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The effect of polychlorinated biphenyls on type 2 diabetes risk is mediated via DNA methylation

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ABSTRACT

Polychlorinated biphenyls (PCBs) have been identified as diabetogens and potential epigenetic regulators. Epigenetic variation is implicated in the pathogenesis of type 2 diabetes (T2D) and is therefore a potential mechanism linking PCB exposure and T2D. We here investigate the effect of PCB exposure on T2D risk and the

Abbreviations: BMI, Body mass index; CPT1A, Carnitine Palmitoyltransferase 1A; DACT, Divide-aggregate composite-null test; DMP, Differentially methylated probe; DMR, Differentially methylated region; DNAm, DNA methylation; EDC, Endocrine-disrupting chemicals; EWAS, Epigenome-wide association study; FDR, False discovery rate; OR, Odds ratio; PC, Principal component; PCA, Principal components analysis; PCB, Polychlorinated biphenyls; T2D, Type 2 diabetes; YFS, The Cardiovascular Risk in the Young Finns Study; ABCG1, ATP-binding cassette sub-family G member 1; MAML2, Mastermind like transcriptional coactivator 2; GHRFR, Growth hormone releasing hormone receptor.

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CPT1A

extent to which this is mediated via DNA methylation (DNAm) using two epigenome-wide association studies and mediation analysis.

Exposure to PCBs, especially congeners 74, 99, 118, 138 and 183, increased the risk of T2D (range of ORs 1.38–1.54). Five CpG sites were identified as potential mediators. Together, methylation at these sites was estimated to mediate 40 % (95 % CI: 20, 60 %) of the effect of PCBs on T2D. The strongest evidence was obtained for cg00574958, located in the first intron of the *CPT1A* gene.

This study is among the largest to date assessing the link between PCBs and T2D, and the first to investigate the mediation by DNAm. The evidence for methylation at cg00574958 as a mediator of the PCB–T2D relationship supports the widely-replicated link of *CPT1A* DNA methylation to a range of metabolic phenotypes including obesity, dyslipidemia and T2D and indicates a possible future target for epigenetic intervention.

1. Introduction

Type 2 diabetes (T2D) is a major and increasing global health challenge, with a predicted prevalence of over one billion people by 2050. (Ong et al., 2023) While genetic variation also plays a role, environment, particularly age-associated sedentary lifestyle and adiposity, are important in T2D pathogenesis. (Ahmad et al., 2022) Furthermore, mounting evidence suggests that other factors, including endocrine-disrupting chemicals (EDC) increase T2D risk. (Hinault et al., 2023).

Polychlorinated biphenyls (PCBs) are synthetic EDCs characterized by long degradation times and accumulation in animals and humans via the food chain. (Gore et al., 2015) Although use of PCBs in open applications was banned in Europe in 1976 (76/769/EEC) and a global ban was enforced by the Stockholm Convention in 2001, bioaccumulation and fat-solubility make PCBs an ongoing health issue. (COP Decisions & Recommendations on PCBs, 2025) The chemical obesogen hypothesis, suggested first in the early 2000 s (Baillie-Hamilton, 2002; Grün et al., 2006) and later confirmed in further studies, points to the crucial role of environmental pollutants as obesogens and diabetogens (Hinault et al., 2023; Gore et al., 2015), predisposing to obesity, insulin resistance and T2D. (Lee et al., 2014; Song et al., 2016).

Previous studies have linked PCBs to human epigenetic variation (primarily DNA methylation) across the life-course. (Curtis et al., 2021; Georgiadis et al., 2019) As epigenetic variation in different tissues has been reproducibly implicated in the pathogenesis of T2D, this suggests a potential mechanistic pathway between environmental exposures and T2D risk. (Ling, 2020) A recent *meta*-analysis of prospective European cohorts found genomic sites with differential methylation involving 65 genes, including the widely-replicated *CPT1A, TXNIP* and *ABCG1*, in association with T2D. (Fraszczyk et al., 2022) Mendelian randomization studies have provided further insights into the causal role of DNA methylation in T2D risk. (Cardona et al., 2019; Juvinao-Quintero et al., 2023) However, to date, no study has combined information on PCB exposure, epigenetic variation and T2D to explore the potential of the epigenome to function as a mechanistic pathway linking PCBs to T2D. Here, we assessed the relationship between PCBs and T2D and the extent to which it is mediated by DNAm in a Finnish population-derived cohort characterized by background exposure to PCBs. In two epigenome-wide association studies (EWAS), we assessed the links between different PCB congeners and DNAm, and between DNAmand T2D risk. We then applied a recently developed method of epigenetic mediation analysis to identify sites of differential methylation potentially mediating the effects of PCB exposure on T2D. (Liu et al., 2022).

2. Materials and methods

2.1. The cardiovascular risk in young Finns study

The Cardiovascular Risk in Young Finns Study (YFS) is a prospective cohort study commenced in 1980, when 3596 children and adolescents belonging to 6 birth cohorts (born in 1962, 1965, 1968, 1971, 1974, 1977) from five Finnish cities (Helsinki, Kuopio, Oulu, Tampere, Turku) and their surrounding rural areas were recruited using population register sampling. (Raitakari et al., 2008) The participants have been followed up altogether 8 times until 2018. The study was approved by the local ethics committees. All participants (or their parents, when the participants were younger than 18 years) provided written informed consent. The Ethical Committee of the Hospital District of Southwest Finland approved the study. The study has been conducted in accordance with the Declaration of Helsinki.

2.2. Analysis sample

The data analysis was conducted for complete cases, i.e., participants who had data on the exposure, mediator, outcome and relevant confounders available. Whenever possible, data on covariates were augmented using measurements from other follow-up visits to maximize the sample size.

2.3. Polychlorinated biphenyl (PCB) measures

Ten PCB congeners (74, 99, 118, 138, 153, 156, 170, 180, 183 and 187; pg/ml) were measured from serum samples drawn at the 2001 follow-up when the participants were 24–39 years old. PCB measurement consisted of liquid–liquid extraction, silica column clean-up and analysis by the Agilent 7010 gas chromatograph triple quadrupole mass spectrometer as described previously. (Koponen et al., 2013) Concentrations below the quantification limit (LOQ) of 5 pg/ml were assigned value 2.5 pg/ml. Table 1 presents the numbers and proportions of observations below the quantification limit in the entire analysis sample and the ranges of the congeners in pg/ml. After quality control (Supplemental Material, Quality control), PCB concentrations were log-transformed and standardized (mean 0, SD 1).

2.4. T2D outcome

Participants were followed until T2D (binary: yes/no) onset, death, emigration or end of follow-up (2018), whichever occurred first (Supplemental Material, T2D follow-up; Fig. S1). We defined T2D onset as the first instance of any of the following: fasting glucose level ≥ 7 mmol/L (measured in 2001, 2007, 2011 and 2018), haemoglobin A1c level $\geq$

Table 1
Numbers and proportions of PCB measurements below the quantification limit and the range [minimum–maximum] of each congener in a sample of n = 1216 participants in the prospective Cardiovascular Risk in Young Finns Study.

PCB	N (%)	Range
PCB74	120 (9.9)	[2.5–525.4]
PCB99	62 (5.1 %)	[2.5–155.1]
PCB118	6 (0.5)	[2.5–172.2]
PCB153	0 (0)	[18.9–1033.5]
PCB138	0 (0)	[10.3–671.9]
PCB156	21 (1.7)	[2.5–105.0]
PCB187	5 (0.4)	[2.5–198.0]
PCB183	79 (6.5)	[2.5–92.4]
PCB180	0 (0)	[22.3–678.4]
PCB170	0 (0)	[9.7–313.0]

Abbreviations: PCB, polychlorinated biphenyl.

6.5 % (2011, 2018), self-reported diabetes (2001, 2007, 2011 and 2018), self-reported glucose-lowering medication (2007, 2011 and 2018), diagnosis in the Finnish Hospital Discharge Register (available until 2018), diagnosis in the Finnish Primary Care Register (Avohilmo; available until 2018) or registered imbursement for diabetes medication (Drug Reimbursement Registry, Finnish National Social Insurance Institution; available until 2016). (Sund, 2012; Furu et al., 2010) The outcome of interest was the risk of T2D during the follow-up period starting in 2011 (main analysis) or 2001 (additional analyses). This approach corresponds to modelling the average risk of T2D onset by the end of follow-up across the six birth cohorts and the follow-up time.

2.5. Potential confounders

In all analyses, birth year cohort (1962, 1965, 1968, 1971, 1974, 1977) was considered as a confounder because age is associated with risk of T2D and the historical discontinuation of PCB use in Finland since the 1970 s has predisposed the birth cohorts to different levels of exposure from birth to late adolescence. We adjusted for sex (male/female), smoking status (current smokers/current non-smokers) and education level (comprehensive school/secondary non-academic education/academic education) in all statistical analyses. In a secondary analysis, data were stratified by sex. Smoking was defined based on questionnaire data as a binary variable indicating current smoking, largely from the 2011 questionnaire and augmented using smoking history reported in 2007 and 2018 (see Supplemental Material, Definition of smoking). Participants who reported to smoke "Daily", "Weekly", or "Less than weekly", were categorized as current smokers, while those who reported to be "Never smokers" or "Quitted smoking" were categorized as current non-smokers. Education level was used as a proxy for socio-economic status, classified into comprehensive school, secondary non-academic and academic education, assessed primarily from the 2001 questionnaire and augmented with the 2007 questionnaire. We investigated the role of BMI in additional sensitivity analyses by standardizing the PCB levels with BMI, calculated as weight (kilograms) divided by height (meters) squared, both measured at the baseline (2001) follow-up visit. In addition, we stratified the sample by obesity status (clinic-measured BMI $>$ 30 kg/m^2) during the T2D follow-up period between 2011 and 2018. A sensitivity analysis for lipids was conducted by transforming the measured total cholesterol and triglyceride levels from mmol/l to mg/dl, calculating the total serum lipids using the Phillips formula ($total\ lipids\left(\frac{mg}{dl}\right) = 2.27{*}total\ cholesterol + triglycerides + 0.623$) and scaling the PCB levels by total lipids. Additional sensitivity analysis were performed by accounting for other potential confounding factors: geographic location (North-Eastern: Oulu, Kuopio/South-Western: Helsinki, Turku, Tampere, (Palo et al., 2009; Juonala et al., 2004) as reported in 2001); self-reported medication for hypercholesterolemia and hypertension at the time of the PCB measurements in 2001 (yes/no); physical activity levels (Mansikkaniemi et al., 2012) (questionnaire-based MET-index, primarily assessed at the 2011 visit and augmented by year 2007 and 2018 questionnaires in case of missing data); and alcohol use (questionnaire-based weekly alcohol use, calculated in standard doses of 12 g of pure ethanol, coded into drinks per day; assessed at the 2011 visit, augmented by year 2007 and 2018 questionnaires in case of missing data).

2.6. DNA methylation data and preprocessing

DNAm was assessed from whole blood samples in a subset of 1529 participants (42.5 % of the original sample) attending the 2011 follow-up visit. (Harville et al., 2021) Genomic DNA was extracted from EDTA-blood samples using the Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). Genome-wide DNA methylation levels were quantified using the Infinium MethylationEPIC array. For all samples, the average detection p-values across the probes were $<$0.01. We used the Normal-exponential Out-Of-Band method to correct for dye-correction and background fluorescence biases and a Beta-mixture quantile normalisation to correct for type II probe bias. (Triche et al., 2013) After removing cross-reactive probes (Pidsley et al., 2016) and probes with detection p-value $>$ 0.01 in at least one of the samples, 770,881 common probes remained. In all analyses, M−values were used, obtained through transforming the beta values as $M = \log_2(\beta/(1-\beta))$. The blood cell composition was estimated from the DNAm data with the Houseman method (Houseman et al., 2012).

2.7. Statistical methods

Fig. 1 presents a directed acyclic graph (DAG) describing the overarching mediation hypothesis of the role of PCBs in T2D via DNA methylation. Pathways (i) and (ii) describe the *total* and *direct effects,* i.e., the effect of PCB exposure on T2D risk with and without accounting for the mediator, respectively. The *indirect effect* is comprised of two parts, pathway (iii), (effect of PCB exposure on DNAm), and pathway (iv) (effect of DNAm on T2D risk). The proportion mediated describes the extent to which DNAm mediates the effect of PCB exposure on T2D risk.

2.8. Principal components analysis for PCBs

As the levels of the 10 PCB congeners were highly correlated with each other (Fig. 2**A**), they were summarized in terms of a lower-dimensional summary exposure measures obtained by principal components analysis (PCA). Prior to PCA, each PCB level was log-transformed and normalized to mean 0 and standard deviation 1. The scree plot in Fig. 2**B** visualizes the variances explained by each principal component (PC). The first principal component explains 84.6 % of the variation in the PCB levels. Each of the PCBs had a positive loading to the first component (range 0.28–0.34). The second component (PC2) explained 8.3 % of the variation. PCB congeners 74, 99 and 118 had high positive loadings on PC2. Lastly, the third principal component explained 3.2 % of the variation in the PCBs. We also investigated the levels of individual PCB congeners separately.

2.9. Estimation of total effects

As a first step of analysis, the total effects of the PCB congeners and their principal components on risk of T2D were estimated using logistic regression, treating T2D as a binary variable to avoid introducing bias from the uncertain T2D onset times due to the sparse and irregular

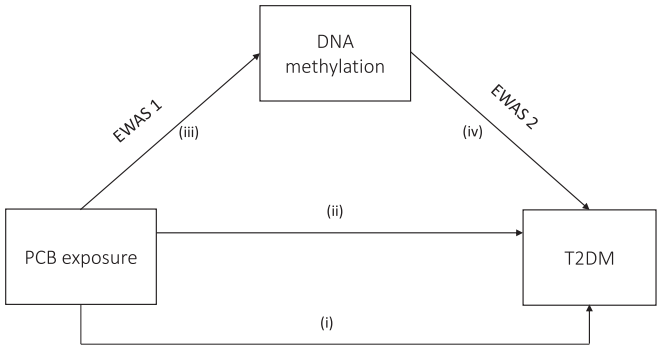

Fig. 1. Directed acyclic graph describing analyses undertaken and proposed mediation via DNA methylation. Pathways (i) and (ii) are the total and direct effects, describing the effect of PCB exposure on T2D risk with and without accounting for the mediator, respectively. Pathway (iii) is the effect of PCB exposure on DNAm and (iv) the effect of DNAm on T2D risk. Together the pathways (iii) and (iv) comprise the indirect effect. *Abbreviations: PCB, polychlorinated biphenyl; T2D, type 2 diabetes; EWAS, epigenome-wide association study.*

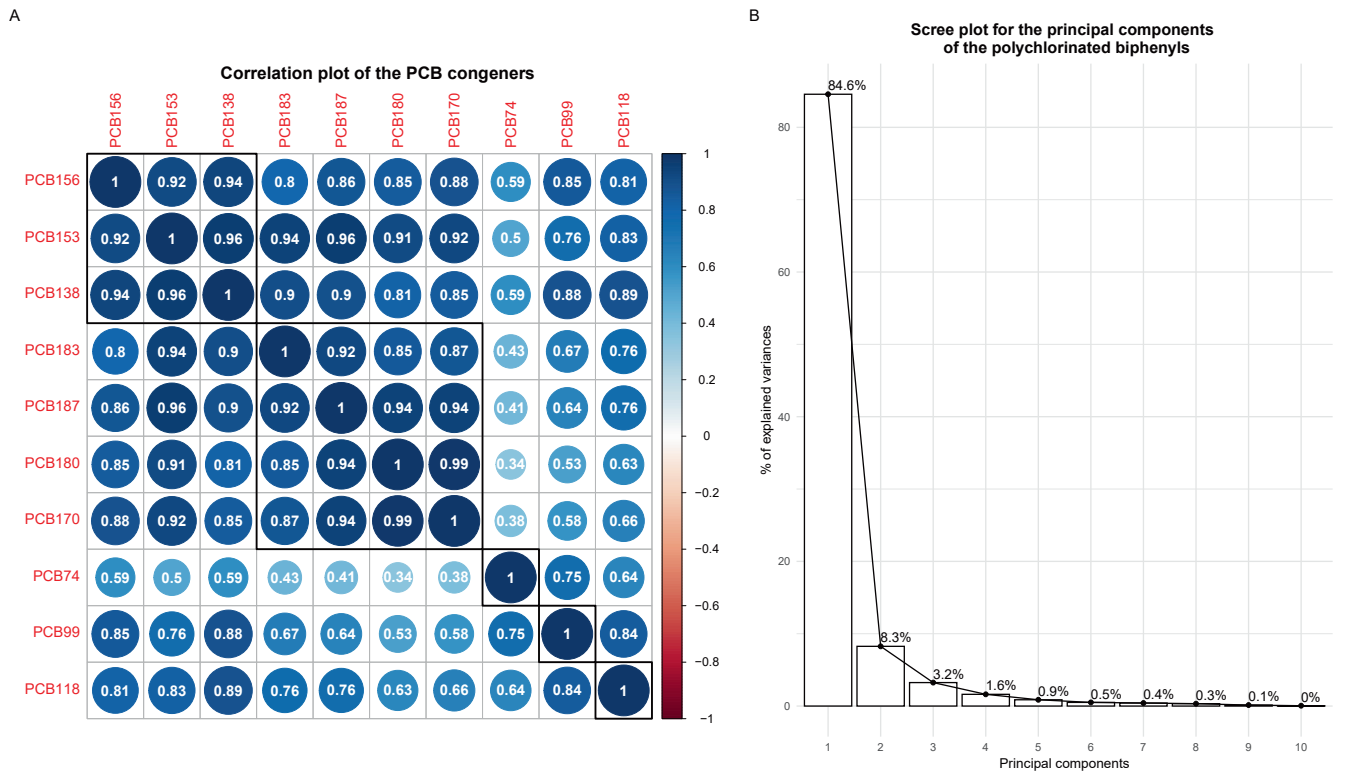

Fig. 2. PCB congeners measured in 2001 in the prospective Cardiovascular Risk in Young Finns Study, based on n = 2282 participants who had any data on PCBs. (A) Correlations of the of the levels of 10 PCB congeners, ordered and clustered using hierarchical clustering with complete linkage and 5 clusters; (B) Scree plot describing the variances explained by the 10 first PCB principal components.*Abbreviations: PCB, polychlorinated biphenyl.*

follow-up schedule.

In principle, especially in the linear model framework, when using the product-of-coefficients approach in mediation analysis, the sum of the estimated direct effect and indirect effects should equal the total effect. However, in practice this does not necessarily hold due to the non-collapsibility of the odds ratio and the fact that our mediation effects (cf. **Section "Mediation analysis"**) were estimated from models involving additional covariates, such as the technical covariates for DNA methylation. Thus, to obtain an estimate for the proportion mediated in the mediation analyses, the total effect was calculated as the sum of the direct effect and the indirect effect.

2.10. Epigenome wide association studies (EWAS)

2.10.1. Identification of differentially methylated probes (DMPs)

The pathways (iii)-(iv) were investigated with two EWAS analyses covering all 770,881 probes (CpG sites). The results for CpG sites with a nominal p-value threshold of <0.00005 are presented; results with FDR (false discovery rate)-adjusted p-value <0.05 (Benjamini-Hochberg) are also highlighted. (Benjamini and Hochberg, 1995).

Step 1 EWAS (pathway iii). Step 1 EWAS assessed the effect of PCB exposure on DNA methylation. For each probe, we estimated the effect of the first three PCB PCs simultaneously (principal components regression) and the effect of each PCB congener separately using the lmFit function in R package limma. (Ritchie et al., 2015) An empirical Bayes analysis was performed using the eBayes function. In addition to the confounders described above, the EWAS were adjusted for blood cell composition (CD4 + and CD8 + T-cells, B-cells, monocytes, granulocytes and natural killer cells, estimated from the DNAm data with the Houseman method (Houseman et al., 2012) and technical covariates (sentrix position, sample plate, both treated as categorical variables by dummy coding).

Step 2 EWAS (pathway iv). Step 2 EWAS tested the effect of DNA methylation on T2D risk. For each probe, multiple logistic regression was used to model the effect of the M−value on T2D risk using function ewaff-sites in R package ewaff. In addition to the adjustments used for Step 1 EWAS (including technical covariates and blood cell composition), also the PCB levels were adjusted for.

Identification of candidate mediators. To identify potentially mediating CpG sites, we utilized the divide-aggregate composite null test (DACT), which provides a composite p-value based on the distributions of the p-values of both EWAS. (Liu et al., 2022) The DACT method is based on the idea that in any mediation setting the null hypothesis is a composite hypothesis consisting of three components. Denoting the effect of PCB on a probe as β_1 (pathway (iii) in Fig. 1) and the effect of a probe on T2D risk as γ_1 (pathway (iv)), the composite null hypothesis includes the following cases for each CpG site: (a) PCB has no effect on DNAm, which does have an effect on T2D risk ($\beta_1 = 0$, $\gamma_1 \neq 0$); (b) PCB exposure has an effect on the DNAm, which does not have an effect on T2D risk ($\beta_1 \neq 0$, $\gamma_1 = 0$); (c) neither PCB affects DNAm nor DNAm affects T2D risk ($\beta_1 = 0$, $\gamma_1 = 0$). If any one of the three null hypotheses remains unrejected, no mediation is concluded for the CpG site in question. In contrast, both effects being present ($\beta_1 \neq 0$, $\gamma_1 \neq 0$) supports the conclusion of mediation. In practice, for both EWAS steps, the proportion of non-nulls was first estimated to calculate empirical probabilities of the three null cases, subsequently scaled into relative weights w_1, w_2 and w_3. (Liu et al., 2022) These weights were then used to build the combined DACT p-value for each probe as: $DACT_j = w_1 p_{\beta_j} + w_2 p_{\gamma_j} + w_3 Max\left(p_{\beta_j}, p_{\gamma_j}\right)^2$; where p_{β_j} is the p-value related to β_1 and p_{γ_j} is the p-value related to γ_1 for the jth probe. The empirical null distribution framework (Jin and Cai, 2007) was applied for the DACT p-values. (Liu

et al., 2022) All CpG sites with combined DACT p-value below 0.00005 were treated as potential mediators in the formal mediation analysis.

2.10.2. Identification of differentially methylated regions (DMRs)

DMRs associated with the first two principal components of PCB variation in Step 1 EWAS were identified using the DMRcate tool using the default settings and p-value threshold of 0.05. (Peters et al., 2021) DMRs comprised 3 or more consecutive probes showing a coordinated shift in DNA methylation at FDR-corrected $p < 0.05$. Bedtools was used to intersect DMRs with individual probes. (Quinlan and Hall, 2010).

2.10.3. Gene ontology and pathway enrichment analyses

For each EWAS (Step 1, Step 2 and DACT-combined), a list of DMPs with a p-value <0.0005 were investigated in more detail in gene ontology and pathway analyses. Each DMP was annotated to their nearest gene using the GREAT tool. (McLean et al., 2010) Gene ontology analyses were performed using HOMER for the genes that had a DMP within 5 KB of their transcription start site. (Zhang and Chen, 2011) Specifically, we used the findMotifs tool to obtain promoter motif abundance. For pathway enrichment analysis, we investigated KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways.

2.11. Mediation analysis

We used multiple-mediator analysis to estimate the direct and indirect effects simultaneously for all CpG sites identified as potential mediators. (VanderWeele and Vansteelandt, 2014) Causal mediation effects can be estimated from observational data subject to four identifiability conditions: (1)—(3) no unobserved confounders of the effect of PCB exposure on T2D risk, the effect of DNA methylation on T2D risk, or the effect of PCB exposure on DNA methylation; (4) no confounders of the association between DNA methylation and T2D risk are affected by PCB exposure. (VanderWeele, 2016) To this end, we adjust the analysis by the confounders. It is believable that assumption (4) is not violated in our analysis.

In practice, the following two models were used to estimate the direct and indirect effects simultaneously for all K CpG sites identified as potential mediators:

$(a) logit(p(T2D)) = \theta_0 + \sum_{k=1}^{K} \theta_{1k} M_k + \theta_2 X + \boldsymbol{\theta}_3' \boldsymbol{C}$

$(b) \boldsymbol{M} = \boldsymbol{\eta}_0 + \boldsymbol{\eta}_1 X + \boldsymbol{\eta}_2' \boldsymbol{C}$, where X is the level of PCB exposure, vector $\boldsymbol{C}$ contains the confounders and $\boldsymbol{M} = (M_1, \cdots, M_K)$ is a K-dimensional vector of M values, modelled using a multivariate linear model. Parameter θ_2 is the direct effect on the log-odds scale, estimated using logistic regression. Under rare outcomes, such as T2D in our cohort, the indirect effect is approximated by the product of η_{1k} (effect of PCB exposure on DNA methylation) and θ_{1k} (effect of DNA methylation on T2D risk). (VanderWeele, 2016) For the PCB-specific models, X is the individual PCB level, whereas for the principal components regression models, $\boldsymbol{X}$ contains the first three PCs as summary measures. Similar models were used to estimate the direct and indirect effects for each identified CpG site separately. Following the regression-based approach by VanderWeele and Vansteelandt (VanderWeele and Vansteelandt, 2014); we calculated the total indirect effect as $\sum_{k=1}^{K} \theta_{1k} \eta_{1k}$ and the proportion mediated as $\sum_{k=1}^{K} \theta_{1k} \eta_{1k} / (\sum_{k=1}^{K} \theta_{1k} \eta_{1k} + \theta_2)$, where the denominator, i.e., the sum of the direct effect and indirect effects, is considered as the total effect in the mediation analysis. For each mediated effect and the proportion mediated, the standard errors were calculated based on the delta method. (Valeri and VanderWeele, 2013) Additionally, bootstrapping-based confidence intervals, robust for any potential violations of normality, were realized for the mediation effects and the proportion mediated. (MacKinnon et al., 2004)

2.12. Additional analyses

A set of secondary analyses were conducted. First, we conducted the main analyses with all T2D cases, including those diagnosed before the DNA methylation assessment. Second, analyses were conducted for males and females separately. Third, given the potential of BMI and serum lipid levels to induce measurement error to the PCB levels measured in serum, we provide a set of additional sensitivity analyses accounting for BMI and lipids. The BMI standardization was conducted by fitting a flexible smoothing spline model of BMI on log transformed toxicant concentrations and calculating for each participant the residual as the difference between their measured and predicted log toxicant concentration. Lipid standardization was conducted by scaling the PCB concentrations by total serum lipid concentration. The lipid standardized variables were additionally standardized by BMI using the same residual method as above. We first investigated the correlations between BMI and each of the unstandardized PCB congeners, and then compared the total effects between the original congeners and their PCs and the BMI-standardized congeners and their PCs. Mediation analyses were performed for those PCs and PCBs that exhibited clear total effects both with and without BMI standardization.

Lastly, a set of sensitivity analyses accounting for various covariates were performed: the mediation analyses were adjusted separately for geographic location (Eastern versus Western Finland), use of alcohol, physical activity levels, and self-reported use of hypertension and hypercholesterolemia medication at the baseline. The detailed results of these analyses are presented in Supplemental Material, Additional analyses.

The quality of the DNAm data was assessed by performing an EWAS for self-reported smoking in 2011 and comparing the results to previously well-replicated associations (Supplemental Material, EWAS for smoking, Fig. S2). (Joehanes et al., 2016) In accord with expectations, methylation variation at cg05575921 was strongly associated with exposure to smoking ($p < 10^{-120}$), providing confidence in the quality of the final methylation dataset. To assess potential biases due to outcome definition, we evaluated differences in adherence to study visits and primary source of T2D diagnosis (visit- versus register-based information) by exposure levels. We found that PCB exposure was neither associated with study adherence nor the source of T2D diagnosis (Supplemental Material, Outcome detection; Fig. S3, Tables S1-S3). Further, the strength of evidence for the indirect effects was investigated by a permutation test approach (for details, see the Supplemental Material, Permutation test). All analyses were conducted using complete-case data. Lastly, we thus present comparisons of the non-participants and participants in Supplemental Material, Attrition analysis.

3. Results

3.1. Study overview

The formation of the study population is detailed in Fig. S4. Participants with missing T2D status (no registry data or no follow-up visits, n = 7), type I diabetes (n = 20) or gestational diabetes (n = 4) were excluded. After further removing participants with missing data on PCBs, DNAm or confounders, 1241 participants remained (33.7 % of the original sample; Fig. S4). In total, 96 were diagnosed with T2D by 2018. Of these, 25 were diagnosed before assessing DNAm, leaving 71 T2D cases and 1145 T2D non-cases in the main analysis. Table 2 summarizes the participant characteristics.

3.2. PCB exposure

The six YFS birth cohorts differed in PCB levels measured in 2001, the oldest participants having the highest PCB levels in alignment with historical PCB use in Finland (Table S1). Individual PCB levels were moderately or highly correlated, except for PCB74, which exhibited weak to moderate correlation with the other congeners (Fig. 2**A**). The three first principal components (PCs) explained 96.1 % of the total variation in the PCBs (Supplemental Material**, PCA;** Fig. 2**B**; Table S4).

Table 2
Characteristics and PCB levels in a sample of n = 1216 participants in the prospective Cardiovascular Risk in Young Finns Study reported as mean ± standard deviation or as median [interquartile range] or as n (percentage); p-values reported for comparison between T2D cases and non-cases.

	All (n = 1216)	T2D cases (n = 71)	T2D non-cases (n = 1145)	p-value
Basic characteristics				
Age in 2001	31.8 ± 5.0	33.9 ± 4.3	31.6 ± 5.1	0.0001[a]
Birth year	1969.2 ± 5.0	1967.1 ± 4.3	1969.4 ± 5.1	0.0001[a]
Year at censoring/T2D diagnosis	2017.8 ± 1.1	2014.7 ± 3.1	2017.99 ± 0.2	<0.0001[a]
Age at censoring/T2D diagnosis	48.5 ± 5.1	47.6 ± 5.6±	48.6 ± 5.1	0.05[a]
Male	536 (44.0 %)	36 (50.7 %)	500 (43.7 %)	0.24[b]
Smokers in 2011	266 (21.9 %)	13 (18.3 %)	253 (22.1 %)	0.45[b]
Socio-economic status				0.29[b]
Comprehensive school	86 (7.1 %)	8 (11.3 %)	78 (6.8 %)	
Secondary non-academic	894 (73.5 %)	52 (73.2 %)	842 (73.5 %)	
Academic	236 (19.4 %)	11 (15.5 %)	225 (19.7 %)	
Blood cell composition, proportion				
CD8 + T cells	0.06 ± 0.04	0.06 ± 0.05	0.06 ± 0.04	0.60[a]
CD4 + T cells	0.17 ± 0.06	0.17 ± 0.05	0.17 ± 0.06	0.10[a]
NK cells	0.07 ± 0.05	0.08 ± 0.06	0.07 ± 0.04	0.54[a]
B-cells	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.008[a]
Monocytes	0.06 ± 0.02	0.06 ± 0.03	0.06 ± 0.02	0.77[a]
Granulocytes	0.58 ± 0.08	0.58 ± 0.08	0.58 ± 0.08	0.70[a]
PCB levels (pg/ml)				
PCB74	10.2 [7.2; 15.3]	14.0 [7.2; 23.0]	10.1 [7.2; 15.0]	0.0012[a]
PCB99	14.5 [9.6; 21.1]	18.5 [11.4; 30.8]	14.2 [9.5; 20.7]	0.0004[a]
PCB118	25.2 [17.0; 37.7]	32.5 [18.4; 57.2]	24.8 [16.9; 37.2]	0.0007[a]
PCB153	177.6 [123.0; 263.5]	209.0 [129.9 331.9]	178.3 [123.0; 260.5]	0.013[a]
PCB138	111.4 [75.7; 162.4]	139.4 [80.8; 230.4]	110.9 [75.4; 160.2]	0.002[a]
PCB156	16.6 [11.3; 25.2]	18.6 [11.7; 33.5]	16.5 [11.3; 24.6]	0.034[a]
PCB187	29.8 [18.9; 45.9]	37.1 [20.4; 59.0]	29.4 [18.8; 45.4]	0.015[a]
PCB183	12.1 [8.0; 17.9]	15.4 [9.3; 22.0]	11.9 [8.00; 17.64]	0.001[a]
PCB180	124.5 [87.8; 191.0]	137.4 [99.1; 236.4]	123.9 [87.7; 189.6]	0.050[a]
PCB170	55.6 [39.2; 84.1]	65.7 [43.1; 109.0]	55.4 [39.1; 83.3]	0.022[a]
Principal components of PCBs				
PC1	0.03 ± 2.89	1.00 ± 3.47	−0.03 ± 2.84	0.02[c]
PC2	0.01 ± 0.88	0.29 ± 0.88	−0.01 ± 0.88	0.0058[c]
PC3	−0.01 ± 0.58	0.06 ± 0.50	−0.01 ± 0.58	0.32[c]
Covariates used in sensitivity analysis				
BMI in 2001	25.0 ± 4.3	29.3 ± 5.7	24.7 ± 4.1	<0.0001[c]
Total lipids in 2001 (mg/dl)	545 [483, 634]	634 [501, 766]	545 [475, 633]	<0.0001[a]
Overweight or obese during the T2D follow-up (2011–2018)[d]	840 (69.1 %)	62 (87.3 %)	778 (68.0 %)	0.0006[b]
Participants from Western Finland[e]	819 (66.1 %)	53 (74.6 %)	746 (65.3 %)	0.11[b]
Participants from Eastern Finland[e]	420 (33.9 %)	18 (25.4 %)	397 (34.7 %)	
Use of hypercholesterolemia medication[f]	6 (0.5 %)	2 (2.8 %)	4 (0.4 %)	0.004[b]
Use of hypertension medication[f]	23 (1.9 %)	7 (9.9 %)	16 (1.4 %)	<0.0001[b]
Physical activity, MET-index[g]	19.5 [3.0; 32.6]	11.8 [3.0; 21.8]	19.5 [5.0; 32.6]	0.0084[a]
Alcohol use, drinks per day[h]	0.4 [0.1; 1.0]	0.6 [0.1; 1.3]	0.4 [0.1; 1.0]	0.022[a]

Abbreviations: T2D, type 2 diabetes; PCB, polychlorinated biphenyl; BMI, body mass index.
[a] based on Wilcoxon signed rank test;
[b] based on Chi-Square test;
[c] based on *t*-test;
[d] data on BMI during the T2D follow-up was missing from n = 1 participant;
[e] data on geographic location was missing from n = 2 participants.
[f] no missing data on self-reported medication use
[g] data on physical activity was missing from n = 9 participants
[h] data on alcohol use was missing from n = 2 participants

Individual PCBs had similar loadings (range 0.28–0.34) on the first component, which thus describes the overall PCB exposure (Table S4). The lower-chlorinated (containing 4–5 chlorine atoms) PCBs 74, 99 and 118 had large positive loadings on the second component and the loadings of higher-chlorinated (6–7 chlorine atoms) PCBs 156, 170,180 and 187 were clearly negative (Table S4). The higher-chlorinated congeners, being more resistant to metabolic degradation and having longer elimination half-lives, were more dependent on age, whereas lower-chlorinated PCBs with shorter half-lives reflecting more recent exposure, had smaller correlations with age (Supplemental Material, PCB congeners and age; Fig. S5A-B). (Montano et al., 2022; Ritter et al., 2011)

3.3. PCB levels and T2D risk

The unadjusted T2D risk was highest in the older birth cohorts (Fig. S3, Table S2). The average T2D diagnosis year was 2014.7, and the diabetes cases and non-cases were on average 47.6 and 48.5 years old at the time of diagnosis or the end of the follow-up, respectively (Table 2). The levels of each PCB congener were higher in the T2D cases compared to the non-cases (Table 2).

In the adjusted models, the second principal component (PCB PC2) had the clearest total effect on T2D with an OR of 2.28 (95 % CI 1.60, 3.26), while the first and third components were not associated with T2D (Table 3). In line with this, congeners with any positive loadings to the second PC (74, 99, 118, 138 and 183) were associated with elevated odds of T2D (total effect OR range 1.33–1.52), whereas the remaining congeners with any negative loadings did not affect the T2D risk (total effect OR range 0.89–1.14) (Table 3, Table S4).

Table 3
Total effects of the three first PCB principal components and individual PCB congeners on T2D risk based on a logistic regression model adjusted for birth year, sex, education and smoking status in a sample of n = 1216 participants in the prospective Cardiovascular Risk in Young Finns Study reported as odds ratios and 95 % confidence intervals.

PCB	OR (95 % CI)	p-value
PC1	1.01 (0.91, 1.12)	0.89
PC2	2.28 (1.60, 3.26)	0.0000056
PC3	1.43 (0.86, 2.37)	0.17
PCB74	1.42 (1.09, 1.85)	0.01
PCB99	1.37 (1.04, 1.81)	0.023
PCB118	1.52 (1.16, 2.00)	0.0025
PCB153	1.14 (0.86, 1.52)	0.36
PCB138	1.33 (1.00, 1.76)	0.052
PCB183	1.40 (1.04, 1.88)	0.028
PCB156	0.92 (0.69, 1.24)	0.60
PCB187	1.10 (0.82, 1.48)	0.51
PCB180	0.89 (0.64, 1.22)	0.46
PCB170	0.99 (0.72, 1.35)	0.93

Abbreviations: PCB, polychlorinated biphenyl; PC, principal component; OR, odds ratio.

3.4. EWAS

3.4.1. PCB exposure and DNA methylation (Step 1 EWAS)

The main EWAS was carried for the first three PCs (Fig. 3, Figs. S6-S7). The numbers of CpG sites with unadjusted p-value <0.00005 (alternatively, with FDR p-value <0.05) were 123 (2), 151 (19) and 27 (0) for PCs 1–3, respectively (Table 4, Excel Table S1a-c). The congener-specific EWAS revealed four FDR-significant CpG sites for PCB118, two for each of PCB153, 156, 170 and 187, and three for PCB180, while the number of CpG sites with p-value <0.00005 varied between 59 and 131 (Fig. S8, Tables S5-S6).

We also identified 721 and 345 DMRs for PC1 and PC2, respectively. PC1-associated DMRs contained up to 38 CpG sites while the strongest evidence of association by p-value was obtained for an unannotated DMR on Chr6 (Chr6:30094980–30095802; $p = 1.50 \times 10^{-14}$) and *ELAVL4* ($p = 3.16 \times 10^{-13}$) (Excel Table S2a). PC2-associated DMRs contained up to 39 CpG sites. The three DMRs showing the strongest evidence for an association with PC2 by p-value overlapped *VARS* ($p = 5.65 \times 10^{-25}$), *RNF5/AGPAT1* ($p = 2.01 \times 10^{-23}$) and *CPT1A* ($p = 1.84 \times 10^{-18}$) (Excel Table S2b).

3.4.2. DNA methylation and T2D (Step 2 EWAS)

In total, 50 CpG sites were identified as associated with T2D with an uncorrected $p < 0.00005$, including cg00574958 in *CPT1A* (also found in Step 1 EWAS for PC2) (Table S7). The summary EWAS results for the effects of DNA methylation on T2D risk are displayed as a volcano plot (Fig. 3) and Q-Q-plot (Fig. S7).

3.4.3. Identification of potentially mediating CpG sites

Probes with the strongest evidence for a relationship between variable DNAm and T2D and DNAm and PC2 were identified using DACT and carried forward to the mediation analysis. In addition, PCB congener-specific DACT analyses were performed for congeners that showed association with T2D (PCBs 74, 99, 118, 138 and 183; Table 3).

Of the DACT results for PCB PC2, five CpG sites were identified with a p-value <0.00005 (Fig. S9). Subsequently, formal mediation analyses were conducted for the 5 identified CpG sites (Table S8). These included cg00574958 in *CPT1A*, identified amongst the highest-ranking CpG sites in both EWAS steps. Given the pivotal role of *CPT1A* in both Step 1 and Step 2 EWAS, we present the map of the *CPT1A* gene in human genome assembly GRCh37 in Fig. 4**A**. Furthermore, the average DNAm levels for the T2D cases and non-cases as well as the effects of PC2 on DNAm levels are presented in Fig. 4**B**.

In the congener-specific analyses, the number of DACT p-values <0.00005 (alternatively, FDR-significant p-values) for congeners 74, 99, 118, 138 and 183 was 12 (2), 14 (2), 15 (1), 17 (0) and 15 (0), respectively. Of these CpG sites, five overlapped between all congeners: cg00574958, cg05325763, cg09737197 and cg17058475 in *CPT1A* and cg16740586 in *ABCG1* (Supplemental Material, Congener-specific results; Excel Table S3).

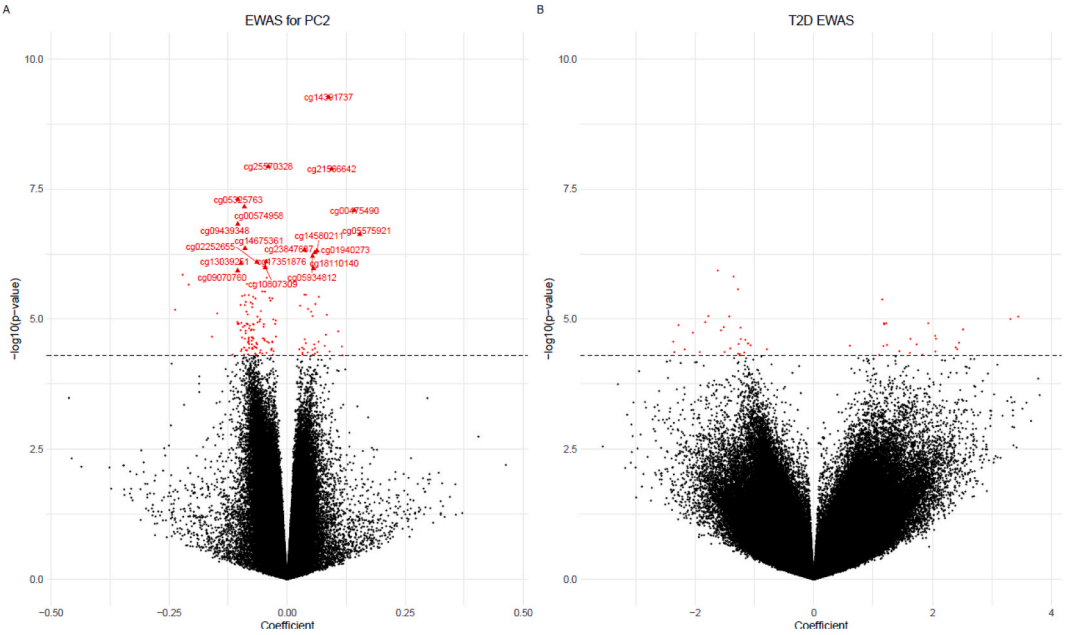

Fig. 3. Volcano plots describing the epigenome-wide effects of the second principal component of PCB exposure on DNA methylation and the epigenome-wide effects of DNA methylation on T2D in a sample of n = 1216 participants in the prospective Cardiovascular Risk in Young Finns Study. The dotted line represents the p-value limit of 0.00005, p-values are based on lmFit function in the R package limma (left-hand figure) and ewaff.sites in the R package ewaff (right-hand figure). The p-values below limit 0.00005 have been marked with red dots and FDR-significant p-values with red triangles. Abbreviations: PC, principal component; PCB, polychlorinated biphenyl; T2D, type 2 diabetes; EWAS, epigenome-wide association study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
FDR-significant CpG sites identified from an epigenome-wide association study of DNA methylation for the principal components of the PCBs in a sample of n = 1216 participants in the prospective Cardiovascular Risk in Young Finns Study.

PC	CpG site	chr	Gene name	Coeffient	Standard error	P-value	FDR-corrected p-value	Individual PCB congeners with FDR-significant associations
PC1	cg09097948	1	*RP11-382E9.1*	−0.018	0.003	1.20E-08	0.0086	153, 156, 170, 180, 187
PC1	cg09764233	10	*FAM149B1; RP11-152 N13.12; DNAJC9*	−0.0096	0.002	2.24E-08	0.0086	153, 156, 170, 180, 187
PC2	cg14391737	11	*PRSS23*	0.0876	0.01	5.45E-10	0.00042	
PC2	cg25570328	2	*SULT1C2*	−0.0400	0.007	1.17E-08	0.0034	
PC2	cg21566642	2		0.0941	0.02	1.30E-08	0.0034	
PC2	cg05325763	11	*CPT1A*	−0.104	0.02	5.06E-08	0.0097	118
PC2	cg00574958	11	*CPT1A*	−0.0906	0.02	6.96E-08	0.010	118
PC2	cg00475490	11	*PRSS23*	0.142	0.03	8.16E-08	0.010	
PC2	cg09439348	2	*CALM2*	−0.105	0.02	1.50E-07	0.017	
PC2	cg05575921	5	*AHRR*	0.154	0.03	2.37E-07	0.023	
PC2	cg14675361	13	*LMO7;RP11-29G8.3*	−0.0892	0.02	4.39E-07	0.034	
PC2	cg23847637	1	*SLC16A1*	0.0368	0.01	4.70E-07	0.034	
PC2	cg14580211	5	*C5orf62*	0.0631	0.01	4.87E-07	0.034	
PC2	cg01940273	2		0.0589	0.01	5.27E-07	0.034	
PC2	cg05934812	5	*AHRR*	0.0538	0.011	6.22E-07	0.037	
PC2	cg17351876	1	*TMCC2*	−0.0444	0.01	7.91E-07	0.041	
PC2	cg02252655	5	*LHFPL2*	−0.0640	0.01	8.07E-07	0.041	
PC2	cg13039251	5	*PDZD2*	−0.0979	0.02	8.51E-07	0.041	
PC2	cg10807309	6	*VARS*	−0.0461	0.009	1.02E-06	0.046	
PC2	cg18110140	15	*PPCDC*	0.0559	0.01	1.07E-06	0.046	
PC2	cg09070760	17	*NLRP1*	−0.105	0.02	1.18E-06	0.048	

Abbreviations: PC, principal component; PCB, polychlorinated biphenyl

3.5. Mediation analysis

Table 5 presents the results of the multiple-mediator analysis. The OR for the direct effect of PC2, when accounting for all five CpG sites simultaneously, was 1.77 (95 % CI: 1.15, 2.73) and the OR for the total indirect effect of the five mediators was 1.45 (1.27, 1.68). The total effect was thus 2.58 (1.66, 4.02) and the proportion mediated 40 % (0.20, 0.60). The coefficients of the mediation analysis are visualized in Fig. 5.

The confidence intervals based on bootstrapping were rather similar to those obtained using the delta method (Table S9). The permutation test further provided support that the estimated effects were real rather than stemming from a random chance (Supplemental Material, Mediation analysis results, Fig. S10-S11). The individual indirect effects in the single-mediator models remained similar to those reported in Table 5, each CpG site having a positive indirect effect, the largest being for cg00574958 (OR 1.12 (1.05, 1.20) (Table S8).

3.6. Gene ontology and pathway enrichment analysis

Pathway enrichment analysis for CpG-associated genes of PC1 identified 82 KEGG pathways showing any level of enrichment (Excel Table S4a). These included several cancer-associated, neuronal function, cell signalling, and immune regulation pathways. A total of 57 KEGG pathways showed enrichment following PC2 EWAS, including fatty acid metabolism and biosynthesis of unsaturated fatty acids (Excel Table S4b). Interestingly, Glucagon signalling (hsa04922) was enriched with both Step 1 PC2 and Step 2 (T2D) EWAS associated genes (Excel Table S4c), however, not included in the DACT-EWAS results (Excel Table S4d).

3.7. Additional analyses

3.7.1. All T2D cases

When including all 96 T2D cases that occurred between PCB measurement and the end of follow-up, the estimated total effects remained similar (Table S10). While the congener-specific epigenome-wide effects in Step 1 remained similar (Excel Table S5), Step 2 EWAS identified additional CpG sites, some of which have been linked with T2D in previous studies (Table S11; Supplemental Material, All T2D cases). (Fraszczyk et al., 2022; Cardona et al., 2019; Hillary et al., 2023) Additional 10 candidate mediating CpG sites were identified, of which two (cg05325763 and cg09737197) are in *CPT1A* and one (cg16740586 in *ABCG1)* has been previously associated with T2D (Table S12). (Fraszczyk et al., 2022; Cardona et al., 2019; Hillary et al., 2023)

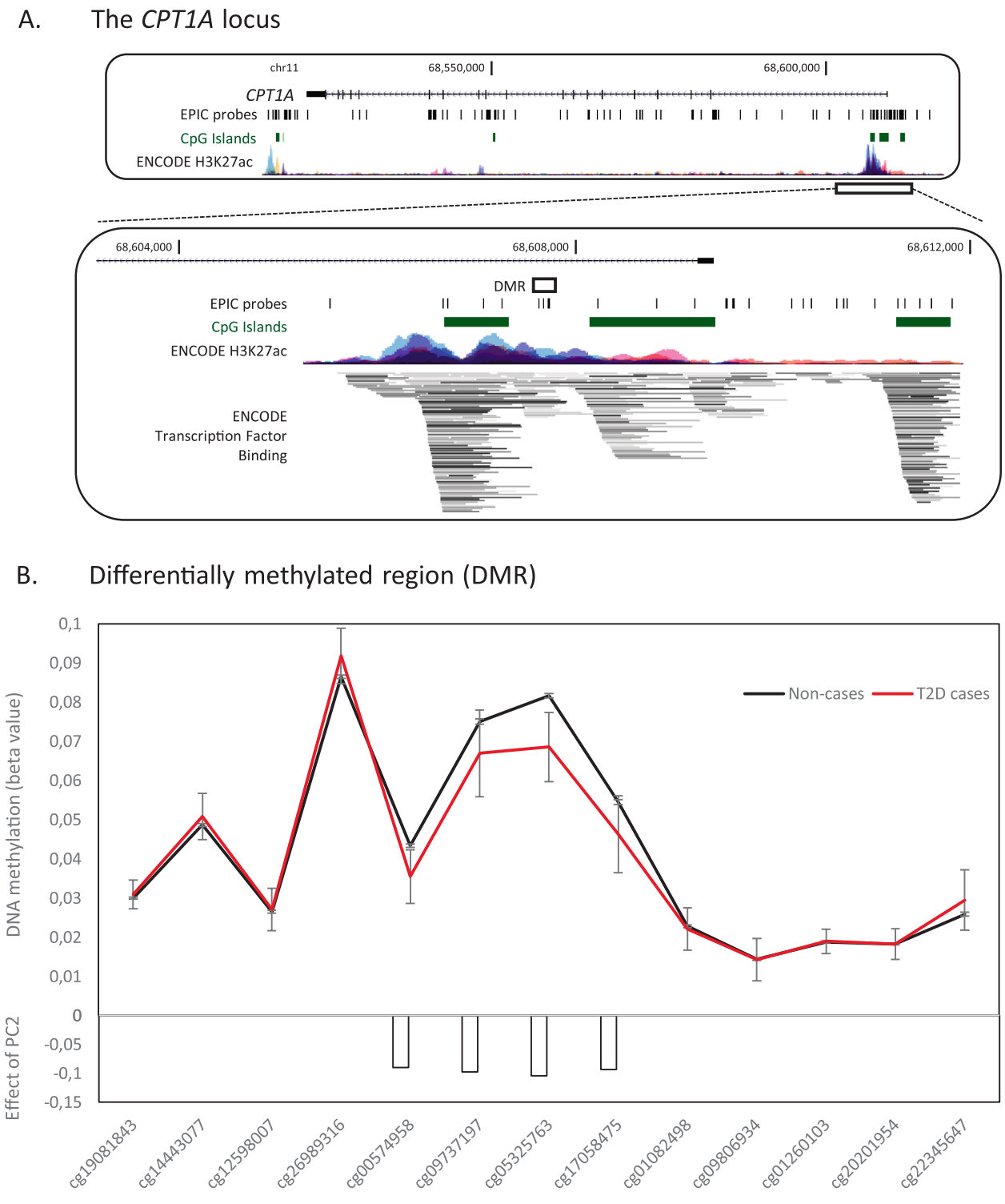

Fig. 4. Detailed DNA methylation map of the *CPT1A* gene. (a) Map of the *CPT1A* gene in human genome assembly GRCh37 (hg19), showing EPIC probe locations, CpG Islands, the location of the DMR, and ENCODE active histone mark and transcription factor binding sites. (b) Mean DNA methylation level at *CPT1A* DMR for the n = 1145 non-diabetic (black line) and n = 71 T2D groups (red line) with the respective standard error of the mean in a sample of n = 1216 participants in the prospective Cardiovascular Risk in Young Finns Study. Four DMPs within the DMR show lower DNA average methylation in the T2D group. The bars at the bottom of the graph are the effects of PC2 on DNAm in the CpG sites based on linear regression models. *Abbreviations: PC, principal component; T2D, type 2 diabetes.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.7.2. Sex-stratification

In the sex-stratified analyses, the PCB levels were higher in males compared to females, and the association between PC2 and T2D was stronger in females than in males, ORs 3.78 (2.08, 6.87) and 1.74 (1.08, 2.81), respectively (Tables S13-S15). (Song et al., 2016) The top EWAS findings were mostly non-overlapping between the sexes (Excel Tables S6-S7; Tables S16-S20, Fig. S12). Altogether 13 and 2 CpG sites had a DACT p-value <0.00005 in males and females, respectively (Table S19).

Table 5
Estimates of mediation effects from the multiple-mediator mediation analysis in a sample of n = 1216 participants in the prospective Cardiovascular Risk in Young Finns Study. The total indirect effect was calculated as the sum of individual (CpG-site specific) log-odds indirect effects, and the total effect is the sum of the direct effect and the total indirect effect on the log-odds scale. Theproportion mediated was calculated as the total log-odds indirect effect divided by the log-odds total effect. Standard errors were obtained using Delta method.

a) Multiple-mediator mediation analysis results				
CpG	**η_{1k}(s.e.)**	**θ_{1k}(s.e.)**	**Indirect effect (s.e.)**	**Indirect effect, OR (95 % CI)**
cg00574958 (*CPT1A*)	−0.09 (0.02)	−1.35 (0.34)	0.12 (0.04)	1.13 (1.05, 1.21)
cg06500161 (*ABCG1*)	0.02 (0.01)	2.56 (0.73)	0.06 (0.03)	1.06 (1.01, 1.12)
cg15791702 (*GHRFR*)	−0.06 (0.02)	−1.30 (0.32)	0.08 (0.03)	1.08 (1.02, 1.15)
cg18833417 (*RN5S449*)	−0.06 (0.02)	−1.01 (0.30)	0.06 (0.03)	1.06 (1.01, 1.12)
cg27627006 (*MAML2*)	−0.04 (0.01)	−1.49 (0.43)	0.06 (0.02)	1.06 (1.01, 1.11)
b) Summary of multiple-mediator mediation analysis				
Effect	**Log-odds scale (s.e.)**	**OR scale (95 % CI)**		
Direct effect (θ_2)	0.57 (0.22)	1.77 (1.15, 2.73)		
Total indirect effect	0.38 (0.07)	1.45 (1.27, 1.68)		
Total effect	0.95 (0.23)	2.58 (1.66, 4.02)		
Proportion mediated	0.40 (0.11) [a]	–		

Abbreviations: OR, odds ratio.
[a] This result corresponds to a 95 % CI of (0.19, 0.61) for the proportion mediated.

3.7.3. Role of adiposity and lipids

Some of the higher-chlorinated PCBs (156, 170, 180) were negatively correlated with BMI at the time of measurement, while a positive correlation was found between BMI and PCBs 74, 99, 138 and 183, in alignment with some previous reports (Table S21). (Lee et al., 2014) The PCB variables used in the main analysis and the BMI-standardized residual were highly correlated, while the BMI-standardized PCBs were not correlated with BMI. The loadings in the principal components analysis for BMI-standardized PCBs were nearly identical to the loadings in the PCA reported for the original PCB variables.

The total effects of PCBs 74, 99, 118, 138 and 183 were slightly diluted compared to the original results when standardizing with BMI (Table S22, Table 3). The direction of the total effects of PCBs 156, 170, 180 changed from negative to positive, however, both the original and BMI-standardized total effects of these congeners were characterized by large uncertainty. The total effect of PC2 was reduced, likely due to the changes in the magnitudes and directions of the individual congeners' total effects. In the mediation analysis for BMI-standardized PC2 (Table S23), the indirect effects remained rather similar to those reported in the main analyses, leading to a proportion mediated of 60 %.

A separate comparison of mediation analyses for the original and BMI-standardized PCB congeners 74 and 99 revealed that the proportion mediated remained rather similar between the two approaches. For PCB74, the total and direct effects were slightly diluted in the BMI-standardized analysis compared to that with the original variable, while the indirect effects remained nearly unchanged (Table S24). The proportion mediated was 38 % of the effect of the original PCB variable, and 42 % (for the BMI-standardized residual variable. For PCB118, each of the total, direct and indirect effects diluted in the BMI-standardized analysis compared to the analysis with the original variable (Table S25). While characterized by larger uncertainty, the proportion mediated was rather similar between the two models: 23 % for the original PCB variable and 26 % for the residual variable.

In the analysis of the subcohort who were overweight/obese during the T2D follow-up from 2011 to 2018 (n = 62 T2D cases and 778 non-cases), the results remained very similar to those reported in Table 5, with the proportion mediated slightly increasing (44 %) (Tables S26-S27).

The total effects of the lipid- and BMI-standardized PCBs were diluted compared to the original and only BMI-standardized total effects (Table S28).

3.7.4. Sensitivity analyses for additional covariates

The geographic location was associated with PCB levels, Western Finns having higher levels than Eastern (Table S29). DNA methylation levels of two of the mediators, cg00574958 and cg27627006, also differed between Eastern and Western Finns, while the incidence of T2D was similar (Table S29). The results of the mediation analysis remained similar after adjusting for geographic location (Table S30). Adjusting the mediation analyses for self-reported use of medications or alcohol use did not affect the total, direct or indirect effects, or the estimate for proportion mediated (Tables S31-S33). While the indirect effects

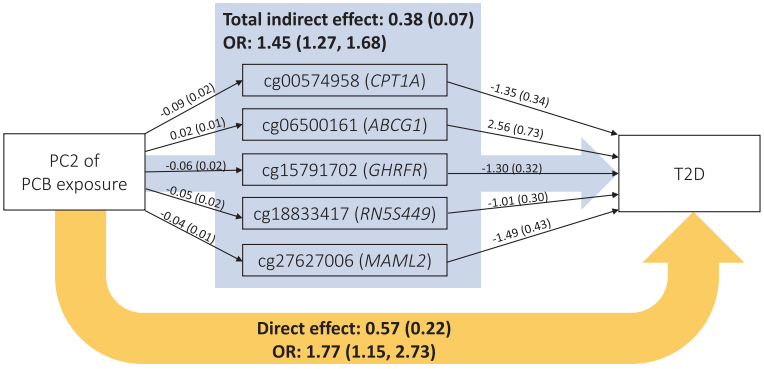

Fig. 5. Visualisation of the results of the mediation analysis (cf. Table 5) for the second principal coordinate of PCB exposure on T2D risk via five CpG sites identified as potential mediators in in a sample of n = 1216 participants in the prospective Cardiovascular Risk in Young Finns Study. The small CpG-site specific arrows describe the effects of PCB exposure on DNA methylation (left-hand side) based on linear models and effects of DNA methylation on T2D (right-handed side) on the log-odds scale based on logistic regression model; the numbers in parentheses are the standard errors of the effects. For the odds ratio scale, the confidence intervals are presented in the parentheses. *Abbreviations: PC, principal component; PCB, polychlorinated biphenyl; T2D, type 2 diabetes; OR, odds ratio.*

remained similar to the main analyses when accounting for physical activity, the direct effect and total effect were slightly increased and thus, the proportion mediated decreased to 36 % (Table S34).

4. Discussion

In this prospective population-based cohort study, we explored the relationship between exposure to PCBs and the risk of T2D in adulthood and examined the extent to which this relationship is mediated via DNA methylation. Our findings add to the growing evidence linking PCB exposure with a range of adverse metabolic outcomes, including T2D, and highlight the role of DNA methylation at key metabolic genes as a mediator of this effect.

4.1. PCB exposure and incident T2D

The putative mechanisms by which PCB exposure may induce adverse metabolic outcomes include inflammatory and oxidative stress responses. (Hoyeck et al., 2022) However, previous studies linking PCB exposure to T2D risk have been somewhat inconsistent, particularly regarding congener-specific effects, likely due to many factors, including imprecision in exposure measures (e.g. dosage effects), complexity of outcome aetiology, tissue- or age-specific effects, and various study design factors (sample size, ethnicity, analytical approach). (Lee et al., 2014; Gang et al., 2022) There are over 200 individual PCB congeners, with most studies measuring only a few. The exposure measures vary, some studies focusing solely on individual congeners while others assess summary measures. (Gang et al., 2022) Further, co-exposure to other chemicals is rarely considered. Despite these variations, evidence is mounting for a link between PCB exposure and dysregulated glucose metabolism, including risk of T2D. (Suarez-Lopez et al., 2015; Tornevi et al., 2019)

Here, we measured 10 PCB congeners in the serum of 1241 participants. The congener-specific analyses showed an elevated risk of T2D with increasing levels of PCB74, 99, 118, 138 and 183, of which PCB74, 99 and 118 with 4–5 chlorine atoms had large positive loadings to PC2 and showed more modest association with age. Similar effects were not observed for the 6–7 chlorines containing PCBs 153, 156, 170, 180, 187. These findings both align with and contradict previous studies. For example, Li et al. (2023) found that PCB118 and PCB138 were associated with incident T2D. They also reported evidence for PCB153 and PCB180, not apparent in our dataset after adjustment for confounding factors, including age. (Li et al., 2023) The difference between the associations of lower- and higher-chlorinated congeners with age and T2D is possibly due to their volatility and bioaccumulation. Congeners with more chlorine atoms are more resistant to degradation, and their exposure route is mainly from food via the environmental cycle, whereas congeners with fewer chlorine atoms are metabolized more rapidly and exposure to those takes place closer to actual PCB-containing products, e.g. via indoor air. (Montano et al., 2022)

Due to the moderate-to-high correlation between the congeners, we derived three principal components accounting for 96 % of variation in PCB levels. Despite PC1 approximating the total PCB concentration, we found little evidence for a link between PC1 and T2D. Instead, pathways associated with cancer were captured by PC1 (Excel Table S4a). Of the three principal components investigated, only PC2 (comprising positive loadings especially for the lower-chlorinated congeners, and negative loadings for the higher-chlorinated congeners) increased the T2D odds, suggesting that a combination of certain congeners rather than their total affects the T2D risk. PC2 was therefore of greatest interest for the DNAm mediation analysis.

4.2. PCB exposure and T2D are associated with DNAm

Generally, only a few individual methylation CpG sites showed strong signals for a link with PCB exposure after FDR correction, with evidence for between 27 and 151 associations for each congener-specific EWAS at a nominal p-value of <0.00005. Several CpG sites were identified as commonly differentially methylated in association with increasing PC2 or congener specific exposures, including cg00574958 (PC2, PCB74, PCB99, PCB118) and cg05325763 (PC2, PCB118), both located within the *CPT1A* gene, and previously linked to a range of metabolic, lipid and other outcomes, including incident T2D. (Cardona et al., 2019) Of note, variation in methylation at cg05575921 in *AHRR*, robustly linked also with smoking in previous studies (Joehanes et al., 2016); was amongst our top hits for PC2 despite accounting for smoking in our analyses. It is possible that both chemical exposures affect *AHRR* via different downstream pathways. On the other hand, heavy smoking is linked with activation of certain CYP enzymes that could increase metabolism and excretion of PCBs. (Moon et al., 2016) Methylation at cg05575921 has also been previously linked with perinatal exposure to PCB congener 170. (Su et al., 2019)

Other noteworthy congener-specific associations included cg16740586 (PCB118) in *ABCG1*, which has been previously linked with T2D risk. (Seo et al., 2023) Interestingly, PCB congeners 153, 156, 170, 180 and 187 (none of which showed evidence of an association with T2D risk) also showed overlap in the EWAS findings, including at cg09097948 and cg09764233 that were also FDR-significant for PC1. Of the FDR-significant CpG sites for PC2, intronic cg00475490 in *PRSS23* and intergenic cg21566642 methylation have been associated with PCB exposure in previous studies, and cg23847637 in *SLC16A1* with exposure to polybrominated biphenyls. (Curtis et al., 2021; Curtis et al., 2019; Pittman et al., 2020)

In a parallel analysis, EWAS of DNA methylation on T2D risk generated nominal ($p < 0.00005$) evidence of an association with methylation levels of 51 CpG sites, including cg00574958 within *CPT1A*, cg09294084 in *MCF2L* and cg14476101 in *PHGDH*, which have been associated with T2D in previous studies. (Fraszczyk et al., 2022; Cardona et al., 2019)

4.3. CPT1A methylation mediates effect of PCB on T2D

In total, we identified five potentially mediating CpG sites that together explained 40 % of the total effect of PC2 on T2D. Of these sites, cg00574958, located in *CPT1A,* had the largest indirect effects as well as clear biological relevance. We found that higher levels of PC2 decreased the methylation of cg00574958 and lower DNA methylation in cg00574958 increased risk of T2D, leading to a positive indirect effect. While the association between PCBs and cg00574958 is novel, this site has previously been associated with T2D and blood HbA1c levels, implying its biological relevance. (Fraszczyk et al., 2022; Cardona et al., 2019) Furthermore, its causal role in T2D is also supported by findings from a Mendelian randomisation study, however, the evidence for this has been inconsistent. (Cardona et al., 2019; Juvinao-Quintero et al., 2023)

Poor glycemic control underpinning type 2 diabetes is associated with a metabolic switch from glycolysis-associated energy production to fatty acid oxidation (FAO). (Shepherd and Kahn, 1999) CPT1A is the rate-limiting enzyme of FAO, ensuring adequate fatty acids bioavailability. (Houten et al., 2016) The CpGs of interest are located between two CpG islands in the first intron of the *CPT1A* gene within a well-defined enhancer, known to bind several transcription factors involved in lipid metabolism (SREBPs, PPAR-γ, USF1). (Eberlé et al., 2004; Huang et al., 2012; Lee et al., 2006)

In addition, variable methylation of cg06500161 in *ABCG1* has been found to be associated with T2D, HbA1c and adiposity. (Fraszczyk et al., 2022; Campanella et al., 2018; Dayeh et al., 2014) *ABCG1* helps maintain lipid homeostasis as part of reverse cholesterol transport pathway which relocates excess cholesterol from cells of peripheral tissues to the liver. (Xu et al., 2022) The identified mediators also include three novel CpG sites: cg27627006 in *MAML2,* cg18833417 in *RN5S449*, and cg15791702.

The CpG site cg27627006 is located in the active promoter region of the *MAML2* gene. Interestingly, genetic variation within *MAML2* has been linked to increased risk of T2D. (Vujkovic et al., 2020) The CpG site cg18833417 is, in addition to *RN5S449*, located in the intron of *RAB31*. Variation in *RAB31* DNA methylation has been shown to associate with early life adiposity development. (Meir et al., 2023) Moreover, *RAB31* has been implicated in glucose and insulin metabolism. (Lodhi et al., 2007) The CpG site cg15791702 is located in intron of a processed transcript of growth hormone releasing hormone receptor (*GHRFR*) gene, which encodes growth hormone involved in glucose, insulin and fatty acid metabolism. (Sharma et al., 2020) In summary, the identified mediator CpG sites are located within genes with known or putative links to fatty acid, lipid, glucose and insulin metabolism, central to T2D pathogenesis.

4.4. Accounting for the role of adiposity and serum lipid levels

It has been suggested that both BMI or, more generally, adiposity and serum lipid levels could induce measurement error in PCB levels due to their lipophilic nature. (Lee et al., 2014; Taylor et al., 2013) It has also been suggested that PCBs could instead be obesogenic and have the potential to disturb lipid metabolism, thus leading to dyslipidemia. (Baillie-Hamilton, 2002; Grün et al., 2006; Taylor et al., 2013; Lee et al., 2011) In these scenarios, the role of adiposity and lipids in our causal framework and thus their appropriate treatment would be different. In the first scenario, adiposity and lipids would be (non-causal) confounders and the measurement error induced by them should be accounted for, while in the latter case they have an additional mediating role and should not be accounted.

Our rigorous sensitivity analyses for the role of BMI and serum lipids showed that the total effects of the PCBs on T2D are slightly diluted when accounting the PCB measurements for these factors. However, our data do not allow concluding whether the dilution is due to mediation or confounding, or both. We surmise that the increase in proportion mediated in the mediation analysis with the BMI-standardized PC2 is likely exaggerated due to the different interpretation of PC2 in relation to T2D compared to the PC2 used in the main analysis. Reassuringly, in the congener-specific analyses, the proportion mediated did not change as notably upon BMI standardization. Overall, despite the variation in the results with and without BMI standardisation, the role of the main CpG sites identified in the EWAS remained unchanged.

Dissecting the role of adiposity and lipids in observational human framework is virtually impossible due to the dynamic nature of PCB exposure and excretion and changes in adiposity and DNA methylation, and progress of T2D over time. The measured PCB levels serve merely as a proxy of the "true" unmeasurable exposure, and as suggested by earlier studies, the truth could lie somewhere between the results with and without BMI and lipid standardization. (Lee et al., 2014; Taylor et al., 2013)

Cardiometabolic diseases are highly comorbid, with methylation at cg00574958 previously linked with obesity and other cardiometabolic traits. (Campanella et al., 2018) Due to the complex comorbidities and interconnected pathogeneses of these diseases, the mechanistic pathway reported here is likely intertwined with those of other cardiometabolic conditions that we were unable to assess in this study.

4.5. Limitations and strengths

While this study provides valuable new insights, it has limitations. Firstly, we assessed DNA methylation in blood. In terms of T2D pathogenesis, other cell types or tissues, such as adipose tissue, pancreatic islets or liver, could be more relevant, but assessing more central tissues is challenging in large human studies. (Olsson Lindvall et al., 2021) Many DNA methylation sites in whole blood are, however, known to covary across tissues and may therefore act as suitable proxies for epigenetic variation in more central tissues. (Ma et al., 2014) PCBs were assessed from serum, although also adipose tissue stores these toxicant. (Pollack et al., 2021) However, in general PCB concentrations in blood have been reported to reflect the lifetime systemic burden associated with dose and duration of exposure. (Raffetti et al., 2020) In addition, the smoking levels that we controlled and investigated in the separate supplemental EWAS were assessed using a questionnaire and are thus prone to reporting bias. However, the results of the smoking EWAS, conducted to assess the validity of DNA methylation, were reassuringly in concordance with previous reports on smoking behaviour and DNA methylation.

Second, instead of the population-level incidence of T2D, we estimated the risk of T2D bounded by our sparse measurement time frame and variable diagnostic criteria. This outcome thus lacks direct interpretability within a broader population; however, the biological interpretation on epigenetic mediation should remain valid. While the prospective population-derived cohort is a strength of the study, the resulting T2D incidence and subsequently, the power for Step 2 EWAS to identify T2D-associated differential methylation are lower than in some previously reported case-control studies. In the secondary analyses, where all T2D cases, including those whose diagnosis was before the DNA methylation assessment, were included, the statistical power can be slightly improved. However, the temporal ordering of the observations poses a risk of reverse causality.

Third, the decreasing levels of human exposure to PCBs may diminish the contemporary relevance of our findings. Exposure to PCBs has decreased globally substantially due to bans on importing and manufacturing. (Kiviranta et al., 1999) In Finland, for example, biomonitoring studies show that median serum PCB concentrations have decreased 96 % in 18-year olds between 1980 and 2018 (Rantakokko personal communication). Nevertheless, our observations provide a foundation for future studies to replicate our findings and further investigate more contemporary EDCs, such as PFAS compounds or phthalates, with the aim to determine whether their effects on T2D or other cardiometabolic diseases are mediated via similar pathways. Further understanding of contributions of various chemicals could be augmented by conducting chemical mixture assessments, as co-exposure to various other chemicals, including other persistent organic pollutants and endocrine disruptors, can not only occur via similar exposure routes in the food chain but also be relevant in terms of T2D risk. Epigenome-wide effects of various other POPs as well as their mixtures and their similarity to those reported here remain to be studied. In addition, it cannot be ruled out that e.g. the use of certain medications, in particular those that induce weight change could have played a role in the associations between PCBs, DNA methylation and T2D.

Fourth, this study was conducted in Finnish participants with a typical Finnish background exposure to PCBs. The generalizability of our findings to populations with different genetic backgrounds or different sources and levels of PCB exposure remains uncertain.

This study also has notable strengths. While mounting evidence has linked PCBs with T2D and DNA methylation, and DNA methylation with T2D, to our knowledge the relationships between all three have not previously been explored simultaneously. To our understanding, the sample size in the PCB EWAS is the largest reported to date. We followed T2D cases prospectively using a variety of different criteria, including biological measurements, self-reporting and registry data. We used recent methodological advancements, which provides better statistical power and more accurate type 1 error rate compared to other widely-used mediation methods (Liu et al., 2022). We performed two large EWAS, and our results thus comprehensively describe epigenome-wide effects of PCBs as well as the contribution of DNA methylation on T2D.

5. Conclusions

In this prospective study, we found that a specific profile of PCB exposure is associated with an increased risk of T2D. We demonstrated that this effect is partly mediated by DNA methylation at key metabolic

genes, including *CPT1A* and *ABCG1*, previously demonstrated to be differentially methylated in association with a range of exposures and metabolic outcomes. Given the well-characterized role of *CPT1A* in the metabolic switch from glycolysis to fatty acid oxidation in diabetes, this provides a plausible biological link between PCB exposure, epigenetic regulation and T2D.

6. Data sharing

The data used in this study comprises health related participant data and their use is therefore restricted under the regulations on professional secrecy (Act on the Openness of Government Activities, 612/1999) and on sensitive personal data (Personal Data Act, 523/1999, implementing the EU data protection directive 95/46/EC). Due to these legal restrictions, the data from this study cannot be stored in public repositories or otherwise made publicly available. However, data access may be permitted on a case-by-case basis upon request only. Data sharing requires a data-sharing agreement. Investigators can submit an expression of interest to the corresponding author.

CRediT authorship contribution statement

Noora Kartiosuo: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kari Auranen:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Visualization, Conceptualization. **Toby Mansell:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis. **Boris Novakovic:** Writing – review & editing, Software, Resources, Methodology, Formal analysis, Visualization, Investigation. **Jaakko Nevalainen:** Writing – review & editing, Supervision, Methodology. **Katja Pahkala:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Suvi Rovio:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Juha Mykkänen:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Jorma Viikari:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Markus Juonala:** Writing – review & editing, Resources, Data curation, Conceptualization. **Panu Rantakokko:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Hannu Kiviranta:** Writing – review & editing, Methodology, Data curation. **Jari Kaikkonen:** Writing – review & editing, Data curation, Conceptualization. **Terho Lehtimäki:** Writing – review & editing, Methodology, Data curation. **Emma Raitoharju:** Writing – review & editing, Methodology, Conceptualization, Data curation. **Pashupati P. Mishra:** Writing – review & editing, Software, Methodology, Data curation. **Mika Kähönen:** Writing – review & editing, Funding acquisition, Data curation. **Anne-Louise Ponsonby:** Writing – review & editing, Conceptualization. **Sam Tanner:** Writing – review & editing, Methodology, Conceptualization. **David Burgner:** Writing – review & editing. **Olli Raitakari:** Writing – review & editing, Supervision, Resources, Funding acquisition, Data curation, Conceptualization. **Richard Saffery:** Writing – review & editing, Supervision, Resources, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109779.

Data availability

The data that has been used is confidential.

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