**NPPA Promoter Hypomethylation Predicts Central Obesity Development: A Prospective Longitudinal Study in Chinese Adults**

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**Keywords**
Atrial natriuretic peptide · Central obesity · DNA methylation

**Abstract**

**Introduction:** Atrial natriuretic peptide plays a potential role in obesity with unclear molecular mechanisms. The objective of this study was to examine the association between its coding gene (*natriuretic peptide A* [NPPA]) methylation and obesity. **Methods:** Peripheral blood DNA methylation of NPPA promoter was quantified at baseline by targeted bisulfite sequencing for 2,497 community members (mean aged 53 years, 38% men) in the Gusu cohort. Obesity was repeatedly assessed by body mass index (BMI) and waist circumference (WC) at baseline and follow-up examinations. The cross-sectional, longitudinal, and prospective associations between NPPA promoter methylation and obesity were examined. **Results:** Of the 9 CpG loci assayed, DNA methylation levels at 6 CpGs were significantly lower in participants with central obesity than those without (all \( p < 0.05 \) for permutation test). These CpG methylation levels at baseline were also inversely associated with dynamic changes in BMI or WC during follow-up (all \( p < 0.05 \) for permutation test). After an average 4 years of follow-up, hypermethylation at the 6 CpGs (CpG2 located at Chr1:11908348, CpG3 located at Chr1:11908299, CpG4 located at Chr1:11908200, CpG5 located at Chr1:11908182, CpG6 located at Chr1:11908178, and CpG8 located at Chr1:11908165) was significantly associated with a lower risk of incident central obesity (all \( p < 0.05 \) for permutation test). **Conclusions:** Hypomethylation at NPPA promoter was associated with increased future risk of central obesity in Chinese adults. Aberrant DNA methylation of the NPPA gene may participate in the mechanisms of central obesity.

**Introduction**

Obesity, a leading but modifiable risk factor for metabolic and cardiovascular complications, has become a global public health crisis [1–4]. It still affects about 46%...
of adults and 15% of children in China now despite decades of control efforts made, indicating unclear complicated mechanisms of this debilitating disorder [4]. Fundamentally, obesity reflects the imbalance between energy intake and expenditure. Atrial natriuretic peptide (ANP) has been suggested to play a potential role in maintaining weight through enhancing lipolysis, lipid mobilization, oxidation, and inhibiting appetite [5–10]. Bodyweight was significantly increased in ANP knockout mice [11]. In humans, the circulating level of ANP and the polymorphisms of its coding gene – natriuretic peptide A (NPPA) have been associated with obesity [12–15], and its related complications, e.g., hypertension [16, 17], atherosclerosis [18], diabetes [19, 20], and eating disorders [21]. However, the clinical implications of ANP are still limited [22]. A better understanding of the underlying molecular mechanisms of the energy-regulating effect of ANP may promote its clinical translation. DNA methylation, an important epigenetic modification that links the environment to the fixed genome and regulates gene function and expression [23], may provide some insights into the mechanisms that we are searching for. In fact, DNA methylation status changed dramatically in our lifetime and dysregulated DNA methylation was previously related to obesity in epigenome-wide association studies (EWAS) [24–28]. Although no genome-widely significant CpG locus was found, NPPA methylation was found to be associated at gene level with obesity in an EWAS [28]. However, no candidate gene study, to the best of our knowledge, was conducted to examine NPPA methylation and obesity. Therefore, we aimed to examine the association, cross-sectional, longitudinal, and prospective, between NPPA promoter methylation and obesity in a systematic manner, leveraging a prospective longitudinal cohort of middle-aged and elderly Chinese adults. The prospective analysis may guide causal inference for the role of NPPA promoter methylation in obesity.

Methods

Study Participants

The Gusu cohort, initiated in 2010, is a community-based prospective longitudinal study of cardiovascular disease and its risk factors in middle-aged and elderly Chinese adults as described previously [29]. In brief, via cluster random sampling, 8 communities were selected from the total 39 communities in the Gusu District, Suzhou, China. In the selected communities, residents who met the inclusion criteria (i) age: ≥30 years, (ii) ethnicity: Han were recruited and invited to participate in the baseline examination. The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The protocols were approved by the Soochow University Ethics Committee (approval NO. SUDA20100601H02). Written informed consent was obtained from all study participants. Figure 1 describes the selection of study participants included in the current analysis. Of 2,706 individuals participating in the baseline examination, after excluding...
participants with missing data on NPPA methylation (n = 208) and
waist circumference (WC) (n = 1), a total of 2,497 participants with
complete data on NPPA methylation, WC, and body mass index
(BMI) included in the cross-sectional analysis. All surviving par-
ticipants were invited to participate in the follow-up examination
4 years later. After further excluding participants who denied to
participate (n = 175) or not received anthropometry (n = 241) in
the follow-up examination conducted in 2014, 2,081 participants
were included in the longitudinal analysis. Of these, participants
free of obesity (n = 1,778) were included in the prospective analy-
sis for the association between NPPA methylation and risk of obe-
sity during follow-up and those free of central obesity (n = 977) at
baseline were included in the prospective analysis for the associa-
tion between NPPA methylation and risk of central obesity during
follow-up.

Quantification of NPPA Promoter Methylation
DNA methylation levels in the promoter region of the NPPA
gene were quantified by target bisulfite sequencing as previously
described [30], using genomic DNA isolated from peripheral
blood mononuclear cells. In brief, based on the genomic coordi-
nates of the NPPA promoter in Genome Reference Consortium
Human Build 37 (GRCh37), we carefully designed the primers to
detect the maximum CpG loci within the CpG islands. The tar-
geted sequence (Chr1:11908117–11908380, reverse strand, rela-
tive to TSS: −540 bp to −277 bp) was illustrated previously [31].
Following primer validation, genomic DNA was bisulfite-treated
using the EZ DNA Methylation-Gold Kit (Zymo Research, Inc.,
Irvine, CA, USA) according to the manufacturer’s protocol, which
converts unmethylated cytosine into uracil and leaves methylated
cytosine unchanged. The treated samples were amplified, barcod-
ed, and sequenced by Illumina Hiseq 2000 (Illumina, Inc., San Di-
egego, CA, USA) using the paired-end sequencing protocol accord-
ing to the manufacturer’s guidelines. Methylation level at each
CpG dinucleotides was calculated as the percentage of the methyl-
ated alleles over the sum of methylated and unmethylated alleles.
For quality control, the samples with bisulfite conversion rate
<98% and the cytosine sites with average coverage less than 20×
were filtered out. DNA methylation levels were finally quantified
at 9 CpG loci in the NPPA promoter. The average of the 9 CpG
sites was used to represent the methylation level of the targeted
region.

Follow-Up Examination and Definition of Obesity/Central
Obesity
Bodyweight (kg) and height (cm) were measured when par-
ticipants wore light clothes and no shoes by trained staff at both
baseline and follow-up examinations. BMI was calculated by di-
viding weight in kilograms by the square of height in meters (kg/
\( m^2 \)). WC was measured at the level of 1 cm above the umbilicus.
According to the recommendations of the Working Group on
Obesity in China [32], obesity was defined as BMI ≥28 kg/m² and
central obesity was defined as WC ≥85 cm for men and ≥80 cm for
women. Incidences of obesity and central obesity were defined as

Fig. 2. A flowchart illustrating the statistical plan.
participants who were free of obesity and central obesity, respectively, at baseline but had obesity or central obesity at the follow-up examination.

Assessment of Confounding Variables

Data on sociodemographic, lifestyles, and metabolic factors were repeated measured at baseline and follow-up examinations. Demographic data including age, sex, and education level were obtained by questionnaires administered by trained staff. The education level was recorded as years a person stays in the education system and the highest level of educational quantifications they hold. Cigarette smoking was classified as current, former, and never smoking. Current smoking was defined as having smoked at least 100 cigarettes in the subject’s entire life, having smoked cigarettes regularly, and smoking currently. Former smoking was defined as having smoked at least 100 cigarettes in the subject’s entire life, having smoked cigarettes regularly in the past, and not smoking currently. Never smoking was defined as never smoked or having smoked fewer than 100 cigarettes in their lifetime by the end of examination. Alcohol consumption was classified as current drinkers or not. The current drinkers are those who had drunk any alcoholic beverages more than 12 times in the past year. According to the standard protocol, 3 consecutive sitting blood pressure measurements (30 s between each) were taken by trained staff using a standard mercury sphygmomanometer and a cuff of the appropriate size [33], after the participant had been resting for at least 5 min in a relaxed sitting position. All participants were required to have avoided exercise, drinking, smoking, and tea for at least 30 min before the measurement. The first and fifth Korotkoff sounds were recorded as systolic blood pressure and diastolic blood pressure, respectively. All participants completed 3 blood pressure measurements and the mean of the 3 measurements was used in statistical analyses. According to the Chinese guidelines for the management of hypertension, participants with a systolic blood pressure ≥140 mm Hg and/or a diastolic blood pressure ≥90 mm Hg or under antihypertensive treatment in the last 2 weeks were diagnosed with hypertension [34]. Fasting glucose was measured by standard laboratory methods [29]. Diabetes was defined as fasting glucose ≥7.0 mmol/L or use of hypoglycemic medication in the last 2 weeks [35].

Statistical Analysis

Baseline characteristics of study participants were presented according to obesity and central obesity, respectively. Group differences in these characteristics were compared using a Student’s t test, Mann-Whitney-U test, or the χ² test as appropriate. To examine whether potential selection bias existed, we compared the baseline characteristics between participants excluded and included in the analysis. To carefully examine the association between NPPA promoter methylation and obesity in a systemic manner, as illustrated in Figure 2, we tested the cross-sectional, longitudinal, and prospective associations of NPPA promoter methylation with obesity and central obesity, respectively. Complete case analysis was used to deal with missing data. Multiple testing was controlled by applying a permutation test which has been widely used in correction for multiple hypotheses [36]. The p value generated from the permutation test of less than 0.05 was considered statistically significant. In addition to single CpG associations, the joint association of multiple CpG sites with obesity was also examined by using gene-based analysis approach which was typically applied in genetic and epigenetic studies to test the joint effect of multiple CpGs or SNPs on a complex phenotype. All analyses were conducted using R version 4.0.2.

Cross-Sectional Analysis

As the effect of DNA methylation at a single CpG site could be very small, testing the individual effect of a single CpG site on a complex trait such as obesity may not be efficient. Therefore, we examined both the single CpG and gene-based associations of the 9 CpGs with obesity. The average methylation level of the 9 CpG sites was used as a surrogate for the methylation level of the targeted region. The mean methylation level was presented and compared using t test between participants with and without obesity/central obesity. To examine the association between single CpG methylation and obesity, we constructed a linear regression model in which WC and BMI was the dependent variable individually and DNA methylation level at each CpG locus was the independent variable, adjusting for age, sex, education level, cigarette smoking, and alcohol consumption. To examine the gene-based association between DNA methylation at multiple CpG sites at NPPA promoter and obesity, we constructed a similar regression model with the average methylation level as the independent variable. We also employed the weighted truncated product method (wTPM) as previously described [37] to combine p values of all CpGs that reaches a preselected threshold (raw p < 0.1 in this study). The regression coefficient of each individual CpG was included as weight in the wTPM statistics. This method has been evaluated by simulation studies [38] and applied to epigenetic analysis [39]. To facilitate data interpretation, we similarly constructed a logistic regression model with prevalent central obesity and obesity as the dependent variable individually to examine whether NPPA promoter methylation was associated with risks of prevalent obesity/central obesity. BMI was additionally adjusted for in the analyses for the association between NPPA promoter methylation and WC/central obesity.

Longitudinal Analysis

We further examined whether NPPA promoter methylation at baseline could predict the dynamic and the change in WC and BMI during follow-up. In specific, to further examine the associations of NPPA promoter methylation with dynamic WC and BMI during follow-up, we constructed a linear mixed regression model with a random intercept in which repeated measures of WC and BMI were the dependent variables individually (at baseline and follow-up), single CpG methylation at baseline was the independent variable, adjusting for age, sex, education level, cigarette smoking, current drinking at baseline and follow-up examinations, and follow-up time, with participants as the random effect. The mixed model was used here to account for the repeated measurements of WC and BMI in both examinations and performed using the R package “lme4” [40]. To examine the associations of NPPA promoter methylation with the changes in WC and BMI during follow-up, we constructed a linear regression model in which WC and BMI change (follow-up minus baseline) was the dependent variable individually, single CpG methylation was the independent variable, adjusting for abovementioned baseline covariates. The gene-based association of NPPA promoter methylation with dynamic WC and BMI was similarly examined. BMI was additionally adjusted for in the analyses for the associations between NPPA promoter methylation and dynamic or changes in WC.
Table 1. Baseline characteristics of study participants according to exhibition of obesity and central obesity, respectively

| Characteristics                        | N          | Central obesity | p value | Obesity | p value |
|----------------------------------------|------------|-----------------|---------|---------|---------|
|                                        |            | without | with    |         | without | with    |
| Participants, n                        | 2,497      | 1,174   | 1,323   | <2.20e−16 | 2,126   | 371     |
| Age, years                             | 2,497      | 51 (43–57) | 55 (48–60) | <2.20e−16 | 53 (43–59) | 56 (47–60) | 3.19e−04 |
| Sex, men (%)                           | 2,497      | 433 (36.88) | 528 (39.91) | 1.13e–01 | 791 (37.21) | 170 (45.82) | 2.01e−03 |
| Education, n (%)                       | 2,485      |          |         |         |         |
| Elementary school or below             | 403 (16.21) | 671 (27.00) | 1.13e−01 | 3891 (35.86) | 183 (7.36) | 4.87e−02 |
| Middle school                          | 498 (20.04) | 407 (6.38) |          | 114 (4.59) |
| High school                            | 203 (8.17) | 177 (7.12) |          | 56 (2.25) |
| Bachelor’s degree or above             | 66 (2.66)  | 60 (2.41)   |          | 17 (0.68) |
| Smoking                                | 2,497      | 274 (23.34) | 307 (23.20) | 3.71e−01 | 486 (22.86) | 95 (25.61) | 3.59e−01 |
| Current smoking, n (%)                 | 2,497      | 201 (17.12) | 263 (19.88) | 8.60e−02 | 384 (18.06) | 80 (21.56) | 1.27e−01 |
| Former smoking, n (%)                  | 2,497      | 31 (2.64)   | 48 (3.63) |          | 65 (3.06) | 14 (3.77) | – |
| Current drinking, n (%)                | 2,497      | 75.5 (71.2–79.0) | 88.0 (84.3–93.0) | <2.20e−16 | 81.0 (75.0–86.0) | 94.0 (89.5–98.0) | <2.20e−16 |
| WC, cm                                 | 2,497      | 22.58 (21.25–24.14) | 26.28 (24.68–28.13) | <2.20e−16 | 23.94 (22.09–25.63) | 29.56 (28.70–30.88) | <2.20e−16 |
| Diabetes, n (%)                        | 2,497      | 70 (5.96)   | 147 (11.11) | 7.22e−06 | 174 (8.18) | 43 (11.59) | 4.05e−02 |
| Hypertension, n (%)                    | 2,497      | 375 (31.94) | 733 (55.40) | <2.20e−16 | 881 (41.44) | 227 (61.19) | 2.43e−12 |
| SBP, mm Hg                             | 2,497      | 124.0 (116.0–136.0) | 131.3 (122.0–142.0) | <2.20e−16 | 128.0 (118.0–138.7) | 132.0 (124.0–144.0) | 3.06e−10 |
| DBP, mm Hg                             | 2,497      | 82.0 (78.0–88.0) | 86.0 (80.0–92.2) | <2.20e−16 | 84.0 (80.0–90.0) | 86.0 (82.0–93.3) | 7.05e−15 |
| Antihypertensive medication, n (%)     | 2,497      | 177 (15.08) | 445 (34.39) | <2.20e−16 | 462 (21.73) | 160 (43.13) | <2.20e−16 |
| Fasting glucose, mmol/L                | 2,497      | 5.00 (4.60–5.50) | 5.30 (4.80–5.80) | <2.20e−16 | 5.10 (4.70–5.60) | 5.40 (4.90–6.00) | 5.40e−11 |

Data are presented as the medians and first and third quartile (Q1–Q3) or n (%). p values indicate the differences between 2 groups performed by Mann-Whitney U test and χ² test as appropriate. There are 12 missing values in the variable of the education level. SBP, systolic blood pressure; DBP, diastolic blood pressure.
Prospective Analysis

In participants free of obesity or central obesity at baseline, we further examined whether NPPA promoter methylation at baseline could predict future risk of obesity or central obesity, respectively, by constructing a logistic regression model in which incident obesity/central obesity (yes/no) was the dependent variable and single CpG methylation was the independent variable, adjusting for follow-up time and abovementioned baseline covariates. The gene-based associations of NPPA promoter methylation with future risk of central obesity and obesity were similarly examined. BMI was additionally adjusted for in the analyses for the associations between NPPA promoter methylation and incident central obesity.

Sensitivity Analysis

To examine whether diabetes and hypertension affect our results, we conducted sensitivity analyses by excluding participants with prevalent diabetes or hypertension at baseline.

Results

Baseline Characteristics of Study Participants

A total of 2,497 participants (median aged 54 [Q1–Q3: 45–59] years, 38.49% of men) were included in the current study. Of them, 371 (14.85%) and 1,323 (52.98%) participants have been diagnosed with obesity and central obesity, respectively. Their clinical characteristics are shown in Table 1. Participants with obesity/central obesity, as expected, were more likely to be older, have lower educational level, and have prevalent diabetes and hypertension than those without (p < 0.05). No significant difference in other listed variables was found. Some variables, for example, age and prevalent diabetes seemed to be different between participants included and excluded (online suppl. Table S1; for all online suppl. materials, see www.karger.com/doi/10.1159/000521295). Although the main exclusion is missing data on NPPA methylation, selection bias cannot be prevented.

Cross-Sectional Associations between NPPA Methylation and Obesity/Central Obesity

Of the 9 CpG sites assayed, DNA methylation levels at 6 and 4 CpG loci were significantly lower in participants with central obesity and obesity, respectively, than those without (p < 0.05, Table 2; Fig. 3). Of them, 6 CpG loci remained to be inversely associated with WC or BMI after multivariable adjustment and correction for multiple testing (all p < 0.05 for permutation test). The average methylation level of all CpGs was significantly associated with lower WC (β = −0.28, 95% confidence interval [CI]: −0.54 to 0.02, p = 3.61 × 10^-22). The results of the wTPM showed that hypermethylation of the 9 CpGs at the NPPA promoter was negatively correlated with WC.

Table 2. The levels of NPPA promoter methylation in participants with and without central obesity/obesity

| CpG loci | Genomic position, GRCh37 | Relative to TSS, bp | Central obesity | Obesity | p value | permutation test p value | p value | permutation test p value |
|----------|--------------------------|-------------------|----------------|---------|----------|--------------------------|----------|--------------------------|
| CpG1     | Chr1:11908353            | −513              | 28.99 ± 5.21   | 28.71 ± 5.40 | 7.21 × 10^-5 | 7.19 × 10^-5 | 28.67 ± 5.22   | 27.89 ± 5.17 | 7.76 × 10^-3 | 8.01 × 10^-3 |
| CpG2     | Chr1:11908348            | −508              | 93.30 ± 2.43   | 93.12 ± 2.60 | 1.12 × 10^-2 | 1.15 × 10^-2 | 93.22 ± 2.48   | 92.84 ± 2.76 | 1.18 × 10^-2 | 6.48 × 10^-3 |
| CpG3     | Chr1:11908299            | −459              | 22.97 ± 3.01   | 22.73 ± 3.86 | 1.18 × 10^-2 | 1.18 × 10^-2 | 22.85 ± 3.02   | 22.75 ± 3.96 | 6.46 × 10^-1 | 6.38 × 10^-1 |
| CpG4     | Chr1:11908200            | −360              | 68.67 ± 6.37   | 67.94 ± 6.57 | 4.85 × 10^-3 | 4.93 × 10^-3 | 68.41 ± 6.36   | 67.54 ± 7.12 | 2.79 × 10^-2 | 1.71 × 10^-2 |
| CpG5     | Chr1:11908182            | −342              | 81.97 ± 4.80   | 81.41 ± 4.97 | 4.34 × 10^-3 | 4.42 × 10^-3 | 81.78 ± 4.79   | 81.08 ± 5.41 | 1.88 × 10^-2 | 8.66 × 10^-2 |
| CpG6     | Chr1:11908178            | −338              | 40.45 ± 6.06   | 39.47 ± 6.16 | 3.22 × 10^-3 | 3.25 × 10^-3 | 40.15 ± 6.08   | 39.56 ± 6.35 | 9.70 × 10^-2 | 1.03 × 10^-2 |
| CpG7     | Chr1:11908168            | −328              | 50.61 ± 6.39   | 50.41 ± 6.47 | 1.95 × 10^-3 | 1.96 × 10^-3 | 50.39 ± 6.48   | 49.75 ± 6.63 | 8.66 × 10^-2 | 7.87 × 10^-2 |
| CpG8     | Chr1:11908165            | −325              | 30.92 ± 6.45   | 30.47 ± 6.38 | 8.30 × 10^-3 | 8.27 × 10^-3 | 30.72 ± 6.40   | 30.45 ± 6.49 | 4.69 × 10^-1 | 4.65 × 10^-1 |
| CpG9     | Chr1:11908142            | −302              | 36.07 ± 7.77   | 36.41 ± 7.72 | 4.09 × 10^-3 | 4.09 × 10^-3 | 36.54 ± 7.67   | 36.47 ± 8.15 | 8.75 × 10^-3 | 8.70 × 10^-3 |
| Average* |                     |                   | 50.50 ± 4.74   | 49.99 ± 4.82 | 7.14 × 10^-3 | 7.20 × 10^-3 | 50.30 ± 4.74   | 49.81 ± 5.07 | 8.37 × 10^-2 | 6.93 × 10^-2 |

Methylation level at each CpG site was calculated as the percentage of the methylated alleles over the sum of methylated and unmethylated alleles. GRCh37, Genome Reference Consortium Human Build 37. *The average of the methylation level at the 9 CpG sites was used to represent the methylation level of the targeted region.
promoter as a whole was significantly associated with WC, BMI, and prevalent obesity (all \( p < 0.05 \), Table 3). The results of all covariates from the complete models for the 9 CpGs and average methylation level are presented in the online supplementary Tables S2–S11.

**Longitudinal Association between Baseline NPPA Promoter Methylation and Dynamic WC and BMI during Follow-Up**

After a median of 4 years of follow-up, more participants, as expected, suffered from hypertension and diabetes, but had lower WC (online suppl. Table S12). However, the longitudinal analysis found similar negative associations to the cross-sectional analysis (Table 4). After adjustment for covariates and multiple testing, hypermethylation at 8 CpG loci was significantly associated with lower levels of either WC or BMI (all \( p < 0.05 \) for permutation test). The average methylation level of all CpGs was also significantly associated with lower levels of WC (\( \beta = -0.25, 95\% \text{ CI}: -0.46 \text{ to } 0.04, p = 2.09e-02 \)) and BMI (\( \beta = -0.22, 95\% \text{ CI}: -0.36 \text{ to } 0.07, p = 2.94e-03 \)). The results of the wTPM uncovered similar gene-based association (all \( p < 0.05 \)). The results of all covariates from the complete models for the 9 CpGs and average methylation level are presented in the online supplementary Tables S13–S22.

**Prospective Associations between Baseline NPPA Promoter Methylation and Obesity/Central Obesity**

Of the 977 participants free of central obesity at baseline, 195 participants developed to central obesity after an average 4 years of follow-up. After multivariable adjust-

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**Fig. 3.** A box plot showing the levels of NPPA promoter methylation in participants with and without obesity/central obesity.
Table 3. The cross-sectional associations of NPPA promoter methylation with obesity/central obesity

| CpG loci | Genomic position, GRCh37 | Relative to TSS, bp | Baseline WC β (95% CI) | p value | permutation test p value | Prevalent central obesity OR (95% CI) | p value | permutation test p value | Baseline BMI β (95% CI) | p value | permutation test p value | Prevalent obesity OR (95% CI) | p value | permutation test p value |
|----------|--------------------------|---------------------|------------------------|---------|-------------------------|--------------------------------------|---------|--------------------------|--------------------------|---------|--------------------------|--------------------------------|---------|--------------------------|
| Single CpG association | | | | | | | | | | | | | | |
| CpG1     | Chr1:11908333 −513       | −0.18 (-0.42 to 0.06) | 1.41e−01               | 8.42e−02 | 0.92 (0.83−1.01)       | 9.16e−02               | 8.90e−02 | −0.20 (−0.33 to 0.06) | 4.59e−03               | <2.00e−16 | 0.88 (0.79−0.98)       | 2.24e−02               | 2.60e−02 |
| CpG2     | Chr1:11908348 −508       | −0.58 (-1.08 to 0.09) | 2.09e−02               | 2.21e−02 | 0.86 (0.71−1.05)       | 1.36e−01               | 1.18e−01 | −0.17 (−0.45 to 0.11) | 2.29e−01               | 2.42e−01 | 0.76 (0.61−0.95)       | 1.60e−02               | 1.70e−02 |
| CpG3     | Chr1:11908299 −459       | −0.29 (-0.61 to 0.04) | 8.50e−02               | 7.74e−02 | 0.93 (0.81−1.06)       | 2.56e−01               | 2.41e−01 | −0.03 (−0.21 to 0.16) | 7.90e−01               | 4.26e−01 | 0.96 (0.83−1.11)       | 6.05e−01               | 6.11e−01 |
| CpG4     | Chr1:11908200 −360       | −0.21 (-0.40 to 0.02) | 3.45e−02               | <2.00e−16 | 0.94 (0.87−1.01)       | 1.02e−01               | 8.90e−02 | −0.10 (−0.21 to 0.00) | 6.01e−02               | <2.00e−16 | 0.91 (0.83−0.99)       | 2.94e−02               | 3.70e−02 |
| CpG5     | Chr1:11908182 −342       | −0.37 (-0.63 to 0.12) | 4.15e−03               | <2.00e−16 | 0.93 (0.84−1.03)       | 1.70e−01               | 1.43e−01 | −0.17 (−0.31 to 0.02) | 2.53e−02               | <2.00e−16 | 0.88 (0.78−0.98)       | 2.24e−02               | 2.80e−02 |
| CpG6     | Chr1:11908178 −338       | −0.22 (-0.43 to 0.02) | 3.19e−02               | 3.40e−03 | 0.93 (0.86−1.02)       | 1.09e−01               | 9.20e−02 | −0.07 (−0.19 to 0.04) | 2.09e−01               | 1.25e−01 | 0.93 (0.85−1.02)       | 1.37e−01               | 1.35e−01 |
| CpG7     | Chr1:11908168 −328       | −0.21 (-0.40 to 0.01) | 3.64e−02               | 9.32e−02 | 0.95 (0.88−1.03)       | 2.29e−01               | 2.10e−01 | −0.07 (−0.18 to 0.04) | 2.14e−01               | 1.56e−02 | 0.94 (0.86−1.02)       | 1.53e−01               | 1.58e−01 |
| CpG8     | Chr1:11908165 −325       | −0.14 (-0.33 to 0.06) | 1.72e−01               | 1.00e+00 | 0.96 (0.89−1.04)       | 3.51e−01               | 3.46e−01 | −0.03 (−0.14 to 0.08) | 6.51e−01               | 8.43e−01 | 0.98 (0.90−1.07)       | 6.01e−01               | 6.02e−01 |
| CpG9     | Chr1:11908142 −302       | −0.08 (-0.24 to 0.09) | 3.35e−01               | 1.00e+00 | 0.97 (0.91−1.04)       | 3.62e−01               | 3.65e−01 | 0.01 (−0.08 to 0.10)  | 7.81e−01               | 1.00e+00 | 0.99 (0.93−1.07)       | 8.71e−01               | 8.75e−01 |
| Gene based association | Average                  | −0.28 (-0.54 to 0.02) | 3.61e−02               | −        | 0.92 (0.83−1.02)       | 1.33e−01               | −        | −0.10 (−0.25 to 0.05) | 1.76e−01               | −        | 0.91 (0.81−1.02)       | 1.14e−01               | −        |
| wTPM     |                          | 1.14e−02               | 1.74e−01               | 1.00e+04 | 2.00e+04               |                          | 2.00e+04 |                          |                          | 2.00e+04 |

The results of all covariates from the complete models for all CpGs and average methylation level were presented in the online supplementary Tables S2–S11. OR, odds ratio. a β indicated the change in WC associated with a per 5% increase in DNA methylation level, adjusting for age, sex, education level, cigarette smoking, alcohol consumption, and BMI. b OR of having central obesity associated with a per 5% increase in DNA methylation level, adjusting for age, sex, education level, cigarette smoking, alcohol consumption, and BMI. c β indicated the change in BMI associated with a per 5% increase in DNA methylation level, adjusting for age, sex, education level, cigarette smoking, and alcohol consumption. d OR of having obesity associated with a per 5% increase in DNA methylation level, adjusting for age, sex, education level, cigarette smoking, and alcohol consumption.
### Table 4. The longitudinal associations of NPPA promoter methylation with dynamic and changes in BMI/WC

| CpG loci   | Genomic position, GRCh37 | Relative to TSS, bp | Dynamic WC | WC changes | Dynamic BMI | BMI changes |
|------------|--------------------------|---------------------|------------|------------|------------|------------|
|            |                          |                     | β (95% CI) | p value    | permutation test | p value    | β (95% CI) | p value    | permutation test | p value    |
| Single CpG association | | | | | | | | | | | |
| CpG1       | Chr1:11908353            | −513                | −0.18      | (−0.37 to 0.02) | 7.70e−02  | (0.55 to 0.06) | 7.50e−02  | 1.66e−02  | 3.80e−03  |                                |            |
| CpG2       | Chr1:11908348            | −508                | −0.54      | (−0.94 to 0.14) | 8.55e−03  | (1.14 to 0.12) | 1.00e−02  | 1.62e−02  | 2.78e−01  |                                |            |
| CpG3       | Chr1:11908299            | −459                | −0.31      | (−0.57 to 0.04) | 2.22e−02  | (−0.84 to 0.16) | 2.10e−02  | 3.48e−03  | <2.00e−16 |                                |            |
| CpG4       | Chr1:11908200            | −360                | −0.17      | (−0.32 to 0.01) | 3.53e−02  | (−0.39 to 0.01) | 3.30e−02  | 6.36e−02  | 2.71e−01  |                                |            |
| CpG5       | Chr1:11908182            | −342                | −0.32      | (−0.52 to 0.11) | 2.89e−03  | (−0.54 to 0.01) | 3.00e−03  | 4.49e−02  | 6.60e−01  |                                |            |
| CpG6       | Chr1:11908178            | −338                | −0.17      | (−0.34 to 0.01) | 4.18e−02  | (−0.44 to 0.02) | 4.50e−02  | 3.06e−02  | 1.80e−03  |                                |            |
| CpG7       | Chr1:11908168            | −328                | −0.19      | (−0.35 to 0.03) | 1.88e−02  | (−0.42 to 0.02) | 1.90e−02  | 3.24e−02  | 2.73e−01  |                                |            |
| CpG8       | Chr1:11908165            | −325                | −0.14      | (−0.30 to 0.01) | 7.58e−02  | (−0.40 to 0.00) | 9.70e−02  | 4.94e−02  | 4.00e−03  |                                |            |
| CpG9       | Chr1:11908142            | −302                | −0.07      | (−0.20 to 0.07) | 3.27e−01  | (−0.36 to 0.03) | 3.32e−01  | 1.15e−01  | 1.00e+00  |                                |            |
| Gene-based association | | | | | | | | | | | |
| Average    |                          |                     | −0.25      | (−0.46 to 0.04) | 2.09e−02  | −0.33      | (−0.60 to 0.06) | 1.63e−02  | −0.22      | 2.94e−03  |                                |            |
| wTPM       |                          |                     | 4.10e−03   | 1.49e−02   | 1.00e−04  | 8.30e−03  |                                |            |

For dynamic analysis, we constructed a mixed regression model with the repeated measures of BMI or WC as the dependent variable. For change analysis, we first calculated the changes in BMI or WC using the follow-up values minus the baseline values and then constructed a linear regression model with the changes in BMI or WC as the dependent variable. The results of all covariates from the complete models for all CpGs and average methylation level in association with dynamic WC and BMI were presented in the online supplementary Tables S13–S22. GRCh37, Genome Reference Consortium Human Build 37.
Table 5. The prospective associations of NPPA promoter methylation at baseline with the risks of central obesity and obesity

| CpG loci | Genomic position, GRCh37 | Relative to TSS, bp | Central obesity unadjusted | adjusted<sup>a</sup> | Obesity unadjusted | adjusted<sup>b</sup> |
|----------|--------------------------|---------------------|---------------------------|-------------------|-----------------|-------------------|
|          |                          |                     | OR (95% CI)                | p value           | OR (95% CI)      | p value           |
|          |                          |                     | p value permutation test   | p value           | OR (95% CI)      | p value           |
| Single CpG association |                      |                     |                           |                   |                 |                   |
| CpG1     | Chr1:11908353            | −513                | 0.83                      | 1.77e−02          | 1.30e−02        | 0.85              | 4.95e−02          | 6.40e−02          |
|          |                         |                     |                            | 0.72−1.00         | 0.62−0.97       | 0.72−1.00         | 0.72−1.00         | 0.72−1.00         |
| CpG2     | Chr1:11908348            | −508                | 0.72                      | 4.47e−02          | 5.20e−02        | 0.62              | 7.37e−03          | 3.00e−03          |
|          |                         |                     |                            | 0.44−0.88         | 0.52−0.99       | 0.44−0.88         | 0.52−0.99         | 0.52−0.99         |
| CpG3     | Chr1:11908299            | −459                | 0.72                      | 2.07e−03          | 2.00e−03        | 0.69              | 1.47e−03          | 5.00e−03          |
|          |                         |                     |                            | 0.55−0.87         | 0.58−0.89       | 0.55−0.87         | 0.58−0.89         | 0.58−0.89         |
| CpG4     | Chr1:11908200            | −360                | 0.88                      | 4.11e−02          | 4.10e−02        | 0.85              | 1.91e−02          | 2.00e−02          |
|          |                         |                     |                            | 0.75−0.97         | 0.78−0.95       | 0.75−0.97         | 0.78−0.95         | 0.78−0.95         |
| CpG5     | Chr1:11908182            | −342                | 0.82                      | 1.85e−02          | 1.50e−02        | 0.78              | 5.77e−03          | 4.00e−03          |
|          |                         |                     |                            | 0.65−0.93         | 0.70−0.97       | 0.65−0.93         | 0.70−0.97         | 0.70−0.97         |
| CpG6     | Chr1:11908178            | −338                | 0.86                      | 2.05e−02          | 1.60e−02        | 0.84              | 1.31e−02          | 1.80e−02          |
|          |                         |                     |                            | 0.73−0.96         | 0.75−0.98       | 0.73−0.96         | 0.75−0.98         | 0.75−0.98         |
| CpG7     | Chr1:11908168            | −328                | 0.90                      | 9.88e−02          | 8.80e−02        | 0.88              | 7.22e−02          | 7.70e−02          |
|          |                         |                     |                            | 0.77−1.01         | 0.80−1.02       | 0.77−1.01         | 0.80−1.02         | 0.80−1.02         |
| CpG8     | Chr1:11908165            | −325                | 0.90                      | 8.32e−02          | 7.30e−02        | 0.87              | 3.70e−02          | 4.10e−02          |
|          |                         |                     |                            | 0.76−0.99         | 0.80−1.02       | 0.76−0.99         | 0.80−1.02         | 0.80−1.02         |
| CpG9     | Chr1:11908142            | −302                | 0.95                      | 3.28e−01          | 3.18e−01        | 0.92              | 1.24e−01          | 1.23e−01          |
|          |                         |                     |                            | 0.82−1.02         | 0.86−1.05       | 0.82−1.02         | 0.86−1.05         | 0.86−1.05         |
| Gene-based association |                   |                     |                           |                   |                 |                   |
| Average  |                      |                     | 0.83                      | 2.37e−02          | –                | 0.79              | 1.04e−02          | –                |
|          |                          |                     |                            | 0.66−0.95         | 0.70−0.97       | 0.66−0.95         | 0.70−0.97         | 0.70−0.97         |
| wTPM     |                          |                     | 5.00e−03                   | 5.00e−03          | 2.57e−01        | 2.14e−01          |

OR, odds ratio; GRCh37, Genome Reference Consortium Human Build 37. Unadjusted, not adjusted for other covariates; Adjusted, adjusted for other covariates. The results of all covariates from the complete models for all CpGs and average methylation level are presented in the online supplementary Tables S23–S32. <sup>a</sup> Adjusting for follow-up time, age, sex, education level, cigarette smoking, alcohol consumption, and BMI at baseline. <sup>b</sup> Adjusting for follow-up time, age, sex, education level, cigarette smoking, and alcohol consumption at baseline.
NPPA Promoter Hypomethylation
Predicts Central Obesity Development

Discussion

In a prospective longitudinal cohort of middle-aged and elderly Chinese adults in the Gusu cohort, our study for the first time systemically examined the cross-sectional, longitudinal, and prospective associations of DNA methylation levels of NPPA promoter with obesity and central obesity. The results showed that DNA methylation levels of NPPA promoter significantly predicted a lower risk of incident central obesity during an average 4 years of follow-up. These identified associations were independent of lifestyles and may not be driven by diabetes or hypertension. Our results may indicate that NPPA promoter methylation may possess the potential to serve as a predictor, or even probably a therapeutic target, for central obesity.

In line with our study, the role of the NPPA gene in central obesity has been suggested by previous studies [14, 15]. For example, a cross-sectional study including 1,507 individuals revealed that the NPPA gene rs5063 and rs198358 polymorphism might contribute to the occurrence of central obesity [14]. Another study including 1,608 individuals revealed that the NPPA gene rs5068 polymorphism might contribute to lower WC [15]. Further, Arora et al. [41] speculated that rs5068, which is located in the 3′ untranslated region, could affect transcript stability and result in higher ANP production. These findings suggest that factors regulating the function of the NPPA gene may conserve the potential to be the molecular mechanisms emerging to be studied that underneath the association between ANP and obesity observed in ours [42] and other population studies [12, 13]. As a modifiable molecular modification to the genome without changes in the genes’ sequence, DNA methylation may affect gene function and repress transcription by altering promoter DNA accessibility and blocking the binding of transcription-activating proteins [43]. We, therefore, examined whether NPPA promoter methylation was associated with obesity. Numerous EWAS studies have been conducted and identified many epigenetic markers of obesity [24–27, 44], but no studies found a genome-wide significant CpG located at the NPPA gene. However, in line with our study, NPPA methylation was found to be associated at the gene level with obesity in an EWAS study [28]. Leveraging an unselected population in the Gusu cohort, we are the first to examine the prospective associations of the NPPA promoter methylation with obesity and central obesity and provide initial evidence for the potential role of the NPPA promoter methylation in the pathogenesis of central obesity.

In this study, we found that the contribution of an individual CpG methylation to central obesity was in general small (mostly <5%), and statistically most CpG sites could not withstand multiple testing correction. Such a small effect size may not be detected by conventional statistical methods, but their combined effects may be large enough to be useful for risk prediction. Therefore, we tested the joint association of multiple CpG methylations, with central obesity and found that the joint contribution of these CpG sites appeared to be much larger. Our results may unravel a molecular mechanism that the NPPA promoter methylation may participate in the pathology of central obesity, and suggest that simultaneously testing...
the joint effects of multiple CpG sites is a powerful approach in epigenetic analysis for complicated diseases, e.g., central obesity.

To the best of our knowledge, our study is the first to examine the prospective association of NPPA promoter methylation with obesity and central obesity in Chinese adults. The strengths of this study include a prospective longitudinal study design, careful and systemic analyses of the associations of NPPA promoter methylation with obesity and central obesity, comprehensive adjustments of many conventional risk factors, and application of a gene-based analytical approach to testing the combined effect of multiple CpG methylations at NPPA promoter on central obesity. However, our study also has several limitations. First, as all observational studies, unobserved confounders may exist in our study. Second, we only included middle-aged and elderly Chinese adults in our study. The generalizability of our findings to other age-groups or populations with different ethnic backgrounds is uncertain. Third, we did not have data on circulating ANP levels in our study. Whether and to what extent NPPA promoter methylation accounts for the molecular mechanisms underlying the association between ANP and central obesity still needs more investigation, although hypomethylation of the NPPA gene was found to be associated with the upregulation of the transcripts of ANP [45]. Fourth, data on medications during follow-up were not comprehensively collected. The influence of drugs impacting weight and alcohol consumption could not be prevented. Fifth, given that DNA methylation is tissue- and cell-type specific, it is unclear whether or to what extent the results derived from peripheral blood could reflect methylation changes in the target organs of central obesity. However, accumulating evidence indicated that epimutations may not be limited to the affected tissue but could also be detected in peripheral blood [30, 46, 47]. For example, the same direction of demethylation of NPPA with ANP was also detected in peripheral blood [48]. Sixth, although the main exclusion of study participants is missing data on NPPA methylation, selection bias cannot be prevented in our study. The data interpretation should be of caution. Last, data on white blood cell-type proportions were not available in our study; we cannot eliminate the influence of cell type on our results. The association between NPPA promoter methylation and obesity should be further studied in more population studies and validated in adipose tissues in the future.

In conclusion, our study demonstrated that hypomethylation at NPPA promoter at baseline was not only significantly associated with a higher WC and BMI but also predicted an increased risk of future central obesity in Chinese adults. These findings indicate that NPPA promoter methylation could serve as a predictor for the identification of individuals at high risk for central obesity during primary prevention, but more evidence is needed to establish the causality between NPPA promoter methylation and central obesity.

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Statement of Ethics

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The protocols were approved by the Soochow University Ethics Committee (approval NO. SUDA20100601H02). Written informed consent was obtained from all study participants.

Conflict of Interest Statement

None of the authors have financial associations that might pose a conflict of interest in connection with the submitted article.

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Author Contributions

J.L., J.Z., and Q.Z. performed the statistical data analysis and drafted the manuscript. H.P. and L.W. developed the concept of the study design and contributed to the draft of the manuscript. L.C., S.M., B.S., and Y.L. obtained the clinical data and critically
reviewed the manuscript. Y.H., R.Z., and M.Z. contributed to the interpretation of the results. All authors contributed to draft the final versions of the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

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

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