Received: 17 December 2019 | Revised: 23 March 2020 | Accepted: 24 April 2020

DOI: 10.1111/bcpt.13424

ORIGINAL ARTICLE

BCPT Basic & Clinical Pharmacology & Toxicology

DNA methylation level in blood and relations to breast cancer, risk factors and environmental exposure in Greenlandic Inuit women

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Funding information
Else og Mogens Wedell Wedellsborgs Fond; Fabrikant Einar Willumsens Mindelegat; International Polar Year Committee, Grant/Award Number: 09, – and 064624; Commission for Scientific Research in Greenland, Grant/Award Number: 2015, – and 111,969; Department for Health and Infrastructure (Greenland Self-government, Nuuk), Aarhus University; Danish Environmental Agency (Miljøstyrelsen), Grant/Award Number: MST-112-00243

Abstract

Several studies have found aberrant DNA methylation levels in breast cancer cases, but factors influencing DNA methylation patterns and the mechanisms are not well understood. This case–control study evaluated blood methylation level of two repetitive elements and selected breast cancer-related genes in relation to breast cancer risk, and the associations with serum level of persistent organic pollutants (POPs) and breast cancer risk factors in Greenlandic Inuit. DNA methylation was determined using bisulphite pyrosequencing in blood from 74 breast cancer cases and 80 controls. Using first tertile as reference, the following was observed. Positive associations for *ATM* in second tertile (OR: 2.33, 95% CI: 1.04; 5.23) and *ESR2* in third tertile (OR: 2.22, 95% CI: 0.97; 5.05) suggest an increased breast cancer risk with high DNA methylation. *LINE-1* methylation was lower in cases than controls. In third tertile (OR: 0.42, 95% CI: 0.18; 0.98), associations suggest in accordance with the literature an increased risk of breast cancer with LINE-1 *hypo*methylation. Among controls, significant associations between methylation levels and serum level of POPs and breast cancer risk factors (age, body mass index, cotinine level) were

Abbreviations: ARNTL, Aryl hydrocarbon receptor nuclear translocator like; ATM, ATM serine/threonine kinase; BMI, Body mass index; BRCA1, BRCA1, DNA repair associated; CDKN1A (p21), Cyclin-dependent kinase inhibitor 1A; CLOCK, Clock circadian regulator; CV, Coefficient of variation; DAGs, Directed acyclic graphs; ER, oestrogen receptor; ESR1, oestrogen receptor 1; ESR2, oestrogen receptor 2; HCB, Hexachlorobenzene; LINE-1, Long interspersed repeat sequences; OCP, Organochlorine pesticide; OR, Odds ratio; p, p′-DDE, Dichlorodiphenyldichloroethylene; p, p′-DDT, Dichlorodiphenyltrichloroethane; PCB, Polychlorinated biphenyl; PER1, Period circadian clock 1; PER2, Period circadian clock 2; PER3, Period circadian clock 3; PFAA, Perfluorinated alkylated acid; PFDA, Perfluorodecanoic acid; PFDoA, Perfluorododecanoic acid; PFHpA, Perfluoroheptanoic acid; PFHxS, Perfluorohexane sulfonate; PFNA, Perfluorononanoic acid; PFOA, Perfluorooctanoic acid; PFOS, Perfluorooctane sulfonate; PFOSA, Perfluorooctanesulfonamide; PFTrA, Perfluorotridecanoic acid; PFUnA, Perfluoroundecanoic acid; POP, Persistent organic pollutants; RAD51, RAD51 recombinase; TP53, Tumour protein p53; β-HCH, β-Hexachlorocyclohexane.

found. Thus, breast cancer risk factors and POPs may alter the risk through changes in methylation levels; further studies are needed to elucidate the mechanisms.

KEYWORDS

arctic inuit, circadian genes, persistent organic pollutants, repetitive elements, tumour suppressor genes

1 | BACKGROUND

Breast cancer is the most common cancer among women worldwide,[1] but 42%-62% of breast cancer cases cannot be explained by known risk factors.[2-4] It is well established that lifestyle and environmental factors play a role in breast cancer risk,[3,5] but genetic factors also influence breast cancer risk. Variations in the two most known and well-studied susceptible breast cancer genes, *BRCA1* and *BRCA2*, only account for 5%-10% of all familial breast cancer cases. However, women carrying variations in one of the two genes have a 60%-80% lifetime risk of developing breast cancer.[6] Other genetic variations with higher frequency in the global population influence breast cancer risk to a smaller extent.[6-8] Still, much of the genetic variations contributing to the development of breast cancer are unknown, and epigenetic variations may elucidate possible risks.

Variations in DNA methylation patterns can alter the gene expression and thereby modify breast cancer risk.[9] Differences in the methylation patterns between breast cancer patients and controls have been observed not only in breast tissue but also in blood samples.[10] In blood, global *hypo*methylation can cause genomic instability and activation of oncogene expression, and *hyper*methylation of the promoter regions can lead to inactivation of tumour suppressor genes, both mechanisms have been suggested to increase breast cancer risk.[10] Even partial inactivation of tumour suppressor genes could contribute significantly to tumour development.[11] In addition to BRCA1 and BRCA2, p53 is one of the most well-known tumour suppressors involved in the DNA repair and cell cycle regulation. The *ATM* gene senses DNA damage and activates checkpoints and DNA repair pathways through rapid phosphorylation of several substrates including p53 and BRCA1. Furthermore, p53 can activate transcription of genes, including the *p21* tumour suppressor gene.[12,13]

Oestrogen and its receptors play an important role in all stages of breast cancer, including development and progression.[14] Whether there is any relation between DNA methylation of the oestrogen receptor (ER) and breast cancer risk, hormone status in the tumour needs further studies.[15-18] Several studies have found a higher *ESR1* methylation in ER-negative tumours compared with ER-positive tumours.[17] In tumour tissue, one study found a strong correlation between *ESR1* methylation and ER negativity in tumours,[17] while another study found that obesity was associated with ER-positive breast cancer among women with unmethylated *ESR1*.[18]

The oestrogen receptors have been observed to interact, directly or indirectly, with another potentially important gene group related to breast cancer, the circadian genes.[19] It was shown that some circadian genes (*PER1*, *PER2* and *PER3*) are deregulated in breast tumour tissue and that expression is highly explained by DNA methylation of the gene promoters.[20] Furthermore, circadian genes have been shown to influence cell proliferation, apoptosis, cell cycles and to interact with several tumour suppressor genes related to breast cancer.[21-24]

The molecular mechanisms by which methylation patterns are established and regulated are complex and not completely understood. Although not consistent, there is some evidence that lifestyle factors, such as body mass index (BMI), smoking, physical activity, alcohol intake and diet, may be correlated with DNA methylation.[25-27] The literature also suggests that environmental chemicals are associated with DNA methylation changes that can affect human health.[28] Global or gene-specific methylation levels have been associated with cadmium, lead, mercury and persistent organic pollutants (POPs), such as dioxins, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers.[28] The results are, however, inconsistent. A cohort study conducted among Japanese women found an inverse association between serum levels of PCBs and global DNA methylation levels in leucocytes,[29] and similar results were seen in studies from Korea[30] and Greenland.[31] Conversely, a Swedish study found in an elderly population that high levels of PCBs were associated with global DNA *hyper*methylation.[32]

We hypothesized that changes in blood DNA methylation of repetitive elements and specific breast cancer-related genes can alter breast cancer risk. To test this hypothesis, using white blood cells, we measured DNA methylation in two repetitive elements (*ALU* and *LINE-1*), five tumour suppressor genes (*ATM*, *BRCA1*, *CDKN1A*, *TP53* and *RAD51*), two isoforms of oestrogen receptors (*ESR1* and *ESR2*) and five circadian genes (*CLOCK, ARNTL*, *PER1*, *PER2* and *PER3*) in blood samples from breast cancer cases and controls among Greenlandic Inuit women. Also, we aimed at investigating associations between breast cancer risk factors, serum levels of POPs and DNA methylation levels.

2 | METHODS

2.1 | Study participants

Participating Greenlandic Inuit women were recruited during 2000-2003 and 2011-2014; a detailed description of the study population has been reported previously.[33] Briefly, breast cancer diagnosis was confirmed by histological samples and ~ 85% of all patients diagnosed with breast cancer in the data collection periods agreed to participate in the study. Controls recruited during 2000-2003 were selected from two cross-sectional studies on healthy persons.[34,35] Controls recruited during 2011-2014 were patients with non-malignant diagnoses at Dronning Ingrid's Hospital, Nuuk.[36] In the inclusion, although not matched, we attempted to obtain similar distribution among cases and controls with regard to age and geographical area (region) of residence. All Greenlandic breast cancer cases are treated at Droning Ingrid's Hospital in Nuuk; however, at other departments (from where controls were included), a higher percentage of patients from Nuuk may be present. The hospital staff responsible for the inclusion was aware of including controls from all regions and in the relevant age groups.

All women were of Greenlandic Inuit descent, defined as being born and having more than two grandparents born in Greenland. Blood samples were obtained when agreeing to participate, and in breast cancer cases before any treatment was initiated. Information on demographic and lifestyle parameters, for example, parity, smoking status, height and weight was collected through questionnaires.

The plasma level of cotinine was used as a biomarker measurement of current tobacco smoking. The Calbiotech Cotinine Direct ELISA Kit was used to measure the plasma cotinine (Calbiotech Inc), and the product protocol was followed. The detection limit was 1 ng/mL, and if the values were below the detection limit, the value was given as 0.5 ng/mL.

The study was approved by the Greenlandic Ethics Committee (2011-050 536) and The Danish Data Protection Agency (2011-41-6371). Before being enrolled, all participants gave written informed consent. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[37]

2.2 | DNA extraction, bisulphite treatment and DNA methylation analyses

Genomic DNA was extracted from whole blood using the QIAamp DNA mini Kit (Qiagen) according to the manufacturer's instructions. Subsequently, 500 ng of each DNA sample was treated with bisulphite using EZ DNA Methylation-Gold Kit (D5007; Zymo Research). Bisulphite-treated DNA was eluted in 30 μL of M-Elution Buffer buffer (D5001-6, Zymo Research) and diluted in further 170 μL of purified water. The samples were stored at − 80°C until use.

The following gene methylation level was measured: 1. two repetitive elements *ALU* and long interspersed repeat sequences (*LINE-1*); 2. five breast cancer-related genes with tumour suppressor function: ATM serine/threonine kinase (*ATM*), BRCA1, DNA repair associated (*BRCA1*), cyclin-dependent kinase inhibitor 1A (*CDKN1A (p21)*), RAD51 recombinase (*RAD51*) and tumour protein p53 (*TP53*); 3. two oestrogen receptor genes: oestrogen receptor 1 (*ESR1*) and oestrogen receptor 2 (*ESR2*); and 4. five circadian genes: aryl hydrocarbon receptor nuclear translocator like (*ARNTL*), clock circadian regulator (*CLOCK*), period circadian clock 1 (*PER1*), period circadian clock 2 (*PER2*) and period circadian clock 3 (*PER3).* The analyses were carried out at Epidemiology, Epigenetics and Toxicology Laboratory, Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Italy.

DNA methylation was quantified using bisulphite-PCR and pyrosequencing. For the repetitive elements (*ALU* and *LINE-1),* a 50 μL PCR was carried out as previously described[38] using 25 μL GoTaq Green Master Mix (Promega), 20 pmol each of the forward and reverse primers, 25 ng bisulphite-treated genomic DNA and water. For the specific genes (*ATM, BRCA1, CDKN1A, RAD51, TP53, ESR1, ESR2, ARNTL, CLOCK, PER1, PER2* and *PER3*), the assays were designed to cover the greatest possible number of CpG sites within the promoter region but considering length of the PCR amplicon, length of the target sequence and primers that avoided CpGs. A 50 μL PCR was carried out in 25 μL Hot Start GoTaq Green Master mix (Promega), 10 pmol each of the forward and reverse primers, 25 ng bisulphite-treated genomic DNA and water. In the supporting information, Table S1 lists the primer sequences and PCR conditions.

A biotin-labelled primer was used to purify the final PCR product. Fifteen μL PCR products were bound to Streptavidin Sepharose High Performance beads (Amersham Biosciences), purified, washed with 70% EtOH, denatured with 0.2 M NaOH and washed with wash buffer (Qiagen, Inc) using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Inc). The pyrosequencing primer (0.3 μM) was annealed to the purified single-stranded PCR product, and pyrosequencing was performed using the PyroMark Q96MD pyrosequencing system (Qiagen, Inc). The methylation level at CpG positions was expressed as the percentage of methylated cytosines, determined as the number of methylated cytosines divided by the sum of methylated and unmethylated cytosines, multiplied by 100% (% 5mC). The data are given as mean of duplicate measurements.

2.3 | Measurement of persistent organic pollutants in serum

Serum POPs and total lipid measurements have been reported in detail previously.[33] PCBs [PCB 99, 101, 105, 118, 128, 138, 153, 156, 170, 180, 183, 187] and organochlorine pesticides (OCPs) [dichlorodiphenyltrichloroethane (p,p′-DDT), dichlorodiphenyldichloroethylene (p,p′-DDE), mirex, β-hexachlorocyclohexane (β-HCH), hexachlorobenzene (HCB), cis- and trans-nonachlor, and oxychlordane] were measured by gas chromatography–mass spectrometry at Le Centre de Toxicologie du Québec, Canada. Perfluorinated alkylated acids (PFAAs) [perfluoroheptanoic acid (PFHpA, C7), perfluorooctanoic acid (PFOA, C8), perfluorononanoic acid (PFNA, C9), perfluorodecanoic acid (PFDA, C10), perfluoroundecanoic acid (PFUnA, C11), perfluorododecanoic acid (PFDoA, C12), perfluorotridecanoic acid (PFTrA, C13), perfluorohexane sulfonate (PFHxS, C6), perfluorooctane sulfonate (PFOS, C8), and perfluorooctanesulfonamide (PFOSA, C8)] were measured by liquid chromatography-tandem mass spectrometry at Department of Environmental Science, Aarhus University, Denmark.

The levels of POPs were summed: ∑*PCB:* PCB 99, 101, 105, 118, 128, 138, 153, 156, 170, 180, 183, 187; ∑*OCP:* p,p′-DDT, p,p′-DDE, mirex, β-HCH, HCB, cis- and trans-nonachlor, oxychlordane; ∑*lipPOP*: ∑PCB, ∑OCP; ∑*PFCA*: PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, PFTrA; ∑*PFSA:* PFHxS, PFOS, PFOSA, and ∑*PFAA:* ∑PFCA, ∑PFSA.

2.4 | Statistics

The statistical analyses were performed using IBM SPSS Statistics 24. The statistical significance level was set at $P \leq .05$, and P-values $\leq .08$ were considered borderline significant. No correction for multiple testing was carried out due to small sample size.

The Mann-Whitney U test was used to determine differences in DNA methylation between cases and controls, since not all DNA methylation variables were normally distributed. To estimate the odds ratio (OR) for the association between methylation levels and breast cancer status, unconditional logistic regression models were used. Methylation levels were considered as categorical variables grouped by tertiles based on the distribution among controls and as continuous variables.

Factors influencing global and gene-specific DNA methylation is not fully understood, and only a few studies have investigated the relations of breast cancer risk factors and gene-specific DNA methylation. Based on published literature and directed acyclic graphs (DAGs) (Figure S1), we identified the following potential confounders: age, BMI, smoking, parity, recruitment period, alcohol intake and physical activity. Due to limited sample size and insufficient information available, we were not able to control for alcohol intake and physical activity. Due to the uncertainty of the relations between the factors and the gene-specific DNA methylation, we chose to analyse the associations with two multivariable logistic regression models. Model 1 included the most important confounders: age (continuous), BMI (groups) and recruitment period (2000-2003 and 2011-2014), while model 2 also included other potential confounders such as cotinine levels (continuous), and parity (continuous) together with age (continuous), BMI (groups), recruitment period (2000-2003 and 2011-2014).

The associations between demographic characteristics, serum POP levels and methylation levels were assessed among the controls using linear regression models.

3 | RESULTS

3.1 | Study population

A total of 97 cases and 94 controls were included in the study; however, due to missing DNA samples, the study population in this study only consisted of 74 breast cancer cases and 80 controls. The participants with missing DNA samples were comparable to the whole population regarding of age, BMI, parity, cotinine levels, menopausal status and breastfeeding history (data not shown).

No significant differences were found between cases and controls regarding age, parity, menopausal status, self-reported smoking status and cotinine level (Table 1). Cases (BMI 26.2 kg/m^2) were leaner than controls (BMI 27.8 kg/m^2); however, the distribution between BMI groups was similar in cases and controls.

3.2 | Association of DNA methylation levels with breast cancer risk

For the repetitive elements, cases had significantly lower *LINE-1* methylation median level compared with controls (cases: 74.44%, controls: 75.70%) (Table 2). Borderline significantly higher methylation median levels of the oestrogen receptor *ESR2* was found in cases compared with controls (cases: 63.82%, controls: 62.38%). Methylation levels did not differ between cases and controls for any of the other measured genes (Table 2).

In Table 3, we report associations between methylation levels (tertiles) and breast cancer risk. Similar tendencies in the results were generally seen across the three models (1: unadjusted, 2: adjusted for age, BMI and recruitment period, 3: adjusted for model 1 plus cotinine levels and parity), even

TABLE 1 Demographic and reproductive characteristics for breast cancer cases and controls

	Cases			Controls			
Parameters	**n (%)**	**Median**	**IQR**	**n (%)**	**Median**	**IQR**	***P*-value**
Demographic factors							
Age (years)	74	52.0	19.0	80	50.0	17.0	.083[a]
≤50	32 (43.2%)			47 (58.8%)			.192[b]
51-55	11 (14.9%)			6 (7.5%)			
56-59	7 (9.5%)			8 (10.0%)			
≥60	24 (32.4%)			19 (23.8%)			
BMI (kg/m^2)	45	26.2	6.2	71	27.8	6.3	**.030**[a]
<25	17 (37.8%)			20 (28.2%)			.129[b]
25-30	22 (48.9%)			30 (42.3%)			
>30	6 (13.3%)			21 (29.6%)			
Smoking status	67			76			
Never	6 (9.0%)			14 (18.4%)			.251[b]
Former	17 (25.4%)			19 (25.0%)			
Current	44 (65.7%)			43 (56.6%)			
Plasma cotinine (ng/ml)	71	44.2	199.5	72	40.5	246.6	.711[a]
Reproductive factors							
Menopausal status	63			71			
Premenopausal	23 (36.3%)			21 (29.6%)			.394[b]
Postmenopausal	40 (63.5%)			50 (70.4%)			
Full-term pregnancies	57	3.0	2.5	63	3.0	3.0	.602[a]
Breastfed	49			64			
Ever breastfed (Yes)	43 (87.8%)			57 (89.1%)			.829[b]

Note: Bold text indicates significant finding.

n, number of subjects with information on the corresponding variable; IQR, interquartile range; *p*-value: testing the difference between cases and controls; BMI, body mass index.

[a]Independent samples t test on ln-transformed variables.

[b]Pearson's chi-square test.

though the level of significance varied due to reduced sample size in the adjusted analyses.

We observed a significantly reduced breast cancer risk with increasing levels of *LINE-1* methylation (Table 3). In the unadjusted model, comparing the second (OR: 0.73, 95%CI: 0.35; 1.54) and third (OR: 0.42, 95%CI: 0.18; 0.92) *LINE-1* tertile to the first, a dose-dependent (*P* for trend ≤ .05) reduced risk was observed (Table 3). Similar estimates were seen in the adjusted models; however, the association was non-significant in the adjusted model 2.

For *ESR2*, a dose-dependent borderline-significant (*P* for trend = .059) increased risk of breast cancer was seen in the unadjusted model but disappeared upon adjustment (Table 3). OR estimates in the second tertile were 1.60 (95% CI: 0.69; 3.72) and in the third tertile 2.22 (95% CI: 0.97; 5.05) (unadjusted model).

For the *ATM* gene, significant and borderline-significant increased breast cancer risk were seen in the second tertile of the unadjusted model (OR: 2.33. 95% CI: 1.04; 5.23) and model 1 (OR: 2.63. 95% CI: 0.99; 6.97), respectively. However, there was no dose response through the tertiles (*P* for trend > .05) (Table 3). Methylation levels of several other genes were significant or borderline significantly associated with breast cancer at the second tertile, including *CDKN1A(p21)* (unadjusted model: OR 0.38, 95% CI: 0.14; 0.98), *RAD51* (model 2: OR 0.38, 95% CI: 0.13; 1.08) and *PER3* (model 2: OR 0.35, 95% CI: 0.11; 1.09) (Table 3). However, there were no dose-response tendencies, the significance varied between the models, and the results for *CDKN1A(p21)*, *RAD51* and *PER3* were not consistent with the results from the analyses with continuous variables (Table S2).

In the analyses with continuous variables, methylation level of the investigated genes was not significantly associated with breast cancer risk; however, borderline-significant associations were found for *LINE-1* (OR: 0.92, 95% CI: 0.83; 1.01), *ATM* (OR: 1.49, 95% CI: 0.96; 2.03) and *ESR2* (OR: 1.02, 95% CI: 1.00; 1.05), in the unadjusted model (Table S2).

TABLE 2 DNA methylation in white blood cell DNA of breast cancer cases and controls

	Cases		Controls		
Methylation (%5mC)	**n**	**Median (Q1-Q3)**	**n**	**Median (Q1-Q3)**	***P***
Repetitive elements					
ALU	74	22.82 (22.37-23.84)	79	22.90 (22.50-24.07)	.301
LINE-1	73	74.44 (72.24-76.89)	77	75.70 (73.33-78.15)	**.017**
Tumour suppressor genes					
ATM	74	1.08 (0.74-1.41)	80	1.06 (0.62-1.28)	.238
BRCA1	69	4.09 (3.30-4.88)	80	3.83 (3.37-4.53)	.456
CDKN1A (p21)	62	2.31 (1.05-4.08)	69	2.07 (0.89-3.27)	.346
TP53	71	3.67 (3.13-4.05)	79	3.72 (3.23-4.08)	.397
RAD51	70	1.47 (1.20-2.07)	79	1.52 (1.15-2.14)	.870
oestrogen receptor genes					
ESR1	73	3.12 (2.39-4.19)	74	3.04 (2.32-3.73)	.511
ESR2	71	63.82 (57.59-72.70)	77	62.38 (53.58-68.82)	**.080**
Circadian genes					
ARNTL	72	3.27 (2.71-3.96)	80	3.49 (2.97-4.15)	.230
CLOCK	71	1.84 (1.33-2.95)	80	2.12 (1.34-3.21)	.363
PER1	72	1.86 (1.21-2.58)	79	1.76 (0.86-2.60)	.241
PER2	73	78.40 (75.08-81.99)	78	79.03 (75.72-82.75)	.404
PER3	73	89.77 (87.98-91.45)	76	89.12 (87.76-91.17)	.744

Note: Mann-Whitney U test was used to compare the methylation level between breast cancer cases and controls. Significant or borderline-significant p-values are given in bold. n: number of subjects in the group with information on the given variable. Q1 and Q3: quartile 1(25th percentile) and quartile 3 (75th percentile).

The estimates were similar in the unadjusted and the two adjustment models. However, the level of significance only persisted for *LINE-1* (OR: 0.89, 95% CI: 0.79; 1.01) in the adjusted model 1, whereas the associations with *ATM* and *ESR2* were not significant in the adjusted models (Table S2). The non-significant results in the adjusted model may be due to the lower sample size due to missing information on the confounder variable for some of the participants.

3.3 | Associations of DNA methylation levels with POPs and breast cancer-related risk factors

Figure 1 shows associations between serum POP levels and methylation levels among controls adjusted for age. Among controls, the majority of the methylation levels were positively associated with the lipophilic POP levels (Figure 1). The methylation levels of the oestrogen receptor genes *ESR1* and *ESR2* and circadian gene *PER1* significantly and positively associated with ∑lipPOP, ∑PCB and ∑OCP, while associations with the tumour suppressor genes *BRCA1* and *TP53* were borderline-significant (Figure 1). The associations between DNA methylation levels and ∑PFAA and ∑PFSA were primarily positive as well, but a different pattern was seen for ∑PFCA being inversely associated with some genes. Methylation levels of tumour suppressor genes *BRCA1* and *TP53* and oestrogen receptor *ESR1* were significantly positively associated with ∑PFAA and ∑PFSA. ∑PFCA was significantly negatively associated with methylation of repetitive element *ALU* and tumour suppressor gene *RAD51* (borderline for repetitive element *LINE-1* and oestrogen receptor *ESR2*), and significantly positively with tumour suppressor gene *CDKN1A(p21)* and circadian gene *CLOCK* (Figure 1). The directions of the associations among cases were similar but mostly weaker and non-significant (not shown).

We further explored the relationship between DNA methylation levels and breast cancer risk factors (age, BMI, smoking, plasma cotinine and menopause) in controls and found that gene-specific methylation levels were significantly associated with age, BMI and cotinine plasma levels (Figure 2). Age was positively associated with methylation levels in the circadian gene *PER3*; BMI was negatively associated with global methylation (*ALU* and *LINE-1*), but positively associated with methylation of the circadian genes *CLOCK* and *PER2*, and cotinine levels were positively associated with tumour suppressor gene *RAD51* (Figure 2). We also analysed the associations between methylation levels and age, BMI, smoking, plasma cotinine and menopause among cases and found similar results (not shown).

TABLE 3 Associations between DNA methylation level and breast cancer risk

	Unadjusted model			Adjusted model 1			Adjusted model 2		
	OR (95% CI)	n (cases/ controls)	*P*-value	OR (95% CI)	n (cases/ controls)	*P*-value	OR (95% CI)	n (cases/ controls)	*P*-value
ALU									
1st Tertile	1.00 (ref)	28/26		1.00 (ref)	20/18		1.00 (ref)	17/15	
2nd Tertile	0.83 (0.38; 1.78)	24/27	.624	0.74 (0.30; 1.84)	16/25	.513	0.68 (0.25; 1.84)	16/22	.443
3rd Tertile	0.79 (0.36; 1.71)	22/26	.544	0.49 (0.15; 1.61)	8/25	.238	0.76 (0.20; 2.93)	7/19	.684
P for trend	.539			.234			.560		
LINE-1									
1st Tertile	1.00 (ref)	33/25		1.00 (ref)	18/21		1.00 (ref)	16/18	
2nd Tertile	0.73 (0.35; 1.54)	26/27	.409	0.51 (0.20; 1.33)	17/24	.168	0.55 (0.19; 1.57)	16/22	.263
3rd Tertile	0.42 (0.18; 0.98)	14/25	**.044**	0.26 (0.08; 0.87)	10/25	**.030**	0.34 (0.09; 1.33)	9/18	.121
P for trend	**.046**			**.023**			.096		
ATM									
1st Tertile	1.00 (ref)	15/27		1.00 (ref)	9/24		1.00 (ref)	9/18	
2nd Tertile	2.33 (1.04; 5.23)	35/27	**.039**	2.63 (0.99; 6.97)	26/24	**.052**	2.39 (0.85; 6.71)	26/20	.097
3rd Tertile	1.66 (0.72; 3.85)	24/26	.236	1.49 (0.47; 4.77)	10/23	.499	0.72 (0.19; 2.75)	6/20	.627
P for trend	.282			.395			.823		
BRCA1									
1st Tertile	1.00 (ref)	19/26		1.00 (ref)	14/19		1.00 (ref)	14/16	
2nd Tertile	0.93 (0.41; 2.13)	19/28	.861	0.71 (0.26; 1.91)	13/27	.493	0.62 (0.22, 1.74)	13/26	.361
3rd Tertile	1.63 (0.74; 3.59)	31/26	.224	1.22 (0.40; 3.76)	14/25	.727	1.28 (0.38; 4.29)	12/16	.686
P for trend	.200			.794			.784		
CDKN1A (p21)									
1st Tertile	1.00 (ref)	24/23		1.00 (ref)	13/20		1.00 (ref)	11/15	
2nd Tertile	0.38 (0.14; 0.98)	9/23	**.045**	0.51 (0.16; 1.59)	8/22	.245	0.52 (0.15; 1.88)	7/20	.323
3rd Tertile	1.21 (0.55; 2.67)	29/23	.639	1.78 (0.65; 4.82)	19/19	.260	2.33 (0.72; 7.53)	18/16	.159
P for trend	.596			.220			.110		
TP53									
1st Tertile	1.00 (ref)	25/26		1.00 (ref)	15/22		1.00 (ref)	14/20	
2nd Tertile	0.92 (0.43; 2.01)	24/27	.843	1.29 (0.50; 3.35)	18/25	.605	1.59 (0.57; 4.47)	17/20	.379
3rd Tertile	0.88 (0.40; 1.94)	22/26	.751	0.99 (0.32; 3.09)	11/23	.989	1.21 (0.34; 4.29)	9/17	.766
P for trend	.750			.980			.681		
RAD51									
1st Tertile	1.00 (ref)	29/27		1.00 (ref)	21/25		1.00 (ref)	21/19	
2nd Tertile	0.75 (0.35; 1.64)	21/26	.473	0.54 (0.21; 1.40)	11/23	.204	0.38 (0.13; 1.08)	11/20	**.070**
3rd Tertile	0.72 (0.33; 1.57)	20/26	.404	0.88 (0.31; 2.50)	22/12	.810	0.43 (0.13; 1.45)	9/18	.173
P for trend	.392			.626			.108		
ESR1									
1st Tertile	1.00 (ref)	23/24		1.00 (ref)	19/20		1.00 (ref)	19/17	
2nd Tertile	0.76 (0.34; 1.74)	19/26	.518	0.68 (0.26; 1.82)	12/23	.444	0.48 (0.17; 1.36)	11/22	.166
3rd Tertile	1.35 (0.62; 2.95)	31/24	.454	1.04 (0.35; 3.08)	14/22	.950	0.87 (0.27; 2.80)	11/17	.810
P for trend	.425			.955			.638		
ESR2									
1st Tertile	1.00 (ref)	15/26		1.00 (ref)	19/20		1.00 (ref)	11/19	

(Continues)

TABLE 3 (Continued)

	Unadjusted model			Adjusted model 1			Adjusted model 2		
	OR (95% CI)	n (cases/ controls)	*P*-value	OR (95% CI)	n (cases/ controls)	*P*-value	OR (95% CI)	n (cases/ controls)	*P*-value
2nd Tertile	1.60 (0.69; 3.72)	24/26	.275	1.56 (0.55; 4.41)	12/23	.403	1.72 (0.58; 5.12)	15/16	.327
3rd Tertile	2.22 (0.97; 5.05)	32/25	**.058**	2.32 (0.66; 8.22)	14/22	.191	1.91 (0.49; 7.46)	13/20	.354
P for trend	**.059**			.190			.313		
ARNTL									
1st Tertile	1.00 (ref)	29/27		1.00 (ref)	17/25		1.00 (ref)	15/20	
2nd Tertile	0.90 (0.42; 1.90)	26/27	.776	0.90 (0.36; 2.25)	15/26	.817	0.83 (0.30; 2.30)	13/24	.720
3rd Tertile	0.61 (0.27; 1.36)	17/26	.227	0.93 (0.34; 2.51)	11/20	.881	1.35 (0.44; 4.13)	11/14	.595
P for trend	.238			.868			.645		
CLOCK									
1st Tertile	1.00 (ref)	26/27		1.00 (ref)	12/25		1.00 (ref)	9/18	
2nd Tertile	1.04 (0.49; 2.22)	27/27	.922	1.09 (0.35; 3.40)	19/23	.877	1.18 (0.34; 4.14)	18/20	.795
3rd Tertile	0.72 (0.32; 1.61)	18/26	.423	0.73 (0.20; 2.74)	13/23	.645	0.83 (0.20; 3.48)	13/20	.799
P for trend	.443			.581			.706		
PER1									
1st Tertile	1.00 (ref)	18/26		1.00 (ref)	10/22		1.00 (ref)	8/19	
2nd Tertile	1.44 (0.64; 3.25)	26/26	.374	1.36 (0.49; 3.77)	17/23	.553	1.66 (0.54; 5.04)	16/20	.375
3rd Tertile	1.50 (0.67; 3.34)	28/27	.322	1.36 (0.50; 3.69)	17/25	.550	2.01 (0.65; 6.34)	16/18	.231
P for trend	.337			.574			.238		
PER2									
1st Tertile	1.00 (ref)	27/26		1.00 (ref)	15/25		1.00 (ref)	14/19	
2nd Tertile	0.96 (0.45; 2.07)	26/26	.923	1.00 (0.38; 2.63)	16/22	.996	0.76 (0.26; 2.24)	14/20	.620
3rd Tertile	0.74 (0.34; 1.64)	20/26	.459	1.00 (0.37; 2.63)	13/22	.994	0.79 (0.25; 2.51)	12/18	.684
P for trend	.467			.994			.683		
PER3									
1st Tertile	1.00 (ref)	27/25		1.00 (ref)	19/22		1.00 (ref)	17/16	
2nd Tertile	0.75 (0.34; 1.65)	21/26	.472	0.40 (0.14; 1.13)	8/23	.084	0.35 (0.11; 1.09)	7/20	**.070**
3rd Tertile	0.93 (0.43; 2.01)	25/25	.846	0.89 (0.34; 2.33)	17/23	.813	0.91 (0.31; 2.64)	16/19	.861
P for trend	.840			.782			.810		

Note: n: number of subjects included in the analysis, OR: odds ratio, CI: confidence interval, bold text indicates significant or borderline-significant findings. In model 1, age, BMI and recruitment period are included as covariates, and in Model 2 cotinine levels and parity were included as well.

4 | DISCUSSION

In the present study, we examined DNA methylation patterns of global repetitive elements and cancer-related genes and associations with breast cancer risk. We demonstrated *hypo*methylation in *LINE-1* repetitive elements in breast cancer cases (Table 2), and a dose-dependent inverse association with breast cancer risk (Table 3). We found an indication of a positive association between DNA methylation of *ATM* and *ESR2* and breast cancer risk (Tables 3 and S2), with a dose-dependent association through the tertiles for *ESR2*. Moreover, DNA methylation levels were associated with exposure to POPs (Figure 1) and lifestyle factors (age, BMI, cotinine levels) (Figure 2).

Measurement of DNA methylation levels in repetitive elements can be used as a surrogate to estimate global methylation. We determined methylation levels of two global methylation markers, *ALU* and *LINE-1*, comprising ~ 10% and ~ 20% of the human genome, respectively.[39] The repetitive elements are generally highly methylated in normal somatic tissues, but in malignant tissues, the elements are methylated to a lesser extent.[40,41] Lower global methylation levels can lead to genomic instability, reactivation of transposable elements and altered gene transcription.[42,43] The methylation levels of *ALU* and *LINE-1* in the present study were similar to levels reported in other studies using the same measurement methods.[44-47] Changes in methylation level do not only occur in malignant tissue; there is growing evidence

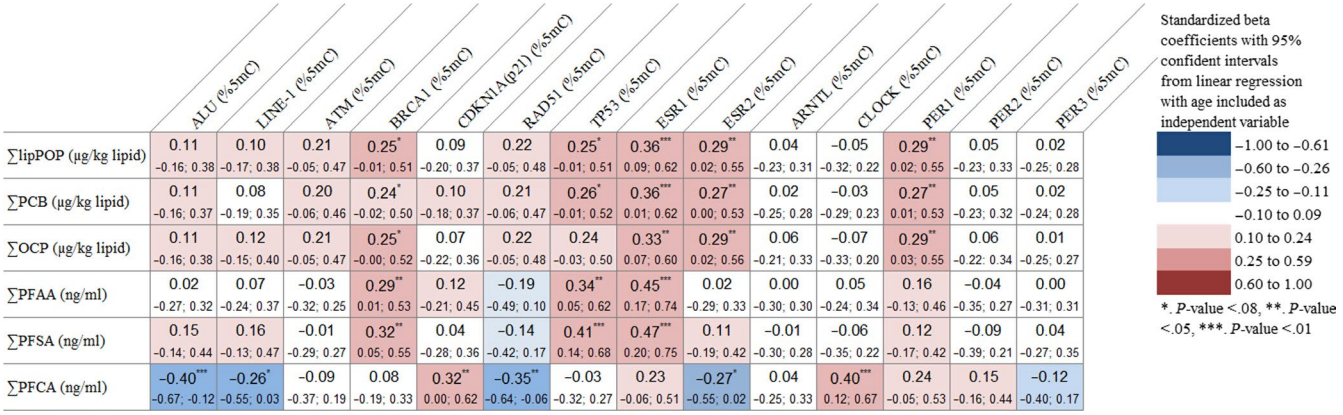

FIGURE 1 Age-adjusted associations between DNA methylation and environmental pollutants among controls. Standardized beta coefficients (in the upper line) with 95% confidence intervals (below in smaller text size) from linear regression including age as covariate. ∑PCB: PCB 99, 101, 105, 118, 128, 138, 153, 156, 170, 180, 183, 187, ∑OCP: p,p′-DDT, p,p′-DDE, mirex, β-HCH, HCB, cis- and trans-nonachlor, and oxychlordane, ∑lipPOP: ∑PCB + ∑OCP, ∑PFCA: PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA and PFTrA, ∑PFSA: PFHxS, PFOS and PFOSA, and ∑PFAA: ∑PFCA + ∑PFSA

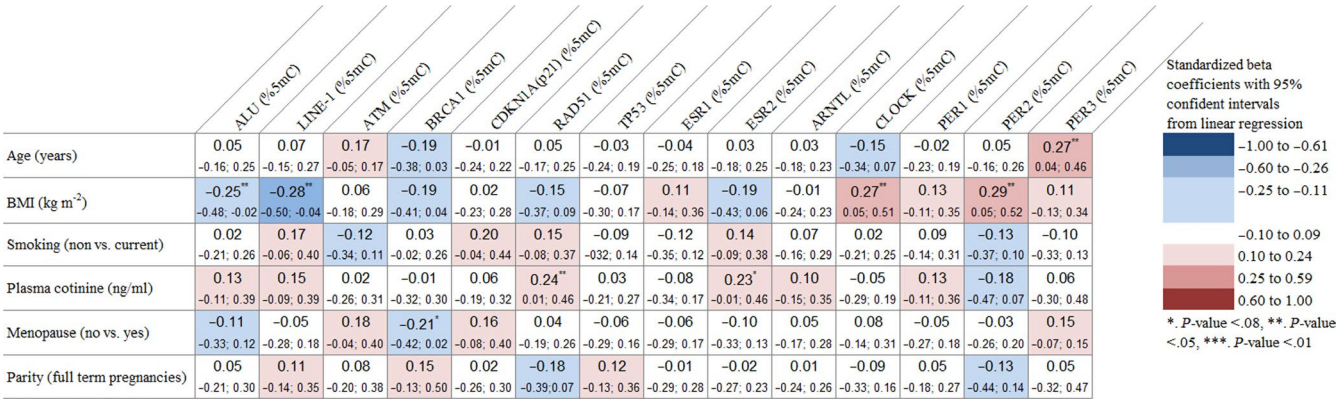

FIGURE 2 Associations between DNA methylation and breast cancer risk related factors among controls. Unadjusted standardized beta coefficients (in the upper line) with 95% confidence intervals (below in smaller text size) from linear regression are reported. However, the analyses were repeated for BMI, smoking, plasma cotinine and menopause with age included as covariate, and the results were similar

supporting more systemic changes, for example, in the white blood cells of cancer patients. Global *hypo*methylation in white blood cells has among others been associated with head and neck cancer,[48] gastrointestinal cancers.[49-52] and urinary tract cancers.[53-55] Similarly, our data suggested that breast cancer risk was associated with DNA *hypo*methylation of *LINE-1* in a dose-dependent manner (Table 3). Several studies have investigated the association between global DNA methylation levels and breast cancer risk using different methods (measurements of repetitive elements, measurement of 5-methyldeoxucytosine etc). However, clear evidence to confirm an association is waiting to be investigated in further studies.[10] A prospective case–control study observed a dose-dependent inverse association between *LINE-1* methylation levels and breast cancer risk.[44] In the same study, an association between methylation and time of blood sampling was observed; for each year closer to the breast cancer diagnosis, the *LINE-1* methylation level decreased by 0.20%. This supports the lower *LINE-1* methylation levels in breast cancer cases in the present study. However, it is in contrast to another large study using data from three cohorts with blood samples drawn before breast cancer diagnosis, not finding that *LINE-1* methylation was related to breast cancer risk.[45] Several case–control studies have reported similar methylation levels of *LINE-1* and *ALU* in both cases and controls.[46,56,57] The global methylation level has been associated with breast cancer risk, using other measures than *LINE-1* and *ALU*.[56,58-61] In a case–control study from the USA, global *hypo*methylation was associated with increased breast cancer risk when measuring 5-methyldeoxucytosine using liquid chromatography-electrospray ionization tandem mass spectrometry.[60] Our study supports the growing evidence of an association between global *hypo*methylation levels in blood cells and breast cancer risk, but the causal relationship needs

to be investigated in prospective studies and optimally with higher statistical power.

We investigated gene-specific methylation levels in 12 breast cancer-related genes. Five genes (*ATM*, *CDKN1A (p21)*, *RAD51*, *ESR2* and *PER3*) were significantly or borderline-associated with breast cancer risk in the tertiled analyses (Table 3). However, for three of the genes (*CDKN1A(p21), RAD51*, *PER3*), no dose-response effect was seen and results were inconsistent across the different models, also the analyses with continuous variables (Table S2), suggesting that the findings may be due to chance and do not reflect true associations. For *ATM* and *ESR2*, the continuous data also showed borderline-significant associations with breast cancer (Table S2), and a dose-response effect was seen for *ESR2* in the tertiled analyses.

ATM encodes the protein ATM, which has an essential role in monitoring and repairing DNA double-strand breaks, and variations in *ATM* have been associated with breast cancer risk.[62] *Hyper*methylation of *ATM* was positively associated with breast cancer risk, both in the tertiled analyses (Table 3) and borderline in the analyses with continuous exposure variables (Table S2). However, the dose-response effect and the significance did not persist in all models. Two other studies investigated methylation of *ATM* in relation to breast cancer risk.[45,63] Both studies found results in line with our data, although the study designs were different. Firstly, the study populations were very different: Flanagan et al[63] included post-diagnostic blood samples from bilateral breast cancer cases, and Brennan et al[45] used data from three cohorts (white or European populations) with pre-diagnostic blood samples. In contrast, we examined post-diagnostic samples from untreated Greenlandic Inuit breast cancer cases. Secondly, we determined the methylation level in the *ATM* promoter, whereas the studies by Flanagan et al and Brennan et al determined intragenic *ATM* methylation. Our results, however, support the hypothesis that DNA *hyper*methylation in tumour suppressor genes leading to reduced tumour suppressor transcription and expression may increase the risk of breast cancer.

Although it is well-known that oestrogen plays a vital role in the development of breast cancer, the role of oestrogen receptor β, encoded by *ESR2*, is not fully understood. Growing evidence supports *ESR2's* role as a tumour suppressor gene.[64] To our knowledge, no other studies have reported on the methylation of *ESR2* in blood and its relation to breast cancer risk, but the methylation of *ESR1,* encoding oestrogen receptor α, has been investigated with conflicting results.[10] Significant *hyper*methylation of *ESR2* has been reported in breast tumour tissue when compared to normal breast tissue,[65] being in line with our data from blood DNA methylation (Tables 3 and Table S2). Taken together, the findings encourage further investigation of *ESR2*, both as a possible blood DNA methylation biomarker and as a tumour suppressor. We did not observe any significant associations between *ESR1* methylation and breast cancer risk (Table 3).

Methylation patterns in differentiated cells are mostly stable and inheritable through generations[30,66,67] but may be altered by environmental and breast cancer-related factors.[66] However, most studies have investigated their effect on global methylation and only a limited number of studies on effects on gene-specific methylation.[28] We investigated the association of POPs with both global and gene-specific DNA methylation and observed that lipPOPs (∑PCB and ∑OCP) were positively associated with methylation levels of five of the genes (*BRCA1*, *TP53*, *ESR1*, *ESR2* and *PER1*). The ∑PFAA and ∑PFSA were positively associated with methylation levels of *BRCA1*, *TP53* and *ESR1*, and ∑PFCA was positively associated with methylation levels of *CDKN1A(p21)* and *CLOCK,* but negatively with *ALU, LINE-1, RAD51* and *ESR2* (Figure 1). We have previously reported an association between serum POP levels and breast cancer risk,[33] and the results reported in the present study could suggest that associations between serum POP levels and breast cancer may be driven by alterations in DNA methylation levels. However, when including methylation levels in the models analysing the association between serum POP levels and breast cancer, the odds ratio estimates and significance levels were very similar to the estimates without methylation levels included in the models (data not shown). The influence of POP exposure on global and gene-specific DNA methylation and the possible link to breast cancer risk needs further investigation, both in large epidemiological studies and in mechanistic studies elucidating the possible pathways and mode of action.

The present study has some limitations. The study sample size is relatively small; therefore, we did not adjust the multiple number of tests carried out. However, the number of tests must be taken into account when concluding on the results, together with the effect size, biological plausibility and consistency of the results both within the study and with other published literature.

We measured the DNA methylation levels in white blood cells. However, it cannot be ruled out that our DNA samples might contain small amounts of circulating free DNA such as DNA from circulating tumour cells or circulating cell-free DNA, as our samples were extracted from blood samples. The number of circulating tumour cells is, however, normally in the order of one cancer cell per 1-10 million normal cells[68] and thus unlikely to influence the results significantly.

Residual confounding may be present. Factors influencing DNA methylation levels are not fully understood, and unidentified confounders may exist. The limited knowledge about associations between DNA methylation levels and breast cancer risk factors makes it difficult to decide on one multivariate model to evaluate the association between DNA methylation levels and breast cancer. We identified several potential confounders (age, BMI, smoking, parity, recruitment period, alcohol intake and physical activity; Figure S1), and in the two models, we did control for the most important confounders;

however, due to the limited samples size and missing information, we were not able to adjust for physical activity and alcohol intake. The estimates from the two models were very similar and did not differ substantially from the unadjusted model either, neither when including all participants nor when the analyses were restricted to those with information on all confounders (data not shown). Breastfeeding has been suggested as a factor potentially affecting DNA methylation levels. Only few cases (n = 6) and controls (n = 7) in the study population had never breastfed, and no associations were found between breastfeeding and DNA methylation (data not shown), which was supported by Collin et al finding no association between breastfeeding and global DNA methylation levels among 1110 controls.[69] Thus, we did not include breastfeeding as a confounder in the analyses. Some of the self-reported variables may be subject to differential information and recall bias; for example, breast cancer cases may recall past exposure to potential risk factors differently than the controls. A potential differential recall bias might affect the self-reported BMI (weight and height) and smoking history. Nevertheless, the variables measured in the laboratory (DNA methylation levels, serum POP levels, etc) cannot be affected by differential misclassification as cases and controls were analysed simultaneously by staff blinded to the breast cancer status.

We used two types of controls in the study. In the first recruitment period (2000-2003), healthy controls from two population-based cross-sectional studies[34,35] and in the second recruitment period (2011-2014), hospitalized controls with non-malignant diseases[36] were included. Using hospitalized controls may introduce bias if the studied exposures are associated with the diseases in the control group. However, the estimates were similar in the two periods (data not shown), indicating that the results were not biased due to the inclusion of hospital controls.

Finally, we report data from a case–control study, which limits the possibilities to conclude any causal relationships.

In conclusion, our findings of an association between global *hypo*methylation (*LINE-1*) and breast cancer risk are in line with the hypothesis and several other studies. The inverse relationship between *LINE-1* methylation levels and breast cancer risk was our most convincing finding, but we did also observe some associations with gene-specific DNA methylation. The association between methylation levels of the tumour suppressor gene *ATM* and breast cancer risk supports previously reported relations, while for the first time we report a possible relation with methylation level of the oestrogen receptor *ESR2* gene. In support of previous reports,[25-32,66] lifestyle factors and environmental exposures significantly associated with methylation levels, which suggest that these factors can modify breast cancer risk through changes in DNA methylation levels. However, with the case–control study design, we cannot determine if the aberrant DNA methylation level is a cause or a consequence of breast cancer. The molecular mechanisms leading to methylation changes in the DNA of blood cells are not clear, and further studies of the mechanisms are needed to understand whether the epigenetic changes are a consequence or cause of the breast cancer.

ACKNOWLEDGEMENTS

The authors thank all the participating women. We gratefully acknowledge the staff at the clinical chemistry hospital laboratory and other involved hospital staff at Dronning Ingrid's Hospital in Nuuk, Greenland. Especially, we wish to thank head nurse Linda Seelk and medical doctors Peder Kern and Ole Lind for their coordination support during the collection period. The authors would also like to thank all the colleagues at the Centre for Arctic Health & Molecular Epidemiology, Department of Public Health, Aarhus University and Epidemiology, Epigenetics and Toxicology Laboratory, Department of Clinical Sciences and Community Health, Università degli Studi di Milano for their scientific support. The study was approved by the Greenlandic Ethics Committee (2011–050536) and The Danish Data Protection Agency (2011–41-6371). All participants gave written informed consent before being enrolled in the study. Financial support was gratefully received from Else og Mogens Wedell Wedellsborgs Fond, Fabrikant Einar Willumsens Mindelegat, The International Polar Year Committee (09–064624), The Commission for Scientific Research in Greenland (2015–111,969), The Department for Health and Infrastructure (Greenland Self-government, Nuuk), Aarhus University and The Danish Environmental Agency (MST-112-00243).

CONFLICT OF INTERESTS

The authors have nothing to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Wielsøe M, Tarantini L, Bollati V, Long M, Bonefeld-Jørgensen EC. DNA methylation level in blood and relations to breast cancer, risk factors and environmental exposure in Greenlandic Inuit women. *Basic Clin Pharmacol Toxicol*. 2020;127:338–350. https://doi.org/10.1111/bcpt.13424