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Circulating RANKL is required for glucose homeostasis during pregnancy

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Article

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Abstract

Background

Gestational diabetes mellitus (GDM) poses a significant health burden on women worldwide, necessitating the search for predictive, diagnostic and therapeutic approaches. Increasing evidence suggests that the receptor activator of NF-κB ligand (RANKL) is closely associated with GDM, and this study explored whether RANKL can be used as a biomarker for GDM.

Methods

We conducted a cross-sectional study on pregnant women attending prenatal examinations in China. 399 pregnancies were finally enrolled, of which 101 were diagnosed with GDM, and the other 298 were non-GDM. Moreover, GDM mouse model was generated with HFD-fed mice. The HFD-fed mice contained two groups, in which one was injected with recombinant protein mouse-derived RANKL and the other was injected equal volume of saline. Tropboblasts were used to investigate the mechanism of RANKL in maternal metabolism homeostasis.

Results

There was a negative correlation between serum RANKL level and the risk of GDM in both crude and adjusted models [OR = 0.993 (95%CI: 0.99–0.996), OR = 0.994 (95%CI: 0.99–0.997)]. Using restricted cubic spline plateau points to determine the optimal effect of serum RANKL. Threshold range is about 800–900 pg/mL. ROC analysis showed that serum RANKL had a high diagnostic value for GDM [AUC = 0.7024 (95% CI: 0.6520–0.7529), $p < 0.0001$, sensitivity = 0.842, specificity = 0.574].

Conclusions

RANKL-treated HFD-fed mice exhibited improvements in glucose and insulin tolerance and serum insulin concentrations in animal model. Meanwhile, RANKL could promote *glucose transported proteins* (*Gluts*) mRNA expression. RANKL might be a diagnostic biomarker of GDM and as a potential drug target for GDM therapy via improving insulin sensitivity.

Introduction

Gestational diabetes mellitus (GDM), characterized by varying degrees of glucose intolerance first identified during pregnancy, is a prevalent complication of gestation, with a global incidence of approximately 10–15% of all pregnancies. This incidence continues to ascend, influenced by a myriad of factors including antecedent history of adverse pregnancy outcomes, ethnic predisposition, genetic determinants, metabolic syndrome, and environmental influences [1–5]. The repercussions of GDM on

maternal health are manifold, encompassing microvascular disease, compromised organ function during the perinatal period, and an escalated risk of metabolic syndromes such as type 2 diabetes and obesity after delivery in later life[6,7]. GDM could lead to potential health risks, ranging from fetal macrosomia and growth restriction to fetal distress, intrauterine fetal demise, and an increased propensity for metabolic disorders for progeny in later life[3]. At presently, the pathogenesis of GDM still remains enigmatic. Hence, the establishment of robust experimental animal models of gestational diabetes, complemented by population studies and deep exploration of GDM and its pathophysiological mechanisms in pregnant women, is critical. The identification of sensitive and reliable biochemical indicators for early detection and efficacious diagnosis of GDM, along with the implementation of timely interventions and treatments in women at high risk of GDM, carries significant clinical and public health implications.

The International Association of Diabetes and Pregnancy Study Groups (IADPSG) standard has been adopted in China, which involves a 75g oral glucose tolerance test since 2011. Given that this method is time-intensive and potentially discomforting for expectant women, scholars are actively seeking stable biomarkers. These include human metabolites such as branched-chain amino acids (BCAAs), alanine, linoleic acid, β-muricholic acid, and cholic acid derivatives; miRNAs like miR-29a, miR-132, miR-195, and miR-223; single-nucleotide polymorphisms such as *TCF7L2*(rs7903146), *MTNR1B*(rs10830963) and *CDKAL1*(rs7754840); and proteins including sex hormone-binding globulin (SHBG) and plasma glycosylated CD59 (PGCD59) [8]. However, the identification of these biomarkers necessitates complex metabolomic and genetic analyses, and the results reported in the literature are often inconsistent due to the complex diversity of biomarkers. This necessitates the exploration of alternative biomarkers to broaden the scope of the field.

In recent decades, bone has been recognized to perform a plethora of physiological functions, including support, protection, mobility, calcium homeostasis, hematopoiesis, and paracrine and endocrine functions of bone cells[9,10]. Furthermore, bone is implicated in energy metabolism processes through the secretion of bone factors and interaction with other tissues[11,12]. One such component is the type II transmembrane glycoprotein known as receptor activator of NF-κB ligand (RANKL), a transmembrane ligand belonging to the TNF superfamily with 317 amino acid residues. By binding to receptor activator of NF-κB (RANK), RANKL can trigger NF-κB signaling, which in turn controls biological processes such as endocrine metabolism, bone remodeling, cell proliferation and differentiation, and apoptosis[13,14]. OPG(Osteoprotegerin) functions as a fusion receptor for RANKL, thereby promoting the proliferation of pancreatic β-cells through the prolactin pathway in diabetic elderly rodents [15]. Mice with placental-specific knockdown of OPG exhibited pronounced glucose intolerance, whereas maternal mice with placental-specific overexpression of OPG demonstrated notable improvement in glucose homeostasis[16]. In women with a history of gestational diabetes mellitus (GDM), circulating levels of OPG were significantly elevated compared to those of normal women and showed positive associations with various metabolic diseases[17,18]. Blocking the activity of the RANK/RANKL pathway in the liver of mice prevents diet-induced glucose intolerance, as reported previously[19]. A study involving 101 GDM and 66

non-GDM individuals revealed higher levels of salivary soluble RANKL (sRANKL) in the GDM group, with serum and salivary sRANKL being higher in the GDM group with gingivitis [20]. However, no significant difference in serum RANKL levels was observed between patients with type 2 diabetes and controls[21,22]. A meta-analysis comprising seven studies investigating RANKL levels found no significant differences between diabetic patients and controls [23]. Interestingly, an inconsistent result was observed, where serum RANKL levels were lower in individuals with GDM [24]. Using both mouse models and human samples, the researchers demonstrates that RANK deficiency reduces the number and impairs the function of T_{reg} cells, potentially contributing to the development of GDM. Furthermore, the activation of the RANK signaling pathway is shown to safeguard pregnancy by regulating the quantity and functionality of T_{reg} cells. These findings highlight the role of RANK in promoting the hormone-mediated development of T_{reg} cells during pregnancy and emphasize the augmented involvement of maternal T_{reg} cells in the pathogenesis of GDM and the restoration of intergenerational metabolic glucose homeostasis[25]. Additionally, an association is observed between the increased number of regulatory T cells and RANKL[26], suggesting a possible dependence on RANKL signaling. Notably, overexpression of RANKL induces systemic expansion of T cells[27]. Thus, it is reasonable to postulate that RANKL, as the ligand for RANK, may hold potential implications in the treatment of GDM. Further investigations in this area could shed light on the therapeutic prospects of targeting RANKL for the management of GDM and its associated complications.

Therefore, the objective of this study is to investigate the impact of RANKL on GDM. Specifically, we aim to address two key questions: (1) Can RANKL serve as a biomarker for the diagnosis and treatment of pregnancy-induced glucose intolerance? (2) What are the underlying pathways involved in the mechanism of glucose homeostasis mediated by RANKL? To achieve these objectives, we conducted a comprehensive analysis of both population-based studies and animal experiments. Through this analysis, we aim to uncover the crucial role of RANKL in regulating GDM during pregnancy. Additionally, this study identifies common research challenges and provides valuable insights for future scientific investigations and study design.

Material and Methods

Study population

A cross-sectional study was conducted at the Shenzhen Longhua Maternity and Child Healthcare Hospital and the Shenzhen Maternity and Child Healthcare Hospital in China, spanning from October 2022 to July 2023. Trained nurses administered questionnaires to collect information on various factors including age, ethnicity, gravidity, education, pre-pregnancy weight, height, menstrual cycle, and the age at first menstruation. Education was classified into three categories: less than college, college, and more than college. Ethnicity encompassed both Han nationality and other nationalities. The formula for calculating pre-pregnancy body mass index (BMI) was weight (in kg) divided by the square of height (in m). BMI was further divided into three sections based on Chinese criteria: <18.5, 18.5–23.9, and ≥ 24

kg/m^2. The number of pregnancies was categorized as one or more than one. Diagnostic information on GDM was obtained from the medical records of the hospitals. Anhui Medical University Animal Care and Use Committee and Use of Laboratory Animals gave the study approval. All included patients gave informed consent for the collection and use of samples (No. 20210327). The study carried out in compliance with the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects.

A total of 512 pregnant women at 24–28 weeks of gestation and 301 women in early pregnancy were interviewed. Eligible participants met the following criteria: age ≥ 18 years, live births, no pre-pregnancy diabetes or previous GDM, and voluntary participation in the study. After excluding individuals who did not provide sufficient serum samples, had multiple pregnancies, or used assisted reproductive technology, a total of 399 women in mid-pregnancy and 262 women in early pregnancy were included in the current study. Among these, 216 had GDM while 445 did not have GDM (**Table S1 and** Table 1).

Following the diagnostic criteria set by China's Ministry of Health and the IADPSG recommendations, pregnant women underwent GDM screening using a 75 g oral glucose tolerance test (OGTT) at second trimester. GDM was diagnosed based on the IADPSG criteria, which included plasma glucose levels of fasting ≥ 5.1 mmol/L, 1 hour ≥ 10.0 mmol/L, and 2 hours ≥ 8.5 mmol/L[3]. Pregnant women with negative OGTT results were included in the control group. Serum samples were separated from the serum tubes and stored at -80°C. Serum RANKL levels were measured using RANKL kits from R&D Systems, United States.

Animal experiments

All ethical regulations and protocols were strictly followed in accordance with the regulations of the Anhui Medical University Animal Care and Use Committee and Use of Laboratory Animals (No. 20210327) and were conducted in accordance with the ARRIVE Guidelines (Animal Research: Reporting of In Vivo Experiments) and the 3Rs principles (Replacement, Reduction, and Refinement) for laboratory animal welfare. Mouse model of GDM have been generated as previously described [28]. Briefly, healthy female ICR mice at 7–8 weeks old and male mice at 9–10 weeks old from Vitalriver (Keaoxieli, China) were selected and housed in a room with a temperature of 22–26°C, relative humidity of 40%-70%, a 12 hours light and darkness alternating cycle as well as a free flow of water. After 1 week of acclimatization to normal feed, male and female mice were randomly combined in cages at a ratio of 2:1. On the following morning, the mice were examined for vaginal pessary or microscopic examination of vaginal secretion smears, and those that were found to have spermatozoa or vaginal pessary were recorded as gestational day 1 (GD1). Pregnant mice that were found to have vaginal pessary were separated, and were fed with high-fat diets (HFD, 45% fat, 20% protein, and 35% carbohydrate, Research Diets, United States) and normal diets (chow, 10% fat, 20% protein, and 70% carbohydrate, Research Diets, United States), respectively, and their body weights were examined continuously during pregnancy. HFD-fed pregnant mice are further divided into two groups: one group is administered with recombinant protein

msRANKL (HFD-msRANKL, 315-11C-10UG Zenbio, China) 1ug/kg (RANKL/body weight) and the other group is administered with an equal volume of saline (HFD-saline as control) through intraperitoneal injection every other day from GD4 to GD18. Similarly, chow-fed pregnant mice are administered with an equal volume of saline (chow-saline, as control) through intraperitoneal injection every other day from GD4 to GD18. At GD18, cervical dislocation euthanasia is performed on all groups of pregnant mice under the condition of isoflurane inhalation anesthesia. Serum is collected from the supernatant after centrifugation of blood at 3500 rcf and 4°C for 10 minutes and stored at -80°C.

Glucose Homeostasis and Serum insulin

Impaired glucose tolerance was detected by intraperitoneal glucose tolerance test (IPGTT) on GD16. Mice were fasted for 12 h and then injected intraperitoneally with glucose (anhydrous D-(C)-glucose, Sinopharm Chemical Reagent Co., Ltd., China) at 0.2% volume fraction, and blood was collected by tail clipping. Blood glucose was measured at 0, 15, 30, 60, and 120 minutes in mice, and the area under curve (AUC) was calculated by trapezoidal cumulative method. Intraperitoneal insulin tolerance test (IPITT) was used on GD17 to detect insulin. After fasting for 6 hours, mice were injected intraperitoneally with 0.75 U/kg (body weight of mice) of insulin (Sigma-Aldrich), and blood was collected from their tails. Glucose was measured at 0, 15, 30, 60, and 120 minutes using a glucose meter (Roche), and the AUC was calculated using the same trapezoidal summation method. Fasting blood glucose (FBG) levels were assessed following a 6-hour fast on GD18, with blood samples collected via retroorbital bleeding following inhalation anesthesia with 3% isoflurane. Tissue samples were collected under anesthesia, which was maintained until the completion of sample collection; all mice were then euthanized by cervical dislocation. The samples were centrifuged for 10 minutes at 4°C at 3500 rcf, and serum was isolated from the supernatant. Insulin kits (Millipore, USA) were used to measure serum insulin levels.

Cell culture

The JEG-3 and HTR-8 cell lines were kindly provided by the Center for Energy Metabolism and Reproduction, Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Biochannel, China) with 10% fetal bovine serum (FBS, Biochannel, China) and 1% penicillin—streptomycin. The JEG3 and HTR8 cell line was maintained in a humidified incubator at 37°C with 5% CO_2. The cells were then separated into four groups: 0ng/ml (DMEM + 10% FBS + 1% penicillin—streptomycin), 25ng/ml (DMEM + 10% FBS + 1% penicillin—streptomycin + 25ng/ml RANKL), 50ng/ml (DMEM + 10% FBS + 1% penicillin—streptomycin + 50ng/ml RANKL) and 100 ng/ml (DMEM + 10% FBS + 1% penicillin—streptomycin + 100ng/ml RANKL). JEG3 and HTR8 cells were incubated with RANKL for 24 h. RANKL was purchased from Peprotech (315-11C; Rocky Hill, USA).

RNA extraction and real-time PCR analysis

Total RNA was extracted from JEG3 and HTR8 cells using TRIzol reagent (Invitrogen, USA) following the manufacturer's guidelines, and the concentration was standardized to 500 ng/μL. RNA purification was performed based on the absorbance ratios at 260 nm and 280 nm. The reverse transcription reactions were prepared according to the instructions provided with the Reverse Transcription Kit (Tolobio, Shanghai). Subsequently, RT-PCR was conducted using SYBR Green Master Mix (Tolobio, Shanghai). The relative mRNA levels were normalized using the comparative Ct method ($2^{-\Delta\Delta Ct}$), with GAPDH serving as the internal reference. The specific primer sequences applied in this study are presented in Table 1.

Table 1
The specific primer sequences.

Symbol	Forward primer	Reverse primer
Glut1	GCAGGAGATGAAGGAAGAGA	GAGTAATAGAAGACAGCGTTGA
Glut2	GATGCTGGCGCTGAGTA	GGCAGAGATGATGACCCTTT
Glut3	GCTCTCTGGGATCAATGCTGTGT	CTTCCTGCCCTTTCCACCAGA
Glut4	CCATCCTGATGACTGTGGCTCT	GCCACGATGAACCAAGGAGTGG
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Statistical Analysis

The means ± SEM are used to present all data. The Kolmogorov-Smirnov test was used to determine whether the data were normal. Multiple group comparisons were made using one-way ANOVA, while two-group comparisons in animal experiments were tested using the student's t-test. In the population study, independent samples t-tests were used to compare quantitative variables, such as age, menstrual cycle, the first menstrual age, the AUC (area under curve) of OGTT, and serum RANKL levels. The chi-square test was employed for qualitative variables, including ethnicity, education, pre-pregnancy BMI, and gravidity, between patients and controls. The correlation between variables (serum RANKL levels and blood glucose) was evaluated using the Spearman Correlation Test. The study employed logistic regression models to calculate the odds ratios (ORs) and 95% confidence intervals (CIs) for the serum RANKL levels (assessed as categorical variables defined by tertiles) and continuous variables on GDM. The adjusted model included maternal ethnicity, education, pre-pregnancy BMI, menstrual cycle, the first menstrual age, and gravidity. Restricted cubic spline regressions was used to explore the nonlinear relationship between serum RANKL and glycemic outcomes. Furthermore, the Receiver Operator Characteristic (ROC) curve was employed to assess the diagnostic value of serum RANKL levels in GDM. A combination of R 4.3.2, GraphPad Prism 8.0.2 and SPSS 26.0 was used for all statistical analyses. For both the population and animal investigations, statistical significance was defined as $p < 0.05$.

Results

Human participants

Serum RANKL levels in women during early pregnancy were 74.62 ± 41.17 pg/ml. There was no significant difference in early serum RANKL levels between GDM and non-GDM pregnant women (p = 0.5279) (Table S1). With regard to the subjects during mid-pregnancy (n = 399), 101 women (25.31%) have been diagnosed with GDM (Table 2). Women with GDM are older compared to subjects without GDM (30.8 ± 0.37vs. 28.24 ± 0.23 years; $p < 0.0001$) and have a higher pre-pregnancy BMI (6.93% vs. 17.45% for < 18.5 kg/m^2, 71.29% vs. 66.11% for 18.5–23.9 kg/m^2, 21.78% vs. 16.44% for ≥ 24.0 kg/m^2; $p < 0.05$). Furthermore, the GDM group has a higher number of pregnancies compared to the non-GDM group (69.31% vs. 47.99%; $p < 0.0001$). No significant differences are observed in ethnicity ($p = 0.149$), education ($p = 0.865$), menstrual cycle ($p = 0.5666$), and the first menstrual age ($p = 0.7025$).

Table 2
Characteristics of Women in Mid-pregnancy

Maternal characteristics	All participants (n = 399)	Non-GDM (n = 298)	GDM(n = 101)	*p*
Age(years)	28.89 ± 0.20	28.24 ± 0.23	30.8 ± 0.37	< 0.0001
Ethnicity				
Minority nationality	37(9.27%)	24(8.05%)	13(12.87%)	0.149
Han nationality	362(90.73%)	274(91.95%)	88(87.13%)	
Education				
Less than college	153(38.34%)	112(37.58%)	41(40.59%)	0.865
college	139(34.84%)	105(35.24%)	34(33.66%)	
More than college	107(26.82%)	81(27.18%)	26(25.75%)	
Gravidity(times)				
1	186(46.62%)	155(52.01%)	31(30.69%)	< 0.0001
≥ 2	213(53.38%)	143(47.99%)	70(69.31%)	
Pre-pregnancy BMI (kg/m^2)				
< 18.5	59(14.79%)	52(17.45%)	7(6.93%)	0.028
18.5–23.9	269(67.42%)	197(66.11%)	72(71.29%)	
≥ 24.0	71(17.79%)	49(16.44%)	22(21.78%)	
Menstrual Cycle(days)	30.43 ± 5.74	30.51 ± 0.36	30.2 ± 0.42	0.567
the first menstrual age(years)	13.66 ± 1.51	13.67 ± 0.09	13.61 ± 0.13	0.703
RANKL (pg/ml)	347.4 ± 25.83	399.8 ± 33.95	192.6 ± 8.53	< 0.0001
HbA1c (%)	4.96 ± 0.37	4.87 ± 0.30	5.22 ± 0.43	< 0.0001
AUC (h*mmol/L)	13.57 ± 0.13	12.46 ± 0.09	16.86 ± 0.22	< 0.0001

BMI: body mass index. AUC: area of under curve.

Serum RANKL levels increase with progression of pregnancy

The RANKL level in the non-GDM group was 76.01 ± 46.11 pg/ml in early pregnancy and significantly increased to 399.8 ± 33.95 pg/ml by mid-pregnancy. The difference between the two groups was extremely significant statistically ($p < 0.0001$). In the GDM group, RANKL levels were 74.62 ± 41.17 pg/ml

during early pregnancy and increased substantially to 347.4 ± 25.83 pg/ml during mid-pregnancy. The difference between the two groups was extremely significant statistically ($p < 0.0001$) (**Figure S1**).

Serum RANKL levels and blood glucose concentrations between Non-GDM and GDM

HbA1c, FBG, one hour OGTT (1hOGTT), two hours OGTT (2hOGTT), and AUC are employed to assess blood glucose levels during pregnancy. The AUC, as depicted in Fig. 1A and **B**, demonstrates significant differences between the two groups ($p < 0.0001$), with GDM exhibiting a higher AUC compared to non-GDM (16.86 ± 0.22 vs. 12.46 ± 0.092 h*mmol/L). Furthermore, the serum RANKL level is higher in non-GDM compared to GDM (399.8 ± 33.95 vs. 192.6 ± 8.53 pg/mL) ($p < 0.0001$) (Fig. 1C).

Correlations of serum RANKL levels with blood glucose

A negative correlation was observed between serum RANKL levels and pregnancy glucose, as well as HbA1c, as indicated in Fig. 2 and Table 3. The AUC exhibited a significant negative correlation with RANKL ($r = -0.214$, $p < 0.0001$). Additionally, FBG, 1h-OGTT, and 2h-OGTT were found to be negative statistically significantly correlated with serum RANKL (FBG: $r = -0.164$, $p < 0.01$; 1h-OGTT: $r = -0.206$, $p < 0.0001$; 2h-OGTT: $r = -0.177$, $p < 0.0001$).

Table 3
Correlations of serum RANKL levels with blood glucose.

	RANKL (pg/ml)	
	Correlation coefficients (*r*)	***p***
AUC (h*mmol/L)	-0.214	< 0.0001
FBG (mmol/L)	-0.164	0.001
1h-OGTT (mmol/L)	-0.206	< 0.0001
2h-OGTT (mmol/L)	-0.177	< 0.0001
HbA1c (%)	-0.111	< 0.05

FBG: fasting blood glucose. AUC: area of under curve. OGTT: oral glucose tolerance test.

Associations of serum RANKL levels with glycemic outcomes

The association between maternal serum RANKL levels and glycemic outcomes is investigated in this study. Table 4 presents the association of RANKL levels with the risk of GDM. Both crude and adjusted models reveal a significant negative correlation between serum RANKL levels (continuous variables) and the risk of GDM [OR = 0.993 (95% CI: 0.99–0.996) for crude models and 0.994 (95% CI: 0.99–0.997) for adjusted models]. In the crude model, Tertile 3 demonstrates a significantly lower risk of GDM compared

to Tertile 2 [OR = 0.034 (95% CI: 0.01–0.111) vs. OR = 0.711 (95% CI: 0.431–1.173), as depicted in Fig. 3]. The dose-response relationship between serum RANKL and GDM risk remains statistically significant even after adjusting for potential confounders [OR = 0.761 (95% CI: 0.434–1.333) for Tertile 2 and 0.032 (95% CI: 0.009–0.110) for Tertile 3, as shown in Fig. 3]. Using restricted cubic spline regression analysis, we found curvilinear associations between continuous serum RANKL levels and the risk of GDM and between serum RANKL levels and glucose of OGTT. Serum RANKL was protective against GDM at high concentrations (> 222.35 pg/ml, n = 194), whereas low concentrations (159.24-222.35pg/ml, n = 106) were risky for GDM (Fig. 4A). The serum RANKL levels at which the glycemic outcomes plateaus among pregnant women was approximately 800–900 pg/ml (Fig. 4).

Table 4
Associations of serum RANKL levels with GDM risk.

RANKL (pg/ml)	Non-GDM	GDM	Crude OR (95%CI)	*p*	Adjusted OR[b] (95% CI)	*p*
RANKL	298	101	0.993(0.990,0.996)	< 0.0001	0.994(0.990,0.997)	< 0.0001
Tertile 1(< 182.71)	80	55	Reference		Reference	
Tertile 2(182.71-252.52)	88	43	0.711(0.431,1.173)	0.181	0.761(0.434,1.333)	0.339
Tertile 3(> 252.52)	130	3	0.034(0.010,0.111)	< 0.0001	0.032(0.009,0.110)	< 0.0001

Logistic regression was used to analysis the associations of serum RANKL levels with GDM risk. Serum RANKL levels were evaluated as categorical variables (defined by tertiles) and continuous variables. GDM: gestational diabetes mellitus. b: adjusted by age, education, pre-pregnancy BMI, ethnicity, gravidity, menstrual cycle, the first menstrual age.

The diagnostic effect of serum RANKL levels on GDM during pregnancy

The diagnostic efficacy of serum RANKL levels in the second trimester for GDM was evaluated using the ROC curve (Fig. 5). The results indicate that serum RANKL levels have a high diagnostic value for GDM, with an AUC of 0.7024 (95% CI: 0.6520–0.7529, $p < 0.0001$), a sensitivity of 0.842, and a specificity of 0.574. After adjusting for covariates including ethnicity, education, gravidity, pre-pregnancy BMI, menstrual cycle, first menstrual age, and maternal age, the diagnostic value remained significant, with an AUC of 0.7854 (95% CI: 0.7380–0.8328, $p < 0.0001$), a sensitivity of 0.861, and a specificity of 0.631 (Fig. 5).

Downregulation of RANKL signaling in the GDM mice

This study used an animal model of GDM to examine RANKL's potential contribution in GDM. Results demonstrate impaired glucose tolerance, as evidenced by IPGTT (Fig. 6A **and B**), and insulin tolerance, as indicated by IPITT (Fig. 6C), in pregnant mice fed a high-fat diet. Additionally, in this study, the concentrations of serum RANKL have been analyzed in pregnant mice. The results revealed significantly higher levels of maternal serum RANKL compared to non-pregnant mice, with a continuous increase observed fromGD9 to GD18 (Fig. 6D). Interestingly, consistent with population studies, serum RANKL concentrations were notably decreased in HFD-fed pregnant mice at GD18 compared to chow-fed pregnant mice (Fig. 6E). These results revealed that RANKL might play a role in metabolism homeostasis during pregnancy.

RANKL could improve pregnancy-induced glucose intolerance

To elucidate the association between RANKL and glucose homeostasis during pregnancy, we administered recombinant protein msRANKL (msRANKL, 1ug/kg) via intraperitoneal injection to HFD-fed pregnant mice every other day from GD4 to GD18. Body weight and body weight gain were not significantly different from controls after the recombinant protein RANKL was administered (Fig. 7A **and B**). Remarkably, maternal glucose tolerance (Fig. 7C **and D**) and insulin tolerance (Fig. 7E **and F**) were significantly improved. Meanwhile, serum insulin concentrations (Fig. 7G) show significant differences between HFD-fed pregnant mice treated with recombinant protein RANKL and HFD-fed or chow-fed pregnant mice treated with saline. These findings suggest that RANKL could enhance glucose homeostasis during pregnancy by improving insulin signaling pathway.

RANKL directly improve glucose transporter proteins upregulation in trophoblasts

In order to investigate the mechanism of RANKL in maternal metabolism homeostasis, we explored the impact of RANKL signaling using two types of trophoblasts, including JEG3 and HTR-8 cells. As shown in Fig. 8A-D, RANKL could significantly increase expression of *glucose transporter proteins (GLUTs)* mRNA ,including *Glut1, Glut2,Glut3*, and *Glut4* in JEG3 cell line. Meanwhile, RANKL also promote expression of *Glut1* (Fig. 8E), *Glut3* (Fig. 8G) and *Glut4* (Fig. 8H) in HTR-8 cell line. However, *Glut2* (Fig. 8F) showed not different in HTR-8 cell line treatment with RANKL. These results indicated that RANKL could directly regulate GLUTs expression in trophoblasts.

Discussion

In this study, RANKL have been firstly demonstrated as a diagnostic biomarker of GDM and a potential drug target for GDM therapy through comprehensive investigations involving both population-based and animal studies. This cross-sectional study found lower levels of RANKL in GDM compared with non-GDM during second trimester. The relationship between blood glucose and RANKL levels was significantly

inverse. Higher RANKL was associated with lower risk of GDM. Our results identify serum RANKL threshold ranges for GDM of about 800–900 pg/ml at 24–28 weeks of gestation. These findings of ROC curve strongly suggest that RANKL holds promise as a diagnostic biomarker for GDM. At the same time, the findings of animal experiments suggest that serum RANKL could improve glucose homeostasis through increasing insulin sensitivity and insulin secretion function in pancreatic β-cell during pregnancy. This highlights the significance of RANKL provide a novel valuable insight into managing GDM and potential therapeutic interventions.

Although, the OGTT represents the gold standard test for diagnosing GDM[29]. However, the implementation of the OGTT has encountered challenges. A study conducted in GDM centers, involving 14,039 pregnant women, demonstrated that using FBG ≥ 5.1 mmol/L as a predictive criterion for the late development of GDM may not be optimal[30]. Additionally, the IADPSG criteria for diagnosing GDM have been shown to diagnose a significantly higher number of patients compared to the American Diabetes Association (ADA) standard. Data from the United Arab Emirates revealed that IADPSG diagnosed approximately three times as many patients with GDM compared to ADA, posing a substantial burden on the country's healthcare resources[31]. Moreover, the reproducibility of IADPSG screening results is poor, with up to 30% of patients with positive screening results testing negative upon retesting[32]. Alternative diagnostic methods, such as measuring Hemoglobin A1c (HbA1c) levels, have limited applicability in clinical settings due to their low sensitivity and specificity. These challenges underscore the need for a more reliable and stable marker to serve as a diagnostic criterion for GDM.

Alternative biomarkers have been investigated for their potential diagnostic role in GDM. One such biomarker is SHBG, which is involved in regulating sex steroid hormones. Low levels of SHBG have been identified as a biomarker for GDM in early pregnancy[8]. However, SHBG levels can be influenced by various hormones, including estrogen, progesterone, insulin, and androgens, as well as conditions affecting hormone regulation, such as menstrual cycle disorders and polycystic ovary syndrome, which may impact the diagnostic utility of SHBG[33]. Another emerging biomarker for GDM is PGCD59, a glycoprotein that is converted from a membrane-bound form called CD59. PGCD59 has demonstrated high sensitivity and specificity in diagnosing GDM during the first and second trimesters[34,35]. However, these findings are based on observational studies and lack validation through population-based intervention experiments and animal studies. MicroRNAs (miRNAs), specifically miR-29a, have also shown potential as biomarkers for GDM. Studies have indicated that miR-29a levels are upregulated in patients with GDM during early pregnancy.[36] Moreover, it has been demonstrated that inhibiting miR-29a in mice can improve hepatic insulin resistance and glucose tolerance[37]. However, challenges in measuring serum miRNAs, including the complexity of the procedure and potential variations in sample handling, limit the clinical applicability of miR-29a as a biomarker for GDM. In summary, while the OGTT remains the gold standard for diagnosing GDM, challenges in its implementation have prompted the exploration of alternative biomarkers. SHBG, PGCD59, and miR-29a have shown promise, but further research is needed to validate their diagnostic utility and overcome limitations associated with their measurement and reproducibility.

T_{reg} cell have been shown to promote insulin sensitivity and glucose tolerance by suppressing pro-inflammatory cytokine production and modulating macrophage polarization in adipose tissue[38]. The deletion of RANK (receptor for RANKL) results in a reduction in the quantity and impaired function of T_{reg} cells, which subsequently contributes to the development of GDM [25]. Recent studies have also implicated RANKL in the pathogenesis of diabetes. Observational studies have reported associations between RANKL and glucose homeostasis. One cross-sectional study demonstrated that hyperglycemia decreases RANKL concentrations[39]. Furthermore, serum RANKL levels were found to be higher in nondiabetic and pre-diabetic patients compared to diabetic patients[40]. Additionally, RANKL levels were higher in non-GDM patients compared to GDM patients[24]. Animal studies have also supported the relationship between serum RANKL and diabetes. For instance, apoE-null 2-type diabetic mice exhibited significantly lower free RANKL levels compared to nondiabetic mice, accompanied by elevated serum OPG levels[22]. These findings are consistent with our own results. To date, there has been a lack of animal intervention experiments investigating the association between RANKL and GDM. We propose for the first time that low levels of RANKL may serve as a diagnostic marker for gestational diabetes.

A distinct metabolic phase occurs during pregnancy, marked by an increase in pancreatic β-cell size in response to higher metabolic demands[41]. However, in GDM, compensatory pancreatic β-cell expansion fails to occur, leading to decreased insulin sensitivity and subsequent hyperglycemia. In order to investigate the possible mechanism of RANKL treatment for GDM, we employed an HFD-fed mouse model. According to our findings, mice given recombinant protein RANKL injections had better insulin and glucose tolerance. These findings suggest that RANKL may enhance glucose homeostasis during pregnancy by improving insulin sensitivity. Interestingly, similar results were observed in another study where OPG-/- mice had higher levels of RANKL in an HFD-fed mouse model, displaying stable insulin sensitivity and improved glucose metabolism compared to WT mice. Furthermore, RANKL treatment promoted differentiation of 3T3-L1 cells and SVF cells into brown adipocytes and increased energy expenditure [42]. It is intriguing that our study's findings in pregnant women are inconsistent with those in the non-pregnant population. Previous literature has shown that RANKL inhibitors (Dinomumab) improve insulin sensitivity, but these studies were conducted in non-pregnant women [43]. Additionally, one study found differences in RANKL levels in normal pregnant women during different trimesters [44], suggesting that the unique metabolic phase of pregnancy may influence the functional role of RANKL in glucose metabolism. Pre-pregnancy overweight and obesity, as well as dietary habits such as increased fat intake, are critical factors in GDM development. Moreover, physical activity during the preconception period is strongly associated with a reduced risk of GDM [45]. In our animal experiments, we used an HFD-fed model as a GDM model and found that RANKL treatment improved glucose homeostasis. Hence, it is reasonable to speculate that the systemic metabolic alterations associated with obesity may also contribute to the beneficial role of RANKL in GDM. It is necessary to conduct more research to clarify the underlying mechanisms.

Our study proposes RANKL as a diagnostic biomarker and therapeutic drugs for GDM, supported by evidence from both population-based studies and animal intervention experiments. However, there are several limitations to consider. First, we employed an HFD-fed model as a GDM model, which may not fully account for the potential influence of systemic glucose metabolism changes related to obesity. Additionally, the lack of information on dietary habits and physical activity during pregnancy limits our ability to assess the impact of lifestyle factors on GDM. Second, RANKL is a transmembrane ligand that can be detected as a soluble molecule in serum, making the source of circulating RANKL difficult to determine. In addition, our study focused on patients with GDM in the second trimester, and it is unclear whether the results can be extrapolated to the entire gestation period. Finally, the smaller sample size of this study may have yielded lower statistical sensitivity. Moving forward, large prospective cohorts and well-designed population-based intervention trials are necessary to validate these findings.

In summary, this study points out serum RANKL enhance glucose homeostasis during pregnancy by increasing insulin sensitivity and insulin levels, and suggests that serum RANKL can be used as a promising biomarker for the diagnosis and treatment of GDM. This research provides novel insights into the diagnosis and treatment of GDM.

Declarations

Acknowledgement

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Data availability

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

Author Contributions

Conceptualization: Fa Zeng, Binbin Huang, Data curation: Wanxin Wu, Formal analysis: Guiyuan Yu, Methodology: Wanxin Wu, Fa Zeng, Funding acquisition: Binbin Huang, Supervision: Fa Zeng, and Yuying Zhang, Visualization: Wanxin Wu, Guiyuan Yu, Writing-original draft: Wanxin Wu, Writing-review & editing: Fa Zeng

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Competing Interests

The authors declare no competing interests.

Ethical approval and consent to participate

The study protocol was reviewed and approved by the Biomedical Ethics Committee (No. 20210327) of Anhui Medical University on March 1, 2021, and approved. Relevant guidelines and regulations are performed on all methods. The designated institutional and licensing committees approved all experimental protocols. Informed consent was obtained from all subjects.

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Figures

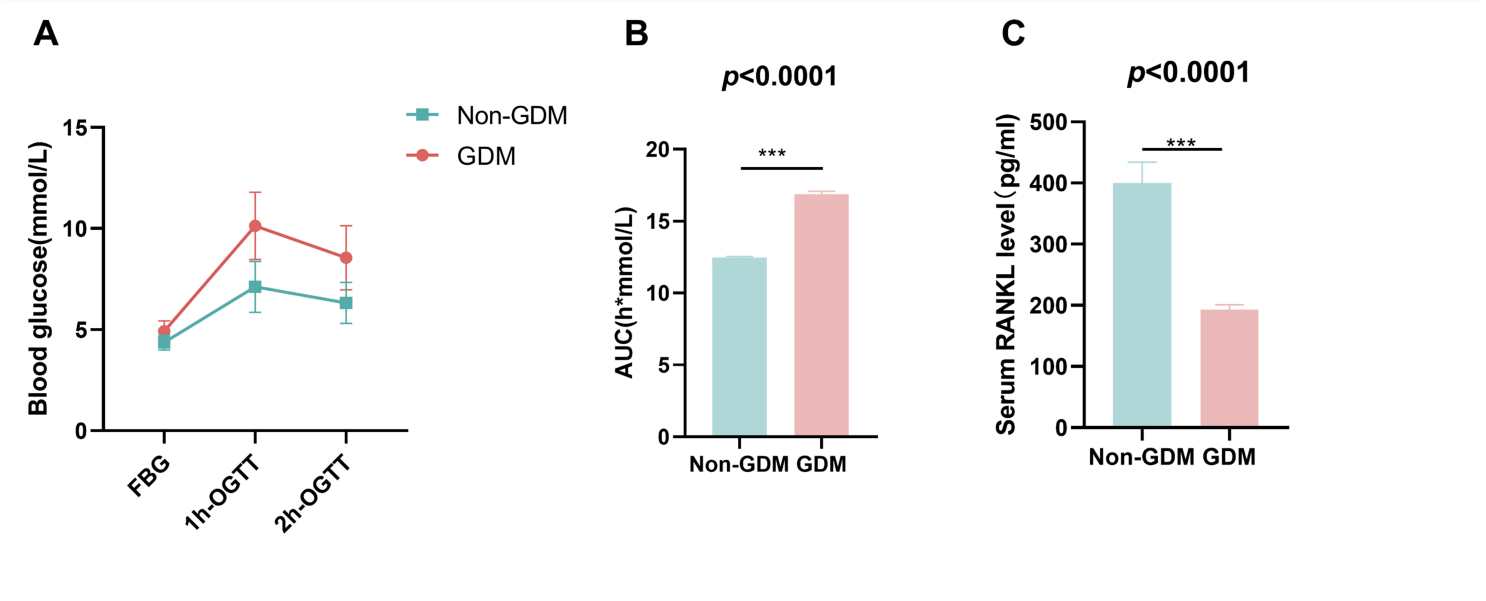


Figure 1

Serum RANKL levels and blood glucose concentrations between non-GDM and GDM. (A) Glucose test of in Non-GDM and GDM at fasting, 1h and 2h. (B) The AUC of Non-GDM and GDM from A. (C) Measure test of serum RANKL in Non-GDM and GDM. FBG: fasting blood glucose. (D) Measure test of HbA1c in

Non-GDM and GDM. FBG: fasting blood glucose. GDM: gestational diabetes mellitus. AUC: area of under curve. OGTT: oral glucose tolerance test. Data was presented as mean ± SEM. n=399. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$.

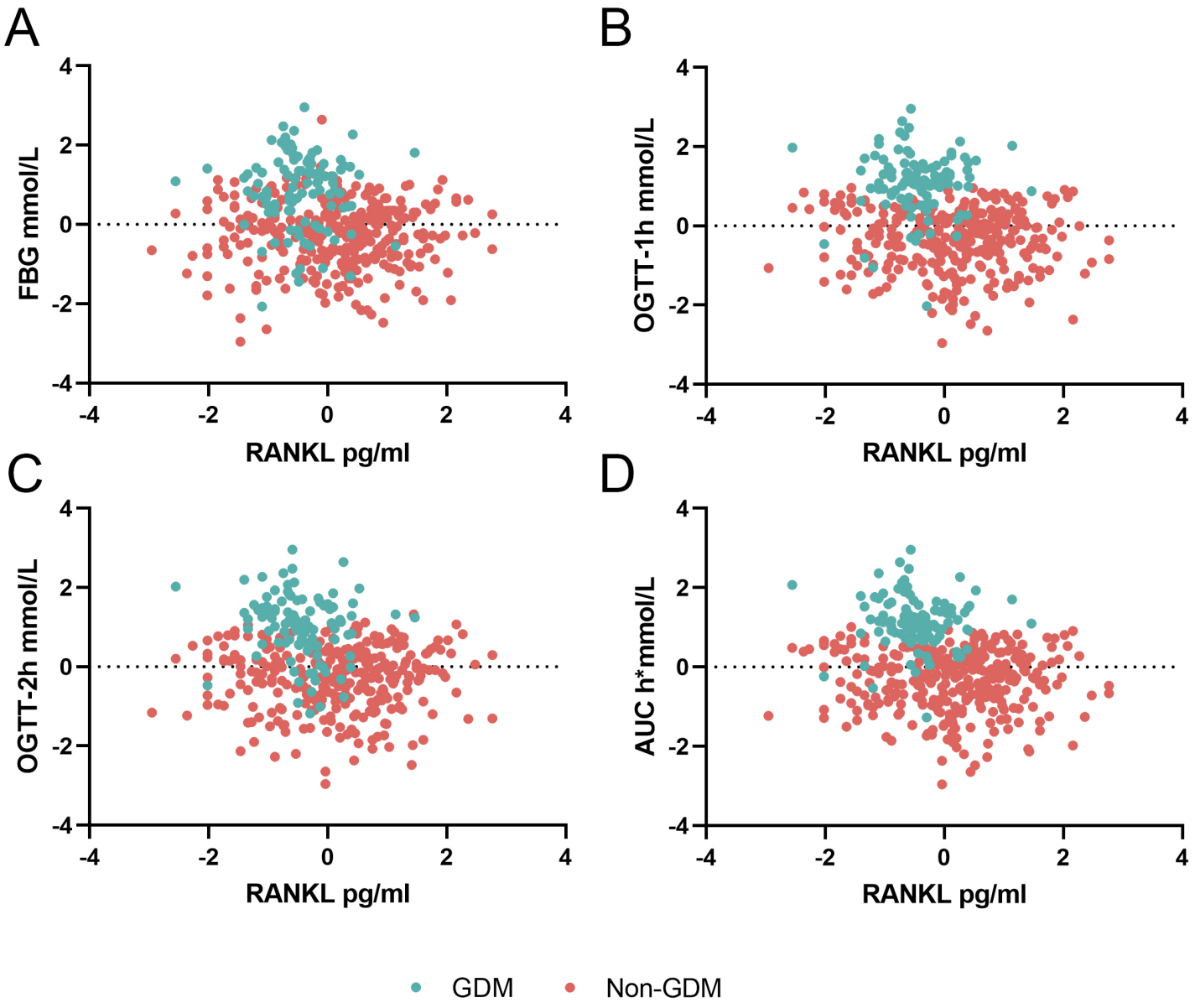


Figure 2

Correlations of serum RANKL levels with blood glucose. Scatter plots reflect the distribution of serum samples from individual pregnant women. FBG: fasting blood glucose. GDM: gestational diabetes mellitus. AUC: area of under curve. OGTT: oral glucose tolerance test. All variables were transformed to a normal distribution by the normal scoring method.

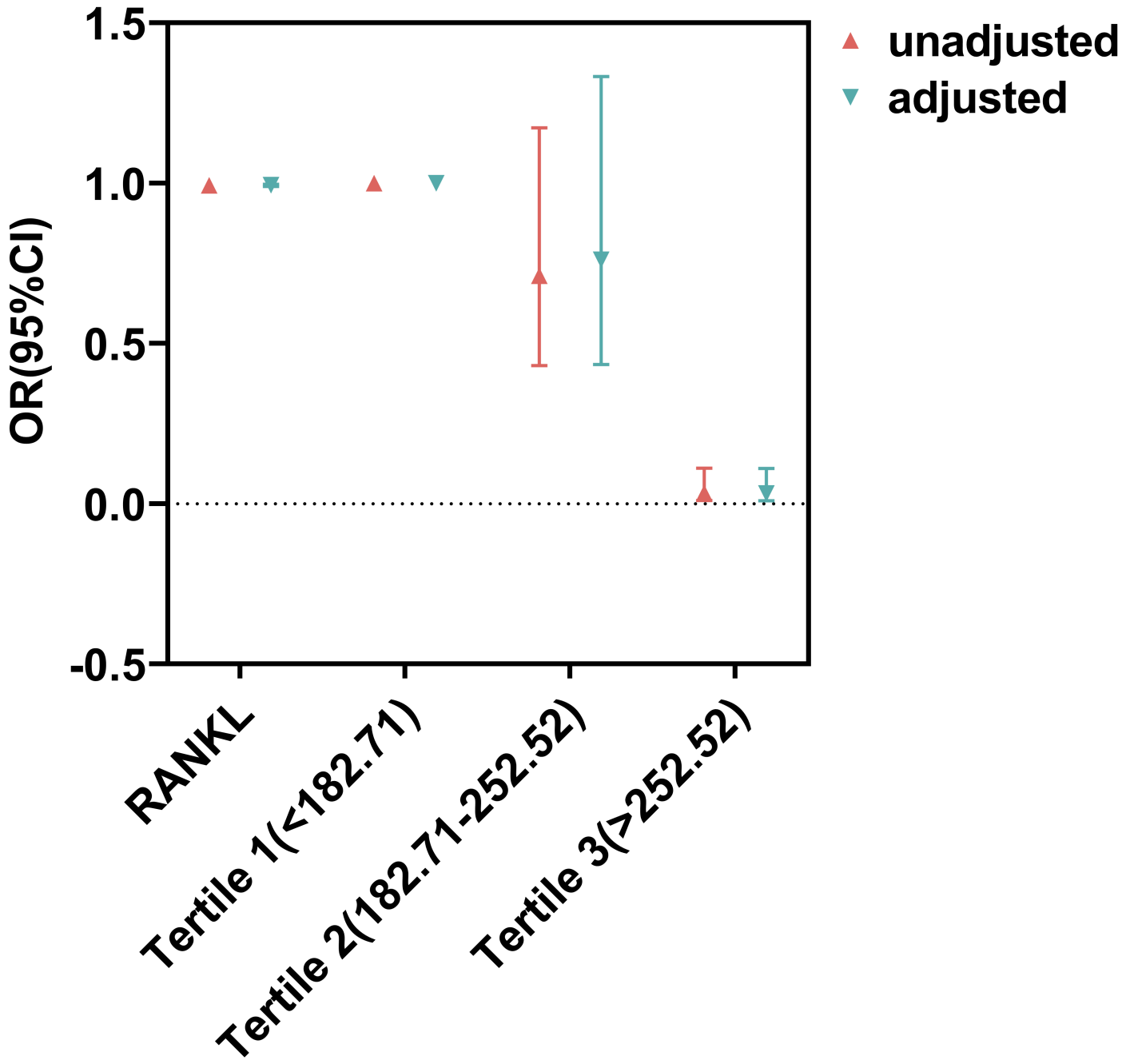


Figure 3

Associations of serum RANKL levels with GDM risk. Logistic regression was used to analysis the associations of serum RANKL levels with GDM risk. Serum RANKL were evaluated as categorical variables (defined by tertiles) and continuous variables. Covariates include age□education□pre-pregnancy BMI, ethnicity□gravidity□menstrual cycle and the first menstrual age.

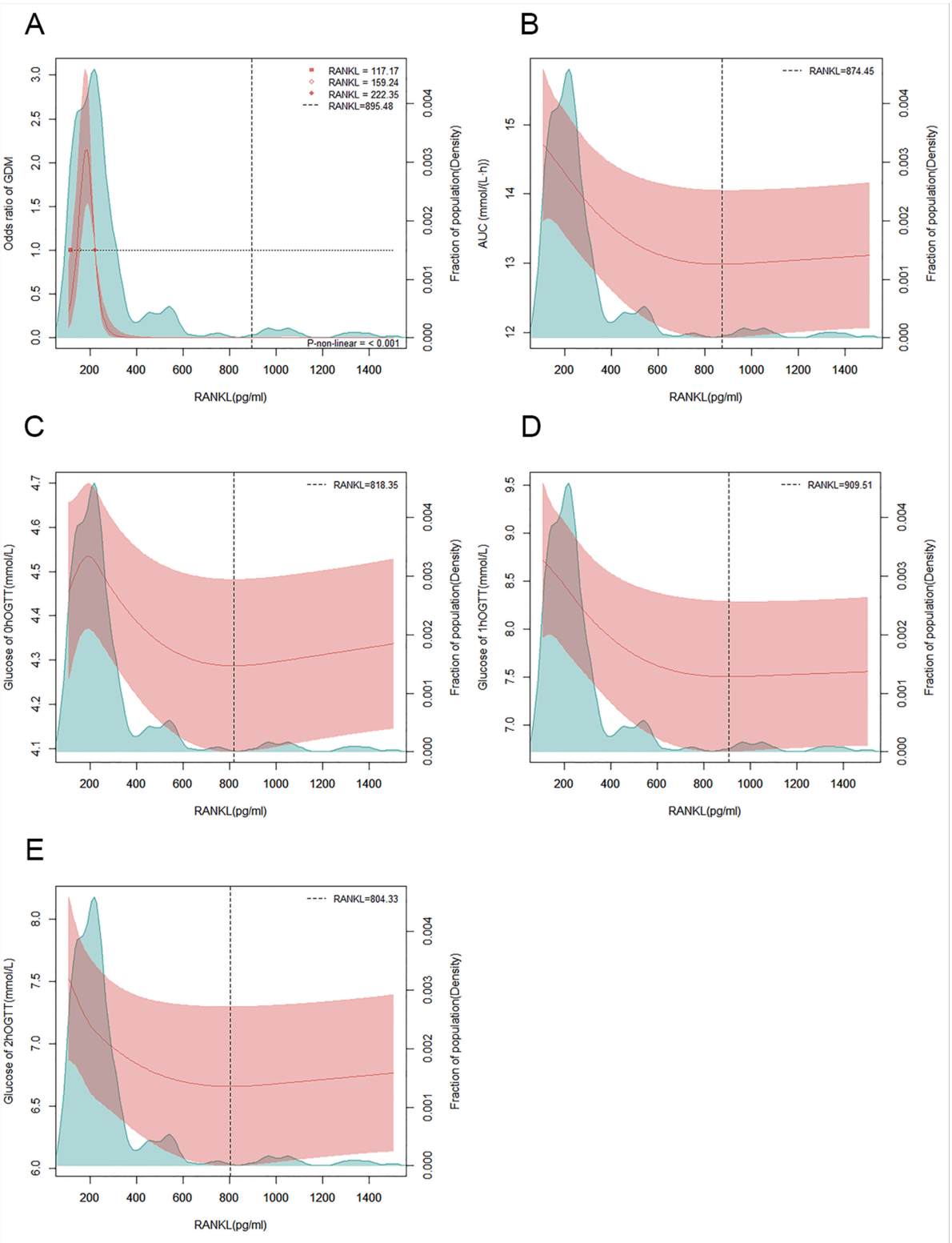


Figure 4

Restricted cubic splines were used to reflect the dose-response relationship between RANKL and glycemic outcomes. (A) Solid red line is odd ratios, with shaded areas showing 95% confidence intervals derived from restricted cubic spline regressions with four knots. Reference lines for no association are indicated by the black dashed line at a hazard ratio of 1.0. Shaded blue areas show the fraction of the population with different levels of RANKL. Vertical line indicates the concentration of RANKL with the

lowest risk of GDM. (B) AUC restricted cubic spline regression with four knots, vertical line indicates the plateau point; shaded red areas are 95% CIs. Shaded blue areas show the fraction of the population with different levels of RANKL. (C) Glucose of 0hOGTT restricted cubic spline regression with four knots, vertical line indicates the plateau point; shaded red areas are 95% CIs. Shaded blue areas show the fraction of the population with different levels of RANKL. (D) Glucose of 1hOGTT restricted cubic spline regression with four knots, vertical line indicates the plateau point; shaded red areas are 95% CIs. Shaded blue areas show the fraction of the population with different levels of RANKL. (E) Glucose of 2hOGTT restricted cubic spline regression with four knots, vertical line indicates the plateau point; shaded red areas are 95% CIs. Shaded blue areas show the fraction of the population with different levels of RANKL. Analyses were adjusted for age□education□pre-pregnancy BMI, ethnicity□gravidity□ menstrual cycle and the first menstrual age. GDM: gestational diabetes mellitus. AUC: area of under curve. OGTT: oral glucose tolerance test.

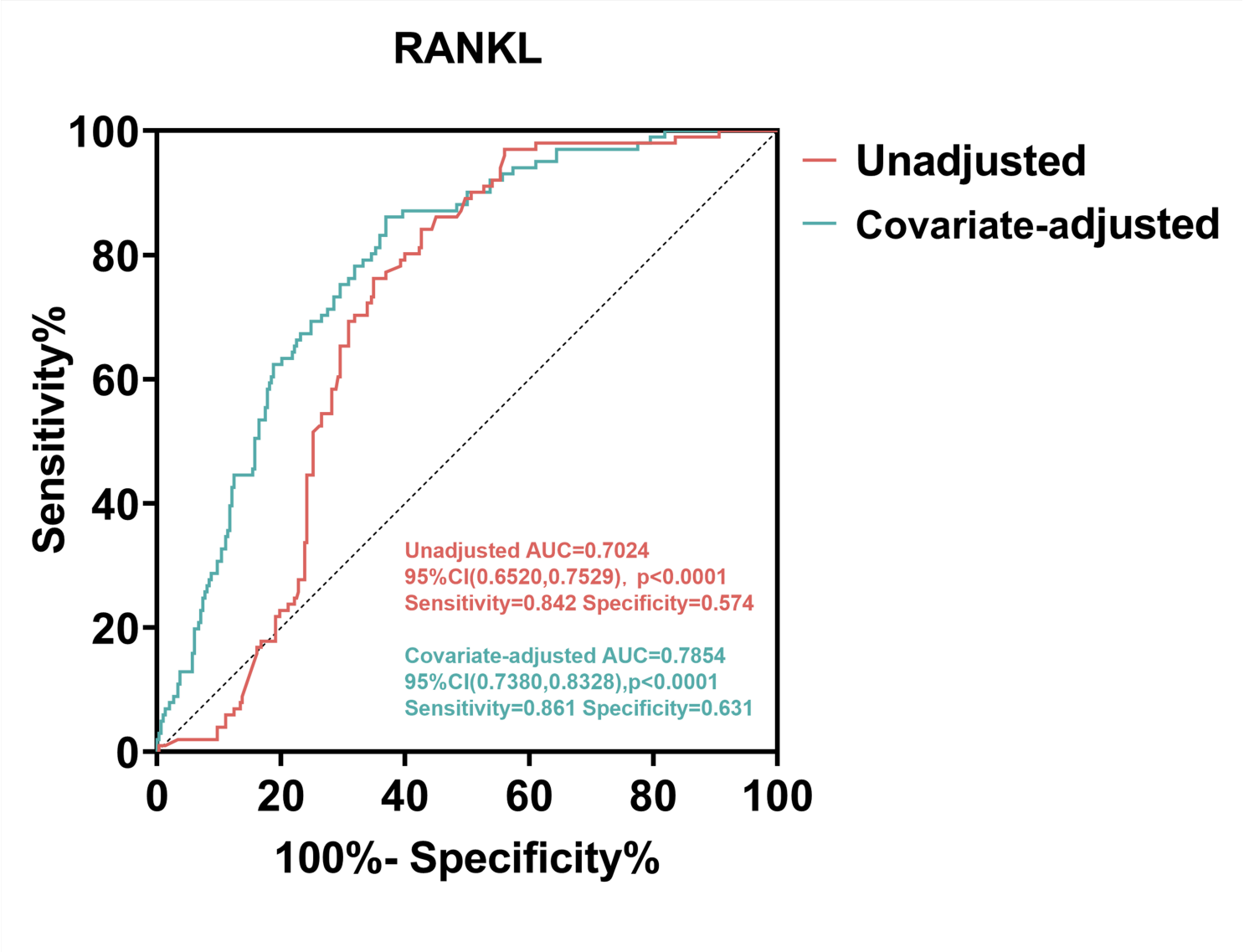


Figure 5

The diagnostic effect of serum RANKL levels on GDM during pregnancy. ROC curve from unadjusted and covariate-adjusted model. Covariate include age、education、pre-pregnancy BMI, ethnicity、gravidity、menstrual cycle、the first menstrual age. AUC: area of under curve.

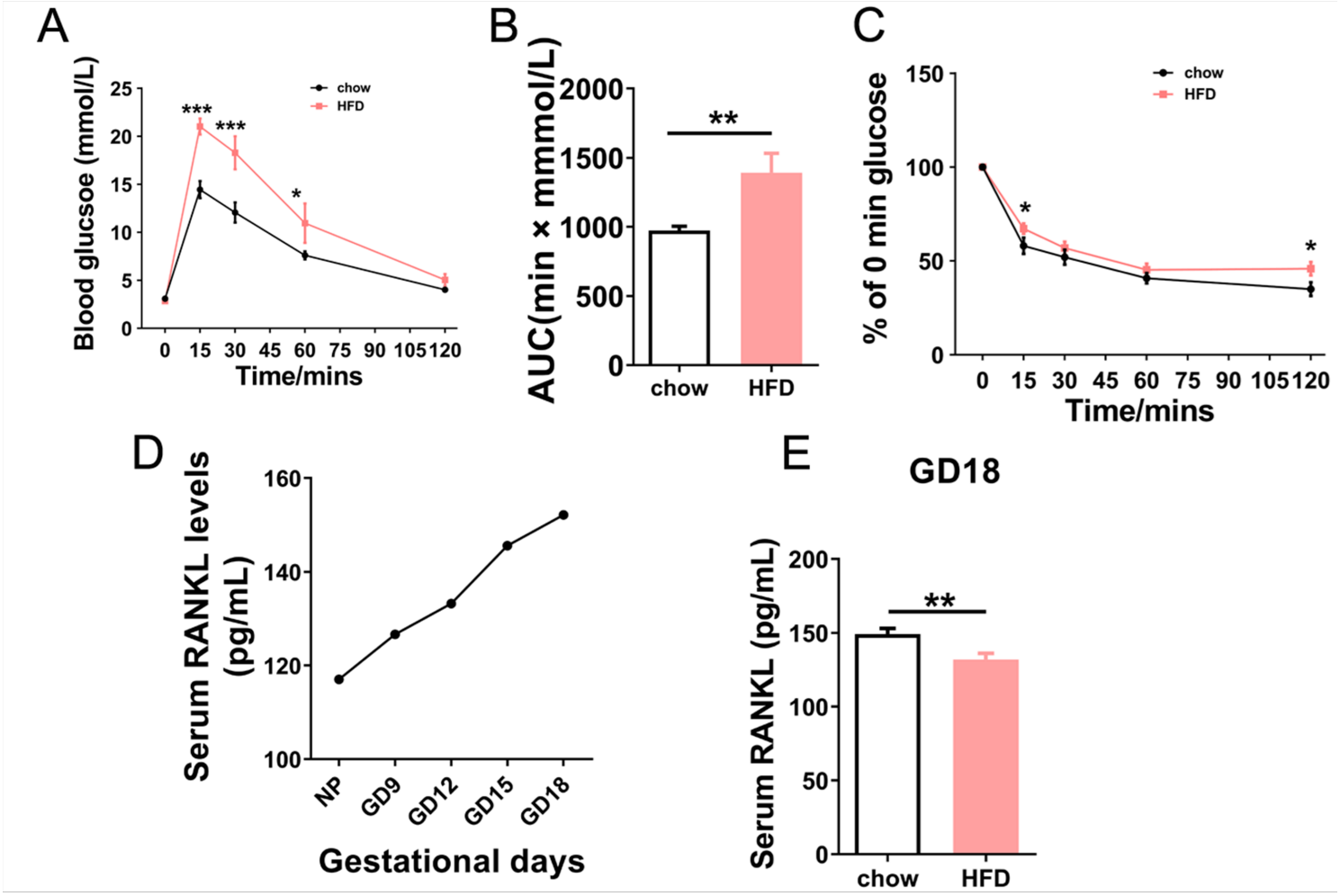


Figure 6

RANKL expression levels in the serum during pregnancy in mice. (A) The IPGTT was measured in chow- and HFD-fed pregnant mice at GD16. (B) The AUC of IPGTT from A. (C) The IPITT was measured in chow- and HFD-fed pregnant mice at GD17. (D) Serum RANKL was measured in chow-fed pregnant mice from NP to GD18. (E) Serum RANKL was measured in chow- and HFD-fed pregnant mice at GD18. Data was presented as mean ± SEM. n=5. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$, black asterisk: HFD vs chow. IPGTT: intraperitoneal glucose tolerance test. HFD: high-fat diets. GD18: gestational day 18. AUC: area of under curve. IPITT: intraperitoneal insulin tolerance test. NP: non-pregnant.

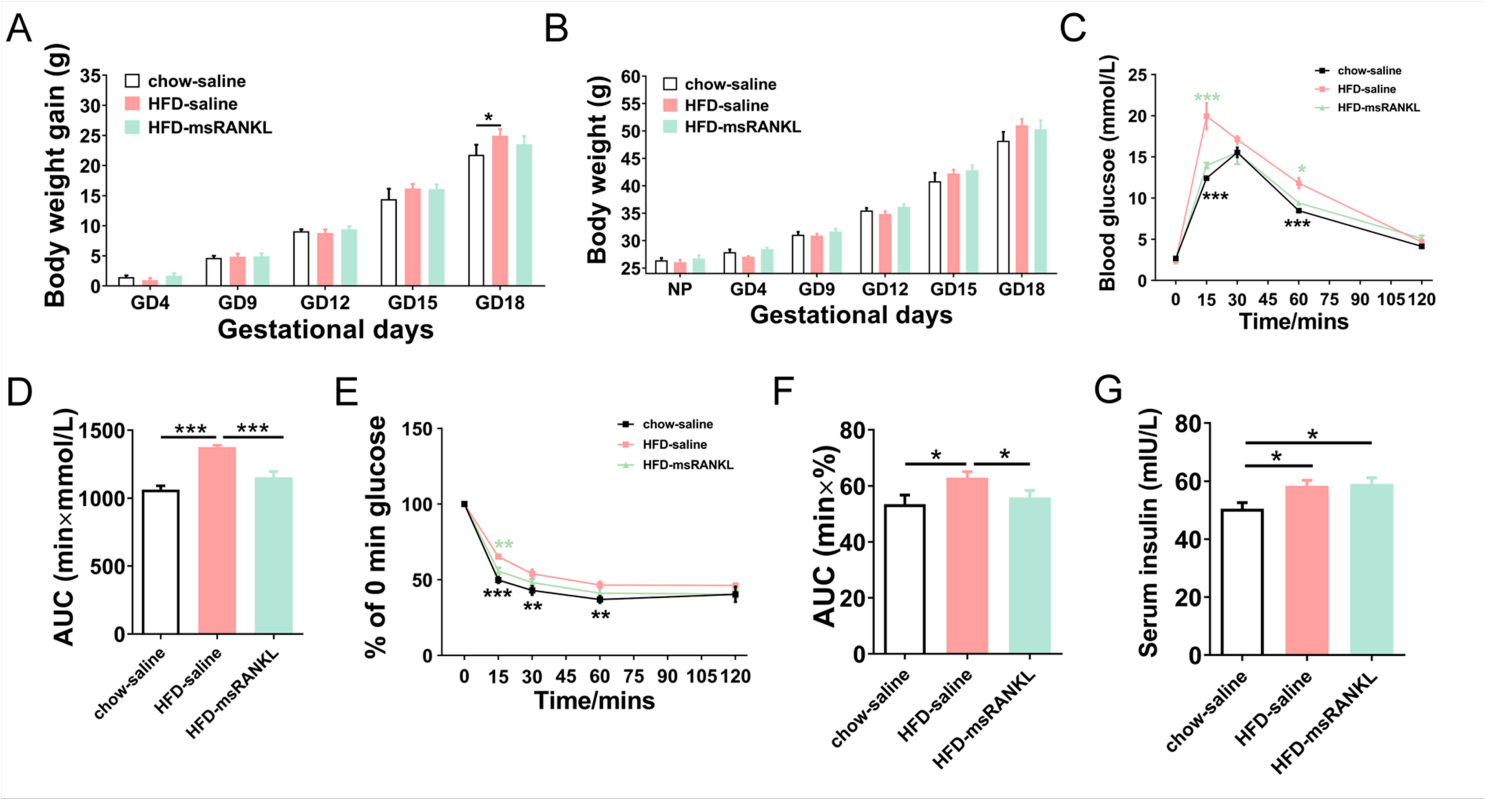


Figure 7

The effect of RANKL on glucose intolerance in pregnant mice. (A)The body weight was measured in pregnant mice treatment with RANKL from NP to GD18. (B) The body weight gains were measured in pregnant mice treatment with RANKL from GD4 to GD18. (C) The IPGTT was measured in pregnant mice treatment with RANKL at GD16. (D) The AUC of IPGTT from H. (E) The IPITT was measured in pregnant mice treatment with RANKL at GD17. (F) The AUC of IPITT from J. (G) Serum insulin was measured in pregnant mice treatment with RANKL at GD18. Data was presented as mean ± SEM. n=4-5. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$, black asterisk: HFD-saline vs chow-saline, green asterisk: HFD-saline vs HFD-RANKL. IPGTT: intraperitoneal glucose tolerance test. HFD: high-fat diets. AUC: area of under curve. IPITT: intraperitoneal insulin tolerance test.

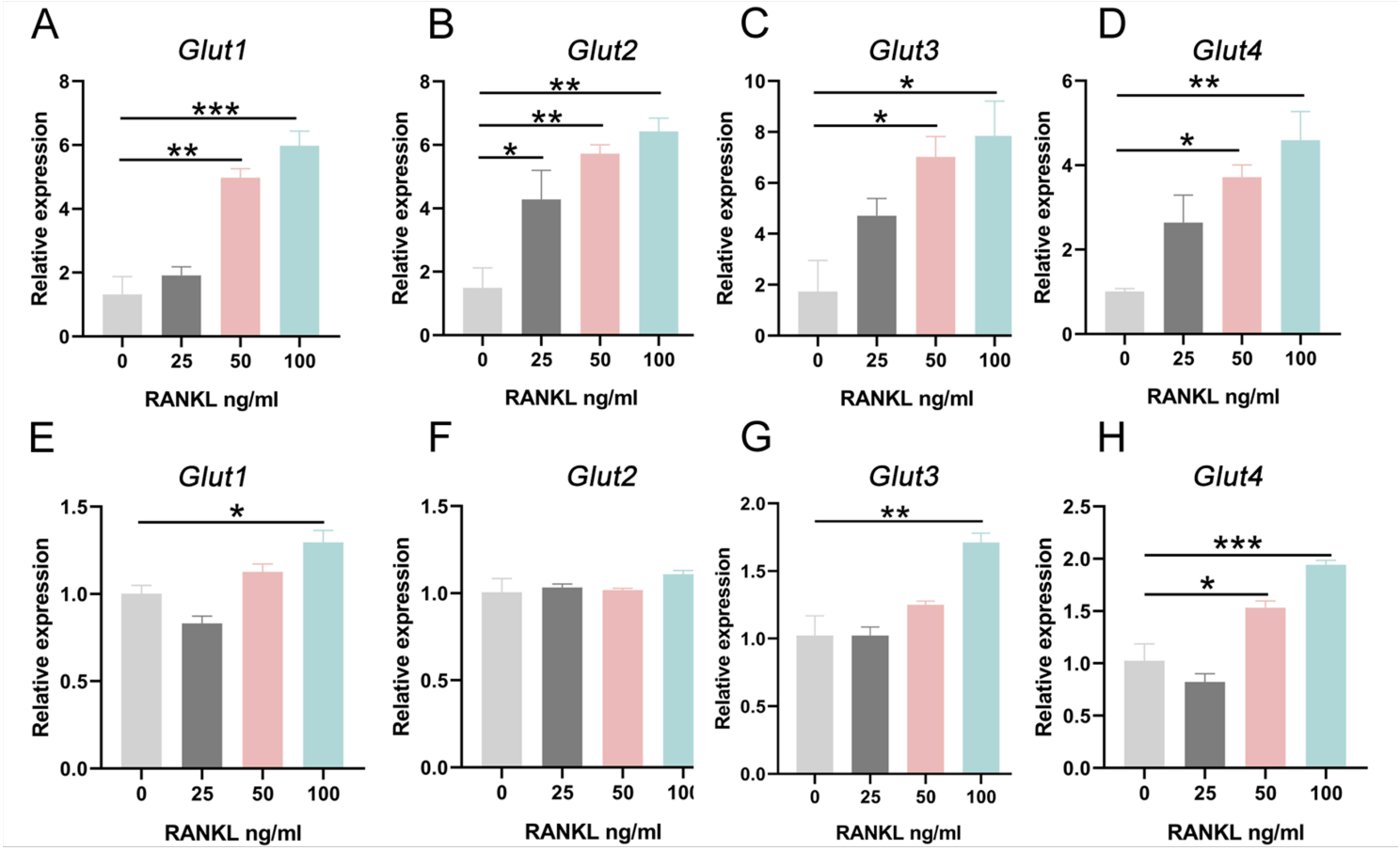


Figure 8

RANKL promoted expression of GLUTs in trophoblasts. Real-time PCR analysis

(A-D) *Glut1*, *Glut2*, *Glut3*, and *Glut4*in JEG3 cell line. (E-H) *Glut1*, *Glut2*,*Glut3*, and *Glut4* in HTR-8 cell line.Values are means ±SD from three independent experiments. *P <0.05, **P < 0.01 and ***P < 0.001compared with controls.

Supplementary Files

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- SupplementaryMaterial.docx