Original Article

Obese status is associated with accelerated DNA methylation change in peripheral blood of senior dogs.

Jumpei Yamazaki [a,b] *, Shinji Meagawa [c], Jaroslav Jelinek [d], Shoko Yokoyama [a], Noriyuki Nagata [e], Masashi Yuki [f], Mitsuyoshi Takiguchi [e]

[a] *Translational Research Unit, Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University, Japan*

[b] *One Health Research Center, Hokkaido University, Japan*

[c] *Department of Pediatrics, MD Anderson Cancer Center, Houston, Tx, USA*

[d] *Coriell Institute for Medical Research, Camden, NJ, USA*

[e] *Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University, Japan*

[f] *Yuki Animal Hospital, 2-99 Kiba-cho, Minato-ku, Aichi, Japan.*

* Corresponding author. Tel.: +81 11 706 5319.

E-mail address: j.yamazaki@vetmed.hokudai.ac.jp (J. Yamazaki).

N18-W9, Kita-ku, Sapporo, Hokkaido, 060-0818

Abstract

Obesity and its associated comorbidities constitute a major and growing health problem worldwide not only involved with people but also dogs and cats. Although few genetic mutations have been associated with obesity in dogs, molecular mechanism remains to be clearly understood. Given the fact that DNA methylation leads to gene expression variability and has plasticity affected by metabolic phenotypes such as obesity in human, the objective of this study is to identify obesity-associated differentially methylated cytosine-phosphate-guanine (CpG) dinucleotide sites in dogs.

With genome-wide DNA methylation analysis using next-generation sequencing for blood samples from fourteen Miniature dachshunds with body condition score (BCS) 4-5 and BCS ≥6, over 100,000 sites could be analysed to identify genomic locations of differentially methylated CpG sites. As a result, 191 differentially methylated CpG sites (89 CpG sites were hypermethylated in BCS ≥6 and 102 were hypermethylated in BCS 4-5) were identified. These sites included promoter regions of Kisspeptin receptor (KISS1R) and Calcyphosine 2 (CAPS2) genes which were subsequently validated by bisulfite-pyrosequencing for another set of 157 dog blood samples. KISS1R methylation levels were found to be higher in BCS ≥6 group than BCS 4-5 in senior (>84 months) dogs. Especially male dogs but not female dogs as well as uncastrated male dogs but not castrated male dogs showed this trend.

DNA methylation of KISS1R gene will be useful for understanding of comprehensive epigenetic change in obese dogs.

Keywords: DNA methylation; Epigenetics; Genome-wide profiling; Obesity, Dog

1. Introduction

Obesity and its associated comorbidities constitute a major and growing health problem worldwide. 1.5 billion people worldwide are overweight or affected by obesity (Wang et al., 2011), which causes 3-4 million deaths based on the higher risk of developing type 2 diabetes (T2DM), cardiovascular disease, metabolic disease, and inflammatory disease (Abdullah et al., 2010; DeFaria Yeh et al., 2007). Obesity is not only involved with people but also dogs and cats. The prevalence of canine obesity is shown to be elevated and related to the presence of overweight/obesity in owners (German, 2006; Montoya-Alonso et al., 2017) . Overweight dogs have a shorter life span and increased susceptibility to diseases such as hip dysplasia and arthritis compared with lean dogs (Lawler et al., 2008). Furthermore, obesity affects the immunometabolic state of dogs resulting in insulin resistance, low-grade chronic inflammation, and dyslipidemia (Herrera Uribe et al., 2016; Tvarijonaviciute et al., 2016; Vitger et al., 2017). Therefore, it has been recognized that obesity is a 'One Health' common problem between people and their pets (Bartges et al., 2017; Tvarijonaviciute et al., 2012).

Molecular mechanism of obesity is primarily investigated only in rodent or human with comparatively fewer data in dogs. Genetic predisposition is rare but identified to involve mutations in fat mass and obesity-associated protein (FTO), melanocortin-4 receptor (MC4R), or peroxisome peroxisome proliferator-activated receptor γ (PPARG) in human as well as dogs (Razquin et al., 2011; van den Berg et al., 2010). In a recent study, a novel deletion in pro-opiomelanocortin (POMC) gene was reported as strongly associated with increased body weight and obesity in Labrador retriever dogs (Mankowska et al., 2017; Raffan et al., 2016). Although there are also reports that described metabolic changes such as adipokine, cytokine, and inflammation markers (Baric Rafaj et al., 2017; Piantedosi et al., 2016; Tvarijonaviciute et al., 2016; Vitger et al., 2017) as well as complete blood count (CBC) and biochemistry

(Radakovich et al., 2017), these are inevitable changes accompanied by obesity and not indicative of the fundamental mechanism that underlies obesity.

DNA methylation at promoter regions of given genes is correlated with gene expression silencing and is now widely accepted to play important roles in many aspects of biology (Bird, 2002). Given the fact that DNA methylation has plasticity and could be affected by metabolic phenotypes, it has recently attracted more attention in changes by obesity. Genome-wide DNA methylation analyses in human identified an association of DNA methylation in an intron 1 of Hipoxia-inducible factor 3 alpha (HIF3A) (Dick et al., 2014), in Insulin receptor substrate 1 (IRS1) target genes (Fradin et al., 2017), and 278 cytosine-phosphate-guanine (CpG) sites (Wahl et al., 2017) with obesity. Importantly, these changes in DNA methylation were correlated with their gene expression, suggesting functional relevance of these specific DNA methylation changes to pathobiological mechanism involved in obesity. On the other hand, there is no studies focusing on the change in DNA methylation by obesity in dogs. Additionally, the fact that species-specific change of DNA methylation involved in obesity possibly exists in dogs dismisses the method of simple extrapolations of the findings from human and rodent model as shown for difference in CpG sites associated with age between human and other mammals (Wang et al., 2020). Genome-wide DNA methylation analysis in dogs is required in this regard. Therefore, the objective of this study is to identify obesity-associated differentially methylated CpG sites in dogs.

2. Materials and methods

2.1. Selection of experimental dogs and blood sampling

We collected residual samples of EDTA-K2-treated blood used for clinical purposes from healthy dogs who had visited a private animal hospital (Yuki Animal Hospital, Aichi, Japan) for vaccination and/or health check. All dogs were considered healthy based on results

of a physical examination and CBC. Fourteen Miniature Dachshund were included for the genome-wide DNA methylation analysis. The age of these dogs ranged from 88 months to 163 months. They included 9 males (3 uncastrated and 6 orchiectomised) and 5 females (2 non-ovariectomised and 3 ovariectomised) (Table 1). 157 dogs were included for the validation cohort. The age of these dogs ranged from 0 months to 197 months. They included 90 males (40 uncastrated and 50 orchiectomised) and 67 females (27 non-ovariectomised and 40 ovariectomised) (Supplementary Table 1). An informed consent was obtained from the owners of each dog for residual blood sample collection to adhere the ethical codes of the Japan Veterinary Medical Association. A nine-point body condition score (BCS) system was employed to allocate scores as follows. BCS 3: Ribs easily palpated and may be visible with no palpable fat. Tops of lumbar vertebrae visible. Pelvic bones becoming prominent. Obvious waist and abdominal tuck. BCS 4: Ribs easily palpable, with minimal fat covering. Waist easily noted, viewed from above. Abdominal tuck evident. BCS 5: Ribs palpable without excess fat covering. Waist observed behind ribs when viewed from above. Abdomen tucked up when viewed from side. BCS 6: Ribs palpable with slight excess fat covering. Waist is discernible viewed from above but is not prominent. Abdominal tuck apparent. BCS 7: Ribs palpable with difficulty; heavy fat cover. Noticeable fat deposits over lumbar area and base of tail. Waist absent or barely visible. Abdominal tuck may be present. BCS 8: Ribs not palpable under very heavy fat cover, or palpable only with significant pressure. Heavy fat deposits over lumbar area and base of tail. Waist absent. No abdominal tuck. Obvious abdominal distention may be present. BCS 9: Massive fat deposits over thorax, spine, and base of tail. Waist and abdominal tuck absent. Fat deposits on neck and limbs. Obvious abdominal distention. Dogs were then categorized into 2 groups based on body condition score: a control group (BCS 4-5) and an overweight/obese group (BCS ≥6) (Laflamme, 1997). Residual 100 to 200 μl of blood samples

were kept at -20 °C degree until genomic DNA extraction using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany).

2.2 Digital restriction enzyme analysis of methylation (DREAM)

Genome-wide DNA methylation analysis was performed with DREAM. DREAM is a method for DNA methylation analysis at tens of thousands of CpG sites across the genome (Jelinek et al., 2012) based on next generation sequencing analysis of methylation-specific signatures created by sequential digestion of genomic DNA with a pair of DNA methylation sensitive/insensitive restriction enzymes (SmaI and XmaI). DREAM could distinguish differences in methylation of >10% with a False Discovery Rate (FDR) of 2.4% (Jelinek et al., 2012). The same principle was previously applied for dogs (Yamazaki et al., 2018) and was performed for 14 blood samples of Miniature dachshunds (Table 1) in this study. Briefly, genomic DNA extracted from the samples was mixed with a set of artificial methylation standards for calibrators (methylation levels of 0%, 25%, 50%, 75% and 100%). These mixes were digested with 100 U of SmaI endonuclease (New England Biolabs, Ipswich, MA) for 8 h at 25 °C. Subsequently, 50 U of XmaI endonuclease (New England Biolabs, Ipswich, MA) were added, and the digestion continued for an additional 16 h at 37 °C. The 3’ recessed ends of the DNA created by XmaI digestion were filled in a dCTP, dGTP, and dATP mix (0.4 mM of each) and 3-dA tails were added to all restriction fragments by Klenow DNA polymerase lacking 3’-to-5’ exonuclease activity (New England Biolabs, Ipswich, MA). Then, Illumina paired-end sequencing adaptors were ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA) followed by size-selection with Agencourt AMPure XP magnetic beads for DNA fragments ranging from 250 bp to 450 bp in size. Size-selected DNA was amplified with paired-end PCR primers (Illumina, San Diego, CA) using KAPA Hifi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA) and 11 cycles of amplification followed by sequencing on NovaSeq

(Illumina, San Diego, CA). Sequencing reads were mapped to SmaI/XmaI sites in the cat genome (CanFam3.1), and signatures corresponding to methylated and unmethylated CpGs were enumerated to calculate methylation frequencies for individual SmaI/XmaI site. The methylation ratio is the ratio of the number of tags starting with CCGGG divided by the total number of tags mapped to a given SmaI/XmaI site. Finally, we corrected methylation levels measured by DREAM based on the values obtained from the spikes in standards. First, we calculated log ratios ln (m/u) and ln (sm/u) for each standard, where m/u is the expected ratio of methylated and unmethylated reads, while sm and u, respectively, are observed numbers of methylated and unmethylated reads. Differences in the 'expected' minus 'observed' log ratios were calculated for each standard. Correction factor c was calculated as an antilog of the average log difference (expected – observed). Corrected methylation values were then computed as $100\% \times [c \times sm/(c \times sm+u)]$ for each CpG site.

We used the University of California, Santa Cruz (UCSC) definition of CpG islands (CGI) (Gardiner-Garden and Frommer, 1987). Promoter regions are defined as being located within 1000 bp from transcription start sites of any genes annotated by Ensembl Gene Predictions – version 99.

2.3. Bisulfite-pyrosequencing

We used bisulfite-pyrosequencing to quantitatively assess DNA methylation (Colella et al., 2003) for promoter regions of canine Kisspeptin receptor (KISS1R) and Calcyphosine 2 (CAPS2) genes. Blood samples from 157 dogs where BCS were available were chosen for bisulfite-pyrosequencing (Supplementary Table 1). Briefly, genomic DNA (500 ng) extracted from the samples was used for bisulfite conversion by using the EZ DNA Methylation-Gold (Zymo Research, Irvine, CA) according to manufacturer's instructions. We selected the primers to amplify fragments that covered the differentially methylated CpG sites identified by Canine

DREAM analysis. Primer sequences and PCR conditions are listed in Supplementary Table 2. We measured levels of DNA methylation as the percentage of bisulfite-resistant cytosines at CpG sites by pyrosequencing using a PSQ24 system with Pyro-Gold reagent Kit (QIAGEN, Hilden, Germany), and the results were analysed using PyroMark Q24 software (QIAGEN, Hilden, Germany).

2.4. Statistical methods

DNA methylation levels between the different groups were compared using the Student's t test. Correlation of DNA methylation levels of KISS1R and CAPS2 with age in months were tested using Pearson correlation coefficients. Adjustment for multiple-testing in Canine DREAM was performed with the procedure of Benjamini and Hochberg (Benjamini and Hochberg, 1995) to identify differential methylation at FDR of 10%. P value < 0.05 was considered statistically significant for DNA methylation levels analysed by bisulfite-pyrosequencing. Statistical analysis was performed using PRISM 6 (Vesion 6.07) (GraphPad Software, Inc., San Diego, CA).

3. Results

Genome-wide DNA methylation analysis

From all 14 samples used for Canine DREAM, 7~15 million unique usable reads after conservative filtering (quality filtered and aligned to the dog genome) were successfully generated for DNA methylation analyses As we used CpG sites that have more than 20 reads to assure quantitative ability, DNA methylation data of approximately 105,000-137,000 CpG sites could be analysed.

We used 79,476 CpG sites where at least 20 reads were obtained in all 14 samples, to analyse the difference in DNA methylation between obese and control dogs. Of those, 28,972

sites are in CGIs and 50,504 sites in non-CGI (NCGIs). Average DNA methylation levels for each CpG sites from six dogs with BCS 4-5 and eight dogs with BCS ≥6 were calculated respectively. By direct comparison of averages of DNA methylation in dogs with BCS 4-5 and BCS ≥6, differential methylation (using the criteria of >10%, $p < 0.04$, false discovery rate 10%) were found at 191 CpG sites (0.2% of the sites analysed) in which 89 CpG sites were hypermethylated in BCS ≥6 group and 102 were hypermethylated in BCS 4-5 group (Figure 1).

Given the fact that DNA methylation at promoter region of genes is associated with gene expression silencing, we filtered the CpG sites at promoter regions that showed differential methylation between BCS 4-5 and BCS ≥6 groups for the following analyses. Among 191 differentially methylated CpG sites, largest difference in DNA methylation were found with the CpG sites located at promoter region of KISS1R (42% in BCS 4-5 and 57% in BCS ≥6, $p=0.0016$) and CAPS2 (73% in BCS 4-5 and 52% in BCS ≥6, $p=0.0007$) genes (Figure 2).

To validate the findings of canine DREAM analysis, we performed bisulfite-pyrosequencing to measure DNA methylation levels of the differentially methylated CpG sites for an additional set of blood samples from 157 dogs also obtained from the same animal hospital. We found that positive correlation of DNA methylation levels of KISS1R and BCS of these dogs as well as inverse correlation of DNA methylation levels of CAPS2 and BCS of these dogs ($p=0.004$ and 0.02 for KISS1R and CAPS2, respectively) (Figure 3a and 3b). Importantly, these DNA methylation trends with BCS were consistent with those in Canine DREAM analysis.

Next, we considered other environmental factors that affect DNA methylation to be eliminated from the analysis. Aging has been well known to strongly correlates with DNA methylation. We also found that DNA methylation levels of these genes were also correlated with age of the dogs (Figure 3c and 3d), suggesting that aging process might affect DNA methylation in addition to effects of obese status. Since BCS ≥6 group were found to be older

than BCS 3-5 group in this study, the difference of DNA methylation between the groups might be overestimated by aging process. Therefore, we analysed the samples of dogs with age of 84 months (7 years old) and older because this age period is known to be “senior” in dogs (Wang et al., 2020) and the samples of dogs used for DREAM analysis also ranged from 88 months and older. As a result, KISS1R methylation levels were still higher in BCS ≥6 group than BCS 3-5 group (Figure 4a). CAPS2 methylation levels were not different between the two groups (Figure 4b).

Finally, we sought for the effect of gender of the sample population and analysed samples from male and female dogs separately. KISS1R methylation was higher in BCS ≥6 male dogs, though it did not reach statistical significance. No difference was observed between BCS ≥6 and BCS 3-5 female dogs (Figure 5). Furthermore, the same trend was found in uncastrated male dogs but not in castrated male dogs in the sample population (Supplementary Figure 1).

4. Discussion

We searched for the differentially CpG sites that were located at promoter regions of dog genes and found KISS1R and CAPS2 as candidates of obesity-associated genes in DNA methylation change. Importantly, the results of a validation cohort using another set of 157 samples subjected to bisulfite-pyrosequencing corresponded to those in the screening cohort in terms of the trend of difference in DNA methylation levels between BCS 3-5 and BCS ≥6 groups, though CAPS2 did not reach statistical significance.

KISS1R, is expressed on gonadotropin-releasing hormone (GnRH) neurons in hypothalamus and also called GPR-54, is a cognate receptor of Kisspeptins (KP) that regulate the reproductive axis (Roseweir and Millar, 2009). Kiss1r showed highly significant puberty-specific differential promoter methylation patterns in puberty in rats (Wyatt et al., 2013),

indicating gene expression change through DNA methylation. Additionally, Kiss1r knock-out mouse showed increased in body weight (Tolson et al., 2014), which is presumably consistent with the finding of the dogs with BCS ≥6 had hypermethylation of these genes in this study. Possible involvement of KISS1R methylation in obesity is also substantiated with the fact that POMC, identified to be mutated in obese Labrador retriever dogs (Raffan et al., 2016), is known to communicate with KP neurons (Backholer et al., 2010). We also found the association between DNA methylation of KISS1R and BCS was more pronounced in male dogs, especially uncastrated male dogs. In fact, human patients with KISS1R mutations showed hypogonadotropic hypogonadism in male (de Roux et al., 2003). A decreased secretion of GnRH from the hypothalamus has been suggested to be one of key factors for hypogonadism in human individuals with obesity and/or type 2 diabetes (Dandona et al., 2008), as evidenced by the low luteinising hormone (LH) and follicle-stimulating hormone (FSH) concentrations in the large majority of these male with low plasma testosterone. As negative correlation was found between testosterone and body mass (Vermeulen et al., 1993), measurement of testosterone concentration of uncastrated male dogs will be interesting to underpin the association of obesity with KISS1R DNA methylation. On the other hand, Kiss1r knockout mice have shown weight gain only in females (Tolson et al., 2014). The effect of gender might vary in the phenotype associated with KISS1R across species of dog, mouse, and human.

The analyses conducted in this study were similar to those in human. Additionally, the number of differential methylation (191 CpG sites) between obese and control dogs in this study is consistent with those identified by Illumina 450k array (450,000 CpG sites to be analysed) frequently used for genome-wide analysis for DNA methylation in human that focused on obesity. For example, Wahl et al. (2017) reported 278 CpG sites as being obesity-associated differentially methylated CpG sites.

In this study, we first analysed blood samples from Miniature Dachshunds to identify differentially methylated CpG sites followed by a validation cohort where blood samples from all breeds were included. Likewise, we found corelation of DNA methylation of KISS1R and BCS only in senior dogs. However, differentially methylated CpG sites associated with obesity may be different in each dog breed or different age period, which could be one of possible reasons that CAPS2 could not be validated in the second set. A larger number of samples in a screening and/or a validation cohort from same breeds or age periods will be needed to address this issue.

Peripheral blood samples were analysed in this study since it is frequently used in human obesity study with an important role in the adverse clinical consequences of obesity (Wahl et al., 2017). However, it is more likely that change, if any, in gene expression through DNA methylation is occurring in the brain, which are not usually available. Although DNA methylation status in somatic tissues such as brain can also be reflected in blood (Gregory et al., 2009; Thompson et al., 2013; Walton et al., 2016), it would be important issue to see if the differential methylation can also be observed in brain tissue, more specifically, on GnRH neurons if possible. Kiss1r is also expressed in multiple non-GnRH brain areas and in several peripheral tissues, including metabolic tissues like fat, liver, and pancreas (Wolfe and Hussain, 2018). Adipocytes and liver cells, which are thought to be involved in metabolism, and thus dysregulation of these cells cause disease. Functional relevance of KISS1R DNA methylation change with gene expression change in these affected tissues should be addressed to contribute to understanding of biology of disease such as diabetes as proposed in human where T2DM is also associated with impaired kisspeptin signalling through glucose intolerance (Tolson et al., 2014).

5. Conclusion

In conclusion, we identified differentially methylated CpG sites that are associated with obese status of senior dogs by a genome-wide analysis of DNA methylation. These findings would be helpful for development of a new biomarker of obesity as well as understanding of biology and mechanism of obesity for both of dogs and human with a translational perspective.

Conflicts of Interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

The authors would like to thank all staff in Yuki Animal hospital (Aichi, Japan) for generously collecting the blood samples from their patients. All dogs and their owners are appreciated as well. This work was supported by Royan Canin Clinical Nutrition Scholarship Program awarded to the recipient, Aozora Yamashita who also helped this project. This work was also supported by and JSPS KAKENHI Grant Number JP18K05964 and 21H02351 (JY). We also acknowledge financial support from the World-leading Innovative and Smart Education (WISE) Program (1801) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Finally, this manuscript is dedicated to the memory of my (JY) sincere friend, Ayako Kamida from Royal Canin Japan.

Appendix: Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi: ...'

Reference

Abdullah, A., Peeters, A., de Courten, M., Stoelwinder, J., 2010. The magnitude of association between overweight and obesity and the risk of diabetes: a meta-analysis of prospective cohort studies. Diabetes Res. Clin. Pract. 89, 309-319.

Backholer, K., Smith, J.T., Rao, A., Pereira, A., Iqbal, J., Ogawa, S., Li, Q., Clarke, I.J., 2010. Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells. Endocrinology 151, 2233-2243.

Baric Rafaj, R., Kules, J., Marinculic, A., Tvarijonaviciute, A., Ceron, J., Mihaljevic, Z., Tumpa, A., Mrljak, V., 2017. Plasma markers of inflammation and hemostatic and endothelial activity in naturally overweight and obese dogs. BMC Vet. Res. 13, 13.

Bartges, J., Kushner, R.F., Michel, K.E., Sallis, R., Day, M.J., 2017. One Health Solutions to Obesity in People and Their Pets. J. Comp. Pathol. 156, 326-333.

Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society: Series B (Methodological) 57, 289-300.

Bird, A., 2002. DNA methylation patterns and epigenetic memory. Genes Dev. 16, 6-21.

Colella, S., Shen, L., Baggerly, K.A., Issa, J.P., Krahe, R., 2003. Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. Biotechniques 35, 146-150.

Dandona, P., Dhindsa, S., Chaudhuri, A., Bhatia, V., Topiwala, S., Mohanty, P., 2008. Hypogonadotrophic hypogonadism in type 2 diabetes, obesity and the metabolic syndrome. Curr. Mol. Med. 8, 816-828.

de Roux, N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L., Milgrom, E., 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc. Natl. Acad. Sci. U. S. A. 100, 10972-10976.

DeFaria Yeh, D., Freeman, M.W., Meigs, J.B., Grant, R.W., 2007. Risk factors for coronary artery disease in patients with elevated high-density lipoprotein cholesterol. Am. J. Cardiol. 99, 1-4.

Dick, K.J., Nelson, C.P., Tsaprouni, L., Sandling, J.K., Aissi, D., Wahl, S., Meduri, E., Morange, P.E., Gagnon, F., Grallert, H., Waldenberger, M., Peters, A., Erdmann, J., Hengstenberg, C., Cambien, F., Goodall, A.H., Ouwehand, W.H., Schunkert, H., Thompson, J.R., Spector, T.D., Gieger, C., Tregouet, D.A., Deloukas, P., Samani, N.J., 2014. DNA methylation and body-mass index: a genome-wide analysis. Lancet 383, 1990-1998.

Fradin, D., Boelle, P.Y., Belot, M.P., Lachaux, F., Tost, J., Besse, C., Deleuze, J.F., De Filippo, G., Bougneres, P., 2017. Genome-Wide Methylation Analysis Identifies Specific Epigenetic Marks In Severely Obese Children. Sci. Rep. 7, 46311.

Gardiner-Garden, M., Frommer, M., 1987. CpG islands in vertebrate genomes. J. Mol. Biol. 196, 261-282.

German, A.J., 2006. The growing problem of obesity in dogs and cats. J. Nutr. 136, 1940S-1946S.

Gregory, S.G., Connelly, J.J., Towers, A.J., Johnson, J., Biscocho, D., Markunas, C.A., Lintas, C., Abramson, R.K., Wright, H.H., Ellis, P., Langford, C.F., Worley, G., Delong, G.R., Murphy, S.K., Cuccaro, M.L., Persico, A., Pericak-Vance, M.A., 2009. Genomic and epigenetic evidence for oxytocin receptor deficiency in autism. BMC Med. 7, 62.

Herrera Uribe, J., Vitger, A.D., Ritz, C., Fredholm, M., Bjornvad, C.R., Cirera, S., 2016. Physical training and weight loss in dogs lead to transcriptional changes in genes involved in the glucose-transport pathway in muscle and adipose tissues. Vet. J. 208, 22-27.

Jelinek, J., Liang, S., Lu, Y., He, R., Ramagli, L.S., Shpall, E.J., Estecio, M.R., Issa, J.P., 2012. Conserved DNA methylation patterns in healthy blood cells and extensive changes in leukemia measured by a new quantitative technique. Epigenetics 7, 1368–1378.

Laflamme, D., 1997. Development and validation of a body condition score system for dogs. Canine practice. 22, 10-15.

Lawler, D.F., Larson, B.T., Ballam, J.M., Smith, G.K., Biery, D.N., Evans, R.H., Greeley, E.H., Segre, M., Stowe, H.D., Kealy, R.D., 2008. Diet restriction and ageing in the dog: major observations over two decades. Br. J. Nutr. 99, 793-805.

Mankowska, M., Krzeminska, P., Graczyk, M., Switonski, M., 2017. Confirmation that a deletion in the POMC gene is associated with body weight of Labrador Retriever dogs. Res. Vet. Sci. 112, 116-118.

Montoya-Alonso, J.A., Bautista-Castano, I., Pena, C., Suarez, L., Juste, M.C., Tvarijonaviciute, A., 2017. Prevalence of Canine Obesity, Obesity-Related Metabolic Dysfunction, and Relationship with Owner Obesity in an Obesogenic Region of Spain. Front Vet Sci 4, 59.

Piantedosi, D., Di Loria, A., Guccione, J., De Rosa, A., Fabbri, S., Cortese, L., Carta, S., Ciaramella, P., 2016. Serum biochemistry profile, inflammatory cytokines, adipokines and cardiovascular findings in obese dogs. Vet. J. 216, 72-78.

Radakovich, L.B., Truelove, M.P., Pannone, S.C., Olver, C.S., Santangelo, K.S., 2017. Clinically healthy overweight and obese dogs differ from lean controls in select CBC and serum biochemistry values. Vet. Clin. Pathol. 46, 221-226.

Raffan, E., Dennis, R.J., O'Donovan, C.J., Becker, J.M., Scott, R.A., Smith, S.P., Withers, D.J., Wood, C.J., Conci, E., Clements, D.N., Summers, K.M., German, A.J., Mellersh, C.S., Arendt, M.L., Iyemere, V.P., Withers, E., Soder, J., Wernersson, S., Andersson, G., Lindblad-Toh, K., Yeo, G.S., O'Rahilly, S., 2016. A Deletion in the Canine POMC Gene Is Associated with Weight and Appetite in Obesity-Prone Labrador Retriever Dogs. Cell Metab. 23, 893-900.

Razquin, C., Marti, A., Martinez, J.A., 2011. Evidences on three relevant obesogenes: MC4R, FTO and PPARgamma. Approaches for personalized nutrition. Mol. Nutr. Food Res. 55, 136-149.

Roseweir, A.K., Millar, R.P., 2009. The role of kisspeptin in the control of gonadotrophin secretion. Hum. Reprod. Update 15, 203-212.

Thompson, T.M., Sharfi, D., Lee, M., Yrigollen, C.M., Naumova, O.Y., Grigorenko, E.L., 2013. Comparison of whole-genome DNA methylation patterns in whole blood, saliva, and lymphoblastoid cell lines. Behav. Genet. 43, 168-176.

Tolson, K.P., Garcia, C., Yen, S., Simonds, S., Stefanidis, A., Lawrence, A., Smith, J.T., Kauffman, A.S., 2014. Impaired kisspeptin signaling decreases metabolism and promotes glucose intolerance and obesity. J. Clin. Invest. 124, 3075-3079.

Tvarijonaviciute, A., Ceron, J.J., de Torre, C., Ljubic, B.B., Holden, S.L., Queau, Y., Morris, P.J., Pastor, J., German, A.J., 2016. Obese dogs with and without obesity-related metabolic dysfunction - a proteomic approach. BMC Vet. Res. 12, 211.

Tvarijonaviciute, A., Ceron, J.J., Holden, S.L., Cuthbertson, D.J., Biourge, V., Morris, P.J., German, A.J., 2012. Obesity-related metabolic dysfunction in dogs: a comparison with human metabolic syndrome. BMC Vet. Res. 8, 147.

van den Berg, L., van den Berg, S.M., Martens, E.E., Hazewinkel, H.A., Dijkshoorn, N.A., Delemarre-van de Waal, H.A., Heutink, P., Leegwater, P.A., Heuven, H.C., 2010. Analysis of variation in the melanocortin-4 receptor gene (mc4r) in Golden Retriever dogs. Anim. Genet. 41, 557.

Vermeulen, A., Kaufman, J.M., Deslypere, J.P., Thomas, G., 1993. Attenuated luteinizing hormone (LH) pulse amplitude but normal LH pulse frequency, and its relation to plasma androgens in hypogonadism of obese men. J. Clin. Endocrinol. Metab. 76, 1140-1146.

Vitger, A.D., Stallknecht, B.M., Miles, J.E., Hansen, S.L., Vegge, A., Bjornvad, C.R., 2017. Immunometabolic parameters in overweight dogs during weight loss with or without an exercise program. Domest. Anim. Endocrinol. 59, 58-66.

Wahl, S., Drong, A., Lehne, B., Loh, M., Scott, W.R., Kunze, S., Tsai, P.C., Ried, J.S., Zhang, W., Yang, Y., Tan, S., Fiorito, G., Franke, L., Guarrera, S., Kasela, S., Kriebel, J., Richmond, R.C., Adamo, M., Afzal, U., Ala-Korpela, M., Albetti, B., Ammerpohl, O., Apperley, J.F., Beekman, M., Bertazzi, P.A., Black, S.L., Blancher, C., Bonder, M.J., Brosch, M., Carstensen-Kirberg, M., de Craen, A.J., de Lusignan, S., Dehghan, A., Elkalaawy, M., Fischer, K., Franco, O.H., Gaunt, T.R., Hampe, J., Hashemi, M., Isaacs, A., Jenkinson, A., Jha, S., Kato, N., Krogh, V., Laffan, M., Meisinger, C., Meitinger, T., Mok, Z.Y., Motta, V., Ng, H.K., Nikolakopoulou, Z., Nteliopoulos, G., Panico, S., Pervjakova, N., Prokisch, H., Rathmann, W., Roden, M., Rota, F., Rozario, M.A., Sandling, J.K., Schafmayer, C., Schramm, K., Siebert, R., Slagboom, P.E., Soininen, P., Stolk, L., Strauch, K., Tai, E.S., Tarantini, L., Thorand, B., Tigchelaar, E.F., Tumino, R., Uitterlinden, A.G., van Duijn, C., van Meurs, J.B., Vineis, P., Wickremasinghe, A.R., Wijmenga, C., Yang, T.P., Yuan, W., Zhernakova, A., Batterham, R.L., Smith, G.D., Deloukas, P., Heijmans, B.T., Herder, C., Hofman, A., Lindgren, C.M., Milani, L., van der Harst, P., Peters, A., Illig, T., Relton, C.L., Waldenberger, M., Jarvelin, M.R., Bollati, V., Soong, R., Spector, T.D., Scott, J., McCarthy, M.I., Elliott, P., Bell, J.T., Matullo, G., Gieger, C., Kooner, J.S., Grallert, H., Chambers, J.C., 2017. Epigenome-wide association study of body mass index, and the adverse outcomes of adiposity. Nature 541, 81-86.

Walton, E., Hass, J., Liu, J., Roffman, J.L., Bernardoni, F., Roessner, V., Kirsch, M., Schackert, G., Calhoun, V., Ehrlich, S., 2016. Correspondence of DNA Methylation Between Blood and Brain Tissue and Its Application to Schizophrenia Research. Schizophr. Bull. 42, 406-414.

Wang, T., Ma, J., Hogan, A.N., Fong, S., Licon, K., Tsui, B., Kreisberg, J.F., Adams, P.D., Carvunis, A.R., Bannasch, D.L., Ostrander, E.A., Ideker, T., 2020. Quantitative Translation of Dog-to-Human Aging by Conserved Remodeling of the DNA Methylome. Cell Syst 11, 176-185 e176.

Wang, Y.C., McPherson, K., Marsh, T., Gortmaker, S.L., Brown, M., 2011. Health and economic burden of the projected obesity trends in the USA and the UK. Lancet 378, 815-825.

Wolfe, A., Hussain, M.A., 2018. The Emerging Role(s) for Kisspeptin in Metabolism in Mammals. Front. Endocrinol. (Lausanne) 9, 184.

Wyatt, A.K., Zavodna, M., Viljoen, J.L., Stanton, J.A., Gemmell, N.J., Jasoni, C.L., 2013. Changes in methylation patterns of kiss1 and kiss1r gene promoters across puberty. Genet Epigenet 5, 51-62.

Yamazaki, J., Jelinek, J., Hisamoto, S., Tsukamoto, A., Inaba, M., 2018. Dynamic changes in DNA methylation patterns in canine lymphoma cell lines demonstrated by genome-wide quantitative DNA methylation analysis. Vet. J. 231, 48-54.

Table 1

Dog information for genome-wide DNA methylation analysis.

Sample	Age (month)	Gender	BCS	KISS1R methylation (%)	CAPS2 methylation (%)
1	88	M	4	42.4	54.1
2	92	F	7	54.7	39.9
3	96	CM	4	46.5	57.3
4	136	F	5	33.5	44.7
5	137	CM	8	57.2	43.1
6	140	M	8	54.5	23
7	142	CM	8	47.7	28.6
8	144	M	5	44.4	39.1
9	159	SF	7	52.9	24.2
10	162	SF	6	64	25.4
11	163	CM	10	59.2	29.7
12	173	SF	5	51.8	55.7
13	183	CM	8	69.6	13.8
14	184	CM	5	31.8	45.8

M: male; CM: castrated (orchiectomised) male; F: female; SF: spayed (ovariectomised) female

Figure Legends

Fig. 1. Difference in DNA methylation levels of BCS 4-5 and BCS ≥6 groups. Volcano plots with the difference in DNA methylation between averages of BCS 4-5 and BCS ≥6 groups on the x-axis, and the unadjusted p-value for each site on the y-axis for sites in CpG island (blue) and non-CpG island (orange). The horizontal line indicates p-value at 0.04.

Fig. 2. Schematic representation of a representative genomic landscape of differentially methylated CpG sites. Original pictures were taken from the University of California, Santa Cruze (UCSC) browser[1]. Promoter regions of dog KISS1R and CAPS2 are shown with DNA methylation levels for the CpG site in both groups along with locations of CpG islands.

Fig. 3. DNA methylation levels of KISS1R and CAPS2 analysed by bisulfite-pyrosequencing. DNA methylation levels of KISS1R (a) and CAPS2 (b) were analysed in 157 samples of blood from dogs of BCS 3-5 and BCS ≥6 groups. Correlations of DNA methylation level of KISS1R (c) or CAPS2 (d) and age of dogs.

Fig. 4. DNA methylation levels of KISS1R and CAPS2 in blood samples from senior dogs. (a) Difference in age of dogs with BCS 3-5 and BCS ≥6. DNA methylation levels of KISS1R (b) and CAPS2 (c) were analysed in 72 samples of blood from senior dogs of BCS 3-5 and BCS ≥6 groups.

Fig. 5. Comparisons of DNA methylation levels of KISS1R and CAPS2 in blood samples from senior male dogs and female dogs. Difference in DNA methylation levels of KISS1R (a)

[1] See: http://genome.ucsc.edu/cgi-bin/hgGateway

and CAPS2 (b) in male dogs with BCS 3-5 and BCS ≥6. Difference in DNA methylation levels of KISS1R (c) and CAPS2 (d) in female dogs with BCS 4-5 and BCS ≥6.

Supplementary Fig. 1. Comparisons of DNA methylation levels of KISS1R and CAPS2 in blood samples from senior male dogs classified by their neuter status. Difference in DNA methylation levels of KISS1R in uncastrated male dogs (a) with BCS 3-5 and BCS ≥6 and castrated male dogs (b) with BCS 4-5 and BCS ≥6.

Figure 1

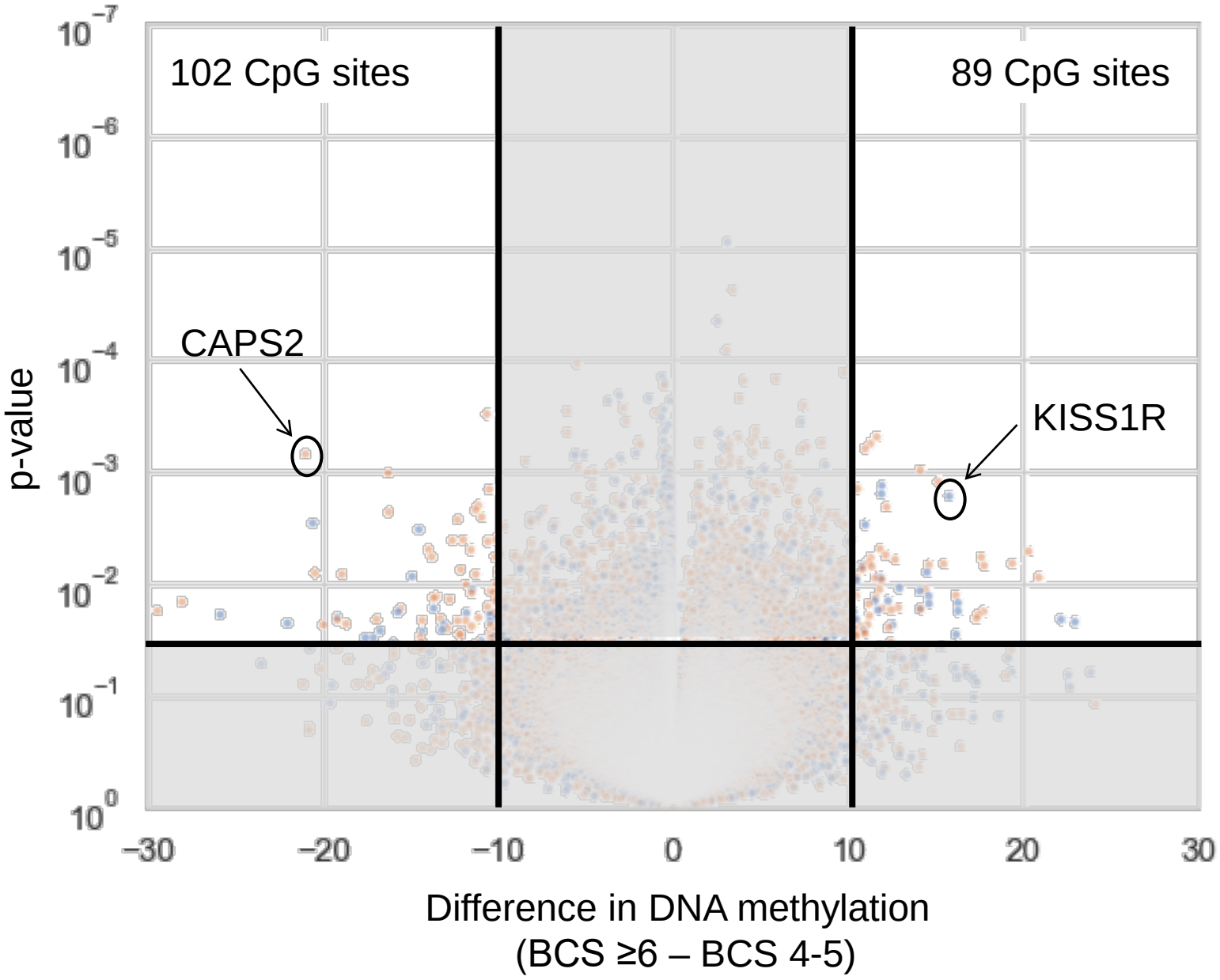

102 CpG sites
89 CpG sites
CAPS2
KISS1R
p-value
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
−30
−20
−10
0
10
20
30
Difference in DNA methylation
(BCS ≥6 – BCS 4-5)

Figure 2

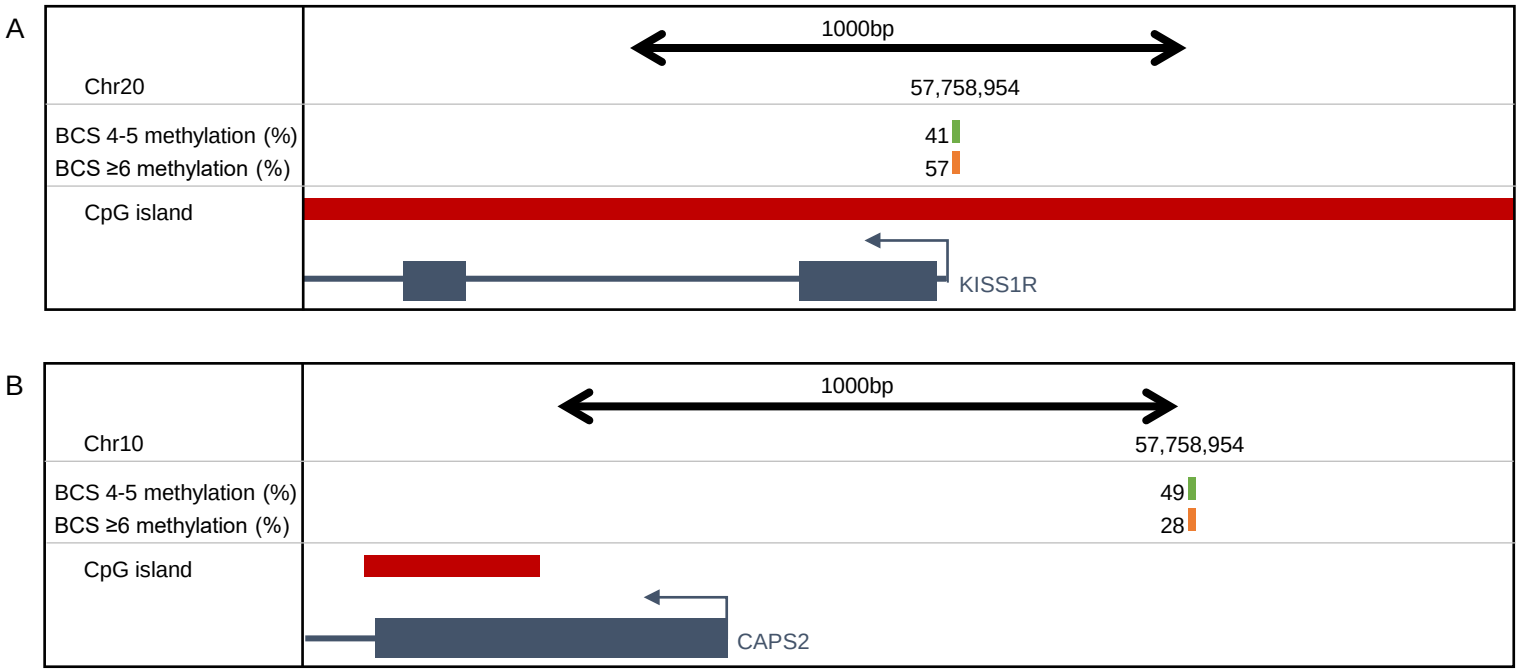

A
1000bp
Chr20
57,758,954
BCS 4-5 methylation (%)
41
BCS ≥6 methylation (%)
57
CpG island
KISS1R
B
1000bp
Chr10
57,758,954
BCS 4-5 methylation (%)
49
BCS ≥6 methylation (%)
28
CpG island
CAPS2

Figure 3

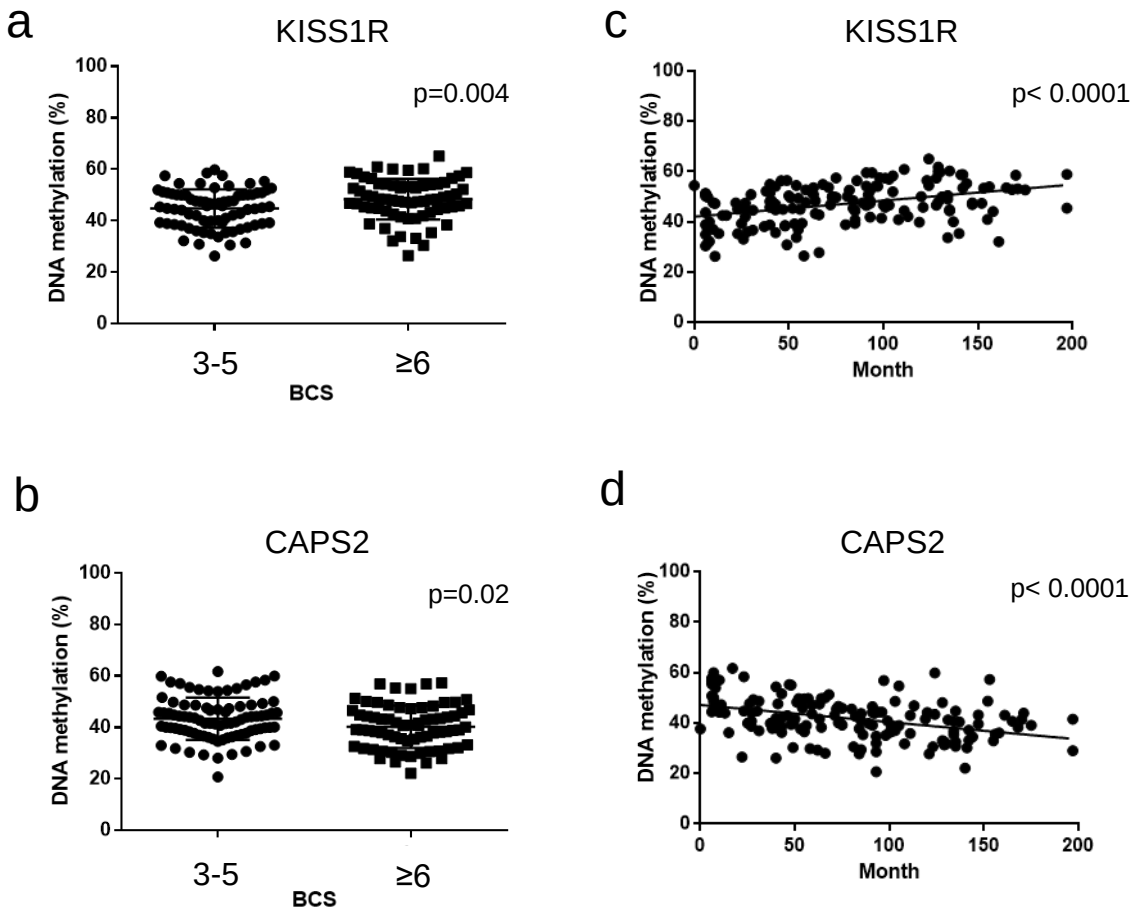

a
KISS1R
p=0.004
DNA methylation (%)
100
80
60
40
20
0
3-5
≥6
BCS
c
KISS1R
p< 0.0001
DNA methylation (%)
0
50
100
150
200
Month
b
CAPS2
p=0.02
DNA methylation (%)
3-5
≥6
BCS
d
CAPS2
p< 0.0001
DNA methylation (%)
Month

Figure 4

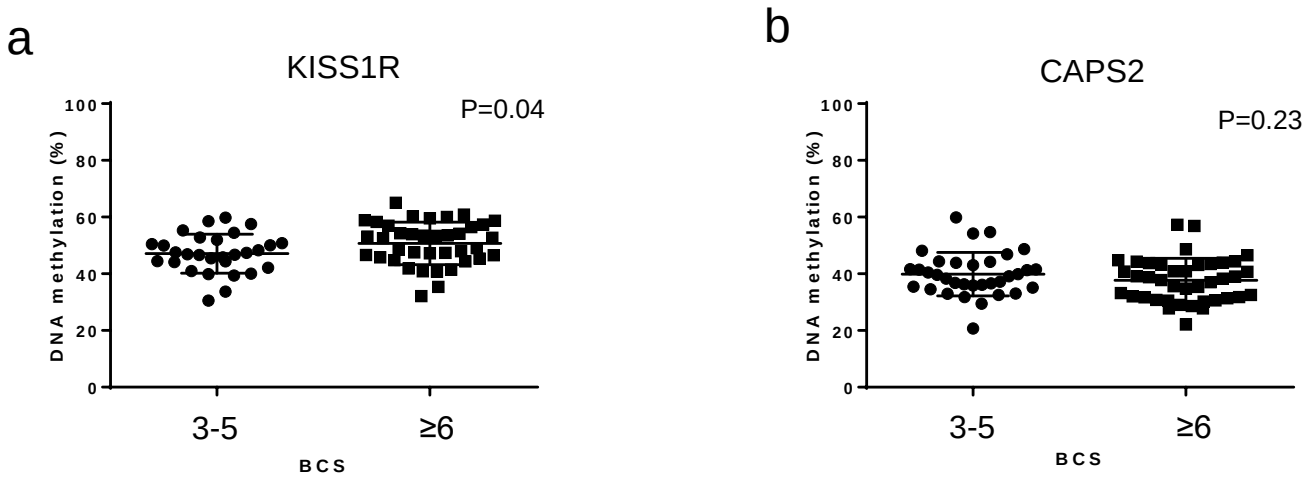
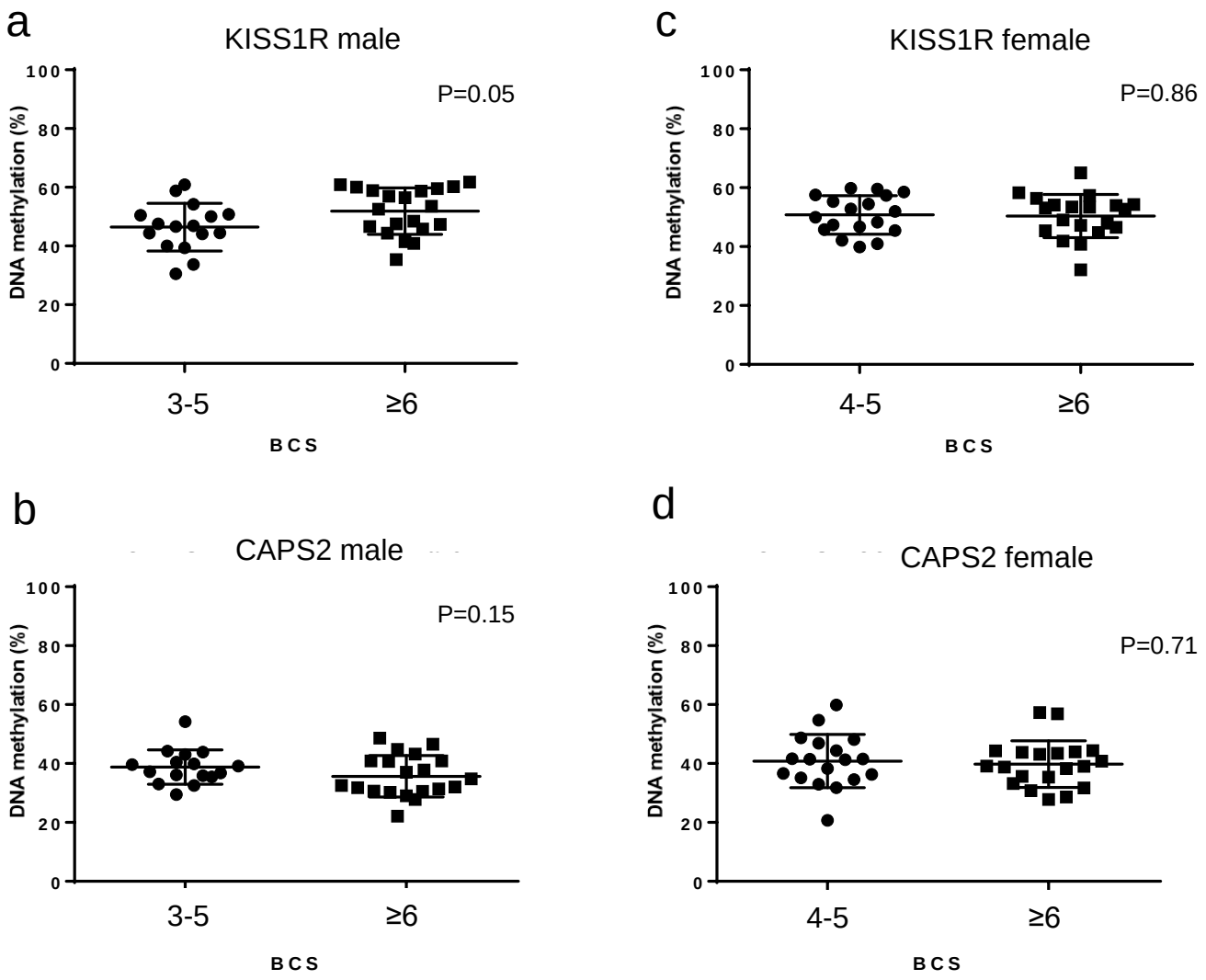

a
KISS1R
P=0.04
DNA methylation (%)
100
80
60
40
20
0
3-5
≥6
BCS
b
CAPS2
P=0.23
DNA methylation (%)
100
80
60
40
20
0
3-5
≥6
BCS

Figure 5


a
KISS1R male
P=0.05
DNA methylation (%)
100
80
60
40
20
0
3-5
≥6
BCS
c
KISS1R female
P=0.86
DNA methylation (%)
100
80
60
40
20
0
4-5
≥6
BCS
b
CAPS2 male
P=0.15
DNA methylation (%)
100
80
60
40
20
0
3-5
≥6
BCS
d
CAPS2 female
P=0.71
DNA methylation (%)
100
80
60
40
20
0
4-5
≥6
BCS

Supplementary Figure 1