

Alma Mater Studiorum Università di Bologna
Archivio istituzionale della ricerca

Assessing the combined effect of extremely low-frequency magnetic field exposure and oxidative stress on LINE-1 promoter methylation in human neural cells

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

Assessing the combined effect of extremely low-frequency magnetic field exposure and oxidative stress on LINE-1 promoter methylation in human neural cells / Gianfranco Giorgi ; Chiara Pirazzini ; Maria Giulia Bacalini ; Cristina Giuliani ; Paolo Garagnani ; Miriam Capri ; Ferdinando Bersani ; Del Re Brunella. - In: RADIATION AND ENVIRONMENTAL BIOPHYSICS. - ISSN 0301-634X. - STAMPA. - 56:(2017), pp. 193-200. [10.1007/s00411-017-0683-8]

Availability:

This version is available at: <https://hdl.handle.net/11585/605269> since: 2017-07-28

Published:

DOI: <http://doi.org/10.1007/s00411-017-0683-8>

Terms of use:

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).
When citing, please refer to the published version.

(Article begins on next page)

This is the final peer-reviewed accepted manuscript of:

Giorgi G, Pirazzini C, Bacalini MG, Giuliani C, Garagnani P, Capri M, Bersani F, Del Re B. Assessing the combined effect of extremely low-frequency magnetic field exposure and oxidative stress on LINE-1 promoter methylation in human neural cells. *Radiat Environ Biophys.* 2017 May;56(2):193-200.PMID: 28258386

The final published version is available online at: <http://dx.doi.org/10.1007/s00411-017-0683-8>

Rights / License:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>)

When citing, please refer to the published version.

Dear Author,

Here are the proofs of your article.

- You can submit your corrections **online**, via **e-mail** or by **fax**.
- For **online** submission please insert your corrections in the online correction form. Always indicate the line number to which the correction refers.
- You can also insert your corrections in the proof PDF and **email** the annotated PDF.
- For fax submission, please ensure that your corrections are clearly legible. Use a fine black pen and write the correction in the margin, not too close to the edge of the page.
- Remember to note the **journal title**, **article number**, and **your name** when sending your response via e-mail or fax.
- **Check** the metadata sheet to make sure that the header information, especially author names and the corresponding affiliations are correctly shown.
- **Check** the questions that may have arisen during copy editing and insert your answers/ corrections.
- **Check** that the text is complete and that all figures, tables and their legends are included. Also check the accuracy of special characters, equations, and electronic supplementary material if applicable. If necessary refer to the *Edited manuscript*.
- The publication of inaccurate data such as dosages and units can have serious consequences. Please take particular care that all such details are correct.
- Please **do not** make changes that involve only matters of style. We have generally introduced forms that follow the journal's style. Substantial changes in content, e.g., new results, corrected values, title and authorship are not allowed without the approval of the responsible editor. In such a case, please contact the Editorial Office and return his/her consent together with the proof.
- If we do not receive your corrections **within 48 hours**, we will send you a reminder.
- Your article will be published **Online First** approximately one week after receipt of your corrected proofs. This is the **official first publication** citable with the DOI. **Further changes are, therefore, not possible.**
- The **printed version** will follow in a forthcoming issue.


Please note

After online publication, subscribers (personal/institutional) to this journal will have access to the complete article via the DOI using the URL: [http://dx.doi.org/\[DOI\]](http://dx.doi.org/[DOI]).

If you would like to know when your article has been published online, take advantage of our free alert service. For registration and further information go to: <http://www.link.springer.com>.

Due to the electronic nature of the procedure, the manuscript and the original figures will only be returned to you on special request. When you return your corrections, please inform us if you would like to have these documents returned.

Metadata of the article that will be visualized in OnlineFirst

ArticleTitle	Assessing the combined effect of extremely low-frequency magnetic field exposure and oxidative stress on LINE-1 promoter methylation in human neural cells	
Article Sub-Title		
Article CopyRight	Springer-Verlag Berlin Heidelberg (This will be the copyright line in the final PDF)	
Journal Name	Radiation and Environmental Biophysics	
Corresponding Author	Family Name	Re 
	Particle	Del
	Given Name	Brunella
	Suffix	
	Division	Department of Pharmacy and Biotechnology (FaBiT)
	Organization	University of Bologna
	Address	via Selmi 3, 40126, Bologna, Italy
	Phone	+39 51 2094202
	Fax	
	Email	brunella.delre@unibo.it
	URL	
	ORCID	
Author	Family Name	Giorgi
	Particle	
	Given Name	Gianfranco
	Suffix	
	Division	Department of Pharmacy and Biotechnology (FaBiT)
	Organization	University of Bologna
	Address	via Selmi 3, 40126, Bologna, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	Family Name	Pirazzini
	Particle	
	Given Name	Chiara
	Suffix	
	Division	Department of Experimental, Diagnostic and Specialty Medicine (DIMES)
	Organization	University of Bologna
	Address	via S. Giacomo 12, 40126, Bologna, Italy
	Phone	
	Fax	
	Email	
	URL	

ORCID

Author	Family Name	Bacalini
	Particle	
	Given Name	Maria Giulia
	Suffix	
	Division	Department of Experimental, Diagnostic and Specialty Medicine (DIMES)
	Organization	University of Bologna
	Address	via S. Giacomo 12, 40126, Bologna, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	

Author	Family Name	Giuliani
	Particle	
	Given Name	Cristina
	Suffix	
	Division	Department of Biological, Geological and Environmental Sciences (BiGeA), Centre for Genome Biology
	Organization	University of Bologna
	Address	via Selmi 3, 40126, Bologna, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	

Author	Family Name	Garagnani
	Particle	
	Given Name	Paolo
	Suffix	
	Division	Department of Experimental, Diagnostic and Specialty Medicine (DIMES)
	Organization	University of Bologna
	Address	via S. Giacomo 12, 40126, Bologna, Italy
	Division	
	Organization	CIG-Interdepartmental Centre "L. Galvani" for Bioinformatics, Biophysics and Biocomplexity
	Address	Piazza di Porta San Donato 1, 40126, Bologna, Italy
	Phone	
	Fax	
	Email	
	URL	
	ORCID	

Author	Family Name	Capri
	Particle	
	Given Name	Miriam
	Suffix	

Division Department of Experimental, Diagnostic and Specialty Medicine (DIMES)
 Organization University of Bologna
 Address via S. Giacomo 12, 40126, Bologna, Italy
 Division
 Organization CIG-Interdepartmental Centre "L. Galvani" for Bioinformatics, Biophysics and Biocomplexity
 Address Piazza di Porta San Donato 1, 40126, Bologna, Italy
 Phone
 Fax
 Email
 URL
 ORCID

Author	Family Name Bersani Particle Given Name Ferdinando Suffix Division DIFA Department of Physics and Astronomy Organization University of Bologna Address via Berti Pichat 6/2, 40127, Bologna, Italy Phone Fax Email URL ORCID
--------	---

Schedule	Received 8 July 2016 Revised Accepted 11 January 2017
----------	---

Abstract Extremely low frequency magnetic fields (ELF-MF) have been classified as "possibly carcinogenic", but their genotoxic effects are still unclear. Recent findings indicate that epigenetic mechanisms contribute to the genome dysfunction and it is well known that they are affected by environmental factors. To our knowledge, to date the question of whether exposure to ELF-MF can influence epigenetic modifications has been poorly addressed. In this paper, we investigated whether exposure to ELF-MF alone and in combination with oxidative stress (OS) can affect DNA methylation, which is one of the most often studied epigenetic modification. To this end, we analyzed the DNA methylation levels of the 5' untranslated region (5'UTR) of long interspersed nuclear element-1s (LINE-1 or L1), which are commonly used to evaluate the global genome methylation level. Human neural cells (BE(2)C) were exposed for 24 and 48 h to extremely low frequency pulsed magnetic field (PMF; 50 Hz, 1 mT) in combination with OS. The methylation levels of CpGs located in L1 5'UTR region were measured by MassARRAY EpiTYPER. The results indicate that exposures to the single agents PMF and OS induced weak decreases and increases of DNA methylation levels at different CpGs. However, the combined exposure to PMF and OS lead to significant decrease of DNA methylation levels at different CpG sites. Most of the changes were transient, suggesting that cells can restore homeostatic DNA methylation patterns. The results are discussed and future research directions outlined.

Keywords (separated by '-') DNA methylation - Epigenetics - LINE-1 - Retrotransposition - Extremely low frequency magnetic field - Oxidative stress

Footnote Information Gianfranco Giorgi and Chiara Pirazzini are co-first authors.

2 **Assessing the combined effect of extremely low-frequency**
3 **magnetic field exposure and oxidative stress on LINE-1 promoter**
4 **methylation in human neural cells**

5 **Gianfranco Giorgi¹ · Chiara Pirazzini² · Maria Giulia Bacalini² · Cristina Giuliani³ ·**
6 **Paolo Garagnani^{2,4} · Miriam Capri^{2,4} · Ferdinando Bersani⁵ · Brunella Del Re¹**

7 Received: 8 July 2016 / Accepted: 11 January 2017
8 © Springer-Verlag Berlin Heidelberg 2017

9 **Abstract** Extremely low frequency magnetic fields
10 (ELF-MF) have been classified as “possibly carcino-
11 genic”, but their genotoxic effects are still unclear. Recent
12 findings indicate that epigenetic mechanisms contribute
13 to the genome dysfunction and it is well known that they
14 are affected by environmental factors. To our knowledge,
15 to date the question of whether exposure to ELF-MF
16 can influence epigenetic modifications has been poorly
17 addressed. In this paper, we investigated whether expo-
18 sure to ELF-MF alone and in combination with oxidative
19 stress (OS) can affect DNA methylation, which is one of
20 the most often studied epigenetic modification. To this end,
21 we analyzed the DNA methylation levels of the 5′untrans-
22 lated region (5′UTR) of long interspersed nuclear element-
23 1s (LINE-1 or L1), which are commonly used to evaluate
24 the global genome methylation level. Human neural cells
25 (BE(2)C) were exposed for 24 and 48 h to extremely low

frequency pulsed magnetic field (PMF; 50 Hz, 1 mT) in 26
combination with OS. The methylation levels of CpGs 27
located in L1 5′UTR region were measured by MassAR- 28
RAY EpiTYPER. The results indicate that exposures to 29
the single agents PMF and OS induced weak decreases and 30
increases of DNA methylation levels at different CpGs. 31
However, the combined exposure to PMF and OS lead to 32
significant decrease of DNA methylation levels at different 33
CpG sites. Most of the changes were transient, suggesting 34
that cells can restore homeostatic DNA methylation pat- 35
terns. The results are discussed and future research direc- 36
tions outlined. 37

Keywords DNA methylation · Epigenetics · LINE-1 · 38
Retrotransposition · Extremely low frequency magnetic 39
field · Oxidative stress 40

Introduction 41

Extremely low frequency magnetic fields (ELF-MF) are 42
pervasive in today’s society. Indeed, people are exposed 43
to increasing electromagnetic fields generated by power 44
lines and ordinary electric and electronic devices on a daily 45
basis. In 2002, the International Agency for Research on 46
Cancer surmised that ELF-MFs increase the risk of neo- 47
plastic malignancies and classified them as “possibly carci- 48
nogenic to humans” (IARC 2002). 49

Various in vivo and in vitro studies have been carried 50
out to understand the molecular mechanisms behind the 51
biological effects induced by ELF-MF, but a clear picture 52
has not yet emerged. Moreover, the assessment of genotox- 53
icity by standard genotoxicity assays has given conflicting 54
results, so the question whether ELF-MF can be involved in 55
carcinogenesis or in cancer progression is still unanswered 56

A1 Gianfranco Giorgi and Chiara Pirazzini are co-first authors.

A2 ✉ Brunella Del Re
A3 brunella.delre@unibo.it

A4 ¹ Department of Pharmacy and Biotechnology (FaBiT),
A5 University of Bologna, via Selmi 3, 40126 Bologna, Italy

A6 ² Department of Experimental, Diagnostic and Specialty
A7 Medicine (DIMES), University of Bologna, via S. Giacomo
A8 12, 40126 Bologna, Italy

A9 ³ Department of Biological, Geological and Environmental
A10 Sciences (BiGeA), Centre for Genome Biology, University
A11 of Bologna, via Selmi 3, 40126 Bologna, Italy

A12 ⁴ CIG-Interdepartmental Centre “L. Galvani”
A13 for Bioinformatics, Biophysics and Biocomplexity, Piazza di
A14 Porta San Donato 1, 40126 Bologna, Italy

A15 ⁵ DIFA Department of Physics and Astronomy, University
A16 of Bologna, via Bertini Pichat 6/2, 40127 Bologna, Italy

(Vijayalaxmi and Prihoda 2009). Some reports suggested that ELF-MF exposure alone is not genotoxic but it can increase DNA damage in the presence of a genotoxic agent. Therefore, further research dealing with co-exposure evaluation should be considered.

Recent evidence suggests that non-genotoxic epigenetic mechanisms, such as DNA methylation, microRNA, long noncoding RNAs, histone code etc, are involved in aging and disease development and, in particular it is known that DNA methylation may play a key role in tumorigenesis and tumor progression (Klutstein et al. 2016).

DNA methylation, the most studied epigenetic mechanism, is a biochemical process where a methyl group is added to DNA nucleotides and, in mammals it typically occurs at cytosines in a CpG dinucleotide. DNA methylation may have a role in the control of gene expression by acting on regulatory elements. Cancer cells often show hypermethylation of the promoter region of specific genes and hypomethylation of the promoter region of repetitive elements, including long interspersed nuclear elements (LINE-1s or L1s) (Klutstein et al. 2016; Cruickshanks et al. 2013; Schulz 2006).

L1 elements constitute approximately 17% of the human genome. A full length L1 element is about 6 kb and consists of a 5' untranslated region (5'UTR) with sense and antisense promoter activity, two open reading frames (ORF1 and ORF2), encoding proteins involved in retrotransposition and 3' untranslated region (3'UTR) with polyadenylation site. Recently, an additional ORF (ORF0) has been reported in the primate lineage and it has been suggested that it could play some positive regulatory role in the retrotransposition process (Denli et al. 2015). After transcription, the L1 retroelement can be inserted into another genomic site by target-primed reverse transcription (TPRT) mechanism. L1 insertion can cause insertional mutagenesis, DNA double-strand breaks, exonisation or shuffling of genetic material, resulting in genetic instability (Iskow et al. 2010).

Several studies have shown an inverse correlation between L1 expression and the methylation status of the CpG island in L1 5'-UTRs (Bourc'his and Bestor 2004). Indeed tumor cells often show both low DNA methylation levels of the L1 5'-UTR promoter region and high L1 retrotransposition activity (Schultz 2006), with consequent alterations of gene expression and genomic instability. Moreover, recent evidence suggests that alterations of the L1 promoter methylation level might be involved in several cell processes, including cell replication timing and chromatin organization (Belan 2013).

Emerging data indicate that changes in L1 5'-UTR methylation levels can be induced by environmental factors (Bollati et al. 2007; Pogribny and Beland 2013). It has been suggested that L1 5'-UTR methylation evaluation should be

included in health risk assessment of environmental (Vrijheid et al. 2014; Chappell et al. 2016).

To the best of our knowledge, only one paper addressed the issue of evaluating the effects of ELF-MF exposure on DNA methylation, reporting that methylation changes occurred in mouse spermatocyte-derived GC-2 cell line under exposure to ELF-MF (Liu et al. 2015).

The aim of this study is to assess whether the exposure to ELF-MF, alone and in combination with oxidative stress (OS), induces changes in methylation of L1 5'UTR region in human cells. A combined exposure was tested to simulate condition of real life, where the simultaneous exposure to ELF fields and other stress agents normally occurs. OS was chosen as co-stressor having been shown to affect DNA methylation (O'Hagan et al. 2011) and to contribute to tumorigenesis and tumor progression (Kryston et al. 2011; Li et al. 2015).

A pulsed magnetic field (PMF) was used since it is produced by several devices and is widely used in clinical applications. Moreover, it was shown to be biologically effective in our previous investigations (Del Re and Giorgi 2013; Del Re et al. 2012).

We used the BE(2)C human cell line, which is representative of neuronal cell type (Biedler et al. 1978) because ELF-MF effects on neuronal cells appear interesting for the risk assessment. Indeed, epidemiological studies suggested a possible relationship between Alzheimer's disease, brain tumors and ELF-MF exposure (Qiu et al. 2004; Li et al. 2009).

Materials and methods

Cell culture and treatments

Neuroblastoma BE(2)C cells were kindly provided by Prof. Della Valle (University of Bologna, Italy), and were maintained in Dulbecco's modified Eagle's medium (DMEM, EuroClone, Milano, Italy), supplemented with 10% heat-inactivated fetal bovine serum (FBS, EuroClone), 100 UI/ml penicillin (Sigma, Ronkonkoma, NY, USA) and 100 µg/ml streptomycin (Sigma), in a humidified 5% carbon dioxide air atmosphere at 37 °C.

24 h before PMF/Sham exposure, BE(2)C cells were seeded into 3 cm petri dish at the density of 75,000 cells/dish.

BE(2)C cells were exposed to 300 µM H₂O₂ (Sigma) for 1 h. Control cultures were treated with equivalent volumes of distilled water. This dose has been largely used in studies dealing with oxidative stress and does not greatly affect the cell viability of our cellular model, as previously reported (Giorgi et al. 2011, 2014).

158 **Exposure system and field characteristics**

159 The exposure system has been previously described (Del
160 Re et al. 2012) and is shown in Fig. 1. It consisted of two
161 systems, each composed by two sets of horizontal Helm-
162 holtz coils of 25 cm diameter, with 40 (20 + 20) turns that
163 were double-wrapped to obtain wound (active coil) or
164 counter-wound configuration. In the counter-wound con-
165 figuration, the current is the same as in the active coil but
166 the MF is zero (sham). The coils are powered by a home-
167 made DC current amplifier, connected with a signal gen-
168 erator Model 33120A (Agilent Technologies, Loveland,
169 CO, USA). Both the active and the sham coils were main-
170 tained in the same 5% CO₂ incubator (B-5060, Heraeus,
171 Hanau, Germany) at a constant temperature of 37 °C, and
172 at a sufficient distance to minimize the stray field from

the active coil in such a way as to have in the Sham coils
a magnetic field $\leq 1/50$ of the field in the active system.
The background field within the incubator was also meas-
ured: the static component of the local magnetic field was
16.9 μT (horizontal component 10.8 μT , vertical com-
ponent 13.0 μT), the AC component was on the order of
0.1 μT , as measured with a very sensitive probe (EMDEX
II, EnerTech Consultants, Campbell, CA).

The system was controlled by means of a PC which,
through an appropriate software and a switching sys-
tem, randomly selected the active and sham coil system.
All experiments were conducted in blind and only at the
end of the experiments was the code decrypted. To have
a field uniformity within 5%, the samples were placed
within a virtual cylinder (about 11 cm in height, and 4 cm
in diameter), centered with respect to the coil system. A
bipolar pulsed-square wave magnetic field was chosen
(Fig. 1b), with an intensity 0-peak of 1 mT, a 50-Hz rep-
etition frequency, and a duty cycle 50%. The rise time
 τ of the square, from peak to peak, was about 0.6 ms,
resulting in an average rate of change of magnetic flux
density of 3.3 T/s. The MF was measured by means of
a Bell gaussmeter (F.W. Bell 7010, Division of Test and
Measurement, Orlando, FL); the error in the magnetic
flux-density values was on the order of 2%.

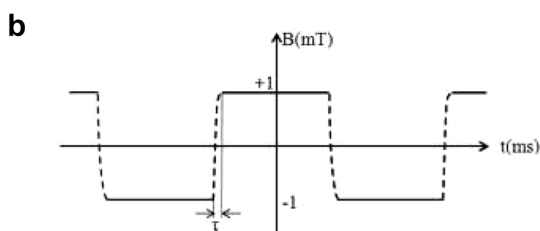
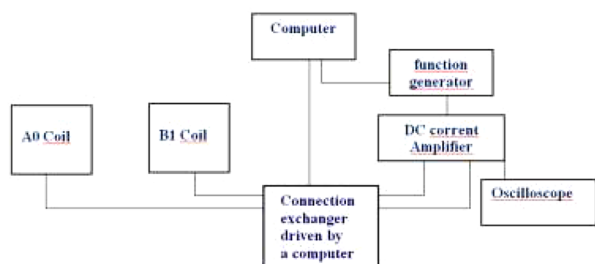
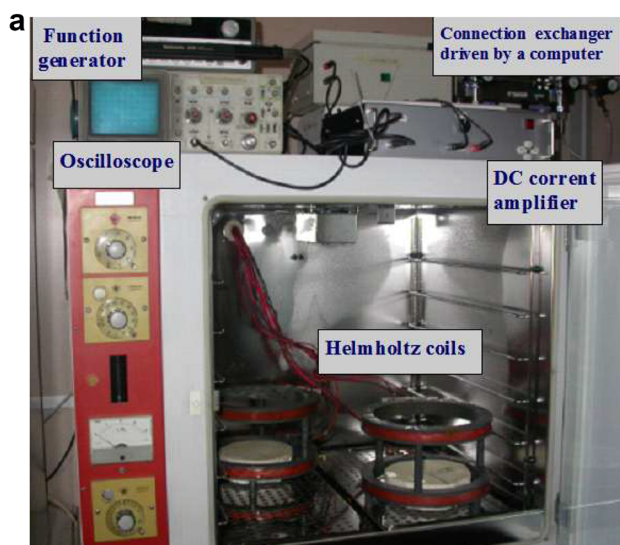


Fig. 1 The exposure system (a) and PMF signal wave shape (b). The rise time of the square was about 0.6 ms

198 **DNA extraction and sodium bisulfite treatment**

Genomic DNA was extracted by QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. 1 μg was treated with sodium bisulfite using the EZ methylation kit (Zymo-Research, Irvine, CA). The treatment converts unmethylated cytosine into uracil, leaving methylated cytosine unchanged. In this way, variations in the sequence are produced according to DNA methylation status of the original DNA molecule.

207 **Methylation analysis**

MassARRAY EpiTYPER technology (Sequenom) was used to quantitatively measure the methylation status of CpG sites within L1 5'UTR region (Accession No. X58075) (Fig. 2). 10 ng of bisulfite-treated DNA were PCR-amplified using the following primers: forward strand primer: AGGAAGAGAGTTTATTAGGGAGTGTTAGATAGTGGG; reverse strand primer: CAGTAATACGACTCACTATAGGGAGAAGGCTTCTATACCCTACCCCAAAAATAAA.

By using these primers, we evaluated DNA methylation levels of 24 CpG units (i.e. regions containing one or multiple CpG sites), containing 28 CpG sites (Table 1).

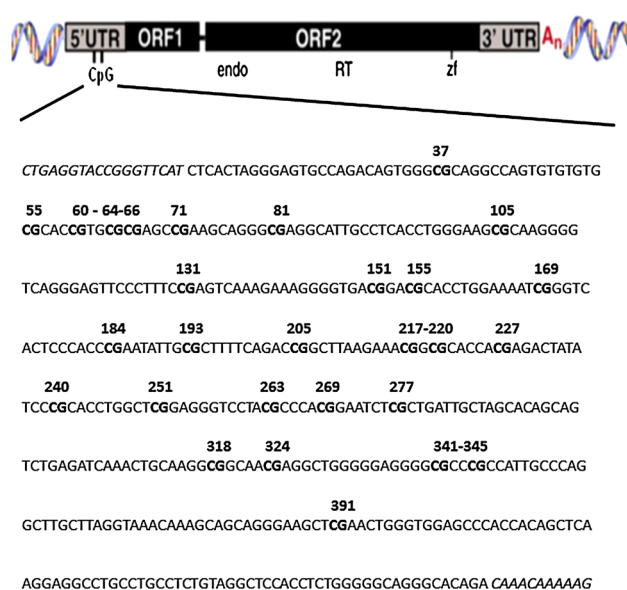


Fig. 2 Schematic structure of an L1 element and CpG sites of the L1 5'UTR region. The sequence represents a 466 base pair fragment (Accession No. X58075). Numbers refer to locations of the CpG units interrogated for their methylation level by MassARRAY EpiTYPER

Table 1 24 CpG units containing 28 CpG sites of the L1 5'UTR region

CpG units	Number of CpG sites
CpG_37	1
CpG_55	1
CpG_60-64-66	3
CpG_71	1
CpG_81	1
CpG_105	1
CpG_131	1
CpG_151	1
CpG_155	1
CpG_169	1
CpG_184	1
CpG_193	1
CpG_205	1
CpG_217-220	2
CpG_227	1
CpG_240	1
CpG_251	1
CpG_263	1
CpG_269	1
CpG_277	1
CpG_318	1
CpG_324	1
CpG_341-345	2

Statistical analysis

Student's *t* test was used to evaluate differences in methylation levels. A *p* value <0.05 was considered to correspond with statistical significance.

Results

To verify whether PMF exposure alone or in combination with OS would affect DNA methylation level of the L1 5'UTR region, BE(2)C cells were exposed or sham-exposed to PMF using the exposure system shown in Fig. 1. In the first hour of exposure, samples were subjected or not to OS (hydrogen peroxide 300 μ M, 1 h). After 24 and 48 h of exposure, DNA methylation was evaluated by MassARRAY EpiTYPER technology, which is a highly accurate and sensitive method for the quantitative analysis of DNA methylation. We focused on a part of the L1 5'UTR region which is 466 bp in length and includes 24 CpG units (Fig. 2). The comparison of methylation levels of all CpGs among all the samples showed that the methylation level of 10 CpG units was modified depending on the type of treatment.

Effects of PMF exposure alone are shown in Fig. 3. After 24 h of PMF exposure, 3 CpGs (37, 217-220, 318) exhibited a significantly increased methylation level as compared to the CpGs from sham exposed samples (Fig. 3a). After 48 h of PMF exposure no significant differences were observed at any CpGs (Fig. 3b).

Results on the effects of OS alone are shown in Fig. 4. After 24 h three CpGs (184, 205, 277) exhibited significantly less methylation as compared to the CpGs from control samples (Fig. 4a). Also in this case, after 48 h no significant differences were observed at any CpG units (Fig. 4b).

Results about the effects of PMF exposure in combination with OS are shown in Fig. 5. After 24 h 5 CpG units (37, 131, 184, 217-220, 318) exhibited significantly less methylation as compared to the CpGs from samples exposed to PMF alone (Fig. 5a). After 48 h only 2 CpG units (60:64:66, 217-220) showed lower methylation levels than control (PMF) (Fig. 5b).

Discussion

Epigenetic processes, including DNA methylation, are a molecular interface mediating the interaction between genome and environment. Changes in global genome methylation have been observed in association with exposure to such factors as air pollution (De Prins et al. 2013), gamma radiation (Kumar et al. 2011) low-levels of benzene

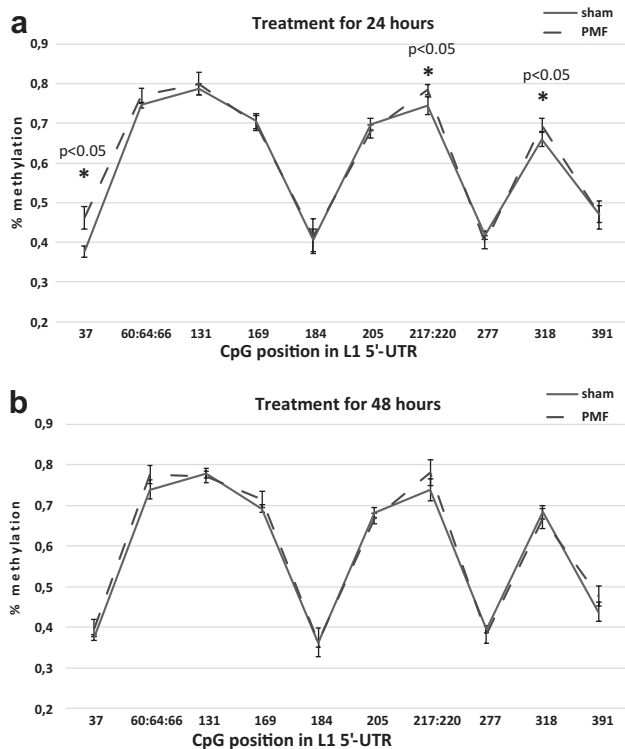


Fig. 3 CpG dinucleotide methylation percentage of L1 5'UTR region in cells exposed to PMF (dashed line) or exposed to sham (control, continuous line) for **a** 24 and **b** 48 h. Error bars represent SEM of the values obtained from three independent experiments

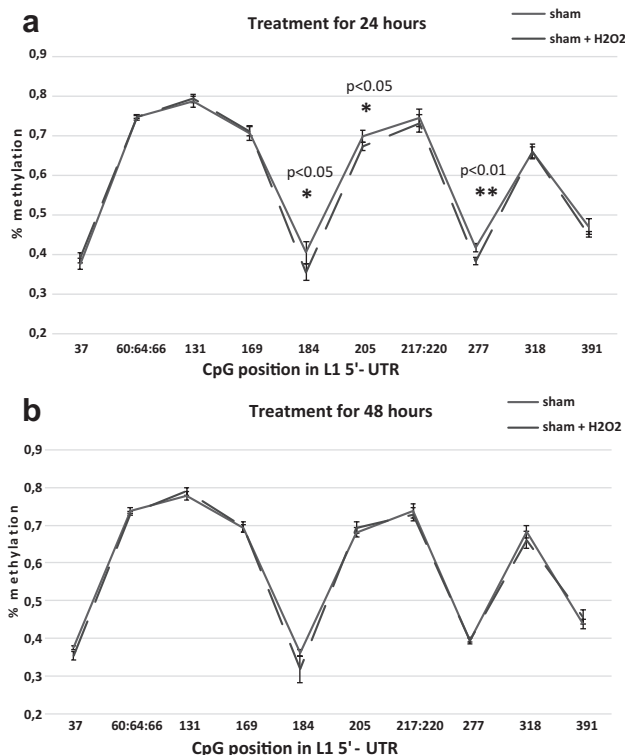


Fig. 4 CpG dinucleotide methylation percentage of L1 5'UTR region in cells subjected to oxidative stress (300 μM H_2O_2 for 1 h) (dashed line) or distilled water (control, continuous line) after **a** 24 and **b** 48 h from the treatment. Error bars represent SEM of the values obtained from three independent experiments

266 (Bollati et al. 2007), cigarette smoke (Liu et al. 2010), syn-
 267 thetic compounds such as perfluoroalkylacids (Watkins
 268 et al. 2014), various genotoxic and non-genotoxic carcin-
 269 ogens (Pogribny and Beland 2013) and nutritional factors
 270 (Bacalini et al. 2014). However, the effects of ELF-MF on
 271 DNA methylation in human cells has never been studied.
 272 Therefore, we analyzed the DNA methylation levels of the
 273 L1 5'UTR region, which is commonly investigated as a sur-
 274 rogate for global genome methylation (Yang et al. 2004), in
 275 BE(2)C cells.

276 We showed that the exposure to PMF can interfere
 277 with DNA methylation inducing a slight increase in
 278 DNA methylation levels of some CpGs located in the L1
 279 5'UTR region. Moreover, we found that OS alone induced
 280 a small and transient decrease of DNA methylation levels
 281 at some CpG units, whereas the combined exposures to
 282 PMF and OS induced a methylation decrease in 10 CpG
 283 units (Fig. 5). Therefore, in the presence of OS, the slight
 284 increase of methylation, induced by the exposure to PMF
 285 alone, disappeared.

286 The relationship between ELF-MF and oxidative
 287 stress has been largely debated and it has been proposed
 288 that ELF-MF can both induce ROS production and acti-
 289 vate antioxidants, depending on the specific conditions

290 tested (Manikonda et al. 2014; Di Loreto et al. 2009).
 291 Here, we observed that PMF synergistically contributes to
 292 OS effects. However, after 48 h of exposure methylation
 293 changes became undetectable. This result seems to be in
 294 line with the most recent evolutionary theories about the
 295 role of DNA methylation changes in humans (Klironomos
 296 et al. 2013; Flores et al. 2013; Giuliani et al. 2015). These
 297 theories suggested that methylation changes seem of crucial
 298 importance for rapid response to new stimuli, and in
 299 particular when new stimuli (in this case PMF+H2O2)
 300 arise. The data suggest that the environmental change
 301 from a normal condition—more than the constant expo-
 302 sure itself—increase DNA methylation variability, at least
 303 at the cellular level. The molecular mechanisms involved
 304 in these changes need to be validated in future studies but
 305 we can speculate as follows. DNA methylation patterns
 306 are dynamic states resulting from a continuous balance of
 307 methylation and demethylation. The ‘maintenance methyl-
 308 transferase’ DNMT1 mainly maintains the methylation
 309 patterns across replication cycles, while de novo DNMT3A
 310 and DNMT3B enzymes mainly introduce methyl groups
 311 into unmethylated sites (Jurkowska et al. 2011). Currently,
 312 not much is known about the effects of electromagnetic

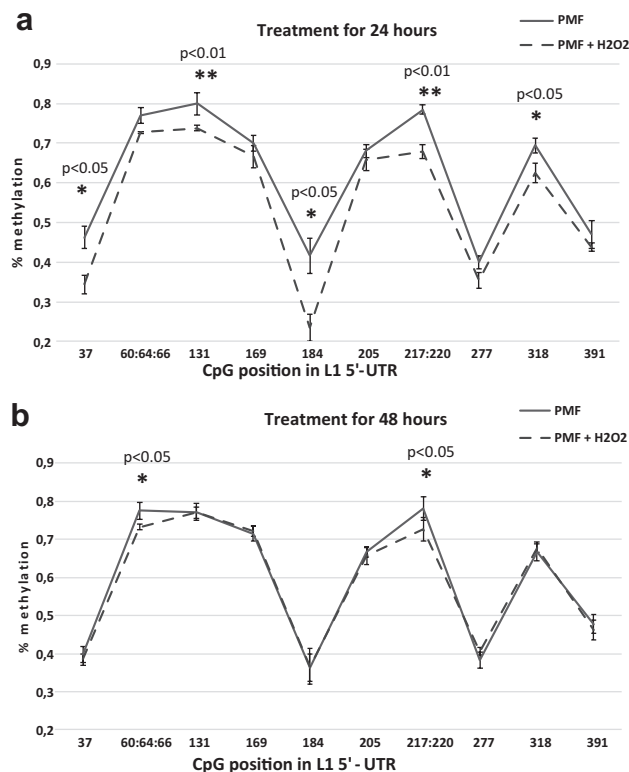


Fig. 5 CpG dinucleotide methylation percentage of L1 5'UTR region in cells exposed to PMF and subjected to oxidative stress (300 μM H_2O_2 for 1 h) (dashed line) or distilled water (control, continuous line) after **a** 24 and **b** 48 h from the treatment. Error bars represent SEM of the values obtained from three independent experiments

313 fields on these enzymes, but a recent paper suggests that
 314 DNMT1 and DNMT3B activity can be modulated by inter-
 315 mittent ELF-MF exposure, depending on the magnetic field
 316 intensity (Liu et al. 2015). Kloypan et al. (2015) found that
 317 OS can induce LINE-1 hypomethylation and they observed
 318 that this effect was mediated through the depletion of
 319 S-adenosylmethionine (SAM) which is the classical methyl
 320 donor for methyltransferases.

321 In our model, therefore, modulation of methyltransferase
 322 could be the mechanism responsible for the observed PMF
 323 effect, according with Liu et al. (2015). In addition, deple-
 324 tion of SAM could be the underlying reason for the OS
 325 effect, according to Kloypan et al. (2015). Finally, in the
 326 combined exposure, the presence of OS could determine
 327 an insufficient quantity of SAM, inhibiting the methyl-
 328 transferase activity and, therefore, masking the increase of
 329 methylation induced by the PMF exposure alone.

330 Our data stimulate two methodological considerations.
 331 The first is about the time of exposure. Most studies on
 332 the relationship between DNA methylation alterations
 333 and environmental factors are epidemiological ones,
 334 which usually do not investigate the effects of differ-
 335 ent exposure times. We analyzed two different exposure

336 times and we found different results, showing that the
 337 effects were transient. Therefore, whenever possible, it
 338 is strongly recommended to analyze various exposure
 339 times. The second consideration is about the CpG sites
 340 that are affected by ELF-MF/OS exposure. The Mas-
 341 sARRAY EpiTYPER approach allows to quantitatively
 342 evaluate DNA methylation levels of multiple adjacent
 343 CpGs, providing more detailed information with respect
 344 to other commonly used approaches, such as the COBRA
 345 (combined bisulphite restriction analysis polymerase
 346 chain reaction) assay. We analyzed 24 CpG units and we
 347 observed that methylation changes occurred preferen-
 348 tially at specific CpG. This observation is in agreement
 349 with findings by Nüsgen et al. (2015), who observed that
 350 some specific CpG units within 5'-UTR L1 region are
 351 more prone to be subjected to methylation modifications.
 352 Our data suggest that it is important to analyse as many
 353 CpG sites as possible, since we do not know which sites
 354 are sensitive in each cell type and eventually affect gene
 355 transcription.

356 Emerging evidences reveal that microvesicles repre-
 357 sent an important mechanism of cell to cell communica-
 358 tion and that they can be involved in epigenetic processes
 359 including DNA methylation (Qian et al. 2015). Recently,
 360 it has been reported that microvesicles are released from
 361 cells upon activation by various stimuli including radia-
 362 tion (Jella et al. 2014) and ELF-MF exposure (Stratton
 363 et al. 2013). This aspect should be investigated, to verify
 364 whether it could be involved in the epigenetic alterations
 365 which we observed.

366 In conclusion, our results suggest that only some CpG
 367 units within L1 5'-UTR region could be subjected to
 368 methylation modification by PMF and OS exposure and
 369 that these alterations are, in any case, transient. The bio-
 370 logical relevance of these transient variations of DNA
 371 methylation levels needs to be elucidated; they are at
 372 the forefront of important mechanisms of what is gener-
 373 ally called "epigenetic stress". We hypothesize that these
 374 variations can explain some conflicting results obtained
 375 until now in in vitro cell systems after ELF exposure.
 376 Further studies are needed to clarify this point and to elu-
 377 cinate the epigenetic effect of ELF-MFs alone and in the
 378 presence of OS, also considering different cell types and
 379 exposure scenarios.

380 **Acknowledgements** This work was supported by RFO (Ricerca
 381 Fondamentale Orientata, Oriented Fundamental Research) grants
 382 from the University of Bologna to BDR and to MC. Funds were also
 383 obtained by Fondazione Pallotti to PG and MC.

384 **Compliance with ethical standards**

385 **Conflict of interest** The authors report no conflicts of interests. The
 386 authors alone are responsible for the content and writing of the paper.

387 **References**

- 388 Bacalini MG, Friso S, Olivieri F, Pirazzini C, Giuliani C, Capri
389 M, Santoro A, Franceschi C, Garagnani P (2014) Present
390 and future of anti-ageing epigenetic diets. *Mech Ageing Dev*
391 136–137:101–115
- 392 Belan E (2013) LINEs of evidence: noncanonical DNA rep-
393 lication as an epigenetic determinant. *Biol Direct* 8:22.
394 doi:10.1186/1745-6150-8-22
- 395 Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS (1978) Mul-
396 tiple neurotransmitter synthesis by human neuroblastoma cell
397 lines and clones. *Cancer Res* 38:3751–3757
- 398 Bollati V, Baccarelli A, Hou L, Bonzini M, Fustinoni S, Cavallo D,
399 Byun HM, Jiang J, Marinelli B, Pesatori AC, Bertazzi PA, Yang
400 AS (2007) Changes in DNA methylation patterns in subjects
401 exposed to low-dose benzene. *Cancer Res* 67:876–880
- 402 Bourc'his D, Bestor TH (2004) Meiotic catastrophe and retrotrans-
403 poson reactivation in male germ cells lacking Dnmt3L. *Nature*
404 431:96–99
- 405 Chappell G, Pogribny IP, Guyton KZ, Rusyn I (2016) Epigenetic
406 alterations induced by genotoxic occupational and environ-
407 mental human chemical carcinogens: a systematic literature
408 review. *Mutat Res Mutat Res* 768:27–45. doi:10.1016/j.
409 mrrrev.2016.03.004
- 410 Cruickshanks HA, Vafadar-Isfahani N, Dunican DS, Lee A, Sproul D,
411 Lund JN, Meehan RR, Tufarelli C (2013) Expression of a large
412 LINE-1-driven antisense RNA is linked to epigenetic silenc-
413 ing of the metastasis suppressor gene TFPI-2 in cancer. *Nucleic*
414 *Acids Res* 41:6857–6869
- 415 De Prins S, Koppen G, Jacobs G, Dons E, Van de Mierop E, Nelen
416 V, Fierens F, IntPanis L, De Boever P, Cox B, Nawrot TS, Schoe-
417 ters G (2013) Influence of ambient air pollution on global DNA
418 methylation in healthy adults: a seasonal follow-up. *Environ Int*
419 59:418–424
- 420 Del Re B, Giorgi G (2013) Cell-host, LINE and environment: three
421 players in search of a balance. *Mob Genet Elem* 3:1–4e24040.
422 doi:10.4161/mge.24040
- 423 Del Re B, Marcantonio P, Gavoçi E, Bersani F, Giorgi G (2012)
424 Assessing LINE-1 retrotransposition activity in neuroblastoma
425 cells exposed to extremely low-frequency pulsed magnetic fields.
426 *Mutat Res* 749(1–2):76–81
- 427 Denli AM, Narvaiza I, Kerman B, Pena M, Benner C, Marchetto MC,
428 Diedrich JK, Aslanian A, Ma J, Moresco JJ, Moore L, Hunter T,
429 Saghatelian A, Gage FH (2015) Primate-specific ORF0 contrib-
430 utes to retrotransposon-mediated diversity. *Cell* 163:583–593.
431 doi:10.1016/j.cell.2015.09.025
- 432 Di Loreto S, Falone S, Caracciolo V, Sebastiani P, D'Alessandro A
433 et al (2009) Fifty hertz extremely low-frequency magnetic field
434 exposure elicits redox and trophic response in rat-cortical neu-
435 rons. *J Cell Physiol* 219:334–343. doi:10.1002/jcp.21674
- 436 Flores KB, Wolschin F, Amdam GV (2013) The role of methylation
437 of DNA in environmental adaptation. *Integr Comp Biol* 53:359–
438 372. doi:10.1093/icb/ict019
- 439 Giorgi G, Marcantonio P, Del Re B (2011) LINE-1 retrotransposition
440 in human neuroblastoma cells is affected by oxidative stress. *Cell*
441 *Tissue Res* 346:383–391. doi:10.1007/s00441-011-1289-0
- 442 Giorgi G, Lecciso M, Capri M, Lukas Yani S, Virelli A, Bersani F,
443 Del Re B (2014) An evaluation of genotoxicity in human neu-
444 ronal-type cells subjected to oxidative stress under an extremely
445 low frequency pulsed magnetic field. *Mutat Res Genet Toxicol*
446 *Environ Mutagen* 775–776:31–37
- 447 Giuliani C, Bacalini MG, Sazzini M, Pirazzini C, Franceschi C,
448 Garagnani P, Luiselli D (2015) The epigenetic side of human
449 adaptation: hypotheses, evidences and theories. *Ann Hum Biol*
450 42:1–9. doi:10.3109/03014460.2014.961960
- IARC Working Group on the Evaluation of Carcinogenic Risks
to Humans (2002) Non-ionizing radiation, Part 1: static and
extremely low-frequency (ELF) electric and magnetic fields.
IARC Monogr Eval Carcinog Risks Hum 80:1–395
- Iskow RC, McCabe MT, Mills RE, Torene S, Pittard WS, Neuwald
AF, Van Meir EG, Vertino PM, Devine SE (2010) Natural
mutagenesis of human genomes by endogenous retrotranspo-
sons. *Cell* 141:1253–1261
- Jella KK, Rani S, O'Driscoll L, McClean B, Byrne HJ, Lyng FM
(2014) Exosomes are involved in mediating radiation induced
bystander signaling in human keratinocyte cells. *Radiat Res*
181:138–145. doi:10.1667/RR13337.1
- Jurkowska RZ, Jurkowski TP, Jeltsch A (2011) Structure and func-
tion of mammalian DNA methyltransferases. *Chembiochem*
12:206–222
- Klironomos FD, Berg J, Collins S (2013) How epigenetic mutations
can affect genetic evolution: model and mechanism. *Bioessays*
35:571–578. doi:10.1002/bies.201200169
- Kloypan C, Srisa-art M, Mutirangura A, Boonla C (2015) LINE-1
hypomethylation induced by reactive oxygen species is medi-
ated via depletion of S-adenosylmethionine. *Cell Biochem*
Funct 33:375–385. doi:10.1002/cbf.3124
- Klutstein M, Nejman D, Greenfield R, Cedar H (2016) DNA
methylation in cancer and aging. *Cancer Res* 76:3446–3450.
doi:10.1158/0008-5472.CAN-15-3278
- Kryston TB, Georgiev AB, Pissis P, Georgakilas AG (2011) Role
of oxidative stress and DNA damage in human carcinogenesis.
Mutat Res 711:193–201. doi:10.1016/j.mrfmmm.2010.12.016
- Kumar A, Rai PS, Upadhy R, Vishwanatha KS, K (2011)
γ-radiation induces cellular sensitivity and aberrant meth-
ylation in human tumor cell lines. *Int J Radiat Biol* 87:1086–
1096. doi:10.3109/09553002.2011.605417
- Li P, McLaughlin J, Infante-Rivard C (2009) Maternal occupational
exposure to extremely low frequency magnetic fields and the
risk of brain cancer in the offspring. *Cancer Causes Control*
20:945–955. doi:10.1007/s10552-009-9311-5
- Li Z, Doho G, Zheng X, Jella KK, Li S, Wang Y, Dynan WS (2015)
Co-culturing with high-charge and energy particle irradiated
cells increases mutagenic joining of enzymatically induced
DNA double-strand breaks in nonirradiated Cells. *Radiat Res*
184:249–258. doi:10.1667/RR14092.1
- Liu F, Killian JK, Yang M, Walker RL, Hong JA, Zhang M, Davis
S, Zhang Y, Hussain M, Xi S, Rao M, Meltzer PA, Schrupp
DS (2010) Epigenomic alterations and gene expression profiles
in respiratory epithelia exposed to cigarette smoke condensate.
Oncogene 29(25):3650–3664
- Liu Y, Liu WB, Liu KJ, Ao L, Zhong JL, Cao J, Liu JY (2015)
Effect of 50 Hz extremely low-frequency electromagnetic
fields on the DNA methylation and DNA methyltransferases in
mouse spermatocyte-derived cell line GC-2. *Biomed Res Int*
2015:237183. doi:10.1155/2015/237183
- Manikonda PK, Rajendra P, Devendranath D, Gunasekaran B,
Channakeshava, Aradhya SR, Sashidhar RB, Subramanyam
C (2014) Extremely low frequency magnetic fields induce
oxidative stress in rat brain. *Gen Physiol Biophys* 33:81–90.
doi:10.4149/gpb_2013059
- Marcantonio P, Del Re B, Franceschini A, Capri M, Lukas S,
Bersani F, Giorgi G (2010) Synergic effect of retinoic acid
and extremely low frequency magnetic field exposure on
human neuroblastoma cell line BE(2)C. *Bioelectromagnetics*
31:425–433
- Nüsken N, Goering W, Dauksa A, Biswas A, Jamil MA, Dimitriou
I, Sharma A, Singer H, Fimmers R, Fröhlich H, Oldenburg J,
Gulbinas A, Schulz WA, El-Maarri O (2015) Inter-locus as
well as intra-locus heterogeneity in LINE-1 promoter methyl-
ation in common human cancers suggests selective demethylation

- 517 pressure at specific CpGs. *Clin Epigenetics* 7:17. doi:[10.1186/](https://doi.org/10.1186/s13148-015-0051-y)
 518 [s13148-015-0051-y](https://doi.org/10.1186/s13148-015-0051-y)
- 519 O'Hagan HM, Wang W, Sen S, Destefano Shields C, Lee SS, Zhang
 520 YW, Clements EG, Cai Y, Van Neste L, Easwaran H, Casero
 521 RA, Sears CL, Baylin SB (2011) Oxidative damage targets com-
 522 plexes containing DNA methyltransferases, SIRT1, and poly-
 523 comb members to promoter CpG Islands. *Cancer Cell* 20:606–
 524 619. doi:[10.1016/j.ccr.2011.09.012](https://doi.org/10.1016/j.ccr.2011.09.012)
- 525 Pogribny IP, Beland F (2013) DNA methylome alterations in chemi-
 526 cal carcinogenesis. *Cancer Lett* 334:39–45
- 527 Qian Z, Shen Q, Yang X, Qiu Y, Zhang W (2015) The role of extra-
 528 cellular vesicles: an epigenetic view of the cancer microenviron-
 529 ment. *Biomed Res Int* 2015:649161. doi:[10.1155/2015/649161](https://doi.org/10.1155/2015/649161)
- 530 Qiu C, Fratiglioni L, Karp A, Winblad B, Bellander T (2004) Occupa-
 531 tional exposure to electromagnetic fields and risk of Alzheimer's
 532 disease. *Epidemiology* 15:687–694
- 533 Schulz WA (2006) L1 retrotransposons in human cancers. *J Biomed*
 534 *Biotechnol* 2006(1):83672
- 535 Speek M (2001) Antisense promoter of human L1 retrotransposon
 536 drives transcription of adjacent cellular genes. *Mol Cell Biol*
 537 21:1973–1985
- 538 Stratton D, Lange S, Inal JM (2013) Pulsed extremely low-frequency
 539 magnetic fields stimulate microvesicle release from human
 540 monocytic leukaemia cells. *Biochem Biophys Res Commun*
 541 430:470–475. doi:[10.1016/j.bbrc.2012.12.012](https://doi.org/10.1016/j.bbrc.2012.12.012)
- 542 Vijayalaxmi TJ, Prihoda (2009) Genetic damage in mammalian
 543 somatic cells exposed to extremely low frequency electro-mag-
 544 netic fields: a meta-analysis of data from 87 publications (1990–
 545 2007). *Int J Radiat Biol* 85:196–213
- 546 Vrijheid M, Slama R, Robinson O, Chatzi L, Coen M, van den Hazel
 547 P, Thomsen C, Wright J, Athersuch TJ, Avellana N, Basagaña
 548 X, Brochot C, Bucchini L, Bustamante M, Carracedo A, Casas
 549 M, Estivill X, Fairley L, van Gent D, Gonzalez JR, Granum B,
 550 Gražulevičienė R, Gutzkow KB, Julvez J, Keun HC, Kogevinas
 551 M, McEachan RR, Meltzer HM, Sabidó E, Schwarze PE, Sir-
 552 oux V, Sunyer J, Want EJ, Zeman F, Nieuwenhuijsen MJ (2014)
 553 The human early-life exposome (HELIX): project rationale and
 554 design. *Environ Health Perspect* 122(6):535–544
- 555 Watkins DJ, Wellenius GA, Butler RA, Bartell SM, Fletcher T, Kel-
 556 sey KT (2014) Associations between serum perfluoroalkyl acids
 557 and LINE-1 DNA methylation. *Environ Int* 63:71–76
- 558 Yang AS, Estéicio MR, Doshi K, Kondo Y, Tajara EH, Issa JP (2004)
 559 A simple method for estimating global DNA methylation using
 560 bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 32:e38

UNCORRECTED PROOF

Author Query Form

Please ensure you fill out your response to the queries raised below and return this form along with your corrections

Dear Author

During the process of typesetting your article, the following queries have arisen. Please check your typeset proof carefully against the queries listed below and mark the necessary changes either directly on the proof/online grid or in the 'Author's response' area provided below

Query	Details Required	Author's Response
AQ1	Author: References "Marcantonio et al. (2010) and Speek (2001)" are given in list but not cited in text. Please cite in text or delete from list.	
AQ2	Author: Fig1 - Figure is poor in quality as its labels are not readable. Please supply a new version of the said figure with legible labels preferably in .eps, .tiff or .jpeg format with 600 dpi resolution.	
AQ3	Author: Fig1 - Figure is poor in quality as its labels are not readable. Please supply a new version of the said figure with legible labels preferably in .eps, .tiff or .jpeg format with 600 dpi resolution.	