PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	Influence of lifestyle on the *FAIM2* promoter methylation between obese and lean children: a cohort study
AUTHORS	Wu, Lijun; Zhao, Xiaoyuan; Shen, Yue; Huang, Guimin; Zhang, Meixian; Yan, Yinkun; Hou, Dongqing; Meng, Linghui; Liu, Junting; Cheng, Hong; Mi, Jie

VERSION 1 - REVIEW

REVIEWER	Gaifen Liu Beijing Tiantan Hospital, Capital Medical College
REVIEW RETURNED	29-Jan-2015

GENERAL COMMENTS	1.How did the sample size estimated? Please address in the methods. 2. The statistical anlaysis needs further work. In the study, the associations of FAIM2 promoter methylation with sedentary behavior is differently in the groups Sedentary Behavior <60 Minutes/Day and >60 Minutes/Day, is there any interaction with Sedentary Behavior?

REVIEWER	Ronja Foraita Leibniz Institute for Prevention Research and Epidemiology - BIPS
REVIEW RETURNED	18-Feb-2015

GENERAL COMMENTS	Obesity-related gene FAIM2 promoter is associated with lifestyle in obese and lean children: a cohort study Comments to the authors The authors claim that there research focus is to investigate the association between methylation levels of the FAIM2 promoter and physical activity. However, the statistical analysis tries to answer the question whether the methylation level is associated with obesity, stratified by physical activity (PA) levels. It would be in fact more interesting to clarify first, if there is an association between obesity and FAIM2 methylation. But the authors argue in the discussion that this question cannot be answered with this data. Statistical methods are missing in the abstract. The introduction should explain in more detail the rationale why PA should have an effect on the methylation of FAIM2 and the rationale why FAIM2 is an intermediate between PA and obesity. The methods section needs to be extended amongst others by name of the study, aim of the study,

	how participants have been recruited, etc. It is a very small study compared to the age range. What is the reason behind this wide range including children and adolescents? How have the anthropometric measures been collected? Have there been trained study nurses or did the participants report their weight and height? Reference 10 seems to be false. At least I wouldn't name the WHO a manufacturer. Fat mass and BMI from children and adolescents are age and sex dependent. It is absolutely necessary to use one of the existing reference systems for BMI that describe the weight status of children (see e.g. Ahrens et al.(2001), Childhood obesity: prevalence worldwide, Springer, p.219-235, for an overview). This has to be corrected in the analysis. It is further necessary to describe the procedure, how BMI is classified into "obese" and "lean". However, the category "lean" is not common use. Please use cut-offs and category names as proposed by the International Obesity Task Force (IOTF) or the WHO. Please refer the name and the reference of the validated questionnaire you used. The assessment of PA is very important for this study and should be explained in more detail. These are amongst others: - are the questions retrospective, - on which time-period focuses the questionnaire (one random day, the last week, …) - how did you make sure that the parents know the PA levels of their children - how are the questions transformed to METs - … Please bring an argument why parents completed the questionnaires of adolescents and not the participants on its own. The statistical analysis does not answer the main research question (see above) that was given in the introduction "(…) to investigate the association of the methylation of FAIM2 promoter with sedentary behaviour and physical activity in the obese and lean children". In the statistics section the research question changed to "() to investigate the difference of methylation levels between obese and lean subjects (…)". The authors used a multiple linear regression (and not a general linear regression) on the methylation levels. The section misses important information, as: - are the methylation levels normally distributed? - were the methylation levels transformed or scaled? - stratification by PA If the outcome is not normally distributed than the authors have to apply other statistical methods. It is absolutely necessary to give beta estimates and confidence intervals as well as the number of observations in each PA category in the tables. A lot of tests have been conducted without considering to adjust the p-value. A method for handling the multiple testing problem has to be applied. The discussion should firstly answer the posed research question. How do the methylation results

	support the question? There might be some differences (maybe not after adjusting for multiple testing), but is there are biological justification? The authors should additionally explain in more detail how the answer fit in with existing knowledge. Furthermore, the study has a lot of limitations that has to be reported and discussed (e.g. age range, assessment of PA…). The reviewer also provided a marked copy with detailed comments. Please contact the publisher for full information about it.

REVIEWER	Tuomas Kilpelainen The Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen, Copenhagen, Denmark
REVIEW RETURNED	23-Feb-2015

GENERAL COMMENTS	The authors have examined whether the methylation status of human FAIM2 gene promoter is modified by sedentary behaviour or physical activity in 59 obese and 39 lean children. The authors report several CpG sites in the FAIM2 promoter whose methylation levels are significantly different between obese and lean children within specific categories of sedentary behavior and physical activity. As the biological mechanisms for FAIM2 and many other recently identified obesity-risk loci, as well as their interplay with lifestyle factors, are poorly understood, the manuscript addresses an important area of research. However, I have several comments and concerns that the authors should adress in their manuscript. 1. The authors have currently tested methylation differences between lean and obese children within categories stratified by physical activity and sedentary behavior. Considering the authors' objective of examining whether methylation changes related to obesity are modified by physical activity and sedentary behaviour, I argue that the correct statistical model would rather involve testing for the interaction between physical activity and methylation levels on the risk of a child being obese. 2. The authors have not accounted for multiple testing in their study. Considering that the authors performed 216 statistical tests to identify methylation differences, the Bonferroni-corrected statistical significance treshold would be P=0.00023. Only three methylation sites reached this significance treshold. 3. FAIM2 is thought to affect energy balance through the brain and is highly expressed in the hypothalamus. The authors are investigating methylation levels in leukocytes. As methylation-changes are highly tissue-specific, the changes found in leukocytes do not necessarily correspond with methylation status in the brain. Further, the authors have not reported whether FAIM2 is at all expressed in leukocytes. 4. Previous studies have studied changes in FAIM2 function in response to changes in diet. Rather than investigating diet, the authors have studied methylation-differences in various strata of sedentary behavior and physical activity. Methylation changes in response to physical activity are likely to be very different to those induced by diet, and it is therefore difficult to make a link between results in the present study and those published previously on

	FAIM2 function. 5. The current title of the paper will need to be changed to clarify that the authors studied changes in the methylation of the FAIM2 promoter. 6. How were the case and control children selected in the present study? 7. The obese children were on average 3.5 years older than the lean children and more of the obese children must have been past puberty. It would be important to control for the stage of puberty in the analyses. 8. In Tables 1-3, it is needed to include sample sizes for obese and lean children within the physical activity and sedentary behaviour strata.

VERSION 1 – AUTHOR RESPONSE

Reviewer: Gaifen Liu
Question 1. How did the sample size estimated? Please address in the methods.

Answer: Reviewer is very careful. Thank you very much! We added the sentences in the methods section, as followed:
The cohort included 59 obese and 39 lean subjects randomly recruited from a cross-sectional survey of Beijing children aged 8–18 years in 2013. The survey was a physical fitness and health surveillance of Beijing school students, and included a questionnaire, medical examination, anthropometric measurement, and collected venipuncture blood samples (n = 3143; boys 50%). The obese diagnosed by the Chinese age- and sex-specific body mass index (BMI) cutoffs (supplementary table S1) and fat mass percentage (FMP)>40. The lean diagnosed by WHO BMI cutoffs and FMP<15. The research budget limited the sample size in the study. In future studies we hope to examine the methylation levels of the FAIM2 promoter in greater sample sizes.

Question 2. The statistical anlaysis needs further work. In the study, the associations of FAIM2 promoter methylation with sedentary behavior is differently in the groups Sedentary Behavior <60 Minutes/Day and >60 Minutes/Day, is there any interaction with Sedentary Behavior?

Answer: We agree with the reviewer's comment. We revised the tables and added beta estimates and confidence intervals. The methylation levels at seven CpG sites showed significant differences between the obese and lean subjects with sedentary behavior <60 minutes/day, but the methylation levels at only two CpG sites showed significant differences between the obese and lean subjects with sedentary behavior≥60 minutes/day. Although the mechanisms are not clear, we think there is significant association between the methylation levels of the FAIM2 promoter and sedentary behavior. The difference in the groups sedentary behavior <60 minutes/day and >60 minutes/day would be examined in our further studies.

Reviewer: Ronja Foraita
Question 1. The authors claim that there research focus is to investigate the association between methylationlevels of the FAIM2 promoter and physical activity. However, the statistical analysis tries to answer the question whether the methylation level is associated with obesity, stratified by physical activity (PA) levels. It would be in fact more interesting to clarify first, if there is an association between obesity and FAIM2 methylation. But the authors argue in the discussion that this question cannot be answered with this data.

Answer: Reviewer is very careful. Thank you very much! We explored that the methylation levels of the FAIM2 promoter were significantly associated with obesity in another study (Wu L et al. Diab Vasc Dis Res. 2015 Feb 12. [Epub ahead of print]), then this study examined the associations between the methylation levels of the FAIM2 promoter and sedentary behavior and physical activity. We added the sentences “the methylation levels of the FAIM2 promoter are significantly associated with obesity” and “The molecular mechanisms by which FAIM2 affects obesity whether is involved in lifestyle are unclear”, and revised the sentence as “This study provides the first evidence that there are significant differences of the associations of the FAIM2 promoter methylation with sedentary behavior and physical activity between the obese and lean children.” in ABSTRACT section. We added the sentence “the methylation levels of the FAIM2 promoter were significantly associated with obesity, but the molecular mechanism by which FAIM2 affects obesity whether is involved in lifestyle has not been clarified” in INTRODUCTION section. We changed the sentence into “The aim of this study was to investigate the differences of the methylation levels of the FAIM2 promoter between the obese and lean subjects according to different sedentary behavior and physical activity.” in INTRODUCTION section. We added the sentence “the methylation levels of the FAIM2 promoter were significantly associated with obesity”, and revised the sentence as “our study demonstrated the differences of the associations of the FAIM2 promoter methylation with sedentary behavior and physical activity between the obese and lean children.” in DISCUSSION section. We revised the sentence as “we explored for the first time that there were significant differences of the associations of the FAIM2 promoter methylation with sedentary behavior and physical activity between the obese and lean children.” in CONCLUSIONS section. We changed the title of the paper to “Influence of lifestyle on the FAIM2 promoter methylation between obese and lean children: a cohort study”.

Question 2. Statistical methods are missing in the abstract.

Answer: Reviewer is very careful. Thank you very much! We added the sentence “The influences of different lifestyles on methylation variations in the obese and lean children were examined by multiple linear regression” in ABSTRACT section.

Question 3. The introduction should explain in more detail the rationale why PA should have an effect on the methylation of FAIM2 and the rationale why FAIM2 is an intermediate between PA and obesity.

Answer: We agree with the reviewer’s comment. We added the sentences “the methylation levels of the FAIM2 promoter were significantly associated with obesity, but the molecular mechanism by which FAIM2 affects obesity whether is involved in lifestyle has not been clarified” and “Because obesity-related lifestyle factors might modify epigenetic patterns and the methylation levels of the FAIM2 promoter are significantly associated with obesity” in INTRODUCTION section.

Question 4. The methods section needs to be extended amongst others by name of the study, aim of the study, how participants have been recruited, etc. It is a very small study compared to the age range. What is the reason behind this wide range including children and adolescents?

Answer: We agree with the reviewer’s comment. We added the sentences “The cohort included 59 obese and 39 lean subjects randomly recruited from a cross-sectional survey of Beijing children aged 8–18 years in 2013. The survey was a physical fitness and health surveillance of Beijing school students, and included a questionnaire, medical examination, anthropometric measurement, and collected venipuncture blood samples (n = 3143; boys 50%). The obese diagnosed by the Chinese age- and sex-specific body mass index (BMI) cutoffs (supplementary table S1) and fat mass percentage (FMP)>40. The lean diagnosed by WHO BMI cutoffs and FMP<15. The research budget limited the sample size in the study. In future studies we hope to examine the methylation levels of the FAIM2 promoter in greater sample sizes.” in the methods section. We added the sentences “There

are a few limitations to this study. First, the age range of the obese and lean subjects is wide and the sample size is small. Studies with greater sample sizes are needed to examine the associations." in discussion section.

Question 5. How have the anthropometric measures been collected? Have there been trained study nurses or did the participants report their weight and height?

Answer: The anthropometric measures have been collected by trained study nurses and the members of Department of Epidemiology, Capital Institute of Pediatrics. Standing height without shoes was measured twice to the nearest 0.1 cm using a wall-mounted stadiometers. Weight (wearing underwear and no shoes) and fat mass percentage were measured using a body composition analyzer (InBody 720, Biospace Co., Ltd. Seoul, Korea). After a rest period of 5 min, blood pressure was measured by auscultation using a standard clinical sphygmomanometer. Measurements were taken on the right arm in a sitting position with the elbow at the level of the right atrium, using an appropriately sized cuff. Systolic blood pressure was determined by the onset of the 'tapping' Korotkoff sounds (K1) and diastolic blood pressure was determined by the fourth Korotkoff sound (K4). Three consecutive measurements were performed and the mean of the three readings was used for analysis.

Question 6. Reference 10 seems to be false. At least I wouldn't name the WHO a manufacturer.

Answer: We agree with the reviewer's comment. We deleted this reference.

Question 7. Fat mass and BMI from children and adolescents are age and sex dependent. It is absolutely necessary to use one of the existing reference systems for BMI that describe the weight status of children (see e.g. Ahrens et al.(2001), Childhood obesity: prevalence worldwide, Springer, p.219-235, for an overview). This has to be corrected in the analysis. It is further necessary to describe the procedure, how BMI is classified into "obese" and "lean". However, the category "lean" is not common use. Please use cut-offs and category names as proposed by the International Obesity Task Force (IOTF) or the WHO.

Answer: Thank you for your valuable suggestion. We corrected in the analysis and added Supplementary table S1. Compared with "marasmus" or "underweight" or "thinness", we think maybe "lean" is more suitable in this study, because we defined "lean" according BMI and FMP. We added the sentences "The obese diagnosed by the Chinese age- and sex-specific body mass index (BMI) cutoffs (supplementary table S1) and fat mass percentage (FMP)>40. The lean diagnosed by WHO BMI cutoffs and FMP<15." in METHODS section.

Question 8. Please refer the name and the reference of the validated questionnaire you used. The assessment of PA is very important for this study and should be explained in more detail. These are amongst others:
- are the questions retrospective,
- on which time-period focuses the questionnaire (one random day, the last week, …)
- how did you make sure that the parents know the PA levels of their children
- how are the questions transformed to METs
- …

Answer: Thank you for your valuable suggestion. The questionnaire was designed by the members of Department of Epidemiology, Capital Institute of Pediatrics. The questionnaire referred the questionnaire used in the study by Meng L et al (Meng L, Liang Y, Liu J, et al. Prevalence and risk factors of hypertension based on repeated measurements in Chinese children and adolescents. Blood Press 2013;22:59-64). We added this reference and the sentence "The questionnaire referred the

questionnaire used in the study by Meng L et al (2013).” in METHODS section.
The data of sedentary behavior and physical activity were collected by questionnaires. Compared with adolescents completing the questionnaires by themselves, parents or guardians reviewed and recorded the data more carefully, so we required parents or guardians to complete the questionnaires. The reliability of questionnaire depends on good communication between the parents and children. Parents or guardians should ask children about sedentary behavior and physical activity in detail and record the data carefully.
Activity with intensity of 3 to 6 work metabolic rates/resting metabolic rates (METs) includes jogging, table tennis, Tai Chi, etc. Activity with intensity of >6 METs includes football, basketball, badminton, etc. We revised the assessment of physical activity in METHODS section, as followed: A validated questionnaire was used to investigate sedentary behavior and physical activity in children. The questionnaires were completed by parents or guardians. The questions were retrospective and the questionnaire collected the data in the last six months. Sedentary behavior was determined by the time spent either watching television or playing video/computer games per day in a week. Moderate physical activity (MPA) was determined by the time spent jogging or table tennis or Tai Chi, etc, per week. High physical activity (HPA) was determined by the time spent football or basketball or badminton, etc, per week. High or moderate physical activity level was determined by the time spent MPA or HPA per week.

Question 9. Please bring an argument why parents completed the questionnaires of adolescents and not the participants on its own.

Answer: Thank you very much! Reviewer is very careful. Compared with adolescents completing the questionnaires by themselves, parents or guardians reviewed and recorded the data more carefully, so we required parents or guardians to complete the questionnaires.

Question 10. The statistical analysis does not answer the main research question (see above) that was given in the introduction “(…) to investigate the association of the methylation of FAIM2 promoter with sedentary behaviour and physical activity in the obese and lean children”. In the statistics section the research question changed to “() to investigate the difference of methylation levels between obese and lean subjects (…)”.

Answer: We agree with the reviewer’s comment. In our study, we stratified the children cohort into six groups, including sedentary behavior <60 minutes/day, sedentary behavior≥60 minutes/day, high physical activity level <30 minutes/day, and high physical activity level ≥30 minutes/day, high or moderate physical activity level <150 minutes/week, and high or moderate physical activity level ≥150 minutes/week according to sedentary behaviour and physical activity. Then we explored the methylation levels of the FAIM2 promoter in the obese and lean subjects in these six groups, respectively. We changed the title of the paper to “Influence of lifestyle on the FAIM2 promoter methylation between obese and lean children: a cohort study”. We revised the manuscript and added the sentences “the methylation levels of the FAIM2 promoter are significantly associated with obesity” and “The molecular mechanisms by which FAIM2 affects obesity whether is involved in lifestyle are unclear”, and revised the sentence as “This study provides the first evidence that there are significant differences of the associations of the FAIM2 promoter methylation with sedentary behavior and physical activity between the obese and lean children.” in ABSTRACT section. We changed the sentence “The aim of this study was to investigate the associations of the methylation of the FAIM2 promoter with sedentary behavior and physical activity in the obese and lean children.” into “The aim of this study was to investigate the differences of the methylation levels of the FAIM2 promoter between the obese and lean subjects according to different sedentary behavior and physical activity” and added the sentence “the methylation levels of the FAIM2 promoter were significantly associated with obesity, but the molecular mechanism by which FAIM2 affects obesity whether is involved in lifestyle has not been clarified” in INTRODUCTION section. We added the sentence “According to

sedentary behavior and physical activity we stratified the subjects into six groups, including sedentary behavior <60 minutes/day, sedentary behavior≥60 minutes/day, HPA level <30 minutes/day, HPA level ≥30 minutes/day, high or moderate physical activity level <150 minutes/week, and high or moderate physical activity level ≥150 minutes/week" in METHODS section. We added the sentence "the methylation levels of the FAIM2 promoter were significantly associated with obesity", and revised the sentence as "our study demonstrated the differences of the associations of the FAIM2 promoter methylation with sedentary behavior and physical activity between the obese and lean children." in DISCUSSION section. We revised the sentence as "we explored for the first time that there were significant differences of the associations of the FAIM2 promoter methylation with sedentary behavior and physical activity between the obese and lean children." in CONCLUSIONS section.

Question 11. The authors used a multiple linear regression (and not a general linear regression) on the methylation levels. The section misses important information, as:
- are the methylation levels normally distributed?
- were the methylation levels transformed or scaled?
- stratification by PA If the outcome is not normally distributed than the authors have to apply other statistical methods.

Answer: Thank you very much! In METHOD section, we changed the statistical method from general linear model to multiple linear regression for calculating beta values and 95%CI.
For the first question, we used a general linear model to investigate the difference of methylation levels of FAIM2 promoter in the obese and lean children, because most of the methylation levels were near to normally distributed. According to the reviewer's comment, we reanalyzed all data used a multiple linear regression, and got same P values.
Second question, the methylation levels were not transformed or scaled.
Third question, we reanalyzed used a multiple linear regression, and got same results.

Question 12. It is absolutely necessary to give beta estimates and confidence intervals as well as the number of observations in each PA category in the tables.

Answer: We agree with the reviewer's comment. We revised the tables and added beta estimates and confidence intervals as well as the number of observations in each PA category.

Question 13. A lot of tests have been conducted without considering to adjust the p-value. A method for handling the multiple testing problem has to be applied.

Answer: We agree with the reviewer's comment. We added the multiple testing and the sentences "We applied a multiple testing to correct for multiple comparisons, the false discovery rate (FDR) (Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Statist. Soc. B 1995;57: 289-300) approach was used; FDR analysis (0.05 as criteria) was applied for six groups (sedentary behavior <60 minutes/day, sedentary behavior≥60 minutes/day, HPA level <30 minutes/day, HPA level ≥30 minutes/day, high or moderate physical activity level <150 minutes/week, and high or moderate physical activity level ≥150 minutes/week) and 36 CpG sites simultaneously (number of test: 36×6=216). In brief, if the original P value was less than P value for FDR, then it represented the statistical significance; otherwise, it suggested the non statistical significance." in METHODS section. Thanks reviewer again!

Question 14. The discussion should firstly answer the posed research question. How do the methylation results support the question? There might be some differences (maybe not after adjusting for multiple testing), but is there are biological justification? The authors should additionally explain in more detail how the answer fit in with existing knowledge. Furthermore, the study has a lot of limitations that has to be reported and discussed (e.g. age range, assessment of PA…).

Answer: We agree with the reviewer's comment. We revised the manuscript and added two paragraphs according to reviewer's comment in DISCUSSION section, as followed:
"Previous studies have shown that lifestyle factors might modify epigenetic patterns and the methylation levels of the FAIM2 promoter are significantly associated with obesity, but the molecular mechanisms by which FAIM2 affects obesity whether is involved in lifestyle are unclear. In this study, we investigated the potential associations of the FAIM2 promoter methylation with sedentary behavior and physical activity in the obese and lean children. The methylation levels of the FAIM2 promoter were significantly different in the obese and lean children when the groups were stratified by sedentary behavior and physical activity. There were significant differences of the associations of the FAIM2 promoter methylation with sedentary behavior and physical activity between the obese and lean subjects. Our results suggest that lifestyle maybe possibly mediate the process of the FAIM2 involved in obesity."
"There are a few limitations to this study. First, the age range of the obese and lean subjects is wide and the sample size is small. Studies with greater sample sizes are needed to examine the associations. Second, the obese subjects were on average 3.5 years older than the lean subjects. We want to eliminate the effect by adjusting for age. It is important to adjust the stage of puberty in the analyses, so we would collect the data of the stage of puberty in future studies. Third, the data of sedentary behavior and physical activity were collected by questionnaires. The reliability of questionnaire depends on good communication between the parents and children. Fourth, there was no gene expression data from leukocytes or tissues in the study. Fifth, our study investigated the methylation levels in peripheral blood leukocytes, but not in hypothalamus or adipocytes."

Reviewer:Tuomas Kilpelainen
Question 1. The authors have currently tested methylation differences between lean and obese children within categories stratified by physical activity and sedentary behavior. Considering the authors' objective of examining whether methylation changes related to obesity are modified by physical activity and sedentary behaviour, I argue that the correct statistical model would rather involve testing for the interaction between physical activity and methylation levels on the risk of a child being obese.

Answer: Thank you very much! We agree with the reviewer's comment. We explored that the methylation levels of the FAIM2 promoter were significantly associated with obesity in another study (Wu L et al. Diab Vasc Dis Res. 2015 Feb 12. [Epub ahead of print]), then this study examined the associations between the methylation levels of the FAIM2 promoter and sedentary behavior and physical activity. We revised the manuscript and added the sentences "the methylation levels of the FAIM2 promoter are significantly associated with obesity" and "The molecular mechanisms by which FAIM2 affects obesity whether is involved in lifestyle are unclear", and revised the sentence as "This study provides the first evidence that there are significant differences of the associations of the FAIM2 promoter methylation with sedentary behavior and physical activity between the obese and lean children." in ABSTRACT section. We added the sentence "the methylation levels of the FAIM2 promoter were significantly associated with obesity, but the molecular mechanism by which FAIM2 affects obesity whether is involved in lifestyle has not been clarified" in INTRODUCTION section. We changed the sentence into "The aim of this study was to investigate the differences of the methylation levels of the FAIM2 promoter between the obese and lean subjects according to different sedentary behavior and physical activity." in INTRODUCTION section. We added the sentence "the methylation levels of the FAIM2 promoter are significantly associated with obesity", and revised the sentence as "our study demonstrated the differences of the associations of the FAIM2 promoter methylation with sedentary behavior and physical activity between the obese and lean children." in DISCUSSION section. We revised the sentence as "we explored for the first time that there were significant differences of the associations of the FAIM2 promoter methylation with sedentary behavior and physical activity between the obese and lean children." in CONCLUSIONS section. We added a

paragraph in DISCUSSION section, as followed:
“Previous studies have shown that lifestyle factors might modify epigenetic patterns and the methylation levels of the FAIM2 promoter are significantly associated with obesity, but the molecular mechanisms by which FAIM2 affects obesity whether is involved in lifestyle are unclear. In this study, we investigated the potential associations of the FAIM2 promoter methylation with sedentary behavior and physical activity in the obese and lean children. The methylation levels of the FAIM2 promoter were significantly different in the obese and lean children when the groups were stratified by sedentary behavior and physical activity. There were significant differences of the associations of the FAIM2 promoter methylation with sedentary behavior and physical activity between the obese and lean subjects. Our results suggest that lifestyle maybe possibly mediate the process of the FAIM2 involved in obesity.”
We changed the title of the paper to “Influence of lifestyle on the FAIM2 promoter methylation between obese and lean children: a cohort study”.

Question 2. The authors have not accounted for multiple testing in their study. Considering that the authors performed 216 statistical tests to identify methylation differences, the Bonferroni-corrected statistical significance treshold would be P=0.00023. Only three methylation sites reached this significance treshold.

Answer: We agree with the reviewer’s comment. Thank you for your valuable suggestion. We added the multiple testing and the sentences “We applied a multiple testing to correct for multiple comparisons, the false discovery rate (FDR) (Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Statist. Soc. B 1995;57: 289-300) approach was used; FDR analysis (0.05 as criteria) was applied for six groups (sedentary behavior <60 minutes/day, sedentary behavior≥60 minutes/day, HPA level <30 minutes/day, HPA level ≥30 minutes/day, high or moderate physical activity level <150 minutes/week, and high or moderate physical activity level ≥150 minutes/week) and 36 CpG sites simultaneously (number of test: 36×6=216). In brief, if the original P value was less than P value for FDR, then it represented the statistical significance; otherwise, it suggested the non statistical significance” in METHODS section. There are four methylation levels at site -975, site -413, sites -362 and -360, and sites-353 and -349 (P=0.00004, 0.00009, 0.0006, and 0.00005, respectively) reached the significance.

Question 3. FAIM2 is thought to affect energy balance through the brain and is highly expressed in the hypothalamus. The authors are investigating methylation levels in leukocytes. As methylation-changes are highly tissue-specific, the changes found in leukocytes do not necessarily correspond with methylation status in the brain. Further, the authors have not reported whether FAIM2 is at all expressed in leukocytes.

Answer: We agree with the reviewer’s comment. The specific tissue to identify epigenetic variations related to obesity should be hypothalamus or adipocytes, but we investigated the methylation levels in peripheral blood leukocytes because of practical difficulties in obtaining tissues from participants. Moreover, this study has not the data of gene expression. In future studies, we hope to examine the expression and the methylation levels of the gene in hypothalamus. We added a paragraph in DISCUSSION section, as followed:
“There are a few limitations to this study. First, the age range of the obese and lean subjects is wide and the sample size is small. Studies with greater sample sizes are needed to examine the associations. Second, the obese subjects were on average 3.5 years older than the lean subjects. We want to eliminate the effect by adjusting for age. It is important to adjust the stage of puberty in the analyses, so we would collect the data of the stage of puberty in future studies. Third, the data of sedentary behavior and physical activity were collected by questionnaires. The reliability of questionnaire depends on good communication between the parents and children. Fourth, there was no gene expression data from leukocytes or tissues in the study. Fifth, our study investigated the

methylation levels in peripheral blood leukocytes, but not in hypothalamus or adipocytes."

Question 4. Previous studies have studied changes in FAIM2 function in response to changes in diet. Rather than investigating diet, the authors have studied methylation-differences in various strata of sedentary behavior and physical activity. Methylation changes in response to physical activity are likely to be very different to those induced by diet, and it is therefore difficult to make a link between results in the present study and those published previously on FAIM2 function.

Answer: We agree with the reviewer's comment. We explored that the methylation levels of the FAIM2 promoter were significantly associated with obesity in another study (Wu L et al. Diab Vasc Dis Res. 2015 Feb 12. [Epub ahead of print]). We added this reference in manuscript. There was no study about FAIM2 function induced by physical activity. We hope to examine FAIM2 function response to physical activity in future studies. We revised the manuscript and added the sentence "the methylation levels of the FAIM2 promoter are significantly associated with obesity" in ABSTRACT section. We added the sentence "the methylation levels of the FAIM2 promoter were significantly associated with obesity, but the molecular mechanism by which FAIM2 affects obesity whether is involved in lifestyle has not been clarified" in INTRODUCTION section. We added the sentence "the methylation levels of the FAIM2 promoter are significantly associated with obesity" in DISCUSSION section.

Question 5. The current title of the paper will need to be changed to clarify that the authors studied changes in the methylation of the FAIM2 promoter.

Answer: We agree with the reviewer's comment. We changed the title of the paper to "Influence of lifestyle on the FAIM2 promoter methylation between obese and lean children: a cohort study".

Question 6. How were the case and control children selected in the present study?

Answer: Thank you very much! We revised the METHODS as followed:
The cohort included 59 obese and 39 lean subjects randomly recruited from a cross-sectional survey of Beijing children aged 8–18 years in 2013. The survey was a physical fitness and health surveillance of Beijing school students, and included a questionnaire, medical examination, anthropometric measurement, and collected venipuncture blood samples (n = 3143; boys 50%). The obese diagnosed by the Chinese age- and sex-specific body mass index (BMI) cutoffs (supplementary table S1) and fat mass percentage (FMP)>40. The lean diagnosed by WHO BMI cutoffs and FMP<15. The research budget limited the sample size in the study. In future studies we hope to examine the methylation levels of the FAIM2 promoter in greater sample sizes.

Question 7. The obese children were on average 3.5 years older than the lean children and more of the obese children must have been past puberty. It would be important to control for the stage of puberty in the analyses.

Answer: We agree with the reviewer's comment. We want to eliminate the effect by adjusting for age. The data of the study has not the stage of puberty. We would like to collect the data of the stage of puberty in future studies. We added the sentence "The obese subjects were on average 3.5 years older than the lean subjects. We want to eliminate the effect by adjusting for age. It is important to adjust the stage of puberty in the analyses, so we would collect the data of the stage of puberty in future studies." in DISCUSSION section.

Question 8. In Tables 1-3, it is needed to include sample sizes for obese and lean children within the physical activity and sedentary behavior strata.

Answer: Thank you for your comments! We added the sample sizes for obese and lean children within the physical activity and sedentary behavior strata in Tables 1-3.

VERSION 2 – REVIEW

REVIEWER	Tuomas Kilpelainen The Novo Nordisk Foundation Center for Basic Metabolic Research, Section of Metabolic Genetics, University of Copenhagen, Denmark
REVIEW RETURNED	23-Mar-2015

GENERAL COMMENTS	The authors have addressed each of my comments and the paper has improved. However, there are still remaining remarks that should be addressed: Response to my previous comment no. 1: I still do disagree about the analysis strategy that the authors have applied in their study. Their results show that after correction for multiple testing, four CpG sites show differential methylation between lean and obese individuals among the individuals who are sedentary. This result does not, as such, suggest that sedentary behavior or physical activity has something to do with the changes in methylation of FAIM2. This is because the authors have not tested for differences in methylation between the categories of sedentary behavior or physical activity. It would now be important to show that 1) methylation levels are regulated by physical activity (PA) overall, i.e. that there are differences in the methylation status of CpG sites between the PA categories; and 2) to test whether there is an interaction between the methylation of CpG sites and PA regarding the obesity status of the children (Model: Obesity = CpG + PA + CpG*PA), i.e. to test whether PA modifies the association between methylation of CpG sites and the level of obesity. Response to my previous comment no. 3: Even if the authors do not have gene expression data from the present sample, they will be able to use publicly available expression databases to confirm whether FAIM2 is or is not expressed in leukocytes.

VERSION 2 – AUTHOR RESPONSE

Reviewer:Tuomas Kilpelainen
Question 1. Response to my previous comment no. 1:
I still do disagree about the analysis strategy that the authors have applied in their study. Their results show that after correction for multiple testing, four CpG sites show differential methylation between lean and obese individuals among the individuals who are sedentary. This result does not, as such, suggest that sedentary behavior or physical activity has something to do with the changes in methylation of FAIM2. This is because the authors have not tested for differences in methylation between the categories of sedentary behavior or physical activity. It would now be important to show that 1) methylation levels are regulated by physical activity (PA) overall, i.e. that there are differences in the methylation status of CpG sites between the PA categories; and 2) to test whether there is an interaction between the methylation of CpG sites and PA regarding the obesity status of the children (Model: Obesity = CpG + PA + CpG*PA), i.e. to test whether PA modifies the association between methylation of CpG sites and the level of obesity.

Answer: We agree with the reviewer's comment. Thank you for your valuable suggestion. We added

supplementary table S4-7 and the sentences “We analyzed the associations between the methylation levels of the FAIM2 promoter and the categories of sedentary behavior or physical activity (supplementary table S4-6). There was no statistically significant difference after multiple testing.” and “There was no statistically significant interaction between the methylation levels of the FAIM2 promoter and physical activity regarding the obesity status after multiple testing (supplementary table S7)” in result section. We added the sentences “Multiple linear regressions were used to investigate the difference of methylation levels between different groups with adjusting for age and gender, or age, gender, and BMI. A logistic regression model was used to investigate the interaction between the methylation levels of the FAIM2 promoter and physical activity regarding the obesity status of the children (the model: Obesity = CpG + PA + CpG*PA).” in method section.

Question 2. Response to my previous comment no. 3:
Even if the authors do not have gene expression data from the present sample, they will be able to use publicly available expression databases to confirm whether FAIM2 is or is not expressed in leukocytes.

Answer: Thank you very much! We agree with the reviewer’s comment. Previous studies showed the expression of FAIM2 in hypothalamus, neuroblastoma cell, lung fibroblast cell, keratinocyte, and fibrochondrocyte, but there is no expression data of this gene in peripheral blood leukocyte. We added the sentence “Previous studies showed the expression of FAIM2 in hypothalamus or some other tissues, but there was no expression data of this gene in peripheral blood leukocyte.” in discussion section.