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PhD Program - XXX Cycle
Experimental and Translational Medicine

PRELIMINAR EVALUATION OF EPIGENETIC MODIFICATIONS FOLLOWING *IN VITRO* HYDROQUINONE EXPOSURE IN MODELS OF HUMAN ACUTE PROMYELOCYTIC LEUKEMIA CELLS (HL-60) AND OF HUMUAN UMBILICAL CORD MESENCHIMAL STEM CELL (hUCMSC)

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ABSTRACT

Benzene, a volatile aromatic hydrocarbon, is extensively used in industry even though it is recognized as a myelotoxin with leukemogenic activity, representing a significant occupational risk. Metabolism of benzene plays a fundamental role in its toxicity, and among the different metabolites, hydroquinone (HQ) is one of the most important, as it accumulates in the bone marrow where it can induce several genetic and epigenetic changes. Nevertheless, the actual mechanisms behind the carcinogenetic effects of benzene and HQ and the role of epigenetic alterations in the process of tumorigenesis are not fully clarified yet.

Aberrant patterns of DNA methylation, including loss of imprinting (LOI), gene-specific hyper- or hypo-methylation and global hypo-methylation are all common in several tumor types, including AML, and are important for transcriptional repression or activation of cancer-associated genes. Most of the studies on the epigenetic effects of benzene conducted so far have been centered on DNA methylation (measured on repeated elements LINE-1 and Alu, used as a surrogate for the entire genome), whereas only very recently a few investigations have analyzed the effects of environmental chemicals on histone modifications.

Several studies have shown a strong correlation between DNA methylation and histone modifications, indicating a cross-talk between DNMTs and histone modifications. For example, trimethylation of H3K27 (H3K27me3) can be associated with increased DNA methylation while de novo methylation does not occur in regions where bi-or trimethylation of the histone 3 lysine 4 (H3K4me2 or H3K4me3) is observed. Conversely, the simultaneous presence of H3K27me3 and H3K4me3 is recognized as a bivalent mark; it was firstly described during development and it appears to play a role in poisoning non-active genes for aberrant transcription in cancer.

Most notably, epidemiological studies on healthy subjects have shown a difference in the pattern of methylation between subjects exposed to benzene (gasoline station attendants) compared to office workers, with a significant reduction in the methylation of LINE-1 and Alu. This profile of global hypo-methylation following exposure to low doses of benzene appears to be qualitatively comparable to what observed in AML and other neoplasms.

Thus, in the present work we attempted to reproduce in vitro the conditions of chronic exposure to benzene found in the peripheral blood of subjects exposed. We started from “low” doses of hydroquinone in vitro (below 15 μM) and we proceeded by progressively lowering the concentration to explore the cellular response to different types of exposure and concentrations, to evaluate if HQ might be able to alter the epigenetic signature in two different biological systems, thereby describing a poorly explored step in the mechanism of toxicity associated with benzene exposure.

We finally set up a chronic treatment 4 weeks-long with HQ 1 μM, corresponding to 110 ng/mL, a concentration within the amount of total HQ (between 20 and 120 ng/mL, corresponding to 2–16 ng/mL of free HQ) found in the blood of subjects exposed to airborne benzene ranging from 1 mg/m3 (around 0,3 ppm) to 80mg/m3 (around 25 ppm). This concentration was used on a stabilized cell line of human acute promyelocytic leukemia (hAPML), HL60, analyzed as a model of hematopoietic cells; exploring the epigenetic events occurring in chromatin, we found the instauration of the distinctive signature combining the repressive H3K27me3 and the activating H3K4me3, with the gradual increase in H3K4me3 levels, on LINE-1 promoter region. We observed the absence of statistically significant variations in DNA methylation and expression levels of LINE-1, despite a decrease in protein levels of UHRF1, DNA methyl-transferases and histone methyl-transferases.

Moving onto a model of normal stem cells, we had to further lower the concentration to avoid cytotoxicity. Thus, human umbilical cord mesenchymal stem cells (hUCMSC) were treated for 4 weeks with HQ 0,1 μM to verify if long-term exposure to low doses of HQ could be able to alter the epigenetic signature in staminal cells outside the hematopoietic compartment. Surprisingly, we found a progressive increase of H3K4me3 in our control cells, with the instauration of the bivalent mark at the third week; the fourth week was non-evaluable, since the cytotoxic effect was prevalent despite the reduced concentration. Most interestingly, at the third week we observed the peculiar inversion in the levels of H3K27me3 and H3K4me3 on LINE-1 promoter region, with higher H3K27me3 in HQ treated cells as compared to the control, and opposite to what observed in HL60. On the other hand, as for HL60 cells no statistically significant variations in DNA methylation was appreciable. These differences were seen not

only on chromatin but also on the profile of mRNA expression: preliminary Principal Component Analysis showed a significant distance between controls in the three biological replicates under consideration.

In conclusion, in vitro treatment with low-dose HQ determined the instauration of a poisoned state of chromatin in LINE-1 sequences in both the models considered, suggesting that prolonged exposure could cause persistent epigenetic alterations.

INTRODUCTION

1. BENZENE

Benzene is a is an important organic chemical compound with the chemical formula C_6H_6. It is a colorless liquid with a sweet odor. It is a highly flammable volatile liquid: at room temperature and atmospheric pressure it evaporates into air very quickly and dissolves slightly in water.

Benzene is a natural constituent of crude oil and is one of the elementary petrochemicals; it is a well-known environmental pollutant, due to its release in the air through cigarette smoke and fumes of motor vehicles (Wallace 1989), by production by incomplete combustion of carbon-rich compounds in the steel and plastics industries. It is also naturally produced in volcanoes or forest fires, cigarette smoke, or at temperatures above 500° C.

It is extensively used in rubber, plastics, paint and petrochemical industries even though it is recognized as a myelotoxin with leukaemogenic activity, representing a significant occupational risk (Khalade et al. 2010).

Indeed, benzene has been classified by the IARC as a carcinogen of group 1 and due to his volatility, it can be easily absorbed by inhalation, dermal contact or ingestion. The mechanism by which benzene induces carcinogenesis is unknown, but strong evidences indicate that, to exert mutagenic and carcinogenic action, benzene must undergo oxidative metabolism and turn into reactive intermediates that include phenol and hydroquinone (Snyder and Hedli 1996, Meek and Klaunig 2010, Zolghadr et al. 2012). Benzene is metabolized mainly in the liver by cytochrome P4502E1 (and most likely by other CYP450), but also in the bone marrow by myeloperoxidase (MPO), leading to formation of various metabolites shown in Figure 1. As evident from the chemical structure of its metabolites, the mechanism of benzene toxicity is not well defined because of the possible role of many intermediate electrophiles (benzene oxide and benzoquinones) and of the reactive oxygen species produced by the redox cycle of catechol, hydroquinone and benzoquinone (Rappaport et al. 2002).

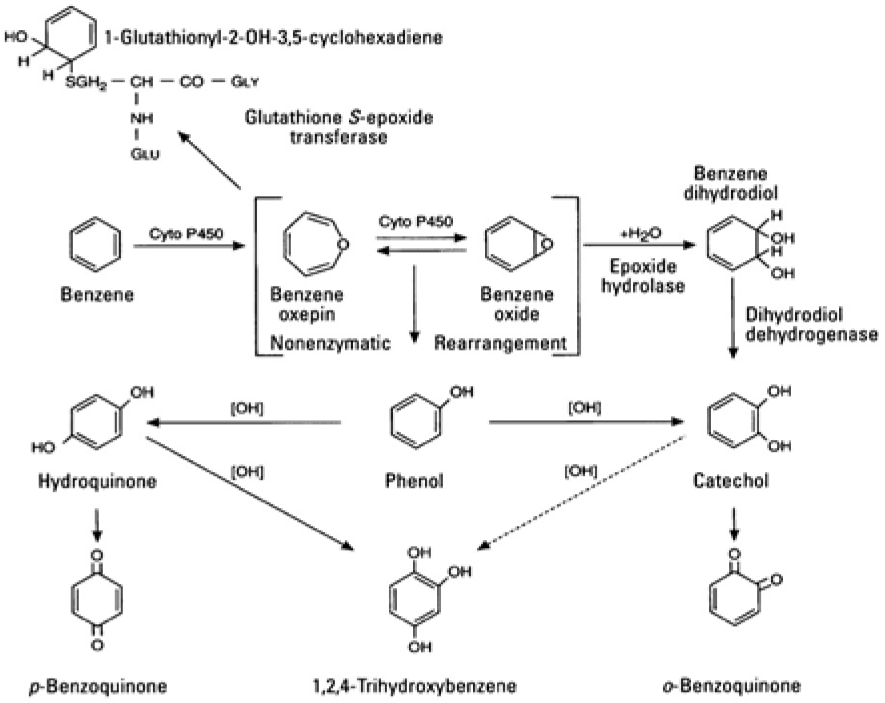


Figure 1 – Benzene metabolism (Snyder and Hedli 1996).

All these phenolic metabolites of benzene, in addition to being metabolized by the liver, can also be oxidized by myeloperoxidase in hematopoietic bone marrow, responsible in particular for the activation of hydroquinone to 1,4-benzoquinone, thus suggesting a possible explanation of the benzene implication in myelotoxicity (Smith 1996). In fact, exposure to high concentrations of benzene leads to toxicity in the hematopoietic system, while acute myeloid leukemia (AML) is the major oncogenic disorder associated with chronic exposure, although other forms of leukemia have been reported (Aksoy 1989, Atkinson 2009, Khalade et al. 2010, Stenehjem et al. 2015).

2. HYDROQUINONE

Hydroquinone (HQ, whose structure is shown in Figure 2), also known as benzene-1,4-diol or quinol, is an aromatic organic compound, having the chemical formula $C_6H_4(OH)_2$. It is one of the most represented benzene metabolites and for this reason it is commonly used to study *in vitro* the effects of benzene on human health.

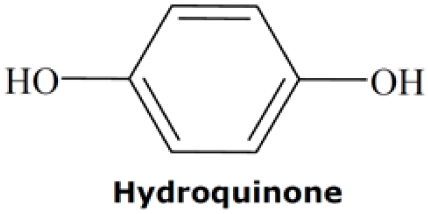


Figure 2 – Chemical structure of hydroquinone (Snyder and Hedli 1996).

Hydroquinone has a variety of uses, principally associated with its action as a reducing agent that is soluble in water. It presents cutaneous and renal toxicity (it is eliminated as a result of glycuronation), but the most toxic effects are in the bone marrow, where it interferes with hematopoiesis through not fully clarified mechanisms (FDA Nomination Profile, 2009).

Several evidences show that a single exposure to high concentrations of hydroquinone (above 30 µM) could be sufficient for the cytotoxic effect (Terasaka et al. 2005a, Terasaka et al. 2005b). On the other hand, the carcinogenesis process due to HQ seems to be induced by continuous exposure to low doses, through a mechanism that is still widely obscure (Lagorio et al. 2013, Snyder and Hedli 1996).

HQ, as its precursor, has been linked to global hypomethylation of DNA and inhibition of the oncosoppressor p53, a significant event for the development of different cancer and leukemia, with an increase in the expression of BCL2, consequent block of BAX activity and activation of the apoptotic process (Westerhof and Kooyers 2005).

In rats exposed to high doses of benzene in the air, in which a myelotoxic effect develops, the concentration in HQ in the blood is about 0,2-1,8 µM, values comparable with those found in subjects exposed to benzene in the occupational air (such as gas stations and traffic police), while in the bone marrow the concentration rises to about 10-60 µM. The concentrations of HQ in the bone marrow of highly exposed people can reach values up to 10-20 µM (Rickert et al. 1979).

3. EFFECTS INDUCED BY BENZENE AND ITS METABOLITES

In people occupationally exposed to benzene, the toxicity is characterized in the early stages by reversible hematotoxicity or, if the exposure is chronic and at high doses, by irreversible damage to the bone marrow. Chronic exposure to benzene is in fact responsible for a broad spectrum of hematotoxic effects including anaplastic anemia, leukopenia and pancytopenia, leading to acute myeloid leukemia (AML) and other forms of leukemia in adults (Aksoy 1989, Atkinson 2009, Stenehjem et al. 2015, Lan et al. 2004). In particular, exposure to benzene and its derivatives has been closely associated with AML in several studies (Khalade et al. 2010).

A common alteration in many types of cancer, including the hematopoietic ones such as AML, is found at the epigenetic level: in particular, changes in the DNA methylation pattern have been extensively observed (with prevalence of global hypomethylation events, as opposed to hypo- and hypermethylation of specific genes) together with loss of imprinting (Bollati et al. 2007, Brait and Sidransky 2011, Hamilton 2011, Liu et al. 2011, Pistore et al. 2017), important processes for transcriptional repression or activation of cancer-associated genes (Chen et al. 2004, Wilson, Power and Molloy 2007). In particular, in acute myeloid leukemia, global DNA methylation levels generally tend to decrease during the various steps of progression from normal to cancer cells, although cases of global hypermethylation are also reported, suggesting the existence of different mechanisms responsible of these alterations that require deeper studies (Lübbert et al. 1992).

DNA methylation of repetitive elements is widely used in research and clinic as an indicator of global genomic methylation level, thanks to their high number of copies interspersed in the genome (Yang et al. 2004, Sahnane et al. 2015): the best-studied families are the long (LINE-1 or L1) and the short (SINEs, in particular Alu) interspersed nuclear elements (Byun et al. 2013). In human studies, differences in DNA methylation of L1 and Alu have been consistently demonstrated in response to a wide range of environmental exposures, including airborne pollutants (Bollati et al. 2007, Baccarelli and Bollati 2009, Peluso et al. 2012, Seow et al. 2012). A study of 2007 first correlated changes in DNA methylation patterns with exposure to low-dose of benzene (Bollati et al. 2007). It highlighted a difference in methylation levels

between the exposed healthy subjects (petrol stations) and the reference group (office workers), with a significant reduction in the first group of 2.33% in the methylation of repeated sequences (LINE- 1 and Alu) used as a surrogate for the whole genome; furthermore, in the first group hypermethylation of the oncosoppressor p15 was observed. This profile resulting from low-dose exposure of benzene appears to be qualitatively comparable to that generally observed during AML and other neoplastic progression.

Liu et al. in 2012 (Liu et al. 2012) demonstrated that in TK6 cells the exposure at different concentration of HQ (2,5; 5; 10,0 and 20,0 μM for 48 h) resulted in the decrease of DNMTs and MBD2 mRNA level, in the global hypomethylation of LINE-1 repetitive sequences and in the increase of MPL at mRNA level. These results indicate that global DNA hypomethylation may result from DNMTs inhibition induced by HQ exposure.

DNA hypomethylation (measured on LINE-1 sequences) was also partially demonstrated *in vitro* in HQ treated cells (Ji et al. 2010). This study suggests that hydroquinone could perform its leukemogenesis toxicity by a mechanism similar to alkylating agents and topoisomerase II inhibitors through a global hypomethylation of DNA.

Conversely, a more recent study demonstrated that HQ exposure could induced transcriptional reactivation of some human retroelements families (such as Alu/L1/HERV) uncoupled from DNA methylation changes (Conti et al. 2016).

Mechanisms explaining DNA demethylation have been largely focused on inhibition of the DNA methyltransferases, but recent evidence suggests a role for the TET family of 5mC dioxygenases in active DNA demethylation. The proposed mechanism entails the conversion of 5mC to 5hmC catalyzed by TET1, followed by deamination to 5hmU and subsequent base excision repair, resulting in an unmethylated cytosine (Tahiliani et al. 2009). Joseph P. Bressler group's studies provide support that this mechanism involving TET1 is responsible for DNA demethylation observed in HEK293 cells exposed to HQ (Coulter, O'Driscoll and Bressler 2013).

Most of the studies conducted so far have been centered on DNA methylation, whereas only a few investigations have analyzed the effects of environmental chemicals on histone modifications: only

recently the relation with benzene exposure was explored (Baccarelli and Bollati 2009, Philbrook and Winn 2015).

Recent studies have demonstrated that specific histone modifications could be altered in response to a variety of environmental pollutants such as metal-rich air particles, arsenic, benzo(a)pyrene, and cigarette smoke (Cantone et al. 2011, Chervona et al. 2012, Liang et al. 2012, Ma et al. 2016, Sundar and Rahman 2016). However, it remains unclear how benzene exposure could alter histone modifications and mediate the cellular response and the molecular mechanism of the transcriptional regulation of specific genes.

Some evidences also correlated *in vitro* exposure to benzene metabolites with generation of γ-H2AX, an early event after the formation of DSBs (Ishihama, Toyooka and Ibuki 2008) and transient instauration of the histone bivalent mark H3K27me3/H3K4me3 on human retroelements (LINE-1) in HL60 cells (Mancini et al. 2017). In a recent study it was demonstrated that exposure to low levels of benzene and its metabolites could correlate with modification of global H3K4me3 in the PBLCs and with the reduction of WBC counts of benzene-exposed workers, indicating that histone modifications play a key role in mediating the hematotoxicity of benzene exposure (Li et al. 2018). Moreover it is demonstrated that after treatment of Primary human lymphocytes (PHLCs) with 0.1, 1.0, and 10.0 mM HQ for 48 h, H3K4me3 mark was enriched in the promoters of several DNA damage responsive (DDR) genes including CRY1, ERCC2, andTP53 (Li et al. 2018).

4. EPIGENETICS

The term ‘epigenetics’ defines the study of inheritable modifications of gene expression that do not involve changes in the primary nucleotide sequence and/or in the number of copies of the DNA (Bird 2002). Genetics play an essential role in defining an organism’s development, morphology and physiology, while epigenetic mechanisms play an essential role in modulating these properties by regulating gene expression. Epigenetic regulation can be either relatively stable (such as X inactivation or imprinting) (Disteche and Berletch 2015) or dynamic (such as the expression of a gene after a stimulus or activation of oncogenes) (Morrison et al. 2016).

Conventionally, all the modifications and/or molecular mechanisms involved in the regulation of gene expression have been considered as the "epigenome", including either DNA methylation (CpG methylation), hPTMs, (post-translational modifications of histones), replacement with histone variants (Suganuma and Workman 2011) and non-coding RNAs (e.g. miRNAs, lncRNAs, ceRNAs) (Malumbres 2013, Ran et al. 2014).

Altogether, these epigenetic marks define a peculiar chromatin profile that delineates the cell identity. In other terms, the heritable instructions that determine spatial and temporal changes of gene activation and repression lead to functional distinct cell types characterized by different phenotypes arisen from the same genotype (Probst, Dunleavy and Almouzni 2009).

5. CHROMATIN ORGANIZATION AND REGULATION OF GENE EXPRESSION

Chromatin is the structure where the DNA is associated with specific proteins and RNA and packaged into the cell nucleus, allowing the segregation of genetic material in a small space (Figure 3). The compaction of DNA in eukaryotic cells is the result of its regular association with histones to form a structure called nucleosome (Kornberg and Lorch 1999). The formation of the nucleosomes is the first stage of a larger and more complex process that allows the DNA to organize in a more condensed structure called chromosome.

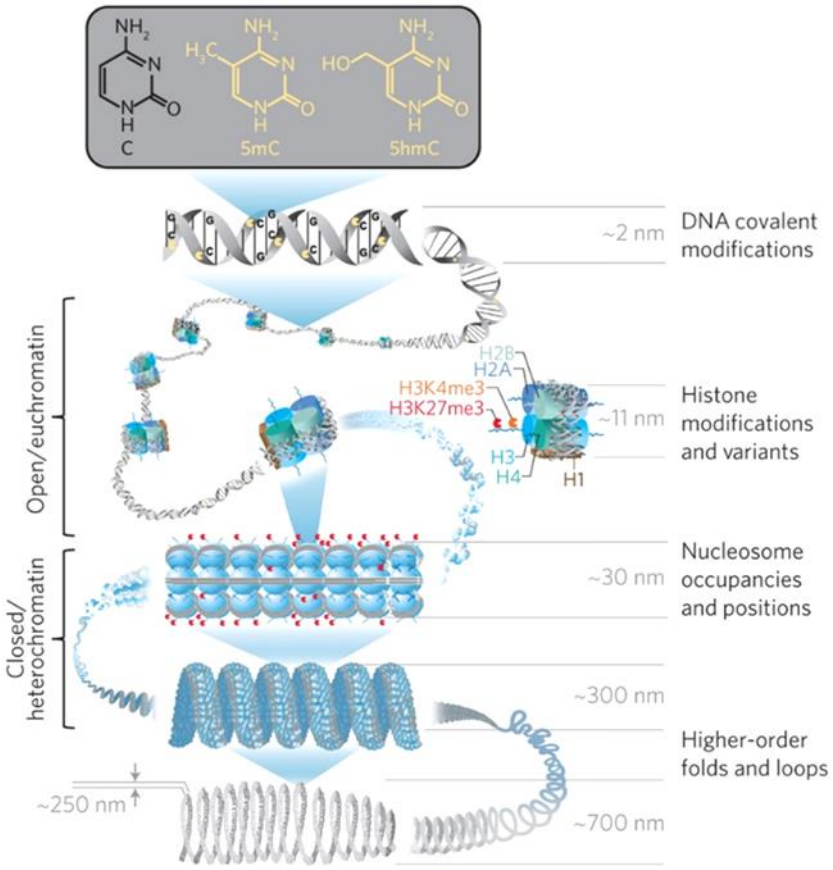


Figure 3 - Chromatin organization and principal modifications (Aguilar and Craighead 2013).

The nucleosome is the basic unit of the chromatin and it is composed of an octamer formed by four heterodimers: two H3-H4 heterodimers associated to give a tetramer, and two heterodimers H2A-H2B to form the second tetramer (Quina, Buschbeck and Di Croce 2006), around which 147 base pairs of DNA are wrapped (Kouzarides 2007).

Each core histone has an N-terminal extension called a "tail" without a defined three-dimensional structure that protrudes outside the nucleosome and becomes accessible. Histone tails are not required for DNA binding with the core but they are fundamental for the regulation of the interactions with the DNA. They are sites of substantial modifications able to change the function of the single nucleosome (Erler et al. 2014): these changes include acetylation and methylation of serine and lysine residues.

Histone H1 is called histone linker because it binds the stretch of DNA that joins two adjacent nucleosomes (Luger and Richmond 1998). H1 induces a close DNA wrapping around the nucleosome and allows the formation of a higher ordered chromatin structure, the 30 nm fiber (Robinson and Rhodes 2006).

Six nucleosomes are arranged in solenoid or zig zag structure and the histone tails stabilize the structure by interacting with the adjacent nucleosomes. Then, the 30 nm fiber forms loops (called domains) containing 40-90 kb of DNA that are blocked at their base by a protein structure called nuclear scaffold. Thus, the DNA turns out to be folded into a more compact structure that reduces its linear length by about 10,000 times while limiting accessibility.

In fact, the association of the DNA with histones allows the condensation of the nucleic acid but, at the same time, reduces the possibility of accessing the DNA. This reduced accessibility can interfere with all cell-life processes such as replication, DNA repair, recombination reactions, formation of centromere and kinetochore, but over all it could interfere with DNA transcription (Li, Carey and Workman 2007). The nucleosome is not a simple static packaging unit but it possesses dynamic properties which are strictly regulated by epigenetic mechanisms, chemical modifications mediated by several protein complexes orchestrating the chromatin remodeling and determining regulation of the gene expression (Li et al. 2007). The transcription initiation could be controlled by different mechanisms: DNA methylation, chromatin structural organization like modifications of histone and nucleosome positioning (Berger 2007), the binding of transcription factors to DNA consensus sequences, interactions between RNApolII specific subunits and gene-specific transcriptional activators (Malik and Roeder 2005) and architectural complexity of the core promoter (regulatory elements, multiple start sites and alternative promoter) (Smale 1997).

Chromatin can be distinguished into two types: euchromatin, less condensed corresponding to the transcriptionally active chromatic regions and therefore rich in genes that are transcribed, and heterochromatin, a more condensed component that constitutes about 10% of the genome and does not seem to present transcription activity.

Heterochromatin is the most common form of gene silencing (Grewal and Moazed 2003) and its name derives from the microscope observation. It is formed by condensed regions and transcriptionally inactive gene. We can distinguish constituent and optional heterochromatin. The constituent is inactive for the whole life of the cell and is mainly found in the telomeric and centromeric regions. The facultative chromatin, instead, is composed by gene that must be silenced in specific moments of the cell cycle or

during the differentiation stages. Moreover, heterochromatin plays an important role in chromosomal segregation and in the maintenance of chromosome stability.

Gene silencing can extend to long regions of DNA and is a mechanism by which genes are turned off transcriptionally. An example is the silencing of the female X chromosome in mammals, inactivated in the early stages of embryogenesis (Brockdorff 2002).

The transition from euchromatin to heterochromatin and / or vice versa, can be modified by different epigenetic mechanisms, such as enzymes that act on the chemistry of the histone tails thus affecting the accessibility of the DNA, by enzymes involved in replication, repair and recombination and transcription processes, by DNA methylation. These mechanisms are dynamic processes that may change in time depending on the stage of the cell cycle, on different external stimuli or during differentiation and development (Reik 2007).

6. CHROMATIN MODIFICATIONS AND FUNCTIONS

As previously mentioned, the dynamic of chromatin structure is regulated by several mechanisms including histone post translational modifications (hPTMs), the presence of different histone variants and DNA methylation (Li et al. 2007).

Chromatin-modifying enzymes cooperate to chromatin organization in a highly-regulated manner (Allis et al. 2007) and could be classified in ‘writers’, ‘erasers’ and ‘readers’ relying on their abilities respectively to add, remove or read different histone and DNA modifications (Treviño, Wang and Walker 2015).

Currently, at least four different DNA modifications (Wu and Zhang 2011) and 16 classes of histone modifications (Kouzarides 2007) are known, even if the attribution a biological function remains unclear for some of them. Between DNA modifications there are 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Ito et al. 2011). Among the histone modifications, the best known are methylation of various lysines (Wu, Connolly and Biggar 2017) and arginines and acetylation of lysine residues.

6.1. DNA METHYLATION

DNA methylation is an epigenetic mechanism of gene transcription regulation mediated by DNA methyltransferase (DNMTs) enzymes that act directly on DNA transferring a methyl group from the carbon-bearing molecule S-adenosyl-methionine (SAM) to position 5 of the cytosine, converting it into 5-methylcytosine (5mC) (Youngblood et al. 2007) as shown in Figure 4.

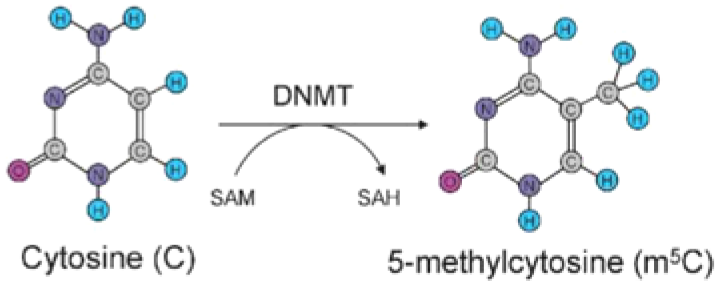


Figure 4 – 5 carbon methylation process of Cytosine, performed by DNMTs (Grønbaek, Hother and Jones 2007).

Notably, 5mC can be actively oxidized to 5-hydroxy-methylcytosine (5hmC) by TET family proteins and can be subsequently deaminated and replaced via BER pathway (Guo et al. 2011). The role of these enzymes in the global DNA de-methylation processes is still not completely understood, although their importance is nowadays well established (von Meyenn et al. 2016).

Methylation typically occurs in CpG sites (cytosine-phosphate-guanine, where cytosine is directly followed by a guanine), which constitute about 1-2% of the genome. These dinucleotides are not uniformly distributed in DNA, but are more frequently found in heterochromatic regions, including telomeres and microsatellites, in the pericentromeric regions, in the proximity of transcription start sites (Deaton and Bird 2011) especially of housekeeping genes and developmental regulators (Meissner 2011). These DNA regions of more than 200 base pairs in length (from 0,2 to 3 kb) are called CpG islands and are characterized by an elevated C/G content (at least 50 %) and a high ratio of CpG dinucleotide (at least 0,6%) (Illingworth and Bird 2009).

In normal cells, CpG methylation plays a fundamental role in the regulation of gene expression necessary for tissue expression and specific development (Deaton and Bird 2011), for the silencing of the inactive X

chromosome, genomic imprinting and protection against the activation of transposable elements, including LINE-1 (Teneng et al. 2011). The methylation status of these CpG sites can have a strong impact on gene activity / expression. In fact, the methylation in the gene promoter region can interfere with the binding of specific proteins committed to transcription, inhibiting it, or favoring the binding of specific repressors, able to "switch off" the genes involved by recruiting enzymes such as histone-deacetylases (HDAC) (Wade 2001).

In the early stages of embryonic development, alternate waves of methylation and demethylation regulate growth and differentiation programs (Haaf 2006). Thereafter, the DNA methylation patterns established during this period remain relatively stable in normal tissues and are maintained constant by a fine regulatory activity exerted by the same DNMTs.

The DNMTs family directly regulates DNA methylation; it includes DNMT1, DNMT3A and DNMT3B (DNMT3L is a regulator protein in which the catalytic site is lost) (Figure 5).

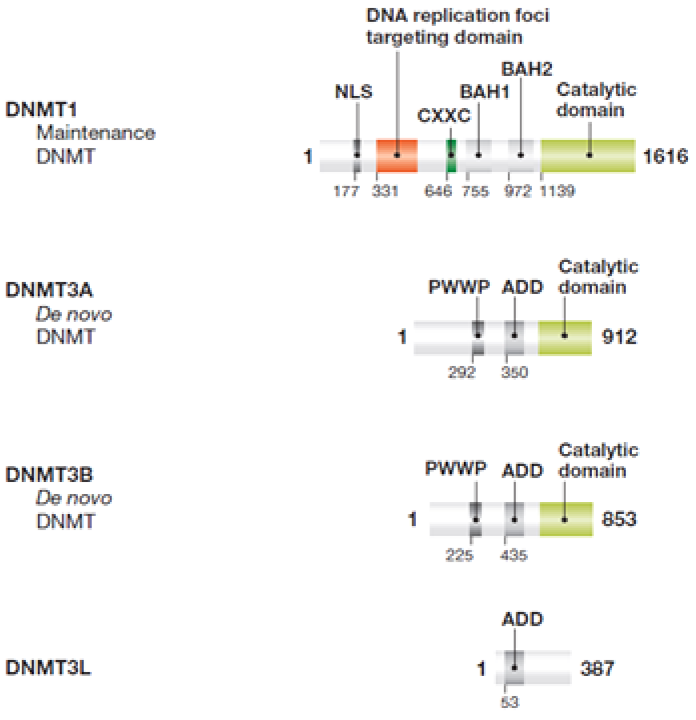


Figure 5 – Structure and domains of DNMTs (Denis, Ndlovu and Fuks 2011).

DNMT3A and DNMT3B are called *de novo* methylases and are fundamental for the deposition of the methylation pattern in the embryonic phase; subsequently this pattern is maintained at each cellular replication by DNMT1 (for this reason called maintenance DNMT) (Li, Bestor and Jaenisch 1992). DNMT1,

together with UHRF1, recognizes the hemi-methylated DNA generated during DNA replication (Sharif et al. 2007); indeed, it is expressed at high levels in cells in active proliferation and depends on the interaction with the SRA domain of UHRF1 for recruitment on hemi-methylated DNA (Avvakumov et al. 2008). DNMT1 has also been shown to possess *de novo* activities in some tumor forms (Jair et al. 2006).

DNMT3A and DNMT3B, act primarily as *de novo* DNA methyltransferases in the establishment of DNA methylation during embryogenesis (Okano et al. 1999). Interestingly, it has been observed that DNMT3A/3B can corroborate DNMT1 in DNA methylation maintenance (Jones and Liang 2009).

Among the factors whose binding is promoted by the presence of methylated dinucleotides, there is a group of proteins with a repressive function that possess a CpG-binding domain (MDB), including for example MeCP2, able to recruit, as already introduced, a complex which also includes HDAC (Jones et al. 1998). This complex recalls other specific heterochromatin proteins, playing an important role in transcriptional repression (Craig 2005).

Methylation plays different roles in cancer, at different levels (Grønbaek et al. 2007). Methylated cytosine can alter the coding regions of the genes inducing point mutations: these cytosines can undergo spontaneous deamination to thymine, thus becoming an endogenous source of mutations when this substitution is not corrected. The C-T transition is in fact responsible for 44% of the point mutations in colorectal cancer and 17% in breast cancer; these data indicate that the different frequency of mutations induced by methylation varies between tissues, probably due to the different efficiency of the mismatch repair mechanisms responsible for the recognition and repair of the mismatch G: T (Sjöblom et al. 2006).

Moreover, tumor cells are generally characterized by an altered methylation pattern in which we can observe global hypomethylation coupled with hypermethylation of specific promoters, in particular of tumor suppressor genes, resulting in transcriptional silencing (Herman and Baylin 2003). Figure 6 effectively represents this apparent paradox (Esteller 2007).

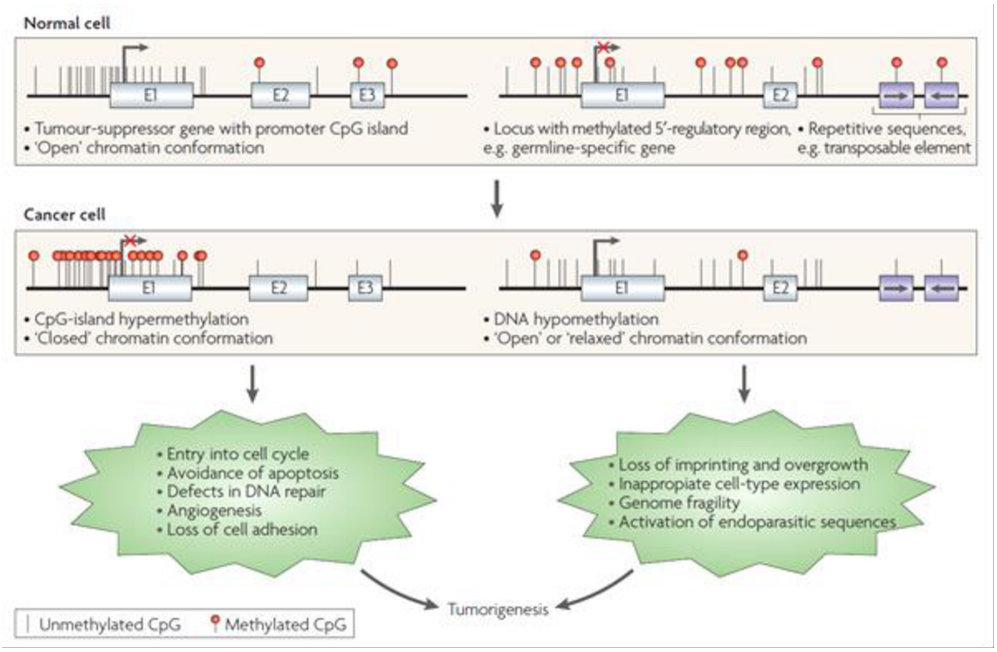


Figure 6 – Differential methylation pattern in normal and cancer cells (Esteller 2005).

In cancer, hypermethylation of specific gene promoters involves a transcriptional repression signal that is also reinforced by other chromatin components such as histones modifications, promoting the formation of a compact and "closed" chromatin structure, inactivating the transcription process. There are numerous examples of genes affected by hypermethylation with consequent transcriptional silencing: MLH1 (involved in colorectal, endometrium and stomach cancer) and BRCA1 (breast and ovarian cancer) coding for factors involved in the shelter of DNA damage, and p15, a cell cycle inhibitor involved in some forms of leukemia (Herman and Baylin 2003, Esteller 2005).

At the same time, in cancer there is a global DNA demethylation: the consequences of these variations are observed at different levels, with re-or hyper-activation of oncogenes, loss of imprinting, inappropriate expression of specific tissue genes and activation of transposable elements (LINE- 1 and Alu) and endoparassitic sequences (e.g. HPV genome in cervical cancer) resulting in genomic instability. Transposon hypomethylation (including LINE-1) promotes an aberrant transcription of flanking genes, especially in the case of sequences inserted in intronic regions where the reactivation of the antisense promoter of LINE-1 determines the expression of chimeric transcripts potentially involved in the tumor development (the oncogene c-MET is the best known and most studied example) (Cruickshanks and

Tufarelli 2009, Weber et al. 2010). Hypomethylation of transposable elements and repeated regions (e.g. microsatellites) also promotes tumor initiation and progression, favoring chromosomal rearrangements and unbalanced translocations (Ehrlich 2009).

Mapping studies have shown a strong correlation between DNA methylation and histone modifications indicating a cross-talk between DNMTs and histone modifications (Hodges et al. 2009). In particular, it seems that the PWWP domain of DNMT3A interacts with a specific residue of the histone H3, lysine 36 precisely, when this is trimethylated (H3K36me3), thus increasing its methyl transferase activity (Dhayalan et al. 2010).

Among the histone modifications of a repressive nature we find also the trimethylation of lysine 9 of histone H3 (H3K9me3) and trimethylation of lysine 27 of the same H3 (H3K27me3), characteristics of heterochromatin. In some types of cancer, H3K27me3 is associated with an increase in DNA methylation, and EZH2 (the enzyme responsible for methylation in K27) has been proposed as one of the possible cross-talk mediators between histone changes and DNMTs (Schlesinger et al. 2007, Viré et al. 2006).

Supporting the interaction between DNA methylation and histone modifications there is also evidence that *de novo* methylation does not occur in regions where bi-or trimethylation of the histone 3 lysine 4 (H3K4me2 or H3K4me3) is observed, modifications that are always associated with transcriptionally active genes (Ooi et al. 2007). Moreover, the nucleosomes flanking the starting site of a gene, besides containing often H3K4me3, show the presence of the histone variant H2A.Z, which excludes the methylation of cytosines in that region (Zilberman et al. 2008).

6.2. HISTONE MODIFICATIONS

As mentioned, the histones H2A, H2B, H3 and H4 are assembled in a double copy to form an octamer around which a 146-bp DNA is wound, thus forming the nucleosome (Quina et al. 2006). Each histone of the core has an N-terminal extension called "tail" that lacks a defined three-dimensional structure and

protrudes outside the nucleosome; this tail structure is important because the residues that compose it are sites of substantial modifications able to change the function of the single nucleosome (Figure 7).

These histone post transcriptional modifications (or hPTMs) are regulated by enzymes that mediate acetylation (Sterner and Berger 2000), methylation (Zhang and Reinberg 2001), phosphorylation (Nowak and Corces 2004), ubiquitination (Shilatifard 2006), SUMOylation (Nathan, Sterner and Berger 2003), ADP-ribosylation (Martinez-Zamudio and Ha 2012), deamination (Cuthbert et al. 2004), proline isomerization (Nelson, Santos-Rosa and Kouzarides 2006) and crotonylation (Tan et al. 2011).

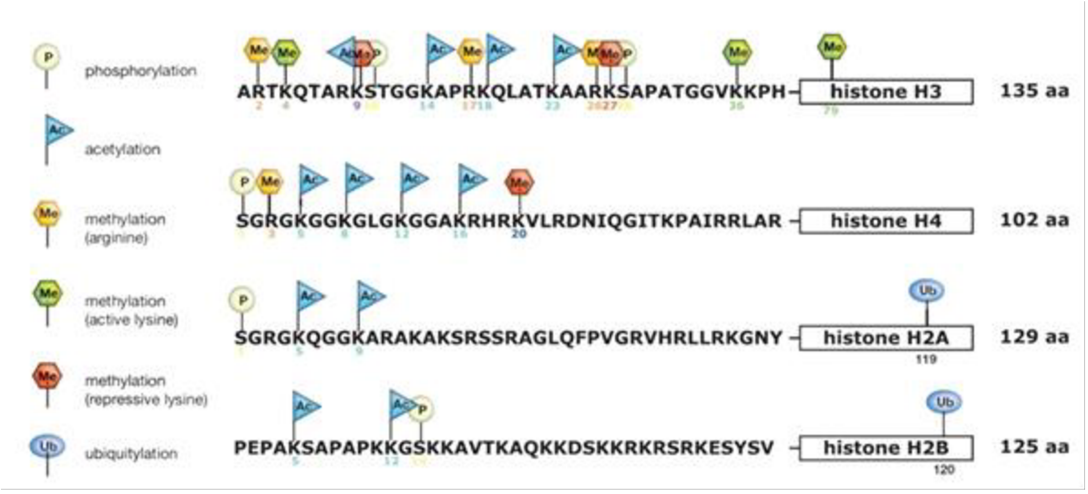


Figure 7 – Representation of some of the possible histone modifications at H3, H4, H2A e H2B (Grønbaek et al. 2007).

Heterochromatin and euchromatin domains are defined by specific pattern of histone modifications. In particular, acetylation of histone 3 and histone 4 (H3 and H4) or di- or trimethylation (me2 or me3) of H3K4 are commonly related to euchromatic regions, while H3K9 and H3K27 methylation are often associated to constitutive heterochromatin and facultative heterochromatin respectively (Li et al. 2007).

The information conveyed by epigenetic modifications plays a critical role in the regulation of all DNA-based processes (such as transcription, DNA repair and replication), promoting or blocking the binding of specific factors involved in these processes. Consequently, abnormal expression patterns or genomic alterations in chromatin regulators can have profound results and can lead to the induction and maintenance of various cancers (Dawson and Kouzarides 2012).

The acetylation of lysine residues has the highest potential to de-condense chromatin since it neutralizes the basic charge of histones and the phosphorylation of serine 10 on H3 is crucial for chromosome condensation and cell-cycle progression during mitosis and meiosis (Eberlin et al. 2008, Fischle et al. 2005, Krishnamoorthy et al. 2006).

In higher-order organisms, the methylation of lysine 9 on the H3 tail is associated with transcriptional repression and is therefore considered a molecular marker of heterochromatin (Nakayama et al. 2001). Also the trimethylation of histone H3 on lysine 27 (H3K27me3) is associate with transcriptional repression (Yamasaki-Ishizaki et al. 2007). Other methylation sites, such as lysine 4 on H3, are instead associated with the increase of transcription (Eissenberg and Shilatifard 2010).

The presence of H3K27me3 coupled with H3K4me3 (two histone marks associated with opposite transcriptional status and actually widely recognized as a bivalent mark) was firstly described in embryonic cells and during development (Iwagawa and Watanabe 2018) but appears to play a role also in poisoning non-active genes for aberrant transcription in cancer (Bernstein et al. 2006, Voigt, Tee and Reinberg 2013). Several studies actually suggest that loss of this bivalent chromatin mark at promoters is accompanied by gene activation (when H3K27me3 is lost) or gene repression (when H3K4me3 is lost) during development, and this event is likely to be a critical step also in cancer pathogenesis, responsible for the activation of crucial genes leading to progression and invasiveness, as demonstrated in colon and ovarian cancer (Chapman-Rothe et al. 2013, Hahn et al. 2014).

The different Histone Methyl Transferases (HMTs) can act on different lysine residues and could transfer up to three methyl groups. The methylated residues are recognized by proteins containing a chromodomain, such as HP1 (heterochromatin protein 1), that bind specifically to H3K9me3 (Lachner et al. 2001, Jacobs and Khorasanizadeh 2002) and is released upon phosphorylation of the adjacent serine 10 during M phase of the cell cycle (Fischle et al. 2005).

SUV39H1 (Suppressor of Variegation 3-9 Homolog 1) is a HMT protein having a chromosomal domain necessary for binding to histone methylation modifications and a SET C-terminal domain responsible for methyltransferase activity (and therefore necessary for modulation of the activity of epigenetic regulation of the protein) (Firestein et al. 2000). Its main function is to tri-methylate lysine 9 of histone H3 (H3K9me3)

at the pericentromeric heterochromatin level and it plays a fundamental role in the organization of chromatin, in the segregation of chromosomes, in mitotic progression (Peters et al. 2001), in the maintenance of genomic stability and in the regulation of mammalian development.

SUV39H1 is also able to interact with DNMT3B (Lehnertz et al. 2003), DNMT1 (Fuks et al. 2003), UHRF1 (Karagianni et al. 2008) and HP1 (Lachner et al. 2001, Fuks et al. 2003), indicating the close correlation between histone modifications and DNA methylation in the maintenance of the heterochromatic state.

The EZH2 gene (Enhancer of Zeste Homolog 2) encodes the catalytic subunit of the Polycomb-group family (PcG). Like SUV39H1, EZH2 also has a SET domain responsible for the methyltransferase activity.

Members of this family form multimeric protein complexes involved in maintaining transcriptionally repressed chromatin status by trimethylation of histone H3 on lysine 27 (H3K27me3) (Cao et al. 2002): this methylation is required in the initial phase inactivation of the X chromosome in embryonic cells (Plath et al. 2003) and is involved in the regulation of processes such as stem cell differentiation and cell proliferation.

An overactivation of EZH2, following overexpression or mutations, has been found in numerous malignant neoplasms including prostate, breast, uterus, stomach, non-small cell lung carcinoma and lymphoma. Its expression is related to aggression, metastasis and poor prognosis (Tan et al. 2014).

Several studies have revealed that EZH2, together with the PcG complex, can directly recruit DNMTs on some specific target genes to repress their transcription, thus suggesting a cross-talk between the two distinct epigenetic silencing mechanisms. In particular, it seems that EZH2 is able to interact with DNMT1 (Viré et al. 2006).

G9a plays its dominant role of transcription suppressor in euchromatin mono- and di-methylating lysine 9 on histone H3 (H3K9me2) and is essential in embryogenic development (Tachibana et al. 2002), in the control of transcription of development genes and in the progression of meiotic prophase in germ cells (Tachibana et al. 2007).

The transcriptional inactivation condition of some genes in euchromatin is also maintained thanks to UHRF1 which, as in heterochromatin, interacts with HMTs, and in particular with G9a. G9a is

overexpressed in many tumors, often in conjunction with UHRF1, where it mediates gene silencing of specific tumor suppressor genes, thus contributing to tumor growth.

Some studies show that, in tumor cells, the double knock-down of SUV39H1 and G9a significantly inhibits cell growth and rapidly leads to a morphological senescence of cells accompanied by telomere abnormalities (Kondo et al. 2008).

7. UHRF1

UHRF1 (Ubiquitin-like protein containing PHD and RING finger domains 1) is a modular multi-domain protein able to mediate interactions between DNA methylation and histone modifications, by interacting directly with DNA and histones (Hashimoto et al. 2009).

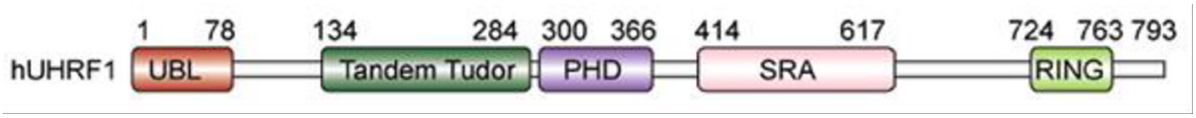


Figure 8 – Structure and domains of UHRF1 (Cheng et al. 2013).

As shown in Figure 8, UHRF1 is characterized by five functional domains fundamental for its interaction with various factors involved in the maintenance of the heterochromatic structure and for the regulation of gene expression (Cheng et al. 2013).

Its ability to bind DNMT1 (Bostick et al. 2007), HMTs and HDACs (Unoki, Nishidate and Nakamura 2004) with great affinity, and to interact with methylated DNA (Avvakumov et al. 2008) and histone modifications (Arita et al. 2012, Rothbart et al. 2013, Rottach et al. 2010, Xie, Jakoncic and Qian 2012), make UHRF1 an essential fulcrum in transcriptional regulation and maintenance of chromatin. The functional ablation of UHRF1 in ESCs (embrionic stem cells) leads to hypomethylation of the genome similar to that induced in ESC cells DNMT1 - / - (Bostick et al. 2007).

The protein is expressed in the late phase G1 and its expression continues then, in a reduced expression, in the phase G2 and M of the cell cycle. Therefore, it plays its major role in cell cycle progression; in particular it is essential in the transition from phase G1 to phase S (Bonapace et al. 2002), as well as playing a fundamental role in DNA damage repair processes in p53-dependent checkpoints (Arima et al. 2004).

In many cancers, such as lung, breast and prostate cancers, UHRF1 is overexpressed (Jenkins et al. 2005) and mediates the transcriptional inactivation of tumor suppressor genes, such as CDH1 (Babbio et al. 2012) and RB1, and at the same time transcriptional activation of oncogenes, such as VEGF (Achour et al. 2008), thus contributing to tumor growth and survival: for these reasons it is a good diagnostic molecular marker (Unoki et al. 2010).

8. TRANSPOSABLE ELEMENTS & LINE-1

Transposable elements (TEs or "jumping genes") are segments of DNA able to move and insert themselves in different positions of the genome (with the possibility of inducing various effects at the gene level), through a mechanism called transposition.

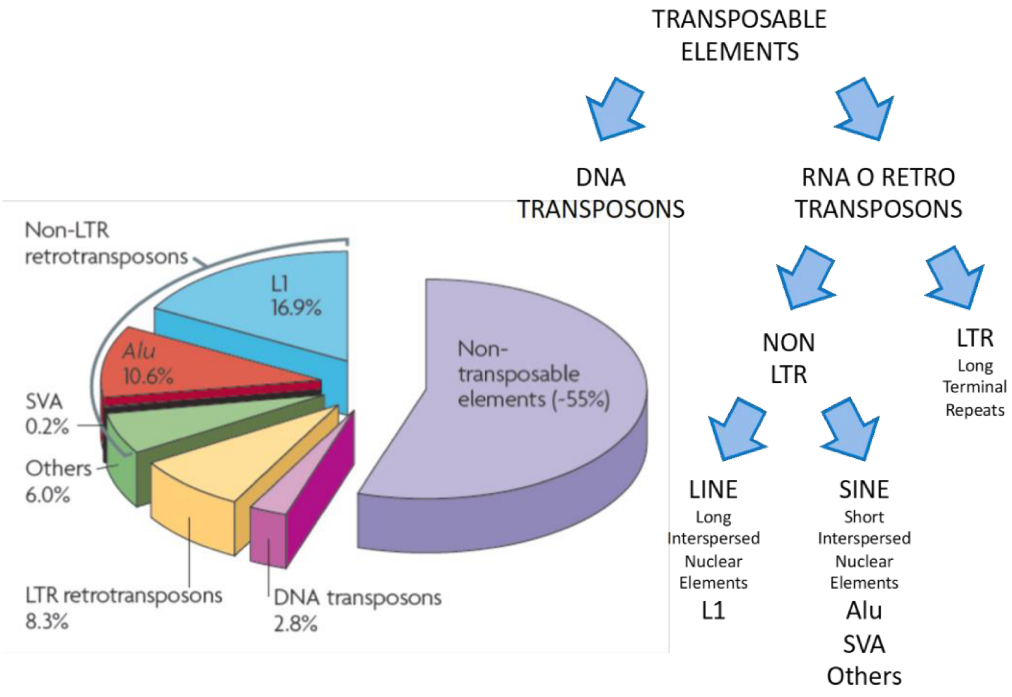


Figure 9 – Human genome compositions and classifications of transposable elements (image modified from (Lander et al. 2001)).

There are several types of transposable elements, classified according to their structure and the mechanisms used for transposition. Among them we could find transposons, retrotransposons, plasmids and virus and retrovirus genomes. As illustrated in Figure 9, about 45% of the human genome is composed of TEs (Smit 1999). Most probably, however, this data is underestimated by the fact that the sequence of many ancient TEs may be very different from the original sequence affecting the identification (Lander et al. 2001).

TEs can be divided into two classes: DNA transposons, able to excuse themselves and reinsert themselves in another position in the genome, and retrotransposons that need an intermediate of RNA to be able to transpose. Moreover, the retrotransposons can in turn be divided into two groups based on the presence or absence of the LTR (Long Terminal Repeats) regions. Among the non-LTR elements we distinguish the LINE-1 (Long Interspersed Nuclear Elements) or L1 and the SINE (Short Interspersed Nuclear Elements) like SVA and Alu.

While the LINE-1 are autonomous thanks to the presence in their sequence of regions coding for an endonuclease and a retro-transcriptase (ORFs), the Alu and the SVAs do not code for proteins and for their retrotransposition activity require the presence of a functional LINE -1 (Kaer and Speek 2013).

The transposition of these elements may have different repercussions on the structure and function of the genome. Among the many ways in which it can induce local genomic instability we find insertional mutagenesis (both LINE-1 and Alu/SVA) and the creation of double strand breaks in DNA (LINE-1) (Schulz 2006). The TEs can also generate genomic rearrangements such as insertion-mediated deletions, ectopic recombination and transduction of flanking sequences; they can also induce gene inactivation through the retrotransposition of gene sequences, creation of new coding sequences, exonization (the use of TEs as exonic variants in the alternative splicing mechanism) and gene duplication. At the level of gene expression, TEs can act on the modulation of gene expression by promoting an alternative splicing; they can then interfere with the elongation process, provide an alternative promoter or an early termination signal (Cordaux and Batzer 2009).

The epigenetic regulation of TEs activity through DNA methylation represents an important defense mechanism of the cell: since they are frequent even in proximity of the genes, the formation of TEs-mediated heterochromatin can involve the flanking genes inducing their transcriptional repression.

The LINE-1 (Long Interspersed Nuclear Elements) or L1 have a length of about 6 kb in the full-length form and are transcribed by the RNA polymerase II. Despite the presence of more than 500,000 copies in the human genome, most of the LINE-1 are inactive due to point mutations, rearrangements or truncations; only about 80-100 copies turn out to be complete and active (Hancks and Kazazian 2012).

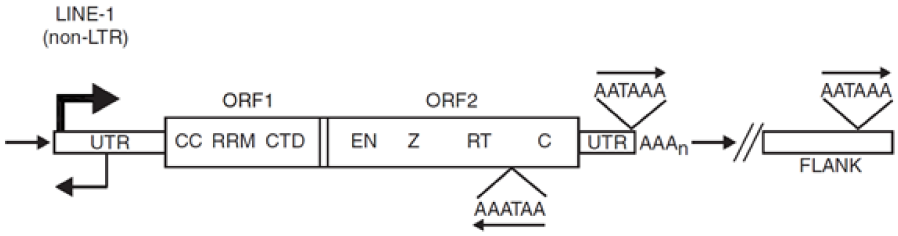


Figure 10 – Structure of a complete LINE-1 (Hancks and Kazazian 2012).

As shown in Figure 10, in the 5 'UTR region of the LINE-1 a strong sense promoter (large black arrow) and an antisense promoter (indicated by the small arrow) are contained. The LINE-1 contain two open-reading non-overlapping frames, ORF1 and ORF2, codifying for two different proteins involved in the processes of retrotransposition.

Thanks to the wide diffusion of LINE-1 in the genome and the fact that the transcriptional repression of these sequences is mainly given by the methylation of the 5'UTR region, they are widely used in the methylation study as a surrogate of the entire human genome.

In normal conditions, the expression of LINE-1 is strictly confined to germ cells, while in somatic cells it is very low or totally absent, with the exceptions of the vascular endothelium cells of male gonads, lymphoblastoids and some neuronal cells, especially at the hippocampus level (Kaer and Speek 2013).

Both the sense and the antisense transcription induced by an altered methylation pattern of the 5'UTR region of the LINE-1 play a fundamental role in the pathogenesis and progression in more advanced phases of different types of tumors, including Chronic Myeloid Leukemia.

As mentioned before, the 5 'UTR region of the LINE-1 contains both a sense promoter (SP), which promotes transcription in the LINE-1 direction, and an antisense promoter (ASP) which instead promotes transcription in the opposite direction. Numerous studies have shown that, when LINE-1 appears to be inserted inverted within genes, usually in intronic sequences, a demethylation of ASP may provide an alternative start site for transcription in a large number of genes such as c-MET (tyrosin kinase receptor activated by HGF), generating a fusion or chimeric transcript that contributes to the transformation, progression and initiation of numerous tumors (Weber et al. 2010).

The retrotransposition of LINE-1 induced by hypomethylation may also inactivate oncosuppressor genes such as APC in colon cancer (Miki et al. 1992) and activation of the LINE-1 promoter inserted upstream of oncogenes may lead to their activation as in the case of c-MYC in breast cancer (Morse et al. 1988).

DNA methylation, however, do not play an exclusive role in suppressing TEs transcriptions: the observed uncoupling between *Alu*/L1/HERV transcriptional activation and DNA hypomethylation confirm the recent finding that DNA methylation is not the only epigenetic mechanisms of suppressing SINE transcription (Varshney et al. 2015). Transposable elements (TEs) reactivation and retrotransposition events, in fact, could be due to loss of DNA methylation, but also to histone modifications (and thus decondensation of the chromatin structure) in response to exposure to ubiquitous environmental stressors (carcinogens or suspected carcinogens) (Miousse et al. 2015). The activity of LINE-1 (and more generally of the TEs) indeed, can be influenced by external stimuli including ionizing radiation, heavy metals, environmental pollutants and demethylating agents (Belancio, Deininger and Roy-Engel 2009).

AIM

This PhD project arose from the hypothesis that the epigenetic compartment, in particular histone post-transcriptional modifications, could be involved in the early response to sub-cytotoxic concentrations of hydroquinone, and that these events could be linked to its carcinogenic activity. Thus, we focused prevalently on possible chromatin alterations, like histone and DNA methylation, and explored the repercussion on the expression of specific genes.

In fact, most of the studies conducted so far have been centred on DNA methylation, whereas only a few investigations have analysed the effects of environmental chemicals on histone modifications (Baccarelli and Bollati 2009, Philbrook and Winn 2015). Nevertheless, the actual mechanism behind the influence of these aberrations on tumorigenesis remains unclear.

Indeed, aberrant patterns of DNA methylation have been extensively observed in several tumour types, including AML and prostate cancer cells (Lübbert et al. 1992, Bollati et al. 2007, Hamilton 2011, Liu et al. 2011, Pistore et al. 2017, Hoffmann and Schulz 2005); however, of high interest for our hypothesis are recent epidemiological evidences showing, on healthy subjects, an alteration in the pattern of methylation between subjects differently exposed to benzene (gasoline station attendant vs office workers), with a significant reduction in the first group in the methylation of repeated elements LINE-1 and Alu, used as a surrogate for the entire genome (Fustinoni et al. 2012). In fact, while several evidences show that a single exposure to high concentrations of hydroquinone (above 30 μM) could be sufficient for the cytotoxic effect (Terasaka et al. 2005a, Terasaka et al. 2005b), the carcinogenesis process due to HQ seems to be induced by continuous exposure to low doses, through a mechanism that is still widely obscure (Lagorio et al. 2013, Snyder and Hedli 1996).

Giving these findings, the purpose of our study was to reproduce *in vitro* exposure conditions as close as possible (relatively to our system) to the ones found in the peripheral blood of people chronically exposed to airborne benzene. Our goal consisted in exploring the cellular response during a long-term treatment, to evaluate if HQ might be able to alter the epigenetic signature, thereby describing a poorly explored step in the mechanism of toxicity associated with benzene exposure.

We started from what are considered low doses of hydroquinone *in vitro* (below 15 μM) and we proceeded lowering the concentrations considering the work from Kerzic et al. (Kerzic et al. 2010). We

chose 1 µM, corresponding to 110 ng/mL, a concentration within the range of total HQ (20-120 ng/mL, corresponding to 2–16 ng/mL of free HQ) found in the blood of workers exposed to airborne benzene levels ranging from 1 mg/m3 (around 0,3 ppm) to 80mg/m3 (around 25 ppm) (Kerzic et al. 2010), and reiterated the exposure daily for 4 weeks.

This type of exposure was performed on two cellular models:

- ✓ a stabilized cell line of human acute promyelocytic leukemia (hAPML), HL60, as a model of hematopoietic cells; this cell line is MPO-positive and therefore able to metabolize HQ to BQ. We focused firstly on searching for sub-cytotoxic concentrations and evaluating different types of treatments, in the effort to move towards a long-term treatment scheme that could somehow recall exposures that can be experienced in working environments, as previously described.
- ✓ a long-term treatment on commercial available human umbilical cord mesenchymal stem cells, hUCMSC, as a model normal stem cells, to verify if a long-term exposition to low doses of HQ could be able to alter the epigenetic signature in staminal cells distinct from the hematopoietic compartment. Since the aim of the present study was the analysis of the effects of sub-cytoxic concentrations of HQ, when moving to mesenchymal stem cells we had to further reconsider the chosen concentration, lowering it to 0,1 µM.

MATERIALS & METHODS

CELL CULTURES

The MPO-positive AML cell line, HL60, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and routinely cultured in suspension at 37 °C and 5% CO2 in RPMI medium supplemented with 10% foetal bovine serum (Euroclone, MI, IT) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

The Human Umbilical Cord-Derived Mesenchymal Stem Cells (ATCC, Rockville, MD, USA), hUCMSC, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and routinely cultured in suspension at 37 °C and 5% CO2 in Mesenchymal Stem Cell Basal Medium (ATCC, Rockville, MD, USA) supplemented with Mesenchymal Stem Cell Growth Kit - Low Serum (ATCC, Rockville, MD, USA).

Hydroquinone (HQ, ≥99%) was purchased from Sigma-Aldrich, stocked at RT and dissolved in complete medium immediately before each treatment.

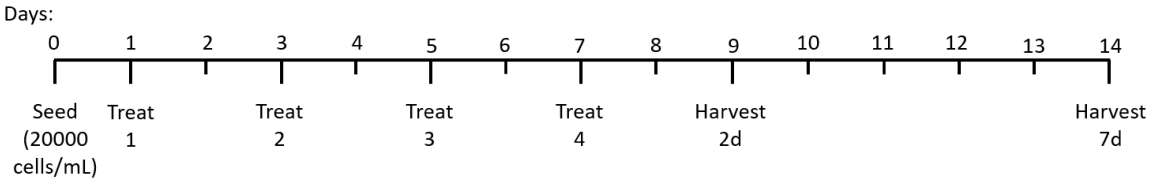
Decitabine (DAC, Sigma-Aldrich) served as a positive control for global DNA methylation: stock solutions (50 mM) were prepared in DMSO and stored at −20 °C. Working dilutions were prepared before use.

TREATMENT SETTINGS – HL60

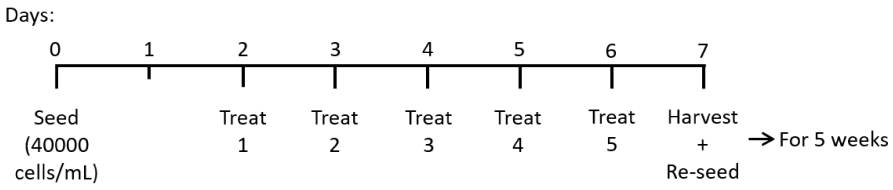
To analyze cell growth, viability and the epigenetic effects cells were seeded at low density and treated 24 h later following the different settings:

Single treatment: cells were treated once with different concentrations of HQ (1, 5, 15 and 25 µM) and harvested 1, 2, 3, 5 and 7 days later; detailed analysis was performed only after treatments with HQ 5 and 15 µM.

Repeated treatment: cells were treated four times every 48 h with different concentrations of HQ (1, 5 and 15 µM) and harvested 2 and 7 days after the fourth treatment; detailed analysis was performed only after treatments with HQ 1 and 5 µM.



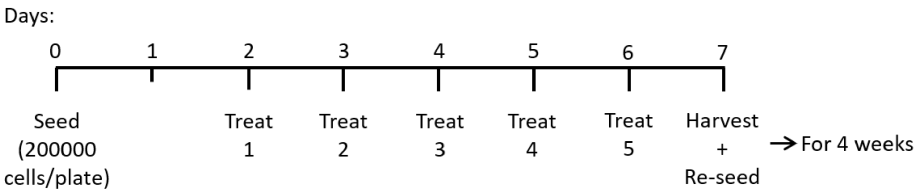
Long-term treatment: cells were treated five times a week for five weeks with a single concentration of HQ (1 µM). Every seven days cells were counted, reseeded at low density and harvested. The dose of 1 µM of HQ used in our long-term exposure corresponds to 110 ng/mL HQ, a concentration within the range of total HQ found in the blood of workers exposed to airborne benzene (Kerzic et al., 2010); that was between 20 and 120 ng/mL, which correspond to free HQ between 2 and 16 ng/mL, for airborne benzene exposures ranging from 1 mg/m3 (around 0.3 ppm) up to a maximum of 80 mg/m3 (around 25 ppm).



TREATMENT SETTINGS – hUCMSC

To analyse cell growth, viability and the epigenetic effects cells were seeded only to perform the long-term treatment:

Long-term treatment: cells were treated five times a week for four weeks with a single concentration of HQ (0,1 µM). Every seven days cells were counted, reseeded at low density and harvested. Compared to HL60 long-term treatment, we decided to reduce the dose of HQ to 0,1 µM (that corresponds to one tenth of 110 ng/mL HQ) because after a single treatment the concentration of 1 µM seemed to have a slight effect on cell growth.



FLOW CYTOMETRY

Cell were harvested at the different end points, rinsed with PBS and fixed in 70% EtOH at −20 °C for at least 20 min. After a further rinse in PBS, DNA was stained with Propidium Iodide in PBS (PI, Sigma-Aldrich, final concentration 50 µg/mL) in the presence of RNAse (Sigma-Aldrich, 30 U/mL).

Samples were analyzed in collaboration with dr. Marzia Bruna Gariboldi (Biotechnology and Life Science Department of Insubria University) at the FACScan flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA) equipped with a 15 mW, 488 nm and air-cooled argon ion laser.

At least 10.000 events were analyzed for each sample and all data were processed using CellQuest software (Beckton Dickinson). Fluorescent emission of PI was collected through a 575 nm band-pass filter and acquired in log mode: the percentage of apoptotic cells was determined based on sub-G1 peaks detected in monoparametric histograms.

SDS PAGE AND WESTERN BLOT

Cell lysates were prepared in RIPA buffer (Sigma-Aldrich) containing protease inhibitor cocktail (Roche, Basel, CH). Protein concentration was determined by Qubit method (quant-iT Protein Assay kit, Invitrogen,

Life Technologies, Carlsbad, CA, USA) and equivalent amounts of total cell lysate (30 µg) were added with sample buffer 2× (Laemmli, Sigma-Aldrich). The samples were sonicated and boiled 5 minutes at 95°C, separated by 10% acrylamide SDS-page under denaturing conditions and transferred onto nitrocellulose membrane (Hybond-ECL, GE Healthcare, Fairfield, CT, USA). Membranes were incubated overnight with primary antibodies.

After incubation with secondary anti-rabbit/mouse antibody conjugated to horseradish peroxidase (Sigma Aldrich), chemiluminescence on membranes was detected by ECL reagents (ECL detection reagent, GE Healthcare) and acquired on films (Hyperfilm-ECL, GE Healthcare).

RNA EXTRACTION AND RETROTRANSCRIPTION

Cell were harvested at the different end points and washed by using 1X PBS (Sigma Aldrich).

RNA was extracted using Trizol reagent (Life Technologies) following the manufacturer's instructions. RNA was quantified by spectrophotometer or Q-bit (Life Technologies).

0,5 to 5 µg of RNA was treated with 4U of TURBO DNAseI (Ambion) for 30 minutes at 37°C and then RNA was extracted by standard phenol:chloroform procedure.

Extracted RNA was used either for RNA-seq analysis or retrotranscribed for PCR experiments.

0,5 to 1 µg of RNA was retrotranscribed using SSIII Superscript (Life technologies) with oligodT. cDNA was diluted at 5ng/µl and used for the PCR analysis.

DNA EXTRACTION

DNA from cells was extracted using SNET buffer protocol. DNA was quantified by Qbit (Life technologies) and prepared for pyrosequencing analysis.

DNA BISULFITE CONVERSION AND PYROSEQUENCING ANALYSIS

Bisulfite modification of genomic DNA (300 ng) was performed with an EpiTect Bisulfite Kit (Qiagen, Hilden, DE) according to the manufacturer's protocol. PCR products were analyzed by pyrosequencing using PyroGold reagents on a PyroMark Q96 ID system (Qiagen) in collaboration with dr. Daniela Furlan at the Ospedale di Circolo di Varese.

Pyrogram outputs were analyzed by the Pyromark ID 1.0 software (Qiagen) to determine the percentage of methylation of each of the four CpG sites analyzed within the 5'UTR of LINE-1 elements (GenBank ID M80343.1, primers in Supplementary Table 1).

POLYMERASE CHAIN REACTION (PCR)

Primers were designed by using the open-access tool Primer 3 software (http://fordo.wi.mit.edu/website) choosing amplicons of approximately 75-135bp (see primers table). The selected sequences were validated in silico by using primerBLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to confirm the specificity of the target.

SEMI-QUANTITATIVE PCR

The semi-quantitative PCR was performed using the HotStart GoTaq polymerase (Promega Inc.) following the manufacturer's instructions. The amplicons were then separated on 1,5% agarose gel and visualized by ethidium bromide staining (Sigma Aldrich) at luminometer.

QUANTITATIVE REAL-TIME PCR

Quantitative real-time PCR was performed in iCycler iQ (BioRad) by using 2X iQ™SYBR Green Supermix (BioRad). Data were analyzed by averaging triplicates Ct. Levels of RNA expression were determined by Gene Expression Analysis for iCycler iQ Real-Time PCR Detection System v1.10 (Bio-Rad) according to the 2-ΔΔCq method. Levels of RNA expression of selected genes were normalized to the internal control reference gene (GAPDH).

Post-PCR melting curves were used to assess the quality of primer pairs.

CHROMATIN IMMUNOPRECIPITATION (CHIP)

Chromatin immunoprecipitation (ChIP) assays were performed based on a modification of previously published methods from our laboratory (Babbio et al., 2012). Briefly, cells were cross-linked by adding 1% formaldehyde to the culture medium: the cross-linking reaction was quenched by adding 0,125 M glycine and collected in PBS 1X. Cell pellets were re-suspended in lysis buffer (5mM PIPES pH8, 85mMKCl, 0,5% NP40, 1X Proteinase inhibitors) and sonicated for sonicated 14 times for 10 s on ice (BRANSON S250 digital sonicator, Branson, Danbury, CT, USA).

Sonicated chromatin was pre-cleared for 1 hour at 4°C using 60µl of pre-washed sepharose beads (KPL). Pre-cleared chromatin was quantified using Qubit® dsDNA BR Assay Kit (Life Tecnologies). 10µg of chromatin was incubated overnight at 4°C in dilution buffer (1% SDS, 10mM EDTA pH8, 50mM Tris-HCl pH8, 1X proteinase inhibitors) with 10µg of specific antibodies.

Twenty percent of the total lysate was used for input control.

Antibody coupled-chromatin was incubated using 60µl pf pre-washed protein G sepharose beads (KPL) for 2 hours at 4°C. The beads were extensively washed and the DNA was extracted by the phenol:chloroform:isoamyl alcohol, ethanol precipitated and resuspended in water.

DNA was extracted using phenol/chloroform/isoamyl alcohol, precipitated in ethanol and resuspended in H2O. Chromatin immunoprecipitation products were amplified using GoTaq Hot-Start Polymerase (Promega, Madison, Wi, USA) and specific primers following the manufacturer's protocol.

To better compare the ChIP results, densitometric analysis was performed using ImageJ program and normalized on input signals as follow: IP) / Densitometric value (INPUT)) X 20 (% of the INPUT)

The resulting value represents the % of the recovery of the antibody. This value indicates the amount of the specific DNA sequence recovered by a specific antibody.

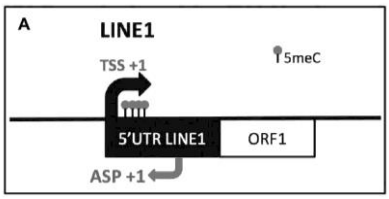
RNA SEQUENCING AND DATA ANALYSIS

Indexed libraries were prepared from 1 µg/ea purified RNA by using TruSeq Stranded Total RNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. Libraries were sequenced (paired-end, 2x100 cycles) at a concentration of 8pmol/L per lane on HiSeq2500 platform (Illumina) with >70 million sequence reads/sample. The raw sequence files generated (.fastq files) underwent quality control analysis using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the quality checked reads were then aligned to the human genome (hg19 assembly) using TopHat2 (version 2.0.13), with standard parameters. A given mRNA was considered expressed when detected by ≥10 reads. Differentially expressed mRNAs were identified using DESeq (version 1.6.3). Firstly, gene annotation was obtained for all known genes in the human genome, as provided by Ensemble (GRCh37) (https://support.illumina.com/sequencing/sequencing_software/igenome.ilmn). Using the reads mapped to the genome, we calculated the number of reads mapping to each transcript with HTSeq-count (version 0.6.0). These raw read counts were then used as input to DESeq for calculation of normalized signal for each transcript in the samples, and differential expression was reported as Fold Change along with associated adjusted p-values (computed according to Benjamini-Hochberg).

PRIMERS AND OLIGONUCLEOTIDES

GENE NAME	FOR	REV	SEQUENCING	METHODS
UHRF1	CCTCACCAAGGAAGCTGTGT	GGAGAAAAATCGGAGTCGTG		PCR
G9a	TGCGTGCTGTTATTCCTGTC	TGATCTTCTCTGTGCGGATG		PCR
EZH2	TTGTTGGCGGAAGCGTGTAAAATC	TCCCTAGTCCCGCGCAATGAGC		PCR
SUV39H1	GCTATGACTGCCCAAATCGT	ACACGTCCTCCACGTAGTCC		PCR
DNMT1	GAGCTACCACGCAGACATC	CGAGGAAGTAGAAGCGGTTG		PCR
DNMT3a	CTGGAGTCCAACCCTGTGAT	CTTTGCCCTGCTTTATGGAG		PCR
DNMT3b	AAGAGTTGGGCATAAAGGTAGG	GCTGGATTCACATTTGAGAGAT		PCR
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG		PCR
LINE-1	GGCCAGTGTGTGTGCGCACCG	CCAGGTGTGGGATATAGTCTCGTGG		ChIP
GAPDH	TACTAGCGGTTTTACGGGCG	TCGAACAGGAGGAGCAGAGAGCGA		ChIP
LINE-1	GAGTTAGGTGTGGGATATAGT	CAAAAAATCAAAAAATTCCCTTTCC	GGTGTGGGATATAGTT	Pyro

LINE-1 CYTOSINES POSITION (PYROSEQUENCING)



ANTIBODIES

PROTEIN NAME	SUPPLIER	DILUTION/QUANTITY	APPLICATION
UHRF1	IGBMC, monoclonal	1:4000	WB, ChIP
G9a	Cell Signaling, polyclonal	1:1000	WB
EZH2	Cell Signaling, monoclonal	1:1000	WB
SUV39H1	GeneTex, polyclonal	1:1000	WB
DNMT1	Abcam, monoclonal	1:500	WB
DNMT3a	Cell Signaling, monoclonal	1:1000	WB
DNMT3b	Active Motif, monoclonal	1:1000	WB
GAPDH	Millipore, monoclonal	1:1000	WB
Anti Rabbit	Pierce	1:5000	WB
Anti Mouse	Pierce	1:5000	WB
IgG	Santa Cruz	10µg	ChIP
H3K4me3	Active Motif, polyclonal	10µg	ChIP
H3K27me3	Active Motif, polyclonal	10µg	ChIP

RESULTS

MODEL OF HEMATOPOIETIC CELLS – HL-60 CELLS

Since the aim of this study was to investigate the processes that takes place in bone marrow following benzene exposure, both benzoquinone (BQ) and hydroquinone (HQ) were good candidates.

HL-60 is a cell line derived from a single patient with acute promyelocytic leukemia and it is widely used as *in vitro* model system for studying cellular and molecular events that are involved in proliferation and differentiation of normal and leukemic cells (Collins 1987). For this reason we chose this cell line also because HL-60 cell line is MPO positive and can thus potentially metabolize HQ to BQ allowing us to analyse the combined effects of both benzoquinone and hydroquinone.

In the first part of the study, in order to set up a long-term treatment with low-doses of hydroquinone within the levels of total HQ found in peripheral blood of benzene-exposed workers, we started with a single and a repetitive exposure using different concentrations of HQ to evaluate possible effects on the epigenetic signature.

As previously described in the introduction, the toxicity of this compound is defined by the concentrations of HQ used in the various types of treatment and by the number of exposures: for short-acute treatments it is possible to use higher concentrations, while for long-term treatments it is necessary to reduce the doses in order to avoid final cytotoxic effects. Exploring *in vitro* the differences between short and long-term exposure to HQ, in the effort to investigate if low-doses might be sufficient to alter the epigenetic signature, we developed three treatment settings (detailed explanation of the settings is reported in material and methods).

Firstly, we evaluated cell viability to choose sub-cytotoxic concentrations in each setting.

Starting from a single exposure, in the survival curve obtained after treatment with different doses, shown in Figure 1, we observed that the concentrations 1 µM and 5 µM have a growth profile similar to the untreated sample: it is interesting to note that low doses (1 and 5 µM) seem to confer a proliferative advantage, while rising to 15 µM a modest cytotoxic effect is observed, that becomes evident 72 hours after the treatment. The 25 µM shows a relevant cytotoxic effect already after 24h. Given these

considerations, we empirically chose the 5 µM and 15 µM concentrations for the single treatment and the 1 µM and 5 µM concentrations for the repeated treatment.

For the chronic treatment, we chose 1 µM concentration corresponding to 110 ng/mL, a level very similar to the highest amount of total HQ (between 20 and 120 ng/mL, corresponding to 2–16 ng/mL of free HQ) found in the blood of workers of a rubber product manufacturing and finishing facility located near Shanghai, China (Kerzic et al. 2010), exposed to airborne benzene levels ranging from 1 mg/m3 (around 0.3 ppm) up to a maximum of 80mg/m3 (around 25 ppm). Interestingly, the highest blood levels of total HQ were observed even at very low levels of air benzene exposure (≤1 ppm), levels that have been shown to cause hematotoxicity (Lan et al. 2004).

Moreover, the work from Kerzic et al. shows that free concentration of HQ in the blood does not vary with exposure to airborne benzene, remaining constant, contrary to what is observed for total and protein-bound HQ, that are directly proportional to exposure.

HQ accumulates in the bone marrow after benzene exposure (Rickert et al. 1979, Greenlee, Sun and Bus 1981).

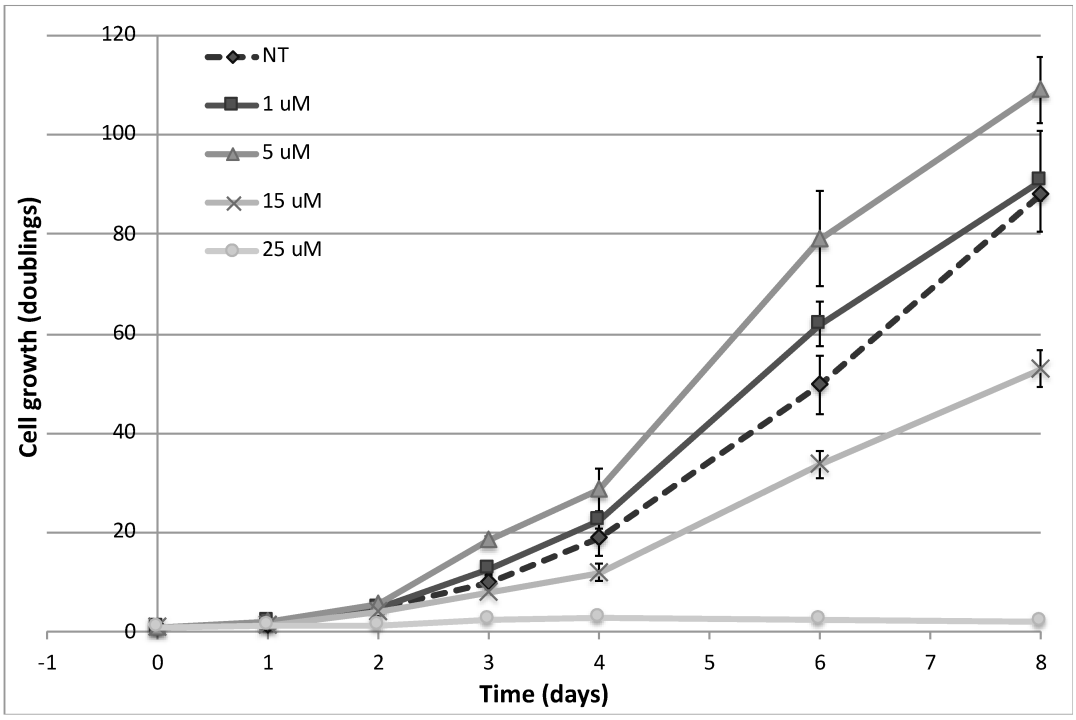


Figure 1. Survival curve of HL60 (cells n°*10^6) obtained after a single treatment with different doses of HQ (average of three independent biological replicates).

The selected doses were used to assess in each treatment the alterations either in histone modifications (hPTMs) on chromatin and in DNA methylation levels to identify possible changes in the epigenetic compartment.

SINGLE TREATMENT

1. SURVIVAL CURVE AND FLOWCYTOMETRY ANALYSIS

Figure 2A shows the growth curve of HL-60 cells treated with a single dose of HQ (5 and 15 µM). Notably, the cells (both control and HQ exposed) are still in active proliferation 120h after the single treatment. Moreover, we can observe that HQ doses used do not alter significantly the cells growth (except for HQ 15 µM at 8 days), confirming what observed during the preliminary study for the choice of HQ doses. To further exclude cytotoxicity of the selected doses of the compound, we also evaluated the effect on cell cycle progression and apoptosis: flow cytometry analysis evidenced that single exposure is not able to alter significantly the percentage of both cycling and apoptotic cells as shown in Figure 2B.

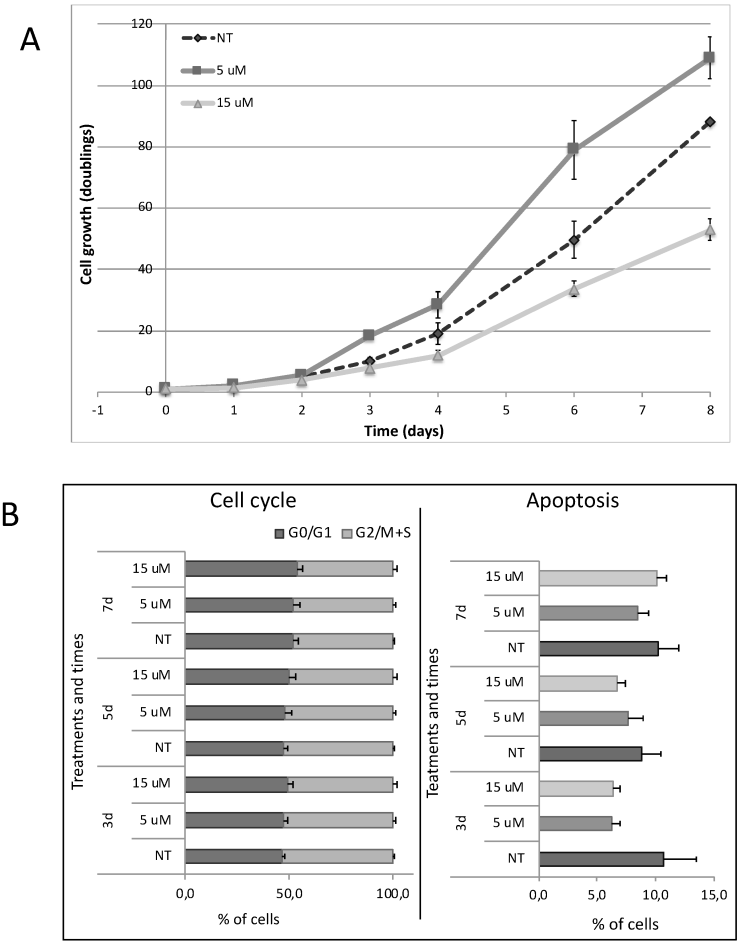


Figure 2. Survival curve (A, cells n°*10^6) and flowcytometry analysis (B) of HL60 cells treated with a single dose of 5 µM and 15 µM of HQ (average of three independent biological replicates).

2. VARIATIONS IN DNMTS, UHRF1 AND HMTS LEVELS INDUCED BY SINGLE TREATMENT WITH HQ

Given our interest in the epigenetic compartment, we explored the variations occurring at protein levels in the components necessary for the maintenance of DNA and histone methylation (UHRF1, DNMTs and some HMTs) following a single exposure to HQ.

2A. EVALUATION OF THE PROTEIN LEVELS

To verify the effects of HQ on the protein levels of the DNA methylation enzymes, UHRF1 and histone methylation enzymes, we proceeded to specifically examine the levels of DNMT1, DNMT3a, DNMT3b, UHRF1, EZH2, SUV39H1 and G9a through Western Blot analysis. An antibody directed towards GAPDH was used as a control. As Figures 3A show, after a single treatment (5 and 15 µM), we could detect an effect prevalently 5 days after the exposure: a visible reduction of variable entity, more pronounced in UHRF1 and DNMT1, is visible. These differences were still partially present two days later.

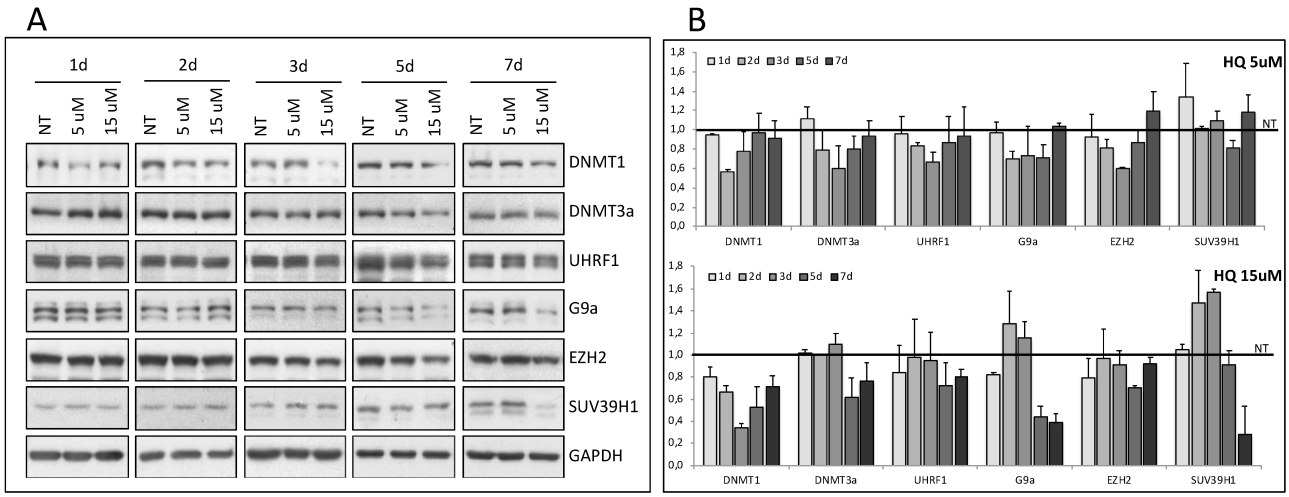


Figure 3. Western Blot panels (A) are representative of the average variations observed in three independent biological replicates: DNMT3b is not shown because it was not detectable in any condition. The densitometry graphs (B) show the variations at the different times or concentrations, each compared to basal levels (NT): the results are shown as mean + SD of two independent biological replicates. *P ≤ 0.05.

1, 2 and 3 days after the single treatment, in fact, the protein levels of DNMT1, DNMT3a and UHRF1 did not seem to vary significantly compared to the untreated, except for DNMT1 after 2 days from treatment with HQ 5 µM and after 3 days from treatment with HQ 15 µM. At 5d instead, we could observe a dose dependent reduction of UHRF1, DNMT3a and DNMT1 levels, as clearly shown by the densitometric analysis in Figure 3B. DNMT3b is not detectable in any condition and will not be analyzed in any treatment settings.

Since the control of gene regulation is mediated not only by DNA methylation, but also by histone PTMs, we analyzed the protein levels of three main histone methyl transferases (HMTs): G9a (H3K9me1-2), EZH2 (H3K27me1-2-3) and SUV39H1 (H3K9me3). These histone changes carry a transcriptional repression signal. As shown in Figure 3, at 1d and 2d no significant variations were observed, while at 3d a slight decrease of EZH2 was visible only at the highest concentration. At 5d the reduction seemed to be more pronounced, with a dose dependent trend, as clearly evidenced by the densitometric analysis; this trend seemed evident also for G9a but not for SUV39H1, whose basal protein levels are however much lower than the other two factors.

2B. EVALUATION OF THE EXPRESSION LEVELS

To better investigate the effects of the single exposure to HQ on the factors previously analyzed by Western Blot, we evaluated the expression of the relative mRNAs through quantitative PCR.

The analysis of the different Cts, normalized for the house-keeping gene GAPDH and referred to the NT, determined the two graphs shown in Figure 4 separately for the samples treated with 5 µM and 15 µM concentration of HQ.

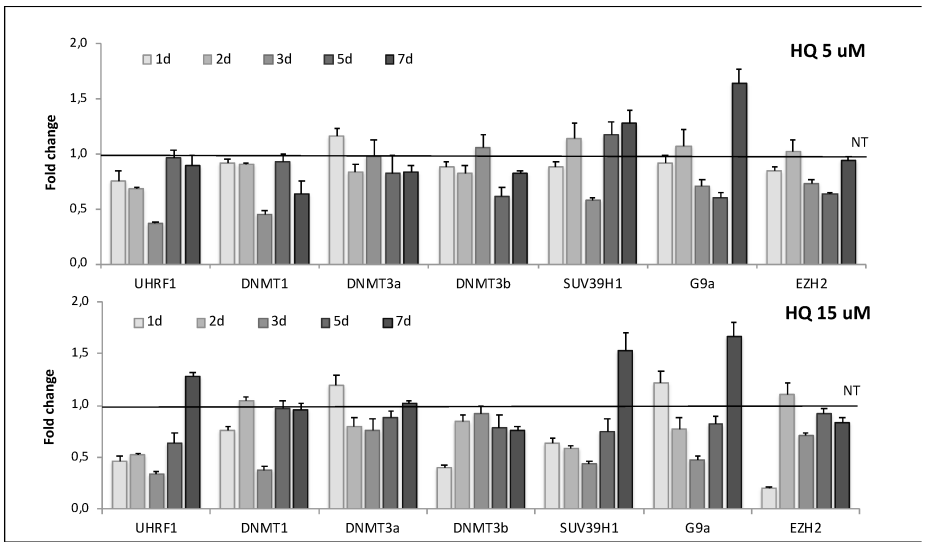


Figure 4. qPCR analysis of factors involved in epigenetic modifications after single treatment (average of three independent biological independent biological replicates).

As shown in Figure 4, a single exposure to 5 µM HQ is able to induce a significant reduction of UHRF1 mRNA levels at 1d, 2d and 3d, compared to control, while after 5 days from the treatment UHRF1 expression level seemed to return comparable to the NT. Concerning DNMTs, the levels of all the enzymes did not seem to change significantly within all the analyzed times, although they presented a general decreasing trend in expression, particularly evident only in some cases such as for DNMT3b 5d after single treatment and DNMT1 after 3d and 7d from the treatment. For the samples treated with HQ 15 µM we could observe a strong reduction of UHRF1 mRNA levels compared to NT (about 50%) in every collection point, confirming the trend observed with the 5 µM concentration. Finally, we observed a more pronounced reduction of the different DNMTs compared to 5 µM concentration.

Concerning HMTs, the expression levels of SUV39H1 showed the greatest variations, almost decreasing by half respect to the NT already after 1 day and remaining constantly below the basal levels until 7d, where we observed an increasing of the expression of this factor, especially in the 15uM treated samples. G9a and EZH2 showed a fluctuating behavior. G9a in fact seemed to be subject to short term (2d, 3d and 5d) reduction in expression levels, reverted at 7d, where we observed an increase of the expression in both the concentrations; EZH2 in HQ 5 µM samples showed a similar behavior to G9a, while in the 15 µM treated samples we could highlight a strong decrease in the expression level only 1 day after from the treatment.

3. EFFECT OF THE EXPOSURE TO HQ ON GLOBAL METHYLATION

To evaluate if the transcriptional and protein-related changes could induce an alteration in global genome methylation levels, we utilized the sodium bisulfite technique followed by Pyrosequencing.

In particular, we investigated DNA methylation status on the 5'UTR region of LINE-1, vastly utilized in research and clinic for the evaluation of methylation levels (Yang et al. 2004, Sahnane et al. 2015), after exposure to HQ.

We considered the methylation levels of 4 cytosines within the 5'UTR region of LINE-1 and compared them to the data obtained from normal blood (Ctrl-) and the data obtained in the same cell line HL-60 treated with Decitabine (DAC) 0,3 µM (Ctrl+), corresponding to the IC50. DAC is a well-known hypomethylating agent that we used it as positive control. Comparing the percentages of methylation of our Ctrl- (Blood) with non-treated HL-60 (NT), we could observe, in first instance, that the cell line used in this project resulted to be hyper-methylated. Another preliminary consideration is that, as expected, DAC was able to induce a strong reduction in cytosine methylation levels (Figure 5A).

Based on Western Blot results, we decided to analyze 5d and 7d with both 5 µM and 15 µM, but we could not detect statistically significant variations in both the time-points: we could only observe a slight tendency towards a dose dependent reduction 5 days after the exposure (Figure 5B), reduction that was lost in the samples harvested two days later, where the methylation levels seemed to return comparable to the NT (Figure 5B).

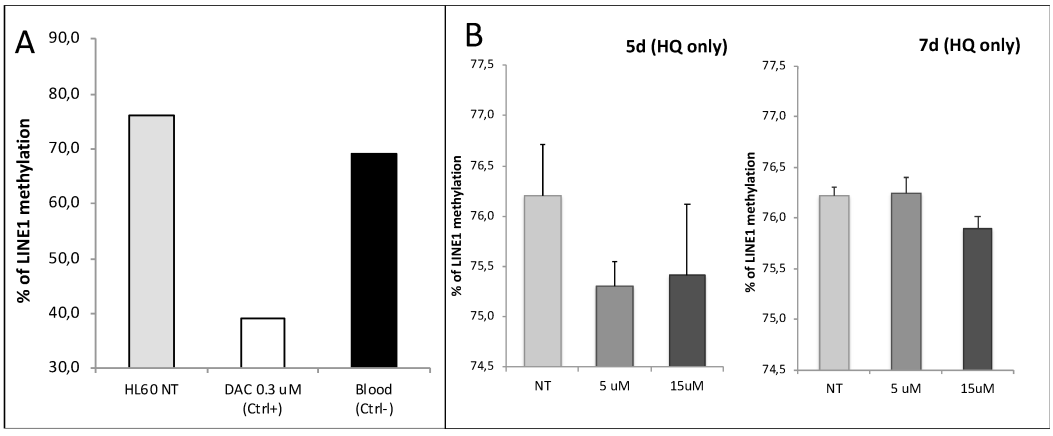


Figure 5. Methylation levels of four different cytosines in the 5'UTR region of LINE-1 evaluated through Pyrosequencing analysis. (A) comparison of HL60 with normal blood (Ctrl-) and with the same cell line HL-60 treated with Decitabine (DAC) 0,3 µM (Ctrl +). (B) Methylation levels at 5d and 7d after single treatment (average of three independent biological replicates).

We also evaluated the expression of LINE-1 after a single treatment with HQ. Transcriptional levels were evaluated through qPCR on the same region used for the Pyrosequencing analysis (corresponding to the promoter region of the sequence); variations in expression were compared to basal levels (NT). As for DNA methylation, we observed no significant variations in LINE-1 transcriptional levels, as shown in Figure 6.

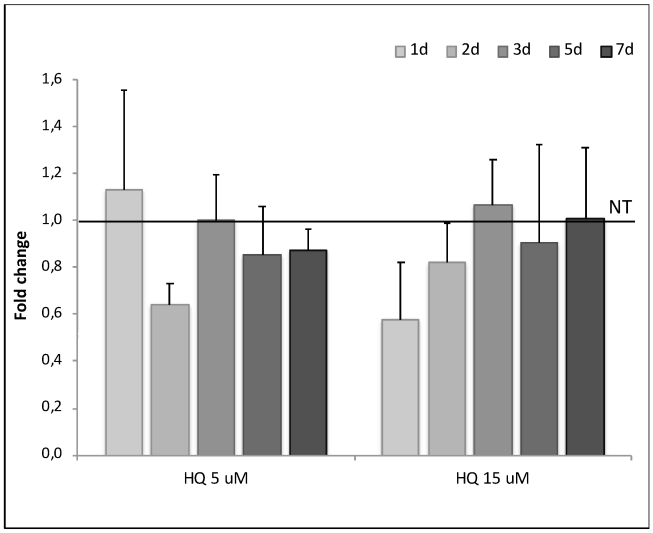


Figure 6. Transcriptional levels of 5'UTR region of LINE-1 evaluated through Pyrosequencing analysis (average of three independent biological replicates).

4. EVALUATION OF HISTONE MODIFICATIONS IN THE PROMOTER REGION (5'UTR) OF LINE-1

Focusing our attention on chromatin, given the variations observed in HMTs proteins, we performed ChIP experiments on LINE-1 5'UTR (the same region evaluated by Pyrosequencing and qPCR); we evaluated possible changes in the levels of the regulatory epigenetic marks H3K9me3, H3K9me2, H3K27me3, H3K4me3.

The first step was to optimize the sonication protocol of the chromatin to obtain fragments between 300 and 600 bp. At the end of each sonication the chromatin was checked, using a 0,8% agarose gel, to test the success of the experiment (Figure 7).

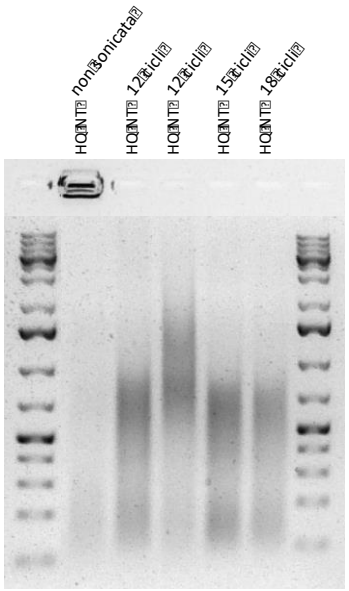


Figure 7. Evaluation of chromatin sonication.

We observed that the best sonication pattern was obtained after 12 cycles (lane 2). 15 and 18 cycles in fact determined a too high degree of fragmentation (lanes 4 and 5). Lane 3 is an example of a non-optimal sonication.

Subsequently, the chromatin was immunoprecipitated using specific antibodies directed against regulatory histone PTMs marks (activating mark: H3K4me3; repressing marks H3K9me3, H3K9me2, H3K27me3), on the 5'UTR promoting region of LINE-1, which is transcriptional repressed in these cells. Immunoprecipitation with anti-IgG antibody was performed as a control. The result of the amplification of the immunoprecipitated DNA is shown in Figure 8 and 9. The promoter region of the gene for GAPDH was analyzed as a control.

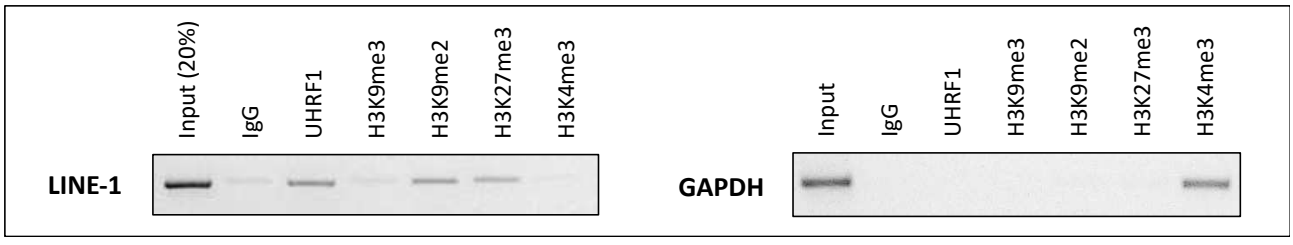


Figure 8. Levels of H3K9me3, H3K9me2, H3K27me3 and H3K4me3, as well as UHRF1 binding, were evaluated in the 5'UTR region of LINE-1 through chromatin immunoprecipitation in basal conditions.

Under basal conditions, as expected, on the promoter sequence of LINE-1, we observe the presence of UHRF1 and the repressive tri-methylation of H3K27 coupled with the absence of activation marker such as H3K4me3 (Figure 8).

Data on H3K9 showed the prevalence of bi-methylation vs. tri-methylation: this could be due to the hypothesis that the sequences analyzed by PCR are in fact the more conserved intronic LINE-1, where bi-methylation is prevalent, but also because HL-60 displays very low levels of SUV39H1 (the HMT deputed to H3K9 tri-methylation). H3K4me3 was generally absent, as expected in transcriptionally-repressed and hyper-methylated sequences.

For these reasons we decided to focus our attention only on the double histone mark H3K27me3/H3K4me3, known to be fundamental during development (Iwagawa and Watanabe 2018) but also during cancer (Chapman-Rothe et al. 2013, Hahn et al. 2014).

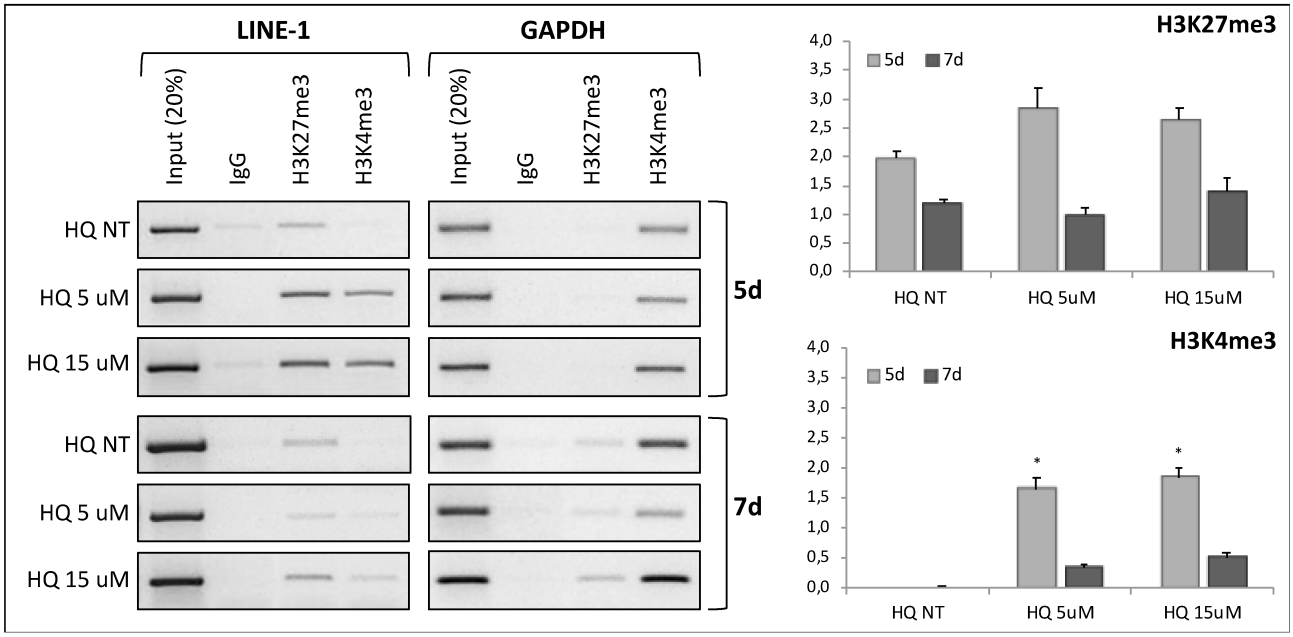


Figure 9. After single treatment, levels of H3K27me3 and H3K4me3 were evaluated in the 5'UTR region of LINE-1 through chromatin immunoprecipitation (densitometric analysis on the right, average of three independent biological replicates). The chromatin status was evaluated also on a constitutively active gene (GAPDH). PCR panels are representative of the average variations observed; densitometry analysis of LINE-1 are shown as mean + SD of three independent biological replicates and are normalized versus IgG and Input. *P ≤ 0.05.

Following a single treatment, the situation appears peculiar (Figure 9): after 5 days we could notice an increase of H3K4me3 while the H3K27me3 marker did not seem to vary significantly with both the

concentrations. As for protein and mRNA levels, at the 7d the effect on chromatin was lost, with the restoration of the basal condition.

These alterations, however, as shown above were not sufficient to determine significant variations in LINE-1 5'UTR transcriptional levels, probably due to the maintenance of repressive markers (H3K27me3 and DNA methylation).

Overall, this first analysis on chromatin modifications of LINE-1 sequences showed that even a single exposure to HQ is enough to perturb the structure of non-active DNA portion of the genome. However, these changes in the case of short-term treatments are lost in time, suggesting the ability of HL-60 to revert to the original conformation in the absence of HQ and thus the necessity of a prolonged exposure for the persistence of these alterations.

REPEATED TREATMENT

1. SURVIVAL CURVE AND FLOWCYTOMETRY ANALYSIS

Moving towards a long-term exposure, we increased the number of treatments up to four: cells were treated four times every 48 hours and harvested 2 and 7 days after the fourth treatment. Cell counting showed high toxicity at 15 µM and a slight reduction in cell growth at 5 µM, while 1 µM did not affect cell proliferation. For this reason, as explained previously, we excluded 15 µM in the following experiments. Figure 10A refers to the growth curve of HL-60 subjected to repeated treatment that shows how the cells (both NT and exposed to treatment) are still in active proliferation even 2 days after the fourth treatment and that the doses of HQ used do not exert an impact on cellular growth.

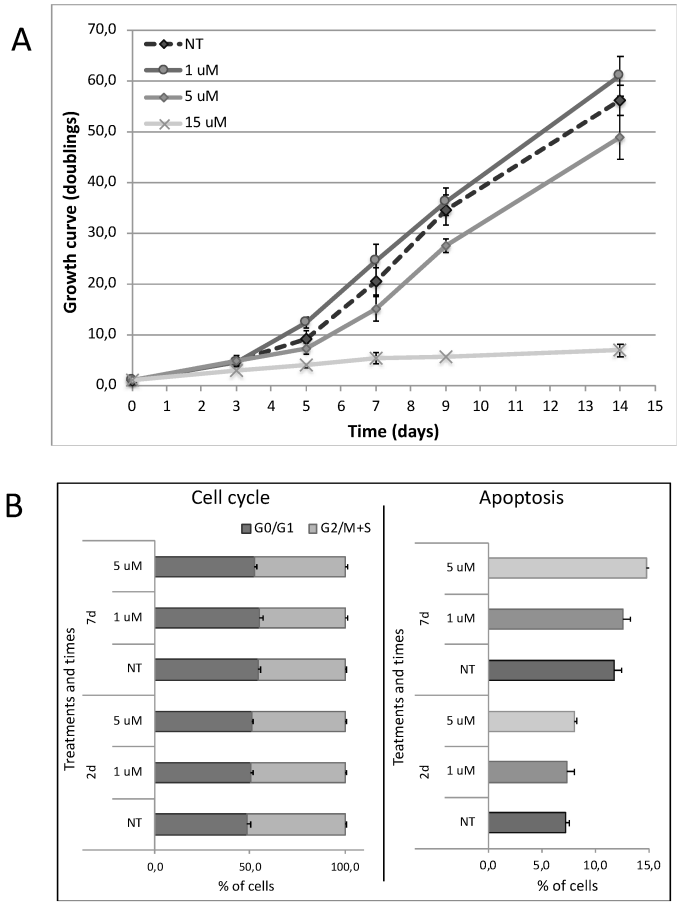


Figure 10. Survival curve (A, cells n°*10^6) and flowcytometry analysis (B) of HL60 cells in the repeated treatment (average of three independent biological replicates).

We also evaluated the effect of the selected sub-cytotoxic doses on cell cycle progression and apoptosis (Figure 10B): flow cytometry analysis evidenced that four treatments with 1 µM and 5 µM of HQ are not able to alter the percentage of cycling and apoptotic cells.

2. VARIATIONS IN DNMTS, UHRF1 AND HMTS LEVELS INDUCED BY REPEATED TREATMENT WITH HQ

As already presented for the single treatment, we explored the variations occurring at protein and expression levels in the components necessary for the maintenance of DNA and histone methylation (UHRF1, DNMTs and some HMTs) following four exposures to HQ, 1 µM and 5 µM.

2A. EVALUATION OF THE PROTEIN LEVELS

To analyze if repeated exposure to HQ could induce possible variations in the same factors analyzed in the single treatment, we proceeded with the evaluation of protein levels; with this analysis we wanted to highlight any differences compared to the effects determined by a single treatment.

We proceeded by Western Blot analysis, specifically examining the levels of the same DNMTs and HMTs previously selected (Figure 11A). GAPDH was again used as a control.

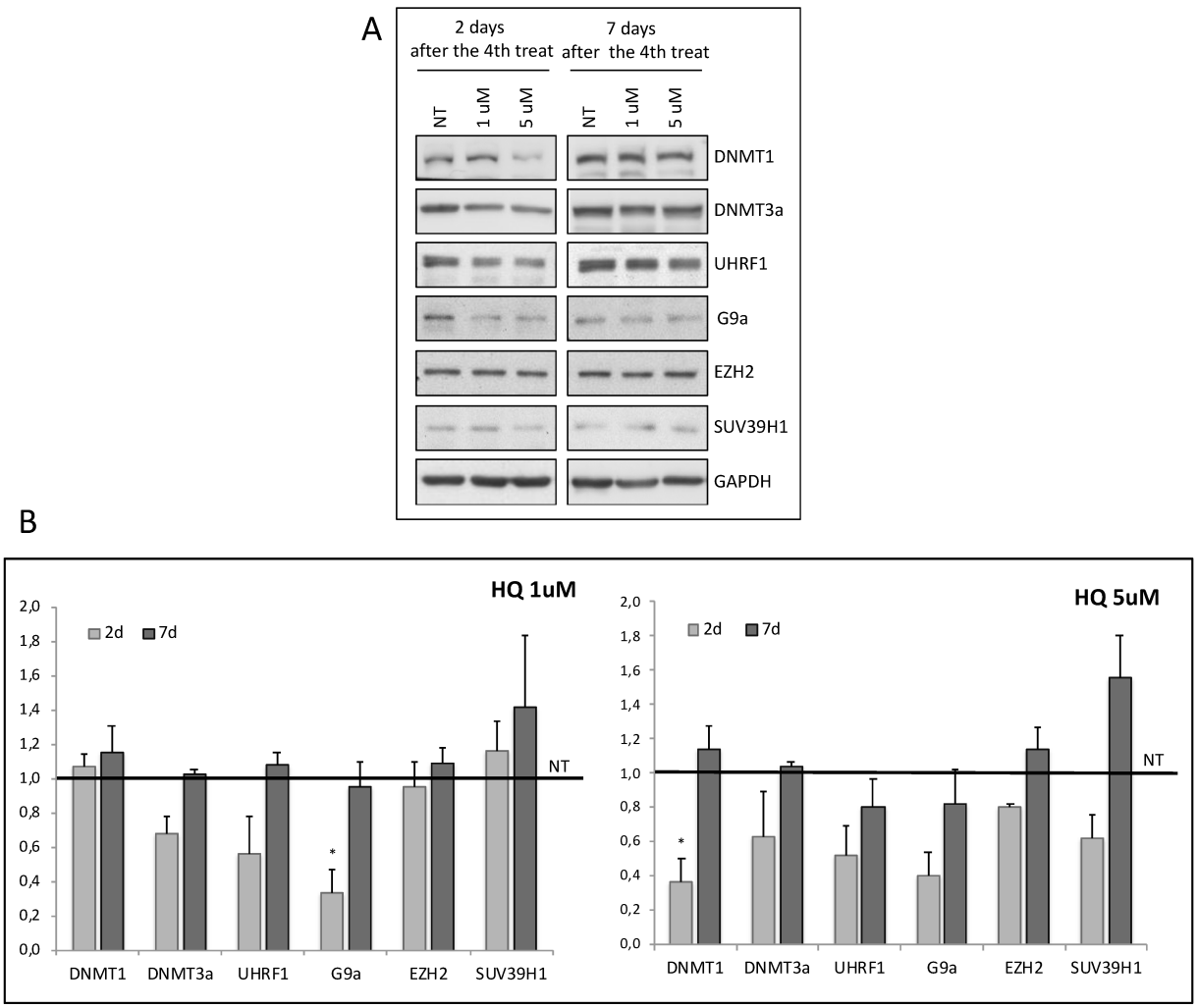


Figure 11. Western Blot panels (A) are representative of the average variations observed in three independent biological replicates: DNMT3b is not shown because it was not detectable in any condition. The densitometry graphs (B) show the variations at the different times or concentrations, each compared to basal levels (NT): the results are shown as mean + SD of two independent biological replicates. *P ≤ 0.05.

After repeated exposure to 1 and 5 µM of HQ, DNMT1 showed a great reduction 2 days after the 4th treatment only with the highest concentration; the levels of the protein however returned comparable to the NT one week after the end of the treatments and remained unvaried in the lowest concentration. The levels of UHRF1 and DNMT3a varied slightly at 2d, but even in this case at 7d the effect was no longer visible. DNMT3b is not detectable under any conditions even in this set of experiments.

Regarding HMTs, we could observe a partial variation in the protein levels only at the highest concentration and only 2days after the end of the treatment, as confirmed by the densitometric analysis (Figure 11B). G9a was the only HMTs showing a statistically significant reduction. Overall, the results 7 days after the treatments displayed the ability of HL-60 to recover to basal level in the absence of HQ.

2B. EVALUATION OF THE EXPRESSION LEVELS

To better investigate the effects of the repeated exposure to HQ on the factors previously analyzed by Western Blot, we evaluated the expression of the relative mRNAs. The two graphs in Figure 12 show separately the samples treated with 1 µM and 5 µM HQ.

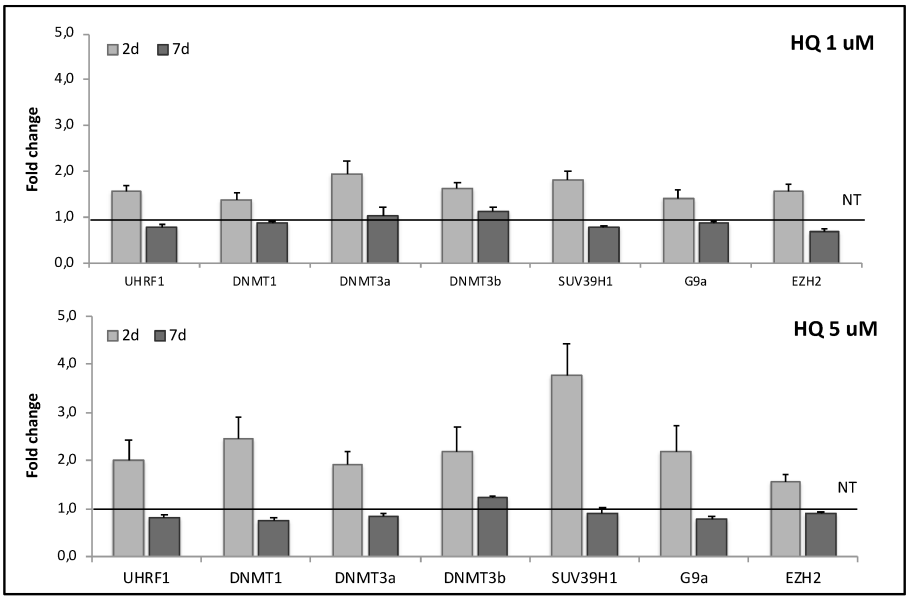


Figure 12. qPCR analysis of factors involved in epigenetic modifications after repeated treatment (average of three independent biological replicates).

Interestingly, after 2 days from the fourth treatment we could observe an increasing in the expression levels of all the investigated factors: the increment seemed to be dose-dependent, since it was more considerable at the highest concentration (especially for DNMT1 and SUV39H1). This trend is in fact opposite to what was shown by Western Blot analysis (Figure 11): the possible explanations of this inverse correlation will be debated in the discussion chapter.

Conversely, the results 7days after the end of the treatments displayed again the ability of HL-60 to recover to basal level in the absence of HQ: ay this time point the levels of expression return comparable to the NT for both samples treated with the two HQ concentrations, with some exception (EZH2 at 1 µM, DNMT1 at 5 µM) where we could observe a slight reduction in mRNA expression.

3. EFFECT OF THE EXPOSURE TO HQ ON GLOBAL METHYLATION

Considering the contrasting results between protein and mRNA levels of the factors analyzed by Western Blot and qPCR in the repeated treatment, we again extended our study to DNA methylation through Pyrosequencing analysis.

The results, shown in figure 13A, indicates the value of the methylation analysis made on the samples collected both 2 days and one week after the last treatment. The effects of the repeated treatment on LINE-1 methylation 2 days after the 4th treatment are controversial, with a slight decrease only at the highest dose, while one week later the levels of methylation were again back to basal. No significance was found even in HQ 5 µM due the low number of samples, coupled with the sensitivity of the instrument.

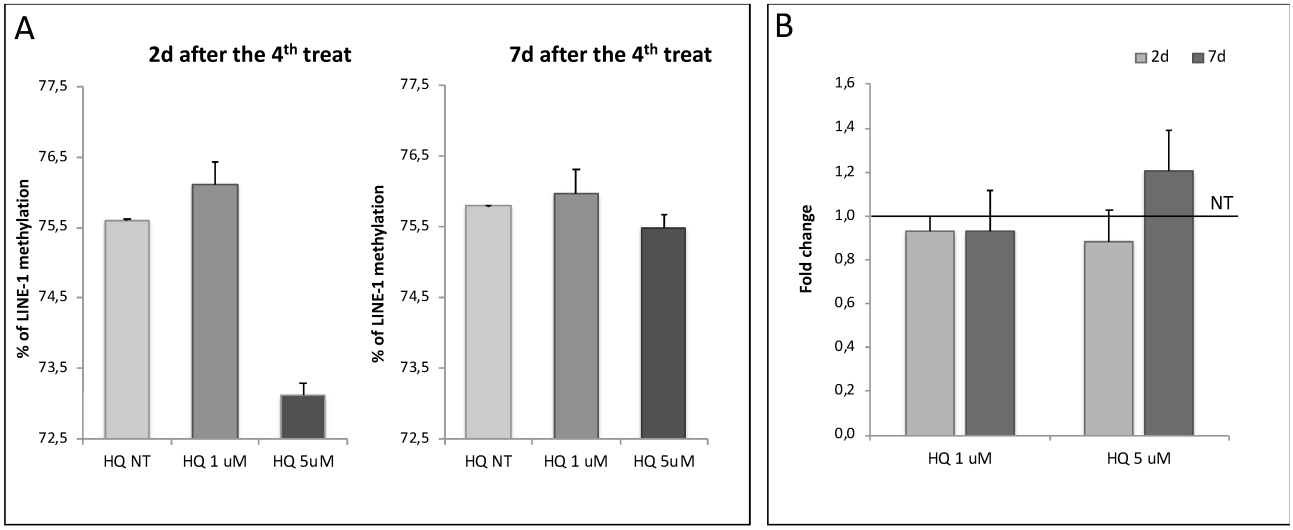


Figure 13. Methylation levels (A) of four different cytosines in the 5'UTR region of LINE-1 evaluated through Pyrosequencing analysis (average of three experiments) and transcriptional levels (B) of the same region of LINE-1 (average of three independent biological replicates).

In correspondence to the absence of alterations on DNA methylation, also for the repeated treatment, we could not observe significant variations in LINE-1 5'UTR transcriptional levels, as shown in Figure 13B.

4. EVALUATION OF HISTONE MODIFICATIONS IN THE PROMOTER REGION (5'UTR) OF LINE-1

As for the single treatment, we focused our attention on chromatin, performing ChIP experiments on LINE-1 5'UTR. The results are shown in figure 14.

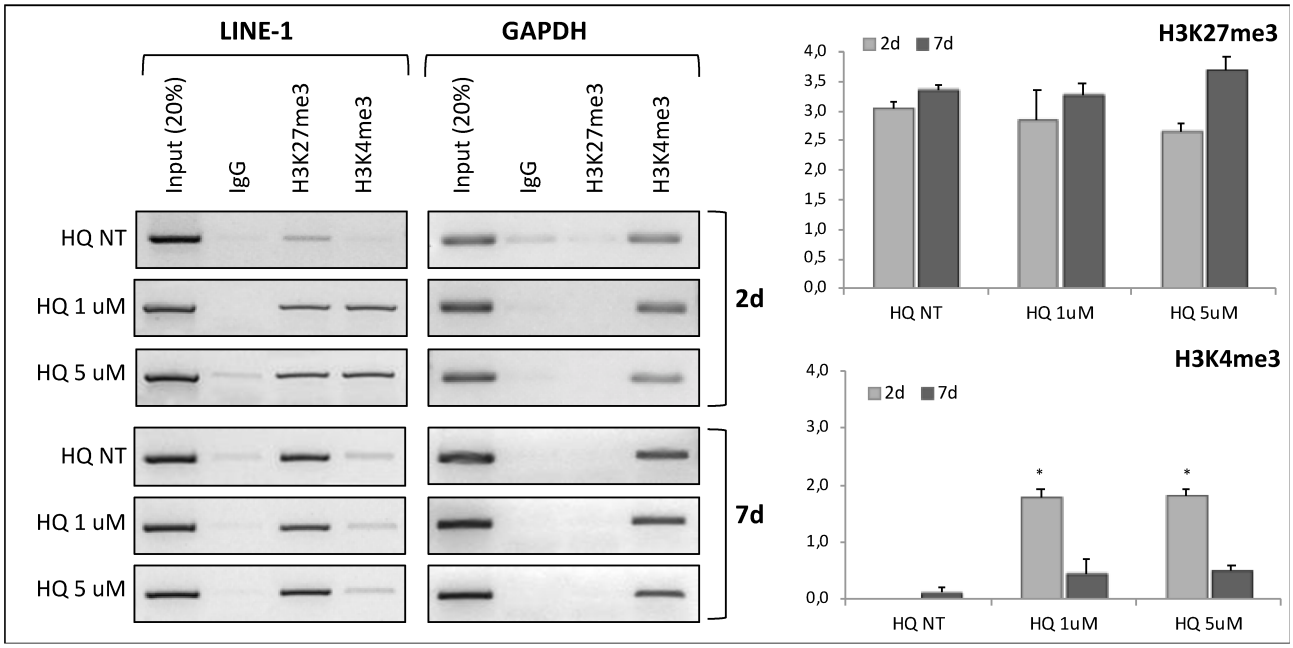


Figure 14. After repeated treatment, levels of H3K27me3 and H3K4me3 were evaluated in the 5'UTR region of LINE-1 through chromatin immunoprecipitation (densitometric analysis on the right, average of three independent biological replicates). The chromatin status was evaluated also on a constitutively active gene (GAPDH). PCR panels are representative of the average variations observed; densitometry analysis of LINE-1 are shown as mean + SD of three independent biological replicates and are normalized versus IgG and Input. *P ≤ 0.05.

Following repeated treatment, at 2d we could observe a situation similar to what observed at 5d of the single treatment: an increase in H3K4me3 again coupled with preservation of H3K27me3. This situation however was lost at 7d, confirming the ability of HL-60 to revert to the original conformation in the absence of HQ and thus the necessity of a more prolonged exposure for the persistence of these alterations.

Again, the persistence of repressive markers (H3K27me3 and DNA methylation) was sufficient to determine the absence of significant variations in LINE-1 5'UTR transcriptional levels as shown above in Figure 14 and 13.

LONG-TERM TREATMENT

1. SURVIVAL CURVE AND FLOWCYTOMETRY ANALYSIS

Given the previous observation on HL-60 ability to revert the molecular effects of a single and repeated treatment with HQ at the end of the exposure, in order to better investigate this ability following prolonged exposure, we provided daily administrations of the lowest concentration (1 µM) for multiple weeks. Cells were treated five times a week for five weeks with a single concentration, 1 µM. Every seven days cells were counted, reseeded at low density and harvested.

As previously described in Materials and Method and in Introduction, the dose of 1 µM of HQ used in our long-term exposure corresponds to 110 ng/ml, a concentration within the range of total HQ found in the blood of workers exposed to airborne benzene (Kerzic et al. 2010) that was between 20 and 120 ng/ml, which correspond to free HQ between 2 and 16 ng/m, for airborne benzene exposures ranging from 1 mg/m^3 (around 0,3 ppm) up to a maximum of 80 mg/m^3 (around 25 ppm).

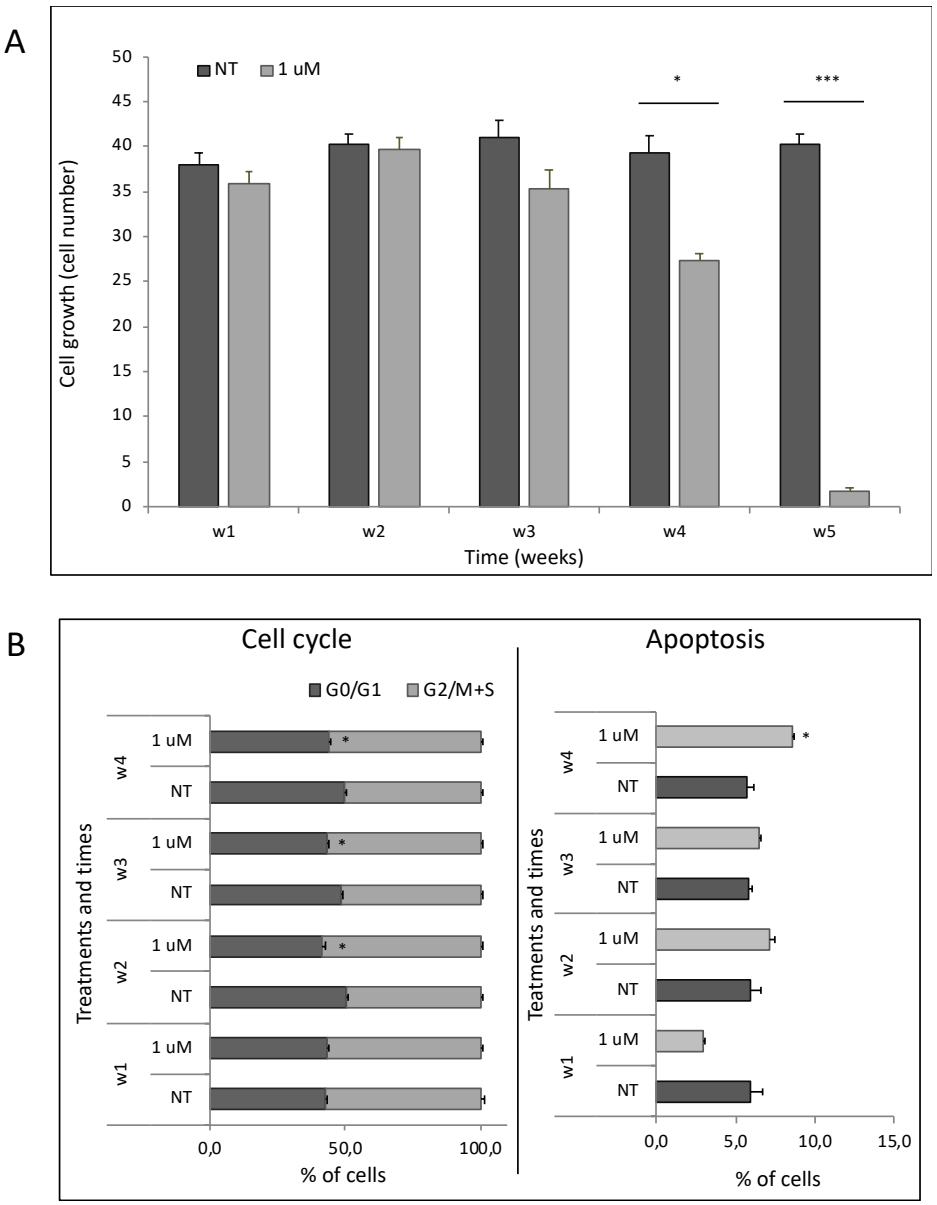


Figure 15. Survival curve (A, cells n°*10^6) and flowcytometry analysis (B) of HL60 cells in the long-term treatment. All results are shown as mean + SD of four independent biological replicates. *P ≤ 0.05; ***P ≤ 0.0001.

As shown in Figure 15A, we did not observe significant effects on cell growth for the first three weeks: a reduction in the cell number was observed starting at the fourth week and became massive the fifth week. The reduced growth affected only the treated cells, while non-treated cells showed no visible effect compared with the previous weeks, underlying that cell toxicity was due to the compound, and not to prolonged cultivation.

Following these observations, we concluded that even a very low concentration of HQ such as 1 µM (within total HQ levels found in peripheral blood of benzene-exposed workers), could not be administered

in vitro for long periods in the selected cell line due to its toxic effect. Thus, we decided to analyze only samples collected up to the fourth week.

Evaluating cell cycle progression and apoptosis by flow cytometry analysis, long-term treatment showed a slight reduction in G0/G1 starting from the second week of treatment and a slight increase of the apoptotic

cells in the fourth week of treatment only (Figure 15B). This feeble increase, however, does not seem to be sufficient to explain the decline in growth observed a week later: further analyses are necessary to better clarify the reason behind the dramatic effect on cell number registered the fifth week.

2. VARIATIONS IN DNMTS, UHRF1 AND HMTS LEVELS INDUCED BY LONG-TERM TREATMENT WITH HQ

To complete our exploration of HQ effects on the components necessary for the maintenance of DNA and histone methylation (UHRF1, DNMTs and some HMTs), we explored the variations occurring at protein levels, mRNA expression, DNA and histone methylation following a long-term exposure to HQ at 1 µM concentration.

2A. EVALUATION OF THE PROTEIN LEVELS

The evaluation of protein levels after long-term exposure concentration is useful to study the chronic effects of low doses of HQ, but also the possible differences compared to the effects determined by a single and a repeated treatment.

By Western Blot analysis, we again specifically examined the levels of DNMT1, DNMT3a, DNMT3b and UHRF1 for the DNA methylation pattern, EZH2, SUV39H1 and G9a as representative for HMTs.

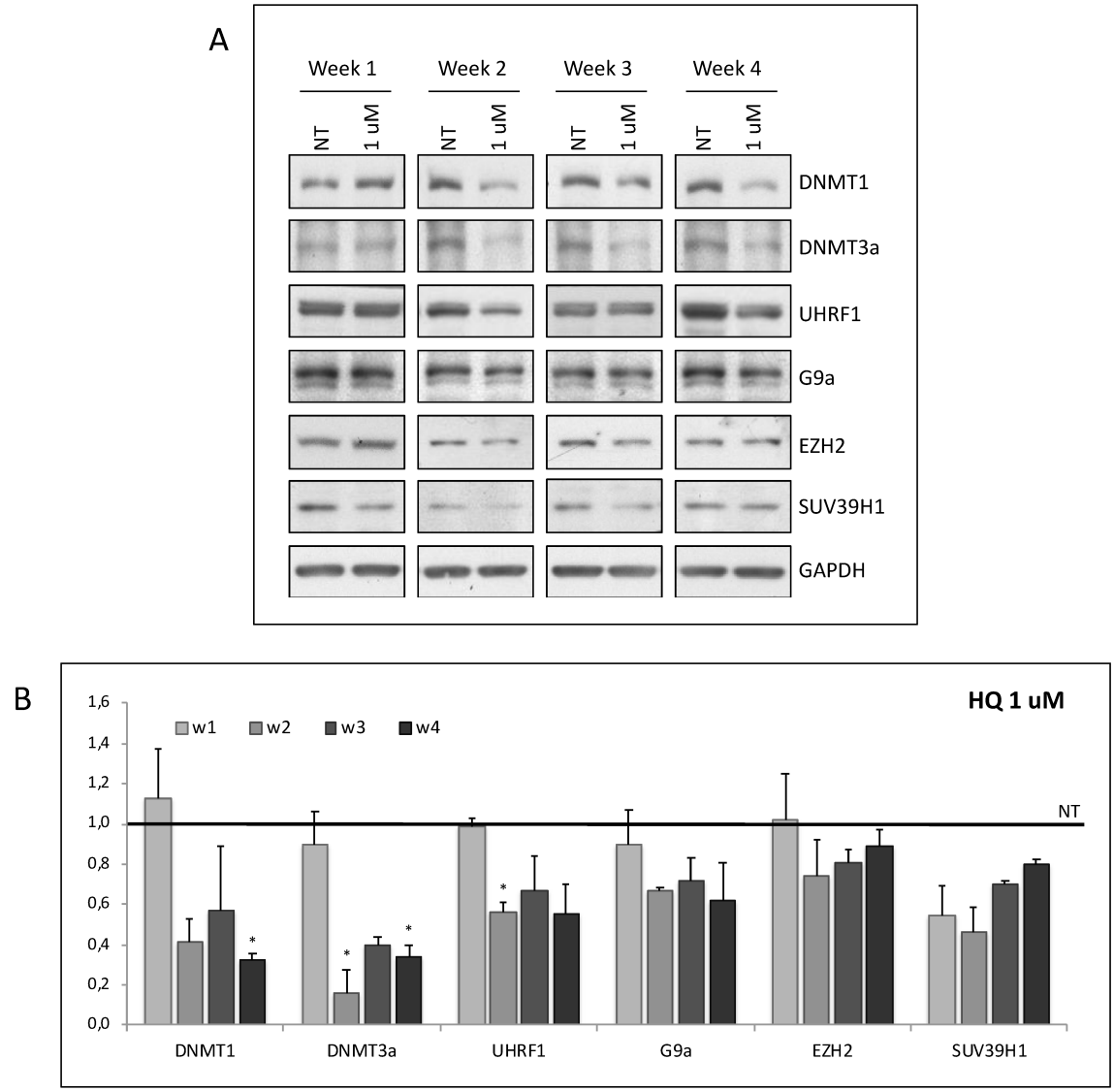


Figure 16. Western Blot panels (A) are representative of the average variations observed in three independent biological replicates: DNMT3b is not shown because it was not detectable in any condition. The densitometry graphs (B) show the variations at the different times or concentrations, each compared to basal levels (NT): the results are shown as mean + SD of two independent biological replicates. *P ≤ 0.05.

As shown in figure 16A, by looking at the effect of a prolonged exposure to a low dose of HQ, it becomes clear that this benzene metabolite is indeed able to down-regulate the epigenetic components analyzed: the reduction was visible starting from the second week of treatment and was maintained in the following weeks. Indeed, after one week we could detect no alteration in the protein levels of all factors, except for SUV39H1.

Looking at the densitometric analysis in Figure 16A, the reduction in protein level is noticeable especially for DNMT1 and DNMT3a and it is maintained, starting from w2, for all the remaining three weeks. The other factors (UHRF1, SUV39H1 and G9a) showed a less pronounced trend during the treatment, but all

were down-regulated. On the contrary, EZH2 seemed to be the less sensible to HQ exposure, with almost no variation during the four weeks.

2B. EVALUATION OF THE EXPRESSION LEVELS

Analysis of the effect of a long-term exposure to HQ on transcriptional levels of the factors previously valuated by Western Blot resulted in the graph shown in Figure 17.

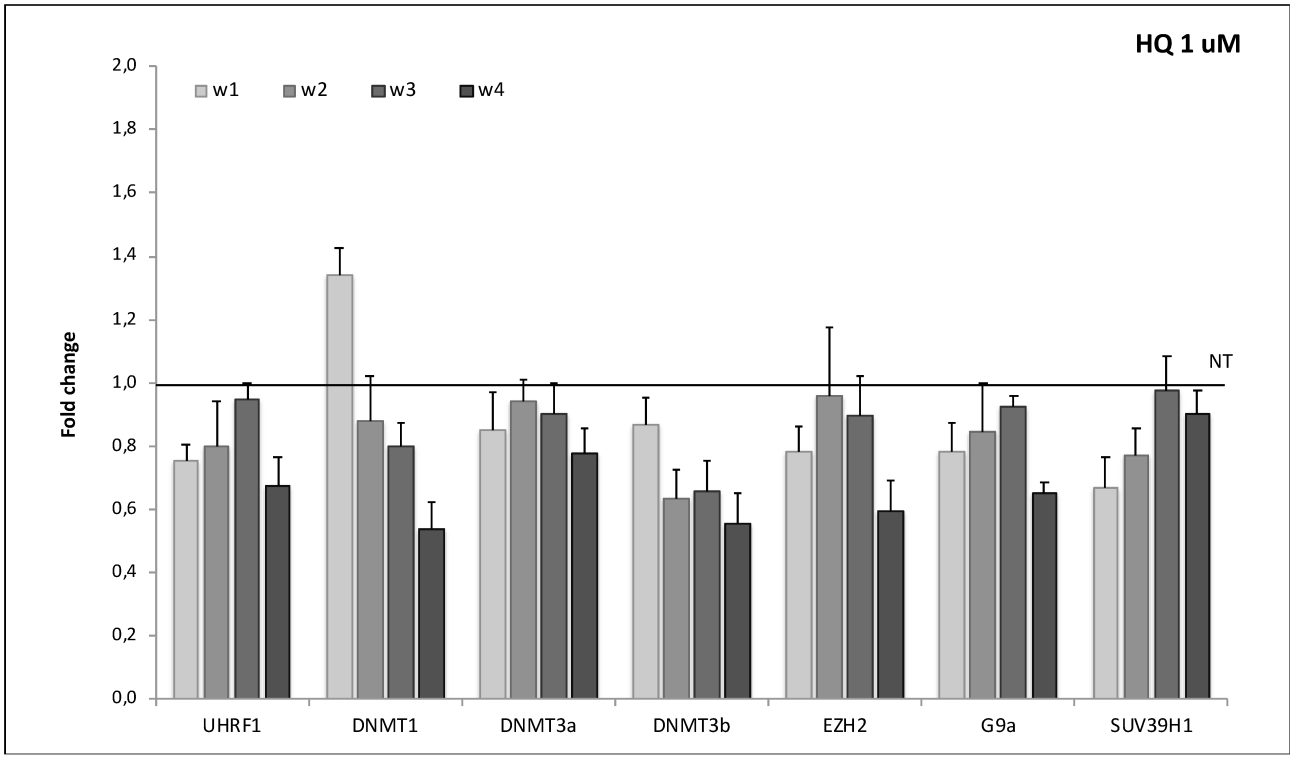


Figure 17. qPCR analysis of factors involved in epigenetic modifications after long-term treatment (average of three independent biological replicates).

The alterations in mRNAs levels are less defined respect to the protein levels: with the progression of the treatment we could observe a tendency towards a decrease in DNMTs transcription (especially DNMT1) and a fluctuating behavior of UHRF1 and the HMTs investigated.

The higher effect on expression levels seemed to be exerted at the fourth week for all the factors, with the exception of SUV39H1. Taking into account all the weeks, DNMT3b seemed to be the factor most

affected by long-term treatment; however, it is necessary to notice that basal expression levels of this enzyme are very low, and the protein is not detectable in HL-60.

3. EFFECT OF THE EXPOSURE TO HQ ON GLOBAL METHYLATION

Considering the robust variation in the levels of the factors analyzed, especially by Western Blot, in the long-term treatment, we performed Pyrosequencing and qPCR analysis to highlight possible variations in LINE-1 5'UTR methylation and transcription.

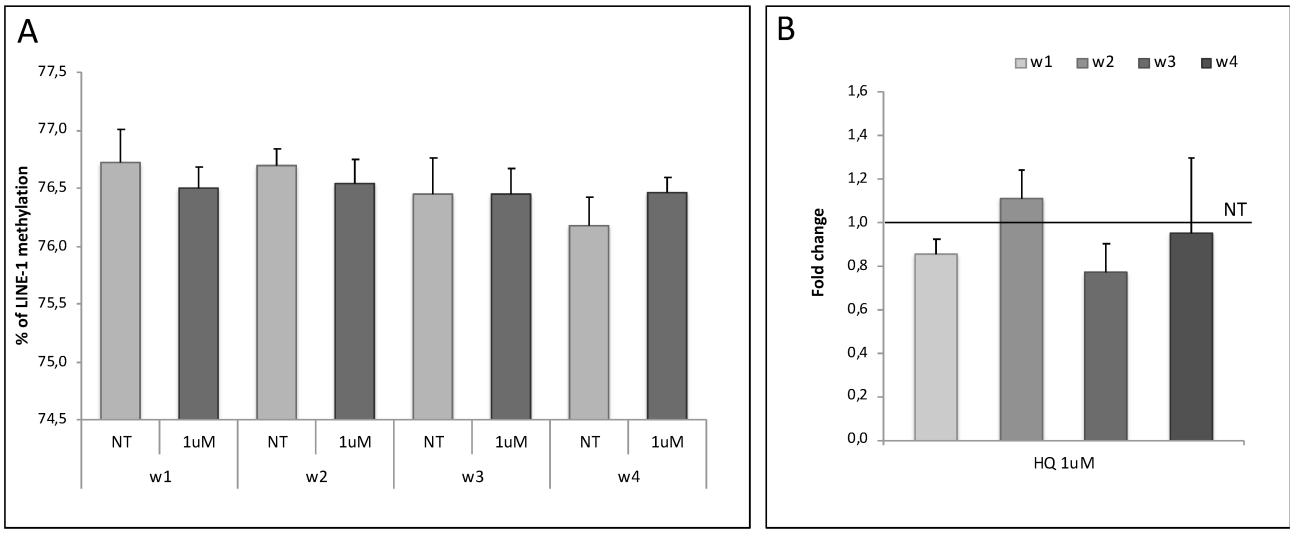


Figure 18. Methylation levels (A) of four different cytosines in the 5'UTR region of LINE-1 evaluated through Pyrosequencing analysis (average of three experiments) and transcriptional levels (B) of the same region of LINE-1 (average of three independent biological replicates).

Despite what observed in Figure 16 about the variation of the epigenetic components analyzed, also for long-term treatment we could not observe significant variations in any of the time-points analyzed (Figure 18A). In correspondence to the absence of alterations on DNA methylation we could observe no significant variations in LINE-1 5'UTR transcriptional levels even at the fourth week (Figure 18B).

4. EVALUATION OF HISTONE MODIFICATIONS IN THE PROMOTER REGION (5'UTR) OF LINE-1

Given the variations observed also in HMTs proteins (Figure 16), we focused our attention on chromatin, performing ChIP experiments. The results for long-term treatment are shown in figure 19.

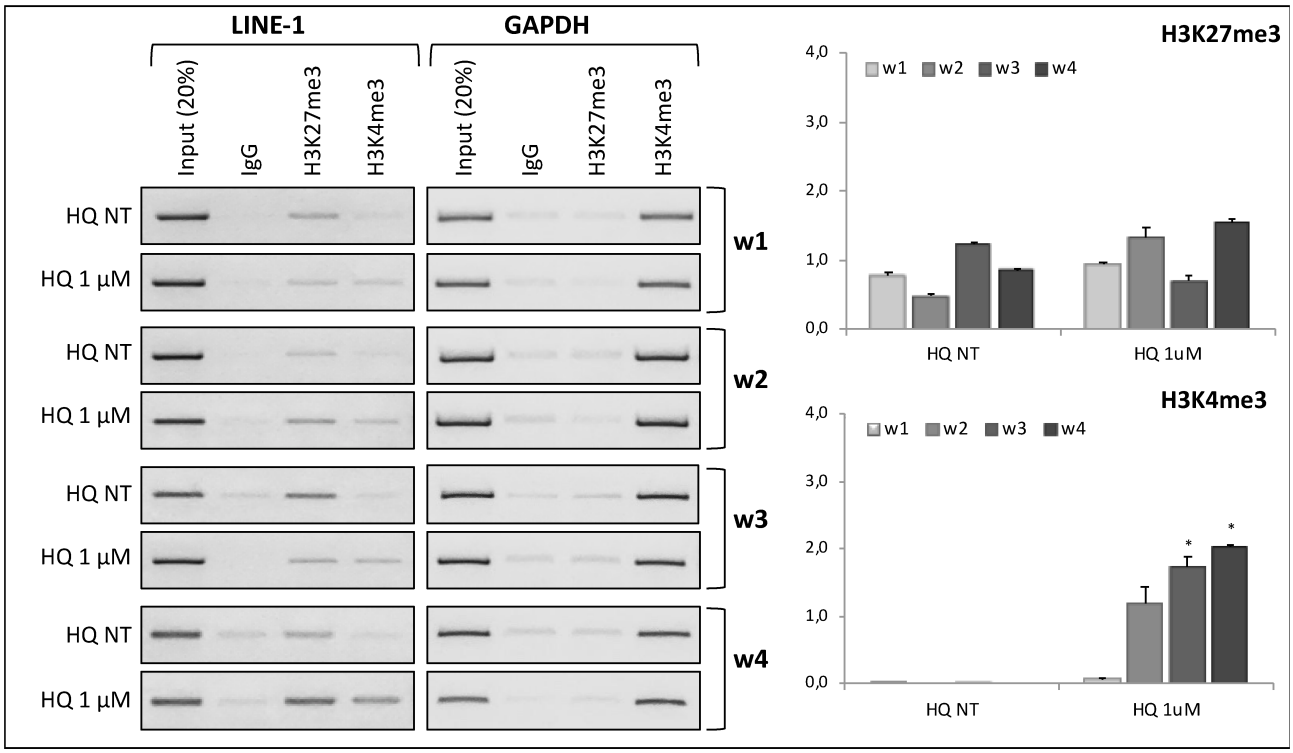


Figure 19. After long-term treatment, levels of H3K27me3 and H3K4me3 were evaluated in the 5'UTR region of LINE-1 through chromatin immunoprecipitation (densitometric analysis on the right, average of three independent biological replicates). The chromatin status was evaluated also on a constitutively active gene (GAPDH). PCR panels are representative of the average variations observed; densitometry analysis of LINE-1 are shown as mean + SD of three independent biological replicates and are normalized versus IgG and Input. *P ≤ 0.05.

Conversely to what observed on DNA methylation, we could detect a progressive effect from week 1 to week 4 with a gradual increase in the levels of H3K4me3; levels of H3K27me3 were stable at all time-points.

In particular, the increasing in H3K4me3 was well defined starting from w2 and became significant at w3 and w4. Unfortunately, we could not evaluate at long-term the persistence of this alterations in the absence of HQ due to the instauration of cytotoxicity.

Overall, these results showed that a long-term treatment with a low dose of HQ was able to alter significantly the chromatin status in the LINE-1 5'UTR region. In the same treatment, however, these alterations were not sufficient to determine significant variations in LINE-1 5'UTR transcriptional levels (as shown above in Figure 18B), probably due to the maintenance of repressive markers (H3K27me3 and DNA methylation): four weeks of exposure, in fact, are not able to enough to determine the removal of persistent cellular signal of inhibition of transposable elements interspersed in intronic sequences.

MODEL OF NON-HEMATOPOIETIC STEM CELLS – HUMAN UMBILICAL CORD MESENCHYMAL STEM CELLS (hUCMSCs)

Stem cells are unique cells: they have the ability to become many different types of cells and to self-renew or multiply while maintaining the potential to develop into other types of cells. They can be highly vulnerable to the mutagenic or toxic effects of drugs or other chemicals early during differentiation interfering with this process (Snyder 2014) with alterations that can lead to cancer.

Thus, to better understand the mechanisms of toxicity associated with long-term exposure to HQ, we decided to use a model of normal stem cells. We chose human umbilical cord mesenchymal stem cells (hUCMSCs) instead of bone marrow MSCs (BM-MSCs) or other stem cells for several reason:

- they can circumvent the ethical concerns associated with other kind of stem cell,
- they display higher proliferation potential than BM-MSCs *in vitro*,
- they can maintain a steady doubling time (DT) over a greater number of passages (until passage 10), whereas BM-MSCs showed notably increased DT after only six passages,
- they are easier to grow compared to other stem cell lines.

Given all these preliminary observations, in the second part of this project thesis we reproduced our long-term exposure to HQ 1 µM in hUCMSCs to evaluate possible effects on the epigenetic signature.

Strikingly, we had to stop our experiment before the end of the first week due to a massive effect on cell growth (data not shown): our treated samples, in fact, showed a drastic decrease in cell number. Given our goal, namely to obtain a model of treatment on non-hematopoietic stem cell to distinguishing possible mechanisms of cell regulation (especially at the chromatin level) from cytotoxic effects (e.g. apoptosis), we had to reconsider the concentration that was determined previously in HL-60.

We re-examined the work form Kerzic et al. to choose the optimal sub-cytotoxic concentration for these cells: therefore, we opted to test two different doses within the range of HQ detected in the blood of exposed workers (Kerzic et al. 2010). Our choice fell on 0,5 and 0,1 µM, corresponding to 55,06 ng/ml and 11,01 ng/mL; these two concentrations are representative of medium and low levels of total HQ detected (for details, see Introduction and results of HL-60 model).

LONG-TERM TREATMENT

To investigate the long-term effects of HQ on hUCMSC, we treated the cells with the two concentrations of the compound (0,1 and 0,5 µM) for five days a week for four weeks.

1. SURVIVAL CURVE AND FLOWCYTOMETRY ANALYSIS

Administration of the two concentrations determined very different effects on cell growth: 0,5 µM produced in hUCMSC a significant reduction in cell number already at the end of the first week, and cells did not survive the second week of treatment, as shown in Figure 20A.

Non-treated cells showed no visible effect up to the fourth week, underlying that cell toxicity was due to the compound, and not to prolonged cultivation. Instead, the 0,1 µM concentration of HQ showed a progressive reduction in the cell number only beginning from the third week. This led us to speculate that in the first and second weeks HQ could induce early events involving mechanisms of cell regulation with an instauration of cytotoxic effects only during the third week, allowing us to investigate potential epigenetic effects during the early response to the compound. Given the observed effects, we decided to analyse only the samples treated with 0,1 µM HQ.

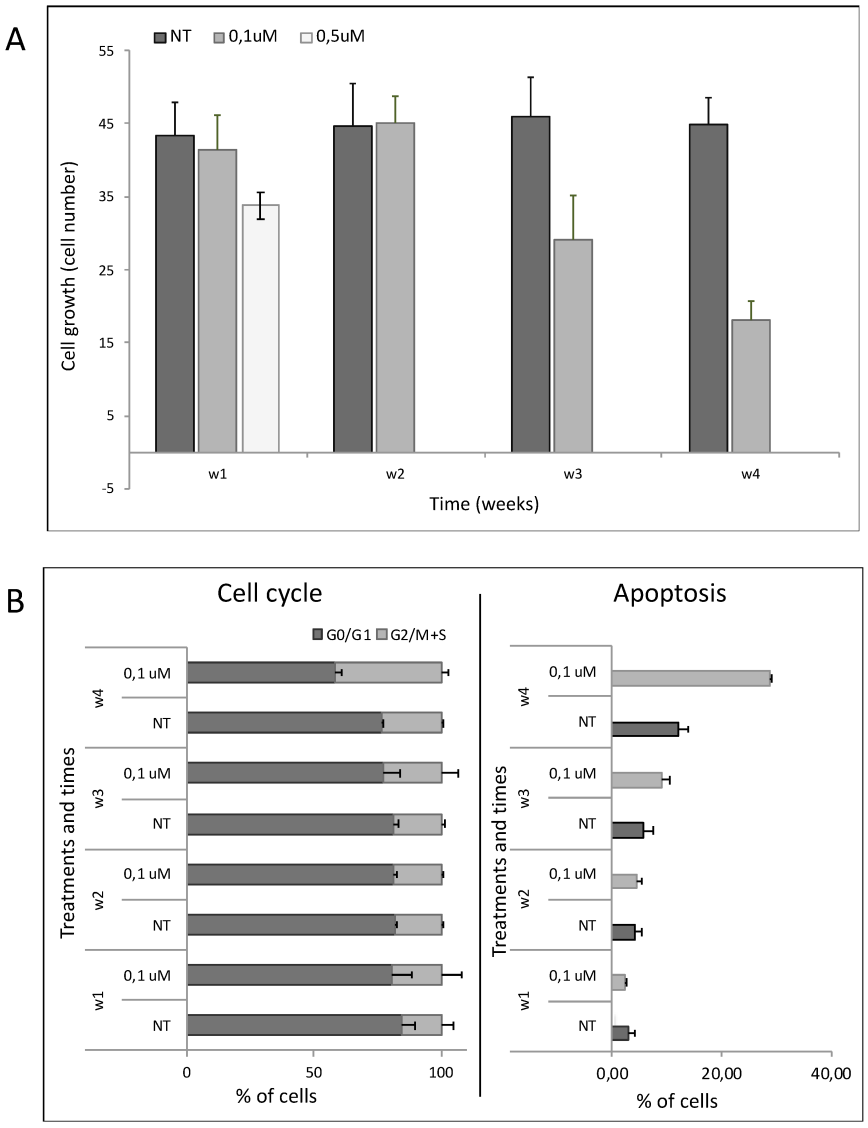


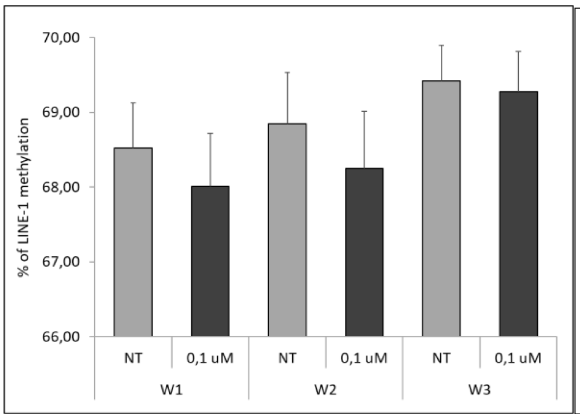
Figure 20. Survival curve (A, cells n°*10^6) and flowcytometry analysis (B) of hUCMSC cells in the long-term treatment HQ (average of three independent biological replicates).

We evaluated the effect of 0,1 µM of HQ on cell cycle progression and apoptosis analyzing samples collected up to the fourth week. Until the third week, they do not seem to be differences between control NT and 0,1 µM treated samples. Flow cytometry analysis evidenced that long term exposure to 0,1 µM of HQ is able to alter significantly the percentage of cycling cells at the fourth week as we could see in figure 20B. At this time point we could observe a reduction in G0/G1 phase and an increasing in G2/M+S. Looking at the percentage of apoptosis at fourth week, we could observe that NT samples had a rate of apoptosis higher than the previous week indicating a possible response of this cell line to long-term cultivation. However, at fourth week the exposure to HQ is able to increase the percentage of apoptosis about twice respect to the NT.

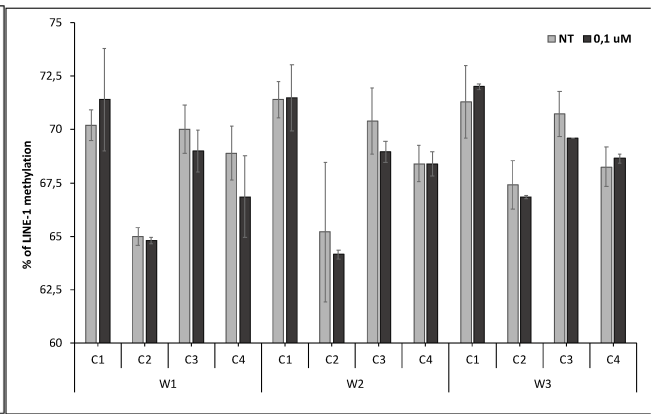
2. EFFECT OF THE EXPOSURE TO HQ ON GLOBAL METHYLATION

We performed Pyrosequencing analysis to highlight possible variations in LINE-1 5'UTR methylation in hUCMSC after a long-term treatment only on samples collected until the third week: at the fourth week the rate of apoptosis was high and, thus, we decided not to analyze this time point. Our aim was indeed to highlight early events not related to cytotoxicity. The results are shown in Figure 21.

A



B



C

	TEST.T
C1	0,360
C3	0,542
C3	**0,034**
C4	0,394

D

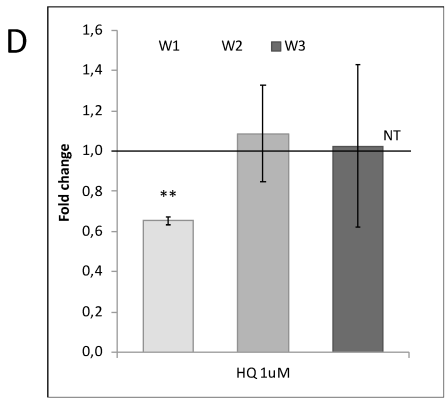


Figure 21. Methylation levels of four different cytosines in the 5'UTR region of LINE-1 evaluated through Pyrosequencing analysis (average of two experiments). (A) shows the mean of methylation value of four cytosines, (B) shows the value for each single cytosine (average of two independent biological replicates) and (C) the p values of the alterations of the single cytosines during the three weeks. Transcriptional levels (D) of the same region of LINE-1 (the results are shown as mean + SD of two independent biological replicates). **P ≤ 0.01

Looking at Figure 21A, in which the mean of the four analysed cytosine is represented, we could not observe significant variations in any of the three weeks of treatment. However, evaluating the single cytosines, we could observe an overall significant reduction of C3 only during the three weeks, although at the single week level the number of experiments was not sufficient to reach significance for minor

alterations like the ones observed. An explanation of this result may lay in the fact that HQ could preferentially alter the methylation levels of this specific cytosine. Further experiments, however, will be needed to confirm this result.

The transcriptional levels of LINE1 (Figure 21D) in hUCMSC following the long term treatment were significantly reduced after the first week, but the expression returned to levels that are comparable to the NT.

3. EVALUATION OF HISTONE MODIFICATIONS IN THE PROMOTER REGION (5'UTR) OF LINE-1

To examine the long-term effects of HQ on hUCMSC, we focused our attention only on the bivalent histone mark H3K27me3/H3K4me3 (investigated also in HL-60), known to be fundamental both during development (Iwagawa and Watanabe 2018) and cancer (Chapman-Rothe et al. 2013).

Again, we decided to concentrate our study only on samples collected until the third week: at the fourth week the cytotoxic effect was prevalent despite the reduced concentration and thus, as for DNA methylation analysis, we decided not to analyze this time point.

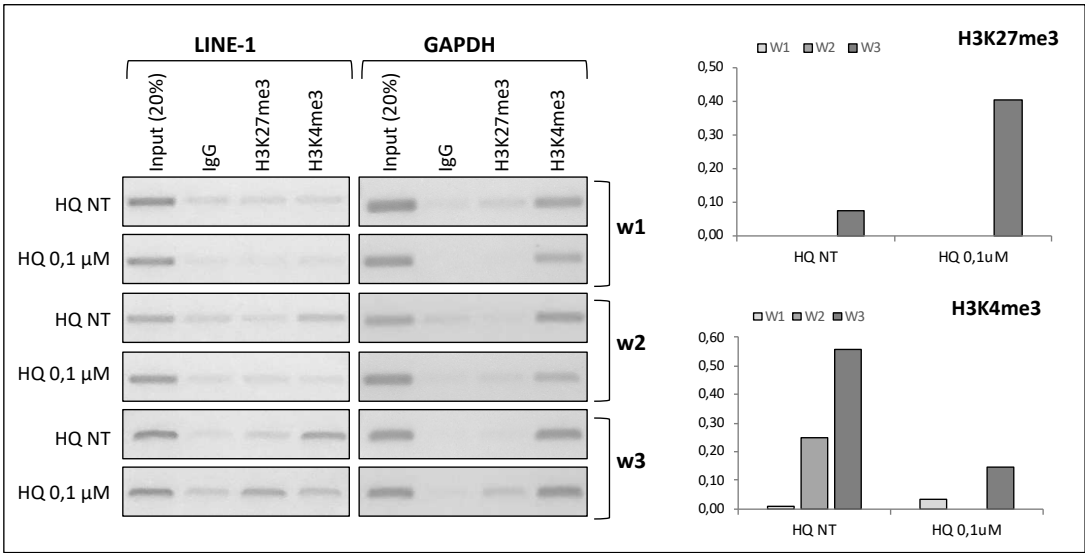


Figure 22. After long-term treatment, levels of H3K27me3 and H3K4me3 were evaluated in the 5'UTR region of LINE-1 through chromatin immunoprecipitation (densitometric analysis on the right is normalized versus IgG and Input). The chromatin status was evaluated also on a constitutively active gene (GAPDH).

As shown in Figure 22, in hUCMSC the chromatin configuration of LINE-1 promoter in basal condition is very different compared to HL-60. Surprisingly, we found the absence of both markers at week 1 in our control cells and a progressive increase of H3K4me3 in the following weeks; we could also observe the presence of low levels of H3K27me3, indicating the potential instauration of the bivalent mark, at the third week. Most interestingly, in HQ treated cells at the third week we observed the peculiar inversion in the levels of H3K27me3 and H3K4me3 levels on LINE-1 promoter region as compared to the control, with a stronger increase in H3K27me3 and a slighter enrichment in H3K4me3. This behaviour, notably, is opposite to what observed in HL-60. In the first week, despite a transcriptional reduction of LINE1 and no modification at methylation levels in the promoter region, we didn't observe alterations in the chromatin modification.

Unfortunately, the presented results are performed only in a single experiment; further biological replicates are necessary to confirm this chromatin status after exposure to HQ and to confirm the dynamics of chromatin in stem cell maintained in culture for several weeks.

4. EVALUATION OF hUCMSC TRANSCRIPTOME

In order to evaluate the effects of long-term exposure to HQ on the transcriptome profile, and thus to enlighten differentially expressed gene potentially involved in response to HQ exposure, we performed RNA-seq experiments in collaboration with Prof. Alessandro Weisz (Lab of Molecular Medicine & Genomics, University of Salerno, Italy).

As mentioned before, our goal was to obtain a stem cell model to study early events in the response to HQ, thus distinguishing possible mechanisms of cell regulation from cytotoxic effects. For this purpose, we performed this preliminary analysis only on samples collected after one week of treatment, in triplicate.

The preliminary Principal Component Analysis showed a significant distance between NT samples in the three biological replicates under consideration, indicating the difficulty in the maintenance of stem cells,

which undergo profound alterations even when cultivated following the guidelines of the supplier (Figure 23). However, the distance between NT and 0,1 µM samples remained constant in the first and the second replicate, possibly indicating the effects directly attributable to hydroquinone. The treated sample of the third replicate turned out to be contaminated from Mycoplasma, so it was not taken into consideration in the following analysis.

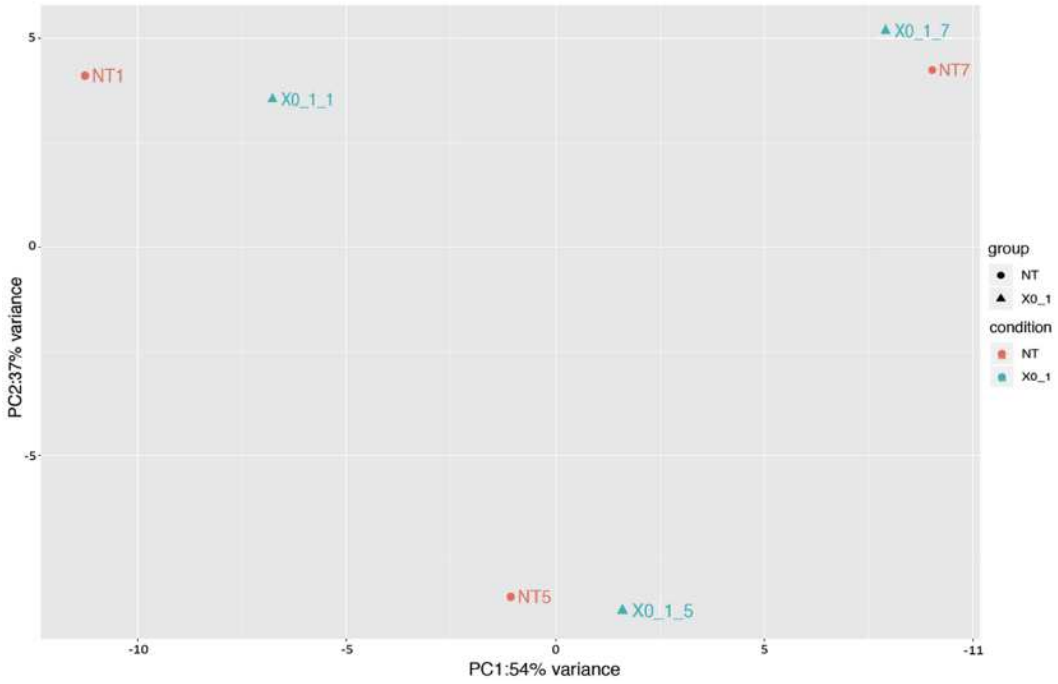


Figure 23 – Principal Component Analysis of three biological replicates analyzed by RNA-seq experiments. NT1 and X0_1_1 are NT and treated samples of first replicate, NT5 and X0_1_5 are NT and treated samples of second replicate, NT7 and X0_1_7 are NT and treated samples of third replicate,

Given these considerations, we decided to analyze the combined expression profile of experiment 1 and 2, to highlight the presence of common genes that display differential expression values in HQ vs control samples. Fold change (FC) variations $\geqslant 2$ and $\leqslant -2$ of the differentially expressed (DE) mRNAs were used to define up-regulated and down-regulated transcripts, respectively. Actually, we detected a total of 153 mRNAs that showed a p-value <0.05 (Appendix A). Further analyses of the involved pathways are still ongoing.

DISCUSSION

In the last few years, several investigations have examined the relationship between exposure to environmental chemicals and epigenetics and have identified numerous agents able to modify the epigenetic marks. Most of the studies conducted so far have been centered on DNA methylation, whereas only recently a few have focused on the effects on histone modifications (Bollati et al. 2007, Baccarelli and Bollati 2009, Wilson et al. 2007, Philbrook and Winn 2015).

In a recent study it was demonstrated that exposure to low levels of benzene and its metabolites could correlate with modification of global H3K4me3 in PBLCs and with the reduction of WBC counts in benzene-exposed workers, indicating that histone modifications could play a key role in mediating the hematotoxicity following benzene exposure: indeed, H3K4me3 mark was enriched in the promoters of several DNA damage responsive (DDR) genes (Li et al. 2018).

Benzene and its metabolites are among the most studied environmental pollutants, due to their association with diverse hematopoietic malfunction, as well as leukaemogenesis.

Global DNA hypo-methylation, accompanied by gene-specific hyper-methylation, was demonstrated in benzene-exposed healthy workers (Bollati et al. 2007); epidemiological studies on healthy subjects showed a difference in the pattern of methylation between the exposed group (gasoline station attendants) and the control group (office workers), with a significant reduction in the first group of 2.33% in the methylation of repeated elements LINE-1 and Alu, used as a surrogate for the entire genome (Fustinoni et al. 2012). Moreover (Lan et al. 2004) demonstrated that the highest blood levels of total HQ were observed even at very low levels of exposure to air benzene (≤1 ppm), levels that have been shown to cause hematotoxicity.

Despite these epidemiological evidences, *in vitro* demonstration of benzene activity on the epigenetic machinery is complicated by the nature of the type of exposure behind these changes: while studies on benzene and its metabolites on acute effects (i.e. apoptosis activation and ROS production) are easily achievable using single high-dose administrations of the agents (Zolghadr et al. 2012, Terasaka et al. 2005a, Terasaka et al. 2005b), chronic exposures at very low doses, comparable to the ones reached in occupational exposure, are more difficult to reproduce in laboratory.

Given these considerations, the aim of the present thesis project was to demonstrate the epigenetic effects of the administration of low (non-cytotoxic) concentrations of hydroquinone in HL-60 cells (a model of hematopoietic cancer cells), according to three different experimental schemes designed to evaluate possible differences between a single administrations at higher doses (in any case sub-toxic, as shown by the preliminary survival curves) and repeated doses over time at milder doses. Finally, the long-term treatment scheme was intended to somehow recall exposures that can be experienced in working environments. Moreover, to better understand the mechanisms of toxicity associated with prolonged exposure to HQ, we replicated the long-term exposure setting also on a second model, namely on human umbilical cord mesenchymal stem cells (hUCMSC) to highlight possible effect on stem cells.

For HL-60, starting from a single exposure, we evaluated cell viability to choose sub-cytotoxic concentrations of HQ in each setting: we empirically chose the 5 μM and 15 μM concentrations for the single treatment, 1 μM and 5 μM concentrations for the repeated treatment. The HQ concentration we used (1 μM) for long-term treatment corresponds to 110 ng/ml, a level very similar to the highest amount of total HQ (between 20 and 120 ng/mL, corresponding to 2–16 ng/ml of free HQ) found in the blood of workers of a rubber product manufacturing and finishing facility located near Shanghai, China (Kerzic et al. 2010), exposed to airborne benzene levels ranging from 1 mg/m3 (around 0.3 ppm) up to a maximum of 80mg/m3 (around 25 ppm). Interestingly, high blood levels of total HQ were observed even at very low levels of airborne benzene exposure (≤1 ppm) (Lan et al. 2004). Moreover, when the ratio of bound to unbound metabolites were compared in subsets of exposed workers, the increase in blood metabolite concentration was nearly all due to an increase in the protein-bound molecule. These results suggest that it is very difficult to define a tight correlation between the level of benzene exposure and the concentration of HQ, especially the free form, in the blood. Considering that HQ has a half-life of at least 20 h (ICPS, INCHEM, SIDS, 2002), that the molecule undergoes rapid redox reactions and that 10% FBS present in the medium contains many HQ binding proteins, the amount of free HQ may vary considerably during the experiments, making it difficult to accurately measure the final concentration of free HQ, very similarly to what observed in blood.

Altogether, we used the dose of 110 ng/mL for the long-term experiments, which is close to the maximum total levels detected in the blood of exposed workers (120 ng/mL), in the effort to mimic *in vitro* a strong occupational exposure, although we could not define the exact working concentration range of free HQ. Evaluating the effects on cell proliferation, following single treatment the concentration of 5 µM seemed to confer a proliferative advantage, while rising to 15 µM a modest cytotoxic effect was observed. This treatment was not sufficient to alter the percentage of apoptotic cells nor the distribution of cells in the different cell cycle phases at none of the analyzed time point; a similar result was observed in the repeated treatment. The situation in the actually still unexplored long-term treatment was very different: a daily administration of a dose as low as 1 µM induced a reduction in the cell number starting at the fourth week and became massive the fifth week. The reduced growth affected only the treated cells, while non-treated cells showed no visible effect compared with the previous weeks, underlying that cell toxicity was due to the compound, and not to prolonged cultivation. The control HL-60 cells started to undergo a reduction in cell growth after 8 weeks of cultivation (data not shown), possibly due to the accumulation of stress factors: one possible explanation of this phenomenon might be that low-dose HQ contributes to the stress load that leads the cells to arrest/undergo senescence/die in a shorter time with respect to controls. Simultaneously, we observed an increase in apoptotic cells at the fourth week and, starting from the second week, a statistical reduction in G0/G1 phase compared to the control. These results showed for the first time that even a very low concentration of HQ such as 1 µM (within total HQ levels found in peripheral blood of benzene-exposed workers (Lan et al. 2004, Kerzic et al. 2010) could not be administered *in vitro* for long periods in the HL-60 cell line due to its cytotoxic effect.

Aberrant expression of DNMTs, accompanied by hyper-methylation of tumor suppressor genes and hypo-methylation of unique genes or repetitive sequences, are the main types of aberration in the DNA methylation machinery occurring in AML and other cancer types (Brait and Sidransky 2011, Hamilton 2011). A study of 2007 first correlated changes in DNA methylation patterns with exposure to low-dose of benzene (Bollati et al. 2007). Liu et al. in 2012 (Liu et al. 2012) demonstrated that in TK6 cells the exposure at different concentration of HQ (2,5; 5; 10,0 and 20,0 µM for 48 h) resulted in the decrease of DNMTs and MBD2 mRNA level, in the global hypo-methylation of LINE-1 repetitive sequences and in the

increase of MPL at mRNA level. These results indicate that global DNA hypo-methylation may result from DNMTs inhibition induced by HQ exposure.

Given these evidences, we focused our attention on alteration in the epigenetic machinery occurring in each setting, exploring the hypothesis that exposure to HQ could determine variations at protein and expression levels in the components necessary for the maintenance of DNA and histone methylation (UHRF1, DNMTs and some HMTs). A single treatment (5 and 15 μM) was in fact able to determine a reduction in protein levels of variable entity, more pronounced in the DNA methylation maintenance compartment (UHRF1 and DNMT1). Also at the mRNA levels the investigated factors presented a general decreasing trend in expression in the first days following the treatment. However, both at protein and transcript levels we could observe only a transitory effect; indeed, the observed alterations were lost at longer time after the treatment. The same trend was evident also after repeated exposure (1 and 5 μM), where a tendency towards global reduction in protein levels 2 days after the 4th treatment was visible; again, these variations were lost one week after the end of the treatments, displaying the ability of HL-60 to recover to basal level in the absence of HQ. Interestingly, at the expression levels we could observe a transient increasing of all the investigated factors: the increment seemed to be dose-dependent. This trend, opposite to what was shown in protein, could be possibly due to post transcriptional mechanisms blocking the translation or to a cellular response to the falling levels of the analyzed factors.

Among the post-transcriptional mechanisms blocking the translation, miRNAs could have a relevant role, their action in fact can lead to the blocking of the translation of mRNAs. For example, the expression of UHRF1 in colorectal cancer is directly regulated by tumor suppressor miR-9 and in Non-small cell lung cancer by miR-193a-3p (Choudhry et al. 2018), miR-126 and miR-126* decreased DNMT1 expression (Solly et al. 2017) in low blast count acute myeloid leukemia (AML), in vitro studies showed that miR-125a-5p could directly suppress the SUV39H1 but also SUV39H1 could induce demethylation of miR-125a-5p, resulting in re-activation of miR-125a-5p in gastric cancer (Cai et al. 2018).

Hypothetically, the action of HQ could interfere with the expression of specific miRNAs involved in the regulation of specific epigenetic factors and vice versa, specific epigenetic factors could regulate the expression of specific miRNAs. Hypothetically, the action of HQ could interfere with the expression of

specific miRNAs involved in the regulation of specific epigenetic factors and vice versa, specific epigenetic factors could regulate the expression of specific miRNAs.

It has been demonstrated that aberrant miRNA profiles associate with chronic benzene poisoning. Since the focus of our study was to evaluate the chromatin modification and the DNA methylation, in this preliminary phase we didn't focus our attention on lnRNAs or miRNAs, but in the future it would be very interesting to evaluate the miRNAs expression profile.

Looking at the effect of a prolonged exposure to a low dose of HQ, it becomes clear that this benzene metabolite is indeed able to down-regulate the epigenetic components analyzed, confirming previous data in literature (Liu et al. 2012): the reduction was visible starting from the second week of treatment and was maintained in the following weeks. The alterations in mRNAs levels are less defined compared to the protein levels, with a tendency towards a decrease in DNMTs transcription (especially DNMT1) and a fluctuating behavior of UHRF1 and the HMTs investigated.

Given the alterations observed in DNMTs and the possible repercussion on chromatin, we investigated the DNA methylation status on the promoter region of LINE-1 and the expression levels of LINE-1. Actually, the decrease in global methylation is largely studied at heavily methylated elements, such as satellite repeats (i.e. Sat2) and retrotransposons (i.e. LINE-1) (Yang et al. 2004, Sahnane et al. 2015). In all the treatment settings we could not detect a significant difference in LINE-1 methylation, even if a slight reduction was appreciable. As for DNA methylation, we observed no significant variations in LINE-1 transcriptional levels in all the treatments. Overall, we could not reproduce *in vitro* the alteration in LINE-1 methylation observed in epidemiologic studies (Bollati et al. 2007), even after a long-term exposure to HQ: in fact, even prolonging the exposure was not sufficient to have statistically significant variations, clearly underlying the difficulties of a similar study in an *in vitro* setting. Another difficulty could be represented by the technical limitation of pyrosequencing: as reported by Bollati et al., the differences in percentage are narrow, and thus a higher number of samples would be needed to statistically reproduce and validate these variations.

These results could also indicate that global DNA hypo-methylation may only partially result from DNMTs inhibition induced by HQ exposure, as shown by different studies in which a mechanism of active de-methylation involving TET1 was suggested (Tahiliani et al. 2009, Coulter et al. 2013).

Conversely to DNA methylation, by focusing our attention on chromatin histone modifications we could observe a seemingly more pronounced effect of HQ prolonged exposure. Our attention focused mainly on the bivalent histone mark H3K27me3/H3K4me3, known to be fundamental during development (Iwagawa and Watanabe 2018) but also during cancer (Chapman-Rothe et al. 2013, Hahn et al. 2014).

Most interestingly, in HL-60 cells we could notice an increase of H3K4me3 while the H3K27me3 marker did not vary significantly, both in the single and the repeated treatments, indicating that even a single exposure to HQ is enough to perturb the structure of non-active DNA portion of the genome; the enrichment in H3K4me3 is in accordance to the results obtained by Li et al (Li et al. 2018). For both treatments, however, the effect on chromatin was lost at longer times, with the restoration of the basal condition, again confirming the ability of HL-60 to revert to the original conformation in the absence of HQ stimulus; this reversibility could be interpreted as a cellular mechanism of defense against environmental insults that can produce permanent modifications in chromatin, ultimately leading to alteration in the transcriptional levels of targeted genes/sequences.

Confirmation of the influence of HQ on histone modifications came from analysis of the long-term treatment samples, where we could detect a progressive effect from week 1 to week 4 with a gradual increase in the levels of H3K4me3 and the maintenance of H3K27me3 levels. Unfortunately, we could not evaluate at long-term the persistence of the alterations in the absence of HQ due to the instauration of cytotoxicity, as previously discussed.

Overall, the discussed results lead us to a paper publication (Mancini et al. 2017) demonstrating, after long-term treatment, the transient instauration of the distinctive signature combining the repressive H3Lys27 tri-methylation mark and the activating H3Lys4 tri-methylation mark, indicating a tendency toward a poisoned chromatin conformation. The persistence of H3K27me3, coupled with enrichment of H3K4me3, clearly shows the transition in these sequences towards a more permissive conformation following exposure to HQ, as illustrated in the hypothetical model in Figure 1.

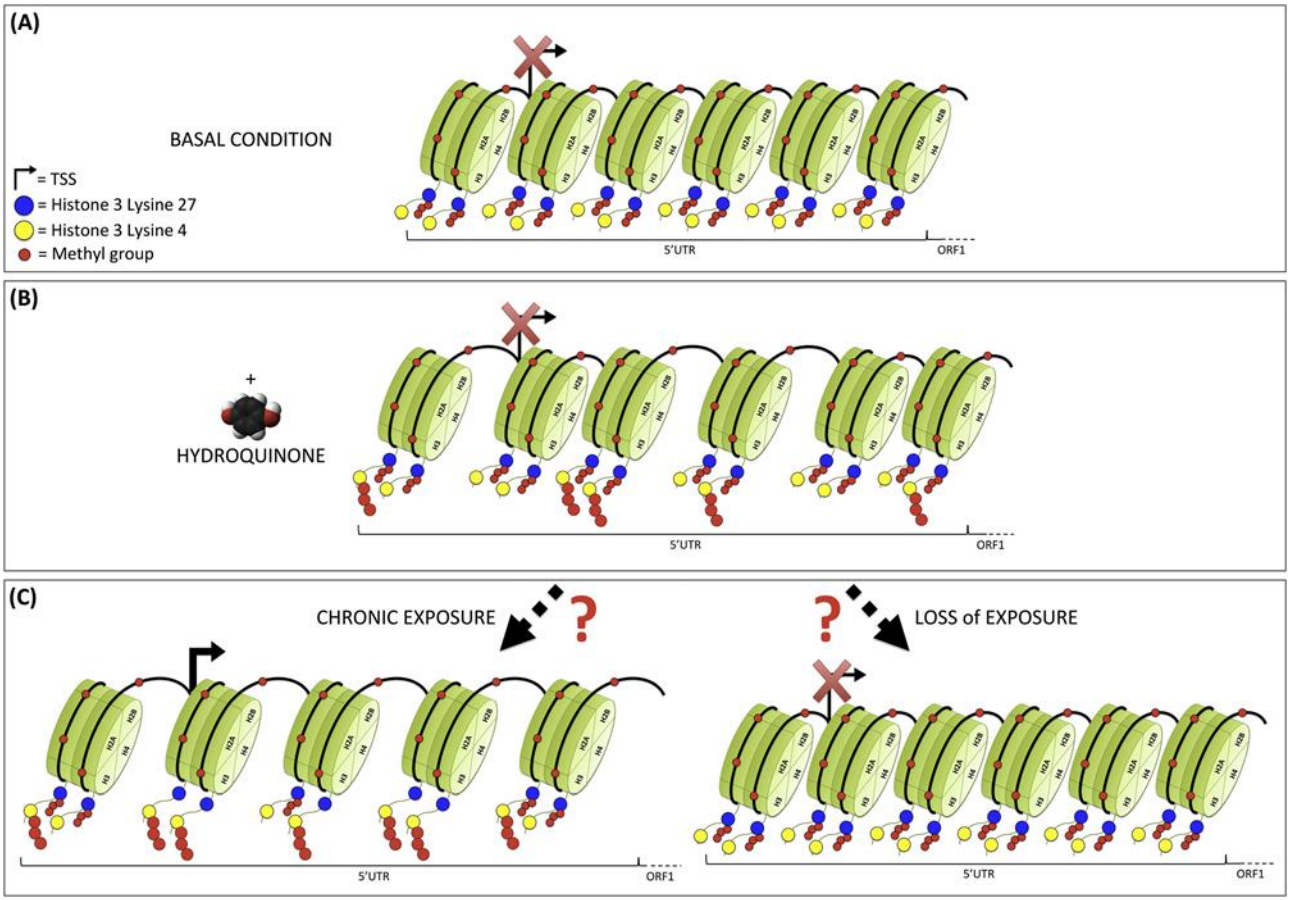


Figure 1 - Hypothetical model of chromatin alteration on LINE-1 5'UTR after treatment with HQ. (A) In basal condition, we observed the presence of repressive marks, such as H3K27 trimethylation (as well as UHRF1 and H3K9me2, not reported in figure); H3K4me3 was generally absent, as expected in transcriptionally-repressed sequences. (B) After treatment with HQ we observed the appearance of H3K4 tri-methylation, coupled with retention of H3K27 tri-methylation, indicating a poisoned chromatin conformation. These variations, however, were not stable in time and were not sufficient to determine significant variations in LINE-1 5-UTR transcriptional levels, probably due to the maintenance of repressive markers (like H3K27me3 and methylation). (C) Following chronic exposure to HQ, LINE-1 sequence could hypothetically lose the repressive marks and activate transcription, while in the absence of the pollutant the original conformation of the chromatin could be restored.

In this figure, we represented what we experimentally observed (1A and 1B) and the two hypothetical fates: if the exposure is removed (Figure 1C, left) we could observe restoration of the initial condition, with loss of trimethylation on K4 and persistence of K27me3; if the exposure becomes chronic (Fig. 1C, right) it might end up with the loss of repressive marks (K27me3) and activation of transcription in the presence of K4me3. The plasticity of this fundamental chromatin bivalent mark (H3K27me3/H3K4me3), firstly described in embryonic cells, is now known to play a role in poisoning non-active genes for aberrant transcription in cancer (Bernstein et al. 2006, Voigt et al. 2013). Several studies actually suggest that loss of this bivalent chromatin mark at promoters is accompanied by gene activation (when H3K27me3 is lost) or gene repression (when H3K4me3 is lost) during development, and this event is likely to be a critical

step also in cancer pathogenesis, responsible for the activation of crucial genes leading to progression and invasiveness, as demonstrated in colon and ovarian cancer (Chapman-Rothe et al. 2013, Hahn et al. 2014). The persistence of H3K27me3 observed by ChIP could be considered responsible for the absence of significant alteration in DNA methylation, even in the presence of H3K4me3, as well as for the absence of meaningful variations of LINE-1 transcriptional levels.

Our *in vitro* results are in agreement with a very recent epidemiological study in which it was demonstrated that exposure to low levels of benzene and its metabolites could correlate with modification of global H3K4me3 in the PBLCs and with the reduction of WBC counts of benzene-exposed workers, indicating that histone modifications play a key role in mediating the hematotoxicity of benzene exposure (Li et al. 2018). Moreover, in that study, it is demonstrated that after treatment of Primary human lymphocytes (PHLCs) with 0.1, 1.0, and 10.0 mM HQ for 48 h, H3K4me3 mark was enriched in the promoters of several DNA damage responsive (DDR) genes including CRY1, ERCC2, and TP53 (Li et al. 2018), confirming our findings reporting that even one administration of HQ is enough to perturbate the histone modification pattern.

To better understand the mechanisms of toxicity associated with long-term exposure we explored the effects of hydroquinone also on a model of non-hematopoietic stem cells, hUCMSC and we reproduced our long-term exposure setting to verify the repercussions on chromatin configuration. Stem cells in fact, can be highly vulnerable to the mutagenic or toxic effects of drugs or other chemicals early during differentiation interfering with this process (Snyder 2014) inducing alterations that can lead to cancer.

The first important consideration about this cell line comes from the evidence of the effect on cell growth that even the concentration of 1 and 0,5 µM exerted oh hUCMSC. Thus, we had to reduce the concentration used in HL-60 to 0,1 µM. Even after lowering the concentration by a 10-fold factor, nonetheless, we observed a progressive reduction in the cell number in the third and fourth weeks, and at week 4 a significant alteration on the percentage of cycling and apoptotic cells was detected.

As our goal was to obtain a stem cell model to study early events in response to HQ, thus distinguishing possible mechanisms of cell regulation (especially at the chromatin level) from cytotoxic effects (es. apoptosis), the treatment of hUCMSC with 0,1 µM seemed to be a good experimental model. In fact, HQ

at that concentration doesn't affect cell growth until the third week, leading us to speculate that in the first and second weeks HQ could induce early events involving mechanisms of cell regulation with an instauration of cytotoxic effects only during the third week, allowing us investigating potential epigenetic effects during the first week that might alter the progression of the cellular response to further three weeks treatment of low doses of HQ. Given these findings, we decided to carry on the epigenetic analysis only on samples collected up to the third week and the RNA-seq only in the first week.

As for HL-60, we could not observe an overall significant alteration in LINE-1 5'UTR methylation, except for one of the analyzed cytosines (C3): this may suggest a possible HQ specificity towards particular residues.

Surprisingly, in hUCMSC we detected a statistically significative reduction in the expression levels of LINE1 after the first week, but during the second and third week the expression returned to levels that are comparable to the NT.

Concerning the chromatin configuration of LINE-1 promoter in basal condition, we observed a very different basal situation in hUCMSC compared to HL-60. Moreover, it is very interesting to notice that in our control samples, the prolonged culture maintenance induced significant alteration in the analysed histone modifications, with the distinct increase of H3K4me3. Surprisingly, the exposure to HQ seemed to reduce the amount of H3K4me3 and increase the presence of H3K27me3 on LINE-1 promoter region, following an opposite behavior compared to what observed in HL-60.

Unfortunately, these results on hUCMSC were performed only once; further biological replicates are necessary to confirm the chromatin status and the LINE1 expression after exposure to HQ, investigating also other histone modifications in case of the confirmation of the results of the first week, and to confirm the dynamic of chromatin in stem cell maintained in culture for several weeks. It is however very peculiar to underline the apparent difficulty in the maintenance of stem cells, which undergo profound alterations even when cultivated following the guidelines of the supplier. These differences were appreciable not only on chromatin modifications but also on the profile of mRNA expression of the different passages of these cells: analyses of the transcriptome are still ongoing, but the preliminary Principal Component Analysis showed a significant distance between controls in the three biological replicates under

consideration. However, the distance between NT and 0,1 µM samples remained constant: this may indicate that the detected effects could be directly attributable to hydroquinone mechanism of action.

We have found a list of differentially expressed gene but further analysis are necessary to define in hUCMSC transcriptional profile after long-term exposure to low doses of HQ.

In conclusion, future investigations are needed to determine whether long-term exposed subjects might develop stable epigenetic alterations on chromatin that can precede alteration in DNA methylation, and, in turn, whether such alterations could increase the risk of cancer after exposure to benzene and its metabolites. Given the technical difficulties in setting *in vitro* experiments reproducing occupational and environmental exposures, epidemiological and *in vivo* studies are probably the best alternatives.

Additional studies are indeed required to investigate the mechanism by which benzene and its metabolites produce the alterations observed for the first time in this work, as well as the role of these modifications in AML development. On the other side, in non-hematopoietic stem cells the conditions are further aggravated by the complexity arising from the necessity to maintain unaltered in culture the cells; thus, the effects of HQ in a stem cell model would need more in-depth studies.

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