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DNA Methylation on *Interleukin- 6* Correlates with Weight Loss in Obese Women

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Abstract

Introduction

Obesity and its associated comorbidities are major health problems worldwide. Obesity is accompanied by a systemic low-grade inflammation with elevated blood-levels of inflammatory mediators like Interleukin-6. Interleukin-6 was described to be regulated by epigenetic mechanisms such as DNA methylation.

Methods

32 study participants finished a three months weight loss intervention. 16 individuals lost more than 5 % of their initial body weight, four men and twelve women. To avoid a gender bias only the blood samples of the 12 obese women who lost weight compared to 12 obese women without weight loss were analysed. The methylation levels of seven CpG-sites in the promoter and first exon of the *Interleukin-6* gene were assessed using pyrosequencing.

Results

Three CpG-sites showed a significant higher methylation after the weight loss intervention in the responder group compared to the non-responder group. Weight loss and the initial methylation of two CpG-sites correlated negatively. Further, methylation changes of distinct CpGs correlated with the weight loss and the initial BMI.

Conclusion

In this study we found that weight loss influences patterns of CpG methylation on *Interleukin-6*.

In this study, we revealed two CpG-sites which might be promising candidates for developing predictive biomarkers for weight loss response. Further studies are needed to support this findings.

Keywords: Adiposity; Inflammation; Epigenetic; Biomarker; Weight reduction; Promoter Methylation

Introduction

Overweight (body mass index (BMI) ≥ 25 kg/m^2) and obesity (BMI ≥ 30 kg/m^2) are major health problems worldwide. The WHO assessed that in 2014 39 % of adults aged over 18 were overweight or obese worldwide [1]. The associated comorbidities like high blood pressure and diabetes mellitus type 2 cause suffering of the affected patients and enormous burdens for national health care systems.

The often occurring familiar aggregation of obesity cannot only be explained by the shared environmental conditions. Thus, the exposure to chemical reagents, climate conditions and especially the gastro-intestinal microbiota are known to contribute to an individual´s body weight. The heritability of obesity is between 40 and 70 % but risk loci associated with BMI revealed in genome wide association studies can only explain approximately 16 % of this heritability even if their additive effects are considered [2]. Thus, epigenetic mechanisms came into the focus of obesity research during the last years to explain the missing heritability. Epigenetic describes partially heritable changes of the gene expression which are not caused by alterations in underlying the DNA sequence. These mechanisms include the addition of a methyl group to a cytosine which is followed by a guanosine in the sequence (CpG-site). Other mechanisms are structural changes of the chromatin through modifications such as for example the methylation, acetylation or phosphorylation of the histones. Further, microRNAs which do not code for a protein transcript interact with messenger RNA and thus are involved in epigenetic gene regulation.

Several studies have already observed differences in the methylation levels on distinct CpG-sites between obese and lean individuals [3-5] and after a weight loss intervention [6-8]. Epigenome-wide association studies showed correlations between the methylation levels of distinct gene sites and the BMI. The identified genes were for example *hypoxia inducible transcription factor 3A (HIF), peptidase M20 domain containing 1 (PM20D1)* and *matrix metallopeptidase 9 (MMP9)* [3, 5]. Further, different methylation states of distinct CpG-sites between low and high responder to a weight loss program were detected before the start of the intervention [9, 10]. These results provide a hint that DNA methylation is mechanistically involved in weight loss but in general it is not clear whether differences in the DNA methylation are a reason or a consequence of obesity and weight loss.

Epigenetic mechanisms play also important roles in the regulation of genes involved in inflammatory processes. Since overweight and obesity are associated with a systemic low-grade inflammation which triggers comorbidities like diabetes mellitus type 2 or cardiovascular events, inflammation is of special interest in the obesity research. The question which still has to be answered is whether the low-grade inflammation is a reason or a consequence of excessive weight gain.

The elevated inflammatory mediators which are assessed in obese individuals are produced by the enlarged adipocytes themselves [11] and by macrophages which invades the adipose tissue [12, 13].

Several pathways were already revealed which try to explain the underlying mechanisms: a) The enlargement of the adipose tissue leads to regional hypoxia. Hypoxia is known to elevate the expression of inflammatory cytokines and to attract macrophages [13]. b) The inflammatory mediators produced by the enlarging adipocytes attract macrophages which invade in the tissue and produce additional inflammatory chemokines [11]. c) Obesity-associated pro inflammatory signals like high levels of glucose or lipid intermediates trigger the expression of inactive interleukin-1β (IL-1 β). After a repeated signal IL-1β is activated in an inflammasome complex [14] and subsequently induces the nuclear factor kappa B (NF-κB) pathway leading to an increase of inflammatory mediators [15]. d) The stress level of the endoplasmatic reticulum is increased in obesity through the demanded synthetic rate because of the higher intake of nutrients. This induces pro-inflammatory pathways [16].

One of the major inflammatory mediators involved in obesity is *Interleukin-6* (*IL-6*). Studies showed that the circulating level of *IL-6* is elevated in obese individuals up to two to three fold compared to non-obese [17]. Further, the *IL-6* level directly correlates with adiposity and insulin resistance [18] and the *IL-6* level of the adipose tissue of obese individuals strongly correlates with the circulating c-reactive protein (CRP) level as it was shown by Bastard et al [19].

The expression of *IL-6* is known to be regulated by epigenetic mechanisms. Several studies found correlations between the methylation levels of distinct CpG-sites in the promoter region of *IL-6* and the mRNA expression [20-22] suggesting a mechanistic link. For example, Nile and colleagues investigated peripheral blood mononuclear cells (PBMCs) of patients suffering from rheumatoid arthritis and found a significant lower methylation on the CpG-site -1099 compared to healthy controls [23]. The CpG-sites which we investigated in this study have already been described to be associated with mRNA expression. Dandrea and colleagues found that the demethylating agent 5-aza-2´-deoxycytidine (5-Aza) induces the *IL-6* expression in different pancreatic adenocarcinoma cell lines. This increased expression correlates with the methylation level of distinct CpG-sites. They further suggested the region -666 to -426 as a binding site for the methyl-CpG-binding protein 2 which then condenses the structure of the surrounding chromatin by recruiting histone methyl transferases [21]. Results of a study of Poplutz and colleagues elucidated that the changes of the methylation level in the same region correlates with changes of the *IL-6* expression during differentiation of monocytic HL-60 cells [20].

The objective of this study was to investigate the changes in the DNA methylation of seven CpG-sites located in the promoter region and the first exon of the *IL-6* gene during a weight loss intervention in obese individuals. Further, we looked for methylation differences between study participants who lost more than 5 % of their initial body weight (responders) and who lost less (non-responders).

Materials and Methods

Study design and study participants

52 obese (BMI > 30 kg/m^2) participants were enrolled for this study at the clinic in Eggenburg (Austria). The participants were hospitalised for 12 weeks except for the weekends to lose weight. They received individual counselling and a detailed nutritional plan. The weight loss intervention also included individualised physical activity which was adjusted to every participant's mobility, physical health and preference. Thus, the frequency, intensity and type of physical activity intervention showed a high variety between the study participants. Blood samples were taken by venous punctuation at the beginning and at the end of the study. 32 participants finished the 12 weeks of intervention. For characterisation of the study participants see table 1.

		Before intervention		After 3 months	
	All	**Responders**	**Non-responders**	**Responders**	**Non-responders**
Number	52 (f: 37 m: 15)	—	—	12 (f)	12 (f)
Age [y]	43.2 ± 13.6 (range: 18-67)	50.4 ± 3.6 (range: 24-67)	37.8 ± 4.1 (range: 18-58)	—	—
Weight [kg]	131.1 ± 35.2	128.3 ± 10.3	111.0 ± 4.6	119.1 ± 9.7	108.5 ± 4.5
BMI [kg/m^2]	46.1 ± 9.8	47.1 ± 3.4	40.8 ± 2.2	43.7 ± 3.2	39.9 ± 2.2
Weight loss [kg]	—	—	—	-9.2 ± 0.8	-2.5 ± 0.5
Weight loss [%]	—	—	—	-7.2 ± 0.4	-2.2 ± 0.4

Table 1. Characterisation of the study population.

Ethics statement

This study was approved by the ethics committee of Burgenland (EK-number: 31/2009 TOP 8). All study participants signed a written consent.

Sample preparation and pyrosequencing for *IL-6* DNA methylation

The blood samples were collected into PAXgene Blood DNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland) and the DNA was isolated with the PAXgene Blood DNA Kit (PreAnalytiX). The bisulfite conversion was performed using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) and the converted DNA was quantified with a Pico100 (Picodrop Limited, Hinxton, United Kingdom). The primers for the polymerase chain reaction (PCR) and the pyrosequencing were designed with the PyroMark Assay Design Software 2.0 (Qiagen) using the NCBI reference sequence (gene accession number: NM_000600.2) (table 2). For subsequent sequencing the reverse primers were modified with biotin. The PCR was performed under the following conditions: The 25 µl total volume for each reaction contained 12.5 µl PyroMark 2x PCR Master Mix (Qiagen), 2.5 µl CoralLoad (Qiagen) 10x, 5 pmol of each primer and 10 ng of bisulfite converted DNA.

The cycling program was: 10 min 95°C followed by 45 cycles of each 95°C for 30 sec, 50°C for 60 sec and 72°C for 60 sec and a final elongation for 10 min at 72°C. Subsequently, the PCR products were loaded on a 2 % agarose gel. Pyrosequencing was performed on a PyroMark Q24 MDx (Qiagen) following the suppliers manual. The forward strands of each PCR product were sequenced with specific sequencing primers (table 2).

Statistical analyses

The statistical analyses were done using SPSS Statistics 20 (IBM, Armonk, USA).

For the comparison between the groups and the time points the T-test for dependent and independent samples was used. The correlations between the data sets were calculated applying the Spearman rank correlation. A p-value smaller or equal to 0.05 was considered as significant.

CpG-sites		sequence
-610; -628; -664; -666	forward	
		GGTGAAGAAAGTGGT
+13; +20; +27	forward	
		ATGTTTGAGGTTTATTTTGTT

Table 2. Primer sequences.

Results

Weight loss intervention

From the 52 study participants enrolled, 32 finished the three months of intervention period. 16 lost more than 5 % (average: 7.2 ± 0.4 % or -9.2 ± 0.8 kg) of their initial body weight and thus were defined as responders. This responder group included only 4 men which did not enable an analysis by gender. Thus we focused on the female participants and compared their analyses to twelve female non-responders to avoid a gender bias.

IL-6 methylation

Before the start of the intervention no significant differences in the methylation levels of the investigated CpG-sites could be assessed between the responder and the non-responder group. Further, we did not observe significant differences in the methylation levels between the two sampling time points. However, at the second sampling time point three out of seven investigated CpG-sites showed a significant higher methylation in the responder group than in the non-responder group: CpG -666: 3.18 ± 1.42 % vs. 2.15 ± 0.59 %; p = 0.037; CpG -628: 4.58 ± 1.86 % vs. 3.14 ± 0.78 %; p = 0.042; CpG +13: 5.13 ± 2.68 % vs. 3.12 ± 0.35 %; p = 0.025 (figure 1).

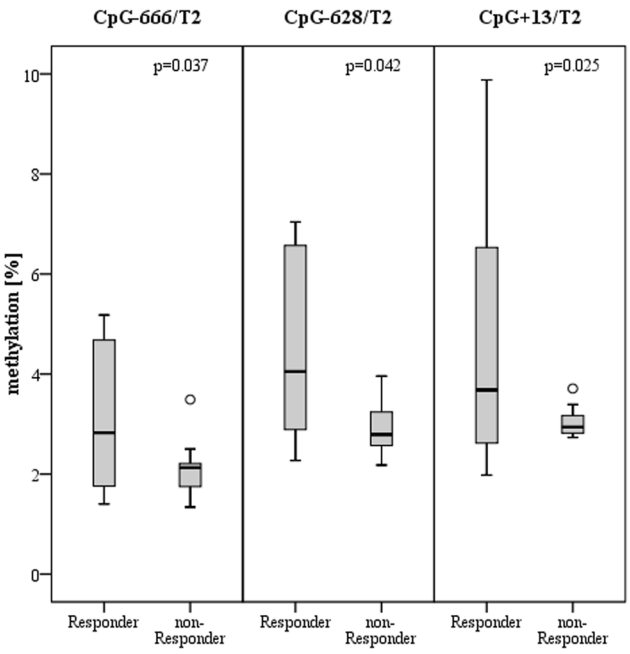

Figure 1. Higher methylation levels in the responder group after the intervention.
The boxplots show a higher methylation level at the CpG-sites -666, -628 and +13 at the second sampling time point after the weight loss intervention in the responder group. Black lines: median; boxes: from 25 to 75 percentile; whiskers: minimal and maximal value respectively; circles: outlier

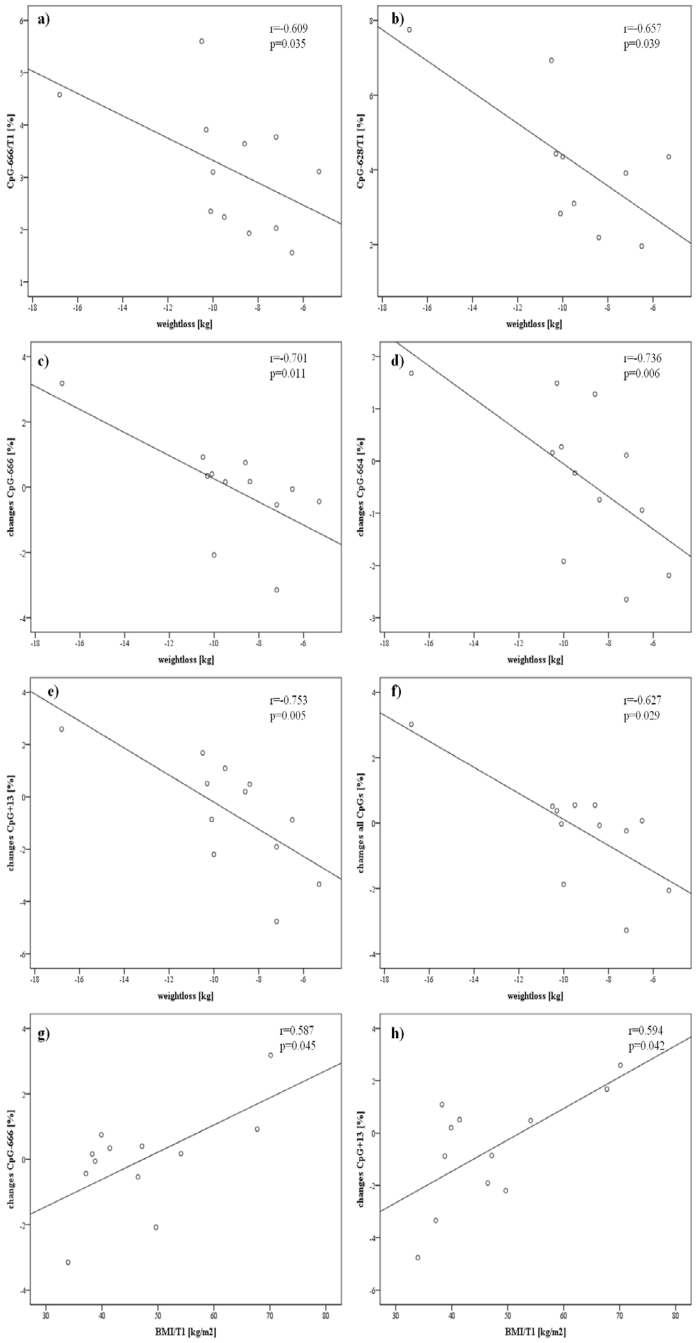

Figure 2. Correlations between CpG-methylation and weight loss and BMI respectively

Correlations between methylation at a) CpG-666 b) CpG-628 at sampling time point 1 (before the intervention) and the weight loss in kg; Correlation between the changes of the methylation from sampling time point 1 to time point 2 (after 3 months of intervention) on c) CpG-666 d) CpG-664 e) CpG+13 f) mean over seven CpGs and the weight loss in kg; Correlation between the changes of the methylation from sampling time point 1 to time point 2 on g) CpG-666 h) CpG+13 and the BMI at time point 1.

Further, the absolute weight loss in kg of the responders correlated negatively with the initial methylation of CpG -666 (r = -0.609; p = 0.035) and CpG -628 (r = -0.657; p = 0.039). We also observed negative correlations between the weight loss and changes in the methylation levels over the study period for CpG -666 (r = -0.701; p = 0.011), CpG -664 (r = -0.736; p = 0.006), CpG +13 (r = -0.753; p = 0.005) and the mean of the seven investigated CpGs (r = -0.627; p = 0.029) (figure 2).

Additionally, the BMI at the beginning of the study showed positive correlations with changes of the methylation level of CpG -666 (r = 0.587; p = 0.045) and CpG +13 (r = 0.594; p = 0.042) (figure 2). No significant correlations between CpG methylations and anthropometric data like body weight or BMI were found in the non-responder group. The mean and the standard deviation of the investigated CpG-sites are displayed in table 3.

CpG-sites	Time point	Responders	Non-responders
-666	1	3.29 ± 1.30 %	2.08 ± 0.69 %
	2	2.95 ± 1.52 %	1.75 ± 0.30 %
-664	1	6.21 ± 1.83 %	4.50 ± 0.56 %
	2	6.10 ± 1.83 %	4.48 ± 0.58 %
-628	1	4.51 ± 2.10 %	2.52 ± 0.84 %
	2	4.36 ± 1.71 %	2.69 ± 0.38 %
-610	1	2.20 ± 1.17 %	1.42 ± 0.56 %
	2	1.94 ± 1.19 %	1.17 ± 0.18 %
+13	1	4.58 ± 2.10 %	3.40 ± 0.81 %
	2	4.11 ± 2.37 %	3.03 ± 0.38 %
+20	1	4.79 ± 2.06 %	3.73 ± 0.89 %
	2	4.21 ± 2.49 %	3.05 ± 0.33 %
+27	1	3.95 ± 1.33 %	3.17 ± 0.46 %
	2	3.47 ± 1.64 %	2.80 ± 0.32 %

Table 3. methylation levels of the investigated CpG-sites.

Discussion

In this study we investigated the methylation levels of seven CpG-sites in the promoter region and the first exon of the *IL-6* gene. The objective of this study is to look for methylation sites which might be suitable biomarkers for the development of obesity and weight loss. In addition, DNA methylation sites might be targets for therapies of obesity and its comorbidities. Since only four men lost more than 5 % of their initial body weight, we excluded the male study participants from further analyses to avoid a gender bias. Even though Zhang and colleagues did not find a differences of *IL-6* CpG methylation between the genders [24], gender differences of DNA methylation are possible and were described for several methylation sites.

Our analyses revealed a higher methylation level at the second sampling time point in responders on the CpG-sites -666, -628 and +13. Further, we found correlations between the weight loss and the initial methylation level of CpG -666 and -628. Significant correlations also exist between weight loss and changes of the methylation level during the intervention period of CpG-sites -666, -664, +13 and the mean of all seven CpGs. Further, the changes of CpG -666 and +13 correlated significantly with the initial BMI. Our findings revealed that the CpG-site -666 is differently methylated in responders compared to non-responders and that its methylation and methylation change respectively correlates with weight loss and BMI. Thus, it is a promising target for further research to develop a predictive biomarker for weight loss response as it was suggested already for *tumour necrosis factor alpha (TNF-α)* [25]. Similar applications are possible for the CpG-site +13.

The region of the *IL-6* promoter which was investigated in our study has also been analysed before but due to the low methylation levels the CpG-sites -666 and +13 were often not further evaluated.

Low methylated CpG-sites and small methylation changes are often neglected in epigenetic studies because some methods does not allow sufficient accuracy and thus significant differences are overseen. The inter-individual variations often overlay the intra-individual changes during an intervention. Analysing of correlations is more robust against this effect. In our study, we saw significant small changes on low methylated CpG-sites especially in the analyses of correlations with anthropometric data.

However, we also detected significant differences between responders and non-responders after the intervention but not before. These results show that the responders are not only more sensitive to weight loss but also to changes of the CpG methylation. It seems that an individualised weight loss intervention influences the methylation of *IL-6* in an individual manner according to the weight loss success. This might be the consequence of a greater methylation plasticity which summarises the methylation and demethylation events of an individual during development and growth. A link between epigenetic plasticity and phenotypical adaptability is the most likely mechanism why different phenotypes can emerge from the same genotype. Possible small methylation differences between responders and non-responders before the intervention might be masked through the high inter-individual variance and became emphasised during the weight loss. The strong correlations between weight loss and changes in the

CpG-methylation further suggest a mechanistic association between these events. Of course, the molecular mechanisms underlying have to be elucidated in further studies as well as if they are a consequence or a reason for successful weight loss.
The methylation differences we saw in this study might also be influenced by cofounders. Smoking was shown to alter DNA methylation but also epigenome wide studies did not describe this effect for *IL-6* [26]. A study of Besingi and Johannson found an enrichment of methylation changes of genes involved in the "positive regulation of *IL-6*-mediated signalling pathway" but not on *IL-6* itself [27]. Stress is also a factor which was associated with obesity and is discussed to influence epigenetic mechanisms but there is no study which shows an altered DNA methylation on the *IL-6* gene. This might also be because of the difficulties to measure stress objectively. Obesity often interacts with other diseases and this than might impact DNA methylation of *IL-6* or the obesity-associated altered *IL-6* methylation modifies the susceptibility to other diseases. For example, a study on asthmatic patients saw that 80% of the patients with a high *IL-6* blood level were obese and showed a more severe asthma [28]. DNA methylation was not assessed but an epigenetic cause is plausible.

Additionally, studies are needed to investigate which impact nutrition or physical activity on its own have on the methylation level of *IL-6*. Whereas the influence of diet is described in several studies [24, 29, 30], no effect of physical activity on *IL-6* methylation was found until now [24].

Milagro and colleagues draw similar conclusions about epigenetic and phenotypical adaptability. They investigated more than 27,000 CpG-sites in PBMCs of obese women before and after eight weeks of an energy-restricted diet. Even though they found that the number of different methylated sites between high and low responders decreased the high responders showed more changes in the CpG-methylations. These results indicate a higher epigenetic plasticity in the high responder group similar to our data. Interestingly, the CpG-sites which show significant methylation differences between high and low responders after the weight loss intervention were located in or near genes involved in inflammatory processes [10]. This points to the crucial role inflammatory process play in obesity and weight loss.

The biological impact of small changes in DNA methylation especially on CpG-sites showing already a low methylation level are discussed controversially. Of course, whether CpG-methylation has an impact on mRNA expression depends on the biological system, the DNA region and its location in relation to a gene or promoter.

We analysed the mRNA blood levels of the study participants but the values were near the limit of detection and thus below the limit of quantification. A possible reason for this low *IL-6* mRNA blood levels might be the medical treatment of obese individuals. In industrialised countries, implications of obesity like elevated blood pressure or elevated blood triglycerides are commonly treated with medication which often also have anti-inflammatory properties.

However, the consequences of a methylation change are not important in the usage as biomarker. DNA methylation was already suggested as biomarker, especially in cancer research [31, 32] but also for weight loss intervention [25, 33, 34]. Especially, the blood level of inflammatory mediators can change quickly in response to sudden inflammatory events and thus cover an obesity-associated low-grade inflammation. Additional, IL-6 levels undergo a circadian rhythm whereas DNA methylation does not [35]. Further, it was described that the sampling method impacts the analysed blood-levels of *IL-6*. Generally, the mRNA and protein expression underlies a multitude of regulatory processes which are affected by many different factors and environmental impacts making it very complex to find associations to the target parameter. Since DNA methylation reacts more slowly it might be a more adequate and robust biomarker. For weight loss, CpG-methylation in the promoter region of *TNF-α* have been already suggest as a predictive biomarker [25].

Of course, further studies are needed to elucidate the underlying mechanisms of these novel findings of correlations between weight change and CpG-methylation. For the development of biomarkers for the progression and intervention success of obesity, we identified the CpG-sites -666 and +13 as promising candidates.

Conclusion

In this study we found different CpG-methylations in the promoter region of *IL-6* in responders and non-responders to an energy-restricted weight loss diet after three months of the intervention. Further, we saw correlations between the methylation levels of distinct CpG-sites before the treatment and the weight loss as well as correlations between the changes of body weight and CpG-methylation. Further studies will be needed to elucidate the underlying mechanisms and consequences of these results. Independently, these findings might provide an important basis for the development of biomarkers for weight loss response and revealed possible targets for prevention- and therapy-strategies. Especially the CpG-sites -666 and +13 are promising candidates.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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