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

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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.

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

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

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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**

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6 **Paolo Garagnani<sup>2,4</sup> · Miriam Capri<sup>2,4</sup> · Ferdinando Bersani<sup>5</sup> · Brunella Del Re<sup>1</sup>**

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**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

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(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

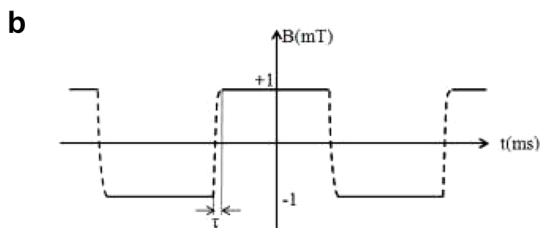
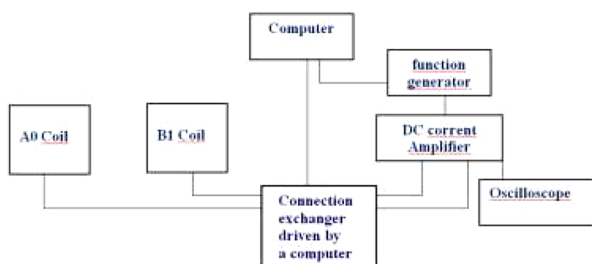
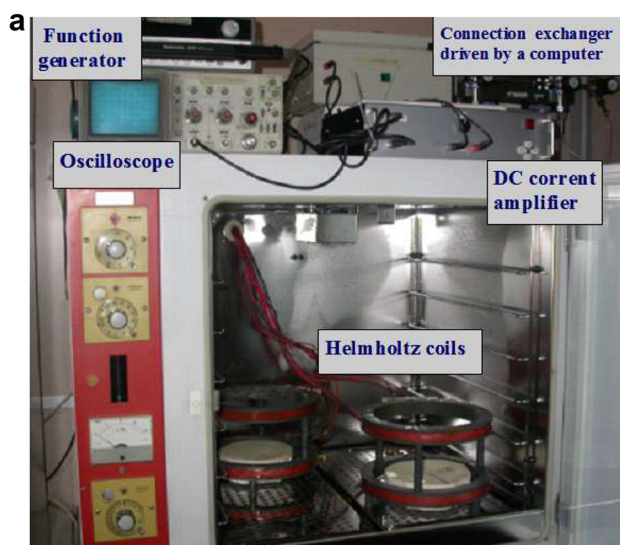
Neuroblastoma BE(2)C cells were kindly provided by Prof. Della Valle (University of Bologna, Italy), and were maintained in Dulbecco's modified Eagle's medium (DMEM, EuroClone, Milano, Italy), supplemented with 10% heat-inactivated fetal bovine serum (FBS, EuroClone), 100 UI/ml penicillin (Sigma, Ronkonkoma, 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 H<sub>2</sub>O<sub>2</sub> (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% CO<sub>2</sub> 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



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

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  $\mu\text{T}$  (horizontal component 10.8  $\mu\text{T}$ , vertical com-  
ponent 13.0  $\mu\text{T}$ ), the AC component was on the order of  
0.1  $\mu\text{T}$ , 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  
 $\tau$  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%.

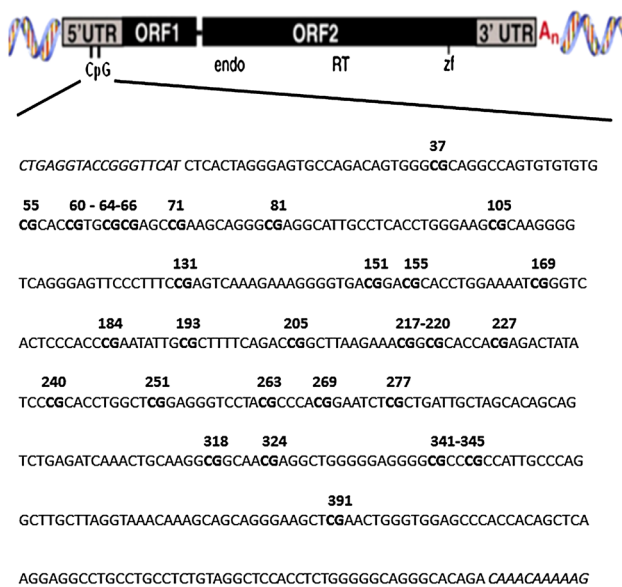
**DNA extraction and sodium bisulfite treatment**

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

**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 bisulphite-treated DNA were  
PCR-amplified using the following primers: forward strand  
primer: AGGAAGAGAGTTTATTAGGGAGTGTTAGAT  
AGTGGG; reverse strand primer: CAGTAATACGACTCA  
CTATAGGGAGAAGGCTTCTATACCCTACCCCA  
AAAATAAA.

By using these primers, we evaluated DNA methylation  
levels of 24 CpG units (i.e. regions containing one or multi-  
ple 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  $\mu$ 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.

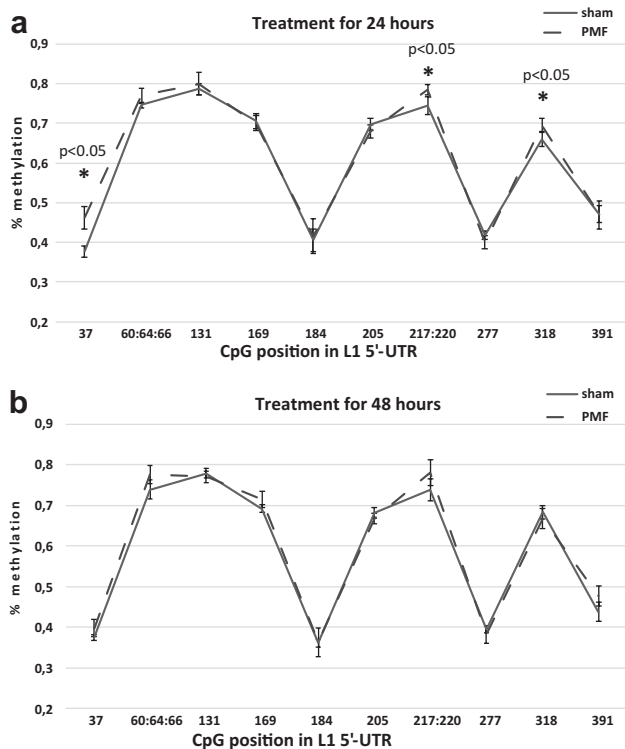
Effects of PMF exposure alone are shown in Fig. 3. After 24 h of PMF exposure, 3 CpGs (37, 217-220, 318) exhibited a significantly increased methylation level as compared to the CpGs from sham exposed samples (Fig. 3a). After 48 h of PMF exposure no significant differences were observed at any CpGs (Fig.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

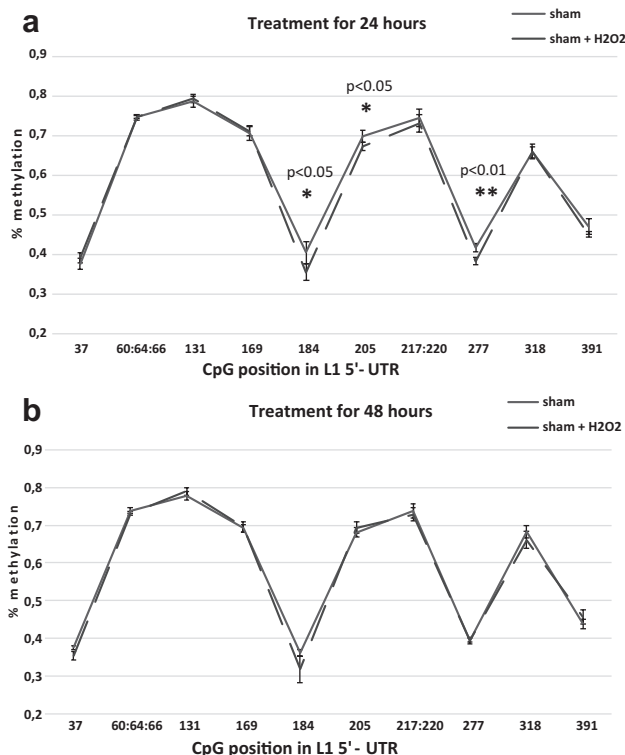
Results about the effects of PMF exposure in combination with OS are shown in Fig. 5. After 24 h 5 CpG units (37, 131, 184, 217-220, 318) exhibited significantly less methylation as compared to the CpGs from samples exposed to PMF alone (Fig. 5a). After 48 h only 2 CpG units (60:64:66, 217-220) showed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



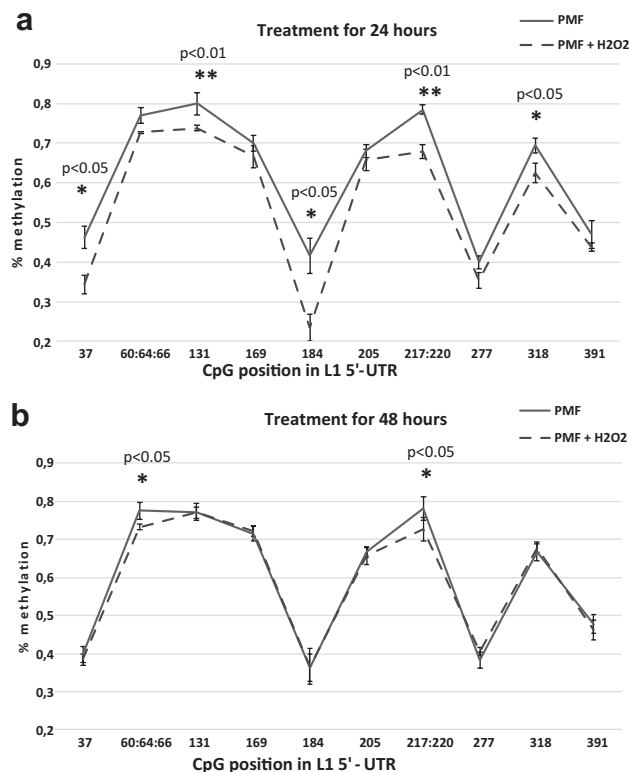
**Fig. 4** CpG dinucleotide methylation percentage of L1 5'UTR region in cells subjected to oxidative stress (300  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 1 h) (dashed line) or distilled water (control, continuous line) after **a** 24 and **b** 48 h from the treatment. Error bars represent SEM of 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  $\mu$ M  $H_2O_2$  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 cidate the epigenetic effect of ELF-MFs alone and in the  
 378 presence of OS, also considering different cell types and  
 379 exposure scenarios.

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384 **Compliance with ethical standards**

385 **Conflict of interest** The authors report no conflicts of interests. The  
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