Epigenetic effects of environmental chemicals: association between methylation of long interspersed nuclear elements (LINEs-1) and exposure to heavy metals and persistent organic pollutants (POPs)

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The global reduction of 5-methyldeoxycytosine levels (5-mdC) in CpG sites of the genome constitutes a common epigenetic phenomenon in neoplasia. Genome-wide DNA hypomethylation has been shown to increase genome instability, potentially leading to higher cancer risk. Environmental chemicals may increase the risk of diseases (including cancer) by altering the expression of genetic information through epigenetic mechanisms such as those linked to CpG methylation. The repetitive sequences of DNA are heavily methylated in normal tissue and, among these, LINE-1 is often used as a surrogate marker for the study of global DNA methylation levels.

Are the levels of LINE-1 methylation in human leukocyte DNA influenced by exposure to a series of toxic environmental chemicals ???

EXPERIMENTAL DESIGN

Envirogenomarkers: Breast Cancer and Lymphoma case-control study within 2 cohorts from Italy and Sweden

Analysis of 600 blood samples (300 from Italy and 300 from Sweden) for % of methylation at three CpG islands of LINE-1
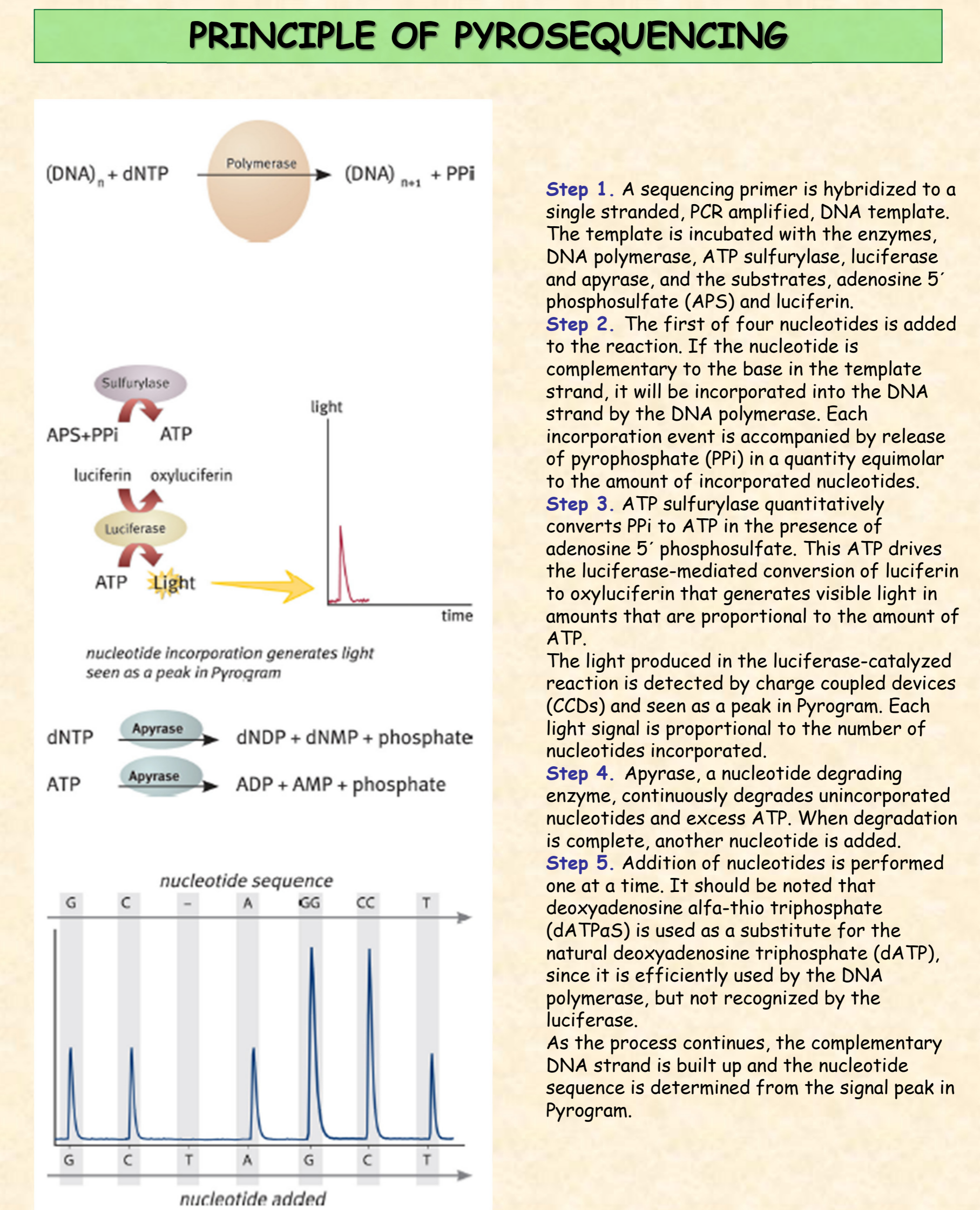
Methods:

A. Blood measurements of Persistent Organic Pollutants (POPs) and Heavy Metals (Lund University)
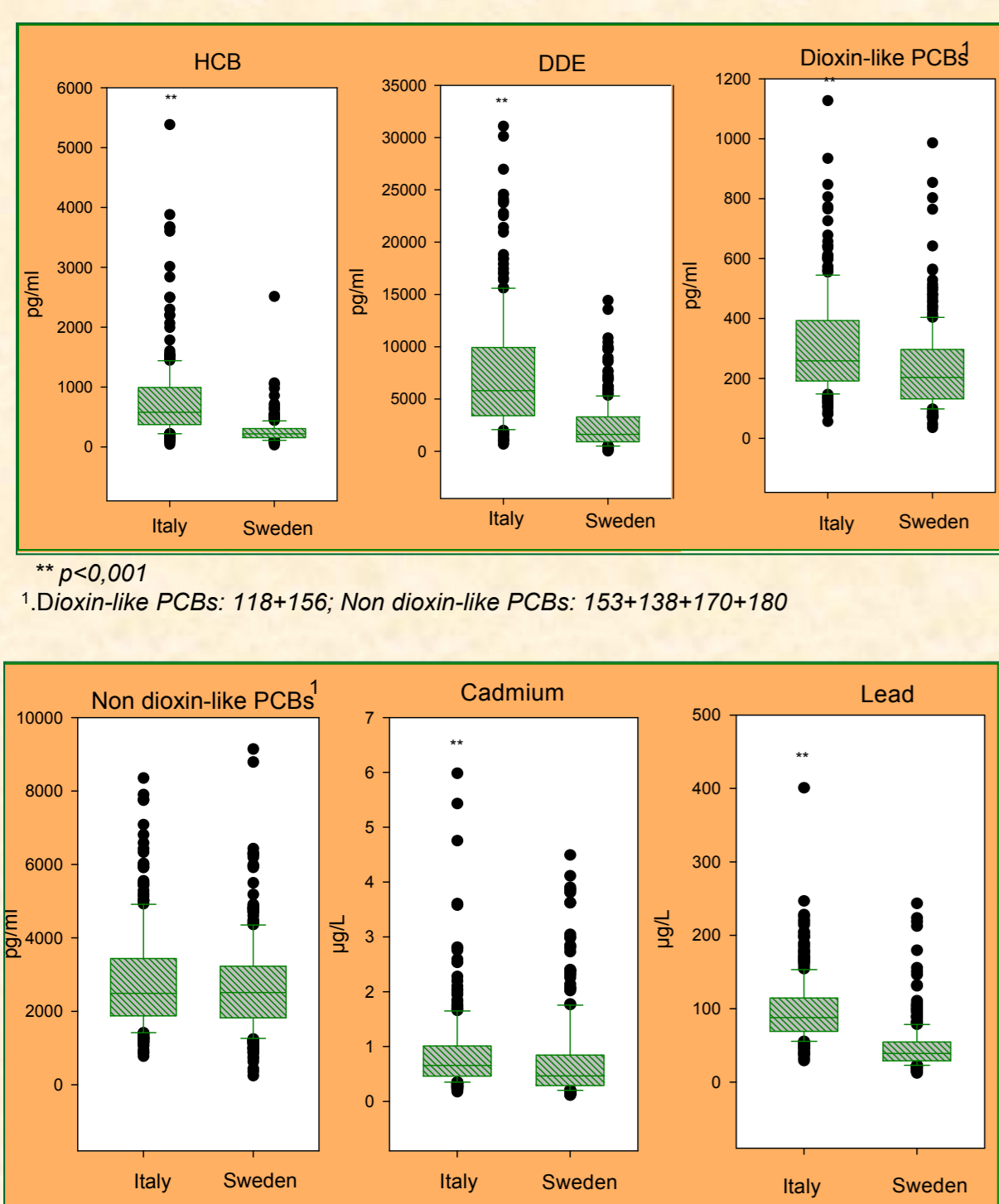
B. LINE-1 methylation
- Isolation of blood DNA
- DNA-bisulfite treatment
- PCR-Pyrosequencing of three CpG sites of LINE-1

Statistical analysis: Comparison between % of LINE-1 methylation and exposures

PRINCIPLE OF PYROSEQUENCING

Step 1. A sequencing primer is hybridized to a single stranded, PCR amplified, DNA template. The template is incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates, adenosine 5' phosphosulfate (APS) and luciferin.
Step 2. The first of four nucleotides is added to the reaction. If the nucleotide is complementary to the base in the template strand, it will be incorporated into the DNA strand by the DNA polymerase. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotides.
Step 3. ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5' phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP.
The light produced in the luciferase-catalyzed reaction is detected by charge coupled devices (CCDs) and seen as a peak in Pyrogram. Each light signal is proportional to the number of nucleotides incorporated.
Step 4. Apyrase, a nucleotide degrading enzyme, continuously degrades unincorporated nucleotides and excess ATP. When degradation is complete, another nucleotide is added.
Step 5. Addition of nucleotides is performed one at a time. It should be noted that deoxyadenosine alfa-thio triphosphate (dATPaS) is used as a substitute for the natural deoxyadenosine triphosphate (dATP), since it is efficiently used by the DNA polymerase, but not recognized by the luciferase.
As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peak in Pyrogram.

The Envirogenomarkers project aims at the investigation of a new generation of biomarkers and at the study of the role of environmental agents in human cancer

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Exposure levels of POPs and heavy metals in Italy and in Sweden

** p<0.001
[1] Dioxin-like PCBs: 118+156; Non dioxin-like PCBs: 153+138+170+180

In general, Italy presents higher exposure levels than Sweden

Spearman correlation analysis: Methylation of LINE-1 versus exposure to POPs and Heavy Metals

A. All samples (Italy and Sweden)

ALL SAMPLES		log1st	log2nd	log3d	[1]log vector
log HCB	Correlation Coefficient	,236	,302	,192	,184
	Sig. (2-tailed)	,000	,000	,000	,000
	N	530	530	530	529
log DDE	Correlation Coefficient	-,193	,274	,181	,161
	Sig. (2-tailed)	,000	,000	,000	,000
	N	530	530	530	529
log(118+156)	Correlation Coefficient	,171	0,04	,067	-,010
	Sig. (2-tailed)	,000	,316	,026	,819
	N	530	530	530	529
log (153+138+170+180)	Correlation Coefficient	-,022	-,031	,076	-,006
	Sig. (2-tailed)	,618	,472	,081	,892
	N	530	530	530	529
log cadmium	Correlation Coefficient	,111	,190	,102	,116
	Sig. (2-tailed)	,007	,000	,013	,005
	N	584	584	584	583
log lead	Correlation Coefficient	-,210	,347	,090	,217
	Sig. (2-tailed)	,000	,000	,030	,000
	N	584	584	584	583

p<0.05 / p<0.1 / p<0.2

[1] Log vector: the log transformed value of a geometric estimation of the average methylation of the three CpG sites
**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Strong association of methylation with exposure at all sites

B. Comparison between the two cohorts

ITALY ALL SAMPLES		log1st	log2nd	log3d	log vector
log HCB	Correlation Coefficient	,014	-,077	-,034	-,041
	Sig. (2-tailed)	,831	,227	,593	,524
	N	245	245	245	244
log DDE	Correlation Coefficient	-,013	-,115	-,015	-,080
	Sig. (2-tailed)	,834	,072	,810	,211
	N	245	245	245	244
log (118+156)	Correlation Coefficient	-,065	-,193	-,019	-,144
	Sig. (2-tailed)	,309	,002	,766	,024
	N	245	245	245	244
log (153+138+170+180)	Correlation Coefficient	,043	-,120	,050	-,052
	Sig. (2-tailed)	,502	,061	,438	,416
	N	245	245	245	244
log cadmium	Correlation Coefficient	-,010	,083	,031	,030
	Sig. (2-tailed)	,869	,156	,600	,605
	N	297	297	297	296
log lead	Correlation Coefficient	-,048	-,126	-,068	-,095
	Sig. (2-tailed)	,412	,029	,241	,102
	N	297	297	297	296

SWEDEN ALL SAMPLES		log1st	log2nd	log3d	[1]log vector
log HCB	Correlation Coefficient	-,154	-,014	,184	-,024
	Sig. (2-tailed)	,009	,817	,002	,685
	N	285	285	285	285
log DDE	Correlation Coefficient	-,060	,026	,138	,033
	Sig. (2-tailed)	,310	,666	,020	,577
	N	285	285	285	285
log(118+156)	Correlation Coefficient	,147	-,050	,128	-,060
	Sig. (2-tailed)	,013	,399	,030	,315
	N	285	285	285	285
Log (153+138+170+180)	Correlation Coefficient	-,051	-,014	,128	-,013
	Sig. (2-tailed)	,390	,820	,031	,830
	N	285	285	285	285
log cadmium	Correlation Coefficient	-,071	,051	,116	,034
	Sig. (2-tailed)	,234	,389	,051	,562
	N	287	287	287	287
log lead	Correlation Coefficient	,082	,010	-,013	,055
	Sig. (2-tailed)	,167	,869	,824	,350
	N	287	287	287	287

- Evidence of association between methylation and exposure
- Apparent differences between the two cohorts

Linear Regression model: Association of LINE-1 methylation with exposure controlling for different demographic parameters

A. All samples (Italy and Sweden)

ALL SAMPLES	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	1,372	,174		7,901	,000
cohort	-,121	,020	-,362	-5,905	,000
type	-,007	,012	-,021	-,551	,582
disease	-,094	,015	-,281	-6,314	,000
sex	-,052	,020	-,130	-2,576	,010
age	-,002	,001	-,119	-2,674	,008
bmi	,000	,002	-,003	-,072	,943
smoking status	,009	,010	,047	,964	,336
log HCB	,054	,033	,107	1,654	,099
log DDE	,001	,025	,004	,056	,955
log(118+156)	-,216	,073	-,296	-2,967	,003
log(153+138+170+180)	,185	,071	,220	2,608	,009
log cadmium	-,002	,027	-,004	-,081	,935
log lead	-,041	,034	-,065	-1,221	,223

(Dependent variable: logvector)

B. Comparison between the two cohorts

ITALY ALL SAMPLES	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	1,499	,256		5,845	,000
type	,000	,018	,000	,001	,999
disease	-,173	,021	-,489	-8,143	,000
sex	-,066	,030	-,144	-2,238	,026
age	-,001	,001	-,039	-,639	,523
bmi	-,002	,003	-,041	-,749	,455
smoking_status	,003	,014	,015	,238	,812
log HCB	,055	,040	,089	1,361	,175
log DDE	-,015	,038	-,026	-,396	,692
log(118+156)	-,206	,107	-,230	-1,914	,057
log(153+138+170+180)	,194	,102	,198	1,897	,059
log cadmium	-,011	,040	-,016	-,262	,793
log lead	-,110	,058	-,106	-1,879	,061

SWEDEN ALL SAMPLES	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	,995	,228		4,360	,000
type	-,009	,016	-,032	-,556	,579
disease	-,002	,021	-,007	-,089	,929
sex	-,041	,027	-,131	-1,486	,138
age	-,002	,001	-,139	-1,965	,050
bmi	,000	,002	,012	,190	,850
smoking_status	,009	,014	,057	,701	,484
log HCB	,056	,063	,094	,890	,374
log DDE	,035	,034	,104	1,049	,295
log(118+156)	-,101	,100	-,177	-1,016	,311
log(153+138+170+180)	,044	,102	,069	,434	,665
log cadmium	,019	,035	,048	,544	,587
log lead	-,002	,038	-,004	-,059	,953

(Dependent variable: logvector)

- Significant correlation between methylation of LINE-1 and gender, age and exposure to PCBs
- The associations seem to be valid only in the Italian cohort
- Lead exposure appears to be a confounder of the methylation levels, in Italy

Multinomial Logistic: Methylation of LINE-1 versus exposure

Italian cohort

ITALY ALL SAMPLES quartile vector		B	Std. Error	Wald	df	Sig.	Exp(B)	95% Confidence Interval for Exp(B)	
								Lower Bound	Upper Bound
1rst quartile	sex	1,756	,633	7,692	1	,006	5,788	1,674	20,019
	age	,038	,034	1,246	1	,264	1,039	,971	1,111
	logHCB	-1,090	,872	1,560	1	,212	,336	,061	1,859
	logdioxin-like	5,657	2,365	5,720	1	,017	286,385	2,776	29540,502
	lognon dioxin-like	-6,062	2,324	6,806	1	,009	,002	,000	,221
	loglead	2,388	1,382	2,986	1	,084	10,892	,726	163,480
2nd quartile	sex	1,171	,678	2,982	1	,084	3,224	,461	12,171
	age	-,004	,036	,011	1	,916	,996	,461	1,068
	logHCB	-1,831	,983	3,470	1	,062	,160	,461	1,100
	logdioxin-like	3,146	2,416	1,696	1	,193	23,248	,461	2645,675
	lognon dioxin-like	-1,607	2,299	,489	1	,484	,200	,461	18,146
	loglead	,050	1,466	,001	1	,973	1,051	,461	18,593
3rd quartile	sex	1,554	,612	6,441	1	,011	4,731	1,425	15,713
	age	,045	,030	2,185	1	,139	1,046	,986	1,110
	logHCB	-1,632	,793	4,233	1	,040	,195	,041	,926
	logdioxin-like	3,746	2,038	3,378	1	,066	42,336	,780	2298,383
	lognon dioxin-like	-2,842	1,996	2,027	1	,155	,058	,001	2,916
	loglead	,456	1,248	,133	1	,715	1,578	,137	18,209

a. The reference category is the 4th higher quartile.
b. This parameter is set to zero because it is redundant.

Dependent variable: methylation in quartile vector123 (versus 4th)
Independent variables: POP and heavy metal exposures in addition to demographic parameters
Controlling for: disease, type, bmi, smoking status, DDE and cadmium

Verification of the linear regression model

FINDINGS: Methylation of LINE-1 is significantly correlated with the serum levels of exposure to dioxin-like (known for their endocrine disruptor activity) and non dioxin-like POPs. A similar, inverse association was also observed between DNA methylation and erythrocyte lead levels in subjects of the Italian cohort, who on average suffered higher exposure than their Swedish counterparts. Furthermore, there's an evidence that males present increased levels of global methylation than females.

OUR STUDY SUGGESTS THAT CUMULATIVE EXPOSURE TO PCBS AND HEAVY METALS MAY ALTER THE CpG METHYLATION PATTERN AND AFFECT THE EPIGENETIC REGULATION OF CELLULAR FUNCTIONS