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Epigenome-wide DNA methylation signatures associated with combined hormonal contraceptive use

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Abstract

Background: Estrogens and progestogens exert widespread regulatory effects on gene and protein expression through receptor binding and epigenetic regulation. The systemic epigenetic effects of these steroids are poorly understood. Moreover, the effects of menstrual cycle phase and hormonal contraceptive use on epigenome-wide DNA methylation in relation to depressive symptoms have not been systematically investigated.

Methods: Peripheral blood samples were collected from 116 healthy females aged 18-33 years while using hormonal contraceptives or naturally cycling at the early follicular or periovulatory phase at baseline and follow-up. The hormonal milieu changed only for starters and discontinuers between two sampling points, whereas participants in the naturally cycling groups provided samples at the same menstrual cycle phase at both time points. Linear mixed models were fitted to examine the relationship between hormonal contraceptive use, hormonal group trajectories, and DNA methylation levels across the whole methylome. Differentially methylated CpG sites and regions were further examined in relation to circulating endogenous and synthetic hormone concentrations as well as depressive symptom levels.

Results: Groups representing different hormonal milieus of the early follicular and periovulatory phases, continuous contraceptive use, as well as initiation and discontinuation of contraceptive use, were associated with differential methylation at several CpG sites and regions, including loci annotated to *RUNX1*, *STAT3* and *LCN8*. These DNA methylation signatures showed significant correlations with circulating synthetic hormone concentrations, but not with depressive symptoms and endogenous hormone levels.

Conclusion: This study revealed epigenome-wide DNA methylation correlates of hormonal contraceptive use and trajectories across hormonal groups. While highlighting a potential relationship between synthetic sex hormones and peripheral DNA methylation, the findings warrant replication in larger cohorts and clinical studies to further understand the biological mechanism behind the interaction between sex hormones and women's mental health.

Background

Hormonal contraceptives are one of the most preferred birth control methods worldwide (United Nations, 2022). They shift the hormonal environment from natural ovarian cycling toward relatively stable exposure to synthetic hormones (Lewis et al., 2019). Combined hormonal contraceptives (CHC) contain both a synthetic estrogenic compound, such as ethynyl estradiol (EE) and estetrol (E4), and a progestin (P), such as drospirenone or levonorgestrel. Both endogenous sex steroids, produced primarily by the ovaries, and their exogenous counterparts exert widespread physiological effects by binding to steroid receptors across many systems, including the central nervous system (Bencker et al., 2025; Harding & Heaton, 2022; Hwang et al., 2021; Santos et al., 2026). They regulate cellular function through sex steroid signaling that also interacts with the epigenome (Kovács et al., 2020; Retis-Resendiz et al., 2021). Characterizing epigenetic differences across hormonal milieus, such as distinct menstrual cycle

phases and hormonal contraceptive use, may therefore provide insight into biological mechanisms linking hormonal status to physical and mental health in females.

Fluctuations in endogenous sex steroids across the menstrual cycle and use of hormonal contraceptives have long been associated with mood changes, despite inconsistent findings across studies (Fruzzetti & Fidecicchi, 2020; Romans et al., 2012). Although approximately 60% of women experience premenstrual mood symptoms (Hantsoo et al., 2022) and up to 35% of hormonal contraceptive users report mild affective symptoms (Hampson, 2023), the mechanisms underlying hormone-related variations in mood remain insufficiently understood (Comasco et al., 2025; Song et al., 2023). Given the association between DNA methylation signatures and mood (Paoli et al., 2022) as well as the interaction between epigenetic machinery and sex steroids (Kovács et al., 2020; Retis-Resendiz et al., 2021), peripheral DNA methylation may serve as a potential marker of hormone-associated mood changes.

DNA methylation involves the addition of a methyl group to a cytosine residue, most commonly at CpG sites, where cytosine is followed by guanine, modulating gene expression in response to environmental and physiological signals (Tsankova et al., 2019). DNA methyltransferases (DNMTs) regulate DNA methylation patterns. While DNMT1 maintains existing methylation marks, DNMT3a and DNMT3b establish de novo methylation. Estrogens could affect DNA methylation directly near estrogen response elements via coregulators or indirectly by modulating expression of different DNMTs (Kovács et al., 2020). Although evidence on the relationship between other sex steroids and DNA methylation is scarcer than that for estrogens, progesterone has been shown to regulate the expression of *DNMT3a* and *DNMT3b* (Retis-Resendiz et al., 2021).

Several studies have reported associations between sex steroids and DNA methylation across various tissues. Among them, estradiol (E2) has been studied more abundantly (Bjune et al., 2022; Sklias et al., 2021), including a genome-wide characterization of DNA methylation in the hippocampus upon estradiol administration (Sinha et al., 2021). EE administration has been associated with hypomethylation of the *BDNF* promoter in the hippocampus (Cheong et al., 2018). While maternal progestin exposure has been linked to DNA methylation changes in the offspring brain (Soyer-Gobillard et al., 2023), progesterone (P4) exposure has altered DNA methylation in endometrial fibroblasts (Houshdaran et al., 2020). Epigenome-wide studies in humans reported that E2 and P4 levels have been associated with DNA methylation in male blood at genes linked to mental disorders and nervous system diseases (Harbs et al., 2023), E2 was associated with differential methylation in transgender women assigned male at birth (Shepherd et al., 2022), and menstrual cycle phases were also associated with differential methylation in endometrial tissue, but not in blood (Mortlock et al., 2019).

However, none of the studies examined the effect of CHC use and menstrual cycle phase on DNA methylation in relation to mood symptoms using a hypothesis-free and epigenome-wide approach in repeated female blood samples. The current study sought to determine whether current CHC exposure is associated with DNA methylation differences using time-varying exposure status across all assessments; to examine DNA methylation trajectories associated with continued CHC use, CHC

initiation, and CHC discontinuation relative to naturally cycling females; and to assess cross-sectional baseline DNA methylation differences between menstrual cycle phases and current CHC users in a female cohort comprising naturally cycling females, continuous CHC users, staters, and discontinuers sampled at baseline and follow-up. Additionally, this study examined the relationship between hormonal contraceptive and menstrual cycle-associated differential methylation, depressive symptoms, and circulating endogenous and exogenous sex steroids.

Methods

Participants

The current study included 118 assigned females at birth who were recruited at the Department of Psychiatry, University Hospital Tuebingen, for a larger study investigating neural and behavioral effects of combined oral contraceptives (Kimmig et al., 2025). Participants included German-speaking, healthy females of self-reported European ancestry (including 6 mixed European ancestry) between the ages of 18 and 33.

The participants were categorized into five groups based on their self-reported hormonal status as i) naturally-cycling participants during the early follicular phase (fNC), ii) naturally cycling participants during the periovulatory phase (oNC), iii) long-term combined hormonal contraceptive users (HC), iv) combined hormonal contraceptive users who discontinued (dHC) and v) naturally cycling participants during the early follicular phase who started taking hormonal contraceptives at follow up (sHC; Fig. 1).

Afterward, these categories were confirmed by sex steroid levels measured in blood (Kimmig, Bischofberger, et al., 2022), excluding 2 participants due to the presence of synthetic hormones in blood during a self-reported naturally cycling phase. Except for two participants in the dHC group, one using a vaginal ring and the other a triphasic pill, all hormonal contraceptive users were on a combined monophasic pill with varying formulations involving different progestins such as levonorgestrel or dienogest.

The study was approved by the Ethics Committee of the Medical Faculty of the University of Tuebingen (331/2016BO2 and 103/2024 BO2). Informed consent was obtained from all participants, and they received a monetary reimbursement for their participation.

Protocol

The study protocol, including neuroimaging and behavioral measures, is described in detail by Kimmig, Bischofberger, et al. (2022); Kimmig et al. (2025); Kimmig, Wildgruber, et al. (2022). Briefly, the study applied strict criteria, excluding participants with a history of mental or gynecological disorders, such as endometriosis or polyendocrine metabolic ovarian syndrome (PMOS), pregnancy, or heavy smoking behavior, described as consuming more than 7 cigarettes per day. The measurement sessions were

planned during a specific hormonal phase for each category, such as the active phase from the third day onwards for the hormonal contraceptive users and within 6 days after the onset of menses for the females in the early follicular phase. The ovulation status was confirmed via urine luteinizing hormone tests for the periovulatory group. After an average of four months (range: [2.4, 7.2]), individuals participated in a follow-up session, when the hormonal phase remained the same for fNC, oNC, and HC groups, but varied for discontinuers (dHC) and starters (sHC). Participants were assessed three months after the change in contraceptive use to allow sufficient time for washout after discontinuation or adjustment after initiation. The present hypotheses and analysis approach were preregistered before the epigenome-wide methylation analyses were conducted (osf.io/af5qs).

Measures

Participants completed the Beck Depression Inventory-II (BDI-II) at both assessments, which was used to measure levels of depressive symptoms of the past two weeks by summing 21 items with values ranging from 0 to 3 (Hautzinger et al., 2009). In a subsample of the current cohort, dHC had higher levels of depressive symptoms at baseline compared to HC (Kimmig, Friedrich, et al., 2022).

DNA methylation

Peripheral venous blood was collected in two 9-mL ethylenediaminetetraacetic acid (EDTA) tubes (Sarstedt, Germany) at both baseline and follow-up measurements. The plasma extracted from one EDTA tube for hormonal quantification was replaced with phosphate-buffered saline (PBS) solution and both tubes were stored at -80°C until further processing. DNA was isolated using the QIAamp DNA Blood Maxi kit (Qiagen, Germany) from whole blood, quantified via Qbit 4 Fluorometer (ThermoFisher Scientific, USA), and bisulfite converted with EZ-96 DNA Methylation-Lightning Kit (Zymo Research, USA) following the manufacturer's instructions. DNA methylation levels were determined by means of the Infinium MethylationEPICv2.0 BeadChip array and iScan scanner (Illumina, USA). The bisulfite conversion and subsequent processing were conducted for all samples together at Life & Brain (Bonn, Germany) in accordance with the respective manuals provided by the manufacturer.

The preprocessing of raw methylation data followed an adapted version of the CPACOR pipeline (Lehne et al., 2015) and an open-access pipeline for analysis EPICv2 array (Katrinli, 2024). Raw idat files were processed using the minfi package (Aryee et al., 2014) and ENmix package (Xu et al., 2016). Probes with detection p-values $>1e^{-16}$ or bead counts < 3 were considered low quality and filtered if fewer than 95% of samples passed quality criteria. No sex mismatches and samples with < 95% call rate were detected. Data preprocessing consisted of out-of-band background correction, RELIC dye-bias correction (Xu et al., 2017), quantile normalization, and probe-type bias adjustment using regression on correlated probes (RCP; (Niu et al., 2016)). Additional filtering removed non-cg (e.g., ch), unmapped (chr0), cross-hybridizing probes, and problematic replicate probes (Peters et al., 2024), as well as probes on chromosome Y.

Principal component analysis (PCA) of control probe intensities was performed to adjust for technical biases in the EWAS models (control PCs). Leukocyte compositions were predicted for six cell types (CD8 and CD4 T cells, natural killer (NK) cells, B cells, monocytes and neutrophils) with Robust Partial Correlation (RPC) method (Salas et al., 2018) using the Epidish package (Zheng et al., 2018). PCA of cell type proportions was conducted for each participant to reduce dimensionality. The first two principal components (cell PCs), explaining 80% of total variance, were included in EWAS models. Smoking was adjusted by mean-centered beta values from the cg05575921 probe in the *AHRR* gene (Langdon et al., 2021; Pośpiech et al., 2024).

Hormonal assessment

Plasma concentration of endogenous sex steroids, estradiol (E2) and progesterone (P4), as well as their exogenous counterparts, ethinylestradiol (EE) and progestins (P; levonorgestrel, dienogest and chlormadinone acetate), were assessed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described by Kimmig et al. (2025) and Su et al. (2025).

All CHC formulations used by participants contained EE in combination with a single progestin component. For subsequent analyses, measured concentrations of the different progestins were combined into a single progestin variable. Overall, there was no difference in the six different progestin types reported among long-term users, discontinuers and starters of CHC. (Table 1). For four participants, the reported progestin component could not be quantified with the available assay. For one participant using a norgestimate-containing CHC, the measured levonorgestrel concentration was retained as the available progestin measure, consistent with the metabolism of norgestimate into active metabolites, including levonorgestrel (Hammond et al., 2003). The missing values for EE and P were imputed as 0 in naturally cycling females. Within- and between-group differences in hormonal concentrations were previously described (Kimmig, Friedrich, et al., 2022; Kimmig et al., 2025).

Table 1
Sample Characteristics

Age	**Overall** N = 116[1]	**oNC** N = 26[1]	**fNC** N = 26[1]	**HC** N = 26[1]	**dHC** N = 27[1]	**sHC** N = 11[1]	**p-value**[2]
	24.2 (3.2)	**24.6 (2.9)**	**24.2 (3.4)**	**24.2 (3.0)**	**24.4 (3.7)**	**22.7 (3.0)**	**0.4**
BMI	21.62 (2.64)	21.56 (2.16)	21.17 (1.83)	21.14 (1.71)	23.12 (3.79)	20.31 (2.55)	0.075
Ancestry							0.6
European	110 (95%)	25 (96%)	24 (92%)	26 (100%)	25 (93%)	10 (91%)	
Mixed	6 (5.2%)	1 (3.8%)	2 (7.7%)	0 (0%)	2 (7.4%)	1 (9.1%)	
Education							0.7
1	2 (1.7%)	0 (0%)	0 (0%)	0 (0%)	2 (7.4%)	0 (0%)	
2	73 (63%)	14 (54%)	18 (69%)	17 (65%)	16 (59%)	8 (73%)	
3	41 (35%)	12 (46%)	8 (31%)	9 (35%)	9 (33%)	3 (27%)	
Progestin*							0.7
Chlormadinone acetate	3 (4.7%)	NA	NA	1 (3.8%)	2 (7.4%)	0 (0%)	
Desogestrel	2 (3.1%)	NA	NA	0 (0%)	1 (3.7%)	1 (9.1%)	
Dienogest	24 (38%)	NA	NA	10 (38%)	11 (41%)	3 (27%)	
Etonogestrel	1 (1.6%)	NA	NA	0 (0%)	1 (3.7%)	0 (0%)	
Levonorgestrel	33 (52%)	NA	NA	15 (58%)	11 (41%)	7 (64%)	
Norgestimate	1 (1.6%)	NA	NA	0 (0%)	1 (3.7%)	0 (0%)	

[1]Mean (SD); n (%), [2]Kruskal-Wallis rank sum test; Fisher's exact test. 1: No higher education entrance qualification, 2: Higher education entrance qualification, 3: Higher education degree. *among only CHC users (HC, dHC and sHC)

Statistical Analyses

All statistical analyses were performed in the R statistical environment (version 4.5.1, (R Core Team, 2025)). To investigate epigenome wide-DNA methylation levels in relation with hormonal contraceptive use and hormonal groups, two linear mixed models were applied using the lmer function of lme4 (v.2.0–1 (Bates et al., 2015)) and lmerTest (Kuznetsova et al., 2017) with DNA methylation levels as the independent variable, sampling time (baseline vs follow-up) and hormonal status or their interaction as the fixed effects and subject as a random factor, while controlling for age (Hannum et al., 2013), smoking (Langdon et al., 2021), cell type proportions (i.e., cell PCs) and technical components (first ten control PCs) as described above. The models were not corrected for ancestry or population stratification due to predominantly European ancestry of the sample ($p = 0.6$, Table 1, Supplementary Fig. 1).

To estimate DNA methylation differences associated with current CHC intake, the first model compared samples collected during CHC use with samples collected in the absence of CHC use, using a dummy-coded CHC-status variable. Each sample was coded as exposed when the participant was using CHC at that assessment (CHC+) and as unexposed otherwise (CHC-), allowing the status of starters and discontinuers to vary over time. To test the association between DNA methylation and hormonal group trajectories, the second model included five hormonal groups and their interaction with time as a predictor to examine longitudinal DNA methylation differences across CHC-use trajectories, including continued use, initiation, and discontinuation in comparison to naturally cycling females. Results with convergence issues and singularity are excluded from further analyses.

Additionally, baseline DNA methylation differences across fNC, oNC, and HC groups were evaluated using linear regression with the base function lm. Statistical significance was assessed using coefficient-level p-values extracted from the summary function for the CHC-use model, whereas for longitudinal and baseline hormonal group models, p-values were obtained from the anova function implemented in base R. The summary statistics of the longitudinal hormonal group model, derived by summary function in R, were used to report baseline group differences and group differences in methylation change over time. No additional post hoc pairwise comparisons were performed due to multiple-testing burden.

Genomic inflation was inspected using the qqman package (Turner, 2018). The results were additionally corrected for bias and inflation using *BACON* only in the CHC-use model, for which coefficient-level test statistics were retained (van Iterson et al., 2017). Multiple testing correction was performed using the Benjamini-Hochberg method (False Discovery Rate, FDR) within each model, and probes with FDR-adjusted p-values lower than 0.05 were described as differentially methylated positions (DMPs). Annotation was based on the manufacturer-provided manifest file supplemented with the updated annotations from Peters et al. (2024). Differentially methylated regions (DMRs) were determined using the comb-p tool within the ENmix package, with a seed p-value of 0.01 and a distance of 500 base pairs, encompassing at least 2 CpG sites and a Sidak-corrected p-value lower than 0.05.

Follow-up linear mixed models and linear models were fitted to examine the association between DNA methylation levels at identified DMRs and DMPs and depressive symptoms or circulating sex steroids

using lmer function (Kuznetsova et al., 2017) and the base lm function while accounting for different hormonal groups with additional fixed time and random subject factors for longitudinal designs (DNA methylation ~ hormone/BDI(within) + hormone/BDI(between) + hormonal groups + time + (1| subject)). For DMPs, beta values of individual CpG sites were used, whereas for DMRs, mean beta values across all CpG sites within each region were calculated. All variables were scaled before follow-up analyses to obtain standardized estimates. To disentangle associations with intraindividual hormonal changes from those reflecting between-person differences, hormone concentrations were decomposed into within-person and between-person components. Mean-centered values of each subject represented intraindividual deviations, while person-specific means captured between-person differences. Collinearity was evaluated using variance inflation factors, which were below 2.22 across all models. Additionally, DMPs and DMRs identified in the longitudinal CHC-use models were compared between androgenic and non-androgenic CHC users using Wilcoxon rank sum test. These analyses were corrected for multiple testing using the Benjamini-Hochberg method for each predictor variable (e.g, individual hormones, symptoms or androgenicity) across a set of DMP or DMR.

Results

The groups were similar regarding age, BMI, and education (all $p > 0.07$, Table 1). dHC had higher levels of depressive symptoms at baseline compared to HC, fNC, oNC and sHC groups ($p < 0.05$ for all). Additionally, discontinuers reported decreased levels, while sHC reported increased levels of depressive symptoms at follow-up compared to baseline ($p < 0.001$ for dHC and $p = 0.009$ for sHC; Supplementary Fig. 2). The mean concentration of endogenous and exogenous hormones for each hormonal group confirmed the hormonal profile assigned to that group, with high variation of hormonal levels across individuals (Supplementary Fig. 3). Briefly, oNC had higher endogenous sex steroids than the fNC, HC, dHC, and sHC groups at both time points, and the sHC group had higher EE levels than the HC group at follow-up.

Epigenome-wide association of hormonal contraceptive use

Current CHC intake was associated with 24 DMPs independently of sampling time point (ß: [-0.3, 0.27], $0.04 < R^2 < 0.12$, $p_{FDR} < 0.05$, Supplementary Table 1). Considering moderately early deviation in the QQ-plot with a lambda value of 1.1, the BACON-correction was applied to account for potential inflation (Supplementary Fig. 4), after which 12 DMPs remained (ß: [-0.29, 0.23], pFDR < 0.05, Fig. 2A, Supplementary Table 1). 58.3% of the 12 DMPs were hypomethylated when participants were taking oral contraceptives for at least 3 months (Fig. 3, Supplementary Table 1). None of the DMPs were associated with depressive symptoms or endogenous hormone levels after multiple testing correction ($R^2 < 0.04$ and $p_{FDR} > 0.153$ for all, Supplementary Table 1). However, the methylation levels of 7 DMPs were associated with individual deviations in ethinylestradiol and 5 DMPs with deviations in progestin concentrations, with hormones explaining 0,5 to 3% of variance in methylation levels of the single CpG sites at genes *RUNX1*, *STARD3*, *LCN8*, and *ERC2* (ß: [-0.24, 0.27], $p_{FDR} < 0.038$ for all, Supplementary Table

1). Among them, two CpG sites at *RUNX1* and one site at *STARD3* were inversely associated with both exogenous hormones, while CpG sites at *LCN8* and *ERC2* were positively associated with both hormones.

The DMR analysis revealed 25 DMRs with current CHC use ($p_{šidák} < 0.05$ for all, Fig. 4A, Supplementary Table 2). The mean methylation across any DMR was not significantly associated with depressive symptoms, estradiol, or progesterone levels (Supplementary Table 2). 4 DMRs at *ACAP1*, *RUNX1, PPP1R27* and *STARD3* were negatively associated with changes in ethinylestradiol and progestin concentrations in the blood (ß: [-0.2, -0.07], $R^2 < 0.019$, $p_{FDR} < 0.012$ for all with EE and ß: [-0.27, -0.10], $R^2 < 0.021$, $p_{FDR} < 0.017$ for all with P), whereas DMRs at *TCRB* and *LCN8* were positively associated with variations in both hormones (ß = 0.13 and 0.12, $R^2 = 0.009$ and 0.007 with EE, respectively, $p < 0.001$ with EE for both; ß = 0.17 and 0.15, respectively, R2 = 0.007 and $p \leq 0.001$ for both with P). No differences in methylation levels at the identified DMPs and DMRs were observed between users of androgenic and non-androgenic CHCs after correction for multiple testing (all $p > 0.009$ and $p_{FDR} > 0.140$).

Epigenome-wide association of different hormonal groups

To investigate the association between genome-wide DNA methylation and the five characterized hormonal groups (fNC, oNC, HC, dHC, and sHC) as well as change in methylation over time, the main effects of group variable and the interaction between time and group were examined. The comparison between five hormonal groups revealed no significant DMPs, but 21 DMRs ($p_{šidák} < 0.031$ for all, Fig. 4B, Supplementary Table 3). The mean methylation of these DMRs was not significantly linked to depressive symptoms, estradiol, or progesterone ($R^2 < 0.047$, $p_{FDR} < 0.059$ for all), with only three DMRs remaining in a significant association with synthetic hormones after multiple testing correction. Two were positively linked to within-subject changes in ethinylestradiol and all three differentially to shifts in progestin concentration, explaining little variation in methylation of *LCN8*, *PTH2R*, and an intergenic region (ß: [-0.18, 0,15], $R^2 < 0.007$, $p_{FDR} < 0.042$ for all, Supplementary Table 3).

The interaction of five different hormonal states and sampling time was associated with 10 DMPs (34,3 < Wald's $Chi^2 < 38.7$, $0.01 < R^2 < 0.11$, $p_{FDR} < 0.043$ for all, Fig. 2B, Supplementary Table 4) and one DMR. This DMR included 5 CpG sites that fully overlapped with the DMR associated with current CHC intake at *TCRB* (T-cell receptor beta; $p_{šidák} < 0.001$) and was positively associated with intrapersonal variations in ethinylestradiol and progestin concentrations (ß: 0.14 and 0.17, R^2: 0.01 and 0.008, respectively, $p < 0.001$ for both), but not with depressive symptoms or endogenous hormones. The change in the methylation of top DMP (cg05157335) from baseline to follow-up was significantly different in oNC, dHC and sHC compared to fNC ($p < 0.17$ for all, Fig. 5A). Only DMP that overlapped with the CHC-use model was cg02233071 at the *RUNX1* gene (Fig. 5B). The remaining DMPs exhibited heterogeneous baseline and longitudinal differences in methylation levels relative to the fNC group (Supplementary Fig. 5). None of the DMPs were significantly correlated with depressive symptoms or endogenous sex hormones after multiple testing correction (pFDR > 0,115 for all, Supplementary Table 4). The methylation in cg02233071

at *RUNX1*, cg05157335, and cg11069824 at *CHD5* were negatively linked to changes in ethinylestradiol and progestin concentrations, explaining around 1% of the variance (ß: [-0.23, -0.14], $p_{FDR} < 0.046$ for all, Supplementary Table 4).

Baseline group differences in epigenome-wide DNA methylation

No CpG sites were differentially methylated at baseline across fNC (including fNC and sHC), oNC and HC (including HC and dHC) groups after FDR correction. However, 10 DMRs were identified ($p_{šidák} < 0.006$ for all, Supplementary Fig. 6). One DMR with 3 CpG sites at the *GFER* gene showed an insignificant negative association with estradiol concentrations at baseline (ß = -0,26, p = 0.022, $R^2 = 0.054$, p_{FDR} = 0,208). Other tested associations between mean methylation levels of 10 DMRs and depressive symptoms or sex steroid concentrations did not reach statistical significance, even before FDR correction ($p > 0.053$ for all).

Discussion

This study explored peripheral DNA methylation signatures associated with combined hormonal contraceptive (CHC) use in healthy females and demonstrated differential methylation of several CpG sites and regions across the epigenome in peripheral blood. In addition, methylation patterns differed across menstrual cycle and CHC-use groups and their trajectories from baseline to follow-up, capturing differences associated with between-person natural-cycle phases (fNC and oNC), ongoing CHC use (HC), and within-person CHC initiation (sHC) and discontinuation (dHC). The differentially methylated CpG sites and regions correlated with synthetic hormone concentrations, but not with depressive symptoms, suggesting that changes in depressive symptomatology with the start and discontinuation of CHC were not reflected in CHC-related epigenetic regulation.

The top DMP with current CHC use was annotated to *RUNX1* and the same CpG site was also differentially methylated across five hormonal groups over time, with the latter effect appearing to be primarily driven by CHC use rather than menstrual cycle phase (Fig. 5B). RUNX1 is a member of the Runt-related transcription factor (RUNX) family and involved in hematopoiesis, with its expression levels having been previously shown to be regulated by estradiol and through the menstrual cycle in breast tissue (Haynes et al., 2014; Vivacqua et al., 2015). Nuclear estrogen receptors and RUNX1 have been identified as regulators of differential expression in PMOS and in the development of the female reproductive system (Cunha et al., 2018; Senthilkumar & Arumugam, 2026). Broadening the intricate interaction between estrogens and RUNX1 (Stender et al., 2010; Wall et al., 2013), here we reported epigenetic regulation of the *RUNX1* gene in relation to synthetic sex steroid concentrations (EE and P) in blood. Although it plays a role in neuronal differentiation (Fukui et al., 2018) and methylation levels have been associated with depression, post-traumatic stress disorder (PTSD) and anti-depressant response (Fiori et al., 2026; Wuchty et al., 2021), we did not observe a relationship between DNA methylation levels in significant loci annotated to *RUNX1* and depressive symptom levels in our healthy cohort.

Several genes were consistently identified as differentially methylated in both the current CHC exposure model and the hormonal group models, including *LCN8*, *RUNX1*, *STAT3*, and *TCRB*. *STARD3* was associated with CHC use both in DMP and DMR analyses. STAT3 is a member of the signal transducer and activator of transcription (STAT) protein family and is activated by cytokines to induce an inflammatory state (Yu et al., 2009). Its activation has also been implicated in endometriosis and dienogest was suggested to mitigate the effects of STAT3 activation in endometriotic cells (Choi et al., 2024). However, DNA methylation levels at a single CpG site (cg25867318) were significantly associated with ethinylestradiol levels in our study. Although evidence linking the other identified genes to sex steroids is limited, *LCN8* and *STARD3* point toward lipid or lipophilic molecule transport that may be biologically relevant in the context of steroid-based reproductive hormones. LCN8 belongs to the lipocalin family that binds to small hydrophobic molecules (Wen et al., 2021) and was associated with PTSD (Mehta et al., 2017), while the same probe (cg14145338) in the current study was associated with folate intake in another EWAS (Mandaviya et al., 2019). Similarly, STARD3 is a steroidogenic acute regulatory (StAR) domain protein and involved in intracellular cholesterol transport, which is notable given that cholesterol is the precursor of estrogens and progestogens (Wilhelm et al., 2017). While STAT3 is involved in astrogliosis and neuroinflammation (O'Callaghan et al., 2014), LCN8 and STARD3 are also expressed in the brain (The GTEx Consortium et al., 2020).

Interestingly, despite the role of estrogens as modulators of DNMT expression and activity (Kovács et al., 2020), the present EWAS models did not reveal broad and large effects for methylation differences associated with CHC exposure and different hormonal groups. One possible explanation is the compensatory or adaptive mechanisms against hormonal fluctuations. For instance, hormonal contraceptives and sex hormones may influence DNA methylation only at specific loci (Mostafa Moradi et al., 2025), in specific cell populations (Fetke et al., 2021), or during sensitive developmental windows (Levine et al., 2016), rather than producing broad or large-scale methylation differences detectable in whole blood. This explanation could be considered more plausible given that much of the evidence on interactions between DNMTs and estradiol had been derived from breast cancer cell models (Kovács et al., 2020), while other studies had largely been conducted in animal brain or liver tissue (Fetke et al., 2021; Nugent et al., 2015). An alternative explanation is that DNA methylation captures only one component of molecular regulation. Sex steroids regulate expression via nuclear receptors and various other epigenetic mechanisms such as miRNAs and histone modifications (Kovács et al., 2020). Consistent with this broad regulatory landscape, a recent population-based study revealed extensive effects of hormonal contraceptives on the blood proteome, with effect sizes exceeding those of age, sex and BMI, warranting correction for CHC use in future proteomic studies (Dordevic et al., 2025). On the contrary, the present study did not provide evidence for similarly large effects of CHC on the blood epigenome, suggesting other regulatory mechanisms may be more prominent in mediating the biological effects of CHC use.

The analyses on hormonal group trajectories were limited by the small group sizes and precluded adequately powered post hoc pairwise comparisons. Model coefficients were instead inspected relative to the early follicular naturally cycling (fNC) reference group in an explorative manner. These revealed

that DNA methylation was affected by CHC use status and change in CHC use. Nevertheless, unexpected longitudinal patterns also emerged, including nominal differences in methylation changes among groups that were expected to remain relatively stable over time, such as the peri-ovulatory naturally cycling (oNC) group and continuous CHC users (HC, Fig. 5 and Supplementary Fig. 5). These findings may reflect greater variability in hormonal exposure within these groups compared to fNC, as estradiol concentrations fluctuate around ovulation (Schmalenberger et al., 2021), while circulating synthetic hormone concentrations depend on factors such as the time interval between pill intake and blood sampling and the number of consecutive days after the inactive phase (Hampson, 2023).

The concentration of exogenous steroids in the peripheral blood was linked to DNA methylation levels at identified loci, highlighting their role in CHC-use associated epigenetic regulation. Although intraindividual variations in ethinylestradiol and progestin concentrations were associated with DNA methylation in the same direction, the direction of these associations varied across loci, with increasing synthetic hormone concentrations associated with increased methylation at some loci such as *LCN8* and *TCRB* and decreased methylation at others, *RUNX1* and *STARD3*. However, these analyses were conducted secondary to DMP and DMR analyses and should therefore be replicated in an independent sample.

CHC use was associated with unfavorable mood changes in this sample, with depressive symptoms improving among discontinuers but worsening among starters. However, discontinuers also reported higher depressive symptoms at baseline than continuous users, which might reflect perceived side effects that might have contributed to their decision to discontinue. As mood was not the primary outcome of this study, these findings were not corrected for multiple testing and should be interpreted cautiously. On the other hand, our findings suggested that peripheral DNA methylation patterns may not capture individual vulnerability to hormone-related mood fluctuations in a healthy sample. Although differentially methylated positions and regions were identified in genes of neural and synaptic function, such as *ERC2* and *NCAM1*, none of the identified DMRs and DMPs showed a robust association between depressive symptoms and DNA methylation. Such associations may be more relevant in clinical populations with current or previous depressive disorders, which remains to be investigated in future studies.

Tissue specificity of DNA methylation should also be considered. Given that CHCs are distributed systemically via the bloodstream, the observed methylation differences in blood may primarily reflect peripheral hormonal exposure and cannot be directly extrapolated to brain tissue. Although some methylation signatures show cross-tissue correspondence between blood and brain, none of the identified DMPs were represented in Image-CpG data (Braun et al., 2019) and only 5 were represented in BECon with no concordance ($r_s < 0.5$ for all (Edgar et al., 2017), limiting our interpretation of cross-tissue concordance due to limited coverage of EPICv2 probes in the current databases. Regardless, three CpG sites comprising DMRs in *PIWIL2* (cg10137597 and cg18135555) and TF& *SRPRB* (cg16414030) showed high correlation between live brain tissue and peripheral blood ($r > 0.85$ for all; (Braun et al., 2019). Notably, STAT3, PRXL2A and TF proteins were also reported to be differentially abundant in human

neural progenitor cells after EE and levonorgestrel treatment (Thilmany et al., 2026). This suggests potential convergence between CHC-associated DNA methylation differences observed in peripheral blood and hormone-induced alterations in neuronal protein expression.

To our knowledge, this is the first study to investigate epigenome-wide DNA methylation differences with CHC use in a repeated-measures design and between CHC users and females at different menstrual cycle phases. Given the limited prior evidence, the study was exploratory in nature and was strengthened by the assessment of both endogenous and synthetic sex hormones using state-of-the-art LC-MS/MS methodology. Several limitations should nevertheless be considered. First, participants used different CHC formulations, which may have introduced additional variability into the data and hindered the generalizability of current findings. The sample size limited our ability to adjust for specific formulations or to conduct sufficiently powered post hoc analyses to further clarify group differences over time. Second, although the repeated-measure design provides insight into within-person changes over time, the observational nature of the study precludes causal inference. Establishing whether CHC use and synthetic sex steroids directly induce DNA methylation changes would require experimental or quasi-experimental designs, such as randomized controlled trials and preclinical studies. Third, DNA methylation represents only one layer of molecular regulation, and multi-omic approaches will be necessary to better characterize the biological pathways associated with CHC use and hormone-associated side effects such as mood symptoms (Koh & Hwang, 2019). Additionally, identified DMPs and DMRs, as well as their associations with synthetic hormone concentrations, require replication in independent and larger cohorts. Finally, except for one participant using a vaginal ring, all CHC users used oral contraceptive pills, limiting the generalizability of the findings to other CHC administration routes. However, the participant using a vaginal ring showed hormone concentrations comparable to those observed in pill users, and a recent study reported the same proteomic signatures between oral and vaginal CHC formulations (Dordevic et al., 2025), indicating that these administration routes may share similar biological underpinnings.

Conclusion

This study provides evidence that combined hormonal contraceptive use and different hormonal trajectories are associated with DNA methylation variations in peripheral blood. These DNA methylation signatures were linked to circulating synthetic hormone concentrations, supporting a potential role of exogenous sex steroids in peripheral epigenetic regulation. Additionally, they did not correlate with depressive symptom levels in this healthy sample, suggesting that experimental studies, larger samples with clinical populations and multi-omics approaches are needed to clarify the biological mechanisms underlying hormone-related mood changes in females.

Abbreviations

BDI-II
Beck Depression Inventory-II

BMI
body mass index
CHC
combined hormonal contraceptives
dHC
hormonal contraceptive discontinuers
DMP
differentially methylated position
DMR
differentially methylated region
DNA
deoxyribonucleic acid
DNMT
DNA methyltransferases
E2
estradiol
EDTA
ethylenediaminetetraacetic acid
EE
ethynyl estradiol
EWAS
epigenome-wide association study
FDR
false discovery rate
fNC
early follicular phase
HC
continuous hormonal contraceptive users
LC-MS/MS
liquid chromatography-tandem mass spectrometry
oNC
periovulatory phase
P
progestin
P4
progesterone
PC
principal component
PCA
principal component analysis

PMOS
polyendocrine metabolic ovarian syndrome
PTSD
post-traumatic stress disorder
sHC
hormonal contraceptive starters

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Medical Faculty of the University of Tuebingen (331/2016BO2 and 103/2024 BO2). All participants provided written informed consent.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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Author contributions

MNM processed blood samples, conducted preprocessing and statistical analyses, prepared visualizations, and drafted the original manuscript. LZ developed the analysis scripts and provided methodological guidance. SE assisted with the analyses. ACSK, EC, BD and VN conceptualized the study, acquired funding and supervised the research. ACSK conducted participant recruitment and data collection, curated the data and coordinated the planning and execution of the research project. All authors reviewed and revised the manuscript and approved the final version.

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Availability of data and materials

The datasets analyzed during the current study are not publicly available due to data protection considerations and participant consent restrictions but are available from the corresponding author on reasonable request.

References

1. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, Irizarry RA (2014) Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics 30(10):1363–1369. https://doi.org/10.1093/bioinformatics/btu049
2. Bates D, Mächler M, Bolker B, Walker S (2015) Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw 67(1):1–48. https://doi.org/10.18637/jss.v067.i01
3. Bencker C, Gschwandtner L, Nayman S, Grikšienė R, Nguyen B, Nater UM, Guennoun R, Sundström-Poromaa I, Pletzer B, Bixo M, Comasco E (2025) Progestagens and progesterone receptor modulation: Effects on the brain, mood, stress, and cognition in females. Front Neuroendocr 76:101160. https://doi.org/10.1016/j.yfrne.2024.101160
4. Bjune J-I, Strømland PP, Jersin RÅ, Mellgren G, Dankel SN (2022) Metabolic and Epigenetic Regulation by Estrogen in Adipocytes [Mini Review]. *Frontiers in Endocrinology*, *Volume 13–2022*. https://doi.org/10.3389/fendo.2022.828780
5. Braun PR, Han S, Hing B, Nagahama Y, Gaul LN, Heinzman JT, Grossbach AJ, Close L, Dlouhy BJ, Howard MA, Kawasaki H, Potash JB, Shinozaki G (2019) Genome-wide DNA methylation comparison between live human brain and peripheral tissues within individuals. Translational Psychiatry 9(1):47. https://doi.org/10.1038/s41398-019-0376-y
6. Cheong A, Johnson SA, Howald EC, Ellersieck MR, Camacho L, Lewis SM, Vanlandingham MM, Ying J, Ho SM, Rosenfeld CS (2018) Gene expression and DNA methylation changes in the hypothalamus and hippocampus of adult rats developmentally exposed to bisphenol A or ethinyl estradiol: a

CLARITY-BPA consortium study. Epigenetics 13(7):704–720. https://doi.org/10.1080/15592294.2018.1497388

7. Choi J, Jo M, Lee E, Kim SE, Lee D-Y, Choi D (2024) Dienogest attenuates STAT3 activation in ovarian endometriotic cysts. Eur J Obstet Gynecol Reproductive Biology 294:217–221. https://doi.org/10.1016/j.ejogrb.2024.01.013
8. Comasco E, Epperson CN, Kulkarni J (2025) Psychiatric symptoms on the ovarian hormone roller-coaster. Br J Psychiatry 226(6):329–330. https://doi.org/10.1192/bjp.2025.10298
9. Cunha GR, Robboy SJ, Kurita T, Isaacson D, Shen J, Cao M, Baskin LS (2018) Development of the human female reproductive tract. Differentiation 103:46–65. https://doi.org/10.1016/j.diff.2018.09.001
10. Dordevic N, Dierks C, Hantikainen E, Farztdinov V, Amari F, Hernandes V, De Grandi V, Domingues A, Shomroni FS, Textoris-Taube O, Bahr K, Schmid V, Demuth H, Kurth I, Mülleder F, Pramstaller M, Rainer PP, J., Ralser M (2025) Extensive modulation of the circulating blood proteome by hormonal contraceptive use across two population studies. Commun Med 5(1):131. https://doi.org/10.1038/s43856-025-00856-0
11. Edgar RD, Jones MJ, Meaney MJ, Turecki G, Kobor MS (2017) BECon: a tool for interpreting DNA methylation findings from blood in the context of brain. Transl Psychiatry 7(8):e1187. https://doi.org/10.1038/tp.2017.171
12. Fetke JK, Martinson JW, Flick RW, Huang W, Bencic DC, See MJ, Pilgrim EM, Debry RW, Biales AD (2021) DNA methylation and expression of estrogen receptor alpha in fathead minnows exposed to 17α-ethynylestradiol. Aquat Toxicol 233:105788. https://doi.org/10.1016/j.aquatox.2021.105788
13. Fiori LM, Nagy C, Turecki G (2026) Identification of antidepressant response-related changes to DNA methylation and gene expression. Int J Neuropsychopharmacol 29(2). https://doi.org/10.1093/ijnp/pyag001
14. Fruzzetti F, Fidecicchi T (2020) Hormonal Contraception and Depression: Updated Evidence and Implications in Clinical Practice. Clin Drug Investig 40(12):1097–1106. https://doi.org/10.1007/s40261-020-00966-8
15. Fukui H, Rünker A, Fabel K, Buchholz F, Kempermann G (2018) Transcription factor Runx1 is pro-neurogenic in adult hippocampal precursor cells. PLoS ONE 13(1):e0190789. https://doi.org/10.1371/journal.pone.0190789
16. Hammond GL, Abrams LS, Creasy GW, Natarajan J, Allen JG, Siiteri PK (2003) Serum distribution of the major metabolites of norgestimate in relation to its pharmacological properties. Contraception 67(2):93–99. https://doi.org/10.1016/S0010-7824(02)00473-0
17. Hampson E (2023) Oral contraceptives in the central nervous system: Basic pharmacology, methodological considerations, and current state of the field. Front Neuroendocr 68:101040. https://doi.org/10.1016/j.yfrne.2022.101040
18. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, Klotzle B, Bibikova M, Fan JB, Gao Y, Deconde R, Chen M, Rajapakse I, Friend S, Ideker T, Zhang K (2013) Genome-wide methylation

profiles reveal quantitative views of human aging rates. Mol Cell 49(2):359–367. https://doi.org/10.1016/j.molcel.2012.10.016

19. Hantsoo L, Rangaswamy S, Voegtline K, Salimgaraev R, Zhaunova L, Payne JL (2022) Premenstrual symptoms across the lifespan in an international sample: data from a mobile application. Arch Women Ment Health 25(5):903–910. https://doi.org/10.1007/s00737-022-01261-5
20. Harbs J, Rinaldi S, Keski-Rahkonen P, Liu X, Palmqvist R, Van Guelpen B, Harlid S (2023) An epigenome-wide analysis of sex hormone levels and DNA methylation in male blood samples. Epigenetics 18(1):2196759. https://doi.org/10.1080/15592294.2023.2196759
21. Harding AT, Heaton NS (2022) The Impact of Estrogens and Their Receptors on Immunity and Inflammation during Infection. *Cancers*, *14*(4), 909. https://www.mdpi.com/2072-6694/14/4/909
22. Hautzinger M, Keller F, Kühner C, Beck AT, Steer RA, Brown GK (2009) *Beck Depressions-Inventar: BDI-II ; Revision ; Manual* (2. Aufl ed.). Pearson
23. Haynes BP, Viale G, Galimberti V, Rotmensz N, Gibelli B, Smith IE, Dowsett M (2014) Differences in expression of proliferation-associated genes and RANKL across the menstrual cycle in estrogen receptor-positive primary breast cancer. Breast Cancer Res Treat 148(2):327–335. https://doi.org/10.1007/s10549-014-3181-6
24. Houshdaran S, Oke AB, Fung JC, Vo KC, Nezhat C, Giudice LC (2020) Steroid hormones regulate genome-wide epigenetic programming and gene transcription in human endometrial cells with marked aberrancies in endometriosis. PLoS Genet 16(6):e1008601. https://doi.org/10.1371/journal.pgen.1008601
25. Hwang WJ, Lee TY, Kim NS, Kwon JS (2021) The Role of Estrogen Receptors and Their Signaling across Psychiatric Disorders. *International Journal of Molecular Sciences*, *22*(1), 373. https://www.mdpi.com/1422-0067/22/1/373
26. Katrinli S (2024) *EPICv2_pipeline*. https://github.com/skatrinli/EPICv2_pipeline
27. Kimmig A-CS, Bischofberger JA, Birrenbach AD, Drotleff B, Lämmerhofer M, Sundström-Poromaa I, Derntl B (2022) No Evidence for a Role of Oral Contraceptive-Use in Emotion Recognition But Higher Negativity Bias in Early Follicular Women [Original Research]. Front Behav Neurosci 15. https://doi.org/10.3389/fnbeh.2021.773961
28. Kimmig A-CS, Friedrich P, Drotleff B, Lämmerhofer M, Sundström-Poromaa I, Weis S, Derntl B (2022) To start or to discontinue the pill – changes in progestogens reflected by resting-state connectivity and positive mood. bioRxiv [Preprint]. 2022.2009.2021.508780 https://doi.org/10.1101/2022.09.21.508780
29. Kimmig A-CS, van Egmond LT, Derntl B (2025) Emotion recognition largely unaffected by combined oral contraceptive transitions or their androgenicity. Horm Behav 176:105843. https://doi.org/10.1016/j.yhbeh.2025.105843
30. Kimmig A-CS, Wildgruber D, Gärtner A, Drotleff B, Krylova M, Lämmerhofer M, Sundström-Poromaa I, Derntl B (2022) Lower affective empathy in oral contraceptive users: a cross-sectional fMRI study. Cereb Cortex 33(8):4319–4333. https://doi.org/10.1093/cercor/bhac345

31. Koh EJ, Hwang SY (2019) Multi-omics approaches for understanding environmental exposure and human health. Mol Cell Toxicol 15(1):1–7. https://doi.org/10.1007/s13273-019-0001-4
32. Kovács T, Szabó-Meleg E, Ábrahám IM (2020) Estradiol-Induced Epigenetically Mediated Mechanisms and Regulation of Gene Expression. Int J Mol Sci 21(9). https://doi.org/10.3390/ijms21093177
33. Kuznetsova A, Brockhoff PB, Christensen RHB (2017) lmerTest Package: Tests in Linear Mixed Effects Models. J Stat Softw 82(13):1–26. https://doi.org/10.18637/jss.v082.i13
34. Langdon RJ, Yousefi P, Relton CL, Suderman MJ (2021) Epigenetic modelling of former, current and never smokers. Clin Epigenetics 13(1):206. https://doi.org/10.1186/s13148-021-01191-6
35. Lehne B, Drong AW, Loh M, Zhang W, Scott WR, Tan S-T, Afzal U, Scott J, Jarvelin M-R, Elliott P, McCarthy MI, Kooner JS, Chambers JC (2015) A coherent approach for analysis of the Illumina HumanMethylation450 BeadChip improves data quality and performance in epigenome-wide association studies. Genome Biol 16(1):37. https://doi.org/10.1186/s13059-015-0600-x
36. Levine ME, Lu AT, Chen BH, Hernandez DG, Singleton AB, Ferrucci L, Bandinelli S, Salfati E, Manson JE, Quach A, Kusters CDJ, Kuh D, Wong A, Teschendorff AE, Widschwendter M, Ritz BR, Absher D, Assimes TL, Horvath S (2016) Menopause accelerates biological aging. *Proceedings of the National Academy of Sciences*, *113*(33), 9327–9332. https://doi.org/10.1073/pnas.1604558113
37. Lewis CA, Kimmig A-CS, Zsido RG, Jank A, Derntl B, Sacher J (2019) Effects of Hormonal Contraceptives on Mood: A Focus on Emotion Recognition and Reactivity, Reward Processing, and Stress Response. Curr Psychiatry Rep 21(11):115. https://doi.org/10.1007/s11920-019-1095-z
38. Mandaviya PR, Joehanes R, Brody J, Castillo-Fernandez JE, Dekkers KF, Do AN, Graff M, Hänninen IK, Tanaka T, de Jonge EAL, Kiefte-de Jong JC, Absher DM, Aslibekyan S, de Rijke YB, Fornage M, Hernandez DG, Hurme MA, Ikram MA, Jacques PF, Heil SG (2019) Association of dietary folate and vitamin B-12 intake with genome-wide DNA methylation in blood: a large-scale epigenome-wide association analysis in 5841 individuals. Am J Clin Nutr 110(2):437–450. https://doi.org/10.1093/ajcn/nqz031
39. Mehta D, Bruenig D, Carrillo-Roa T, Lawford B, Harvey W, Morris CP, Smith AK, Binder EB, Young RM, Voisey J (2017) Genomewide DNA methylation analysis in combat veterans reveals a novel locus for PTSD. Acta psychiatrica Scandinavica 136(5):493–505. https://doi.org/10.1111/acps.12778
40. Mortlock S, Restuadi R, Levien R, Girling JE, Holdsworth-Carson SJ, Healey M, Zhu Z, Qi T, Wu Y, Lukowski SW, Rogers PAW, Yang J, McRae AF, Fung JN, Montgomery GW (2019) Genetic regulation of methylation in human endometrium and blood and gene targets for reproductive diseases. Clin Epigenetics 11(1):49. https://doi.org/10.1186/s13148-019-0648-7
41. Mostafa Moradi S, Fatemeh Z, Hooriya V, Zahra A, Navid J, Mohammad S-N (2025) Increased Serum Levels of Inflammatory Markers and Promoter Hypermethylation of BRCA1 and BRCA2 in Oral Contraceptive Users. Curr Cancer Therapy Reviews 21(5):749–755. http://dx.doi.org/10.2174/0115733947310701240726043841

42. Niu L, Xu Z, Taylor JA (2016) RCP: a novel probe design bias correction method for Illumina Methylation BeadChip. Bioinformatics 32(17):2659–2663. https://doi.org/10.1093/bioinformatics/btw285
43. Nugent BM, Wright CL, Shetty AC, Hodes GE, Lenz KM, Mahurkar A, Russo SJ, Devine SE, McCarthy MM (2015) Brain feminization requires active repression of masculinization via DNA methylation. Nat Neurosci 18(5):690–697. https://doi.org/10.1038/nn.3988
44. O'Callaghan JP, Kelly KA, VanGilder RL, Sofroniew MV, Miller DB (2014) Early activation of STAT3 regulates reactive astrogliosis induced by diverse forms of neurotoxicity. PLoS ONE 9(7):e102003. https://doi.org/10.1371/journal.pone.0102003
45. Paoli C, Misztak P, Mazzini G, Musazzi L (2022) DNA Methylation in Depression and Depressive-Like Phenotype: Biomarker or Target of Pharmacological Intervention? Curr Neuropharmacol 20(12):2267–2291. https://doi.org/10.2174/1570159x20666220201084536
46. Peters TJ, Meyer B, Ryan L, Achinger-Kawecka J, Song J, Campbell EM, Qu W, Nair S, Loi-Luu P, Stricker P, Lim E, Stirzaker C, Clark SJ, Pidsley R (2024) Characterisation and reproducibility of the HumanMethylationEPIC v2.0 BeadChip for DNA methylation profiling. BMC Genomics 25(1):251. https://doi.org/10.1186/s12864-024-10027-5
47. Pośpiech E, Rudnicka J, Noroozi R, Pisarek-Pacek A, Wysocka B, Masny A, Boroń M, Migacz-Gruszka K, Pruszkowska-Przybylska P, Kobus M, Lisman D, Zielińska G, Cytacka S, Iljin A, Wiktorska JA, Michalczyk M, Kaczka P, Krzysztofik M, Sitek A, Branicki W (2024) DNA methylation at AHRR as a master predictor of smoke exposure and a biomarker for sleep and exercise. Clin Epigenetics 16(1):147. https://doi.org/10.1186/s13148-024-01757-0
48. R Core Team (2025) *R: A Language and Environment for Statistical Computing*. In R Foundation for Statistical Computing, Vienna Austria. https://www.R-project.org/
49. Retis-Resendiz AM, González-García IN, León-Juárez M, Camacho-Arroyo I, Cerbón M, Vázquez-Martínez ER (2021) The role of epigenetic mechanisms in the regulation of gene expression in the cyclical endometrium. Clin Epigenetics 13(1):116. https://doi.org/10.1186/s13148-021-01103-8
50. Romans S, Clarkson R, Einstein G, Petrovic M, Stewart D (2012) Mood and the menstrual cycle: a review of prospective data studies. Gend Med 9(5):361–384. https://doi.org/10.1016/j.genm.2012.07.003
51. Salas LA, Koestler DC, Butler RA, Hansen HM, Wiencke JK, Kelsey KT, Christensen BC (2018) An optimized library for reference-based deconvolution of whole-blood biospecimens assayed using the Illumina HumanMethylationEPIC BeadArray. Genome Biol 19(1):64. https://doi.org/10.1186/s13059-018-1448-7
52. Santos RLd, Bonfim IM, Cunha TRd, Giesen JAS (2026) Progesterone and vascular function: mechanisms and therapeutic implications in cardiovascular disease. *Steroids*, *235–236*, 109837. https://doi.org/10.1016/j.steroids.2026.109837
53. Schmalenberger KM, Tauseef HA, Barone JC, Owens SA, Lieberman L, Jarczok MN, Girdler SS, Kiesner J, Ditzen B, Eisenlohr-Moul TA (2021) How to study the menstrual cycle: Practical tools and

recommendations. Psychoneuroendocrinology 123:104895. https://doi.org/10.1016/j.psyneuen.2020.104895

54. Senthilkumar H, Arumugam M (2026) Integrative bioinformatics and transcriptomic analysis identifies biomarkers in Polycystic Ovary Syndrome through machine learning approach. J Steroid Biochem Mol Biol 255:106887. https://doi.org/10.1016/j.jsbmb.2025.106887
55. Shepherd R, Bretherton I, Pang K, Mansell T, Czajko A, Kim B, Vlahos A, Zajac JD, Saffery R, Cheung A, Novakovic B (2022) Gender-affirming hormone therapy induces specific DNA methylation changes in blood. Clin Epigenetics 14(1):24. https://doi.org/10.1186/s13148-022-01236-4
56. Sinha P, Rani A, Kumar A, Riva A, Brant JO, Foster TC (2021) Examination of CA1 Hippocampal DNA Methylation as a Mechanism for Closing of Estrogen's Critical Window [Original Research]. *Frontiers in Aging Neuroscience*, *Volume 13–2021*. https://doi.org/10.3389/fnagi.2021.717032
57. Sklias A, Halaburkova A, Vanzan L, Jimenez NF, Cuenin C, Bouaoun L, Cahais V, Ythier V, Sallé A, Renard C, Durand G, Le Calvez-Kelm F, Khoueiry R, Murr R, Herceg Z (2021) Epigenetic remodelling of enhancers in response to estrogen deprivation and re-stimulation. Nucleic Acids Res 49(17):9738–9754. https://doi.org/10.1093/nar/gkab697
58. Song JY, Patton CD, Friedman R, Mahajan LS, Nordlicht R, Sayed R, Lipton ML (2023) Hormonal contraceptives and the brain: A systematic review on 60 years of neuroimaging, EEG, and biochemical studies in humans and animals. Front Neuroendocr 68:101051. https://doi.org/10.1016/j.yfrne.2022.101051
59. Soyer-Gobillard M-O, Gaspari L, Paris F, Courtet P, Sultan C (2023) Effects of prenatal exposure to synthetic sex hormones on neurodevelopment: a biological mechanism [Mini Review]. *Frontiers in Molecular Neuroscience*, *Volume 16–2023*. https://doi.org/10.3389/fnmol.2023.1237429
60. Stender JD, Kim K, Charn TH, Komm B, Chang KCN, Kraus WL, Benner C, Glass CK, Katzenellenbogen BS (2010) Genome-Wide Analysis of Estrogen Receptor α DNA Binding and Tethering Mechanisms Identifies Runx1 as a Novel Tethering Factor in Receptor-Mediated Transcriptional Activation. Mol Cell Biol 30(16):3943–3955. https://doi.org/10.1128/MCB.00118-10
61. Su M, Drotleff B, Janker T, Bürger Z, Kimmig A-CS, Derntl B, Lämmerhofer M (2025) Quantification of Endogenous Steroids and Hormonal Contraceptives in Human Plasma via Surrogate Calibration and UHPLC-MS/MS. Anal Chem 97(25):13496–13503. https://doi.org/10.1021/acs.analchem.5c01912
62. The GTEx Consortium, Aguet F, Anand S, Ardlie KG, Gabriel S, Getz GA, Graubert A, Hadley K, Handsaker RE, Huang KH, Kashin S, Li X, MacArthur DG, Meier SR, Nedzel JL, Nguyen DT, Segrè AV, Todres E, Balliu B, Volpi S (2020) The GTEx Consortium atlas of genetic regulatory effects across human tissues. Science 369(6509):1318–1330. https://doi.org/10.1126/science.aaz1776
63. Thilmany S, Thomas A, Reinders Y, Shakeri F, Vogel M, Sickmann A, Scholl C, Thevis M (2026) Hormonal Contraceptives and Depression: A Proteomic Analysis Using Neuronal Models. Proteom – Clin Appl 20(1):e70017. https://doi.org/10.1002/prca.70017
64. Tsankova N, Renthal W, Kumar A, Nestler EJ (2019) Author Correction: Epigenetic regulation in psychiatric disorders. Nat Rev Neurosci 20(3):187–188. https://doi.org/10.1038/s41583-018-0089-x

65. Turner SD (2018) qqman: an R package for visualizing GWAS results using Q-Q and manhattan plots. J Open Source Softw 3(25):731. https://doi.org/10.21105/joss.00731
66. United Nations, Department of Economic and Social Affairs, Population Division (2022) World Family Planning 2022: Meeting the changing needs for family planning: Contraceptive use by age and method. UN
67. van Iterson M, van Zwet EW, Heijmans BT, the BC (2017) Controlling bias and inflation in epigenome- and transcriptome-wide association studies using the empirical null distribution. Genome Biol 18(1):19. https://doi.org/10.1186/s13059-016-1131-9
68. Vivacqua A, De Marco P, Santolla MF, Cirillo F, Pellegrino M, Panno ML, Abonante S, Maggiolini M (2015) Estrogenic gper signaling regulates mir144 expression in cancer cells and cancer-associated fibroblasts (cafs). Oncotarget 6(18):16573–16587. https://doi.org/10.18632/oncotarget.4117
69. Wall EH, Hewitt SC, Liu L, del Rio R, Case LK, Lin CY, Korach KS, Teuscher C (2013) Genetic control of estrogen-regulated transcriptional and cellular responses in mouse uterus. Faseb j 27(5):1874–1886. https://doi.org/10.1096/fj.12-213462
70. Wen Z, Liu D, Zhu H, Sun X, Xiao Y, Lin Z, Zhang A, Ye C, Gao J (2021) Deficiency for Lcn8 causes epididymal sperm maturation defects in mice. Biochem Biophys Res Commun 548:7–13. https://doi.org/10.1016/j.bbrc.2021.02.052
71. Wilhelm LP, Wendling C, Védie B, Kobayashi T, Chenard MP, Tomasetto C, Drin G, Alpy F (2017) STARD3 mediates endoplasmic reticulum-to-endosome cholesterol transport at membrane contact sites. EMBO J 36(10):1412–1433. https://doi.org/10.15252/embj.201695917
72. Wuchty S, Myers AJ, Ramirez-Restrepo M, Huentelman M, Richolt R, Gould F, Harvey PD, Michopolous V, Steven JS, Wingo AP, Lori A, Maples-Keller JL, Rothbaum AO, Jovanovic T, Rothbaum BO, Ressler KJ, Nemeroff CB (2021) Integration of peripheral transcriptomics, genomics, and interactomics following trauma identifies causal genes for symptoms of post-traumatic stress and major depression. Mol Psychiatry 26(7):3077–3092. https://doi.org/10.1038/s41380-021-01084-3
73. Xu Z, Langie SAS, De Boever P, Taylor JA, Niu L (2017) RELIC: a novel dye-bias correction method for Illumina Methylation BeadChip. BMC Genomics 18(1):4. https://doi.org/10.1186/s12864-016-3426-3
74. Xu Z, Niu L, Li L, Taylor JA (2016) ENmix: a novel background correction method for Illumina HumanMethylation450 BeadChip. Nucleic Acids Res 44(3):e20–e20. https://doi.org/10.1093/nar/gkv907
75. Yu H, Pardoll D, Jove R (2009) STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 9(11):798–809. https://doi.org/10.1038/nrc2734
76. Zheng SC, Breeze CE, Beck S, Teschendorff AE (2018) Identification of differentially methylated cell types in epigenome-wide association studies. Nat Methods 15(12):1059–1066. https://doi.org/10.1038/s41592-018-0213-x

Figures

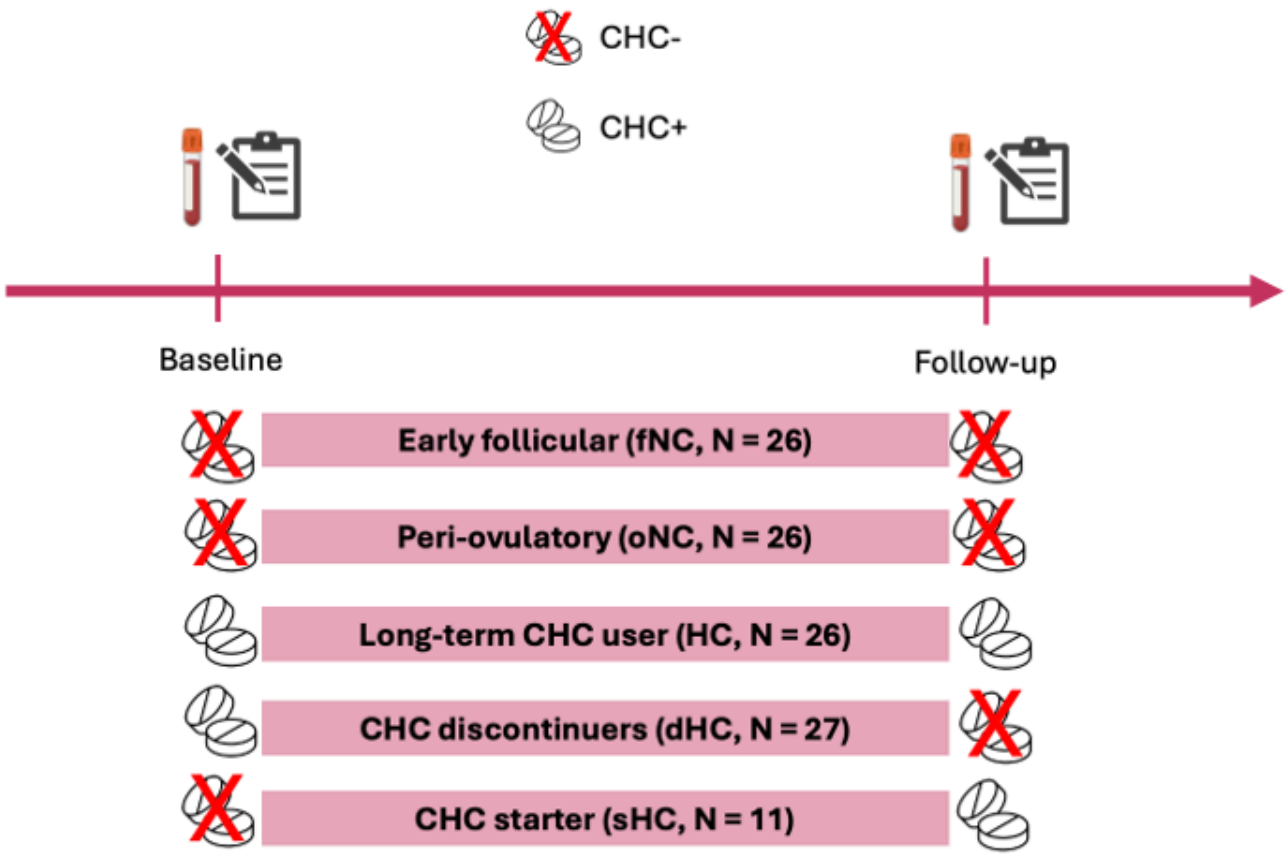


Figure 1

Study Design

Blood samples were collected at two time points, with an average interval of four months apart. Naturally cycling participants were assessed either in the early follicular or the peri-ovulatory phase at both time points, while long-term CHC users were sampled at the active contraceptive phase at both time points. CHC discontinuers and starters were sampled before and at least three months after the change in CHC use status. The pill with red X symbols marks sampling during the absence of CHC intake, and the pill symbol marks CHC intake. CHC+: sampling during CHC use, CHC-: sampling without CHC use.

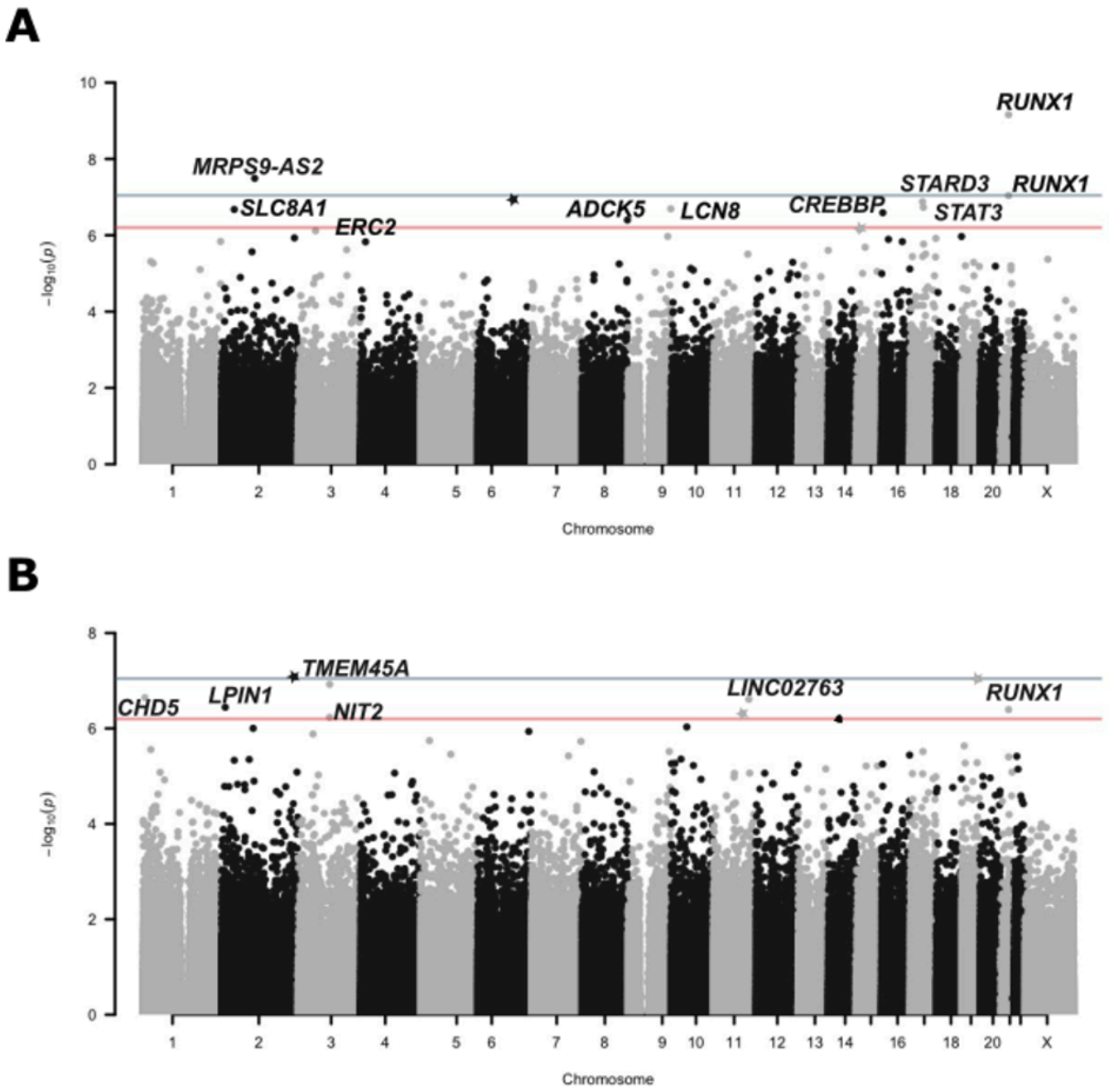


Figure 2

Manhattan plots for CHC intake and the hormonal group trajectories.

Manhattan plots for A) epigenome-wide association analysis of current CHC intake and B) differentially methylated probes associated with group and time interaction. The x-axis shows the chromosomal location of each probe and the y-axis shows -log 10 of the p-values (Bacon-corrected p-values in A). The red line indicates the suggestive line after FDR-corrected p-values. The blue line indicates genome-wide EWAS statistical significance at $p < 9 \times 10^{-8}$. The stars indicate intergenic probes and the triangle a novel transcript without a gene symbol. *ADCK5:* AarF domain-containing kinase 5. *CREBBP*: CREB binding lysine acetyltransferase. *ERC2*: ELKS/RAB6-interacting/CAST family member 2. *MRPS9-AS2*: MRPS9

antisense RNA 2. *LCN8*: Lipocalin 8. *RUNX1*: RUNX family transcription factor 1. *SLC8A1*: solute carrier family 8 member A1. *STARD3*: StAR-related lipid transfer domain containing 3. *STAT3*: Signal transducer and activator of transcription 3. *CHD5*: Chromodomain helicase DNA-binding protein 5. *LPIN1*: Lipin 1. *LINC02763*: Long independently transcribed non-coding RNA 2763. *NIT2*: Nitrilase family member 2. *RUNX1*: RUNX family transcription factor 1. *TMEM45A*: Transmembrane protein 45A.

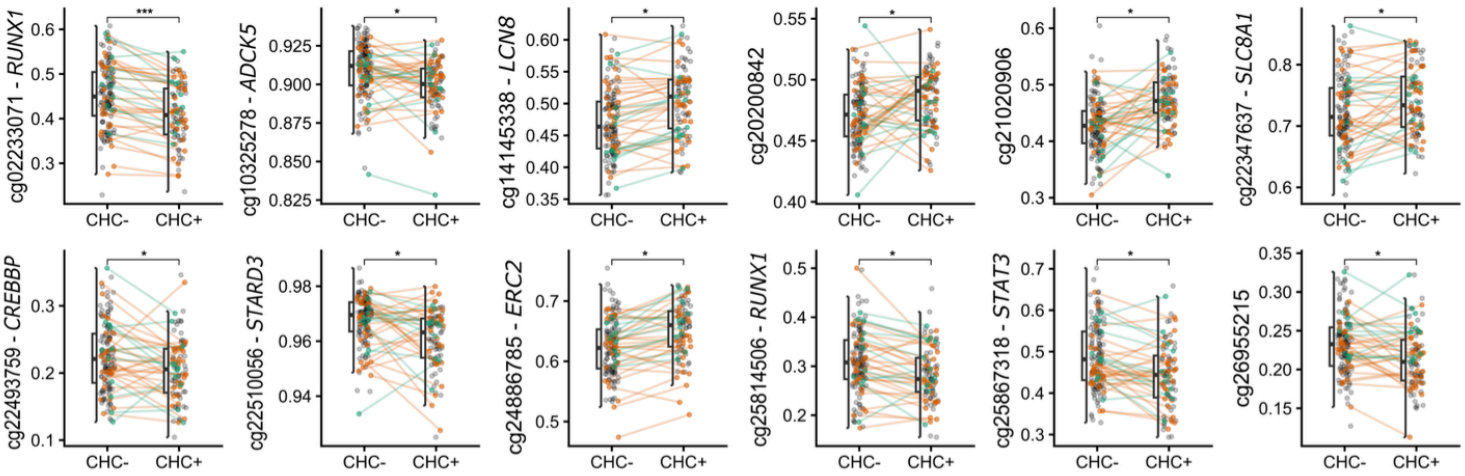


Figure 3

Differentially methylated CpG sites related to current CHC intake.

DNA methylation levels at 12 significant CpG sites are shown according to current CHC exposure status. Points represent individual samples, and lines connect repeated measurements from the same participant, who contributed observations under both CHC- and CHC+ conditions. To clarify the direction of change, starters (CHC- at baseline to CHC+ at follow-up) were colored green, whereas discontinuers (CHC+ at baseline to CHC- at follow-up) were colored orange. The y-axis shows beta values in the respective probe with the nearest annotated gene, indicating 5 DMPs are hypermethylated with CHC use, whereas 7 DMPs are hypomethylated. Asterisks indicate FDR- and Bacon-corrected significance levels: ***p < 0.001 and *p < 0.05. CHC+: sampling during CHC use, CHC-: sampling without CHC use

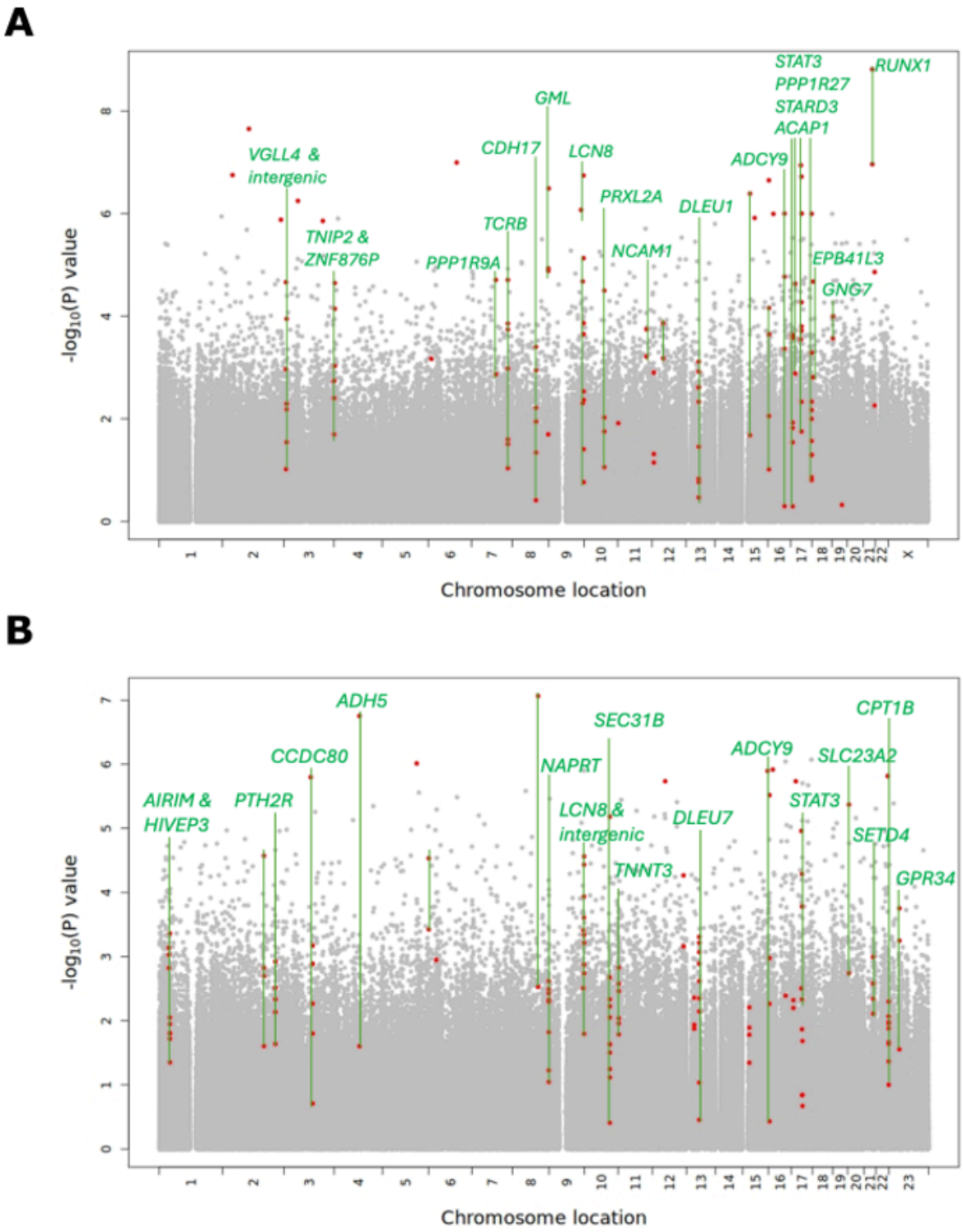


Figure 4

Differentially methylated regions associated with current CHC use and across different hormonal groups.

A) Current CHC use was associated with 25 DMRs. B) 21 DMRs were found across five hormonal groups using p-values obtained from the main group effects slope in linear mixed models. Each CpG site is

depicted as a point, with red points indicating neighboring probes contributing to regional significance before filtering for regions with fewer than 2 CpG sites and Sidak p < 0.05. The x-axis shows the chromosomal location of each site, and the y-axis shows the negative log10 of unadjusted p-values. The neighboring probes constituting each DMR (after filtering) are outlined by green lines, and annotated genes are indicated. The intergenic locations and uncharacterized/novel transcripts were indicated with a green line without annotation. *ACAP1*: ArfGAP with coiled-coil, ankyrin repeat and PH domains 1. *ADCY5:* Adenylate cyclase 5. *CDH17*: Cadherin 17. *DLEU1*: Deleted in lymphocytic leukemia 1. *EPB41L3*: Erythrocyte membrane protein band 4.1-like 3. *GML*: Glycosylphosphatidylinositol-anchored molecule-like. *GNG7*: G protein subunit gamma 7. *LCN8*: Lipocalin 8. *NCAM1*: Neural cell adhesion molecule 1. *PPP1R9A*: Protein phosphatase 1 regulatory subunit 9A. *PPP1R27*: Protein phosphatase 1 regulatory subunit 27. *PRXL2A*: Peroxiredoxin-like 2A. *RUNX1*: RUNX family transcription factor 1. *STARD3*: StAR-related lipid transfer domain containing 3. *STAT3*: Signal transducer and activator of transcription 3. *TCRB*: T-cell receptor beta. *TNIP2*: TNFAIP3 interacting protein 2. *VGLL4*: Vestigial-like family member 4. ZNF876P: Zinc finger protein 876, pseudogene. *ADCY9*: Adenylate cyclase 9. *ADH5*: alcohol dehydrogenase 5. *AIRIM*: AFG2 interacting ribosome maturation factor. *CCDC80*: Coiled-coil domain containing 80. *CPT1B*: Carnitine palmitoyltransferase 1B. *DLEU7*: Deleted in lymphocytic leukemia 7. *GPR34*: G protein-coupled receptor 34. *HIVEP3*: HIVEP zinc finger 3. *LCN8*: Lipocalin 8. *NAPRT*: Nicotinate phosphoribosyltransferase. *PTH2R*: Parathyroid hormone 2 receptor. *SEC31B*: SEC31 homolog B. *SETD4*: SET domain containing 4. *SLC23A2*: solute carrier family 23 member 2. *STAT3*: Signal transducer and activator of transcription 3. *TNNT3*: Troponin T3.

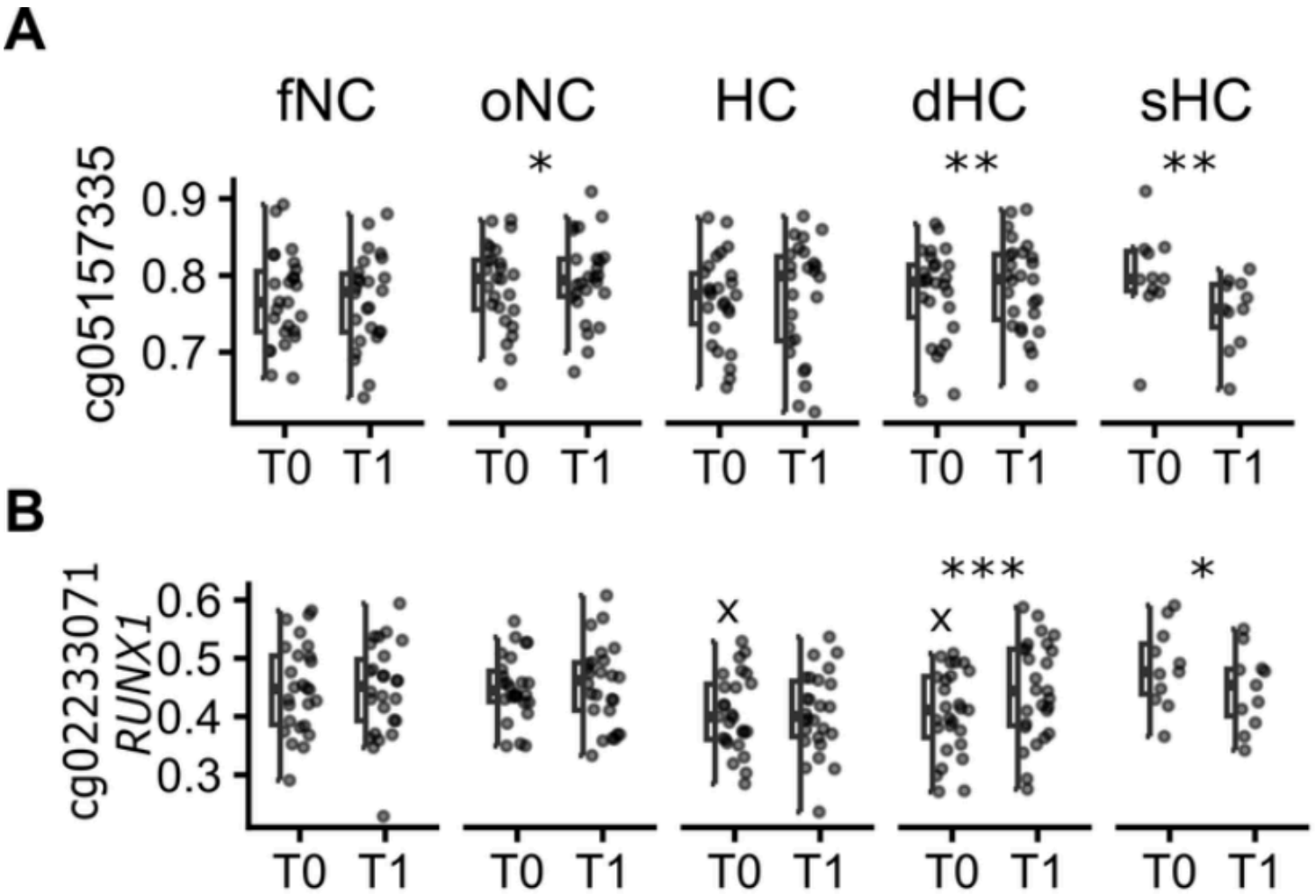


Figure 5

Two DMPs associated with hormonal group trajectories

Boxplots show the distribution of methylation levels within each group at baseline and follow-up. A: For the top DMP from the group*time interaction (cg05157335), the changes in methylation levels differed significantly between oNC, dHC and sHC groups and fNC (ß = 0.15, 0.19 and -0.25; p = 0.017, 0.003 and 0.002, respectively). B: For cg02233071, only DMP common to group*time and CHC-use models, methylation levels were lower among CHC users than in the fNC group at baseline (HC: β = -0.29, p = 0.019; dHC: β = -0.28, p = 0.024). Moreover, discontinuation and initiation of CHC use were associated with methylation changes in opposite directions relative to the fNC group (dHC: β = 0.30, p < 0.001; sHC: β = -0.23, p = 0.012). This pattern is consistent with methylation at cg02233071 being associated with CHC use. “x” indicates a significant baseline difference compared with the follicular-phase naturally cycling (fNC) group, whereas “*” indicates a significant difference in methylation change over time relative to the fNC group. ^{x}p < 0.05, *p < 005, **p < 0.01, ***p < 0.001

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ocEWASadditionalfile1.docx
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