**Title**

Epigenome wide association study of thyroid function traits identifies novel associations of fT3 with *KLF9* and *DOT1L*

**Running Title**

EWAS of thyroid function

**Authors (name and institution)**

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The authors have no disclosures.

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## Abstract

**Context**

Circulating concentrations of free triiodothyronine (fT3), free thyroxine (fT4) and thyrotropin (TSH) are partly heritable traits. Recent studies have advanced knowledge of their genetic architecture. Epigenetic modifications, such as DNA methylation (DNAm), may be important in pituitary-thyroid axis regulation and action, but data are limited.

**Objective**

To identify novel associations between fT3, fT4 and TSH and differentially methylated positions (DMPs) in the genome in subjects from two Australian cohorts.

**Method**

We performed an epigenome-wide association study (EWAS) of thyroid function parameters and DNAm using participants from: Brisbane Systems Genetics Study (median age 14.2 years, n=563) and the Raine Study (median age 17.0 years, n=863). Plasma fT3, fT4 and TSH were measured by immunoassay. DNAm levels in blood were assessed using Illumina HumanMethylation450 BeadChip arrays. Analyses employed generalised linear mixed models to test association between DNAm and thyroid function parameters. Data from the two cohorts were meta-analysed.

**Results**

We identified 2 DMPs with epigenome-wide significant ($p<2.4E-7$) associations with TSH and 6 with fT3, including cg00049440 in *KLF9* ($p=2.88E-10$) and cg04173586 in *DOT1L* ($p=2.09E-16$), both genes known to be induced by fT3. All DMPs had a positive association between DNAm and TSH and a negative association between DNAm and fT3. There were no DMPs significantly associated with fT4. We identified 23 differentially methylated regions associated with fT3, fT4 or TSH.

**Conclusions**

This study has demonstrated associations between blood-based DNAm and both fT3 and TSH. This may provide insight into mechanisms underlying thyroid hormone action and/or pituitary-thyroid axis function.

## Introduction

The thyroid synthesises and secretes hormones, triiodothyronine (T3) and thyroxine (T4), required for growth and the regulation of metabolism (1). Circulating levels of thyrotropin (TSH) and thyroid hormones are tightly regulated via the hypothalamus-pituitary-thyroid (HPT) axis; intraindividual variation is less than interindividual variation, suggesting that individuals have unique set points (2). Variations of thyroid function within the population-based normal range have been associated with adverse health outcomes including atrial fibrillation (3), coronary heart disease (4), stroke (5), mood (6), cognitive disorders (7), body mass index (BMI) (8) and overall mortality (4,9). It is therefore important to improve our understanding of mechanisms behind these variations to facilitate better management of patients.

Genetic factors influence interindividual variation in free T3 (fT3), free T4 (fT4) and TSH levels. From studies comparing monozygotic and dizygotic twins, it is estimated that heritability accounts for up to 65% of variation in fT3, fT4 and TSH (10-12). Genome wide association studies (GWAS) have advanced knowledge in this area by identifying a substantial number of genes that may contribute to thyroid function variance (13-16). However, established loci account for only 21% and 33% of genetic variance in fT4 and TSH respectively (13). Epigenetics could provide a link between genes, environmental exposures and differences in fT3, fT4 and TSH levels.

Epigenetics describes mechanisms that control the regulation of gene expression, including DNA methylation (DNAm) and histone modification, among others and that do not involve a change in the DNA nucleotide sequence (17). DNAm is one of the most commonly described

epigenetic mechanisms (18) and involves the addition of a methyl group to a cytosine in a cytosine-phosphate-guanine (CpG) dinucleotide sequence through the action of DNA-methyltransferases (18,19). DNAm can cause major effects on gene expression levels by influencing the binding of regulatory elements to DNA. These epigenetic features further augment transcriptional regulation dictated by the DNA nucleotide coding and are additional critical regulators of gene expression that are considered to make a significant contribution to complex disease susceptibility (20). The methylation profile of a cell changes during differentiation as certain genes are up- and down-regulated, resulting in the formation of a unique methylome in each cell type (21). The relationship between DNAm and gene expression is complex; methylation within a gene promoter typically represses transcription of the gene, whereas DNAm located within exons or introns is frequently associated with active expression and can influence splicing and activity of alternate promoters (22). In line with this, the DNAm landscape has been found to change profoundly during the process of cell differentiation (23) and across the lifespan (24-27).

Epigenome-wide association studies (EWAS) have been used to investigate variations in DNAm genome-wide and explore relationships between methylation and clinical phenotypes (19,28,29). EWAS can identify differentially methylated positions (DMPs), which are individual CpGs that show differential methylation depending on the phenotype, as well as differentially methylated regions (DMRs) which are segments of adjacent CpGs that show overall differential methylation depending on the phenotype (30). Despite the increasing epigenetics literature in other fields including autoimmune thyroid disease, there have, to our knowledge, been no published EWAS with thyroid function markers as a phenotype.

In this study, we performed EWAS to look for associations between blood-based DNAm and circulating levels of fT3, fT4 and TSH from two Australian-based cohorts to provide further insight into mechanisms underlying thyroid hormone action and/or pituitary-thyroid axis function.

## Methods

*Study participants*

Two population-based cohorts were used in the research, the Brisbane Systems Genetics Study (BSGS) and the Raine Study. The participants from BSGS were recruited as part of a prospective study, the Brisbane Longitudinal Twin Study (BLTS), made up of healthy monozygotic and dizygotic twins and triplets, their singleton siblings and their parents who were recruited in Brisbane, Queensland, Australia (31-33). BLTS participants were enlisted by media appeals, word of mouth and by contacting the principals of primary schools in the greater Brisbane area. The study was approved by the Human Research Ethics committee of the Queensland Institute of Medical Research. All participants provided written informed consent.

The Raine Study (formerly known as the Western Australian Pregnancy Cohort Study) is a prospective multigenerational observation study which recruited pregnant women of 16-20 weeks gestation in Perth, Western Australia between 1989 and 1991. It has followed these participants and their offspring (Generation 2) since birth, as described in detail previously (34). The study was approved by the Human Ethics Committee of the University of Western Australia. All participants and their parents or carers provided written informed consent.

The present study uses plasma, clinical assessment and questionnaire data from Generation 2 participants at age 14 (35) and DNAm data from specimens collected at age 17 (36).

In each cohort, participants were excluded if they had peroxidase antibodies (TPOAb) above the reference range (>6IU/mL) or if plasma samples for thyroid function and DNAm were collected more than 5 years apart. In both cohorts, most participants are of self-reported European ancestry and reside in areas of iodine sufficiency (37).

*Laboratory Data*

In both cohorts, fT3, fT4, TSH and TPOAb were measured on securely archived frozen plasma samples by automated immunoassay using the Abbott ARCHITECT analyser (Abbott Diagnostic, Illinois, USA), as previously described (38). DNA methylation profiles were generated from leucocyte DNA from whole-blood samples using the Illumina Infinium HumanMethylation 450 BeadChip array (Illumina, San Diego, CA), as described previously for BSGS (32) and the Raine Study (36). This array interrogates more than 485,000 CpG sites, targeting gene regions and covering 99% of RefSeq genes, CpG islands, among other sites (32,39).

*Statistical analysis*

Linear mixed models were used to test for association between quantile normalised DNA methylation beta values and each of fT3, fT4 and TSH (natural log transformed), adjusting for sex, age, age squared, difference between ages at which thyroid function and DNAm were measured, and white blood cell composition (CD8T, CD4T, NK, Bcell, Mono, Gran). Random intercepts were fitted to account for plate number and sample position variation. A

nested random effects structure with separate intercepts for each subject within zygosity group within family was used to account for relatedness (within monozygotic twins and triplets or among dizygotic twins and triplets and siblings more generally). All analyses were performed using R version 3.5.2 (including packages lme4, qqman, EasyStrata and data.table).

Results from both cohorts were then meta-analysed using METAL software. The epigenome-wide significance threshold was defined as a *P*-value below 2.4E-7, calculated by using a permutation approach which takes into account the correlation structure of CpGs across the genome(40). The threshold for suggestive associations was defined as a *P*-value below 1E-5 following previous studies which used Illumina methylation arrays to identify suggestive DMPs for preliminary consideration and to guide future hypotheses (41,42). R package coMET was used to generate regional association plots (43).

For the detection of DMRs, association tests of fT3, fT4 and TSH were performed using the results from the meta-analysis that had directionally consistent effects. We examined the association between fT3, fT4 and TSH and DMRs using comb-p software with an analysis window of 300 base-pairs, an autocorrelation lag of 300 bases, a seed *P*-value of 0.001 and a minimum of 3 probes per DMR (42). The corrected *P*-values ($P_{cor}$) were reported after Sidak multiple testing correction.

## Results

*Descriptive statistics*

BSGS comprised 563 participants, 53% were male (Table 1). The median age was 14.2 years [IQR 12.1-17.8] and 442 participants (79%) were in the adolescent age range (10-19 years old) (44). The Raine Study included 863 participants, 53% were male, and had a median age of 17.0 years [IQR 16.9-17.2]. All participants were adolescents.

*EWAS Results*

Miami plot of results of the EWAS meta-analysis of the BSGS and the Raine study for fT3 and TSH, comprising up to 483,254 probes, are shown in Fig. 1 and for fT4 in Supplemental Fig. 1 (QQ plots are presented in Supplemental Fig. 2-; $\lambda$ = 1.005, 1.071 and 1.068 for FT3, fT4 and TSH respectively) (45). The meta-analysis found 6 novel epigenome-wide significant DMPs associated with fT3 and 2 associated with TSH (Table 2), while no significant associations were seen for fT4. Details of DMPs that were below the suggestive threshold for association are presented in Supplemental Tables 1-3 (45).

The epigenome-wide significant DMPs associated with fT3 were cg00024471 on chromosome 3 which lies in intron 1 of tumour protein p63 regulated 1 (*TPRG1*) (Supplemental Fig. 3a), cg02183564 located on chromosome 7 within intron 4 of coiled-coil domain containing 146 (*CCDC146)* (Supplemental Fig. 3b), cg00049440 on chromosome 9 within intron 1 of Kruppel like factor 9 (*KLF9*) (Fig. 2a), cg04173586 on chromosome 19 within intron 1 of the disruptor of telomeric silencing 1-like (*DOT1L*) (Fig. 2b) and two probes in intergenic regions: cg01695994 on chromosome 17 and cg19837174 on chromosome 10 (Supplemental Fig. 3c and 3d respectively) (45). All 6 DMPs had a negative association between DNAm and fT3.

The epigenome-wide significant DMPs associated with TSH were cg03445151 on chromosome 2 in an intergenic region (Supplemental Fig 3e) and cg20065905 on chromosome 17 within the 3' untranslated region of forkhead box K2 (*FOXK2)* (Supplemental Fig 3f) (45).

*Differentially methylated region (DMR) analysis*

Data from the DMR analyses highlighted 4 regions associated with fT3, including an intergenic region in chromosome 4 nearest to Sep(O-Phosphoserine) TRNA: Selenocystine TRNA synthase (*SEPSECS)* associated with increased methylation; 11 associated with fT4 and 8 associated with TSH, including one on chromosome 1, within intron 3 of NOD-, LRR- and pyrin domain-containing protein 3 (*NLRP3*) associated with increased methylation (Table 3).

**Discussion**

In this EWAS of thyroid function we identified 6 novel epigenome-wide significant DMPs associated with fT3 and reduced DNAm and 2 associated with TSH and increased DNAm in a meta-analysis of two independent cohorts of healthy participants. This may indicate that altered methylation at these loci plays a role in HPT axis physiology, or since we studied DNAm from blood, may reflect fT3 action on leucocyte DNAm. It is also possible that fT3 or TSH and DNAm at these sites are not causally related, but have a common association with one or more as yet unidentified variables.

Of the DMPs associated with fT3, cg00049440 is within *KLF9,* previously known as basic transcription element binding protein 1, a member of the Krüppel family of zinc-finger transcription factors. These factors bind to GC-rich regions in the genome (46) and regulate

proliferation, differentiation, development and programmed-cell death (47). *KLF9* is a T3 response gene. T3, via nuclear receptor activation, upregulates *KLF9* mRNA. KLF9 then acts as a transcription activator or repressor (48). It is expressed in a large number of tissues and has many roles including in haematopoiesis (18), hippocampal neurogenesis (49), oligodendrocyte differentiation, myelin regeneration (50), intestinal morphogenesis (47) and is downregulated in many cancers as discussed further below. *KLF9* also helps mediate the neuronal protective role of T3 on neurones exposed to hypoxia (51).

*KLF9* is downregulated in multiple cancers including endometrial (52), oesophageal squamous cell carcinoma (SCC) (53), colorectal cancer (54), hepatocellular carcinoma (HCC) (55), breast cancer (56) and neuroblastoma (57). *KLF9* has been demonstrated to suppress neuroblastoma growth and progression (57), inhibit growth, migration and metastasis of oesophageal SCC (53), inhibit breast cancer metastasis (56) and inhibit proliferation and induce apoptosis of HCC (55). Interestingly, a recent study showed that short-term treatment with T3 in rats with HCC caused a prolonged reduction in the number and burden of HCC compared with untreated rats by induction of genes involved in hepatocyte differentiation including *KLF9* (58). Although the authors hypothesise that this may be due to the restoration of the T3/TR axis, effects on DNAm may be responsible for the persistent effects. This could have therapeutic implications for several cancers.

*DOT1L* is a methyltransferase and is an enzyme well-known to methylate H3K79, an activation histone mark. Histone methylation can alter chromatin structure and may recruit effector proteins to certain chromatin regions (59). *DOT1L*, like *KLF9*, is known to be activated by T3. *Xenopus* metamorphosis is a hormone-dependent period of development

when T3 is high. During metamorphosis, T3 activates *DOT1L* which in turn increases methylation of H3K79 in thyroid receptor (TR) targets, thereby inducing chromatin remodeling and allowing gene activation by TR; it also acts as a TR coactivator (60). Functions of *DOT1L* include DNA repair and cell cycle regulation, and it has an essential role in general embryogenesis, chondrogenesis and cardiac development in mice (61). In the present study, most participants were studied during adolescence, a period of developmental change, during which circulating fT3 levels are higher than in adults (38,62), and it is possible that the observed association between fT3 and DNAm of *DOT1L* is relevant to pubertal development.

Both *DOT1L* and *KLF9* have been demonstrated to play an important role in haematopoesis and *KLF9* with T cell lymphopoesis (51,63). Hypothyroidism is known to be a cause of anaemia and, to a lesser extent, reduced lymphocyte count (64-66). Given DNAm is a tissue specific process and *KLF9* and *DOT1L* are known to have a role in the formation of blood cellular components, it is possible that changes in the levels of DNAm in white blood cells form part of the mechanism by which T3 affects haematopoesis rather than being involved in regulation of circulating T3 levels.

Probe cg19837174 on chromosome 10 is within 1.1kbp of *LINC02656*, which is associated with thyroid hormone administration (67). Other identified DMPs associated with fT3 in this EWAS have no currently known associations with thyroid hormones. Of interest, one of the DMPs which reached the suggestive threshold was cg20146909, on chromosome 1 within intron 1 of leucine rich repeat containing 8 family, member D (*LRRC8D*). *LRRC8D* has also been demonstrated to be directly regulated by thyroid hormone (68).

In our study, increased methylation in cg20065905, which is within *FOXK2*, was associated with higher TSH concentrations; the physiological relevance of this requires further elucidation. *FOXK2* is a member of the forkhead box (FOX) family (69). Although other members of this family, including *FOXE1* and *FOXO1*, have established roles in thyroid physiology and thyroid hormone action, *FOXK2* has no known associations with TSH or thyroid hormones (70-72). *FOXK2* has physiological roles in glycolysis, lipid metabolism and mitochondrial function, which may potentially be relevant to thyroid hormone action and has a reciprocal translocation pattern into the nucleus with the FOXO family in response to insulin (69,73). Increased methylation was also seen in cg03445151 with higher TSH concentrations, which lies within an intergenic region with no known significance to thyroid function.

We identified 23 significant DMRs associated with fT3, fT4 or TSH. A DMR associated with fT3 and increased methylation, within an intergenic region in chromosome 4, was closest to *SEPSECS*, which is important in the selenoprotein biosynthesis pathway (74). Iodothyronine deiodinases, which are crucial for thyroid hormone metabolism, are selenoenzymes and require selenocysteine at their catalytic site (75). Selenium deficiency and mutations affecting selenoprotein synthesis are known to affect thyroid hormone levels (75,76).

A DMR within *NLRP3* was associated with TSH and increased methylation. *NLRP3* is part of the NOD-like receptor family, which are inflammasomes that have been associated with autoimmune thyroiditis pathogenesis (77). *NLRP3* activation is also known to be involved in the pathogenesis of ischaemia-reperfusion liver injury and research has demonstrated that

T3 treatment prior to ischaemia-reperfusion in rats reduced the expression of *NLRP3* and liver injury (78).

In the present study, we identified 6 DMPs associated with fT3 at an epigenome-wide level of significance and 2 associated with TSH, but none with fT4. We also identified 23 DMRs, 4 associated with fT3, 11 associated with fT4 and 8 with TSH. Epigenetic modifications such as DMPs are subject to both genetic and environmental factors. Heritability estimates for fT4 and TSH are higher than those for fT3 in most (10,11) but not all studies (12), and GWAS have been more successful in identifying common genetic variants associated with TSH and fT4 than with fT3. Circulating fT3 appears more responsive than TSH or fT4 to environmental influences such as nutritional state (79), childhood growth and pubertal development (38,66) and non-thyroidal illness (80). It is possible that the identification of 6 DMPs for fT3, 2 for TSH and none for fT4, indicates a greater degree of epigenetic influence from environmental factors on circulating fT3 than on TSH or fT4. Alternatively, since T3 is the active thyroid hormone (whereas T4 is largely a prohormone and TSH the major trophic hormone to thyrocytes), the DMPs associated with fT3 in this study may reflect hematopoietic effects of thyroid hormone, reflected in reduced methylation of leucocyte DNA. T3 has been previously demonstrated to have effects on DNAm. Treatment with T3 in rodent primary cortical neurons exposed to hypoxia reduces hypoxia-mediated DNA hypermethylation by upregulating ten-eleven translocation (TET) genes and downregulating DNA methyltransferase (Dnmt)3a and Dnmt3b (51), required for demethylation. Our significant DMPs showed an association of fT3 with reduced DNAm and TSH with increased DNAm and a number of our results are within genes known to be directly regulated by

thyroid hormones. It is possible that DNAm regulation plays an important role in the actions of T3 and regulation of other genes.

This EWAS of thyroid function has identified novel associations between the level of methylation and fT3 at 6 DMPs and TSH at 2 DMPs and provides a basis for further targeted studies, particularly in relation to probes cg00049440 and cg04173586. Strengths of the study include use of a robust, well-characterised technology platform for detection of differential methylation of CpGs and extensive characterisation of community-based cohorts. The study also has limitations. Firstly, we used whole blood to examine DNAm however methylation varies across tissue types (28), therefore DNAm levels in the pituitary, thyroid and peripheral tissues may differ. Secondly, we used a methylation array that targets more than 485,000 selected CpG sites; however it does not provide the high level of coverage that would be achieved using whole genome bisulfite sequencing. Therefore many CpGs that exist in the genome, but which were not present on the array that we used, may be relevant to thyroid function; other approaches such as whole genome bisulfite sequencing may be needed to fully characterise the association between thyroid hormones and DNA methylation. Thirdly, although we adjusted for major confounders in our analysis, residual confounding cannot be excluded. Finally, our study was observational; although we found significant associations between fT3 and DMPs, we cannot establish whether this reflects a causal relationship. Studies in an independent cohort are required to replicate our findings. Larger studies, with substantially increased numbers of study subjects and therefore increased statistical power are likely to identify additional sites of differential methylation associated with thyroid function, as are analytical platforms which survey more CpGs throughout the genome.

In conclusion, we describe 6 novel DMPs with reduced DNAm associated with increased levels of fT3, 2 novel DMPs with increased DNAm associated with increased levels of TSH and 23 DMRs associated with fT3, fT4 or TSH in whole blood of healthy individuals and highlight novel candidate DMPs and genes. Further research is required to establish the roles of these loci in pituitary-thyroid axis physiology and/or thyroid hormone action and their possible relevance to health outcome and disease. Improved understanding of the relationship between methylation and thyroid function may provide therapeutic targets in the future.

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**Data Availability**

The datasets generated during and/or analysed during the current study are not publicly available, but may be accessed through the corresponding author on reasonable request.

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728 **Table 1 –** Descriptive statistics of study participants

| | **BSGS (n=563)** | **Raine Study (n=863)** |
|---|---|---|
| **Age at DNAm collection, median [IQR] (years)** | 14.2 [12.1-17.8] | 17.0 (16.9-17.2) |
| **Male sex (%)** | 53% | 53% |
| **fT3 (pmol/L)** | 4.92 (0.65) | 5.47 (0.60) |
| **fT4 (pmol/L)** | 12.64 (1.36) | 12.25 (1.25) |
| **TSH (mU/L)** | 1.58 (0.88) | 2.08(0.94) |
| **Time between blood sample taken for DNA and thyroid function (years)** | -0.005 (0.19) | 3.08 (0.51) |

729 Data are shown as mean (SD) unless otherwise stated. Abbreviation: DNAm, DNA

730 methylation; IQR, interquartile range; BSGS, Brisbane Systems Genetics Study.

731 **Table 2 –** Details of the epigenome-wide significant differentially methylated positions from the meta-analysis

| Phenotype | CpG site | Chr | Position (hg19) | Nearest gene | Location | BSGS (n) | BSGS β | BSGS *P*-value | Raine Study (n) | Raine Study β | Raine Study *P*-value | Meta-analysis (n) | Meta-analysis β | Meta-analysis *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| fT3 | | | | | | | | | | | | | | |
| | cg00024471 | 3 | 188692547 | *TPRG1* | Intron 1 | 563 | -2.26 | 5.93E-12 | 863 | -0.44 | 4.11E-2 | 1426 | -0.99 | 2.58E-8 |
| | cg00049440 | 9 | 73026643 | *KLF9* | Intron 1 | 563 | -1.87 | 2.44E-7 | 863 | -1.20 | 1.16E-4 | 1426 | -1.49 | 2.88E-10 |
| | cg01695994 | 17 | 80246403 | *LINC01970* | Intergenic | 563 | -2.75 | 1.33E-13 | 863 | -1.19 | 4.81E-5 | 1426 | -1.81 | 3.31E-15 |
| | cg02183564 | 7 | 76874892 | *CCDC146* | Intron 4 | 563 | -2.63 | 2.29E-13 | 863 | -0.38 | 1.66E-1 | 1426 | -1.24 | 9.69E-9 |
| | cg04173586 | 19 | 2167496 | *DOT1L* | Intron 1 | 559 | -2.11 | 4.64E-18 | 863 | -0.64 | 7.89E-4 | 1422 | -1.22 | 2.09E-16 |
| | cg19837174 | 10 | 6389707 | *LINC02656* | Intergenic | 563 | -1.98 | 5.48E-10 | 863 | -0.80 | 1.38E-3 | 1426 | -1.26 | 1.10E-10 |
| TSH | | | | | | | | | | | | | | |
| | cg03445151 | 2 | 23516881 | *AC012506.1* | Intergenic | 559 | 0.21 | 2.33E-4 | 863 | 0.27 | 5.45E-6 | 1422 | 0.24 | 6.19E-9 |
| | cg20065905 | 17 | 80560980 | *FOXK2* | 3’ UTR | 562 | 0.23 | 1.81E-4 | 863 | 0.23 | 2.45E-4 | 1425 | 0.23 | 1.75E-7 |

732 Abbreviations: Chr, chromosome; n, number; BSGS, Brisbane Systems Genetics Study; UTR, untranslated region.

733 **Table 3 –** Statistically significant differentially methylated regions (DMRs) associated with

734 fT3, fT4 or TSH

| Phenotype | Chr | Position (hg19) | Nearest gene | Location | Probes (n) | Unadjusted *P*-value | Sidak *P*-value ($P_{cor}$) | Direction |
|---|---|---|---|---|---|---|---|---|
| fT3 | | | | | | | | |
| | 10 | 124638874-124639167 | *FAM24B* | Intron | 8 | 1.81E-7 | 1.40E-4 | + |
| | 11 | 65546988-65547172 | *AP5B1* | Exon 2 | 4 | 8.18E-9 | 1.01E-5 | - |
| | 3 | 48694451-48694673 | *CELSR3* | Exon | 4 | 2.83E-8 | 2.90E-5 | + |
| | 4 | 25090491-25090665 | *SEPSECS* | Intergenic | 4 | 1.22E-7 | 1.59E-4 | + |
| fT4 | | | | | | | | |
| | 4 | 186732837-186733060 | *SORBS2* | Various | 7 | 5.86E-12 | 5.78E-9 | + |
| | 4 | 206112-206442 | *ZNF876P* | Exon 1 | 6 | 1.06E-8 | 7.08E-6 | - |
| | 22 | 38092643-38093079 | *TRIOBP* | Intron 1 | 10 | 1.57E-9 | 7.90E-7 | + |
| | 20 | 5485144-5485294 | *LINC00654* | Exon 1 | 5 | 8.51E-8 | 1.25E-4 | - |
| | 10 | 135051233-135051475 | *VENTX* | Exon 1 | 8 | 2.89E-7 | 2.63E-4 | - |
| | 12 | 47225979-47226301 | *SLC38A4* | Exon + Intron 1 | 5 | 2.47E-8 | 1.69E-5 | - |
| | 15 | 91473291-91473569 | *UNC45A/HDDC3* | Exon | 6 | 2.21E-7 | 1.75E-4 | + |
| | 17 | 79380493-79380585 | *BAHCC1* | Intron | 3 | 1.81E-5 | 4.23E-2 | + |
| | 2 | 239008929-239009118 | *ESPNL* | Exon 1 | 5 | 1.14E-6 | 1.34E-3 | + |
| | 7 | 4848814-4848939 | *RADIL* | Intron | 3 | 3.18E-8 | 5.60E-5 | + |
| | 22 | 30476089-30476525 | *HORMAD2-AS1* | Exon 1 | 11 | 3.50E-9 | 1.77E-6 | - |
| TSH | | | | | | | | |
| | 11 | 7110074-7110196 | *RBMXL2* | Exon 1 | 5 | 9.78E-10 | 1.83E-6 | - |
| | 1 | 247611448-247611517 | *NLRP3* | Intron | 3 | 9.90E-11 | 3.28E-7 | + |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 12 | 54446253-54446537 | *HOXC4* | Intron 1 | 6 | 7.73E-7 | 6.23E-4 | + |
| | 13 | 36871878-36872246 | CCDC169 | Exon 1 | 9 | 3.39E-7 | 2.10E-4 | - |
| | 13 | 50703549-50703841 | *DLEU1* | Intron | 3 | 7.44E-8 | 5.83E-5 | + |
| | 20 | 3051954-3052345 | *OXT* | Exon 1 | 9 | 1.13E-9 | 6.62E-7 | - |
| | 4 | 118006619-118006825 | *TRAM1L1* | Exon 1 | 6 | 3.82E-9 | 4.24E-6 | - |
| | 5 | 150325954-150326312 | *ZNF300P1* | Exon 1 | 8 | 9.70E-9 | 6.20E-6 | - |

735 Abbreviations: Chr, chromosome; n, number.

**Figure 1.** Miami plot of meta-analysis of EWAS for fT3 (top panel) and for TSH (bottom panel). The x-axis shows chromosome position, and the y-axis the -$\log_{10}$ *P*-values. The epigenome-wide significance threshold is represented by the horizontal red lines (*P* = 2.4E-7) and the threshold for suggestive association shown by the blue horizontal lines (*P* = 1.0E-5).

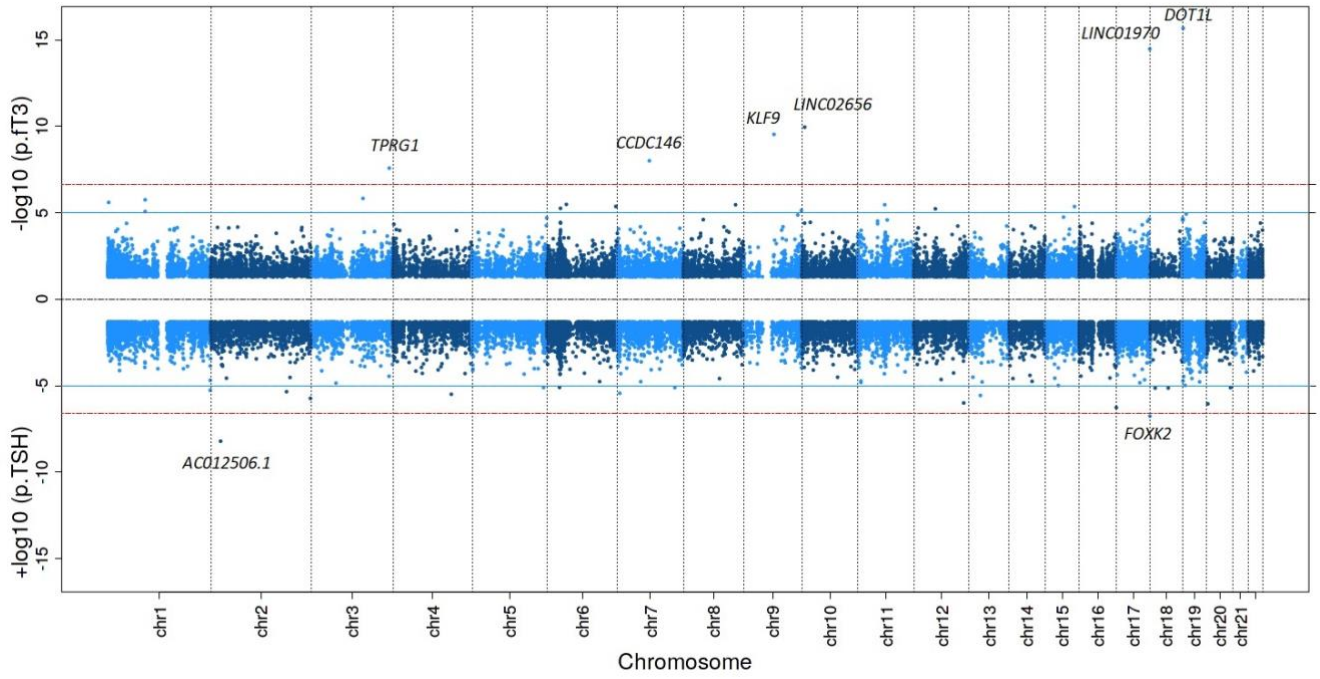
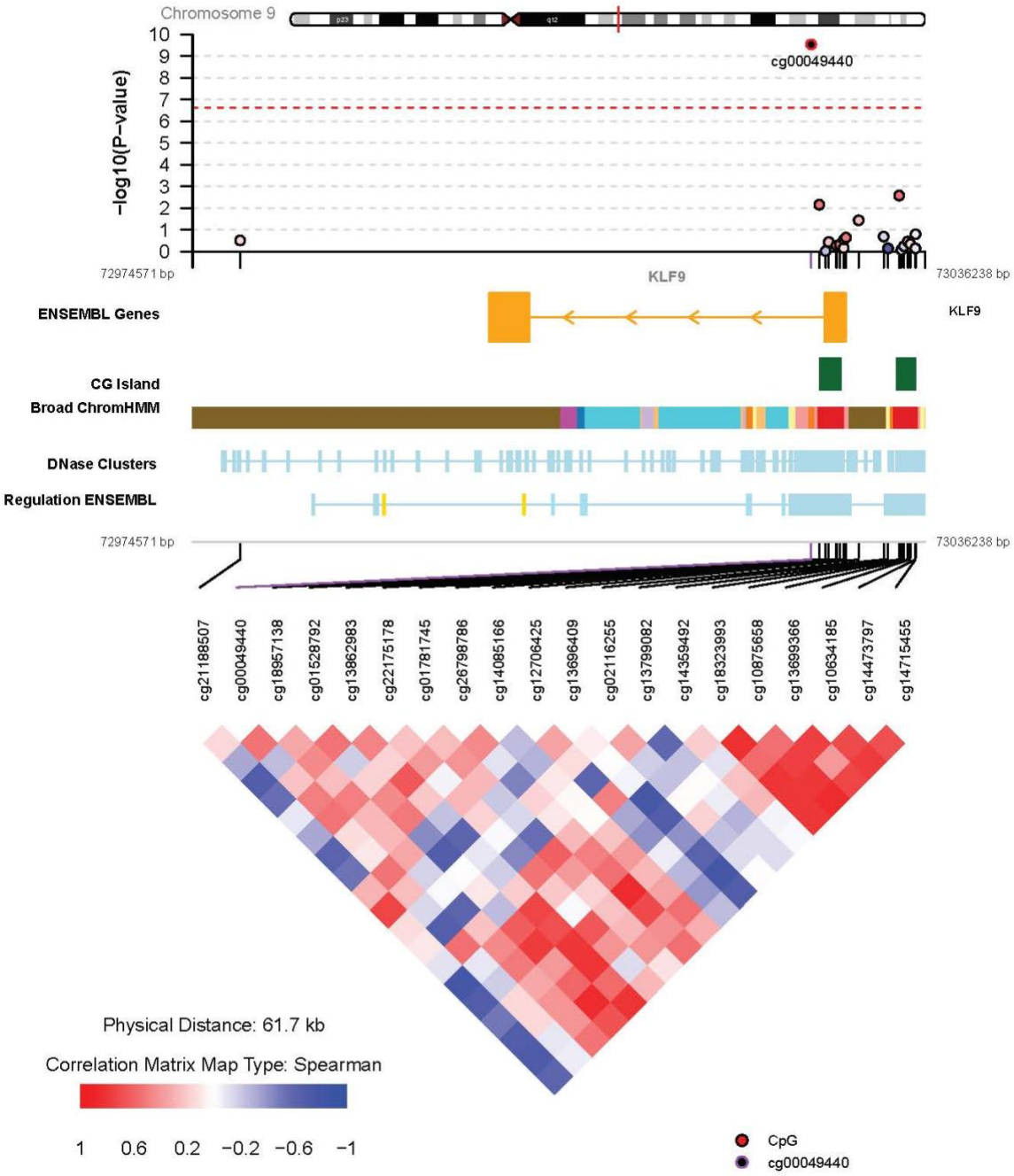


**Figure 2.** Local association plots describing the genomic region for each of the significant DMP (top panel), the functional annotation (middle panel) and the pattern of co-methylation at individual CpG sites at a) cg00049440 and b) cg04713586. Co-methylation relationships are derived from BSGS participants.

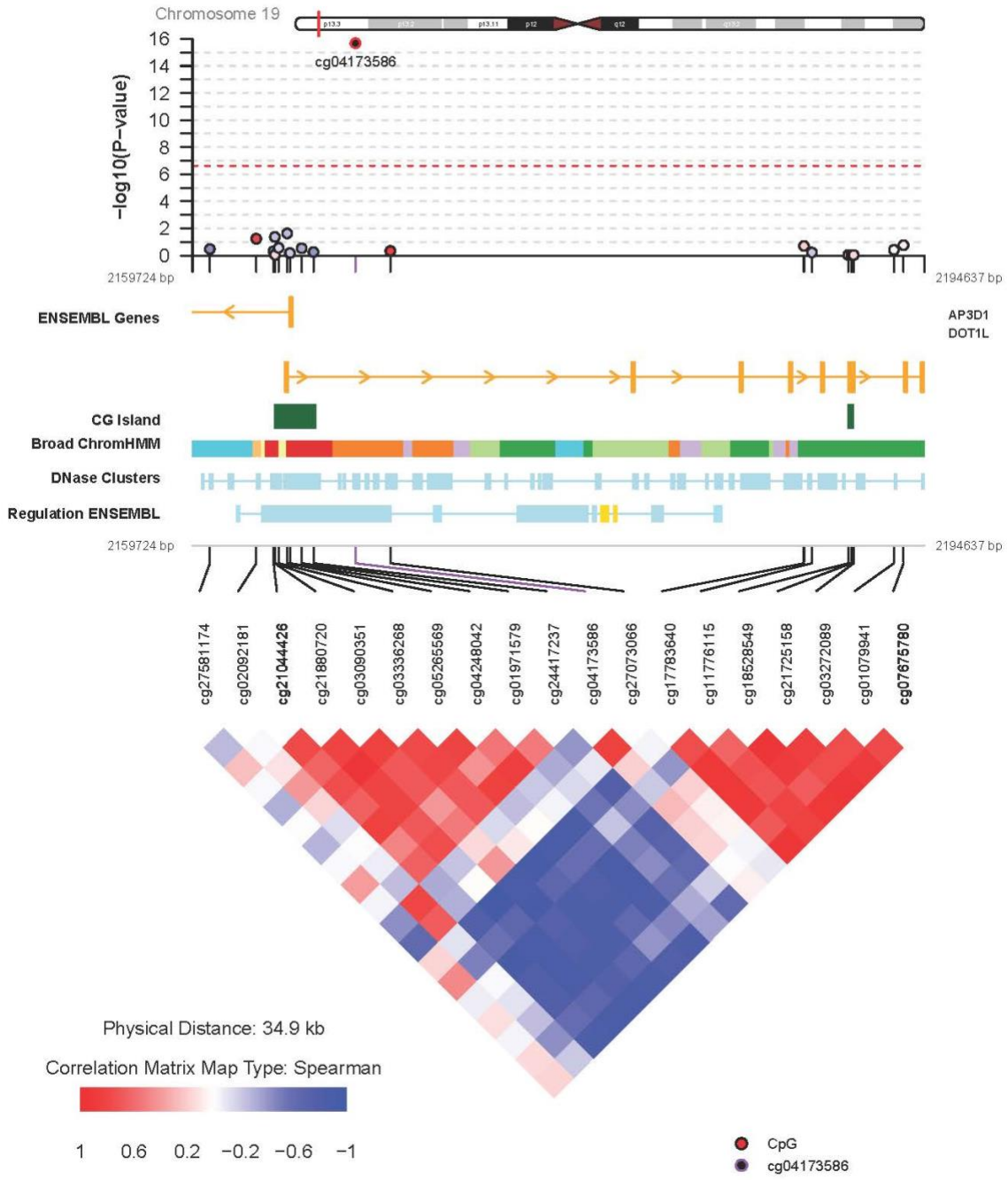
**a)**



750

751

**b)**


Chromosome 19
cg04173586
−log10(P−value)
16
14
12
10
8
6
4
2
0
2159724 bp
2194637 bp
ENSEMBL Genes
AP3D1
DOT1L
CG Island
Broad ChromHMM
DNase Clusters
Regulation ENSEMBL
2159724 bp
2194637 bp
cg27581174
cg02092181
cg21044426
cg21880720
cg03090351
cg03336268
cg05265569
cg04248042
cg01971579
cg24417237
cg04173586
cg27073066
cg17783640
cg11776115
cg18528549
cg21725158
cg03272089
cg01079941
cg07675780
Physical Distance: 34.9 kb
Correlation Matrix Map Type: Spearman
1
0.6
0.2
−0.2
−0.6
−1
CpG
cg04173586