nutrients

MDPI

Research Manuscript

Epigenetic Profiling in the Saliva of Obese Pregnant Women

Chiara Mandò[1*], Silvio Abati[2], Gaia Maria Anelli[1], Chiara Favero[3], Anais Serati[1], Laura Dioni[3], Marta Zambon[4], Benedetta Albetti[3], Valentina Bollati[3,5], Irene Cetin[1,4]

[1] Department of Biomedical and Clinical Sciences, Università degli Studi di Milano, Milano, Italy

[2] Dept. of Dentistry, University Vita-Salute San Raffaele, Milano, Italy

[3] EPIGET LAB, Department of Clinical Sciences and Community Health, Università degli Studi di Milano, 20122, Milan, Italy

[4] Department of Woman, Mother and Child, Luigi Sacco and Vittore Buzzi Children Hospital, ASST Fatebenefratelli-Sacco, Milano, Italy

[5] Occupational Health Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

*** Corresponding Author:** Chiara Mandò, Associate Professor of Nutritional Sciences, Department of Biomedical and Clinical Sciences "Luigi Sacco", Università degli Studi di Milano, Milano, Italy

Abstract:

Maternal obesity is associated with inflammation and oxidative stress, strongly impacting the intrauterine environment with detrimental consequences for both mother and offspring. The saliva is a non-invasive biofluid reflecting both local and systemic health status. This study aimed to profile the epigenetic signature in the saliva of obese (OB) and normal-weight (NW) pregnant women.

Sixteen NW and sixteen OB Caucasian women with singleton spontaneous pregnancies were enrolled. microRNAs were quantified by OpenArray Platform. The promoter region methylation of Suppressor of Cytokine Signaling 3 (SOCS3) and Transforming Growth Factor Beta 1 (TGF-Beta1) was assessed by Pyrosequencing.

754 microRNAs were evaluated: 20 microRNAs resulted differentially expressed between OB and NW. microRNAs Pathway Enrichment Analysis showed a significant association with TGF-Beta Signaling pathway (miTALOS) and with fatty acids biosynthesis/metabolism, Lysine degradation, ECM-receptor interaction pathways (DIANA-miRPath). Both *SOCS3* and *TGF-Beta1* were significantly down-methylated in OB vs NW.

These results help to clarify impaired mechanisms involved in obesity and pave the way for the understanding of specific damaged pathways. The characterization of the epigenetic profile in the saliva of pregnant women can represent a promising tool for the identification of obesity-related altered mechanisms and of possible biomarkers for early diagnosis and treatment of pregnancy adverse conditions.

Keywords: pregnancy; maternal obesity; GDM; oxidative stress; inflammation; epigenetics; miRNA; DNA methylation; saliva; periodontal disease

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Nutrients* **2022**, *14*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

1. Introduction

The obesity epidemic is expected to involve almost 21% of women all over the world by 2025 **[1]**, representing a serious risk for women of reproductive age. Maternal obesity during pregnancy represents a danger for short and long-term health outcomes for both mothers and children **[2-4].** Indeed, up to 50% of obese pregnant women develop Gestational Diabetes Mellitus (GDM) **[5]** and the offspring of obese and diabetic mothers are at higher risk of developing metabolic and cardiovascular diseases in early childhood and later in their adulthood **[6]**, with possible transgenerational effects **[7]**. Moreover, newborns of obese and diabetic mothers have been recently shown to be hypoxic, acidemic, and to have increased oxidative markers compared to normal-weight pregnancies **[8, 9]**.

The metabolic and hormonal balance is dysregulated in obesity and diabetes because of excessive adipose tissue deposition and intracellular fatty acids, leading to alterations of the immune cell profile and inflammaging **[10]**. This metabolic-dependent pro-inflammatory background increases oxidative stress and insulin resistance that further contribute to metabolic dysregulation, in a deleterious vicious circle. Pregnancy itself is characterized by a physiological generalized low-grade inflammation, and maternal obesity can contribute to additional lipotoxicity, inflammation, and oxidative stress that may strongly impact the intrauterine environment, with significant effects on fetal health and metabolism **[8, 11, 12]**. We previously reported alterations in the placentas of obese mothers, showing oxidative stress and dysregulated placental metabolome **[13-15]**. These data suggest that obesity-related increased inflammation and oxidative stress may determine a cascade of events leading to systemic and local dysfunctions and consequent impaired pregnancy outcomes.

Among obesity-related recurring comorbidities, periodontal diseases represent an additional important risk factor for pregnancy outcomes **[16]**. During pregnancy, both obesity and diabetes can cause oral dysbiosis **[17]** which is the primary cause of periodontal diseases **[18]**. In turn, maternal periodontal diseases have been associated with poor obstetric outcomes, such as pre-term birth, premature rupture of membranes, and low birth weight, especially in obese women with pre-gestational obesity and GDM. The systemic inflammatory alterations induced by both obesity/GDM and periodontal diseases can indeed activate adverse responses at the maternal-fetal interface **[19-22]**. We recently reported increased total antioxidant capacity and inflammatory levels in both saliva and blood of obese pregnant women with and without GDM, suggesting a synergic detrimental effect of obesity and periodontal diseases **[23]**.

The saliva is an advantageous, non-invasive, and cost-effective biofluid, reflecting both the local (oral) and systemic health status of the human body **[24]**. Salivary glands are surrounded by capillaries, resulting in a continuous exchange of molecules between blood flow and saliva secretion from the acinus cells. Therefore, the circulating molecules absorbed by salivary glands can affect the saliva composition, mirroring the systemic status **[25]**. Among the multitude of molecules contained in the saliva, microRNAs (miRNAs) are proving promising biomarkers, representing important epigenetic regulators. Indeed, about 500 of these short non-coding RNA molecules have been identified in saliva **[26]**.

Interestingly, alterations of several miRNAs levels have been reported in the maternal circulation depending on pregestational BMI **[27]** and a few salivary miRNAs have been identified as predictors of periodontal disease in both non-diabetic and diabetic non-pregnant patients **[28]**. However, to our knowledge miRNAs profiles in the saliva have never been investigated during pregnancy in the presence of obesity and possible related co-morbidities.

This study aimed to assess miRNAs salivary levels in a group of obese pregnant women that were previously deeply characterized for periodontal diseases comorbidities **[23]**.

Moreover, further epigenetic modifications such as gene methylation are also known to be deregulated in obesity and obesity-related insulin resistance. Therefore, methylation levels of specific genes that are well-known players in this context were assessed in maternal saliva: *SOCS3* (Suppressor Of Cytokine Signalling-3), which is upregulated in response to obesity-related stimuli **[29]** and *TGF-Beta1* (Transforming Growth Factor-Beta1), which is a key cytokine in obesity and insulin resistance and has been reported at higher levels in women with prior history of GDM **[30]**.

The characterization of the epigenetic profile in the saliva of obese women during pregnancy will represent a promising tool for the identification of obesity-related altered molecular mechanisms and possible biomarkers for the early diagnosis and treatment of pregnancy adverse conditions.

2. Materials and Methods

2.1 Study Participants

This is a pilot study performed on a cohort of pregnant women enrolled in the antenatal clinic at the regular checkup in the Obstetric Unit of the L. Sacco Hospital (ASST Fatebenefratelli-Sacco) in Milan. The study was conducted in accordance with the Declaration of Helsinki and in compliance with current Good Clinical Practice guidelines, local laws, regulations, and organizations. The protocol was approved by the hospital ethical committee (Prot. N. 469/2010/52/AP). Participants gave their written informed consent to collect personal data and biological samples. Pregnant women were enrolled during the first trimester and had a regular clinical follow-up during all trimesters. A deep oral health characterization of this population has been previously reported **[23]**.

Only Caucasian women with a singleton spontaneous pregnancy and aged between 18 and 40 years were enrolled. Exclusion criteria were maternal and fetal infections, fetal malformations, chromosomal disorders, maternal alcohol/drugs abuse, and pregestational BMI <18.5 or between 25 and 30.

Thirty-two pregnant women were classified according to their pregestational Body Mass Index (BMI) as normal-weight (NW, n= 16: $18.5 \leq BMI \leq 24.9$ kg/m^2) or obese (OB, n= 16: BMI ≥ 30 kg/m^2). Ten out of sixteen OB women were diagnosed with Gestational Diabetes Mellitus (GDM), based on an oral glucose tolerance test (75 g), according to our clinical

protocol. NW women had uncomplicated pregnancies. Therefore, GDM was an exclusion criterion in this group.

OB patients were followed-up in a dedicated antenatal clinic, providing nutritional counseling on diet and lifestyle, particularly focused on pregnancy weight gain. Obese patients with GDM received further advice to control their glycemia according to the clinical protocol.

2.2 Clinical Data and Biological Samples Collection

Clinical Data: maternal, neonatal, and placental data were recorded at recruitment and after delivery.

Oral and Periodontal Health: a complete clinical oral health examination was performed in the III trimester, as previously reported **[23, 31]**. Pregnant patients were classified as *healthy* with an overall good oral care (no dental soft debris- dental plaque or materia alba or calculus- or nor gingival Bleeding On Probing- BOP or Probing Pocket Depth- PPD >3 mm); or *periodontally diseased* with an overall neglected oral care, which grouped *gingivitis* (soft debris and calculus in ≥6 teeth and gingival bleeding ≤ 5 teeth) and *periodontitis* (Probing Pocket Depth ≥4 mm, soft debris, calculus, and bleeding ≥ 6 teeth).

Saliva Collection: saliva sampling was performed during the third trimester without any stimulation (*passive drool technique*) thus avoiding the use of solutions that could alter physico-chemical features and preventing the employment of tools requiring a specific site of withdrawal. Choosing the same timing of withdrawal for all subjects eludes any fluctuations in the saliva composition related to circadian rhythms. Enrolled women were asked to refrain from eating, drinking sugar or alcoholic beverages, smoking, and performing invasive oral care procedures for at least 1h before collection. They were then asked to rinse their mouth (1 min) with a physiological solution to remove any food residues which could alter pH or promote bacterial growth and afterward not to swallow for 3 minutes and spit in a sterile tube. Collected samples were temporarily stored at -80°C.

2.3 Isolation of extracellular vesicles and miRNA

Saliva samples (1 ml) were thawed on ice to avoid extracellular vesicles (EV) thermal damage. Then, they were centrifuged at 4.000 x g for 30 minutes at 4°C to remove any cell debris and aggregates. Supernatants were ultracentrifuged at 110.000-x g for 75′ at 4 °C in order to pellet EV, then stored at -20°C.

MiRNAs isolation from the obtained EVs was performed with the combination of miRNeasy kit and RNeasy Cleanup Kit (Qiagen Hilden, Germany), according to the manufacturer's protocol. They were eluted in 20 μL of Nuclease-Free Water and stored at -80 °C until used. EV-miRNAs quality and integrity were assessed through the "2100 Bioanalyzer RNA system" with pico kit (Agilent Technologies) but the concentration (ng/μL) was assessed by Quantus Fluorometer (Promega, Italia).

2.4 Screening ofmiRNA expression

15 ng miRNAs were reverse transcripted (RT), preamplified (16 cycles), and analyzed by real-time PCR with the QuantStudio™ 12K Flex OpenArray® Platform (Applied Biosystems) as previously described in detail **[32].** Gene Expression Suite Software (Applied Biosystems) was used to process miRNA profiling expression data from the "TaqMan™ OpenArray™ human MicroRNA panel" (ThermoFisher Scientific) run. As described in Figure 1 we obtained 758 Crt values for each subject, which included 754 unique miRNAs and four internal controls (ath-miR159a, RNU48, RNU44, and U6). For each amplification curve, we obtained an AmpScore value, a quality measurement that indicates the low signal in the amplification curve linear phase (range: 0–2). MiRNAs with Crt value > 28 or AmpScore <1.24 or missing were considered not amplified, and thus the corresponding Crt value was set to 29. MiRNAs that were not amplified in all subjects (n = 420) were excluded, resulting in 334 miRNAs being included in the statistical analysis. MiRNA expression was determined using the relative quantification 2-ΔCrt **[33]**. Among the endogenous controls analyzed (i.e. Ath-miR159a, U6-rRNA, RNU44, RNU48), U6-rRNA and RNU48 were chosen for the normalization by the NormFinder algorithm **[34]**, due to their stability in the comparison between samples.

2.5 DNA Isolation

Total DNAwas extracted using QIAamp® DNA Mini and Blood Mini Kit (Qiagen; Valencia, CA, USA) following the supplementary protocol ('Isolation of genomic DNA from saliva and mouthwash using the QIAamp® DNA Blood Mini Kit'). Briefly, saliva (160 µL) was thawed on ice, and added with 640 µL of PBS (Phosphate Buffered Saline). Samples were then centrifuged at 1.800 x g for 10 minutes, their supernatant to remove any cell debris and aggregates. The pellets were lysed and DNA extracted with a spin-column based method. DNA was eluted in 44 µL of Buffer AE (10 mM Tris·Cl; 0.5 mM EDTA; pH 9.0) for a final concentration of 25 ng/ µL.

2.6 Bisulfite conversion and DNA methylation

500 ng di genomic DNA was bisulfite converted using the EZ DNA Methylation Direct Kit (Zymo Research, Orange, CA, USA), in accordance with the manufacturer's protocol. Converted DNA was eluted in 30 µl of Elution Buffer.

Analysis of DNA methylation was performed by PyroMark MD Pyrosequencing System (Qiagen, Milano, Italy) as previously described **[35, 36]**.

The bisulfite-treated genomic DNA samples were amplified with PCR; CpG sites were analyzed within the promoter regions of the following genes: Suppressor of Cytokine Signalling 3 (SOCS3) and Transforming Growth Factor Beta 1 (TGF-Beta1). Detailed information concerning primer sequences and genomic regions is listed in Table 1. The percentage of 5-methylcytosine (% 5mC) was reported as the percentage of methylated cytosine divided by the sum of methylated and unmethylated cytosines. Every sample was tested

twice for each marker to confirm reproducibility and to increase the precision of the findings.

Table 1. Pyrosequencing assay information.

Gene	Chromosome Position	CpG Sites		Primers: Forward (F) Reverse (R) Sequencing (S)	Sequencing Length	T° Annealing
SOCS3	CHR17:6354421-76354821	2	F	TTGGGTGATTTTTTTATAGGAGT	25	52
			R	bio-TCCCCCCAAAAAAACCTATT		
			S	GAGATGTTGAAGAGTGGTTA		
TGF-Beta1	chr19:41353024-41353458	2	F	GGTTTGTTTTTTGAGTTTT	23	54
			R	bio-CTACAAAAACTAAAAATCTCCC		
			S	TATTTATTTTTTGGTATTAG		

2.7 Statistical Analysis and Prediction Tools

Maternal, neonatal, and placental characteristics and periodontal health parameters were compared between groups by Student t-test or Mann-Whitney U test for independence samples according to data distribution (assessed by Kolmogorov-Smirnov test). Differences between groups in the frequencies of periodontal disease, mode of delivery, neonatal weight centile, and fetal sex were evaluated by the chi-square test for independence or Fisher's Exact Test.

Multivariable linear regression models were applied to verify the association between BMI groups and miRNA expression in the screening and validation phases. MiRNA expression values were log2-transformed to achieve a normal distribution. Regression models were adjusted for age, smoking habit, GDM, and gestational age at saliva withdrawal. Due to the high number of comparisons, we applied a multiple comparison correction method based on the Benjamini-Hochberg False Discovery Rate (FDR) to calculate the FDR P-value. In the screening phase, the criterion used to identify the top miRNAs were a P-value < 0.05, a FDR P-value <0.20 and Fold Change (FC)< 0.5 or FC> 2. A Volcano plot was produced to select miRNAs characterized by more than ± 2-fold case-control differences (FC 2 or < 0.5) with a p-value < 0.05 from linear regression models.

To evaluate the associations between methylation levels (SOCS3, TGF-Beta1) and BMI groups we used multiple linear regression models adjusted for maternal age, smoking habits, and GDM. We reported marginal means and 95% CI.

All statistical analyses were performed using the statistical package SPSS, v.27 (IBM; Armonk, NY, USA) and SAS 9.4 statistical software (SAS Institute Inc., Cary, NC).

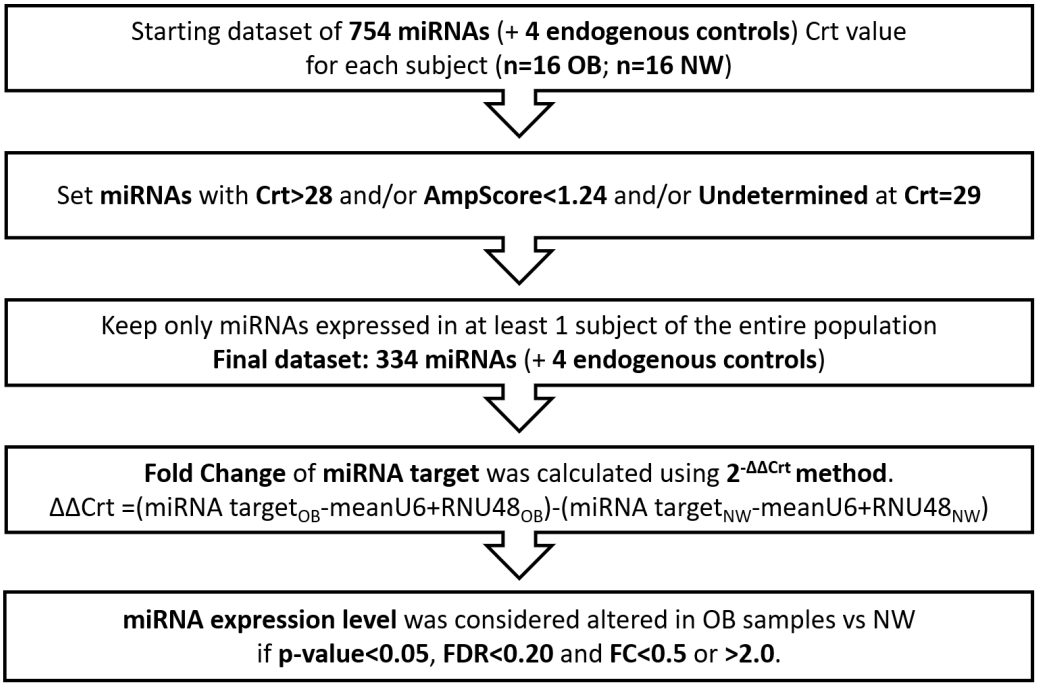

Figure 1. Data Processing Workflow.

miRNAs Pathway Enrichment Analysis: miTALOS v2 tools [available at: http://mips.helmholtz-muenchen.de/mitalos] were used to get insights into tissue-specific miRNA regulation of biological pathways. Indeed, miTALOS integrates five different miRNA target prediction tools and two different resources (KEGG and NCI), being able to identify a specific association between miRNAs and signaling pathways **[37]**.

We integrated our analysis by using DIANA-miRPath v3 [available at: https://diana-lab.e-ce.uth.gr/html/mirpathv3/index.php?r=mirpath], which uses data from KEGG and TarBase resources, to perform miRNAs pathway enrichment analysis. We thus investigated how these pathways are combined in a posteriori bioinformatic analysis (Union and Intersection of Pathways search). The results are shown as miRNA/pathways interaction heat maps **[38]**.

For both miTALOS v2 and DIANA-miRPath v3, the significance of the association (p-value) between miRNAs and the resulting pathways is calculated with Fisher's exact test; then, results for multiple pathways are corrected using the EASE score and False Discovery Rate (Benjamini) **[39-41]**.

3. Results

3.1. Maternal and Oral Health Characteristics and Delivery Data

Table 2 resumes maternal and oral health characteristics.

Pregestational BMI was significantly higher in the OB group compared to NW ($p < 0.001$), according to inclusion criteria. Gestational Weight Gain (GWG) was significantly lower in OB vs NW ($p = 0.035$), in compliance with IOM indications (upper limits: + 9 kg for OB, +16 kg for NW). However, OB women gained overall excessive weight than recommended for OB, as shown by the percentages reported in Table 2 (Maternal GWG to IOM Upper Limit). Maternal basal glycemia was higher in the OB group ($p = 0.034$).

Unstimulated saliva was collected at a similar gestational age and its flow rate had a normal range in both groups **[42]**.

Concerning the overall oral status, a higher percentage of mothers with periodontal disease was observed in the OB group (68.8%) compared to NW (56.2%), though not significantly. Among the recorded measures of dental and periodontal health, the number of teeth was significantly lower ($p = 0.027$) in the OB vs NW group. The dental plaque index percentage was significantly higher in OB women vs NW ($p = 0.024$).

Table 2. Maternal and Periodontal Health Characteristics.

	NW n=16	All OB n=16	*P-value*
Maternal Age, years [B]	30.94 ± 3.89	33.25 ± 4.61	*ns*
Maternal Pregestational BMI, kg/m² [B]	21.02 ± 2.25	36.47 ± 5.53	**<0.001**
Maternal GWG, kg [B]	13.38 ± 4.41	9.13 ± 5.63	**0.035**
Maternal GWG to IOM Upper Limit, % [A]	83.61 ± 27.54	101.37 ± 62.57	*ns*
Maternal Basal Glycemia, mg/dL [B]	81.50 ± 5.48	92.69 ± 15.18	**0.034**
GA at Saliva Withdrawal, mL [A]	33.24 ± 1.73	32.99 ± 2.52	*ns*
Saliva Flow-Rate, mL/min [A]	0.48 ± 0.22	0.47 ± 0.17	*ns*
Periodontal Health [C]			
healthy, n (%)	7 (43.8)	5 (31.2)	*ns*
periodontal disease, n (%)	9 (56.2)	11 (68.8)	
Number of Teeth [B]	27.60 ± 0.74	25.31 ± 3.01	**0.027**
BOP, % sites [B]	17.98 ± 19.76	39.57 ± 38.10	*ns*
PPD, mean [A]	2.38 ± 0.45	2.63 ± 0.88	*ns*

Plaque Index, % [A]	25.61 ± 18.97	52.90 ± 40.32	**0.024**
Calculus, % [A]	27.79 ± 23.65	44.53 ± 35.81	*ns*

Values expressed as mean ± standard deviation were analyzed according to their distribution with independent samples **A.** Student t-test or **B.** Mann-Whitney U test or **C.** Chi-Square Test for Independence or Fisher's Exact Test; statistical significance vs normal-weight women.

BMI: Body Mass Index; GWG: Gestational Weight Gain at delivery; IOM: Institute of Medicine; Maternal Basal Glycemia: maternal fasting glycemia refers to the first value of the Oral Glucose Tolerance Test (OGTT; physiological value ≤ 92 mg/dL) performed between 24 and 28 weeks of gestation; GA: Gestational Age; Flow Rate: ratio between mL of saliva and minutes of withdrawal; Oral Disease: gingivitis and/or periodontitis; PPD: Probing Pocket Depth; BOP: gingival Bleeding On Probing

All the enrolled women delivered at term, without obstetrical complications. No difference was recorded in the mode of delivery, although a higher percentage of cesarean section was observed in the OB group (NW: 12.5%; OB: 43.8%), in agreement with other epidemiological data from obese pregnancies.

Table 3 reports neonatal and placental data at delivery.

Neonates were similar in terms of gestational age and biometric parameters (weight and centiles); as expected, a higher number, though not significant, of LGA (Large for Gestational Age) babies were observed from OB mothers. Fetal sex frequencies did not differ between the two study groups.

Placentas were significantly heavier (p= 0.035) and thicker (p= 0.050) in the OB than in the NW group.

Table 3. Neonatal and Placental Data at Delivery.

	NW n=16	**All OB n=16**	***P-value***
GA at Delivery, wks [A]	39.76 ± 0.87	39.33 ± 1.18	*ns*
Neonatal Weight- **N**, gr [A]	3331.25 ± 294.60	3488.75 ± 356.62	*ns*
Neonatal Weight Centile [A]	47.56 ± 24.19	61.00 ± 28.72	*ns*
AGA, n (%) [C]	15 (93.8)	11 (68.8)	*ns*
LGA, n (%) [C]	1 (6.3)	5 (31.3)	
Neonatal Sex, n [C]			
Males, n (%)	9 (56.3)	7 (43.8)	*ns*
Females, n (%)	7 (43.8)	9 (56.3)	

Placental Weight, gr [A]	439.00 ± 79.05	509.29 ± 80.81	**0.035**
Neonatal/Placental weight [B]	7.78 ± 1.66	6.97 ± 1.63	*ns*
Placental Area, cm^2 [B]	281.94 ± 64.06	266.16 ± 45.33	*ns*
Placental Thickness, cm [A]	1.61 ± 0.37	1.97 ± 0.50	**0.050**

Values are expressed as mean ± standard deviation. Data were analyzed according to their distribution with independent samples **A.** Student t-test or **B.** Mann-Whitney U test or **C.** Chi-Square Test for Independence or Fisher's Exact Test; statistical significance vs normal-weight women.
GA: Gestational Age; Neonatal Weight Centile: calculated using INeS Charts [http://www.inescharts.com/index.aspx] referring to ***[43]****; AGA: Appropriate for Gestational Age; LGA: Large for Gestational Age.*

3.2 miRNAs Profile in Maternal Saliva

In maternal saliva, the presence of 754 miRNAs was evaluated by a simultaneous quantitation with *TaqMan OpenArray Human MicroRNA Panel* which contains the most biologically relevant miRNAs in miRBase [*https://www.mirbase.org/*]. Data were adjusted for gestational age at saliva withdrawal, maternal age and smoking habits, and GDM association as covariates.

The expression of 334 miRNAs was detectable in the saliva of at least one subject of our study population. Among these miRNAs, 31 were differentially expressed (FC< 0.5 or > 2.0) with statistical significance ($p < 0.05$) between OB and NW mothers, as shown by the Volcano plot (**Figure 2**).

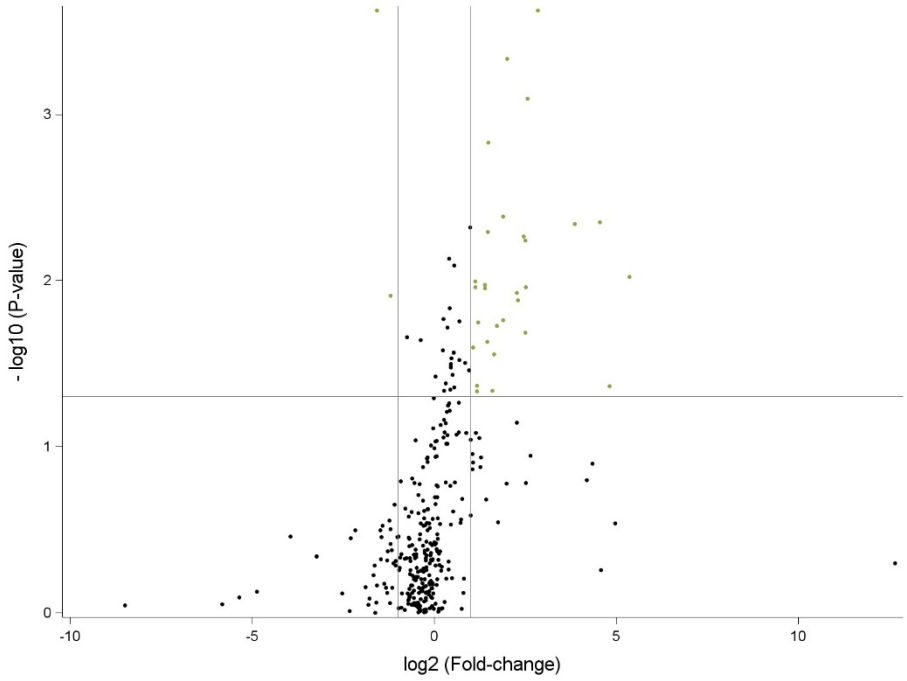

Figure 2. Volcano Plot showing Differentially Expressed miRNAs between OB and NW women. The graph reports the 334 detectable miRNAs (all black and green dots). The horizontal line states the –log10 of the p-value 0.05; the two vertical lines indicate the log2

of the FC values 0.5 and 2.0. As a result, miRNAs with a significantly different expression vs NW are displayed **in green** (n=31). In particular, the upper left quadrant reports the downregulated miRNAs, while the upper right one all the upregulated miRNAs.

Following FDR correction **20 miRNAs** were accepted for the downstream analysis (FDR< 0.20). In particular, 2 miRNAs out of 20 (*hsa-miR-505* and *hsa-miR616*) had FC< 0.5, thus resulting downregulated in the OB group. FC> 2.0 indicated upregulated levels for 18 miRNAs in OB women. FC, p-value, and FDR are summarized in **Table 4.**

Table 4. Up- and Down- Regulated miRNAs in Maternal Saliva.

miRNA name	Fold Change (FC)	P-value	False Discovery Rate P-value (FDR)
hsa-miR-505 ↓	0.335	0.0002	0.0396
hsa-miR-616 ↓	0.434	0.0123	0.1873
hsa-miR-618 ↑	7.141	0.0002	0.0396
hsa-miR-206 ↑	3.985	0.0005	0.0514
hsa-miR-376a ↑	5.870	0.0008	0.0671
hsa-miR-517c ↑	2.788	0.0015	0.0989
hsa-miR-133a ↑	3.703	0.0041	0.1599
hsa-miR-512 ↑	23.295	0.0044	0.1599
hsa-miR-302d ↑	14.427	0.0046	0.1599
hsa-miR-520b ↑	2.755	0.0051	0.1599
hsa-miR-1254 ↑	5.443	0.0054	0.1599
hsa-miR-133b ↑	5.634	0.0057	0.1599
hsa-miR-1285 ↑	40.956	0.0095	0.1859
hsa-miR-635 ↑	2.178	0.0102	0.1859
hsa-miR-551b ↑	2.614	0.0106	0.1859
hsa-miR-548b-5p ↑	5.692	0.0110	0.1859

hsa-miR-1256 ↑	2.169	0.0110	0.1859
hsa-miR-302c ↑	2.622	0.0111	0.1859
hsa-miR-184 ↑	4.817	0.0118	0.1873
hsa-miR-548c-5p ↑	4.912	0.0131	0.1909

Fold Change (FC), p-value and False Discovery Rate (FDR) of the 20 miRNAs with significantly different expression levels in OB vs NW. Data were analyzed with a multiple linear regression analysis adjusted for gestational age at saliva withdrawal, age, smoking habits and GDM association. Results are presented in descending order of statistical significance. (↓: downregulated; ↑: upregulated miRNA in OB vs NW).

To get insights into the miRNA profiling results, miTALOS and DIANA–miRPath were used for *miRNAs Pathway Enrichment Analysis.*

First, all the 20 miRNAs were inserted together in miTALOS. Interestingly, the union analysis (i.e. *combinations of pathways related to the inserted miRNAs*) showed a significant association between the 20 miRNAs and a specific pathway involving *TGF-Beta Signaling* (p=0.040).

An additional analysis was performed by DIANA–miRPath v3. A heat map depicting the force of association was obtained after inserting the 20 miRNAs differentially expressed in OB vs NW maternal saliva. 4 pathways matches were pointed out by theDIANA-miRPath *Pathway Enrichment Analysis* (**Figure 3** and **Table 5**).

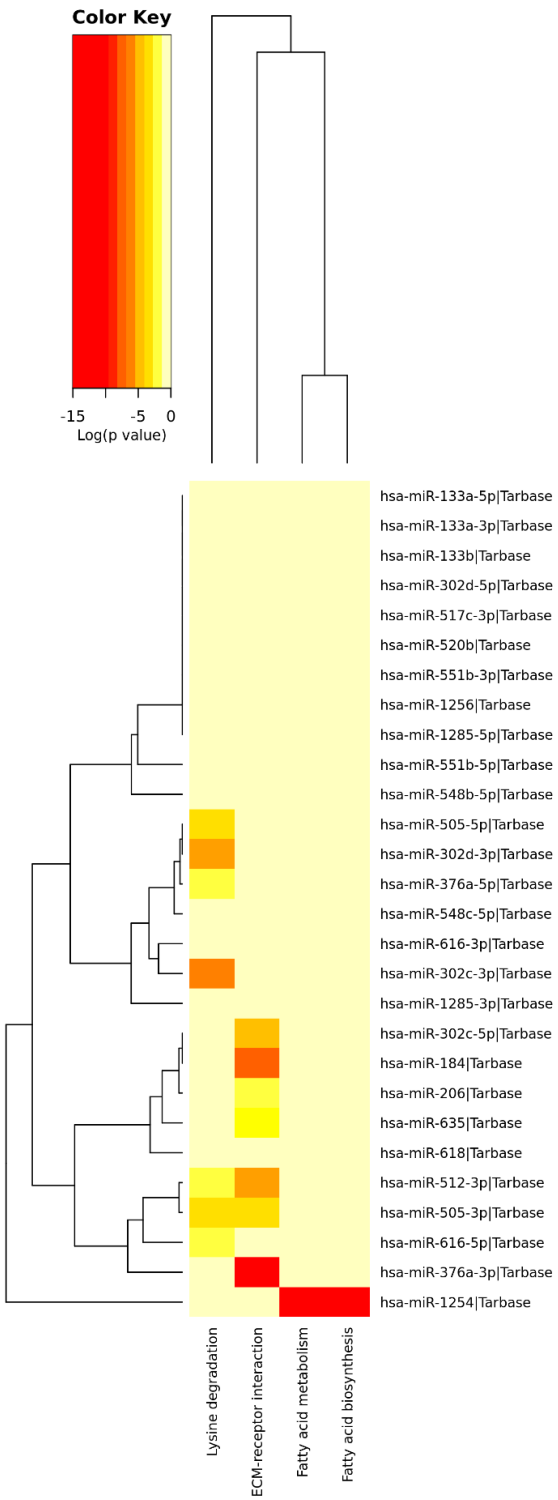

Figure 3. Heat-Map by DIANA-miRPath v.3. The analysed miRNAs are listed on the right. The 4 specific pathways, resulted significant from miRNAs p*athway enrichment analysis*, are listed in the bottom. The force of association [Log(p-value)] between the analysed miRNAs and the 4 pathways is indicated by the color key reported on the upper left corner.

Table 5. Union Analysis Performed with DIANA-miRPath v.3.

		Pathways information	p-values	miRNAs
	Fatty acids biosynthesis	Synthesis of fatty acids. Fatty acids are generally excessive in the Western diet.	2.1444×10^{-11}	hsa-miR-1254
	Fatty acid metabolism	Anabolic and catabolic processes involving fatty acids or related molecules. Fatty acids are generally excessive in the Western diet.	0.0002	hsa-miR-1254
	Lysine degradation	Catabolism of lysine (from dietary up-taken or intracellular proteins). Lysine is generally excessive in the Western diet.	1.7153×10^{-7}	hsa-miR-505-5p; hsa-miR-302d-3p; hsa-miR-376a-5p; hsa-miR-302c-3p; hsa-miR-512-3p; hsa-miR-505-3p; hsa-miR-616-3p
	ECM-receptor interaction	Tissue and organ morphogenesis; maintenance of cell and tissue structure and function; adhesion, migration, differentiation, proliferation and apoptosis; force-transmitting physical link with the cytoskeleton.	$< 1 \times 10^{-325}$	hsa-miR-302c-5p; hsa-miR-184; hsa-miR-206; hsa-miR-635; hsa-miR-512-3p; hsa-miR-505-3p; hsa-miR-376a-3p

3.3 DNA Methylation in Maternal Saliva: TGF-Beta1 and SOCS3

Methylation data were corrected for maternal age, smoking habits, and GDM association. Both *TGF-Beta1* (Transforming Growth Factor-Beta1) and *SOCS3* (Suppressor Of Cytokine Signalling-3) were differentially methylated, with significantly decreased methylation levels in OB mothers compared to NW ($p = 0.019$ and $p = 0.025$ respectively) (**Table 6**).

Table 6. TGF-Beta1 and SOCS3 Methylation Levels.

	Mean OB (95% CI)	**Mean NW (95% CI)**	**p-value**
TGF-Beta1 (% 5mC)	0.44 (0.24 – 0.63)	0.86 (0.61 – 1.11)	0.019
SOCS3 (% 5mC)	60.95 (57.20 – 64.70)	68.50 (63.75 – 73.25)	0.025

Results from a multiple logistic regression analysis adjusted for age, smoking habits and GDM association. Data are shown as mean with 95% Confidence Interval (CI) of methylated cytosines percentage (% 5mC); $p < 0.05$ vs NW.
TGF-Beta1: Transforming Growth Factor-Beta 1; SOCS3: Suppressor Of Cytokine Signalling 3

4. Discussion

Maternal obesity and Gestational Diabetes (GDM) have been associated with lipotoxicity, inflammaging, and increased oxidative stress both at systemic and intrauterine levels **[44, 8, 10]**. We previously reported modifications of the placental metabolic signatures possibly reflecting changes of the intrauterine metabolic obese environment, as well as mitochondrial alterations in both placenta and maternal circulation of obese (OB) pregnancies, indicating an excessive oxidative and lipotoxic intrauterine/systemic environment that damages mitochondria, leading to further abnormal ROS production in a detrimental vicious cycle **[15, 45, 13].** Increased antioxidant capacity and C-Reactive Protein levels in both maternal plasma and saliva of obese women with and without GDM were also reported, confirming saliva as an effective fluid mirroring the systemic status, representing an interesting non-invasive tool for the evaluation of metabolic signatures during pregnancy **[23].**

The obesogenic environment can influence pregnancy outcomes via epigenetic mechanisms, including microRNAs (miRNAs) and DNA methylation **[46, 47].** Alterations occurring in maternal obesity and GDM can affect epigenetic modifications of different maternal and fetal tissues, leading to alterations in several cellular pathways that can affect the future development in the offspring of obesity, diabetes, and other metabolic and cardiovascular diseases **[3, 47, 48].** Furthermore, the upregulation of specific placental-derived miRNAs, associated with post-embryonic development, lipid, and glucose homeostasis, have been recently reported in umbilical cord blood of fetuses from OB

mothers, suggesting that the maternal obesogenic environment could affect miRNA expression and alter metabolism in their offspring **[49].** Studies on maternal circulating miRNAs are currently in progress aiming at identifying specific miRNA profiles associated with pregnancy pathologies **[50-53]**, some of them proposing specific miRNAs as early biomarkers for gestational diseases such as preeclampsia **[50],** obesity **[51, 52]**, or GDM **[53].** Nevertheless, to our knowledge, this is the first study investigating the miRNAs profile and DNA methylation in the saliva of obese compared to normal-weight pregnant women.

Mothers included in this study population had previously been carefully classified for periodontal comorbidities (gingivitis and periodontitis) **[23].** Clinical conditions of the enrolled patients were well characterized during regular prenatal checks, with obese women receiving specific nutritional and lifestyle counseling in a dedicated clinic. Indeed, OB gained significantly less weight during pregnancy compared to normal-weight mothers. Nevertheless, obese women exceeded gestational weight gain IOM recommendations, possibly worsening the obese-related adverse systemic and local environment. In fact, the poorest overall periodontal health status was observed in the OB group. Moreover, both placental weight and thickness were increased in OB suggesting a trend to a lower placental efficiency, giving rise to a higher, though not significant, mean neonatal weight centile. This obesogenic context also resulted in alterations of the saliva epigenetic profile. In particular, we reported a set of 20 up- or down-expressed miRNAs and decreased methylation levels of the genes *SOCS3* and *TGF-Beta1* in the saliva of obese vs normal-weight mothers.

miRNAs profile

Among the most promising biofluids for biomarker detection, saliva shows a very high number of detectable miRNAs, having a unique spectrum of these non-coding RNAs **[54].**

In order to get insights into tissue-specific miRNA regulation of biological pathways, we performed a *pathway enrichment analysis* by integrating different miRNA target prediction tools, enabling us to identify specific associations with signaling pathways. In particular, saliva miRNAs showing significant differences between OB and NW mothers matched pathways involving fatty acids biosynthesis and metabolism, Extracellular Matrix (ECM) – receptor interaction, and lysine degradation.

Fatty Acids Biosynthesis and Metabolism:

We found significant associations between miR-1254 and fatty acids biosynthesis and metabolism. Interestingly, our group recently reported lower levels of LC-PUFA (Long Chain-Polyunsaturated Fatty Acids) derivatives, arachidonic acid, and DHA, opposite to significantly increased saturated palmitic acid levels, in obese placentas **[15].** These data can indicate a disruption of the physiologic LC-PUFA biomagnification linked to maternal obesity, similarly to alterations occurring in pregnancy pathologies characterized by

placental dysfunction, such as intrauterine growth restriction. Moreover, changes in fatty acids biosynthesis and metabolism can damage cell and organelles membranes, leading to excessive ROS production and oxidative stress, a characterizing condition linked with short and long term adverse consequences for both the mother and the offspring, that has already been shown in the systemic and intrauterine environment of obese and GDM pregnancies **[3, 13, 44].** Although this evidence is derived from different tissues, the present results may be suggestive of a disarranged fatty acid metabolism in obese pregnancies mediated by miRNA epigenetic alterations that can be also detected in the oral non-invasive fluid during the third trimester of pregnancy.

Extracellular Matrix:

Pathway enrichment analysis showed a significant match between hsa-miR-184, hsa-miR-206, hsa-miR-302c-5p, hsa-miR-376a-3p, hsa-miR-505-3p, hsa-miR-512-3p hsa-miR-635 and the ExtraCellular Matrix (ECM)-receptor interaction pathway.

Intriguingly, human endothelial cells culture exposed to high levels of saturated fatty acids showed alterations in the structure and functionality of components of the ECM **[55].** Similarly, hyperglycemia induces excessive production of ECM collagens and altered proteoglycans by endothelial cells. Indeed, also in patients with type II diabetes, the ECM of blood vessels presented an altered ratio of proteoglycans and a thicker basement membrane vs normoglycemic controls. These changes could represent pathogenic mechanisms for vascular diabetic complications, such as microangiopathies and macroangiopathies, which have a strong inflammatory basis **[56].** Physiologically, ECM remodeling consists of structural and compositional changes and is critical for the differentiation and expansion of adipocytes. Obesity is characterized by an increased adipose tissue deposition. In adipose tissue hypertrophy, ECM synthesis and degradation can be altered and lead to fibrosis, which in turn causes inflammation and mechanical stress to the surrounding tissues **[57].** Both ECM degradation and pro-inflammatory milieu establishment are promoted by the activity of matrix metalloproteinases (MMPs), specific enzymes that destroy components of the ECM while secreting interleukins and cytokines. Interestingly, MMPs are well-known targets of several miRNAs, suggesting their epigenetic regulation **[58].** In turn, pro-inflammatory cytokines such as IL-1 alpha and pathogens associated with periodontal disease (e.g. P. Gingivalis) raise MMPs secretion, thus favoring tissue destruction and disintegration of ECM **[59].** Therefore, the association between ECM pathways and OB-related miRNAs alterations reported in the present study in the saliva of obese pregnant women might be speculated to be part of the dysregulation of the ECM remodeling, due to the obesogenic environment, the linked inflammatory setting and the oral disease condition.

Lysine Degradation:

hsa-miR-302d-3p, hsa-miR-302c-3p, hsa-miR-376a-5p, hsa-miR-505-3p, hsa-miR-505-5p, hsa-miR-512-3p, hsa-miR-616-3p resulted associated with lysine degradation pathway.

Amino Acids (AAs) are necessary building blocks for protein biosynthesis. Lysine cannot be synthesized by human cells and therefore is an essential amino acid. It is crucial for cell

growth and plays a fundamental role in the production of carnitine, which shuttles long-chain fatty acids into mitochondria for energy production and assists in lowering cholesterol levels **[60].**

Coherently to results reported in this manuscript, decreased lysine levels in the adipose tissue of obese non-pregnant subjects have been shown **[61],** suggesting an altered metabolic pattern typical of the obese condition. Desert and colleagues investigated the impact of maternal obesity on the metabolic profiles in pregnant women at delivery, reporting increased lysine levels in the urinary excretion of obese mothers vs normal-weight, thus hypothesizing an enhancement in lysine renal secretion **[62].** In a metabolomics analysis of OB vs NW placentas, we previously showed several amino acid changes, with marked lower amounts of lysine **[15].** Similar amino acid alterations were previously reported in the serum of hyperglycemic mothers, showing a metabolic profile consistent with insulin resistance **[63].** On the other hand, disrupted placental metabolism may contribute to circulating amino acids alterations in obese and GDM women. Indeed, amino acids are actively transported against a concentration gradient across the placenta and their transport may be altered in OB and GDM **[64].** This may impact circulating lysine levels and therefore could induce epigenetic alterations in the amino acid metabolism.

Despite the consistency of this evidence, the underlying mechanism explaining the relation between obesity and lysine catabolism is still unknown. It is tempting to hypothesize that miRNAs could be involved in these mechanisms. Indeed, the lysine degradation pathway has been shown as a possible target of specific miRNAs related to a diabetic microenvironment in a beta-human cell line **[65].** Targeted studies are needed to confirm this hypothesis.

DNA methylation

TGF-Beta1:

Transforming Growth Factor-Beta 1 (TGF-Beta1) is an anti-inflammatory cytokine with widespread and multi-organs effects. It is involved in several biological processes, thus playing an active role in glucose, lipid, amino acids, and redox metabolism and representing a key cytokine in obesity and insulin resistance **[30, 66-68].** Noteworthy evidence showed that TGF-Beta1 signaling inhibition ameliorated the metabolic profile in mice by increasing glucose and insulin tolerance. In turn, elevated TGF-Beta1 levels were associated with glucose intolerance and increased adiposity **[69]** and have been reported in women with prior history of GDM **[70].** Accordingly, in the present study *TGF-Beta1* methylation levels were significantly decreased in the saliva of obese vs lean mothers, supporting its upregulation, even when adjusted for maternal age, smoking habits, and the presence of GDM. It might be hypothesized that OB *TGF-Beta1* higher systemic levels were mirrored in the saliva, representing the confirmation of this biofluid as a striking non-invasive tool for the investigation of local and systemic alterations. Moreover, the *pathway enrichment analysis* by mi-TALOS showed a noticeable significant association between the 20 miRNAs differentially expressed in OB and the *TGF-Beta* signaling pathway. This evidence supports previous results showing

a miRNA-based regulation of *TGF-Beta* signaling **[71]** and represent an interesting starting point to unravel alterations of molecular regulatory mechanisms of this pathway in obesity, leading to the possible impairment of nutrients and redox metabolism, with the consequent promotion of oxidative stress.

SOCS3:

Over the past few years, the Suppressor Of Cytokine Signalling 3 (SOCS3) is emerged as an interesting target to treat metabolic disorders. Indeed, although molecular mechanisms undergoing its activity remain to be unraveled in detail, SOCS3 association with inflammation, cumulative stress, and insulin resistance in obesity and diabetes is well established. For these reasons, SOCS3 inhibition is considered a promising strategy for the treatment of metabolic disorders in different conditions **[29].** In pregnancy, the possible participation of SOCS3 in the attenuation of leptin and insulin signaling has been recently reported, with increased resistance associated with SOCS3 upregulation during physiological pregnancy-induced metabolic changes **[72].**

Here, we report a significant decrease in *SOCS3* methylation levels in the saliva of OB women compared to NW, likely accounting for exacerbated *SOCS3* expression in pregnant women with higher BMI. In non-pregnant individuals, *SOCS3* DNA methylation has been previously shown to be inversely related to body weight and to modulate the impact of overall stress on obesity **[73].** Furthermore, obesity-related inflammation leads to the up-regulation of SOCS3 proteins in several tissues, leading to the inhibition of insulin signaling and therefore representing an interesting mechanism connecting the immune and metabolic system in a delicate and balanced cross-talk **[29, 74].** Results reported in the present study suggest a disruption of this balance, and the altered *SOCS3* DNA methylation may represent an intriguing mechanism undergoing OB metabolic alterations, deserving further future investigations both as a biomarker of the dysmetabolic context in pregnancy and as a possible future target for its treatment.

Strengths and Limitations

This work is a pilot study, therefore presenting a small sample size. Nevertheless, the analyzed population was very well-defined in terms of inclusion and exclusion criteria, clinical characteristics, and associated conditions, which strongly reduced any additional clinical bias impacting results. The strict statistical analysis that was applied included several covariates, allowing to focus on the effect of obesity on the analyzed epigenetic modifications.

Molecular investigations were performed on the saliva. Indeed, this is an advantageous, non-invasive, and cost-effective biofluid. Saliva composition reflects perturbations occurring in the oral cavity, that might vary depending on the local conditions. However, it can also mirror the systemic status, representing an intriguing tool for biomarker non-invasive examination. Moreover, a careful procedure was employed to remove any contaminant and to avoid chemical alterations or the release of inflammatory mediators.

Indeed, the passive drool technique for saliva collection is recommended when the target concentration is low, and the unstimulated collection allows to obtain high purity and great sample quality **[54].**

5. Conclusions

Obesity is characterized by lipotoxicity, inflammation, and oxidative stress, that can strongly impact the intrauterine environment during pregnancy leading to short- and long-term negative outcomes for both the mother and the offspring. Among mechanisms undergoing obese-related injuries, epigenetic modifications gained increasing attention over the past year for their high potential as both disease biomarkers and treatment targets. We investigated the epigenetic signatures of a group of clinically well-characterized obese and normal-weight pregnant women by analyzing their saliva, which represents a promising non-invasive biofluid that can mirror the systemic status of the individual. We found alterations in the expression of 20 different miRNAs and methylation levels of 2 genes involved in obesity-related inflammation and oxidative stress. These results help to clarify some of the mechanisms involved in the impairment occurring in obesity, which can contribute to the increase of the risk of negative outcomes in OB pregnancies. Furthermore, the underneath association between miRNAs and their related molecular pathways, explored by *pathway enrichment analysis*, will help understanding specific damaged pathways. Since miRNAs could be promising biomarkers in the early diagnosis and treatment of pregnancy conditions, these results place the analyzed salivary miRNAs in a clinical context and propose possible molecular mechanisms in which they could be involved.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the 'Medical Ethical and Institutional Review Board' (Prot. N. 469/2010/52/AP).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data that support the findings of this study are available from the corresponding author (Chiara Mandò) on reasonable request.

Author Contributions: conceptualization, I.C. (Irene Cetin), Silvio Abati (S.A.), Chiara Mandò (C.M.), Valentina Bollati (V.B.); **investigation,** G.M.A. (Gaia Maria Anelli), B.A. (Benedetta Albetti) and L.D. (Laura Dioni); f**ormal analysis,** C.F. (Chiara Favero), G.M.A., C.M.; **resources**, M.Z. (Marta Zambon); **writing-original draft**, C.M., G.M.A., A.S. (Anais Serati); **writing-review and editing,** I.C., S.A., C.M., V.B.; **supervision,** I.C., C.M. V.B.; **funding acquisition**, I.C.

All authors have read and agreed to the published version of the manuscript.

Funding: PRIN 2010-2011 prot. 20102chst5_005 "Parto pre-termine: markers molecolari, biochimici e biofisici dell' unità feto-placentare", grant from the Italian Ministry of University and Research to Irene Cetin.

Progetto di Ricerca Finalizzata RF-2016-02362165, "Epigenetic impact of maternal obesity and nutritional status. Nutritional/lifestyle intervention for the improvement of pregnancy outcomes" (EPI-MOM), grant from the Italian Ministry of Health to Irene Cetin.

The authors are thankful to ASM (Associazione Studio Malformazioni) for an unconditioned grant to the Laboratory of Maternal-Fetal Translational Research "Giorgio Pardi."

Acknowledgments: We thank all the midwives and nurses of the Obstetric Unit of Luigi Sacco Hospital (ASST Fatebenefratelli-Sacco Milano) for their expertise and cooperation. We are particularly grateful to all the pregnant women that contributed to the study with their clinical and biological data.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. NCD Risk Factor Collaboration (NCD-RisC). Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. Lancet. 2016 Apr 2;387(10026):1377-1396. doi: 10.1016/S0140-6736(16)30054-X.
2. Schaefer-Graf, U.; Napoli, A.; Nolan, C.J. Diabetic Pregnancy Study Group. Diabetes in pregnancy: a new decade of challenges ahead. Diabetologia. 2018 May;61(5):1012-1021. doi: 10.1007/s00125-018-4545-y.
3. Catalano, P.M.; Shankar, K. Obesity and pregnancy: mechanisms of short term and long term adverse consequences for mother and child. BMJ. 2017 Feb 8;356:j1. doi: 10.1136/bmj.j1.
4. Poston, L.; Caleyachetty, R.; Cnattingius, S.; Corvalán, C.; Uauy, R.; Herring, S.; Gillman, M.W. Preconceptional and maternal obesity: epidemiology and health consequences. Lancet Diabetes Endocrinol. 2016 Dec;4(12):1025-1036. doi: 10.1016/S2213-8587(16)30217-0.
5. Bordin, P.; Dotto, L.; Battistella,, L.; Rosso, E.; Pecci, L.; Valent, F.; Collarile, P.; Vanin, M. Gestational diabetes mellitus yesterday, today and tomorrow: A 13 year italian cohort study. Diabetes Res Clin Pract. 2020 Sep;167:108360. doi: 10.1016/j.diabres.2020.108360.
6. Monteiro, L.J.; Norman, J.E.; Rice, G.E.; Illanes, S.E. Fetal programming and gestational diabetes mellitus. Placenta. 2016 Dec;48 Suppl 1:S54-S60. doi: 10.1016/j.placenta.2015.11.015.
7. Barker, D.J.; Thornburg, K.L.; Osmond, C.; Kajantie, E.; Eriksson, J.G. Beyond birthweight: the maternal and placental origins of chronic disease. J Dev Orig Health Dis. 2010 Dec;1(6):360-4. doi: 10.1017/S2040174410000280.
8. Parisi, F.; Milazzo, R.; Savasi, V.M.; Cetin, I. Maternal Low-Grade Chronic Inflammation and Intrauterine Programming of Health and Disease. Int J Mol Sci. 2021 Feb 9;22(4):1732. doi: 10.3390/ijms22041732.
9. Bianchi, C.; Taricco, E.; Cardellicchio, M.; Mandò, C.; Massari, M.; Savasi, V.; Cetin, I. The role of obesity and gestational diabetes on placental size and fetal oxygenation. Placenta. 2021 Jan 1;103:59-63. doi: 10.1016/j.placenta.2020.10.013.
10. Zavatta, A.; Parisi, F.; Mandò, C.; Scaccabarozzi, C.; Savasi, V.M.; Cetin, I. Role of Inflammaging on the Reproductive Function and Pregnancy. Clin Rev Allergy Immunol. 2022 Jan 15:1–16. doi: 10.1007/s12016-021-08907-9.
11. Kelly, A.C.; Powell, T.L.; Jansson, T. Placental function in maternal obesity. Clin Sci (Lond). 2020 Apr 30;134(8):961-984. doi: 10.1042/CS20190266.
12. Martino, J.; Sebert, S.; Segura, M.T.; García-Valdés, L.; Florido, J.; Padilla, M.C.; Marcos, A.; Rueda, R.; McArdle, H.J.; Budge, H.; Symonds, M.E.; Campoy, C. Maternal Body Weight and Gestational Diabetes Differentially Influence Placental and Pregnancy Outcomes. J Clin Endocrinol Metab. 2016 Jan;101(1):59-68. doi: 10.1210/jc.2015-2590.
13. Mandò, C.; Anelli, G.M.; Novielli, C.; Panina-Bordignon, P.; Massari, M.; Mazzocco, M.I.; Cetin, I. Impact of Obesity and Hyperglycemia on Placental Mitochondria. Oxid Med Cell Longev. 2018 Aug 14;2018:2378189. doi: 10.1155/2018/2378189.
14. Diceglie, C. and Anelli, G.M.; Martelli, C.; Serati, A.; Lo Dico, A.; Lisso, F.; Parisi, F.; Novielli, C.; Paleari, R.; Cetin, I.; Ottobrini, L. and. Mandò, C. Placental Antioxidant Defenses and Autophagy-Related Genes in Maternal Obesity and Gestational Diabetes Mellitus. Nutrients. 2021 Apr 15;13(4):1303. doi: 10.3390/nu13041303.
15. Fattuoni, C.; Mandò, C.; Palmas, F.; Anelli, G.M.; Novielli, C.; Parejo Laudicina, E.; Savasi, V.M.; Barberini, L.; Dessì, A.; Pintus, R.; Fanos, V.; Noto, A.; Cetin, I. Preliminary metabolomics analysis of placenta in maternal obesity. Placenta. 2018 Jan;61:89-95. doi: 10.1016/j.placenta.2017.11.014.
16. Foratori-Junior, G.A.; da Silva, B.M.; da Silva Pinto, A.C.; Honório, H.M.; Groppo, F.C.; de Carvalho Sales-Peres, S.H. Systemic and periodontal conditions of overweight/obese patients during pregnancy and after delivery: a prospective cohort. Clin Oral Investig. 2020 Jan;24(1):157-165. doi: 10.1007/s00784-019-02932-x.
17. Ye, C.; Kapila, Y. Oral microbiome shifts during pregnancy and adverse pregnancy outcomes: Hormonal and Immunologic changes at play. Periodontol 2000. 2021 Oct;87(1):276-281. doi: 10.1111/prd.12386.
18. Kinane, D.F.; Stathopoulou, P.G.; Papapanou, P.N. Periodontal diseases. Nat Rev Dis Primers. 2017 Jun 22;3:17038. doi: 10.1038/nrdp.2017.38.
19. Byun, J.S.; Lee, H.Y.; Tian, J.; Moon, J.S.; Choi, J.; Lee, S.H.; Kim, Y.G.; Yi, H.S. Effect of Salivary Exosomal miR-25-3p on Periodontitis With Insulin Resistance. Front Immunol. 2022 Jan 7;12:775046. doi: 10.3389/fimmu.2021.775046.
20. Ren, H.; Du, M.. Role of Maternal Periodontitis in Preterm Birth. Front Immunol. 2017 Feb 13;8:139. doi: 10.3389/fimmu.2017.00139.
21. Cetin, I.; Pileri, P.; Villa, A.; Calabrese, S.; Ottolenghi, L.; Abati, S. Pathogenic mechanisms linking periodontal diseases with adverse pregnancy outcomes. Reprod Sci. 2012 Jun;19(6):633-41. doi: 10.1177/1933719111432871. Epub 2012 Mar 14. PMID: 22421445.
22. Srinivas, S.K.; Parry, S. Periodontal disease and pregnancy outcomes: time to move on? J Womens Health. 2012 Feb;21(2):121-5. doi: 10.1089/jwh.2011.3023.
23. Zambon, M.; Mandò, C.; Lissoni, A.; Anelli, G.M.; Novielli, C.; Cardellicchio, M.; Leone, R.; Monari, M.N.; Massari, M.; Cetin, I.; Abati, S. Inflammatory and Oxidative Responses in Pregnancies With Obesity and Periodontal Disease. Reprod Sci. 2018 Oct;25(10):1474-1484. doi: 10.1177/1933719117749758.
24. Kaczor-Urbanowicz, K.E.; Martin Carreras-Presas, C.; Aro, K.; Tu, M.; Garcia-Godoy, F.; Wong, D.T. Saliva diagnostics - Current views and directions. Exp Biol Med. 2017 Mar;242(5):459-472. doi: 10.1177/1535370216681550.

25. Yoshizawa, J.M.; Schafer, C.A.; Schafer, J.J.; Farrell, J.J.; Paster, B.J.; Wong, D.T. Salivary biomarkers: toward future clinical and diagnostic utilities. Clin Microbiol Rev. 2013 Oct;26(4):781-91. doi: 10.1128/CMR.00021-13.
26. Weber, J.A.; Baxter, D.H.; Zhang, S.; Huang, D.Y.; Huang, K.H.; Lee, M.J.; Galas, D.J.; Wang, K. The microRNA spectrum in 12 body fluids. Clin Chem. 2010 Nov;56(11):1733-41. doi: 10.1373/clinchem.2010.147405.
27. Tsamou, M; Martens, DS; Winckelmans, E; Madhloum, N; Cox, B; Gyselaers, W; Nawrot, TS; Vrijens, K. Mother's Pre-pregnancy BMI and Placental Candidate miRNAs: Findings from the ENVIRONAGE Birth Cohort. Sci Rep. 2017 Jul 17;7(1):5548. doi: 10.1038/s41598-017-04026-8. Erratum in: Sci Rep. 2018 Apr 11;8(1):6063.
28. Al-Rawi, N.H.; Al-Marzooq, F.; Al-Nuaimi, A.S.; Hachim, M.Y.; Hamoudi, R. Salivary microRNA 155; 146a/b and 203: A pilot study for potentially non-invasive diagnostic biomarkers of periodontitis and diabetes mellitus. PLoS One. 2020 Aug 5;15(8):e0237004. doi: 10.1371/journal.pone.0237004.
29. Pedroso, J.A.B.; Ramos-Lobo, A.M.; Donato, J. Jr. SOCS3 as a future target to treat metabolic disorders. Hormones (Athens). 2019 Jun;18(2):127-136. doi: 10.1007/s42000-018-0078-5.
30. Yener, S.; Demir, T.; Akinci, B.; Bayraktar, F.; Kebapcilar, L.; Ozcan, M.A.; Biberoglu, S.; Yesil, S. Transforming growth factor-beta 1 levels in women with prior history of gestational diabetes mellitus. Diabetes Res Clin Pract. 2007 May;76(2):193-8. doi: 10.1016/j.diabres.2006.08.014.
31. Page, R.C. and Eke P.I. Case definitions for use in population-based surveillance of periodontitis. J Periodontol. 2007;78(suppl 7):1387-1399.
32. Pergoli, L.; Cantone, L.; Favero, C.; Angelici, L.; Iodice, S.; Pinatel, E.; Hoxha, M.; Dioni, L.; Letizia, M.; Albetti, B.; Tarantini, L.; Rota, F.; Bertazzi, P.A.; Tirelli, A.S.; Dolo, V.; Cattaneo, A.; Vigna, L.; Battaglia, C.; Carugno, M.; Bonzini, M.; Pesatori, A.C.; Bollati, V. Extracellular vesicle-packaged miRNA release after short-term exposure to particulate matter is associated with increased coagulation. Part Fibre Toxicol. 2017 Aug 24;14(1):32. doi: 10.1186/s12989-017-0214-4.
33. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8. doi: 10.1006/meth.2001.1262.
34. Andersen, C.L.; Jensen, J.L.; Ørntoft, T.F. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004 Aug 1;64(15):5245-50. doi: 10.1158/0008-5472.CAN-04-0496.
35. Bollati, V.; Baccarelli, A.; Hou, L.; Bonzini, M.; Fustinoni, S.; Cavallo, D.; Byun, H.M.; Jiang, J.; Marinelli, B.; Pesatori, A.C.; Bertazzi, P.A.; Yang, A.S. Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res. 2007 Feb 1;67(3):876-80. doi: 10.1158/0008-5472.CAN-06-2995.
36. Tarantini, L.; Bonzini, M.; Tripodi, A.; Angelici, L.; Nordio, F.; Cantone, L.; Apostoli, P.; Bertazzi, P.A.; Baccarelli, A.A. Blood hypomethylation of inflammatory genes mediates the effects of metal-rich airborne pollutants on blood coagulation. Occup Environ Med. 2013 Jun;70(6):418-25. doi: 10.1136/oemed-2012-101079.
37. Preusse, M.; Theis, F.J.; Mueller, N.S. miTALOS v2: Analyzing Tissue Specific microRNA Function. PLoS One. 2016 Mar 21;11(3):e0151771. doi: 10.1371/journal.pone.0151771.
38. Vlachos, I.S.; Kostoulas, N.; Vergoulis, T.; Georgakilas, G.; Reczko, M.; Maragkakis, M.; Paraskevopoulou, M.D.; Prionidis, K.; Dalamagas, T.; Hatzigeorgiou, A.G. DIANA miRPath v.2.0: investigating the combinatorial effect of microRNAs in pathways. Nucleic Acids Res. 2012 Jul;40(Web Server issue):W498-504. doi: 10.1093/nar/gks494.
39. Vlachos, I.S.; Zagganas, K.; Paraskevopoulou, M.D.; Georgakilas, G.; Karagkouni, D.; Vergoulis, T.; Dalamagas, T.; Hatzigeorgiou, A.G. DIANA-miRPath v3.0: deciphering microRNA function with experimental support. Nucleic Acids Res. 2015 Jul 1;43(W1):W460-6. doi: 10.1093/nar/gkv403.
40. Hosack, D.A.; Dennis, G. Jr; Sherman, B.T.; Lane, H.C.; Lempicki, R.A. Identifying biological themes within lists of genes with EASE. Genome Biol. 2003; 4(10):R70. doi: 10.1186/gb-2003-4-10-r70.
41. Benjamini, Y.; Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. 1995. Series B (Methodological); 57(1), 289–300. http://www.jstor.org/stable/2346101.
42. Iorgulescu, G. Saliva between normal and pathological. Important factors in determining systemic and oral health. J Med Life. 2009 Jul-Sep;2(3):303-7.
43. Bertino, E.; Spada, E.; Occhi, L.; Coscia, A.; Giuliani, F.; Gagliardi, L.; Gilli, G.; Bona, G.; Fabris, C.; De Curtis, M.; Milani, S. Neonatal anthropometric charts: the Italian neonatal study compared with other European studies. J Pediatr Gastroenterol Nutr. 2010 Sep;51(3):353-61. doi: 10.1097/MPG.0b013e3181da213e.
44. Saben, J.; Lindsey, F.; Zhong, Y.; Thakali, K.; Badger, T.M.; Andres, A.; Gomez-Acevedo, H.; Shankar, K. Maternal obesity is associated with a lipotoxic placental environment. Placenta. 2014 Mar;35(3):171-7. doi: 10.1016/j.placenta.2014.01.003.
45. Anelli, G.M.; Cardellicchio, M.; Novielli, C.; Antonazzo, P.; Mazzocco, M.I.; Cetin, I.; Mandò, C. Mitochondrial content and hepcidin are increased in obese pregnant mothers. J Matern Fetal Neonatal Med. 2018 Sep;31(18):2388-2395. doi: 10.1080/14767058.2017.1344209.
46. Franzago, M.; Fraticelli, F.; Stuppia, L.; Vitacolonna, E. Nutrigenetics, epigenetics and gestational diabetes: consequences in mother and child. Epigenetics. 2019 Mar;14(3):215-235. doi: 10.1080/15592294.2019.1582277.
47. Kerr, B.; Leiva, A.; Farías, M.; Contreras-Duarte, S.; Toledo, F.; Stolzenbach, F.; Silva, L.; Sobrevia, L. Foetoplacental epigenetic changes associated with maternal metabolic dysfunction. Placenta. 2018 Sep; 69:146-152. doi: 10.1016/j.placenta.2018.04.006.

48. Guarino, E.; Delli Poggi, C.; Grieco, G.E.; Cenci, V.; Ceccarelli, E.; Crisci, I.; Sebastiani, G.; Dotta, F. Circulating MicroRNAs as Biomarkers of Gestational Diabetes Mellitus: Updates and Perspectives. Int J Endocrinol. 2018 Apr 12;2018:6380463. doi: 10.1155/2018/6380463.
49. Jing, J.; Wang, Y.; Quan, Y.; Wang, Z.; Liu, Y.; Ding, Z. Maternal obesity alters C19MC microRNAs expression profile in fetal umbilical cord blood. Nutr Metab (Lond). 2020 Jul 6;17:52. doi: 10.1186/s12986-020-00475-7.
50. Luque, A.; Farwati, A.; Crovetto, F.; Crispi, F.; Figueras, F.; Gratacós, E.; Aran, J.M. Usefulness of circulating microRNAs for the prediction of early preeclampsia at first-trimester of pregnancy. Sci Rep. 2014 May 8;4:4882. doi: 10.1038/srep04882.
51. Sørensen, A.E.; van Poppel, M.N.M.; Desoye, G.; Simmons, D.; Damm, P.; Jensen, D.M.; Dalgaard, L.T.; The Dali Core Investigator Group. The Temporal Profile of Circulating miRNAs during Gestation in Overweight and Obese Women with or without Gestational Diabetes Mellitus. Biomedicines. 2022 Feb 18;10(2):482. doi: 10.3390/biomedicines10020482.
52. Carreras-Badosa, G.; Bonmatí, A.; Ortega, F.J.; Mercader, J.M.; Guindo-Martínez, M.; Torrents, D.; Prats-Puig, A.; Martinez-Calcerrada, J.M.; Platero-Gutierrez, E.; De Zegher. F.; Ibáñez, L.; Fernandez-Real, J.M.; Lopez-Bermejo, A.; Bassols, J. Altered Circulating miRNA Expression Profile in Pregestational and Gestational Obesity. J Clin Endocrinol Metab. 2015 Nov;100(11):E1446-56. doi: 10.1210/jc.2015-2872.
53. Poirier, C.; Desgagné, V.; Guérin, R.; Bouchard, L. MicroRNAs in Pregnancy and Gestational Diabetes Mellitus: Emerging Role in Maternal Metabolic Regulation. Curr Diab Rep. 2017 May;17(5):35. doi: 10.1007/s11892-017-0856-5.
54. Wren, M.E.; Shirtcliff, E.A.; Drury, S.S. Not all biofluids are created equal: chewing over salivary diagnostics and the epigenome. Clin Ther. 2015 Mar 1;37(3):529-39. doi: 10.1016/j.clinthera.2015.02.022.
55. Camejo, G.; Hurt-Camejo, E.; Olsson, U.; Bondjers, G. Lipid mediators that modulate the extracellular matrix structure and function in vascular cells. Curr Atheroscler Rep. 1999 Sep;1(2):142-9. doi: 10.1007/s11883-999-0010-1.
56. Olsson, U.; Bondjers, G.; Camejo, G. Fatty acids modulate the composition of extracellular matrix in cultured human arterial smooth muscle cells by altering the expression of genes for proteoglycan core proteins. Diabetes. 1999 Mar;48(3):616-22. doi: 10.2337/diabetes.48.3.616.
57. Pessentheiner, A.R.; Ducasa, G.M.; Gordts, P.L.S.M. Proteoglycans in Obesity-Associated Metabolic Dysfunction and Meta-Inflammation. Front Immunol. 2020 May 19;11:769. doi: 10.3389/fimmu.2020.00769.
58. Brovkina, O.; Nikitin, A.; Khodyrev, D.; Shestakova, E.; Sklyanik, I.; Panevina, A.; Stafeev, I.; Menshikov, M.; Kobelyatskaya, A.; Yurasov, A.; Fedenko, V.; Yashkov, Y.; Shestakova, M. Role of MicroRNAs in the Regulation of Subcutaneous White Adipose Tissue in Individuals With Obesity and Without Type 2 Diabetes. Front Endocrinol. 2019 Dec 5;10:840. doi: 10.3389/fendo.2019.00840.
59. Chang, Y.C.; Yang, S.F.; Lai, C.C.; Liu, J.Y.; Hsieh, Y.S. Regulation of matrix metalloproteinase production by cytokines; pharmacological agents and periodontal pathogens in human periodontal ligament fibroblast cultures. J Periodontal Res. 2002 Jun;37(3):196-203. doi: 10.1034/j.1600-0765.2002.00663.x.
60. Tomé, D.; Bos, C. Lysine requirement through the human life cycle. J Nutr. 2007 Jun;137 (6 Suppl 2):1642S-1645S. doi: 10.1093/jn/137.6.1642S.
61. Hanzu, F.A.; Vinaixa, M.; Papageorgiou, A.; Párrizas, M.; Correig, X.; Delgado, S.; Carmona, F.; Samino, S.; Vidal, J.; Gomis, R. Obesity rather than regional fat depots marks the metabolomic pattern of adipose tissue: an untargeted metabolomic approach. Obesity (Silver Spring). 2014 Mar;22(3):698-704. doi: 10.1002/oby.20541.
62. Desert, R.; Canlet, C.; Costet, N.; Cordier, S.; Bonvallot, N. Impact of maternal obesity on the metabolic profiles of pregnant women and their offspring at birth. Metabolomics, Springer Verlag, 2015, 11 (6), pp.1896-1907.
63. Scholtens, D.M.; Muehlbauer, M.J.; Daya, N.R.; Stevens, R.D., Dyer, A.R.; Lowe, L.P.; Metzger, B.E.; Newgard, C.B.; Bain, J.R.; Lowe, W.L. Jr; HAPO Study Cooperative Research Group. Metabolomics reveals broad-scale metabolic perturbations in hyperglycemic mothers during pregnancy. Diabetes Care. 2014; 37(1):158-66. doi: 10.2337/dc13-0989.
64. Jansson, N.; Rosario, F.J.; Gaccioli, F.; Lager, S.; Jones, H.N.; Roos, S.; Jansson, T.; Powell T.L. Activation of placental mTOR signaling and amino acid transporters in obese women giving birth to large babies. J Clin Endocrinol Metab. 2013 Jan;98(1):105-13. doi: 10.1210/jc.2012-2667.
65. Jeffery, N.; Harries, L.W. miRNAs responsive to the diabetic microenvironment in the human beta cell line EndoC-βH1 may target genes in the FOXO, HIPPO and Lysine degradation pathways. Exp Cell Res. 2019 Nov 1;384(1):111559. doi: 10.1016/j.yexcr.2019.111559. Erratum in: Exp Cell Res. 2019 Dec 1;385(1):111654.
66. Liu, H.; Chen, Y.G. The Interplay Between TGF-β Signaling and Cell Metabolism. Front Cell Dev Biol. 2022 Mar 9;10:846723. doi: 10.3389/fcell.2022.846723.
67. Lee, J.H.; Mellado-Gil, J.M.; Bahn, Y.J.; Pathy, S.M.; Zhang, Y.E.; Rane, S.G. Protection from β-cell apoptosis by inhibition of TGF-β/Smad3 signaling. Cell Death Dis. 2020 Mar 13;11(3):184. doi: 10.1038/s41419-020-2365-8.
68. Toyoda, S.; Shin, J.; Fukuhara, A.; Otsuki, M.; Shimomura, I. Transforming growth factor β1 signaling links extracellular matrix remodeling to intracellular lipogenesis upon physiological feeding events. J Biol Chem. 2022 Feb 19;298(4):101748. doi: 10.1016/j.jbc.2022.101748.
69. Yadav, H.; Devalaraja, S.; Chung, S.T.; Rane, S.G. TGF-β1/Smad3 Pathway Targets PP2A-AMPK-FoxO1 Signaling to Regulate Hepatic Gluconeogenesis. J Biol Chem. 2017 Feb 24;292(8):3420-3432. doi: 10.1074/jbc.M116.764910.
70. Zhu, Y.; Tian, F.; Li, H.; Zhou, Y.; Lu, J.; Ge, Q. Profiling maternal plasma microRNA expression in early pregnancy to predict gestational diabetes mellitus. International Journal of Gynecology & Obstetrics, 2015. 130: 49-53. https://doi.org/10.1016/j.ijgo.2015.01.010.

71. Kim, Y.J.; Hwang, S.J.; Bae, Y.C.; Jung, J.S. MiR-21 regulates adipogenic differentiation through the modulation of TGF-beta signaling in mesenchymal stem cells derived from human adipose tissue. Stem Cells. 2009 Dec;27(12):3093-102. doi: 10.1002/stem.235.
72. Zampieri, T.T.; Ramos-Lobo, A.M.; Furigo, I.C.; Pedroso, J.A.; Buonfiglio, D.C.; Donato, J. Jr. SOCS3 deficiency in leptin receptor-expressing cells mitigates the development of pregnancy-induced metabolic changes. Mol Metab. 2014 Dec 19;4(3):237-45. doi: 10.1016/j.molmet.2014.12.005.
73. Xu, K.; Zhang, X.; Wang, Z.; Hu, Y.; Sinha, R. Epigenome-wide association analysis revealed that SOCS3 methylation influences the effect of cumulative stress on obesity. Biol Psychol. 2018 Jan;131:63-71. doi: 10.1016/j.biopsycho.2016.11.001.
74. Lebrun, P.; Van Obberghen, E. SOCS proteins causing trouble in insulin action. Acta Physiol (Oxf). 2008 Jan;192(1):29-36. doi: 10.1111/j.1748-1716.2007.01782.x.