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ORIGINAL ARTICLE

Unravelling the maternal stress-induced orchestrations: *Fndc5* gene expression dynamics across duodenum, stomach, and whole blood in offspring

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ABSTRACT

Objective: Maternal stress is a known risk factor for a variety of adverse outcomes in offspring, including metabolic and behavioural abnormalities. The hormone irisin, encoded by the *Fndc5* gene, is believed to mediate stress's effects on metabolism. Two weeks of restraint stress causes stomach inflammation and increases oxidative stress in rodents. Irisin, coded by the *Fndc5* gene, probably suppresses this oxidative stress. In this study, we examined the effect of early-life maternal stress on *Fndc5* gene expression in the duodenum, stomach and whole-blood offspring.

Materials and Methods: This study consists of three groups: a control, an unpredictable maternal separation (MS), and an unpredictable maternal separation combined with unpredictable maternal stress (MSUS). On postnatal (PND) days 1-14, randomly three hours a day, MS and MSUS were exposed to unpredictable maternal separation. MSUS was subjected to extra unpredictable maternal stress. Mice were sacrificed on PND35. Total RNA was isolated from duodenum, stomach, and whole blood samples by Phenol-Chloroform technique, and HiScript II 1st Strand cDNA Synthesis Kit was used for cDNA synthesis. Fndc5 and Gapdh genes expression level was measured by qPCR using FastStart Universal SYBR Green Master. The data obtained were analyzed using One-Way ANOVA tests in GraphPad Prism.

Results: *Fndc5* gene expression did not differ between groups in the duodenum ($p>0.05$), significantly increased in the MSUS group compared to the control (female $p=0.0089$, male $p=0.0053$) and MS (female $p=0.0206$, male $p=0.026$) groups in the stomach. In whole blood samples, it decreased in MS and MSUS group males ($p=0.0011$). In addition, a significant negative correlation ($p= 0.0003$) has been established between the stomach and whole blood.

Conclusion: The findings assert the role of irisin in transmitting stress-related effects on metabolism, emphasizing the therapeutic potential of targeting the *Fndc5* gene in preventing and treating stress-related disorders.

Keywords: maternal stress, early life stress, irisin, *Fndc5* gene expression, macrophage.

INTRODUCTION

Physical and psychological stress have been linked to various adverse effects on the gastrointestinal tract, including gut motility and permeability changes and an increased risk of gastrointestinal disorders (1). Maternal stress, which is both physical and psychological stress during the lactation period, has been shown to alter the composition of milk (2,3) and impact infant development (2). Maternal stress is widely used as a typical rodent model of early life stress (4-6). Moreover, restraint stress is often used as a model to observe stress effects on the gastrointestinal tract (7,8).

The stomach plays a crucial role in the digestive process by mechanically breaking down food and delivering nutrients to the small intestine through rhythmic contractions (9). The mouse stomach comprises three main parts: the forestomach for food storage, the corpus for food digestion, and the antrum that secretes mucus and certain hormones (10). It contains smooth muscle tissue, which is responsible for the contraction and relaxation of the organ during digestion (11). A recent discovery has shown that the smooth muscle layers of the gastrointestinal tract contain a population of immune cells called muscularis macrophages (12). Macrophages in the gastrointestinal tract constitute the largest population in the body (13,14), and control gastrointestinal motility in health and disease (15). A study has observed macrophages in the muscularis propria. Muscularis macrophages are essential for maintaining the gastrointestinal system's (GI) stability and preventing illness. These immune cells are distinct from mucosal macrophages in that they possess an anti-inflammatory phenotype (12). Psychological stress (16) and acute stress (8,17) have been found to affect various physiological functions of the gastrointestinal tract, including gastrointestinal tract motility (18). In addition, it is known that due to the presence of stress, the expression of gastric monocyte/macrophage markers is increased (7).

Fibronectin type III domain containing 5 (*FNDC5*), a protein found in muscle cells, acts as a precursor for irisin, a 111-amino acid peptide (19). Irisin, the active hormone derived from FNDC5, increases fat burning, improves endurance, speeds up recovery after exercise, and potentially protects against muscle damage during exercise. Despite numerous studies conducted on FNDC5/irisin in skeletal muscle and fat tissue during exercise, almost no studies have investigated FNDC5/irisin expression in the gastrointestinal tract in the presence of early-life chronic stress. Irisin is a crucial mediator of inflammatory response, oxidative stress, and cell apoptosis (20). FNDC5/irisin may protect against oxidative stress by improving mitochondrial function and reducing reactive oxygen species in cells (21). The gastrointestinal system is vulnerable to oxidative stress, which occurs when an imbalance between harmful molecules called reactive oxygen species (ROS) and the body's ability to detoxify them. Factors like diet, exposure to pathogens, inflammation, and chronic stress can contribute to oxidative stress (22). This condition is associated with various gastrointestinal disorders, such as ulcers, inflammatory bowel disease, and some types of cancer (23). Despite the differences in basal irisin levels, exercise-induced irisin secretion is complicated (24). Some studies have questioned whether irisin is an exercise-induced factor (25), and different assay kits account for a large portion of this controversy. This study aims to investigate the relationship between early-life chronic stress and *Fndc5* mRNA expression, the precursor for irisin, specifically in gastrointestinal tissues such as the stomach and duodenum. This research is particularly relevant given the controversy surrounding irisin measurement and its potential modulation by exercise.

Materials and Methods

Animals

The study was performed with 72 *Balb/c* mice aged five weeks (supplied by Nesa Experimental Animals Laboratory). The mice were five weeks old at the start of the study and were housed in a room with controlled temperature (22°C), humidity (55%) and a 12/12 light/dark cycle. The mice had free access to food and water throughout the study. The mice were treated under European regulations for laboratory animal care, and the study was approved by the Nesa Experimental Animals Laboratory (Ethics Committee Approval No: 016).

Maternal stress mice model

The study included three groups of 12 male and 12 female mice: a control group, a group subjected to unpredictable maternal separation (MS), and a group subjected to unpredictable maternal separation combined with unpredictable maternal stress (MSUS). For MSUS, dams and litters were subjected to randomly three hours of proximal separation daily from postnatal days 1 to 14 (26-28). During separation, dams were unpredictably exposed to either 20-minute restraint or 6-minute forced swim stress during the final 20 minutes of a three-hour unpredictable separation. Dams were kept in a 50-mL plastic Falcon tube to induce restraint stress for 20 minutes. Holes were created at the tapering end of the Falcon tube to ensure sufficient air supply. Dams were forced to swim for five minutes in a jar of cold water (18°C) as part of the forced swimming test. Control animals were left undisturbed except for a cage change once a week. Once weaned, pups were reared in social groups (3–4 mice/cage) composed of animals subjected to similar treatment but from different dams to avoid litter effects. Litters were sacrificed at PND35.

RNA isolation and real-time quantitative RT-PCR analysis

All the genetic studies were performed at Ankara Yıldırım Beyazıt University Central Research Laboratory Research and Application Center (MERLAB). From the hearts of sacrificed mice, 0.5 mL of blood was collected using an Etilendiamin tetraasetik asit (EDTA)-washed syringe. Fifty milligrams of the stomach's corpus region and the start zone of the duodenum were removed. Tissues are homogenised by using an ultrasonic homogeniser (Bandelin SONOPULS ultrasonic homogeniser HD 2070, Berlin, Germany). Total RNA was extracted from blood and tissue samples using QIAzol Lysis Reagent (Qiagen, Germany). RNA samples were stored at –80 °C until used. The quantity (absorbance at 260 nm) and quality (ratio of absorbance at 260 nm and 280 nm) of RNA were estimated with NanoDrop 2000 (Thermo Fisher Scientific, Massachusetts, United States of America). All the RNA samples met the following two criteria: 1) The absorbance ratio at 260 nm to 280 nm was within the range of 1.8 to 2.0, and 2) the total RNA concentration was greater than 100 ng/µl. First-strand cDNA synthesis was performed with HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, China) from total RNA. The obtained cDNA samples were diluted 1:5 by adding nuclease-free water. Quantitative real-time PCR was performed in Rotor-Gene Q (Qiagen, Germany) using specific primers (Table 1) and FastStart™ Universal SYBR® Green Master (Rox) (Roche, Germany) with the following cycling conditions in Table 2.

The study utilised SYBR Green, optimised through agarose gel electrophoresis technique and Melting Curve Analysis (Table 2). It enabled the determination of the melting temperature (Tm) of the expected size amplicon of the target and housekeeping gene. Data were collected in the linear amplification range, and each PCR experiment was repeated at least twice. As a result, delta-delta-Ct (2–ΔΔCt) was carried out using the sample's Ct value within the expected Tm range, and the data were normalised using the mean values obtained in the control group.

Statistical analyses

Statistical analysis was performed on the mRNA expression data in control, MS, and MSUS using one-way ANOVA or the Kruskal-Wallis test. Tukey's or Dunn's multiple comparisons post hoc were performed using GraphPad Prism 9.1.0 (GraphPad Software, California, United States). The data distribution was evaluated using a histogram, q-q plot, and the Shapiro-Wilk test. Based on David C. Hoaglin and Boris Iglewicz's approach, outliers were identified and removed. Weight data was analyzed using repeated measure ANOVA followed by Tukey's post hoc. When appropriate, Pearson's or Spearman's correlation was used to assess the

Table 1. Sequence of primers

Primer	Sequence (5'-3'	Amplicon Size (bp)
Fndc5	Forward:ATGAAGGAGATGGGGAGGAA	101
	Reverse: CGGCAGAAGAGAGCTATAAC	
Gapdh	Forward:GAGAAACCTGCCAAGTATGATGAC	105
	Reverse:TAGCCGTATTCATTGTCATACCAG	

Table 2. Amplification and Melting Curve program profile in Rotor-Gene Q

Step	Temperature [°C]	Time [min]	Cycle
Holding	95	10:00	
Cycling	95	00:20	40
		00:45	
Melting curve	60 – 95		

relationship between the data. All tests were set at a significance level of $p < 0.05$.

RESULTS

Maternal Stress-Induced *Fndc5* mRNA Expression Changes in the Gastrointestinal System and Whole Blood of a Mice Model

This study investigated the mRNA expression level of the *Fndc5* gene in mice exposed to maternal stress in the stomach duodenum and blood samples. Compared to the control group, there was no difference in *Fndc5* gene expression in the duodenum ($p>0.05$) (Fig.1A). Interestingly, *Fndc5* gene expression was significantly increased in the stomach of the MSUS group in both females ($p=0.0089$) and males ($p=0.0053$) compared to the control. In addition, it was also found that *Fndc5* gene expression level was excessively increased in both females ($p=0.0206$) and males ($p=0.026$) of the MSUS group compared to MS (Fig.1B). In contrast, in whole blood samples, *Fndc5* gene expression was increased in the female MS group and unchanged in the MSUS group compared to the control group but decreased in MS and MSUS males. In males, this decrease in *Fndc5* mRNA levels in whole blood samples was not statistically significant in the MS group compared to the control ($p>0.05$), whereas it was statistically significant in the MSUS group ($p=0.0011$) (Fig.1C).

The analysis indicates no correlation between the expression level of the *Fndc5* gene in the duodenum and whole blood ($p= 0.508$) (Fig.2A). On the other hand, a significant negative correlation ($p= 0.0003$) has been established between the stomach and whole blood (Fig.2B).

DISCUSSION

Even though several studies have investigated the exercise-associated gene expression level of *Fndc5*, the effects of early-life maternal stress on the expression level of *Fndc5* have yet to be previously examined. Therefore, this study explored the impact on *Fndc5* gene expression level in the stomach, duodenum and whole blood of litters of early maternal separation, maternal forced swim test and maternal restraint stress. Restraint stress has increased the amount of taurine in milk via taurine transports from maternal blood to milk in the mammary gland in lactating mice (3). Therefore, changes in taurine concentration in milk are likely to affect the offspring to some extent. The expected effect on the offspring is believed to be synergistic, with one potential effect being a decrease in milk production. Stress may also decrease hypothalamic prolactin gene expression and plasma prolactin levels, which can reduce milk quantity (22,23). It was observed that during the lactation period, mothers who were separated from their litters, especially those exposed to restraint stress and cold water, avoided breastfeeding their infants compared to the control group. It is believed that they were weaned due to stress and that breastfeeding was avoided due to the pain it caused.

Macrophages play a vital role in the immune system by acting as defenders and cleaners. Based on their

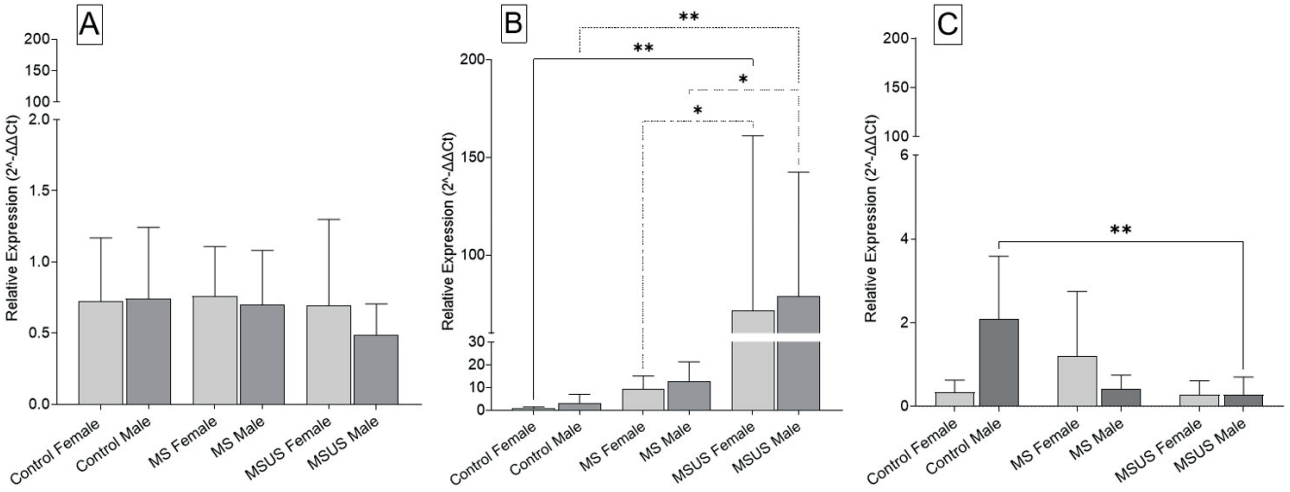

Figure 1. Comparison of *Fndc5* mRNA expression levels in the duodenum **(A)**, stomach **(B)**, and whole blood **(C)** of mice from different groups

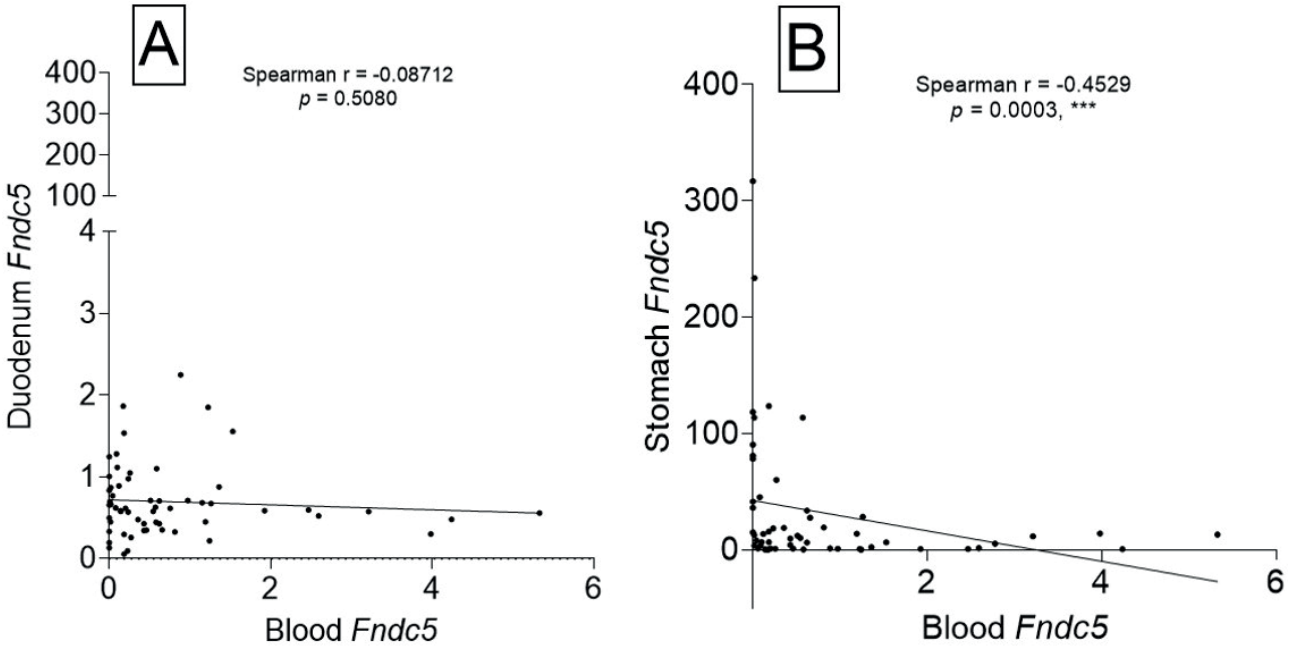

Figure 2. Correlation of *Fndc5* gene expression between Blood and Duodenum **(A)**, Blood and Stomach **(B)**.

activation state, they have two primary forms: M1 and M2 macrophages. M1 macrophages act as attackers by releasing inflammatory molecules to exterminate threats like infections or damaged cells and recruit other immune cells. These macrophages play a crucial role in fighting infections but can also cause tissue damage if inflammation persists. On the other hand, M2 macrophages act as healers by promoting tissue repair, reducing inflammation, and facilitating wound healing. Additionally, different types of M2 macrophages perform specialised functions for repairing and dampening inflammation (29).

Mitochondria are the powerhouses of the cell, responsible for energy production. However, damaged mitochondria can leak harmful reactive oxygen species (ROS) that contribute to oxidative stress (30). Mitophagy is a cellular process where damaged mitochondria are targeted for degradation, helping maintain healthy mitochondria and reducing oxidative stress (30,31). Through mitophagy, aged and damaged mitochondria are selectively eliminated through specific retention and engulfment of mitochondria for subsequent lysosomal degradation. Mitophagy is a critical cellular quality control mechanism that maintains homeostasis in normal physiology and under stress (31). Maintaining a balance between M1 and M2 macrophages and ensuring efficient mitophagy is crucial for a healthy immune response and preventing chronic inflammation and oxidative stress (32). Two weeks of restraint stress causes stomach inflammation and increases oxidative stress in rodents (7). According to studies, taurine supplementation offers defence against diseases linked to mitochondrial defects, including ageing, mitochondrial diseases, metabolic syndrome, cancer, cardiovascular diseases, and neurological disorders (33). Due to oxidative stress, M1 macrophages increase glycolysis and inhibit oxidative phosphorylation, preventing them from polarising to M2 macrophages. M1 and M2 macrophage polarization is critical in maintaining a healthy immune response. However, an imbalance or dysregulation in their activity can result in several diseases. When M1 macrophages are excessively or continuously activated, they release inflammatory molecules that can harm the tissues and promote chronic inflammation (29,33).

Macrophages in the gastrointestinal tract constitute the largest population of macrophages in the body (13,14), and control gastrointestinal motility in health and disease (15). A study has observed macrophages in the muscularis propria, and they suggested that these muscularis macrophages are not only immunomodulatory but also play a housekeeping role and are involved in maintaining normal motility in the healthy gut (15). Psychological stress (16) and acute stress (8,17) have been found to affect various physiological functions of the gastrointestinal tract, including gastrointestinal tract motility (18).

In the presence of stress, the expression of gastric monocyte/macrophage markers is increased (7). Mitochondrial metabolism plays an essential role in regulating macrophage function (34). Studies have demonstrated that metabolism can influence the macrophage phenotype. In active M1 macrophages, mitochondrial oxidative phosphorylation (OXPHOS) is blocked, preventing them from changing into M2 phenotypes. Most cell types rely on mitochondrial OXPHOS as their primary cellular energy production, but this process is blocked in active M1 macrophages (34). Furthermore, a study in mice has shown that M2 macrophages are critical in preventing the development of diabetes-induced delayed gastric emptying (35). In addition, according to Meng L. et al. (32), taurine stabilises energy metabolism and repairs inflammatory damage, helping to prevent chronic diseases and complications. Excessive M1 polarisation of macrophages can lead to the development of inflammatory diseases, but inhibiting M1 polarisation is a protective mechanism against diseases. Taurine transporters in the membrane of macrophages facilitate the transfer of taurine from the extracellular environment to the cytoplasm, which can weaken methionine metabolism and reduce S-adenosylmethionine (SAM) in macrophages. Low SAM is directly sensed by leucine carboxy methyltransferase LCMT-1 and methylesterase PME-1, inhibiting leucine-309 residue of catalytic C-subunit (PP2Ac) methylation, which is required for M1 polarisation. Activation of PTEN-induced kinase 1 (PINK1) promotes the elimination of mitochondria by macrophages via mitophagy for metabolic adaptation, but taurine can block PINK1-mediated mitophagy flux by inhibiting SAM-dependent PP2Ac methylation, resulting in a high mitochondrial density of the M2 phenotype and a low density of the M1 phenotype. This ultimately inhibits the conversion of energy metabolism to glycolysis, which is required for M1 polarisation (36). In addition, Choi et al. (33) found that an increase in taurine content in milk can reduce mitochondrial oxidative stress and increase oxidative phosphorylation in the litter during times of stress. Irisin mediates macrophage proliferation and induces M2 polarisation. Irisin modulates macrophage activity by reducing the overproduction of reactive oxygen species (ROS) (35). *Fndc5* and irisin suppress oxidative stress (18) mediated by mitochondrial dysfunction in macrophages. According to our observations, the MSUS group subjected to maternal and restraint stress showed a markedly higher expression level of *Fndc5* than the control and MS groups (Fig.1). We suggest that overexpression of *Fndc5* mRNA in the stomach of MSUS groups whose mothers were exposed to extra stress may have occurred to reduce oxidative stress.

Irisin, the active hormone derived from FNDC5, promotes converting white adipose tissue (fat storage) into beige or brown adipose tissue. Brown/beige fat cells are more metabolically active and burn energy for heat production. By increasing the amount of brown/beige fat, irisin helps the body burn more calories during and after exercise. In addition, irisin may also encourage muscle cells to use fat as a fuel source during exercise. Irisin stimulates the creation of new mitochondria within muscle cells (24,37). Mitochondria are the powerhouses of cells where energy is produced (31,38). Irisin enhances the function of existing mitochondria, making energy production more efficient. It allows muscles to work harder and for longer during exercise (30,39). By boosting mitochondrial health, irisin might protect muscle cells from oxidative stress and damage that can occur during intense exercise (24). On the other hand, research on the effect of chronic stress on irisin levels has produced mixed results. While some studies indicate a decrease in irisin with chronic stress (24,40), others have shown no significant alteration (25,41). While directly measuring irisin protein levels would be ideal in the long term, focusing on *Fndc5* mRNA expression in the current study has the following advantages: (1) Transcriptional reflection of the *Fndc5* gene is an earlier indicator of changes in irisin production compared to measuring the protein level of mature irisin. (2) Quantifying *Fndc5* mRNA using qPCR is a well-established and sensitive technique. It allows for a more controlled and potentially more sensitive measurement than techniques for mature irisin protein, which can be more susceptible to degradation and variability. (3) Measuring *Fndc5* mRNA in specific tissues (stomach and duodenum) allows pinpointing potential changes in local production within the GI rather than reflecting systemic irisin levels from other tissues. It provides a more precise picture of how early life stress might affect *Fndc5* expression within GI. In summary, accurately measuring irisin levels can be difficult, and this challenge might be one reason for conflicting research results. It is well-known that stress can negatively impact

the digestive system. However, as it is difficult to accurately measure the irisin level (24), this study investigates the expression of *Fndc5* mRNA in the stomach, duodenal tissue, and blood samples of mice exposed to early-life maternal stress.

Energy metabolism and muscle mass are regulated by testosterone in males (42). The positive impact of testosterone on men's body composition and metabolism is widely recognised, but the exact mechanisms behind these effects still need to be fully comprehended. These mechanisms may be linked to myokines, hormones secreted by the skeletal muscles. One myokine, irisin, has shown potential for beneficial metabolic effects (43). Infants exposed to higher levels of stress in early life due to maternal anxiety and a reduction in breastfeeding interaction may experience an earlier onset of puberty (44,45). This study's limitation is that no tests were explicitly performed for early puberty, and testosterone, taurine and irisin levels were not measured. Based on the knowledge in the literature that early-life chronic stress leads to early puberty, in this study, male mice exposed to early-life maternal stress may have increased testosterone levels due to early puberty. Increased testosterone levels due to precocious puberty (46) in the MS and MSUS groups do not affect *Fndc5* gene expression in duodenal and stomach tissue. However, it causes a significant decrease in whole blood samples in males. (Fig.1). Our study indirectly confirms that maternal stress is the cause of early puberty in males. Probably, the high levels of testosterone in males with maternal stress (MS) and maternal separation with unpredictable stress (MSUS) could lead to a decrease in *Fndc5* gene expression. In our study, we could not identify the precise source of *Fndc5* mRNA expression in the blood. However, we hypothesise that adipose tissue, brain, liver, and heart tissues likely played a role in this expression. Further research is needed to understand how *Fndc5* mRNA expression in various tissues contributes to *Fndc5* mRNA expression levels in the blood and its impact on normal bodily functions and disease processes.

As a final result, in maternal stress, *Fndc5* gene expression may increase in the stomach to protect against oxidative stress-induced disorders and keep macrophages in the M1 phase. Presumably, increased *Fndc5* gene expression may increase irisin protein, which both reduces M1 polarisation by blocking oxidative stress and enhances M2 polarisation by mediating macrophage proliferation (Fig.3). Stress can cause significant damage in our body, especially in our stomach, but this damage may be compensated at the molecular level thanks to molecules such as irisin and taurine.

The diagram visually represents the pathways affected by maternal stress and the role of Fndc5/Irisin expression in modulating macrophage metabolism. Maternal stress activates oxidative stress (solid arrows), which enhances glycolysis linked to M1 macrophage polarisation and reduces oxidative phosphorylation associated with M2 macrophage polarisation. The introduction of increased Fndc5/Irisin expression (dashed arrows) blocks the effects of oxidative stress, subsequently reducing glycolysis and enhancing oxidative phosphorylation, potentially reversing the effects on macrophage polarisation. This visual aid underscores the complex interplay between stress responses and immune regulation.

Author contribution

Study conception and design: KKB and AB; data collection: KKB, ANB, MHD, and SNI; analysis and

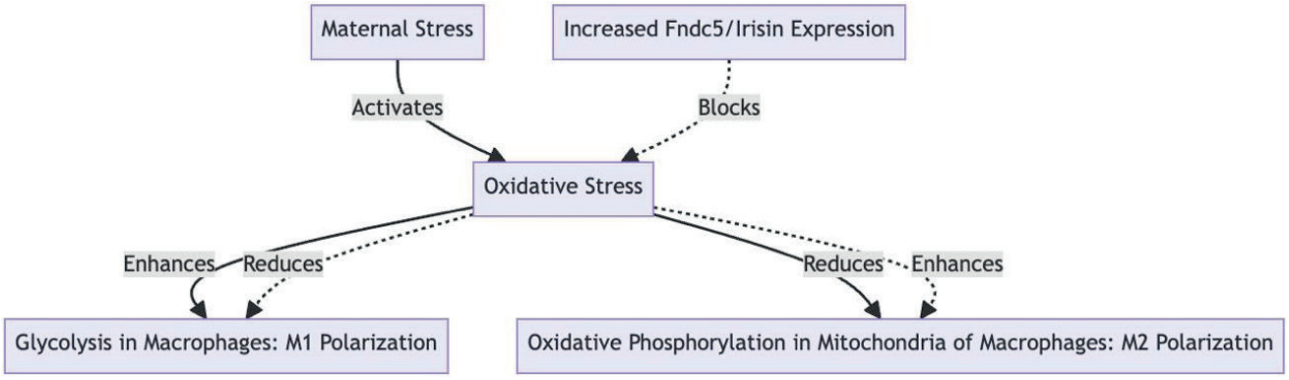

Figure 3. Predicted pathway depicting the potential mechanisms underlying M1/M2 macrophage balance disruption in the gastric mucosa following maternal stress

interpretation of results: KKB, ANB and AB; draft manuscript preparation: KKB, ANB and AB. All authors reviewed the results and approved the final version of the manuscript.

Ethical approval

The study was approved by the Clinical Research Ethics Committee of Nesa Experimental Animals Laboratory (Protocol no. 016, 02 August 2023).

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Conflict of interest

The authors declare that there is no conflict of interest.

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