which support the hypothesis that the placenta is the major source of the high CD13 levels measured in maternal serum [44]. We also found that the leptin concentration and the L/A ratio were increased in Ob-maternal serum at delivery. This finding confirms the concept that these two parameters are obesity risk markers [45,46].

In conclusion, this characterization of Ob-hA-MSCs shows that antigen CD13, by influencing the adipogenic potential of these cells, could be an in utero risk factor for obesity. Our data strengthen the hypothesis that high serum CD13 and mesenchymal stem cell CD13 are markers of obesity.

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## Author Disclosure Statement

The authors declare no financial conflict of interests.

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## MATERNAL OBESITY AND PREGNANCY COMPLICATION.

Obesity was recognised as a risk factor in pregnancy more than 50years ago. (1)

Since then, numerous retrospective, prospective and case-control studies have demonstrated the association between maternal obesity and various pregnancy complications that may occur in the preconception period, gestational and postnatal.

### *Hypertensive disorders. Pre-eclampsia*

The hypertensive disorders are undoubtedly among the most frequently observed complications in obese women. Hypertension may be present before conception, or arise during the early periods of pregnancy (gestational hypertension), with or without evidence of pre-eclampsia.

Pre-eclampsia occurs in 5-7% of healthy nulliparous women and is the second leading cause of maternal death in the U.S. and remains the most common cause of fetal death. (2)

Obesity, insulin resistance and hypertriglyceridemia cofactors are important for the development of endothelial dysfunction, which plays a central role in the pathogenesis of pre-eclampsia. Endothelial dysfunction reduces the secretion of prostacyclin and increases the production of peroxidase that results in vasoconstriction and platelet aggregation. (3)

Large studies have shown that obese women have a two or three times greater probability of developing pre-eclampsia compared to normal weight. [4-5] A systematic review of 13 studies by O'Brien et al. that included more than a million women, have shown that the risk of pre-eclampsia increases with increasing BMI. [4]

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ORIGINAL ARTICLE

# Preeclampsia in women with chronic kidney disease

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***Objective*: Women with chronic kidney disease have an increased risk of developing preeclampsia and its severe complications. Currently, there are no assessments available in order to quantify such risk. The aim of the study is to establish the incidence of superimposed preeclampsia in women with chronic kidney disease according to Serum creatinine (SCr) level. *Methods*: Pregnant women with chronic kidney disease were retrospectively identified from January 2000 to July 2010. We defined two groups according to SCr: Group 1: SCr ≤ 125 µmol/l; Group 2: SCr > 125 µmol/l. Incidence of preeclampsia, early preeclampsia (delivery <34 weeks), gestational age (GA) at diagnosis and delivery outcome were assessed. *Results*: Ninety-three nephropatic women were considered for the analysis. Group 2 ($n = 14$) compared with Group 1 ($n = 79$) had an increased incidence of preeclampsia (78.6% vs. 25.3%; $p < 0.0001$), an increased rate of pregnancy complications as early preeclampsia (82% vs. 38%; $p < 0.03$), a lower GA at diagnosis (29 ± 2 vs. 33 ± 1 weeks; $p < 0.04$) and a lower GA at delivery (30 ± 2 weeks vs. 34 ± 1; $p < 0.04$). *Conclusion*: Women with chronic kidney disease and an increased creatinine threshold have a high risk of developing preeclampsia and delivering preterm.**

**Keywords:** Early preeclampsia, gestational age, kidney function, serum creatinine

## Introduction

Preeclampsia (PE) affects at least 2–5% of pregnancies in developed countries and it is a major cause of maternal and perinatal morbidity and mortality [1].

Many maternal conditions are at increased risk of PE, like chronic hypertension [2], diabetes [3,4], obesity [5], thrombophilia [6] and renal disease [7].

Although PE is a multisystem disorder, kidney deserves particular attention because it is a regulator of blood pressure and plasma volume, playing an important role during pregnancy [7].

One of the markers used to assess kidney function is the serum creatinine (SCr [8]).

The aim of our study is to describe the incidence and severity of superimposed PE in women with chronic kidney disease according to pregnancy SCr levels.

## Methods

All the women affected by chronic kidney disease, referred to our Centre from January 2000 to July 2010, were retrospectively identified. Women with a diagnosis of miscarriage during the first trimester were excluded from our analysis.

We evaluated two groups according to the first available SCr sample in the first trimester: Group 1: women with normal or near-normal renal function (SCr ≤ 125 µmol/l; 1.4 mg/dl) and Group 2: women with a mild or severe renal impairment (SCr > 125 µmol/l; 1.4 mg/dl [9]).

In women known to be normotensive and without proteinuria beforehand, PE was diagnosed in the presence of hypertension (blood pressure ≥140/90 mmHg on at least two occasion and at least 4–6 h apart) and proteinuria (excretion of ≥300 mg of protein every 24 h) after 20 weeks of gestation [2].

Superimposed PE in women with pre-existing hypertension and proteinuria was defined by a significant increase of blood pressure and proteinuria [10,11]. The presence of increased transaminases levels, thrombocytopenia and hyperuricemia was also considered as a diagnosis criteria [12].

Pre-existing hypertension and proteinuria were diagnosed on the basis of clinical history, past medication use or occurrence in the first trimester.

Gestational age (GA) was assessed by the last menstrual period and confirmed by ultrasound.

Maternal and neonatal outcomes were assessed by analysing proteinuria before delivery, proteinuria normalized by estimated glomerular filtration rate (eGFR) (calculated by Cockcroft–Gault Equation [13]), the mean systolic and mean diastolic blood pressure at admission, platelets count before delivery, number of antihypertensive drugs and neonatal outcome as: birth weight, respiratory distress syndrome (RDS) and admission to neonatal intensive care unit (NICU).

Small for gestational age fetuses (SGA) were defined by the presence of an abdominal circumference <10th centile according to local curves [14].

The incidence of maternal complications was reported for each group (hemolysis elevated liver low platelets (HELLP) syndrome, thrombocytopenia and need for dialysis during pregnancy and puerperium). PE was subdivided into early PE, that required delivery before 34 weeks, and late PE, that required delivery at or after 34 weeks [15].

Differences between groups were assessed using independent $t$-test and $\chi$-square test for continuous and categorical variables, respectively. A $p$ value less than 0.05 was considered statistically significant.

SPSS 17.0 package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

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Table I. Renal diseases affecting our sample.

| Renal disease | Overall $n=93$ $n$ (%) | Group 1 $n=79$ $n$ (%) | Group 2 $n=14$ $n$ (%) |
|---|---|---|---|
| Diabetic nephropathy | 39 (41.9) | 34 (43) | 5 (35.7 |
| Lupus nephritis | 29 (31.2) | 27 (34.2) | 2 (14.3) |
| End-stage renal disease not well specified | 11 (11.8) | 5 (6.4) | 6 (42.9) |
| Ig A nephropathy | 6 (6.5) | 6 (7.6) | – |
| Autosomal-dominant polycystic kidney disease | 4 (4.3) | 3 (3.8) | 1 (7.1) |
| Renal calculi | 2 (2.2) | 2 (2.6) | – |
| Renal agenesis | 1 (1.1) | 1 (1.3) | – |
| Cystinuria | 1 (1.1) | 1 (1.3) | – |

## Results

One-hundred women with chronic kidney disease were referred to our Centre from January 2000 to July 2010.

Among these, 7 (7%) had a miscarriage and they were excluded from our analysis.

The remaining 93 women (93%) were divided into the two described groups: 79 (84.9%) in Group 1 and 14 (15.1%) in Group 2.

All the women were Caucasian; the mean maternal age was comparable between the two groups (31 ± 0.6 vs. 30 ± 1.1; $p$ = NS).

Frequency of each renal disease affecting our sample is reported in Table I.

Thirty-one women (33.3%) had a diagnosis of PE. Among these, 20 (25.3%) were in Group 1 and 11 (78.6%) in Group 2. The difference between the two groups was statistically significant ($p < 0.0001$).

The mean GA at diagnosis of PE was significantly lower in Group 2 than in Group 1 (29 ± 2 vs. 33 ± 1 weeks; $p < 0.04$). Among women with PE, early PE was significantly higher in Group 2 than in Group 1 (82% vs. 38%; $p < 0.03$) and GA at delivery was 30 ± 2 and 34 ± 1 weeks, respectively ($p < 0.04$).

Clinical and biochemical characteristics of both groups were shown in Table II.

Among 31 preeclamptic women, 2 women developed HELLP syndrome (1 in Group 1 and 1 in Group 2) and 8 women developed thrombocytopenia (4 in Group 1 and 4 in Group 2). Two women (one for each group) terminated the pregnancy because of the severity of the disease. No intrauterine demise occurred. In all cases, a caesarean section was performed. Twenty-nine alive neonates were born from preeclamptic women.

In Table III, the main characteristics of fetal and neonatal outcome are reported. Newborns in Group 2 were smaller than in Group 1. The rate of RDS and admission to NICU was almost twice higher in Group 2 than Group 1.

## Discussion

This study confirms that women with pre-existing renal impairment are at increased risk of developing PE during pregnancy.

We found a high frequency (33.3%) of PE among nephropatic women, as confirmed by data previously reported [16–18].

Women with chronic kidney disease have been reported to have a good maternal and fetal outcome in case of normal blood pressure, little or no proteinuria and normal or near-normal SCr [7].

Table II. Main clinical and biochemical characteristics of preeclamptic women in both groups.

| Clinical and biochemical characteristics | Group 1 $n=20$ | Group 2 $n=11$ | $p$ value |
|---|---|---|---|
| Proteinuria/eGFR | | | |
| Mean ± Standard Error (ES) | 24.9 ± 8.3 | 340 ± 91.9 | <0.01 |
| Proteinuria (gr/24 h) | | | |
| Mean ± ES | 2291 ± 869 | 8280 ± 2200 | <0.03 |
| 300–4900 mg/24 h | | | |
| $n$ (%) | 19 (95) | 2 (18) | <0.0001 |
| ≥5000 mg/24 h | | | |
| $n$ (%) | 1 (5) | 9 (82) | |
| Diastolic blood pressure (mmHg) | | | |
| Mean ± ES | 87.2 ± 2.5 | 90 ± 4.1 | NS |
| Sistolic blood pressure (mmHg) | | | |
| Mean ± ES | 139 ± 3.7 | 142 ± 8.4 | NS |
| Platelets (u/µl) | | | |
| mean ± ES | 207840 ± 19110 | 163454 ± 23145 | NS |
| Antihypertensive drugs | | | |
| onotherapy | | | |
| $n$ (%) | 11 (52.4) | 1 (9.1) | <0.02 |
| Politherapy | | | |
| $n$ (%) | 9 (42.8) | 10 (90.9) | |

Table III. Fetal and neonatal outcome in preeclamptic pregnancies.

| Analysed parameters | Overall $n=29$ | Group 1 $n=19$ | Group 2 $n=10$ |
|---|---|---|---|
| SGA n (%) | 6 (20.7) | 2 (10.5) | 4 (40) |
| APGAR 5 min median | 9 | 8 | 9 |
| Birth weight (gr)[a] mean ± SE | 2413 ± 226 | 2715 ± 220 | 1280 ± 266 |
| RDS $n$ (%) | 9 (31) | 5 (26.3) | 4 (40) |
| NICU $n$ (%) | 10 (34.5) | 5 (26.3) | 5 (50) |

[a]Significanltly lower birth weight in Group 2 compared to Group 1, $p < 0.05$.
NICU, neonatal intensive care unit; RDS, respiratory distress syndrome; SGA, small for gestational age fetuses.

The current purpose is to investigate how the risk of developing PE is associated with a renal function marker risk factor (SCr). This issue has been investigated before reporting a higher incidence of PE and severity of the feto-maternal outcome among women with increased SCr levels [9]. In order to validate the previous reported results in a heterogeneous retrospectively recruited population, the same cut-off have been used in the current study.

Recent studies well demonstrated the relationship of the severity of the syndrome and the GA at delivery [19,20]. Women with SCr level >125 µmol/l developed PE more frequently and with a lower GA at diagnosis. The incidence of early PE was also higher in Group 2.

Furthermore, higher levels of SCr were associated with higher levels of proteinuria, an increased need for antihypertensive drugs, dialysis, and a higher frequency of complications, reflecting the impaired renal function predisposition.

According to these data, SCr value at the beginning of pregnancy seems to play a role in prediction of PE and feto-maternal outcome. The study has several limitations. The heterogeneity of the population referred and the use of SCr as a single factor would decrease the predictive value of this model in a larger population. Nevertheless, SCr has been reported before as a single risk factor for PE in women with kidney disease, independently from the underlining condition [9].

A prompt hypertension treatment adjustment and prophylactic measures have been reported to improve pregnancy outcome in women with severe chronic disease [21,22]. The booking SCr levels could identify women at a higher risk, in order to address the proper counselling and target the subgroup which would benefit from such interventions. Conversely, women with SCr <125 μmol/l have an increased risk of PE compared to the normal population but with a better obstetric outcome.

***Declaration of interest***: The authors report no conflicts of interest.

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**Metabolic syndrome in patients and hematological diseases**.

The term metabolic syndrome (MS) defines a clustering of cardiovascular risk factors, formerly known as syndrome X. There is some debate about the diagnostic criteria; but the most widely accepted framework is that defined by the National Cholesterol Education Program Adult Treatment Panel III, which requires the simultaneous occurrence of at least three of abdominal obesity, arterial hypertension, hyperglycemia, hypertrigliceridemia and low high-density lipoprotein cholesterol (HDL-C). The prevalence of MS increases with age and varies depending on genetic factors. An abnormally high prevalence has been observed in patients with heterogeneous conditions, such as solid organ transplant recipients, AIDS patients and long-term cancer survivors[1-2].

As some of the pathogenetic factors possibly involved include cyclosporine A, corticosteroids and cancer chemoradiotherapy, it is possible that MS may also be a complication in hematological patients. Some of the characteristics of MS have been reported with a certain frequency in thalassemia patients, and are mainly attributed to iron overload. Impaired hemostasis is a feature of MS rather than a factor predisposing to its development. In oncohematology, an abnormally high

prevalence of MS features has been observed in survivors of pediatric acute lymphoblastic leukemia.

In addition to corticosteroid- and cancer therapy-related hypogonadism, hypothyroidism and defective growth hormone incretion are other factors related to the development of MS. Moreover, the highest frequency of MS is observed in hematopoietic stem cell transplantation (HSCT) recipients. Pediatric patients and allogeneic HSCT recipients have been the subject of foremost investigations; but adult patients and autologous HSCT recipients have also been studied more recently. A wide range of factors may contribute to the development of MS in HSCT recipients. Unfortunately, the real entity of the problem is far from clear because of the retrospective design of the studies, the limited size of their populations and their heterogeneous selection criteria, thus making it difficult to determine whether MS is a transient and possibly reversible phenomenon or a true late effect of the procedure.

***Haemophilic disorders.***

Hemophilia is characterized by spontaneous and provoked mucocutaneous, joint, muscle, gastrointestinal, and central nervous system bleeding, leading to major morbidity and even mortality if left untreated or undertreated. With the pioneering of safe plasma-derived and recombinant coagulation factor

and the initiation of prophylactic infusions of clotting factor, people with hemophilia are now living longer with fewer bleeding and infectious complications. As their life expectancy and lifestyles closer match those of their healthy peers, so does their risk for common public health issues, including overweight, obesity, and the ensuing complications.

Obesity is a major contributing factor to the global burden of chronic disease and disability. According to the WHO, obesity rates have increased at least threefold since 1980 in parts of North America, the United Kingdom, Eastern Europe, the Middle East, the Pacific Islands, Australia, and China.[3-4]

***Thromoembolic disorders***

Pregnancy is a hypercoagulable state and obesity increases the risk of thrombosis by promoting venous stasis, increasing blood viscosity and promoting activation of the coaugulation cascade. The greatest risk is at term, and expecially in association with cesarean delivery[5].

In a retrospective study of 683obese women and 660 normal weight women the incidence of thromboembolism disease was 2.3 % in the obese group and 0.6% in the normal group[6].

Thromboembolism is the leading cause of maternal death in pregnancy and is likely to increase in incidence as a consenquences of higher rates of maternal obesity in developed nations.

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Haemophilia



# Management of patients with factor V deficiency: open issues from the challenging history of a woman with anaphylactic transfusion reactions

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In its severe forms, congenital factor V (FV) deficiency is a rare bleeding disorder (estimated prevalence 1:1 000 000) caused by homozygous or compound heterozygous mutations of FV gene [1]. Unlike haemophilia, but similarly with other recessive coagulation disorders, mucosal haemorrhages (epistaxis, menorrhagia and oral cavity bleeding) are the most common symptoms and bleeding phenotype is not clearly predicted by the residual FV activity. The genetic background is widely heterogeneous, as most patients/families show private mutations [1]. The mainstay of treatment is fresh frozen plasma (FFP), because no specific FV concentrate is still available. Concerns regard the residual risk of bloodborne infections, in particular in children, and volume overload, requiring caution in elderly patients or in those with cardiovascular or renal disease. Moreover, FFP-related immunologic complications, including inhibitors, allergy/anaphylaxis and the transfusion-related acute lung injury (TRALI), are challenging, as few data are available concerning alternative treatment, like platelet transfusions [1,2] or, more recently, recombinant activated factor VII (rFVIIa) [2–4]. We discuss these open issues of management of FV-deficient patients reporting the gynaecological history and the successful pregnancy of a woman with severe FV deficiency (residual activity 1%), whose treatment was complicated by anaphylactic reactions to FFP.

Her coagulation defect was diagnosed in the childhood, owing to recurrent epistaxis and FV deficiency detected in an older sister. Parents were not consanguineous and consistently, molecular analysis revealed compound heterozygous defects (p.Arg712Stop and the previously unreported c.3510–3511 delAA). She experienced menorrhagia since the menarche. In spite of tranexamic acid administration, FFP and red blood cell (RBC) transfusions were needed on several occasions. At the age of 18, the patient was admitted because of severe abdominal pain. Haemoglobin was 6.5 g $dL^{-1}$ and an ultrasound scan revealed free intraperitoneal fluid and a remarkable enlargement of the left ovary, containing a scarcely defined mass. RBC and FFP were administered and the patient underwent exploratory laparotomy. Haemoperitoneum was confirmed and drained (about 700 mL) and a partial resection of the left ovary was carried out. A haemorrhagic follicle cyst was diagnosed at histological examination. Combined oral contraceptives (COC) were then started. Owing to poor compliance to treatment, a similar episode of heamoperitoneum led to a new laparotomy and to the excision of the residual left ovary 10 months later. Full adherence to COC was associated with significant reduction of menstrual blood loss and cessation of RBC transfusion requirement, thereafter. In 1998, when she was 23 years old, the patient experienced an anaphylactic reaction while receiving FFP before tooth extraction. In spite of premedication with antihistamine and steroids, the same reaction occurred 1 year later, when FFP was given because of severe bleeding after a bite of the tongue. The persistence of bleeding led to the

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usually by daily infusions of 15–20 mL $kg^{-1}$ [1,9]. In our case, owing to the limited plasma availability, this was achieved at surgery and FV activity fell below 5% after 30 h. Although rFVIIa was not administered from the start of the surgery, this agent was shown as an effective and safe haemostatic treatment to cover the 5 days following Caesarean section, as recommended in women with IBD [5]. According to our literature search (Table 1), other six patients with congenital FV deficiency have been treated with rFVIIa [2–4,11,12], all but two [4,12] because of FFP-related immunological complications. In the latter, advantages of rFVIIa are obvious, enabling haemostatic efficacy independent of inhibitor titre and absence of anamnestic response. However, avoiding volume overload and infectious risks are other relevant clinical issues leading to the choice of rFVIIa [4,12]. In all cases, standard doses (80–120 μg $kg^{-1}$) and interval of administration similar to haemophilia with inhibitors were used. However, the unknown mechanism of action of rFVIIa in this setting and the lack of validated tools for monitoring patients' response to treatment make it difficult to define the optimal dose, regimen and duration of treatment. In this respect, usefulness of thromboelastographic techniques [4] is debated. Our treatment was the first in a patient who underwent pelvic surgery and 22 rFVIIa doses were administered over 5 days. Concerns for thromboembolic risk are highlighted when rFVIIa is used in off-label indications [1]; in our patient, who showed concomitant risk factors (puerperium, obesity), elastic stockings were prescribed and no adverse event occurred.

In conclusion, FV deficiency is considered a moderately severe bleeding disorder, with less remarkable symptoms than haemophilia with the same factor levels [1]. However, management of FV-deficient patients, especially women, may be more challenging, because specific replacement is still lacking and a series of risks are associated with the only available treatment, FFP. Although clinical experience is still limited, rFVIIa is likely to provide an alternative approach, in particular for patients with FFP-related immunological complications.

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# HAIRY CELL LEUKEMIA DURING PREGNANCY AND TREATMENT WITH INTERFERON-ALPHA: A CASE REPORT AND LITERATURE REVIEW

*Quaglia F., et al*

*CLINICAL PRACTICE POINTS*

Hairy cell leukemia (HCL) is a chronic lymphoid leukemia recognized as a clonal B-cell malignancy. It was first described by Bouroncle et al. in 1985 as leukaemic reticuloendotheliosis[1]and it was renamed HCL by Schrekand Donnelly because it is characterized by the presence of abnormal cells with hairlike cytoplasmatic projections on the surface.[2] It is a rare form of leukemia, with an incidence rate less then 1/100000 cases per year. It affects mainly the male gender (male/female ratio 4:1) with a median age at diagnosis of 55-56 years and a Caucasian preponderance.[3]

*INTRODUCTION*

HCL is extremely rare in pregnancy. Only few cases were reported in literature and they were summarized in Table1.

Treatment options include purine analogues (cladribine), INF-α, splenectomy, or monoclonal antibodies (rituximab).

Cladribine is often use as first line therapy , with complete remission rate of 76-80%.[456]. However, it is not indicated in pregnancy because of its

teratogenic effects and because it improves the risk of perinatal mortality.[7] Rituximab is a monoclonal antibody, producing an overall response rate of 64% in HCL; it acts synergistically with cladribine.[8]

We found a single case reporting the use of rituximad and cladribine in a pregnant woman with HCL, without complications for both the mother and the fetus; in this case, the decision was guided by the advanced gestational age and the need for a rapid improvement of platelet count. [9]

An other therapeutic option is splenectomy, that permits an increase in blood cell count. Four cases of splenectomy in pregnant women developing HCL were described in literature (10-13)

However splenectomy does not produce a remission of the pathology; moreover, there are potential risks of maternal hemorrhage and infections(REF). It is a real possibility only in the early gestation when the risks are lower.

INF-α has a documented response rate of 75%-90% (14-15). INF-α is a pleyotropic cytokine with immunomodulatory properties. Even if it has been considered dangerous for a long time, it does not inhibit DNA synthesis and is not mutagenic in vitro or teratogenic in animal studies; moreover, previous studies showed that it does not reduce fertility (16-19). Negative effects are registered in the monkey only for doses higher than those used in pregnancy

[17.20] Moreover, it was demonstrated that placenta produces INF and INF-like molecules (throphoblast INF) [(21)].

We have already reported four cases of successful pregnancies in women affected by essential thrombocythemia treated with INF-α. [(22)]. Moreover, three previous cases of pregnant women with HCL showed good tolerance, uncomplicated pregnancy and delivery, and normal child development after delivery [(23-24)]. Many adverse side effects are reported as a consequence of INF-α therapy, such as depression, fatigue and flu-like syntoms;[9] 9by the way, no adverse effects were recorded in our case and in cases decrypted before.

## DISCUSSION

We report a case of successful pregnancy in a 30 years-old woman affected by HCL, treated by alfa-interferon. A 30 years-old pregnant woman, gravida I at 8 weeks of gestation, was referred to The High Risk Pregnancies Unit of the Department of Obstetrics & Gynecology (University of Naples "Federico II") for a progressive pancytopenia. On her initial blood count, white cell count was 3540/mm^3, platet 50.000/mm^3, hemoglobin 8.7 gr/dl, and neutrophil count was 45% with the presence of cells with cytoplasmic protusions.Immunophenotyping of B lymphocytes of peripheral blood and bone marrow aspirate showed a population of CD19+, CD103+, CD11c++,

CD25+ B cells (1,5%) compatible with a diagnosis of Hairy Cell Leukemia. (Figure1) Therefore, our management was based on blood transfusion (when hemoglobin was less then 8 gr/dl) and prednisone 25 mg/die during the whole first trimester. The woman required five blood-transfusions. At 12 weeks of gestation, the possibility of a therapy with interpheron-alpha was discussed. A therapy with INF-α (Intron-A 3 M.U.I. three times per week) and prednisone 10 mg/die has been performing until delivery. After one month, a progressive increase of hematological parameters was observed and the patient did not receive other blood transfusions. INF-α was well tolerated. At 34 weeks of gestation, she was admitted to the hospital because of threatened preterm labor. Liggins' prophylaxis and tocolytic therapy were performed. A female baby was delivery at 36 weeks of gestation with a birthweight of 2850 grams and Apgar score 9 and 10 at 1 and 5 minutes, respectively.Placental size was normal and there was not any infiltration of hairy cells.There was no complication during vaginal delivery. Woman could perform breastfeeding because of the low interferon concentration in breast milk.

She was discharged after nine days. Two years after, the patient underwent a teraphy with Cladribine, because of the reaapearance of splenomegaly and pancytopenia. Actually she is in clinical remission. She is currently being

followed in the hematologic clinic. Her child had a normal growth and neurodevelopment at two years-old.

## CONCLUSION

Even if nowadays HCL is a rare event during pregnancy, its management could become a consistent problem because of the trend toward delaying pregnancy until later life.

Unfortunately malignancies during pregnancies represents a difficult problem because of the teratogenic potential of most antineoplastic agents.

Even if INF-α is actually considered the second line therapy for HCL, because of the slow onset of action and the risk for adverse side effects[ix], we think that it is a valid terapheutic option in pregnant women affected by HCL because of the lower risks for both the mother an the fetus; other therapeutic options should be considered only when INF-α fails.

Fig.1

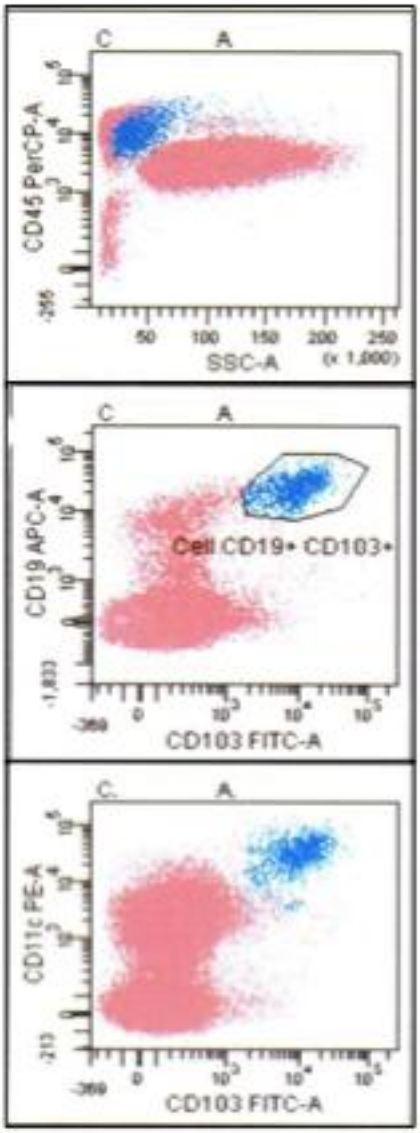
C
A
CD45 PerCP-A
SSC-A
(x 1,000)
CD19 APC-A
Cell CD19+ CD103+
CD103 FITC-A
CD11c PE-A
CD103 FITC-A

Table 1

| Reference | Age | Gestational age (GA) at diagnosis | Type of therapy | GA at delivery | Obstetrical complications | Type of delivery | Birthweight | 5 min APGAR score | Breastfeeding |
|---|---|---|---|---|---|---|---|---|---|
| Stiles et al, 1998 | | | | 38 ws | | Caesarean section | 2875 gr | 9 | |
| Kopec et al, 2005 | 36 | 14ws | INF-α | 39 ws | none | Caesarean section | 3460 gr | 10 | no |
| Baer et al, 1992 (Pz 3) | 32 | 14 ws | INF-α | Full term (non specificate le settimane) | None | TC | Not specified | Not specified | Not specified |
| Baer et al 1992 (Pz 4) | 37 | 22 ws | INF-α | 34 ws | None | Not specified | 1584 gr | Not specified | Not specified |
| Williams et al, 1987 | 34 | 7 ws | None | 37 ws | None | Caesarean section | Not specified | Not specified | Not specified |
| Patsner et al, 1994 | 36 | Diagnosis before pregnancy | | Evacuation of pregnancy | | | | | |

*Submission will be in process*

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**SYSTEMIC LUPUS ERYTHEMATHOSUS-ASSOCIATED THROMBOCYTOPENIA IN PREGNANCY: IS SPLENECTOMY NECESSARY AT THE TIME OF DELIVERY?**

*F.Quaglia, et al.*

**Abstract**

**Background**: Systemic Lupus Erythematosus (SLE) associated thrombocytopenia in pregnancy is a rare condition associated with potential harm to the mother, the fetus and/or newborn, if the thrombocytopenia is severe (< 50,000/$mm^3$). Controversy persists regarding the role and the timing of splenectomy in patients with SLE-associated refractory immune thrombocytopenia in pregnancy. This report describes the use of splenectomy at the time of the Cesarean Section (CS) in a patient with refractory SLE-associated thrombocytopenia.

**Case**: A 19-year-old gravida 2, para 1, woman with SLE- associated thrombocytopenia diagnosed at age 16, developed a platelet count of 10,000/ $mm^3$ at the $18^{th}$ week of gestation. She had been asymptomatic until that point, except for a previous spontaneous abortion at the $8^{th}$ week of gestation in 2008 During this admission she was treated initially with methylprednisolone and enoxaparin but following an episode of epistaxis she received intravenous immunoglobulins (IVIG). She was discharged home with a platelet count of 52,000/ $mm^3$. She was readmitted on the $34^{th}$ week's gestation with a platelet count of 15,000/$mm^3$ unresponsive to

steroids and IVIG; she underwent a CS and an open splenectomy. Following surgery, she continued to receive maintenance dose steroids. She had a partial response to the splenectomy (platelet count on discharge 63,000/mm$^3$).

**CONCLUSION:** Splenectomy at the time of the CS is a safe therapeutic option for women with SLE-associated refractory thrombocytopenia during pregnancy.

## INTRODUCTION

Thrombocytopenia, defined as a platelet count less than 150,000/mm$^3$, is encountered in 7% to 8% of normal pregnancies [1]. This form of thrombocytopenia, defined gestational thrombocytopenia, is benign because it is not associated with increased bleeding tendency in the mother or with thrombocytopenia in the newborn. Typically, no therapy is required and normal vaginal delivery is the norm [2]. However, the acute or chronic autoimmune thrombocytopenias of pregnancy, among these the SLE-associated thrombocytopenia, may have a significant impact on the mother and/or the fetus/newborn [3, 4]. Therefore, the rheumatologist and obstetrician treating such patients are confronted with complex therapeutic decisions, including the choice of medications

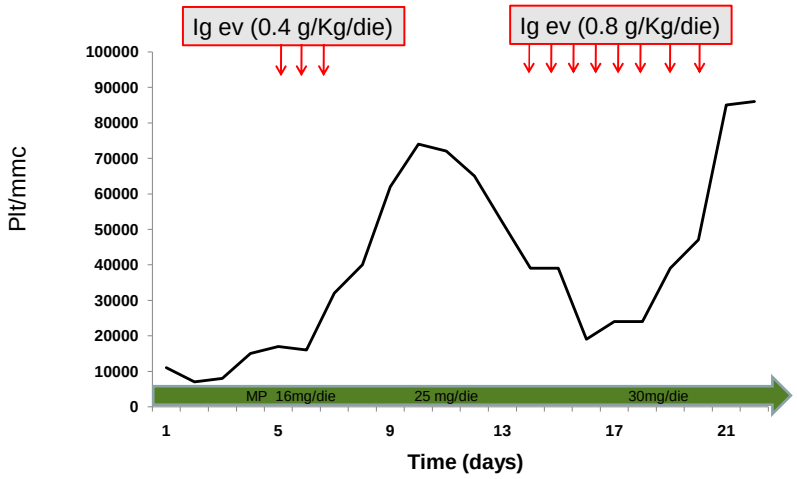
during the pregnancy, the timing and route of the delivery, the possibility of bleeding in the newborn from thrombocytopenia, and in those cases of thrombocytopenia refractory to medical treatment whether splenectomy should be undertaken to correct the immune-mediated thrombocytopenia in the pregnant woman. In the following case report, we describe our approach to a young pregnant patient with SLE-associated thrombocytopenia refractory to medical treatment, which included splenectomy at the time of the CS, and we outline a potential decision tree based on the pertinent review of the available literature.

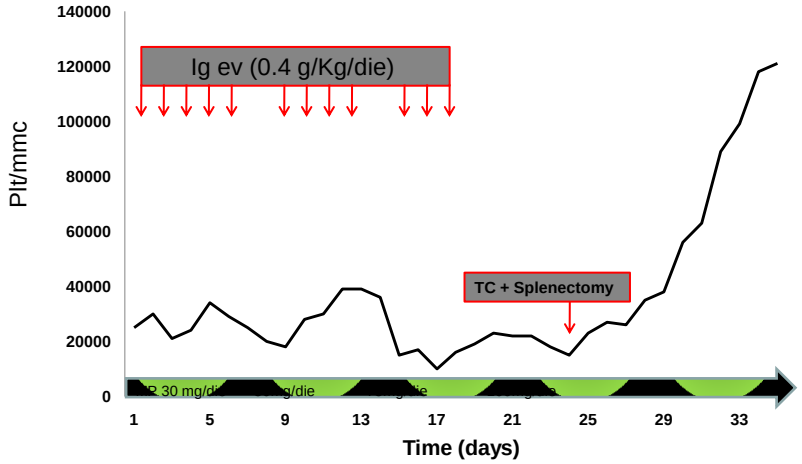
## CASE REPORT

The patient is a 19-year-old woman, gravida 2, para 0, first diagnosed with SLE in 2006 at age 16 when she had developed thrombocytopenia. At the time of the diagnosis of SLE, she had been treated with bolus Intravenous (IV) methylprednisolone with a complete response (platelet count > 150,000/$mm^3$). She was treated subsequently with 6mg/day of methylprednisolone for one year at which time her platelet count had normalized. She remained asymptomatic with a normal platelet count, and in early 2008 she became pregnant but suffered a spontaneous abortion at the $8^{th}$ week of gestation. Following this event she remained asymptomatic. In November 2008, she became pregnant again. At this time, she underwent an

extensive hematologic and immunologic work up that showed normal thyroid and liver function tests, positive antinuclear antibodies (ANA), Anti-Extractable nuclear antigen (ENA) antibodies, normal $C_4$ with decreased $C_3$, increased anticardiolipin (IgM) antibodies, elevated anti-β-glycoprotein, and a platelet count of 204,000/$mm^3$. Vaginal and rectal swabs were negative for Streptococcus Agalactiae. She was given hydroxychloroquine, aspirin, and enoxoparin subcutaneously 40 mg/day. Her platelet count remained relatively stable ranging between 80,000 and 100,000/$mm^3$ until the 18$^{th}$ week of gestation when her blood work revealed a platelet count of 11,000/$mm^3$. At this time (2/13/2009), she was admitted to out department for further diagnostic work up and for appropriate treatment. Her laboratory work-up showed elevated ACA, ANA with decreased $C_3$ level, normal PT, PTT, fibrinogen level (270mg/dl), and ATIII levels (95%). Heparin-induced thrombocytopenia was excluded by assaying anti heparin/PF4-antibodies titer. Her hemoglobin and hematocrit were 10.5 g/dl and 33%, respectively. She had anti-Ro/SSA and anti-La/SSB antibodies. Her rheumatoid factor was 23 IU/ml (upper limit of normal 15.9)(Tab 1). On 2/15/2009 her platelet count decreased to 9,000/$mm^3$; at this time, she experienced epistaxis. She was treated with eight units of platelet concentrates and the methylprednisolone was increased from 16 mg to 24mg/day. In addition, she received intravenous IgG 400mg/day for five days. Her platelet count rose to

52,000/mm$^3$ by March 8, 2009 when she was discharged home on methylprednisolone 6 mg/day, hydroxychloroquine, and aspirin. Her interval follow up examinations confirmed a satisfactory progression of her pregnancy with decreased platelet counts ranging between 20,000 and 25,000/mm$^3$ without any bleeding event. (Fig 1). On her 34$^{th}$ week of gestation (May 20, 2009), she was admitted to our department with a treatment plan that included another course of IVIG with pulsed steroids in preparation for an elective CS. However, she failed to respond to the treatment (Fig 2). Based on her failure to respond to the IVIG therapy, the hematology consultant suggested that she should undergo splenectomy at the time of the CS. On 6/3/2009, her platelet count just before the CS was 15,000/mm$^{3.}$ She received eight units of platelets before a midline laparotomy through which a transverse segmental CS was done followed immediately by the splenectomy. The intraoperative and postoperative course was uneventful. Her postoperative platelet count rose to a nadir of 63,000/mm$^3$ on 6/10/2009 when she was discharged home. The treatment plan at this time included the use of Rituximab. A healthy newborn with a normal platelet count was delivered via CS.

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## Discussion

This case report provides the opportunity to review several issues associated with the decision-making process concerning the management of SLE patients who develop thrombocytopenia during the pregnancy.

**Systemic Lupus Erythematosus in Pregnancy:** fertility rate in SLE patients has been reported to be similar to that of the normal population [3]. Earlier reports recommended that SLE patients avoid or even terminate pregnancy because of the potential negative effects of pregnancy on the course of disease [4, 5]. Some investigators believe that the rate of flares during pregnancy is similar to the rate observed in non-pregnant SLE patients, whereas other investigators suggest that pregnancy may increase disease activity [6, 7]. A more recent prospective outcome study of planned pregnancies in patients with SLE shows that, if one excludes early spontaneous and induced abortions, patients with SLE can have a live birth rate of 96% without maternal or neonatal death, although with a high rate of premature births, with a 27% incidence of modest to moderate lupus flares [8]. The two most important factors associated with worse fetal outcome in SLE are lupus activity during the pregnancy and the presence of antiphospholipid (aPL) antibodies (anticardiolipin antibody and lupus anticoagulant) [9, 10]. Pregnancy in patients with SLE is typically safe, if the

disease has been inactive on 20 mg or less of prednisone for at least one year. Our patient had been asymptomatic with normal renal function off steroids for over one year therefore when she inquired about becoming pregnant again she was advised that a potential pregnancy would not expose her to excessive risks.

**Gestational versus SLE associated thrombocytopenia**

The overall incidence of thrombocytopenia, defined as a platelet count less than 150,000 $mm^3$, in pregnancy varies between 5.1% and 8% [11]. The most common cause is gestational thrombocytopenia which typically accounts for more than 75% of cases [12]. However, thrombocytopenia is also common in SLE patients and has been reported to be present in 14% of pregnant SLE patients [13]. Clearly, several aspects of the thrombocytopenia present in the patient reported here, such as its preexistence before the pregnancy, the severe degree of the thrombocytopenia, and the response to the steroid treatment in 2006, suggested an SLE-associated thrombocytopenia instead of gestational thrombocytopenia. Of note, the patient's platelet count during the first trimester remained moderately low but within the range of values observed before her pregnancy. This is consistent with the available evidence suggesting that pregnancy does not worsen the course of immune mediated thrombocytopenia [14]. However, at

the time of the second trimester, her platelet count had decrease to 9,000/$mm^3$; at this time she had one episode of bleeding (epistaxis) requiring treatment with IVIG. She had a moderate response to the IVIG with a rise of the platelets count to 52,000/$mm^3$ on discharge from our department, and a decrease to the 20,000s range thereafter.

Should she have been considered to have refractory SLE- associated thrombocytopenia at this time based on the decrease in platelet count while still on medical management? Based on the relatively short lived response to the treatment with IVIG with an almost immediate return of her platelet count to values below 25,000/$mm^3$, she was unlikely to have a positive response to another course of IVIG, as in fact occurred when she was admitted for the elective C-section on the 34$^{th}$ week of gestation. Since she was in the second trimester of pregnancy should she have had a laparoscopic splenectomy at this time to avoid the possible bleeding hazards to her and her fetus associated with platelet concentrations below a critical level? There has been at least one case report suggesting that laparoscopic splenectomy in the second trimester of pregnancy is a viable therapeutic option for the treatment of refractory immune-mediated thrombocytopenia in pregnancy [15]. Furthermore, if laparoscopic splenectomy is performed in the second trimester, the patient is very likely to have a spontaneous delivery.

Our hematology consultant felt that it was safe to allow the patient to continue her pregnancy with a platelet count above 20,000/$mm^3$ and that the patient should undergo delivery via C-section after the $34^{th}$ week of gestation after another course of IVIG, if needed. Due to the inadequate response to the repeated course of IVIG, the hematologist advised that the patient have an open splenectomy at the time of the C-section, after transfusion of platelet concentrates to raise the platelet count above 50,000/$mm^3$. Obviously, three issues are raised by the suggestions made by our hematologist: $1^{st}$ the route of delivery; $2^{nd}$ the need and timing for splenectomy; $3^{rd}$ the overall outcome of splenectomy for thrombocytopenia associated with SLE and the ability to predict the response to the splenectomy in this patient.

The first question about the route of delivery concerns whether a vaginal delivery instead of the CS could have been done after having increased the platelet count above 50,000/$mm^3$ with transfusion of platelets, delaying the splenectomy to a later time after having observed whether the pregnancy itself had an effect on the patient's thrombocytopenia. In the past, it has been common practice to perform cesarean section on mothers with SLE-associated thrombocytopenia to decrease the potential risk on intracranial hemorrhage caused by a vaginal delivery [16, 17]. It has also been suggested that fetal platelet count less than 50,000/$mm^3$ may place the fetus at greater risk of intracranial hemorrhage therefore CS should be considered in these

instances [10]. Based on the potential relationship between fetal platelets count and the risk of intracranial bleeding, some authors have suggested the use of either fetal scalp blood sampling or percutaneous umbilical vein sampling to determine the fetal platelet count and to select the mode of delivery [18, 19]. However, more recent data doe not support a correlation between fetal scalp platelet counts and neonatal platelet count at birth and it provide evidence that percutaneous umbilical vein sampling is associated with a high incidence of complications [20]. Furthermore, the universal application of CS delivery to pregnant patients with immune-mediated thrombocytopenia has not been shown to decrease the incidence of intra-ventricular hemorrhage [21]. A review of 601 infants born to women with immune thrombocytopenia showed that severe neonatal thrombocytopenia was present in 72/601 (12%) and that the neonatal intracranial hemorrhage is extremely rare and unrelated to the mode of delivery [21].

The second issue is whether splenectomy was indicated at the time of the CS at the 34$^{th}$ week of gestation or whether the patient should have had the splenectomy in the second trimester. The role and timing of splenectomy in pregnant SLE-patients with thrombocytopenia refractory to medical treatment, including IVIG, remains controversial because of the very limited literature available to support an evidence-based approach to these patients. One report, which includes five patients with immuno thrombocytopenic

purpura (ITP) treated with splenectomy at the time of CS in the third trimester of gestation, proposes that splenectomy following CS is a very good option for mothers with severe resistant ITP [22]. This approach is also supported by another case report suggesting that splenectomy combined with CS at the 34th week of gestation can provide remission of ITP [23]. However, another case report supports laparoscopic splenectomy during the 2nd trimester has a therapeutic option since this approach may afford the patient the possibility of vaginal delivery in the 3rd trimester [24]. Obviously, it is not possible to identify the 'best" evidence-based approach to these patients based on occasional case reports. However, since laparoscopic splenectomy is safe in the 2nd trimester, it seems reasonable to adopt this approach that may prevent the need for a CS at the term of pregnancy.

The third issue regards whether there is evidence to support the efficacy of splenectomy to treat thrombocytopenia associated with SLE and whether there are useful predictors to identify responders versus non-responders to splenectomy in patients with SLE-associated thrombocytopenia in pregnant and non-pregnant patients. Hall and his associates in 1985 reviewed the efficacy of splenectomy to treat thrombocytopenia associated with SLE; they concluded that splenectomy is not effective at curing the thrombocytopenia observed in patients with SLE; therefore, they suggested that it should be used only as a very last resort after having exhausted all other therapeutic

modalities [25]. However, a more recent review of this issue from the Mayo clinic shows that splenectomy is effective in treating SLE-associated thrombocytopenia by providing an 88% early partial or complete response and a 64% sustained response rate [26]. The pathogenesis of thrombocytopenia in SLE is believed to result from either increased platelet clearance mediated by anti-platelet autoantibodies (anti-GPIIb/IIIa antibodies), a mechanism similar to that observed in idiopathic thrombocytopenic purpura (ITP) or by autoantibodies to thrombopoietin receptor (TPOR), which inhibits thrombopoietin-dependet magakaryogenesis. The presence of anti-TPOR antibodies has been associated with the lack of response to IVIG and may be predictive of a poor response to splenectomy in patient with SLE-associated thrombocytopenia [27].

## Conclusions

In conclusion, based on a thorough review of the available literature, we conclude that patients with SLE-associated thrombocytopenia refractory to medical treatment during pregnancy should undergo testing for the presence of anti-GPIIb/IIIa and anti-TPOR antibodies early during their pregnancy. If patients have anti-GPIIb/IIIa and absent anti-TORP antibodies and they fail to respond to IVIG, they should undergo laparoscopic splenectomy in the

second trimester of gestation followed by vaginal delivery at completion of gestation. Open splenectomy immediately following CS in the third trimester remains an available, safe choice, if splenectomy has not been done in the second trimester.

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*Submission will be in process.*

***Obesity and infection***

The world is currently experiencing an obesity epidemic as declared by the World Health Organization.

The traditional view is that behaviour leading to overeating and under-activity is the major contributing factor for this worldwide epidemic. However, several microbes are linked to obesity in animals and humans. On the one hand, various microbes, including animal and human viruses, bacteria, parasites and scrapie agents, increase adiposity in several animal models. Some of these microbes show an association with human obesity, but conclusive evidence for a causative role of microbes in human obesity is lacking. On the other hand, obese individuals show an altered response to infections. Obesity is often associated with impaired immune function, which may lead to increased susceptibility to infection with a number of different pathogens. Hence, certain microbes appear to induce obesity, whereas, obesity itself may exacerbate certain other infections. Linking the two phenomenon is the immunological property of adipocytes and their progenitors.

For instance, proliferating pre-adipocytes share embryonic origin with immune cells and exhibit phagocytic activity. Taken together it appears that there is a close interrelationship between adipose tissue, inflammatory response, immune system and infections. Hence, it is conceivable that in

response to certain infections, adipose tissue expands similar to the expansion of cells of the immune system. The impaired immune function of adipose tissue in obesity may exacerbate infections. Considering the global obesity epidemic, it is necessary to further investigate both phenomena.

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*Research Article*

# *In Vitro* Resistance to Macrolides and Clindamycin by Group B *Streptococcus* Isolated from Pregnant and Nonpregnant Women

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*Background*. Despite the introduction of screening bases intrapartum prophylaxis, *Streptococcus agalactiae* is still an important etiological agent of perinatal infections. The increasing rate of resistance and the differences in resistance pattern among countries suggest that a program of surveillance at the institutional level is important in determining optimal prophylaxis. In contrast, knowledge on GBS epidemiology in Italy is limited, and no data are available in the Southern region of the country. We sought to determine the occurrence of resistance to macrolides and clindamycin of GBS isolates in pregnant and nonpregnant women. *Methods*. Between 2005 and 2008, 1346 vaginal and 810 rectovaginal swabs were obtained from pregnant and not-pregnant women. *Results*. The occurrence of macrolides and clindamycin resistance was 16.5% in 2005 increasing up to 69.9% in 2008. A high percentage of isolates was resistant to tetracycline through all the study period with no statistically significant annual. *Conclusions*. In our cohort, an increase of *in vitro* resistance of GBS to macrolides and clindamycin is clearly evident. The discordance with reports from different countries emphasize the crucial role of microbiological methods in setting possible therapeutic strategies.

## 1. Introduction

Among Gram-positive bacteria, *Streptococcus agalactiae*, also known as Group B *Streptococcus* (GBS) is considered a common commensal of the female urogenital tract and rectum [1] whose importance is referred to severe neonatal pathologies by perinatal transmission from women to newborns. Neonatal infections by GBS are usually distinguished in early onset (occurring in the first 7 days of life) and late onset (occurring between 7 days of life and 3 months of age): this temporal distinction reflects differences in the spectra of infection [2, 3].

In studies carried out in the 1970s, GBS emerged as the leading cause of neonatal morbidity and mortality, with a frequency of 2-3 cases per 1000 live births and case-fatality ratios of 50% [3, 4]. The vaginal colonization prevalence among pregnant women varies in European countries between 10 and 20%, and the incidence of neonatal infections ranges from 0.5 to 2 per 1000 live births [5–8].

Between 1996 and 1997, the American College of Obstetricians and Gynecologists, the Centers for Disease Control and Prevention, and the American Academy of Pediatrics produced recommendations for prevention of perinatal GBS disease. These guidelines recommended the use of culture-based screening for GBS colonization between 35 and 37 weeks gestation and the antibiotic prophylaxis of all colonized women [9, 10].

The intrapartum antibiotic prophylaxis (IAP) for GBS carriers indicates the use of penicillin (or ampicillin). For penicillin-allergic women without a history of anaphylaxis, angioedema, respiratory distress, or urticaria, cefazolin is the preferred agent. Vancomycin and clindamycin are recommended for penicillin-allergic women at high risk for anaphylaxis. The introduction of IAP for GBS carriers has

been associated with a substantial decline in the incidence of early-onset neonatal infections [11].

The purpose of the present study was to assess the antibiotic susceptibility patterns of GBS isolates obtained from a heterogeneous female population (pregnant and nonpregnant) in a region of Southern Italy and to evaluate whether statistically significant changes in GBS antibiotic resistance regarding macrolides and clindamycin occurred in the years in order to generate local data for the development of rational interventions for prevention of GBS infection in our country.

## 2. Materials and Methods

*2.1. Study Population.* In the period from January 2005 up to December 2008, a total of 2156 biological samples (1346 vaginal swabs from nonpregnant women and 810 rectovaginal swabs from pregnant women at 35–37 weeks of gestation) were collected in the Microbiology Laboratory of University Hospital "Federico II", Naples, Italy. All women gave their consent to take part in the study. Therapeutic protocols were not modified for women enrolled in the study.

*2.2. Processing of Samples, Culture of Microorganisms, and Identification Analysis.* All swabs were maintained in the Stuart transport medium and transported to the Microbiology Laboratory. Swabs were plated on several agar media, including Columbia colistin-nalidixic acid (CNA) agar with addition of 5% of sheep blood, MacConkey agar, Sabouraud agar, and chocolate agar and incubated at 37°C overnight in aerobic or microaerobic conditions and were examined microscopically to evaluate the preservation of *Lactobacillus* microbial status.

Bacteria were identified by conventional methods (Gram stain, catalase test) and automated system (Vitek II, bioMérieux, France). The identification of the Lancefield antigen was obtained by Streptococcal Grouping Kit (Oxoid, Hampshire, England).

*2.3. Antimicrobial Susceptibility Testing Method.* To check the sensitivity to antimicrobial agents, an automated microdilution method (Vitek II) was utilized. The susceptibility criteria were in accordance with the National Committee for Clinical Laboratory Standards Interpretative Criteria [12]. Antibiotics tested were as follows: amoxicillin/clavulanic acid, ampicillin, cefaclor, cefotaxime, ceftriaxone, clindamycin, erythromycin, penicillin, teicoplanin, tetracycline, trimethoprim/sulfamethoxazole, vancomycin, levofloxacin, azithromycin, clarithromycin, quinupristin/dalfopristin, and linezolid.

*2.4. Statistical Analysis.* Statistical analysis, including comparison of proportions and chi-squared test, was applied throughout the study. A $P < 0.05$ was considered statistically significant.

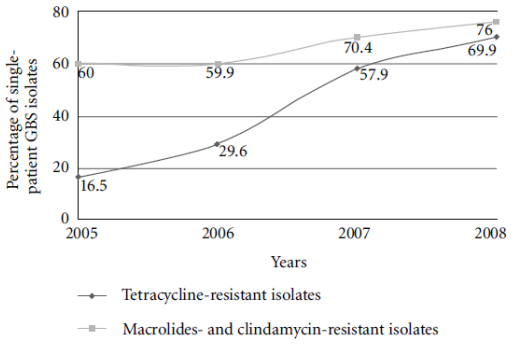


FIGURE 1: Annual increment (%) of resistant isolates during the study period.

## 3. Results

In the study period, a total of 879 GBS from all samples (2156 between vaginal and rectal-vaginal swabs) were isolated. The distributions of swabs, positive cultures, and patients in the period of study are indicated in Table 1.

The distribution of single-patient resistant GBS isolates is showed in Table 2 as well as the susceptibility pattern over the study period. The antibiotic susceptibility profiles indicate that isolates showed sensitivity to beta-lactams, glycopeptides, quinolones, quinupristin-dalfopristin, trimethoprim-sulfamethoxazole, and linezolid.

The number of isolates resistant to tetracycline was high through all the study period, indicating not statistically significant fluctuations. Instead, the increment of resistance to macrolides and clindamycin was statistically significant through the study period ($X_2$ for trend = 8.100, $P = 0.004$). The annual increment in percentage of macrolides- and clindamycin-resistant isolates during the study period is indicated in Figure 1.

The GBS isolates resistant to tetracycline showed a MIC value $\geq 16\,\mu$g/mL through all the study period. The MICs obtained for macrolides and clindamycin range from $\leq 0.25\,\mu$g/mL to $\geq 8\,\mu$g/mL.

## 4. Discussion

In our experience, in accordance with CDC 2010 guidelines, penicillin and ampicillin are still the first choice for IAP, followed by first-generation cephalosporins as cefazolin in penicillin allergic women. In fact all GBS isolates were susceptible to these antibiotics. The prevalence of isolates resistant to macrolides and clindamycin is considerably high (55%) and has increased significantly from 16.5% to 70% during the study period ($P < 0.05$).

Although very few women GBS positive give birth to babies who are infected with GBS, antenatal screening is routinely performed to reduce the rate of early-onset infections in newborns. However, there is still controversy about its prevention since antenatal screening and treatment

Table 1: Distribution of swab type, positive cultures, and number of infected patients.

| Year | Total swabs *N* (%) | | Total positive cultures *N* (%) | |
|---|---|---|---|---|
| | Vaginal-rectal | Vaginal | Vaginal-rectal | Vaginal |
| 2005 | 76 (7.7%) | 153 (13%) | 23 (6.4%) | 62 (11.9%) |
| 2006 | 192 (19.5%) | 212 (18.1%) | 51 (14.3%) | 97 (18.6%) |
| 2007 | 345 (35.1%) | 357 (30.4%) | 134 (37.5%) | 146 (28%) |
| 2008 | 370 (37.6%) | 451 (38.4%) | 149 (41.7%) | 217 (41.6%) |
| | Total 2156 | | Total 879 | |

Table 2: Distribution (number and percentage) of resistant GBS strains during the 4-year study period.

| | 2005 positive cultures (85) | | 2006 positive cultures (162) | | 2007 positive cultures (280) | | 2008 positive cultures (366) | |
|---|---|---|---|---|---|---|---|---|
| | *N*° | % | *N*° | % | *N*° | % | *N*° | % |
| AMC | 0 | — | 0 | — | 0 | — | 0 | — |
| AMP | 0 | — | 0 | — | 0 | — | 0 | — |
| CEC | 0 | — | 0 | — | 0 | — | 0 | — |
| CTX | 0 | — | 0 | — | 0 | — | 0 | — |
| CRO | 0 | — | 0 | — | 0 | — | 0 | — |
| CLI | 14 | 16.5 | 48 | 29.6 | 162 | 57.9 | 256 | 69.9 |
| ERY | 14 | 16.5 | 48 | 29.6 | 162 | 57.9 | 256 | 69.9 |
| PEN | 0 | — | 0 | — | 0 | — | 0 | — |
| TEC | 0 | — | 0 | — | 0 | — | 0 | — |
| TET | 51 | 60 | 97 | 59.9 | 197 | 70.4 | 278 | 76 |
| SXT | 0 | — | 0 | — | 0 | — | 0 | — |
| VAN | 0 | — | 0 | — | 0 | — | 0 | — |
| LVX | 0 | — | 0 | — | 0 | — | 0 | — |
| AZM | 14 | 16.5 | 48 | 29.6 | 162 | 57.9 | 256 | 69.9 |
| CLR | 14 | 16.5 | 48 | 29.6 | 162 | 57.9 | 256 | 69.9 |
| Q-D | 0 | — | 0 | — | 0 | — | 0 | — |
| LZD | 0 | — | 0 | — | 0 | — | 0 | — |

AMC = amoxicillin-clavulanic acid; AMP = ampicillin; CEC = cefaclor; CTX = cefotaxime; CRO = ceftriaxone; CLI = clindamycin; ERY = erythromycin; PEN = penicillin; TEC = teicoplanin; TET = tetracycline; SXT = trimethoprim-sulfamethoxazole; VAN = vancomycin; LVX = levofloxacin; AZM = azithromycin; CLR = clarithromycin; Q-D = quinupristin-dalfopristin; LZD = linezolid.

may carry disadvantages for the mother and the baby. The usual recommendation for prevention of GBS transmission from colonized women to their infants during labour is to administer intravenous penicillin or ampicillin every 4 h for the duration of labour [11].

On the maternal side, IAP's risks are allergic reactions. Even if there are some anecdotal reports of maternal mortality due to anaphylaxis, usually allergic reactions are not severe and mainly with maculopapular rushes [11, 13, 14].

On the fetal/neonatal side, there is no risk for anaphylaxis resulting from IAP, but there is a growing concern about the development of antibiotic resistance among GBS isolates and other pathogens. The increased resistance may have two effects: exposure of neonates to antibiotic-resistant pathogens with development of intractable sepsis and reduction of the chance to prevent maternal fetal transmission by GBS.

Two recent published surveys have demonstrated that in England and France neonatal infections are still mainly caused by GBS, and the current policy of GBS maternal prophylaxis is not associated with an excessive risk of pathogen resistance [15, 16]. The incidence of early-onset sepsis (EOS) ranged among 0.9 to 1.9/1000 live births, and GBS (58–62%) and *Escherichia coli* (18–25%) were the most common organisms. About the antibiotic resistance, the majority of pathogens (95%) causing EOS were susceptible to commonly used empiric first-line antibiotic combinations.

About the risk of reduced efficacy of IAP for GBS, data are reassuring. Worldwide, there have been only a few reports of penicillin resistance [17, 18] or elevated MIC [19, 20] secondary to the alterations in penicillin-binding proteins (PBP). In the majority of isolates with alteration of PBP, the measured MICs were just at the threshold of susceptibility, but the clinical significance of higher MIC values remains unclear. Elevated MICs to cefazolin also were reported, but as penicillin/ampicillin, the clinical significance of higher MICs to cefazolin among GBS isolates remains unclear [11]. In our experience GBS isolates have not yet developed

any resistance against penicillin and ampicillin and first-generation cephalosporin. This aspect is very reassuring if we consider that these antibiotics are constantly indicated as first choice in women positive for GBS. Their efficacy as IAP was demonstrated for the first time in clinical trials by Boyer and Gotoff [21] in 1986 and by Garland and Fliegner [22] in 1991. On the contrary the efficacy of alternatives to penicillin/ampicillin for allergic women (including cefazolin, clindamycin, erythromycin, and vancomycin) has not been tested in controlled trials. About cephalosporin, it has been supposed that, given the similar activity, pharmacokinetics, and dynamics of cefazolin to penicillin/ampicillin, it could be a second-line antibiotic in penicillin allergic women with low risk of anaphylaxis. As long as allergic women with high risk of anaphylaxis, the guidelines suggest the use of clindamycin/erythromycin or vancomycin although their ability to reach bactericidal levels in the fetal circulation and amniotic fluid are very limited [23–25]. The choice of one or another antibiotics is made on the results of antimicrobial susceptibility testing. These women should receive clindamycin if their GBS isolate is susceptible to clindamycin and erythromycin or if it is resistant to erythromycin but sensitive to clindamycin with negative testing for inducible clindamycin resistance. Otherwise, if susceptibility to both agents is unknown, these women should receive vancomycin. At the moment, erythromycin is no longer considered an alternative for IAP in penicillin-allergic women at high risk for anaphylaxis [11].

Starting from our data, in the next future the problems related with antibiotic resistance will become bigger and bigger and the treatment of allergic women will be a major obstacle. In fact during the study period, the number of colonized women is increased from 85 to 366. If we consider stable the number of allergic women at risk for anaphylaxis, we will have that more and more women will be treated with alternative antibiotics which will be potentially ineffective or will increase the spectrum of resistance.

In reports published, the prevalence of resistance among GBS ranged from 7% to 25% for erythromycin and from 3% to 21% for clindamycin [26, 27]. Resistance to erythromycin was frequently but not always associated with clindamycin resistance. In our series resistance was always to both erythromycin and clindamycin, and the prevalence was considerably higher, ranging from 16.4% to 70% in the study period, showing a statistically significant increment ($P < 0.05$). This finding is very far from previous reports from other nations indicating significant country variations and supporting the usefulness of research about GBS in each population. The increased resistance to macrolides, particularly to erythromycin observed all over the world, can be ascribable to the treatment of *Chlamydia* infections of the lower reproductive tract [28]; however we have no explanation for the higher rate of resistance in our population. An hypothesis is that the variation may be due to differences in techniques as well as characteristics of the population investigated.

The prevalence of GBS-positive women observed in our population is higher even when compared with other Italian studies. In the study of Savoia et al. [29], among 300 pregnant women screened, 73 single-patient GBS isolates were collected and only 3 out of 73 (4.1%) were resistant to erythromycin. Also the lincosamides (lincomycin) were less efficient. Overall the infection prevalence was 18.2% versus 41% observed in our population (879 infected patients out of 2156 patients). In another Italian study by Sensini et al. [6], the prevalence of GBS was even lower (11%). Comparing the numbers, an hypothesis is that the prevalence of infection is growing over the years (11% versus 18.2 versus 41%).

The main limitations of our study are the difference in surveillance population (pregnant and nonpregnant) and, for pregnant women, the lack of clinical data about the pregnancy and neonatal outcome. This aspect could be a starting point for new research since to date whether *in vitro* resistance of GBS has direct clinical implications remains unclear.

## 5. Conclusion

Antibiotics are used for both GBS prevention and treatment. The introduction of IAP for GBS carriers has been associated with a substantial decline in the incidence of early-onset neonatal infections. However, the potential side effect of the protocol is the risk of development of pathogen resistance to antibiotics. Until now GBS isolates remain susceptible to penicillin and ampicillin and first-generation cephalosporin, but resistance to alternative agents as erythromycin and clindamycin is an increasing concern. In fact these agents are suggested in women with high risk of anaphylaxis although their ability to reach bactericidal levels in the fetal circulation and amniotic fluid is very limited. Comparing reports of the literature, epidemiology of infection, and resistance pattern change substantially among countries suggesting the need of local study to map the prevalence of resistant isolates.

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