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Epigenetic modifications of nuclear and mitochondrial DNA are associated with the disturbance of serum iron biomarkers among the metabolically unhealthy obesity school-age children

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Abstract

Background Serum iron biomarkers are disordered on the progression of obesity and its associated metabolic syndrome (MetS). However, limited evidence is explored the interactions between serum iron biomarkers and the incidence of MetS. Thus, the purpose of this study is to discuss whether epigenetic modifications of nuclear and mitochondrial DNA (mtDNA) are associated with the disturbance of serum iron biomarkers among the metabolically unhealthy obesity (MUO) school-age children.

Methods A representative cross-sectional study was performed using the data from 104 obesity school-age children, while the subjects without obesity were as controls ($n=65$). Then, the 104 obesity subjects were defined as metabolically healthy obesity (MHO, $n=60$) and MUO ($n=44$) subgroups according to whether they were accompanied with MetS. Their serum metabolic indicators, transferrin receptor 1 (TFR1), transferrin (TF) and genome-wide methylation were determined by the Elisa method. Moreover, the methylation levels of TFR1 and TF were measured by the Bisulfite sequencing PCR (BSP-PCR). Furthermore, the copy number (mtDNA-CN) and methylation of mtDNA were detected by the RT-PCR, while the semi-long RT-PCR was then used to estimate the lesions of mtDNA.

Results Compared with the control and MHO groups, the levels of MetS related indicators, anthropological characteristics and 8-OHdG were higher, and the concentrations of CAT, GSH-Px, TF, TFR1 and genome-wide methylation were lower in the MUO group in a BMI-independent manner ($P<0.05$). Then, the contents of serum iron were lower in both the MHO and MUO groups than those in the control group ($P<0.017$). Moreover, they were positively related with the contents of serum CAT and GSH-Px, and negatively with 8-OHdG, TF and TFR1 ($P<0.05$). Furthermore, the methylation patterns on the TF, TFR1 and mtDNA were higher in the MUO group than those in the

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MHO and control groups ($P < 0.017$), which were negatively correlated with their serum contents ($P < 0.05$). Meanwhile, the ratio of methylated/unmethylated mtDNA was significantly associated with their mtDNA-CN and lesions ($P < 0.05$).

Conclusions Our findings suggested that the impairments on the epigenetic modifications of nuclear (genome-wide DNA, TF and TFR1) and mtDNA were associated with the disturbance of serum iron biomarkers to involve in the pathophysiology of MetS among the school-age MUO children.

Trial registration This study was approved by the Ethics Committee of Beijing Children's Hospital affiliated to Capital Medical University (No. IEC-C–006-A04-V.06), which was also registered at the website of http://www.chictr.org.cn/showproj.aspx?proj=4673 (No: ChiCTR-OCH–14004900).

Keywords Iron, Metabolic syndrome, Mitochondrial DNA, Methylation, Metabolically unhealthy obesity

Background

Childhood obesity, as an excessive adipose tissue accumulation, has become one of the public health problems to induce the adverse effects on the occurrences of several metabolic syndrome (MetS), namely abdominal obesity, arterial hypertension, dyslipidemia, insulin resistance, abnormal lipid and glucose metabolism and so on, in which over 35% obese children present at least one above MetS [1, 2]. Meanwhile, existing researches also confirm that there are differently significant impacts on children's health, which depends on whether the obesity participants have the MetS [1]. Thus, it should be given the primary importance to clearly identify the different types of obesity such as the metabolically healthy (MHO) and unhealthy obesity (MUO) to provide the related personalized prevention and treatment strategies [3]. Recently, most studies mainly focus on the roles of dietary macro-nutrients during the progression of MUO, such as the types and/or compositions of fats and carbohydrates. Otherwise, less is known about the related roles of micro-nutrients, including the iron [1–3].

As an essential element for almost every living organism, iron deficiency is still the most common disorder according to the data from the World Health Organization (WHO) [4]. There are multiple physiological functions on the development of obesity, in which it can be involved in the proliferation and differentiation of adipocytes, DNA synthesis and etc by acting as an important co-factor for the antioxidant enzymes like the catalase (CAT), cytochromes, hemoglobin, myoglobin and the other non-heme proteins [5, 6]. Meanwhile, iron can also lead to the formations of toxic oxygen free radicals through the Fenton's oxidative reaction to regulate the mitochondrial bio-genesis and dynamics [7]. Thus, appropriate iron intake is considerable to avoid the progressions of obesity, including the MHO and MUO. Recently, many findings highlight that the risks of iron deficiency are consistently increased among the obesity children in a BMI-dependent manner [8]. Otherwise, more detailed hierarchical analysis also demonstrated that the prevalence of iron deficiency was not increased along the process of obesity due to low-grade chronic inflammation [9]. Moreover, Suárez-Ortegón et al. also proved that there were significantly increased iron stores among the MetS population [10]. So it is still inconsistent of the correlations between serum iron and incidence of MHO/MUO. In this condition, our objective was to discuss the associations between serum iron biomarkers and occurrence of obesity among the MHO and MUO school-age children.

It is widely known that there are two different forms of serum iron on their transforming processes, as the ferrous (Fe^{2+}) and ferric iron (Fe^{3+}) [11–13]. Therefore, these disturbances of iron metabolism can generate a large amount of ROS to cause much damages on the nuclear and mitochondrial DNA (mtDNA) to result in a series of health problems, such as the MUO and MHO [14–16]. In this physiological process, many proteins are involved in the iron uptake, storage and efflux, such as the transferrin receptor 1 (TFR1), transferrin (TF) and etc [11–16]. Exactly, two Fe^{3+} atoms can bind with the TF to form the saturation states and transport to the cell surface, which is incorporated with the TFR1 mediated endocytosis. Then, the TFR1 could form a complex with the hemochromatosis proteins to serve as a component of iron sensing machinery [17, 18]. As mentioned, our previous researches already proved that there were disordered levels of methylation on the progression of obesity [19]. Wang Z et al. also confirmed that the results of present investigations unveiled the causality of iron overload on the acceleration of epigenetic clocks for the potential interventions including the TF, TFR1 and etc [20]. Moreover, the disorders of methylation and hydroxymethylation of mtDNA had also been found in the animal models and human tissues from the cancer, obesity, diabetes cardiovascular and neurodegenerative diseases and so on [21]. However, the relationships between these altered DNA methylation of iron-metabolism genes as the TF, TFR1 and mtDNA are still needed to be further verified, so we conducted this representative cross-sectional study to evaluate the epigenetic modifications of nuclear DNA and mtDNA, and then discuss their associations with the disturbance of iron biomarkers among the school-age MUO children.

Materials and methods

Subjects

A representative cross-sectional study was conducted to select 104 obesity school-age children as the experimental subjects by the classification systems of body mass index (BMI) basing on the age and gender from the Centers for Disease Control and Prevention (CDC), while 65 participants with the normal BMI were as the controls by matching the age, gender and etc [22]. All above subjects were carried out at the Department of Clinical Nutrition in the Beijing Children's Hospital from July 2019 to April 2022 according to the strict inclusion and exclusion criteria. Specifically, the including subjects were 7–12 year healthy school-age children without obvious endocrine, autoimmune and genetic metabolic diseases. Conversely, the participants were excluded if they were previously diagnosed as the genetic syndrome coexisting with obesity, Cushing's syndrome, other secondary causes of obesity, chronic somatic diseases (heart failure, encephalitis disseminate dementia and so on) and severe mental disorders. Meanwhile, they were also excluded with the lack of written informed consents from the participants and their guardians. Moreover, the subjects could be actively withdrew if they were loss of interests, follow-up records and the other unknown reasons.

This study was approved by the Ethics Committee of Beijing Children's Hospital affiliated to Capital Medical University (No. IEC-C–006-A04-V.06), which was also registered at the website of http://www.chictr.org.cn/showproj.aspx?proj=4673 (No. ChiCTR-OCH–14004900). Meanwhile, all the subjects and their guardians were granted the written informed consents after they were clearly informed the significance of this survey by the trained investigators.

Measurement of body compositions

The basic characteristics of the included subjects was collected by the trained pediatricians at the Department of Clinical Nutrition in the Beijing Children's Hospital through the face to face method. The individuals' height (Seca 213, Germany) and weight (H-key 350, see-higher, China) were recorded with three repeated measurements to determine the values of BMI, length for age Z score (LAZ), weight for age Z score (WAZ) and BMI for age Z score (BMIZ). Meanwhile, their body compositions including the body fat, muscle mass, visceral fat area (VFA) and waist hip ratio were obtained by the bio-electrical impedance analyses (BIA) using the whole-body impedance (H-key 350, Haikang, China) under the fasting state.

Collection of blood samples

The peripheral blood samples (about 2mL) were consequently obtained from each participant between 8 am and 9 am after the detection of body compositions by the trained professional researchers under the strict uniform protocols. Then, their serum samples were collected under the 3000r/min for 20min at the room temperature as soon as possible to divide into two EP pipes, in which one was left at the Department of Clinical Laboratory in the Beijing Children's Hospital to determine the concentrations of biochemical indicators, while the others were immediately preserved at -20 °C until use.

Determination of serum biological indicators

The serum biological indicators, such as triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), very low density lipoprotein cholesterol (VLDL-C), fasting blood glucose (FBG), alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were measured by the Coulter 5821 automatic biochemical analyzer (Beckman, USA). Then, the fasting insulin (FINS) was detected by the instruments of immunohistochemical chemiluminescence immunoassay (Siemens, Germany) and calculated the indicators of homeostasis model assessments for β-cells function (HOMA-β) and insulin resistance (HOMA-IR) to evaluate the functions of β-cells. Moreover, the serum glycosylated hemoglobin (HbA1c) was tested by the high-performance liquid chromatography (HPLC, Bio-Rad Variant II Turbo, USA).

Definition of MUO and MHO subgroups

Among the 104 obesity school-age children, the MHO ($n=60$) and MUO ($n=44$) subgroups were respectively defined according to the pediatric criteria for the components of MetS by the de Ferranti et al. [23], including the TG ≥ 110 mg/dL, HDL-C < 50 mg/dL, FBG ≥ 100 mg/dL, ALT ≥ 30U/L or AST ≥ 24U/L.

Evaluation of serum oxidative enzyme activity

The contents of serum superoxide dismutase (SOD) (Nanjing Jiancheng Bioengineering Institute, China, no. A001-3), CAT (mlbio. Co., Ltd, Shanghai, China, no.ml095171) and glutathione peroxidase (GSH-Px) (mlbio. Co., Ltd, Shanghai, China, no.ml09526) were evaluated using the commercial kits according to the manufacturer's instructions.

Determination of serum iron, TF and TFR1

The concentrations of serum iron were detected by the inductively coupled plasma mass spectrometer (ICP-MS), while the contents of serum TF (mlbio. Co., Ltd, Shanghai, China, no. ml092732) and TFR1 (mlbio. Co., Ltd, Shanghai, China, no. ml063113) were measured by the commercial assay kits according to the manufacturer's instructions.

DNA extraction and detection of 8-hydroxydeoxyguanosine (8-OHdG)

Within the strictly controlled workplace, DNA samples of all the enrolling subjects ($n = 169$) were extracted from the peripheral blood (approximately 200uL) using the TIANamp Blood Clot DNA kits (TIANGEN. Co., Ltd, China, no. DP335-02). The quantity of purified DNA was evaluated by the NanoDrop 2000 C (Thermo Fisher Scientific, CN). Then, the oxidized DNA injuries as 8-OHdG were measured using the Elisa kits (CUSABIO, China, no.CSB-E10140h). Exactly, the DNA samples were incubated with the diluted 8-OHdG antibody for 30 min at 37 °C, followed by washing three times and incubated with HRP-conjugated streptavidin for 30 min at 37 °C. Finally, the assay was stopped with the TMB and analyzed at 450 nm using the Micro-plate Reader.

Genome-wide methylation and hydroxymethylation

The DNA samples (about 100ng) were used to determine the levels of genome-wide methylation (MethylFlash Methylated DNA Quantification Kits, Epigentek Co. Ltd, USA, no. P-1034-96) and hydroxymethylation (MethylFlash Global DNA Hydroxymethylation (5-hmC) Elisa Easy Kit, Epigentek Co. Ltd, USA, no. P-1032-96) by the related Elisa kits according to the manufacturer' instructions.

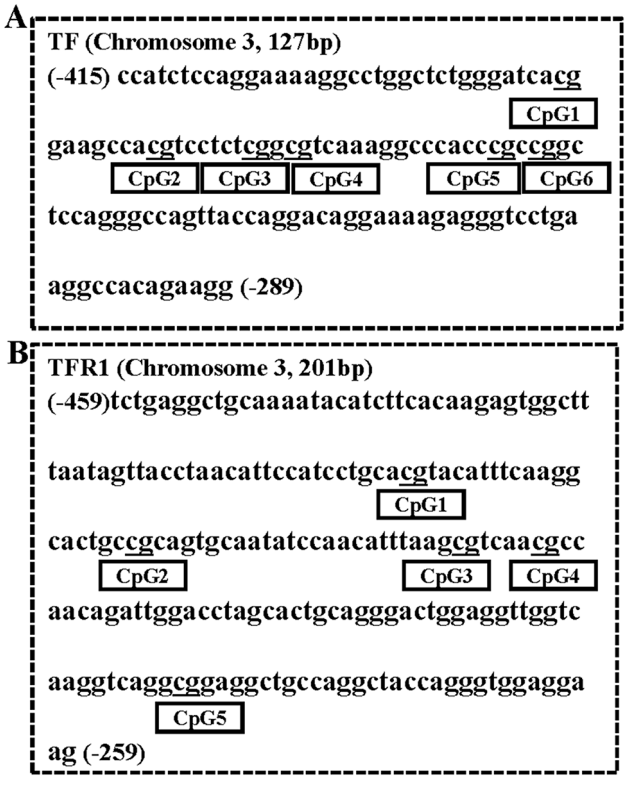

Fig. 1 CpGs sites and sequences in the TF and TFR1. Note: A: TF, B: TFR1. TF: transferrin, TFR1: transferrin receptor 1

Methylation patterns of CpG sites in the TF and TFR1

The DNA samples were bisulfite-converted to determine the methylation levels by the EZ DNA Methylation kits (Zymo Research, Irvine, CA, USA, no. D5002) according to the manufacturer' instructions. The target sequences of CpG islands in the TF (Forward: TTATTTTTAGGA AAAGGTTTGGTTTT, Reverse: CCTTCTATAACCTT CAAAACCCTCT) and TFR1 (Forward-out: TTTTTTT GTATTTTTGAGGTTGTAA AATAT, Forward-in: TTT GAGGTTGTAAAATATATTTTTATAAGA and Reverse: CTTCCTCCACCCTAATAACCTAAC) were obtained using the NCBI Gen-Bank Database (http://www.ncbi.nlm.nih.gov). Their meth-primers were designed basing on the sequences of target regions by the Meth-Primer (http://www.urogene.org/methprimer). Meanwhile, the spans CpG sites were shown in the Fig. 1. Then, the above bisulfite converted DNA samples were determined by the Beijing Genomics Institute (Sangon Biotech (Shanghai) Co., Ltd), in which the PCR reactions were as 95℃ for 10 min, (95℃ for 30 s, 55℃-30 s, 72℃-50 s) × 40 cycles and 72℃ for 5 min.

Evaluation of the contents and damages of mtDNA

The contents of mtDNA were determined using the quantitative real-time polymerase chain reaction (RT-PCR), which was measured by the ratio of MTF3212/R3319 (F: CACCCAAGAACAGGGTTTGT, R: TGGCC ATGGGTATGTTGTTAA) and ND1 (F: ATGGCCAAC CTCCTACTCCT, R: CTACAACGTTGGGGCCTTT), with the control gene as the RPLP0 (F: CAGCAAGTGG GAAGGTGTAATCC, R: CCCATT CTATCATCAACGG GTACAA).

The mtDNA lesions were employed by a semi-long run RT-PCR technique, in which the DNA lesions were on a template strand to block DNA polymerase and result in the decreased amplification of target sequences [24]. Only DNA templates without any DNA damages were amplified. Therefore, the numbers of mtDNA lesions per fragment were calculated basing on the Poisson distribution using the modified equation as Number of mtDNA lesions=-ln($2^{▲Ctshort-▲Ctlong}$). The primers in the above reactions were as ND1-long-F: ATGGCCAACCTCCTA CTCCT, ND1-long-R: GATGAGTGTGCCTGCAAAG A, ND1-short-F: CCTAA AACCCGCCACATCTA and ND1-short-R: GCCTAGGTTGAGGTTGACCA. Then, the above reactions were run in the duplicate and performed as 95℃ 3 min, (95℃ for 30 s, 65℃ for 30 s, 72℃ for 45 s) × 42 cycles and 72℃ for 5 min.

Detection of methylation levels of mtDNA

The methylation levels were determined by the ratios of estimated amounts for the methylated (F: TAGGAATTA AAGATAGATATTGCGA, R: ACTCTCCATACATTTA ATATTTTCGTC) and unmethylated mtDNA (F: GGTA

GGAATTAAAGATAGATATTGTGA, R: ACTCTCCAT ACATTTAATATTTTCATC) in the D-loop [24, 25].

Statistical analysis

The statistical analyses were performed using the SPSS 21.0. Exactly, the normality of data was assessed by the Kolmogorov-Smirnov-Goodness-of-Fit test, in which the continuous descriptive variables were expressed as Mean ± Standard error (SE) and categorical data was expressed as percentage (%) when they were followed the normal distributions. Otherwise, the inter-quartile range (IQR) was chosen to demonstrate the non-normal distributed variables. Besides, the log-transformation was used to determine the methylated/unmethylated mtDNA and contents of mtDNA, because they were not normally distributed. Then, the comparisons of continuous and categorical parameters were respectively performed using the multiple tests of One-way analysis of variance (ANOVA) and Chi-square tests, and the significant comparisons were conducted using the Bonferroni's test to compare the differences between every two groups. Meanwhile, the Mann-Whitney U non-parametric test was chosen to determine the differences among the different groups when the data was not normally distributed. Moreover, the coefficients of Spearman correlation were assessed the relationships between the methylation levels and their related enzymes, biological indicators, growth outcome and body compositions by adjusting the sex, age and the other confounding factors. Furthermore, the residual standard error and semi standardized coefficients were comprehensively evaluated the sensitivity of special values. A two-sided $P < 0.05$ was considered as the statistical significance, while the $P < 0.017$ was chosen as the significant differences under the Bonferroni's correction tests. Meanwhile, we assumed the means of DNA methylation levels (or mtDNA copy numbers) among the MHO, MUO, and control groups, along with the related standard deviation, type 1 error of 0.017, and with current sample size, we could ultimately choose more than 80% power to identify their mean differences.

Results

Basic characteristics of the participants among the different groups

The baseline characteristics of included participants in the different groups were summarized in the Table 1.

Table 1 Basic characteristics of the subjects in different groups

Variables	Control group ($n=65$)	MHO group ($n=60$)	MUO group ($n=44$)	$F/\chi^{2\$}$	*P*
Subjects Characteristics					
Age	10.37 ± 1.43	9.56 ± 1.75	9.32 ± 2.63	0.117	0.733
Gender (Boy, %)	38 (58.46)	35 (58.33)	27 (61.37)	0.119	0.942
Height (cm)	150.34 ± 9.98	153.32 ± 12.61	153.54 ± 12.01	0.073	0.942
Weight (kg)	54.02 ± 6.34	64.03 ± 7.53	70.57 ± 6.69*	4.251	0.033
BMI (kg/m^2)	23.68 ± 1.76	26.67 ± 2.57*	29.01 ± 2.35*	4.869	0.005
Z scores					
LAZ	1.29 ± 0.34	1.81 ± 1.17	2.14 ± 1.46	1.225	0.224
WAZ	1.49 ± 0.51	7.76 ± 3.31*	9.85 ± 3.64*	4.429	0.018
BMI Z	1.08 ± 0.024	2.06 ± 0.29*	2.32 ± 0.27*	5.406	< 0.001
Body composition					
Body fat (kg)	18.31 ± 2.54	25.15 ± 5.67*	28.91 ± 7.44*$^{\#}$	29.012	< 0.001
Muscle mass (kg)	37.05 ± 8.12	36.53 ± 10.52	39.19 ± 12.76	0.829	0.438
Body fat (%)	32.19 ± 3.36	39.21 ± 4.72*	40.77 ± 4.75*	61.460	< 0.001
Muscle mass (%)	64.01 ± 3.18	57.13 ± 4.45*	55.73 ± 4.49*	64.821	< 0.001
Visceral fat area (%)	101.09 ± 12.08	128.73 ± 17.74*	144.79 ± 26.13*$^{\#}$	3.746	0.045
Waist hip ratio	0.86 ± 0.047	0.89 ± 0.064*	0.91 ± 0.092*	4.709	0.011
Maternal characteristics					
Height (cm)	161.52 ± 4.39	160.33 ± 4.35	160.81 ± 4.87	0.161	0.689
Weight (kg)	64.07 ± 9.64	63.15 ± 10.36	62.12 ± 9.96	0.163	0.688
BMI (kg/m^2)	24.56 ± 3.19	24.56 ± 3.85	24.01 ± 3.54	0.358	0.552
Paternal characteristics					
Height (cm)	175.83 ± 4.57	172.82 ± 4.96	173.74 ± 6.23	2.294	0.061
Weight (kg)	77.73 ± 13.14	78.00 ± 11.87	82.47 ± 14.16	1.407	0.249
BMI (kg/m^2)	25.15 ± 3.98	26.07 ± 3.48	27.29 ± 4.35	3.032	0.052

Note$^{\$}$The percent of gender was shown as (%) and analyzed by χ^2 test among the three different groups, while the other variables were shown as mean ± standard deviation (SD) and analyzed by Bonferroni's correction test after the one-way analysis of variance (ANOVA). MHO: metabolically healthy obesity, MUO: metabolically unhealthy obesity, BMI: body mass index, LAZ: length for age Z scores, WAZ: weight for age Z scores, BMI Z: BMI for age Z scores. *Compared with the control group, $P < 0.017$, $^{\#}$Compared with the MHO group, $P < 0.017$

Table 2 Biochemical indicators among the subjects in different groups

Indicators	Control group (*n* = 65)	MHO group (*n* = 60)	MUO group (*n* = 44)	*P*
Glucose metabolism				
HbA1c (%)	5.02 ± 0.32	5.31 ± 0.27	5.48 ± 0.33*	0.047
Fasting glucose (mM)	4.55 ± 0.21	4.99 ± 0.47	4.95 ± 0.40	0.817
Fasting insulin (μlU/mL)	21.59 ± 5.15	27.91 ± 7.42	40.64 ± 8.21*#	0.008
HOMA-β	854.13 ± 145.24	706.72 ± 108.52	565.22 ± 112.05*#	< 0.001
HOMA-IR	5.09 ± 2.14	6.27 ± 2.33	9.12 ± 3.97*#	< 0.001
Lipid metabolism				
TG (mM)	1.03 ± 0.16	1.12 ± 0.28	1.36 ± 0.26*	0.043
TC (mM)	4.02 ± 0.34	4.23 ± 0.62	4.71 ± 0.59*	0.031
HDL-C (mM)	1.62 ± 0.13	1.32 ± 0.25	1.34 ± 0.37	0.542
LDL-C (mM)	2.12 ± 0.34	2.43 ± 0.47	2.84 ± 0.18*	0.032
VLDL-C (mM)	0.21 ± 0.0098	0.22 ± 0.13	0.27 ± 0.13	0.875
Liver function				
ALT (U/L)	16.25 ± 4.02	20.54 ± 3.78	52.46 ± 6.18*#	< 0.001
AST (U/L)	19.05 ± 2.06	22.27 ± 3.06	37.71 ± 5.07*#	< 0.001

Note All above variables were shown as mean ± standard deviation (SD) and analyzed by Bonferroni's correction test after the one-way analysis of variance (ANOVA). MHO: metabolically healthy obesity, MUO: metabolically unhealthy obesity, HbA1c: glycosylated hemoglobin, HOMA-β: homeostasis model assessment of β cell function, HOMA-IR: homeostasis model assessment of insulin resistance, TG: triglyceride, TC: total cholesterol, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol, VLDL-C: very low density lipoprotein cholesterol, ALT: alanine amino transferase, AST: aspartate transaminase. *Compared with the control group, $P < 0.017$, #Compared with the MHO group, $P < 0.017$

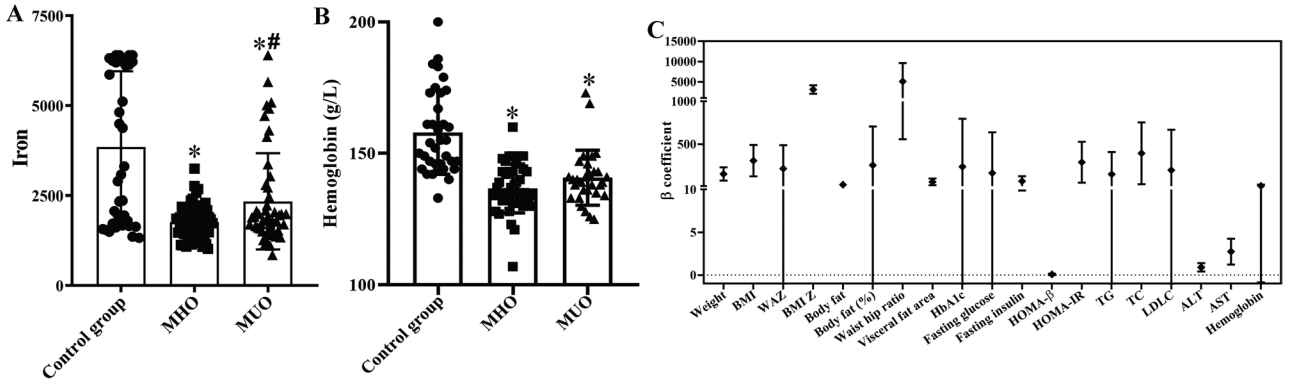

Fig. 2 Identification of serum iron biomarkers and their correlations with the metabolic related indicators. Note: A: Concentrations of iron, B: Concentrations of hemoglobin, C: β-coefficients and 95% CIs of the regression models between the contents of serum iron and metabolic related indicators. All related indicators were analyzed by the Bonferroni's correction tests after the One-way analysis of variance (ANOVA). *Compared with the control group, $P < 0.017$

Of the 169 individuals, 60 (35.50%), 44 (26.04%) and 65 (38.46%) subjects were respectively definted as the MHO, MUO and control groups. Among these three groups, the age, sex, height, LAZ, muscle mass, maternal (height, weight and BMI) and paternal characteristics (height, weight and BMI) were not significantly different ($P > 0.05$). Obviously, comparing with the control group (Tables 1 and 2), the weight, BMI, WAZ, BMIZ, body fat, waist hip ratio, VFA, hemoglobin, FINS, HOMA-β, HOMA-IR, TG, TC, LDL-C, ALT and AST were significantly higher in the MUO and MHO groups ($P < 0.017$), while the body fat and VFA were more serious in the MUO group than those in the MHO group ($P < 0.017$).

Oxidative indicators were related with the biomarkers of serum iron in the different groups

Comparing with the control group, the values for serum iron (Fig. 2A) and hemoglobin (Fig. 2B) were significantly lower in the MHO and MUO groups ($P < 0.017$). Moreover, they were much higher in the MUO than that in the MHO group ($P < 0.017$). Furthermore, there were positive associations between the biomarkers of serum iron and indicators of weight, BMI, BMIZ, WAZ, body compositions, glucose (HbA1c, FBG, FINS, HOMA-β and HOMA-1R) and lipid profile (TC, TG and LDL-C), and hepatic function (AST and ALT) (Fig. 2C, $P < 0.05$).

Obviously, the oxidative indicators (CAT and GSH-Px) were significantly lower (Fig. 3B) and contents of 8-OHdG (Fig. 3A) were higher in the MHO and MUO

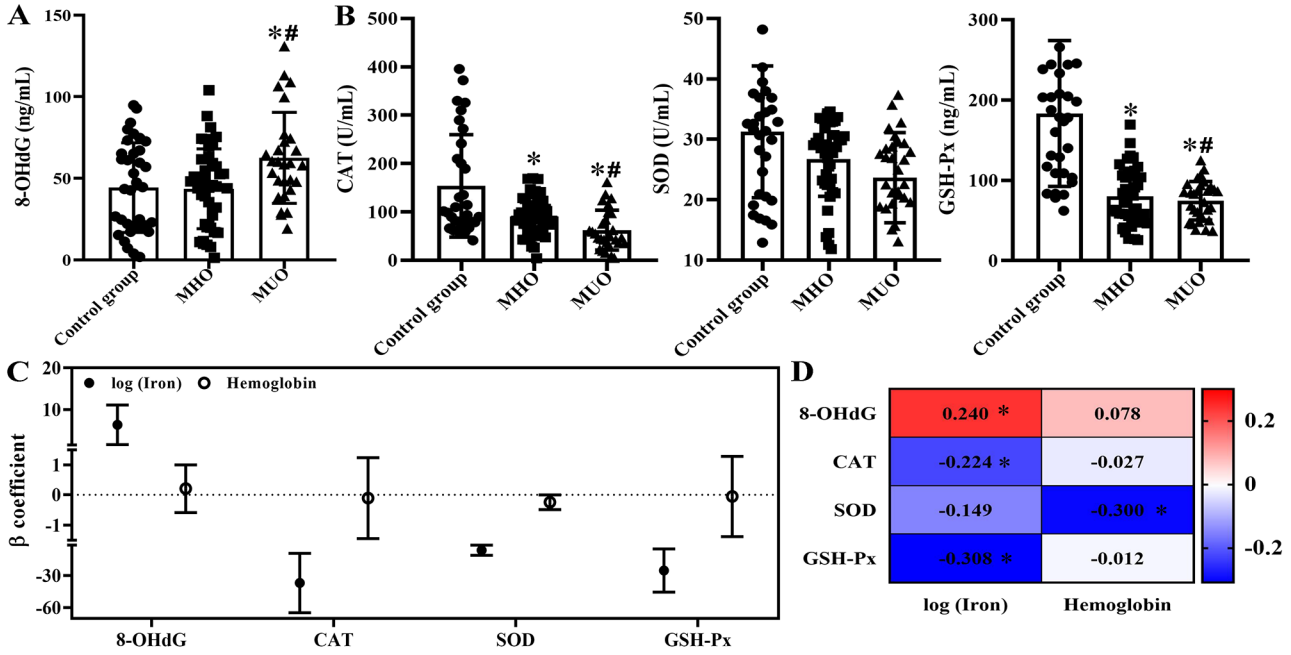

Fig. 3 Concentrations of oxidative indicators and their correlations with serum iron biomarkers. Note: **A**: 8-OHdG, **B**: CAT, SOD and GSH-Px, **C**: Correlations between the contents of serum iron/hemoglobin and related oxidative indicators; **D**: β-coefficients and 95% CIs of the above regression models. All indicators were analyzed by the Bonferroni's correction tests after the One-way analysis of variance (ANOVA). *Compared with the control group, $P < 0.017$, *It displayed that the regression equations were significantly different in the Figure 3D, $P < 0.05$. #Compared with the MHO group, $P < 0.017$

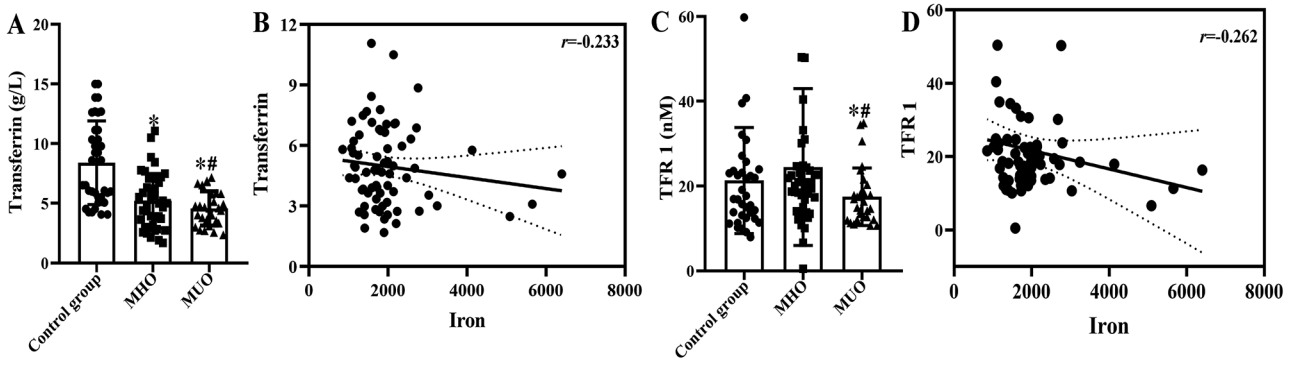

Fig. 4 Identification of significant iron related enzymes and their correlations with serum iron biomarkers in the different groups. Note: **A**: Contents of serum TF, **B**: Correlations between the contents of serum TF and iron, **C**: Contents of serum TFR1, **D**: Correlations between the contents of serum TFR1 and iron. TF: transferrin, TFR1: transferrin receptor 1. The concentrations of serum TF and TFR1 were analyzed by the Bonferroni's correction tests after the One-way analysis of variance (ANOVA). *Compared with the control group, $P < 0.017$, #Compared with the MHO group, $P < 0.017$

groups than those in the control group ($P < 0.017$). Otherwise, there were no significant differences on the contents of SOD. Further compositions demonstrated that the contents of CAT and GSH-Px were much lower and 8-OHdG were higher in the MUO group than those in the MHO group($P < 0.017$). Among those, the disorders of serum iron exhibited an upward trend with the contents of 8-OHdG ($r = 0.240$) and a downward with the increasing levels of CAT (r=-0.224) and GSH-Px (r=-0.308) (Fig. 3C and D, $P < 0.05$). Otherwise, the negative correlations were shown between the concentrations of hemoglobin and SOD ($P < 0.05$).

Identification of iron related enzymes and their correlations with the levels of serum iron in the different groups

Comparing with the control group, the concentrations of serum TF and TFR1 were lower in the MHO and MUO groups (Fig. 4A and C, $P < 0.017$). As expected, it had much significantly lower TF and TFR1 in the MUO group than those in the MHO group ($P < 0.017$). Then, the linear regression analysis was performed to assess the correlations between the contents of serum TF/TFR1 and iron, in which there were significantly negative correlations (TF: Fig. 4B, r=-0.233; TFR1: Fig. 4D, r=-0.262).

Methylation patterns on the wide-genome and CpG sites in the iron related genes among the participants in the different groups

To comprehensively assess the whole-genomic methylation in the Fig. 5A, the patterns of wide-genome methylation and hydroxymethylation were lower in the MHO and MUO groups than those in the control group ($P<0.017$). Then, we obtained a total of 6 CpG units in the TF (Fig. 5B) and 5 CpG units in the TFR1 (Fig. 5C) for further analysis. Comparing with the control group, the methylation levels in these above CpG sites were appeared higher in the MHO and/or MUO groups ($P<0.017$). Further comparisons between the MHO and MUO subgroups showed that the methylation levels on the CpG 1, CpG 2 and average of TF, and CpG 1 and CpG 5 of TFR1 were much higher, while the methylation contents of CpG 2 and CpG 3 in the TFR1 were lower in the MUO group than those in the MHO group ($P<0.017$). The above significant differences of these examined CpGs showed there were strong inter-correlations between the methylation levels and their contents (Fig. 5D and G), in which the CpG sites in the TF ($P<0.05$, Fig. 2H and J, CpG 1: r=-0.289, CpG 2: r=-0.283, CpG 3:: r=-0.258, CpG 4: r=-0.264, CpG 6: r=-0.252 and average: r=-0.301) and TFR1 ($P<0.05$, Fig. 2I and K, CpG 1: r=-0.306, CpG 3:: r=-0.262) were negatively correlated with their related enzymes.

Assessment of mtDNA-CN and damages of mtDNA among the different obesity subjects

As shown in the Fig. 6A and B, the significant increases of mtDNA-CN (ND1 and MTF3212/R3319) were shown in the MHO and MUO groups than those in the control group ($P<0.017$). Moreover, they were much higher in the MUO group than those in the MHO group ($P<0.017$). Otherwise, the significant differences were not found on the lesions of mtDNA among the control, MHO and MUO groups ($P>0.05$, Fig. 6C). However, these parameters were negatively correlated with

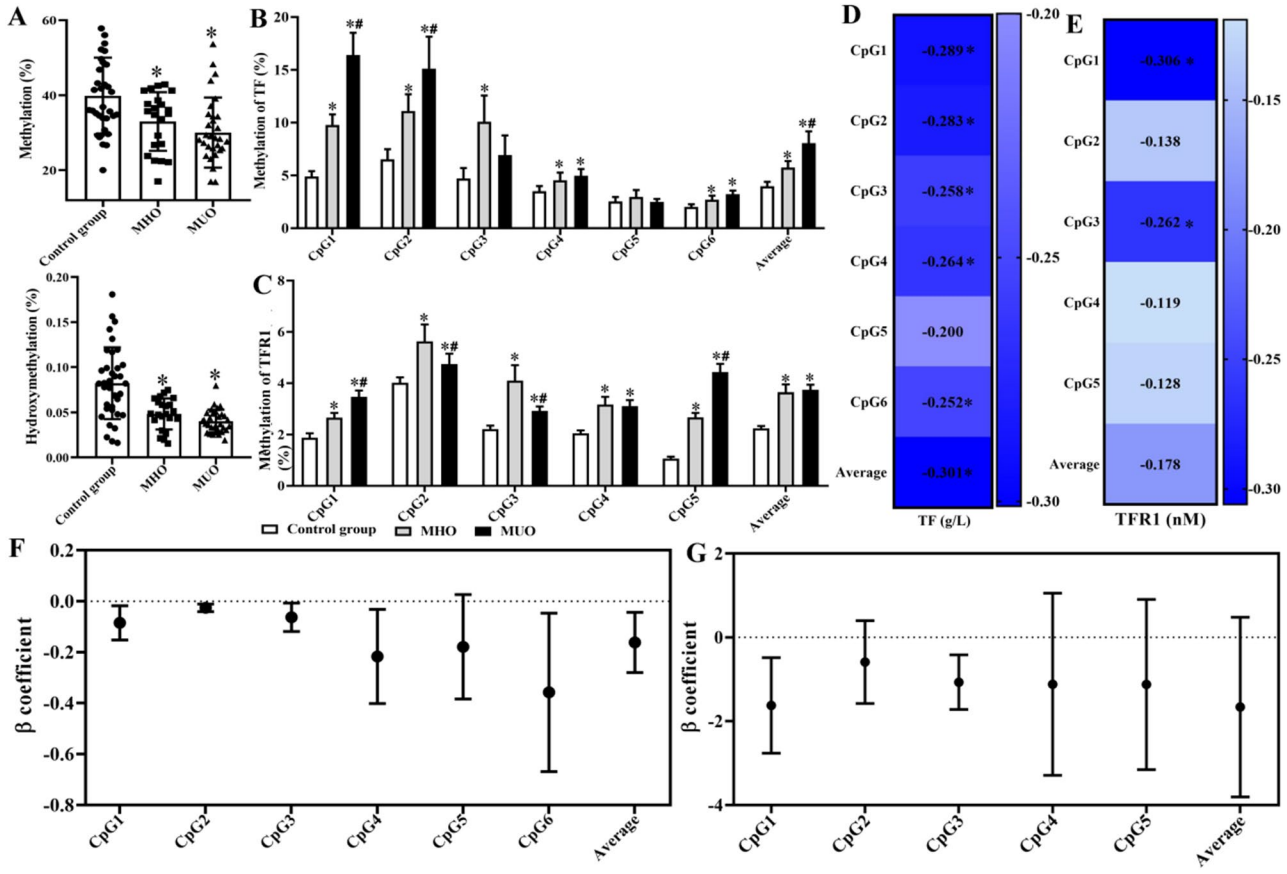

Fig. 5 Methylation patters on the genome-wide DNA, TF and TFR1, and their correlations with their expressions in the different obesity groups. Note: **A**: Methylation levels in the genome-wide DNA, **B**: Hydroxymethylation levels in the genome-wide DNA, **C**: Methylation levels of CpGs sites in the TF, **D**: Methylation levels of CpGs sites in the TFR1, **E** and **F**: Correlations between the methylation levels of different CpGs sites in the TF/TFR1 and their contents respectively. **G** and **H**: β-coefficients and 95% CIs of the above regression models. TF: transferrin, TFR1: transferrin receptor 1. All indicators were analyzed by the Bonferroni's correction tests after the One-way analysis of variance (ANOVA). *Compared with the control group, $P<0.017$. *It displayed that the regression equations were significantly different in the Fig. 5D, $P<0.05$. #Compared with the MHO group, $P<0.017$

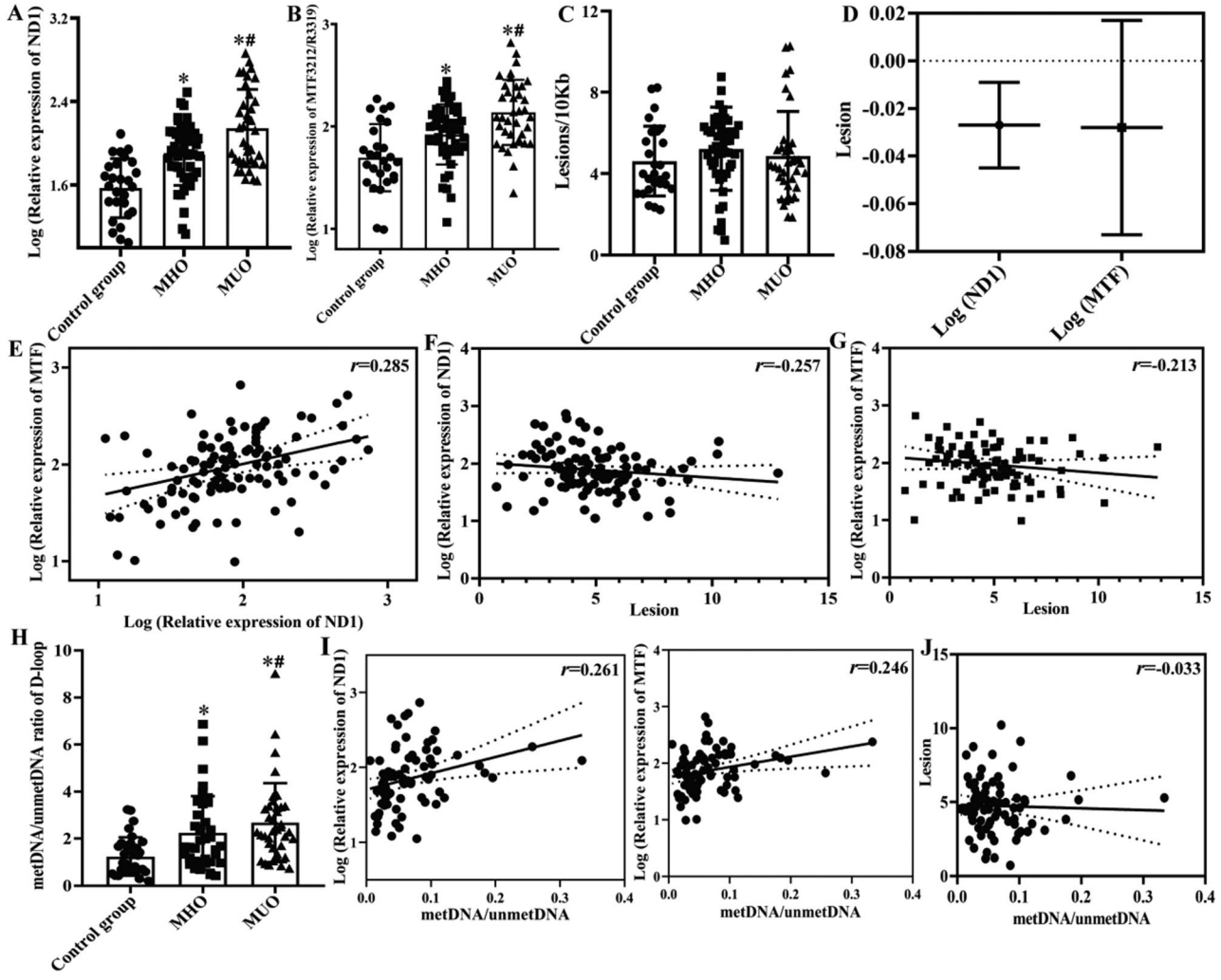

Fig. 6 Assessment of differential copy numbers, lesions and methylation levels of mtDNA among the different obesity groups. Note: **A**: Relative expressions of ND1, **B**: Relative expressions of MTF3212/R3319; **C**: mtDNA lesions; **D**; β-coefficients and 95% CIs of the regression models between the expressions of ND1/MTF3212/R3319 and mtDNA lesions; **E**: Correlations between the expressions of ND1 and MTF3212/R3319; **F**: Correlations between the expressions of ND1 and mtDNA lesions; **G**: Correlations between the expressions of MTF3212/R3319 and mtDNA lesions; **H**: Methylation levels in the D-loop region; **I**: Correlations between the methylation levels of mtDNA and expressions of ND1/MTF3212/R3319 and mtDNA lesions. All above indicators were analyzed by the Bonferroni's correction tests after the One-way analysis of variance (ANOVA). *Compared with the control group, $P < 0.017$, # Compared with the MHO group, $P < 0.017$

mtDNA-CN (ND1: r=-0.257 and MTF3212/R3319: r=-0.213) ($P < 0.05$, Fig. 6D and G).

Methylation in the D-loop region and their correlations with mtDNA-CN

Compared with the control group, both the MHO and MUO groups had a higher DNA methylation ratio in the D-loop region ($P < 0.017$). Then, further comparisons showed that the methylation in the D-loop region was much higher in the MUO group than that in the MHO group ($P < 0.017$, Fig. 6H). Meanwhile, the patterns of DNA methylation in the D-loop region were positively correlated with the mtDNA-CN ($P < 0.05$, Fig. 6I, ND1: $r = 0.270$ and MTF3212/R3319: $r = 0.259$). However, there was no significant correlation between the methylation in the D-loop region and mtDNA lesions ($P > 0.05$, Fig. 6J).

Discussions

As previously mentioned, there was lower iron absorption and transportation on the progression of obesity, which could be triggered through the increasing contents of hormone hepcidin and inflammation, and lower concentrations of serum TF and TFR1. Then, it could precisely reduce the contents of serum circulating iron [25]. Both Zhao and Del Giudice et al. proved that there were lower concentrations of serum iron and TF among the obesity population [26, 27]. Meanwhile, Manios et al. also found that there was a negative correlation between TF

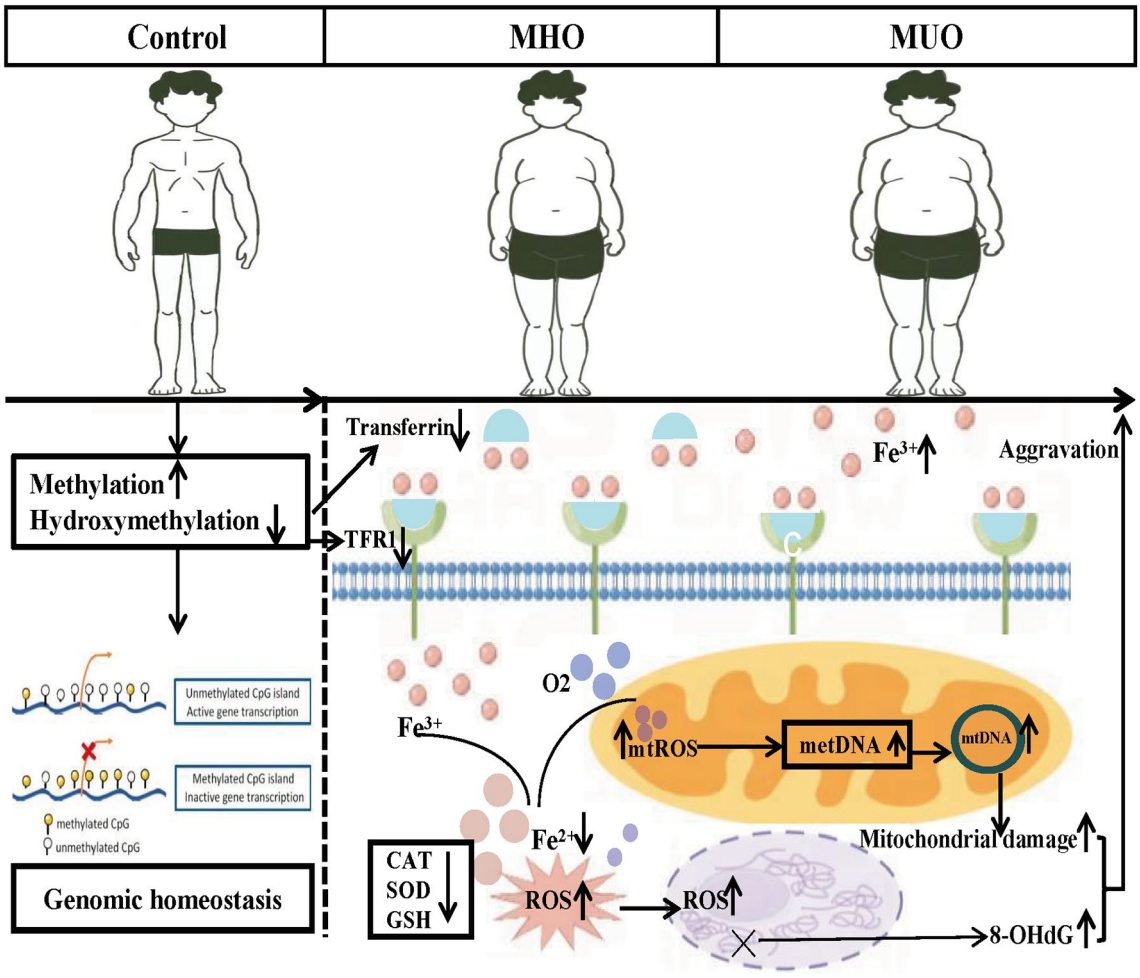

Fig. 7 Obesity related epigenetic modifications on the nuclear DNA and mtDNA could cause the disturbance of serum iron biomarkers to induce the occurrence of MetS. The MHO was adaptive with the abnormal epigenetic modifications of nuclear (whole-genome, TF and TFR1) and mtDNA to result in the decreases of iron related biomarkers in a BMI-independent manner. Then, it might conversely affect the oxidative stress to aggravate the occurrence of MetS with the increasing serum iron biomarkers

saturation and adiposity [28]. Otherwise, Sal et al. demonstrated there were no significant differences on the concentrations of serum ferritin and iron [29]. So the contents of serum iron were significantly various under the different degrees of obesity with the inconsistent conclusions [29]. Conversely, as the development of obesity with MetS especially the hepatic injury, it could be resulted in the disorder of ferritin releases into the bloodstream to increase the contents of serum iron, which was induced the oxidative environment to cause the occurrences of insulin resistance and necrotic signals among the obesity subjects to aggravate more severe hepatic damages. Besides, nearly one-third of MetS patients were suffered from the hyperferritinemia, as accompanying by the dysmetabolic iron overload syndrome [30]. These profound alterations on the energy homeostasis were closely linked to the distinct changes of iron homeostasis [31]. Our results in this study were in agreement with the above researches, in which the iron levels were lower in the MHO and MUO groups than those in the control group among the school-age children. Otherwise, comparing with the MHO group, it was higher in the MUO group. Moreover, there were positively correlations between the serum iron and concentrations of CAT and GSH-Px, while they were negatively correlated with the contents of ALT, AST, TF and TFR1 using the linear models independently of the age, sex and the other confounding factors ($P < 0.05$). Therefore, the disturbance of serum iron might present with two sides facing opposite directions with the obesity-related iron deficiency, in which the MHO was on one side and MUO was on the other.

Recently, there were many genes involving in the iron absorption and transportation, especially the TF and TFR1. They could be regulated by the genetic, hormonal, epigenetic and environmental factors, in which the epigenetic mechanisms could reversibly inhibit the gene expressions on the patters of DNA methylation [32–34].

Our study also indicated that the levels of genome-wide methylation and hydroxymethylation were lower in the MHO and MUO groups than those in the control group. Meanwhile, the hydroxymethylation levels were much lower on the progression of MUO. Moreover, comparing with the control group, the methyaltion levels in many CpG sites in the TF and TFR1 were appeared higher both in the MHO and/or MUO groups. Furthermore, the methylation levels on the CpG 1, CpG 2 and average in the TF and CpG 1 and CpG 5 in the TFR1 were much higher in the MUO group than those in the MHO group, while the other CpG units (CpG 2 and CpG 3 of the TFR1) were appeared with the lower methylation levels. Totally, the above significant differences of these examined CpGs showed they were strongly correlated with the expressions of related enzymes. Therefore, the occurrence of obesity as MHO could cause the disorder of whole genome methylation and lead a decrease on the gene homeostasis to affect the expressions of TF and TFR1, which then could exacerbate the occurrences of obesity with MetS as in the MUO group. Meanwhile, the disorders of Fe^{2+} and Fe^{3+}, as the co-factor of multitude enzymes, were resided both in the cytosol and mitochondrial matrix. Then, they could directly catalyze the free radical formation via the Fenton's oxidative reaction to mediate the lipid peroxide generation [32–36]. Thus, the iron import, export, storage, and turnover could have significant impacts on the glucose and lipid metabolisms. As a consequence, the iron metabolism mainly leads to the disorders of DNA damages as the 8-OHdG and mtDNA to further aggravate the hepatic damages among the obesity school-age children with MetS, which could be proved by our study. In our results, there were significant increases of 8-OHdG, mtDNA-CN and mtDNA methylation on the D-loop region in the MUO group than those in the MHO group, which were positively correlated with the indicators on the hepatic injury such as ALT and AST. Nevertheless, there were many limitations in this research. Firstly, the sample size was small, which could decrease our power to find the significant differences. Therefore, our conclusions still need to be further confirmed by many studies with much larger samples in the future. Secondly, the whole blood was used to study the DNA methylation and their associations with metabolic outcomes. This can not exclude the potential issues arising from the tissue specificity nature in the iron metabolism. Finally, it was lack of data on the gene expressions such as TF and TFR1 in this study due to the shortages of whole blood samples.

In summary (Fig. 7), our findings suggested that obesity could affect the impairments on the epigenetic modifications of nuclear DNA (such as whole-genome, TF, TFR1 and etc.) and mtDNA to cause the disturbance of serum irons in a BMI-independent manner, which might conversely affect the oxidative stress to aggravate the progression of MetS among the school-age obesity children. Moreover, it also emphasized the importance of individually clinical treatments with the iron dysfunction at the different obesity degrees.

Abbreviations

MetS	Metabolic Syndrome
mtDNA	mitochondrial DNA
mtDNA-CN	mtDNA Copy Numbers
MHO	Metabolically Health Obesity
MUO	Metabolically Unhealthy Obesity
CAT	Catalase
DMT1	Divalent Metal Transporter 1
TFR1	Transferrin Receptor 1
TF	Transferrin
FPN	Ferroportin
BMI	Body Mass Index
VFA	Visceral Fat Area
BIA	Bio-Electrical Impedance Analyses
UA	Uric Acid
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransaminase
TG	Triglyceride
TC	Total Cholesterol
HDL-C	High Density Lipoprotein Cholesterol
LDL-C	Low Density Lipoprotein Cholesterol
VLDL-C	Very Low Density Lipoprotein Cholesterol
FBG	Fasting Blood Glucose
FINS	Fasting Insulin
HbA1c	Hemoglobin
HOMA-β	Homeostasis Model Assessment for β Cell Function
HOMA-IR	Homeostasis Model Assessment for Insulin Resistance

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Author contributions
LLX, XL, YQL and PL were responsible for the study design and data accuracy. LLX and JY collected the biological samples. XYJ and WLY performed the statistical analysis. KMQ and PL revised the whole manuscript. All the authors reviewed and approved the final manuscript.

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Data availability
No datasets were generated and/or analyzed in the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Ethics Committee of Beijing Pediatric Research Institution, Beijing Children's Hospital affiliated to Capital Medical University, which was registered at the website as http://www.chictr.org.cn/showproj.aspx?proj=4673 (No: ChiCTR-OCH-14004900). The details of all subjects had been removed from their clinical case descriptions to ensure the anonymity. Meanwhile, the informed consents were obtained both from the participants and their guardians.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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