

Original Research

Investigation of Regulatory Functions in Non-Diabetic Macrosomia: A Combined Analysis of Clinical Characteristics and Small-Scale Exosome Sequencing

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Academic Editor: Paolo Ivo Cavoretto

Submitted: 16 June 2025 Revised: 20 August 2025 Accepted: 1 September 2025 Published: 26 November 2025

Abstract

Background: Macrosomia is a significant perinatal complication with potential risks for both mother and child. Although diabetes is a known major risk factor, specific clinical and metabolic factors contributing to macrosomia in non-diabetic pregnancies are not fully understood. Therefore, this study aimed to explore the clinical characteristics and potential metabolic risk factors associated with non-diabetes-related neonatal macrosomia. Additionally, this study aimed to examine the relationship between metabolic dysregulation and the presence of exosomes in umbilical cord blood. **Methods:** A retrospective analysis of 356 non-diabetic pregnant women (170 non-diabetic pregnant women with macrosomic infants and 186 normal pregnant women) was conducted. Additionally, umbilical cord blood plasma samples were collected from 16 participants (8 macrosomia and 8 normal deliveries). After separating exosomes from plasma, RNA was extracted and sequenced. Weighted gene co-expression network analysis (WGCNA) was used to explore the correlation between clinical characteristics and gene expression. **Results:** Among the baseline characteristics, the pre-pregnancy body mass index (BMI) and overall weight gain in non-diabetic mothers with macrosomic infants were significantly higher than those in the normal group ($p < 0.05$). The lipid profiles revealed that triglyceride (TG) and low-density lipoprotein (LDL) levels were significantly elevated, whereas the high-density lipoprotein (HDL) levels were significantly decreased ($p < 0.05$). Logistic regression analysis showed that pre-pregnancy BMI, gestational weight gain, LDL levels, and alkaline phosphatase (ALP) levels in the third trimester were risk factors for macrosomia, while primiparas and HDL levels were protective factors. WGCNA analysis revealed that the expression of the *mRNA royalblue* module and the *lncRNA darkgrey* module presented a significant positive correlation with gestational weight gain ($p < 0.05$). Compared to the normal group, the expressions of transmembrane protein 175 (*TMEM175*) and HIF1A antisense RNA 2 (*HIF1A-AS2*) were downregulated, whereas the expressions of phosphoglycerate kinase 1 (*PGK1*) and methionine adenosyltransferase 2B (*MAT2B*) were upregulated in the exosomes derived from the umbilical cord blood plasma in the macrosomic group. **Conclusions:** Messenger RNAs (mRNA) (*TMEM175*, *PGK1*, *MAT2B*) and long non-coding RNAs (lncRNA) (*HIF1A-AS2*) may potentially contribute to the development of fetal macrosomia in non-diabetic pregnancies.

Keywords: exosomal RNAs; macrosomia; non-diabetic pregnant women

1. Introduction

Macrosomia is a perinatal complication characterized by the birth of a full-term infant weighing 4000 grams or more. This condition is associated with increased risk of complications during delivery for both mother and child, and its prevalence varies across different countries. Maternal obesity and diabetes, including pre-gestational and gestational diabetes, are significant risk factors [1,2]. Studies have shown that the etiology of macrosomia includes both non-modifiable factors, such as genetics, fetal sex, parity,

and maternal age, as well as modifiable factors, including maternal nutrition intake, pre-pregnancy body mass index (BMI), and gestational weight gain [3–5]. Additionally, the physiological and pathological mechanisms of macrosomia are related to the excessive supply of nutrients by the mother to the fetus and/or the inability of the fetus to regulate the metabolism of nutrients effectively or efficiently [6]. In a study involving 115,097 singleton live births, the prevalence of macrosomia among women with gestational diabetes mellitus (GDM) decreased between 2012 and 2021. However, the incidence of macrosomia



in women with GDM remained significantly higher than that in non-GDM women. Furthermore, the study found no significant decline in the incidence of macrosomia in non-GDM women during the same period [7]. Macrosomia is also known to increase the risk of adverse maternal outcomes and long-term health problems in the newborn [8,9]. Although there are various interventions for the treatment of diabetic macrosomia, the accurate prenatal prediction and treatment of macrosomia in non-diabetic pregnancies remain a significant hurdle, as the diagnosis is often only confirmed retrospectively [10]. Currently, macrosomia in mothers with GDM is relatively well-researched. However, there is a significant knowledge gap concerning macrosomia in non-diabetic mothers, necessitating more research to clarify the possible etiology and mechanisms [11].

Exosomes are transporters of diverse bioactive molecules, including RNAs [such as long non-coding RNAs (lncRNAs) and messenger RNAs (mRNAs)], DNAs, and proteins that facilitate cell-to-cell communications and the exchange of substances and information between the mother and the fetus [12–15]. As a major organ during pregnancy, the placenta secretes exosomes into the maternal circulation as early as 6 weeks of gestation [16]. Changes in the release of exosomes, their composition, and bioactivity, along with altered patterns of exosomal non-coding RNAs, particularly microRNAs and lncRNAs, can lead to several pregnancy-related complications [17,18]. Yuan *et al.* [19] discovered that exosomal RNAs (including mRNAs, lncRNAs, and circRNAs) were expressed at abnormal levels in the umbilical cord blood of patients with GDM-related macrosomia. The altered expression patterns of these exosomal RNAs suggest that they may participate as biomarkers in predicting macrosomia in patients with GDM. The study evaluated the predictive performance of the individual exosomal RNAs, and they determined that growth differentiation factor 3 (GDF3) demonstrated good predictive performance with an area under the curve (AUC) of 0.78. In another study, lncRNAs were also found to be differentially expressed in umbilical cord blood exosomes of patients with pregnancy complications compared to healthy ones [20]. Lu *et al.* [21] also reported that lncRNAs may participate in the pathogenesis of placental development and may influence fetal growth. They found that lncRNA ubiquitin specific peptidase 2 antisense RNA 1 (*USP2ASI*) was involved in the pathogenesis of nondiabetic fetal macrosomia by regulating cell function. Furthermore, Ren *et al.* [22] reported that lncRNA H19 was significantly correlated with the birth weight of fetuses with intrauterine hyperglycemia. While research has shown an association between exosomal RNAs and macrosomia in women with GDM, the role of these RNAs in non-GDM pregnancies needs further investigation. Therefore, addressing the current research challenges will be crucial to leveraging the full potential of exosomal RNA biomarkers in optimizing

clinical decision-making and improving the outcomes of both mother and baby in non-GDM-related macrosomia.

Weighted gene co-expression network analysis (WGCNA) is an R package that constructs and analyzes gene co-expression networks. It identifies groups of genes with similar expression patterns (modules) and assesses their relationship to biological processes or phenotypes. Relevant networks facilitate network-based gene screening methods to determine genes associated with specific phenotypes, potentially identifying biomarkers or therapeutic targets [23]. Therefore, we can combine the gene expression and clinical indexes to explore the intrinsic molecular mechanism of macrosomia. Currently, research on the risk factors for macrosomia in non-diabetic pregnancies and their association with exosomal RNA is relatively limited. This study aims to investigate the maternal clinical risk factors related to neonatal macrosomia in non-diabetic mothers and to identify key regulatory factors for its occurrence using the WGCNA method.

2. Materials and Methods

2.1 Study Design

A retrospective study was conducted involving pregnant women who received prenatal examinations and gave birth in the Department of Obstetrics, Maternity and Child Health Care of Guangxi Zhuang Autonomous Region, from January 1, 2016, to September 30, 2019. This study was designed and reported in strict accordance with the strengthening the reporting of observational studies in epidemiology (STROBE) checklist. Utilizing the hospital's electronic medical record system, two participant groups were established: the non-diabetic macrosomia group ($n = 170$), which included pregnant women whose newborns had a birth weight >4000 g with no abnormal blood glucose levels during pregnancy; and the normal group ($n = 186$), which served as controls with newborns of normal weight, matched for gestational age. During the third stage of labor, 10 mL of umbilical venous blood samples were collected from 16 pregnant women (8 in the non-diabetic macrosomia group and 8 in the normal group). To minimize the interference of confounding factors, the participants were strictly matched for age and BMI (with a difference of <1) in addition to having comparable gestational age. All subjects underwent an oral glucose tolerance test (OGTT) at 24–28 weeks of gestation in our hospital. According to the diabetes diagnostic criteria recommended by the American Diabetes Association (ADA) guidelines, pregnant women with diabetes (including pre-pregnancy diabetes and gestational diabetes) were excluded from the study. Furthermore, pregnant women with any diseases that could affect neonatal metabolism and growth and development were excluded, such as metabolic disorders (e.g., thyroid dysfunction during pregnancy and Cushing's syndrome), hypertensive disorders in pregnancy, and autoimmune diseases. Baseline data, laboratory test data, delivery data, and

neonatal clinical data of all subjects were collected. The study protocol was approved by the Medical Ethics Committee of Guangxi Medical University (approval number: 2019-SB-067), and written informed consent was obtained from the participants.

2.2 Sample Collection and Processing

During the third stage of labor, 10 mL of umbilical venous blood was collected from non-diabetic pregnant women and transferred into dipotassium ethylenediaminetetraacetic acid (K_2EDTA) vacuum blood collection tubes (BH1359, Bioroyee, Beijing, China), each. All blood samples were centrifuged at $1000 \times g$ at $4^\circ C$ for 15 min. Subsequently, at least 5 mL of plasma was isolated from each sample, aliquoted into multiple 1.5 mL Eppendorf tubes (0030125215, Eppendorf, Hamburg, Germany), and immediately stored at $-80^\circ C$ for subsequent experimental analysis.

2.3 Exosome Isolation and Identification

To eliminate cellular debris and large vesicles, 2 mL of plasma was first centrifuged at $2000 \times g$ for 10 min at $4^\circ C$, followed by a second centrifugation at $10,000 \times g$ for 60 min. The resulting supernatant was then ultracentrifuged at $120,000 \times g$ for 2 h at $4^\circ C$ to enrich exosomes. The obtained pellet was gently rinsed with phosphate-buffered saline (PBS; 6-2554, Tianjingsha Gene Technology Co., Ltd., Beijing, China) and ultracentrifuged again at $120,000 \times g$ for 2 h at $4^\circ C$ to purify exosomes. Finally, the supernatant was carefully removed, and the exosome pellet was gently resuspended in 200 μL of PBS to obtain the exosome suspension, which was subsequently used for downstream experimental analyses.

The microstructure of the exosome was observed using a JEM-1200EX transmission electron microscope (TEM) (JEOL Ltd., Tokyo, Japan). The observation was carried out using an accelerating voltage ranging from 40–120 kV. Image data were acquired and exported. The particle size distribution and concentration of exosomes were measured via nanoparticle tracking analysis (NTA) (Nanosight, Wiltshire, UK). Each sample was analyzed in triplicate, and the mean particle size, modal particle size, and particle size distribution were calculated using NTA software (v3.2, Malvern Panalytical Ltd., Malvern, UK).

PBS (100 μL) was added to the resuspended exosomes, which were then incubated with 10 μL Fluorescein Isothiocyanate (FITC)-conjugated anti-CD63 antibody (557288, BD Biosciences, San Jose, CA, USA) or FITC-conjugated anti-CD81 antibody (551108, BD Biosciences, San Jose, CA, USA). Flow cytometry analysis was performed using an Accuri C6 flow cytometer (AccuriC6, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) according to the manufacturer's instructions.

2.4 RNA Extraction and RNA Sequence

Plasma samples (3 mL) were extracted from umbilical venous blood using the Qiagen exoRNeasy/Plasma Midi Kit (77044, Qiagen, Hilden, North Rhine-Westphalia, Germany) following the manufacturer's instructions. RNA sequencing was conducted by LC-Bio Technologies Co., Ltd. (Hangzhou, Zhejiang, China) on the Illumina HiSeq 2500 platform. Total RNA extracted from the samples was utilized to construct strand-specific whole-transcriptome libraries with the SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Takara Bio USA, San Jose, CA, USA). After verifying the sequencing libraries met the required standards through quality control, paired-end 150-bp (PE150) sequencing was performed. Raw sequencing data were initially processed using Cutadapt (v3.4, Marcel Martin, Stockholm, Sweden, <https://cutadapt.readthedocs.io/>) to remove adapter sequences introduced during library preparation and filter out low-quality reads, obtaining high-quality valid data. These clean reads were then aligned to the human reference genome GRCh38 using Hisat2 (v2.2.1, Johns Hopkins University, Baltimore, MD, USA, <https://dabehwankimlab.github.io/hisat2/>). Subsequently, transcript-level expression profiles for mRNA and lncRNA were generated based on the corresponding genome annotation file (general transfer format, GTF).

2.5 WGCNA Algorithm

The Batch effect was eliminated using the R package limma (<https://bioconductor.org/packages/limma/>). Co-expression networks of mRNA and lncRNA were constructed with the WGCNA package (v1.47, <https://cran.r-project.org/web/packages/WGCNA/>) in R (v4.4.3, Foundation for Statistical Computing, Vienna, Austria). The WGCNA analysis workflow strictly followed the methodology described by Huang *et al.* [24], which encompasses critical steps for the selection of soft-thresholding parameters, module identification, and screening of key genes. Specifically, the pickSoftThreshold function was employed to determine the appropriate soft threshold parameter (β) for approximating the scale-free network topology. The selected β was required to ensure that the scale-free topology index R^2 was ≥ 0.8 with a relatively high average connectivity. Based on this soft threshold, a weighted adjacency matrix was constructed, and modules were subsequently identified. Module eigengenes (MEs) were calculated for each module, and their correlations with clinical features were evaluated to identify gene modules significantly associated with clinical phenotypes. Gene modules showing significant correlations with clinical features ($r \geq 0.3$, $p < 0.05$) were selected for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using the clusterProfiler R package. Hub genes were determined by calculating Gene Significance (GS) and Module Membership (MM). GS reflects the correlation between a gene's

Table 1. Clinical and biochemical characteristics of the maternal participants.

Patient characteristics	Macrosomia (n = 170)	Normal (n = 186)	<i>p</i>
Maternal age (years)	31.27 ± 4.73	29.59 ± 4.01	<0.001 ^a
Ethnicity			0.904 ^b
Han	109.00 (64.12)	115.00 (61.83)	
Zhuang	54.00 (31.76)	63.00 (33.87)	
Others	7.00 (4.12)	8.00 (4.30)	
Blood pressure (mmHg)			
SBP	114.91 ± 10.70	116.48 ± 10.12	0.154 ^a
DBP	73.00 (66.00–78.00)	75.00 (69.00–81.00)	0.012 ^c
Primiparous			0.008 ^d
Yes	52.00 (30.59)	83.00 (44.62)	
No	118.00 (69.41)	103.00 (55.38)	
Mode of delivery			<0.001 ^d
Vaginal	76.00 (44.71)	165.00 (88.71)	
Caesarean section	94.00 (55.29)	21.00 (11.29)	
Height (cm)	160.18 ± 4.00	159.78 ± 3.64	0.330 ^a
Pre-pregnancy BMI (kg/m ²)	22.91 ± 3.57	20.86 ± 2.94	<0.001 ^a
Pre-pregnancy BMI categories (kg/m ²)			<0.001 ^b
Underweight (<18.5)	12.00 (7.06)	42.00 (22.58)	
Normal (18.5–24.9)	116.00 (68.24)	129.00 (69.35)	
Overweight (25–29.9)	37.00 (21.76)	14.00 (7.53)	
Obese (>30)	5.00 (2.94)	1.00 (0.54)	
Overall weight gain (kg)	15.36 ± 4.05	13.70 ± 3.10	<0.001 ^a
Weight gain			
First trimester	0.00 (–0.53 to 1.00)	0.00 (–1.00 to 1.00)	0.199 ^c
Second trimester	9.00 (7.50–11.00)	8.50 (7.00–10.00)	0.006 ^c
Last trimester	6.00 (4.00–7.80)	5.35 (4.00–6.50)	0.009 ^c
TC (mmol/L)	5.53 ± 1.22	5.46 ± 2.43	0.748 ^a
TG (mmol/L)	2.69 ± 1.47	2.06 ± 1.28	<0.001 ^a
HDL (mmol/L)	1.79 ± 0.33	1.90 ± 0.40	0.004 ^a
LDL (mmol/L)	3.03 ± 0.91	2.75 ± 0.84	0.002 ^a
ALP (U/L)			
12 weeks	50.78 ± 22.21	48.96 ± 28.09	0.500 ^a
32 weeks	164.82 ± 50.03	149.08 ± 57.16	0.006 ^a

^a, *t*-test; ^b, Fisher's Exact Test; ^c, Mann-Whitney U test; ^d, Chi-square test. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ALP, alkaline phosphatase.

expression level and a specific clinical trait or phenotype, while MM quantifies the importance of genes within a module.

2.6 Statistical Analysis

Statistical analyses were performed using SPSS software (v25.0, IBM Corp., Armonk, NY, USA). The Shapiro-Wilk test was employed to evaluate the normality of continuous variables. Continuous variables with a normal distribution were presented as mean ± standard deviation (SD), and between-group comparisons were analyzed using the independent-samples *t*-test. Those deviating from a normal distribution were presented as median and interquartile range (IQR; 25th–75th percentiles, Q1–Q3), with group comparisons conducted via the Mann-Whitney U test. Cat-

egorical variables were described as counts and percentages [n (%)], and between-group comparisons were performed using the Chi-square test or Fisher's Exact test. Graphs were generated using R software and Adobe Illustrator (v28.0, Adobe Inc., Mountain View, CA, USA). All statistical tests were two-sided, and *p* < 0.05 was considered statistically significant.

3. Results

3.1 The Baseline Data of the Subjects

Maternal age (*p* < 0.001), diastolic blood pressure (DBP) (*p* = 0.012), primiparity (*p* = 0.008), and mode of delivery (*p* < 0.001) had significant impacts on the occurrence of macrosomia (Table 1). Compared to the control group, the pre-pregnancy BMI (22.91 ± 3.57 vs. 20.86 ±

Table 2. Clinical parameters of neonatal.

	Macrosomia (n = 170)	Normal (n = 186)	<i>p</i>
Neonatal gender			<0.001 ^a
Male	125.00 (73.53)	96.00 (51.61)	
Female	45.00 (26.47)	90.00 (48.39)	
Neonatal			
Birth weight (kg)	4.14 ± 0.37	3.19 ± 0.36	<0.001 ^b
Birth length (cm)	53.22 ± 3.24	50.29 ± 1.62	<0.001 ^b
Chest circumference (cm)	36.20 ± 1.37	33.06 ± 1.46	<0.001 ^b
Head circumference (cm)	35.49 ± 1.16	32.96 ± 1.22	<0.001 ^b
Placenta			
Length (cm)	22.39 ± 2.19	20.02 ± 1.71	<0.001 ^b
Breadth (cm)	20.81 ± 2.68	18.55 ± 1.20	<0.001 ^b
Thick (cm)	2.57 ± 0.47	2.38 ± 0.38	<0.001 ^b
Weight (kg)	0.77 ± 0.31	0.60 ± 0.09	<0.001 ^b

^a, Chi-square test; ^b, *t*-test.

Table 3. Associations between clinical characteristics and risk of macrosomia.

Variables	Adjust OR (95% CI)	<i>p</i>
Maternal age (years)	1.068 (0.999–1.142)	0.054
Pre-pregnancy BMI (kg/m ²)	1.235 (1.124–1.358)	<0.001
Primiparous	0.524 (0.287–0.954)	0.035
Overall weight gain (kg)	1.330 (1.177–1.503)	<0.001
Weight gain (kg)		
Second trimester	1.218 (1.004–1.477)	0.046
Last trimester	1.189 (1.008–1.402)	0.039
TG (mmol/L)	1.204 (0.967–1.499)	0.097
HDL (mmol/L)	0.180 (0.080–0.407)	<0.001
LDL (mmol/L)	1.827 (1.298–2.572)	0.001
32 weeks ALP (U/L)	1.007 (1.003–1.012)	0.002

OR, odds ratio; CI, confidence interval.

2.94, $p < 0.001$) and overall weight gain (15.36 ± 4.05 vs. 13.70 ± 3.10 , $p < 0.001$) in non-diabetic mothers with macrosomic infants were higher. Weight gain was also significantly higher during the second [9.00 (7.50–11.00) vs. 8.50 (7.00–10.00), $p = 0.006$] and last trimesters [6.00 (4.00–7.80) vs. 5.35 (4.00–6.50), $p = 0.009$]. Notably, significant differences were observed in maternal blood lipid profiles [including high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG)] and alkaline phosphatase (ALP) levels in the last trimester between the two groups (all $p < 0.05$). Additionally, significant differences in clinical characteristics related to the placenta were identified between the macrosomia and control groups; the macrosomia group also had a higher proportion of male infants (Table 2).

3.2 Logistic Regression Analysis of Risk Assessment Parameters for Fetal Macrosomia in Non-Diabetic Mothers

This study utilized logistic regression analysis to identify factors independently associated with macrosomia in non-diabetic mothers. The chosen method of delivery was excluded from the analysis as it was based on fetal weight estimation. Although DBP showed a statistically significant difference in univariate analysis ($p < 0.05$), its values remained within the range of normal physiological fluctuations. Moreover, there was a lack of sufficient evidence to support its role as an independent risk factor for macrosomia in non-diabetic pregnant women. Therefore, DBP was excluded from the multivariate regression analysis conducted in this study. After adjusting for the confounding effect of maternal age [25], the results showed that non-diabetic pregnant women with a higher pre-pregnancy BMI (odds ratio [OR] = 1.235, 95% confidence interval [CI]: 1.124–1.358, $p < 0.001$), excessive overall weight gain (OR = 1.330, 95% CI: 1.177–1.503, $p < 0.001$) and weight gain during the second (OR = 1.218, 95% CI: 1.004–1.477, $p = 0.046$) and third trimesters (OR = 1.189, 95% CI: 1.008–1.402, $p = 0.039$), higher LDL levels (OR = 1.827, 95% CI: 1.298–2.572, $p = 0.001$), and elevated ALP levels in the third trimester (OR = 1.007, 95% CI: 1.003–1.012, $p = 0.002$) were more likely to deliver macrosomic infants (Table 3). However, primiparas (OR = 0.524, 95% CI: 0.287–0.954, $p = 0.035$) and elevated HDL levels (OR = 0.180, 95% CI: 0.080–0.407, $p < 0.001$) in non-diabetic mothers had a lower risk of macrosomic births. No multicollinearity was observed among these predictors.

3.3 Identification of the Umbilical Venous Blood-Derived Exosomes

Exosomes were successfully extracted from the umbilical venous blood. First, we analyzed the size and shape of the exosomes isolated from plasma (Fig. 1A). Next, we recorded the median size of the vesicles, the majority of which were between 60 nm and 100 nm (Fig. 1B). Flow cytometry analysis confirmed that the isolated vesicles were exosomes, as indicated by the specific biomarkers CD63 and CD81 (Fig. 1C).

3.4 Construction of the Co-Expression Network of Umbilical Venous Blood-Derived Exosomal mRNA and lncRNA Corresponding to Clinical Traits

We eliminated batch effects in two batches of the sequenced mRNA and lncRNA data using the R limma package. The datasets, containing 19,349 mRNAs and 9625 lncRNAs, were selected for analysis after removing the missing values of gene expression from the raw data, and the WGCNA package was filtered. Firstly, Soft threshold power values were selected in the mRNA and lncRNA groups. When $\beta = 20$, the R^2 scale was set at 0.9360 to obtain a higher average connectivity degree in the mRNA group (Fig. 2A). Conversely, when $\beta = 30$, the R^2 scale was

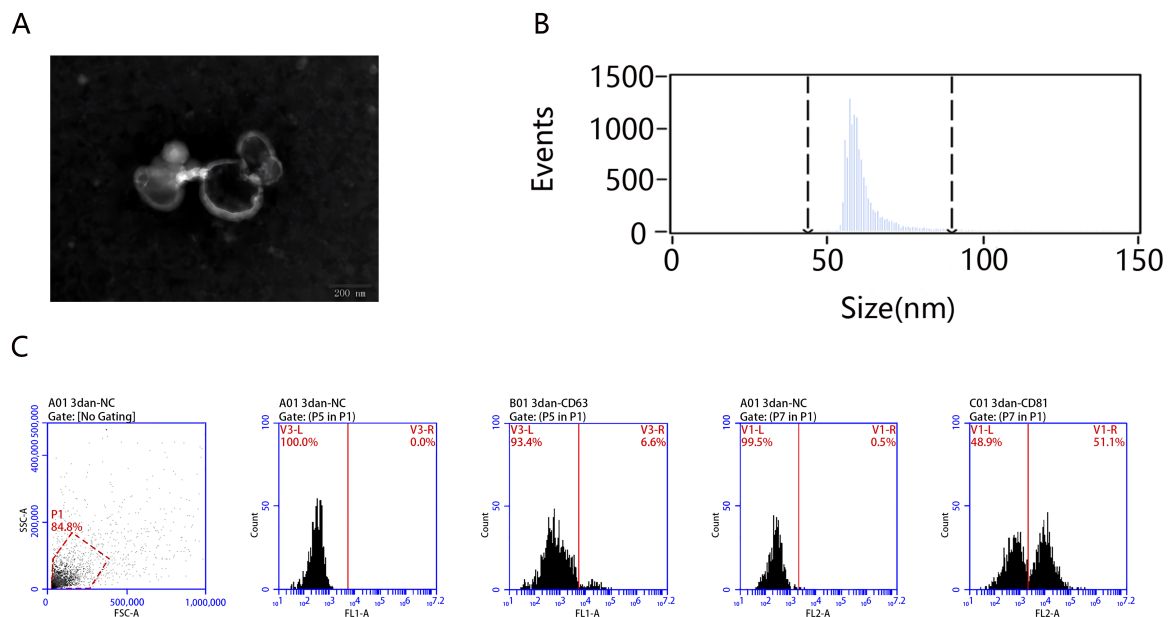


Fig. 1. Characterization of isolated exosomes. A representative TEM image of isolated exosomes. (A) Exosomes ranged in size from 20–80 nm (scale bar: 200 nm). (B) Exosomes isolated from the plasma derived from the cord blood of pregnant women with macrosomic neonates were evaluated by NanoSight. The plot shows a broad size distribution. (C) Flow cytometry of exosomes expressing CD63 and CD81. TEM, transmission electron microscope; FSC-A, Forward Scatter-Area; FL1-A, FLuorescence 1-Area.

set at 0.8012 to obtain a higher average connectivity degree in the lncRNA group (Fig. 2B). Therefore, the β value determined distinct gene co-expression modules in mRNA and lncRNA of the exosome. The cluster dendrogram of all selected genes was clustered with the adjacency matrix.

This study performed correlation analysis between the eigengene patterns within the co-expression modules and the clinical phenotype dataset (including continuous variables such as maternal age, pre-pregnancy BMI, overall weight gain, weight gain during first/second/last trimesters, and neonatal weight) (Fig. 3A,B). Key modules were identified by calculating the correlation coefficient “r” and p -value between module eigengenes and clinical traits. The mRNA module-trait relationship heatmap (Fig. 3A) revealed that *lightyellow* module was significantly positively correlated with pre-pregnancy BMI (“r” = 0.56, p = 0.020), *green* module was significantly positively correlated with weight gain during first trimester (“r” = 0.57, p = 0.020), and *royalblue* module exhibited a significant positive correlation with overall weight gain (“r” = 0.65, p = 0.006). The lncRNA module-trait relationship heatmap (Fig. 3B) showed that the *darkgrey* module was significantly correlated with weight gain during the first trimester (“r” = 0.60, p = 0.009). Based on the “r” and p values, the *mRNA royalblue* module and *lncRNA darkgrey* module, which have the highest correlation with clinical phenotypes, were selected as key modules for further investigation into their association with macrosomia occurrence in non-diabetic pregnancies.

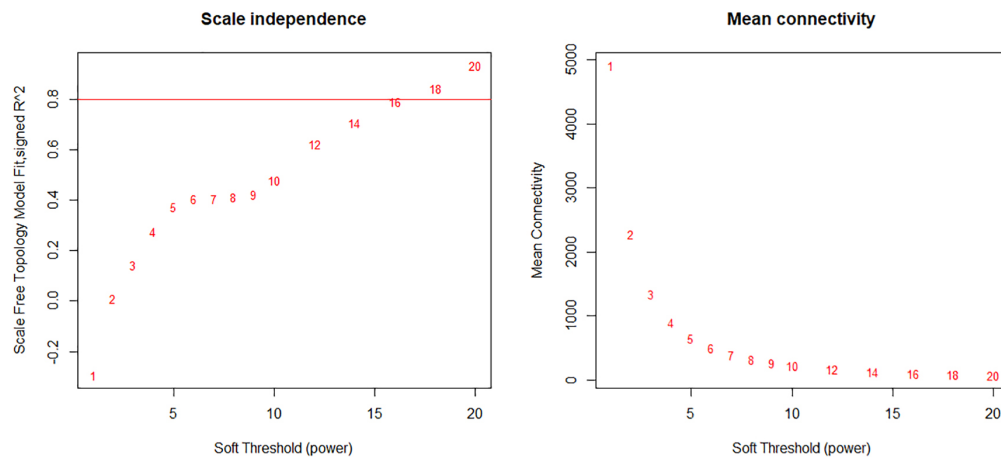
3.5 KEGG Functional Enrichment Analysis of mRNA and lncRNA Gene Modules

The analysis of KEGG pathways revealed various important signaling pathways corresponding to the clinical traits, including metabolic pathways such as the oxytocin signaling pathway, aldosterone synthesis and secretion, and cortisol synthesis and secretion (Fig. 4).

3.6 Hub Genes Analysis of mRNA and lncRNA Gene Modules

Hub gene interaction networks were constructed for the *mRNA royalblue* module and the *lncRNA darkgrey* module, respectively (Fig. 5). Scatter plots depicting GS versus MM were also generated (Fig. 6). The criteria for screening hub genes in this study were set as $|MM| \geq 0.88$ and $|GS| \geq 0.35$. Through hub gene screening, core genes such as transmembrane protein 175 (*TMEM175*), phosphoglycerate kinase 1 (*PGK1*), and methionine adenosyltransferase 2B (*MAT2B*) in the *mRNA royalblue* module and HIF1A antisense RNA 2 (*HIF1A-AS2*) in the *lncRNA darkgrey* module were found to be closely associated with neonatal weight gain. Their MM and GS values were significantly higher than those of other genes (Table 4). By calculating relative expression levels, it was found that compared to normal-weight newborns, the expression of *TMEM175* and *HIF1A-AS2* was down-regulated, while the expression of *PGK1* and *MAT2B* was up-regulated in non-diabetes-related macrosomia cases (Fig. 7). These findings suggested that these genes may play important regulatory roles in the development of macrosomia in non-diabetic preg-

A



B

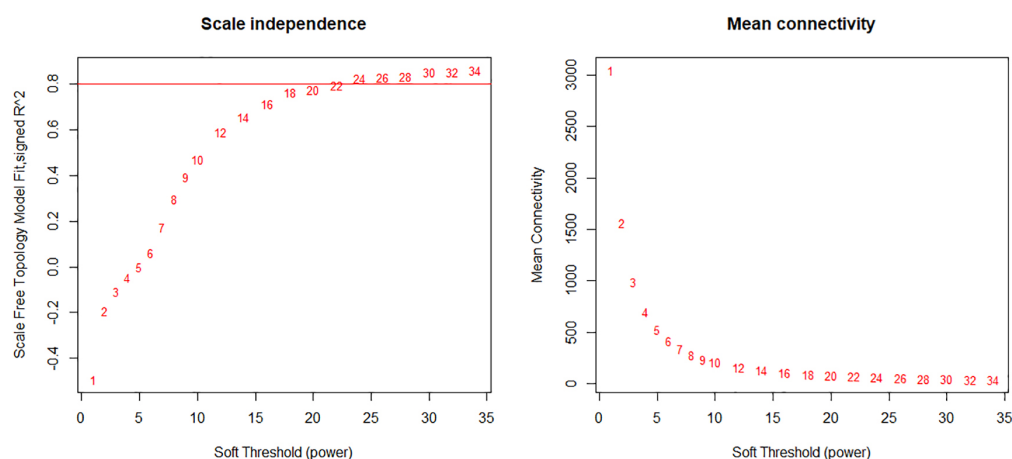


Fig. 2. Analysis of network topology of various soft-thresholding powers. mRNA (A) and lncRNA (B) power value filter. The left panel shows the scale-free fit index (y-axis) as a function of the soft-thresholding power (x-axis). The right panel displays the mean connectivity (degree, y-axis) as a function of the soft-thresholding power (x-axis). mRNA, messenger RNAs; lncRNA, long non-coding RNAs.

nancies. They potentially influence fetal growth and development through complex network regulatory mechanisms, thereby promoting the occurrence of macrosomia.

4. Discussion

By integrating clinical risk factors with exosome transcriptome analysis, our study confirmed that elevated pre-pregnancy BMI, excessive gestational weight gain, and dyslipidemia were closely related to the occurrence of macrosomia in non-diabetic pregnancies. The core genes *TMEM175*, *PGK1*, *MAT2B*, and *HIF1A-AS2* may remodel the nutrient transport patterns at the maternal-fetal interface by regulating mitochondrial metabolism, glucose metabolism, lipid metabolism, and placental development, ultimately leading to fetal overgrowth.

4.1 Clinical Risk Factors for Macrosomia in Non-Diabetic Pregnancies

Our study found that excessive gestational weight gain was a high-risk factor for macrosomia, aligning with the findings of earlier studies [26,27]. A meta-analysis indicated that excessive gestational weight gain was an independent risk factor for macrosomia compared with moderate weight gain [28]. A retrospective study also found that accelerated weight gain rate in obese pregnant women before 24 weeks of gestation was associated with an increased risk of macrosomia [29]. Our study also confirmed that the pre-pregnancy BMI of the macrosomia group was significantly higher than that of the control group, verifying the protective effect of lower pre-pregnancy BMI against macrosomia [30]. Research has reported that multiparous women were more likely to give birth to macrosomic infants

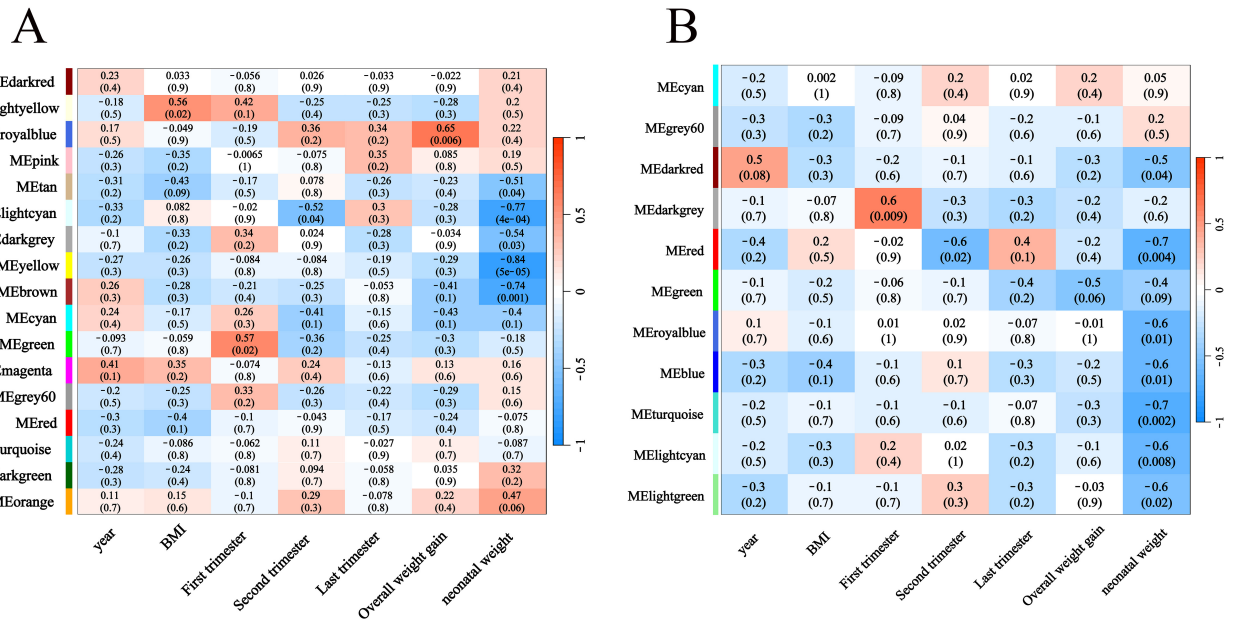


Fig. 3. Module-trait associations for mRNA (A) and lncRNA (B). Each row corresponds to a module eigengene, and the columns correspond to clinical indexes. Red indicates a positive correlation, and blue indicates a negative correlation. Each cell contains the corresponding correlation and *p*-value.

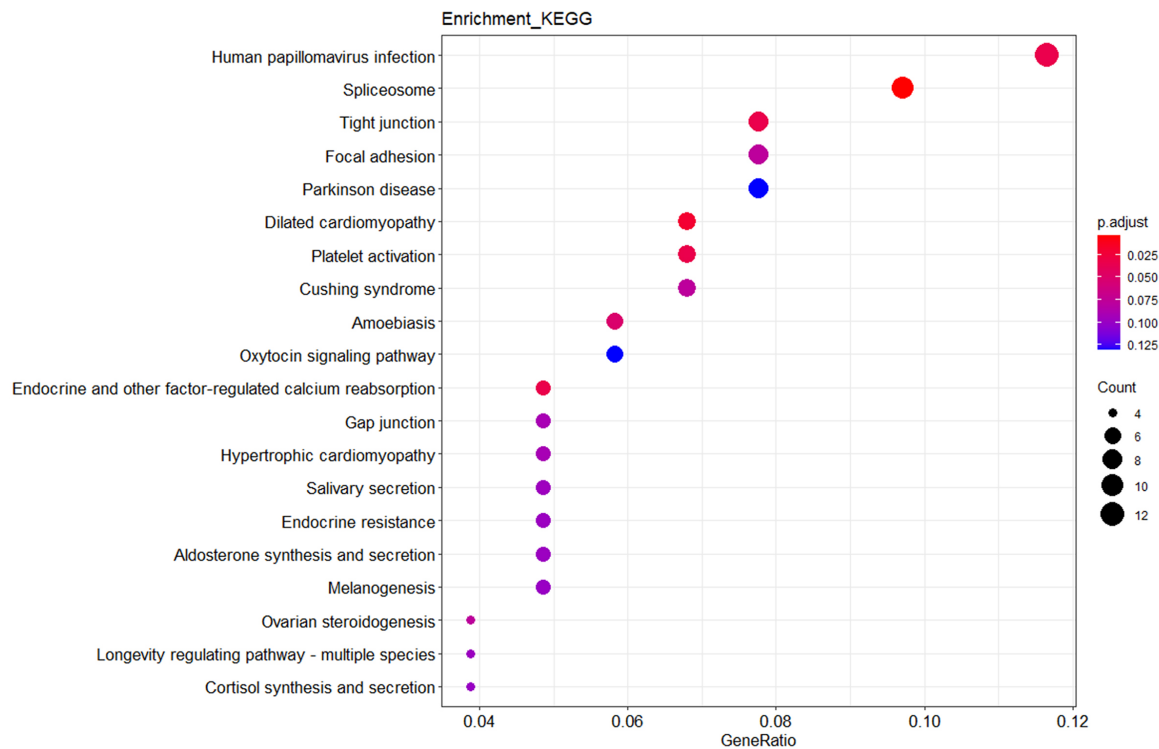


Fig. 4. KEGG enrichment. KEGG analysis revealed that the enriched pathways were the oxytocin signaling pathway, aldosterone synthesis and secretion, and cortisol synthesis and secretion. KEGG, Kyoto Encyclopedia of Genes and Genomes.

[31], which was also observed in our study. Our study also found that the cesarean section rate is significantly higher in non-diabetic macrosomic infants, further validating that fetal weight is a key factor in the decision-making process

for delivery methods [32]. Omaña-Guzmán *et al.* [33] indicated that elevated DBP during pregnancy may be negatively correlated with fetal growth. Although this study found that maternal DBP was lower in the macrosomia

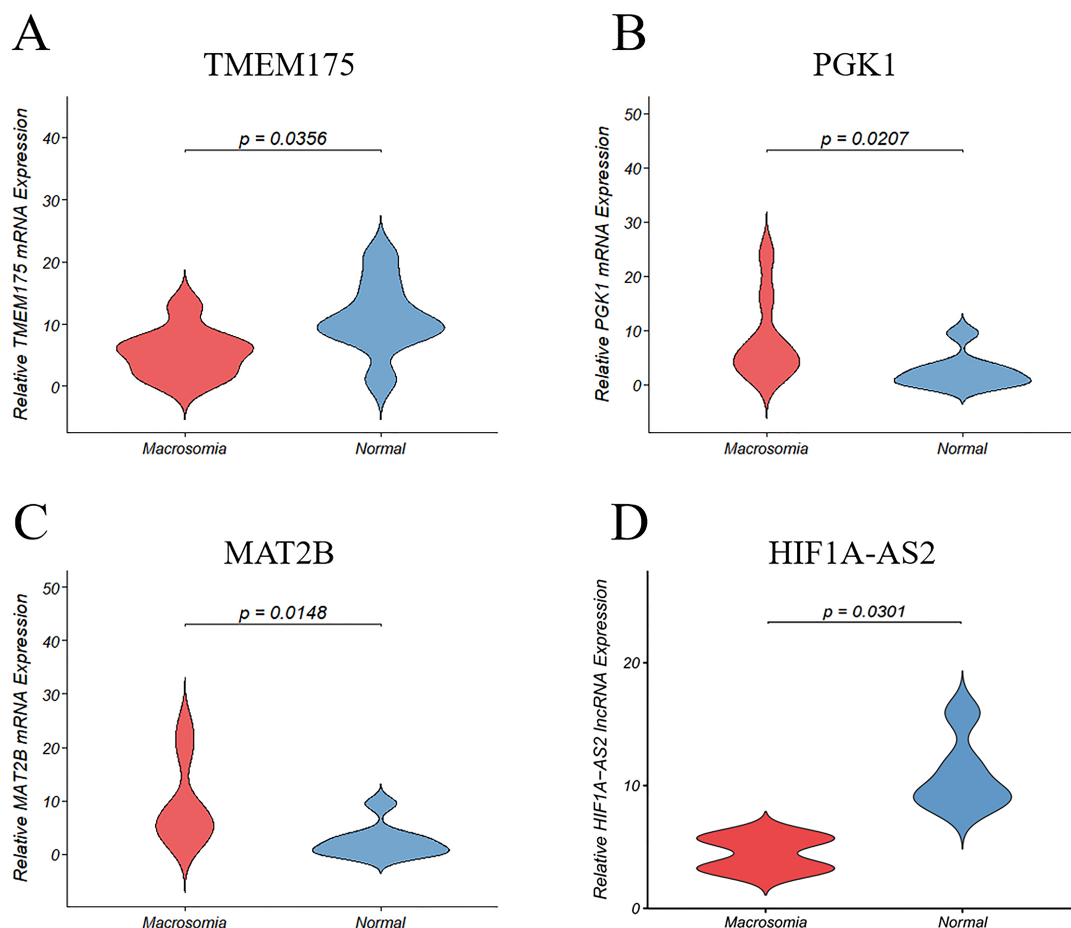


Fig. 7. The expression levels of *TMEM175* (A), *PGK1* (B), *MAT2B* (C) in the *mRNA royalblue* module and *HIF1A-AS2* (D) in the *lncRNA darkgrey* module in umbilical cord blood.

Table 4. Hub genes for the modules correlate with neonatal weight.

Gene symbol	GS	<i>p</i>	MM	<i>p</i>
<i>mRNA royalblue</i>				
<i>TMEM175</i>	0.369	0.005	0.997	<0.001
<i>PGK1</i>	0.357	0.007	0.999	<0.001
<i>MAT2B</i>	0.356	0.007	0.998	<0.001
<i>lncRNA darkgrey</i>				
<i>HIF1A-AS2</i>	0.387	<0.001	0.922	<0.001

TMEM175, transmembrane protein 175; *PGK1*, phosphoglycerate kinase 1; *MAT2B*, methionine adenosyltransferase 2B; *HIF1A-AS2*, HIF1A antisense RNA 2.

ALP levels and adverse effects on fetal outcomes can be complex and remain controversial. A study has found that pregnant women with extremely high levels of serum ALP levels gave birth to normal-weight infants [38]. This may imply that ALP alone does not affect macrosomia in infants, and it should be used together with other indicators to dictate clinical management [40]. These findings highlight the significance of managing pre-pregnancy weight, monitoring gestational weight gain, and regulating lipid levels

for preventing macrosomia in non-diabetic mothers. In particular, lipid level regulation can be accomplished through a balanced diet rich in unsaturated fats and dietary fiber, along with the moderate physical activity advised by health-care providers, to mitigate the effects of maternal obesity on the child [41]. Moreover, neonatal gender seems to be related to macrosomia in non-diabetic mothers. Placental samples showed higher methylation levels in males compared to females [42]. Studies indicated a higher proportion of male neonates among macrosomic births in non-diabetic pregnancies [1,4,43], consistent with our findings. Nevertheless, the specific regulatory mechanisms involved require further investigation.

Fetal macrosomia is more prevalent in mothers with pre-gestational diabetes and GDM compared to non-diabetic mothers; thus, mothers with GDM carry a higher risk. This increased risk is primarily due to the effects of maternal hyperglycemia, which leads to excess glucose transfer to the fetus and subsequent fetal hyperinsulinemia and fat storage [44,45]. In non-diabetic mothers, fetal macrosomia is a complex condition resulting from the interplay of multiple factors such as dyslipidemia and gestational weight gain [35,45,46]. Moreover, an increase in maternal

glucose metabolism in non-diabetic mothers can contribute to macrosomia [47]. A significant number of patients with GDM may also suffer from obesity, a factor associated with fetal macrosomia. The combination of maternal obesity and GDM appears to have a synergistic effect, increasing the risk even further [48]. However, obesity is an independent risk factor, and research indicates that a significant portion of infants with macrosomia are born to women who are obese without diabetes [49]. This suggests that BMI is a major contributor to macrosomia in both diabetic and non-diabetic mothers.

4.2 Exosome Transcriptome Analysis and Associated Pathways

Currently, the application of exosomes in the research of the mechanism of pregnancy complications remains quite limited, and related explorations have not been extensively conducted [16]. Notably, the feasibility of exosomal RNA as a non-invasive biomarker also deserves attention. Although the initial equipment investment for isolation techniques (e.g., ultracentrifugation) is relatively high, the popularization of commercial kits has significantly reduced the cost of single detection. Moreover, exosomes are widely present in body fluids such as blood, urine, and milk, making sample collection convenient and non-invasive, which is suitable for large-scale population screening [50]. Additionally, combined with multi-omics analysis (such as small RNA sequencing), its specificity and sensitivity can be improved, providing a multi-dimensional solution for the early diagnosis and assessment of pregnancy complications [51].

In our study, RNA sequencing of umbilical venous blood exosomes was used to construct a co-expression network of mRNA and lncRNA. By analyzing continuous variables such as maternal age, pre-pregnancy BMI, and gestational weight gain, we found that the *mRNA royalblue* module and *lncRNA darkgrey* module were highly correlated with gestational weight gain. These modules may participate in physiological processes such as adipocyte differentiation and energy metabolism balance through cooperative regulation of gene expression [52]. As key mediators of intercellular communication, RNA molecules carried by exosomes may affect gene expression patterns in the placenta and maternal metabolic organs via blood circulation [53]. Furthermore, KEGG enrichment analysis revealed associations between macrosomia-related genes and the oxytocin signaling pathway, aldosterone synthesis and secretion, and cortisol synthesis and secretion pathways. As a study has shown, the oxytocin pathway may be involved in weight regulation [54]. Aldosterone is found to be elevated in obese individuals, and it is positively associated with BMI [55,56], while cortisol promoted weight gain by enhancing fat storage and slowing down metabolism [57]. Therefore, we speculate that these three pathways may increase the risk of macrosomia by influencing maternal weight changes

during pregnancy. However, the specific role of exosome RNA remains unclear, and future studies using animal models are needed to verify the causal relationship between exosomes in weight regulation during pregnancy and the occurrence of macrosomia, providing a theoretical basis for early intervention in pregnancy complications.

4.3 Core Genes and Their Potential Regulatory Mechanisms

WGCNA analysis identified *TMEM175*, *PGK1*, and *MAT2B* as hub genes in the *mRNA royalblue* module, and *HIF1A-AS2* as the hub gene in the *lncRNA darkgrey* module. Moreover, all these genes were closely related to gestational weight gain. As a key channel protein regulating the lysosome-mitochondria metabolic axis, *TMEM175* mutations can lead to abnormal lysosomal acidification, which in turn impacts the lysosomal-mediated autophagy process and nutrient-sensing pathways (e.g., the mechanistic target of rapamycin (mTOR) pathway). Consequently, this can lead to cellular metabolic disorders [58,59]. The mTOR pathway has been proven to play a significant role in regulating early stages of embryonic development [60], while *TMEM175* is also capable of modulating mitochondrial function and facilitating mitophagy [59]. Lin *et al.* [61] observed reduced placental mtDNA copy numbers in macrosomic infants born to non-diabetic mothers, indicating a possible link between mitochondrial dysfunction and macrosomia, though the precise mechanisms remain unclear. Additionally, fetal growth mainly depends on the availability of sufficient nutrients and their transportation in the placenta, such as glucose. Glucose has been proven to be a key component promoting cell proliferation [62]. Research has demonstrated that regulating maternal glucose levels is essential for the growth and development of offspring. Furthermore, *PGK1*, a vital enzyme in the glycolytic pathway, influences glucose utilization [63]. The concentration of maternal glucose affects placental glucose transport, which provides energy for normal fetal intrauterine development. Therefore, we infer that *PGK1* may regulate placental nutrient transport via glucose metabolism, and its dysregulation may increase glucose transport, leading to fetal overgrowth and macrosomia. Furthermore, fetal growth and development also require an adequate and balanced supply of amino acids for physiological processes such as protein synthesis. Methionine, an essential sulfur-containing amino acid, is critical for fetal growth and development [64]. Research has shown that methionine adenosyltransferase 2A (*MAT2A*) is involved in methionine metabolism and can catalyze the synthesis of S-adenosylmethionine (SAM) (a precursor of cysteine) [65]. Rubini *et al.* [66] confirmed that cysteine can affect fetal development. Furthermore, studies have reported that overexpression of *MAT2A* can promote lipid accumulation and significantly upregulate the expression levels of adipogenic marker genes such as peroxisome proliferator-

activated receptor γ (*PPAR* γ), sterol regulatory element-binding protein-1c (*SREBP-1c*), and adipocyte protein 2 (*aP2*) [67]. *PPAR* γ , which regulates placental development, may affect macrosomia by modulating placental lipid transport [68]. Research has indicated that in the case of lncRNAs, the upregulation of *HIF1A-AS2* can significantly promote trophoblast proliferation and regulate placental angiogenesis [69]. These two factors are fundamental to placental development and the pathogenesis of macrosomia in infants born to non-diabetic mothers. This may affect the formation and development of the placenta, enhance the efficiency of maternal nutrient transport to the fetus, and lead to excessive weight gain by a fetus exposed to a high-nutrition environment over extended periods of time [70]. However, no studies to date have directly explored the roles of *TMEM175*, *PGK1*, *MAT2A*, and *HIF1A-AS2* in macrosomia in non-diabetic pregnancies. Their specific regulatory mechanisms and long-term effects on fetal development require further investigation.

4.4 Limitations

This study constructed a differential transcriptome network based on systems biology to elucidate associations between differentially expressed RNAs and phenotypes (e.g., gestational weight gain), providing a basis for developing preventive strategies. However, there are several limitations. Firstly, its retrospective design inherently introduces selection bias. The inclusion criteria and patient characteristics may not reflect the broader population, and the availability and completeness of historical data may introduce additional confounding factors, thereby affecting the generalizability of the findings. Secondly, the single-center setting and relatively small sample size limit the robustness and generalizability of findings. Thirdly, there is a lack of comprehensive functional verification and a thorough assessment of clinical practicality. Although associations with exosome RNA were observed, the specific regulatory mechanisms (such as molecular pathways and interaction networks) remain unclear. Moreover, the clinical significance of exosomal RNA as a non-invasive screening biomarker, encompassing indicators such as sensitivity, specificity, and predictive value, requires further exploration. Future research should employ multi-center designs and expand sample sizes to verify result reliability. *In vitro* and *in vivo* functional experiments are needed to clarify the precise regulatory mechanisms of exosomal RNA. Additionally, well-designed prospective clinical trials should be conducted to systematically evaluate their diagnostic accuracy, prognostic value, and clinical applicability, thereby providing robust evidence for potential clinical application.

5. Conclusions

This study identified pre-pregnancy BMI, gestational weight gain, LDL levels, and ALP levels in the last trimester as risk factors for macrosomia in non-diabetic

pregnancies. Through the investigation of umbilical cord blood plasma exosomes, *TMEM175*, *PGK1*, *MAT2B*, and *HIF1A-AS2* were determined to be potential core regulators for macrosomia in infants born to non-diabetic mothers. In the future, additional research must be undertaken to investigate the specific molecular mechanisms of these potential core regulators, to elucidate their roles in metabolic disorders and how they affect the development of macrosomia. Secondly, more clinical indicators and molecular markers may be considered to develop a more comprehensive risk prediction model for macrosomia, thereby improving the ability for early prediction and intervention of macrosomia. Moreover, based on the risk factors identified in this study, targeted gestational intervention studies can be conducted. This could involve the development of appropriate gestational weight management plans and the regulation of blood lipid levels, to determine if the occurrence of macrosomia can be decreased, thereby offering more effective strategies for the clinical prevention of macrosomia.

Abbreviations

ALP, alkaline phosphatase; AUC, area under the curve; BMI, body mass index; CI, confidence interval; DBP, diastolic blood pressure; GDM, gestational diabetes mellitus; GS, Gene Significance; HDL, high-density lipoprotein; KEGG, Kyoto Encyclopedia of Genes and Genomes; LDL, low-density lipoprotein; LGA, large for gestational age; MM, Module Membership; OGTT, oral glucose tolerance test; OR, odds ratio; SAM, S-adenosylmethionine; SBP, systolic blood pressure; SD, standard deviation; TG, triglyceride; WGCNA, weighted gene co-expression network analysis; TC, total cholesterol.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

JY and LS jointly designed the research study. JY, LS, JL, and KC were responsible for data acquisition. YM and DY performed the experimental procedures. JY and LS completed data analysis and interpretation. YL oversaw project supervision, funding acquisition, and provided guidance on research design. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was carried out in accordance with the guidelines of the Declaration of Helsinki. The study was approved by the Medical Ethics Committee of Guangxi Med-

ical University (approval number: 2019-SB-067) and the written informed consent was obtained from the participants.

Acknowledgment

Not applicable.

Funding

This study was supported by the Guangxi Natural Science Foundation Program (2020GXNSFDA297024); the National Natural Science Foundation of China (81960282), the Self-Funded Scientific Research Project of Guangxi Health Commission (Z-A20220479 and Z-A20230789), the “Medical Excellence Award” funded by the Creative Research Development Grant from the First Affiliated Hospital of Guangxi Medical University (201903), and the Guangxi Key Research and Development Program (Guike AB25069096).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/CEOG43904>.

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