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"Assessment of genetic effects on human fibroblasts induced by *in vitro* exposure to continuous and pulsed non-ionizing electromagnetic signals"

"Valutazione degli effetti genetici indotti su fibroblasti umani da esposizione *in vitro* a radiazioni elettromagnetiche non ionizzanti con segnale continuo e impulsato"

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SINTESI

Negli ultimi anni le radiazioni elettromagnetiche non-ionizzanti ed in particolare le radiofrequenze (RF) (3 Khz – 300 GHz), sono ampiamente utilizzate in diversi ambiti, soprattutto in quello industriale, medico e delle telecomunicazioni. Ciò ha determinato un crescente interesse sui potenziali rischi per la salute associati all'esposizione a queste radiazioni.
Le radiazioni non-ionizzanti, a differenza delle ionizzanti, non posseggono energia sufficiente a rimuovere un elettrone da un atomo o molecola o a rompere un legame, ma vengono assorbite attraverso l'eccitazione di livelli roto-vibrazionali delle molecole, con conseguente incremento termico. Attualmente la normativa che regolamenta i limiti di esposizione a tali radiazioni si basa sugli effetti termici che sono stati ben dimostrati e caratterizzati.
Inoltre, poiché ad oggi esistono limitate evidenze scientifiche circa la correlazione fra esposizione a RF e insorgenza di tumori, l'Agenzia Internazionale per la Ricerca sul Cancro (IARC) ha classificato le RF come possibili cancerogeni per l'uomo (gruppo 2 B).
Giacché gli effetti termici sono ben noti, l'attuale interesse scientifico è rivolto all'individuazione di possibili effetti non-termici e di eventuali meccanismi coinvolti nella risposta biologica a tali radiazioni. In particolare, sono stati condotti numerosi studi *in vitro* per valutare i potenziali effetti genotossici delle RF.
I risultati di tali ricerche sono a tutt'oggi molto eterogenei e spesso discordanti, riportando in alcuni casi danno diretto al DNA, effetti aneugenici, instabilità genomica ed in altri casi nessun effetto. Non è quindi ancora stata stabilita una relazione causale tra esposizione a RF e rischio per la salute.
Un altro aspetto ancora poco esplorato è quello relativo agli effetti biologici indotti dalla modulazione del segnale elettromagnetico, impiegata in una vasta gamma di applicazioni (radar, applicazioni wireless, ecc.). I risultati delle scarse ricerche su questo argomento hanno prodotto dati contraddittori e questo parametro fino ad ora è stato poco considerato nella regolamentazione per esposizione a RF.
Un nuovo interessante approccio nello studio della risposta biologica alle RF, è mirato all'individuazione di geni sensibili che possono modulare il loro profilo di espressione in seguito ad esposizione a queste radiazioni. Anche in questo ambito sono stati condotti pochi studi i cui risultati restano poco chiari.

Le principali problematiche, che si evidenziano dalla revisione della letteratura scientifica e che rendono spesso difficile il confronto dei risultati ottenuti nei diversi studi, possono essere ricondotte principalmente all'utilizzo di sistemi di esposizione eterogenei, di differenti frequenze, SAR, modelli cellulari, tempi di esposizione valutati e un insufficiente numero di replicati sperimentali. Inoltre, gli studi in cui è stata valutata l'espressione genica presentano limiti metodologici dovuti alla tecnica utilizzata dei microarray e all'assenza della successiva validazione dei risultati mediante RT-PCR.
Il presente progetto di ricerca ha avuto lo scopo di studiare i potenziali effetti biologici indotti *in vitro* dalle RF, ponendo particolare attenzione alla messa a punto del disegno sperimentale e all'utilizzo di un sistema di esposizione ben caratterizzato.
Il largo impiego della tecnologia wireless, in ambito sia domestico che lavorativo, ha determinato una crescente preoccupazione sui potenziali rischi sulla salute derivanti da questa esposizione, il che ha indirizzato la scelta della frequenza di studio su 2.45 GHz. Gli esperimenti sono stati condotti con un SAR di 2 W/kg, corrispondente al valore limite di esposizione a RF stabilito dalle linee guida. Inoltre, sono stati valutati gli effetti di 2.45 GHz sia in modalità continua (CW) che impulsata (PW).
Nella prima fase di questa ricerca particolare importanza è stata data alla scelta e caratterizzazione del sistema espositivo. Confrontando differenti soluzioni progettuali, il sistema che meglio soddisfaceva i requisiti ottimali allo svolgimento dello studio, è risultato essere il Wire Patch Cell (WPC). Durante il set-up sperimentale, notevole attenzione è stata posta nel garantire che l'energia della radiazione fosse depositata all'interno del campione in modo omogeneo. Al fine di escludere gli effetti termici associati all'esposizione a questa frequenza, prima di procedere alle sedute espositive, sono state effettuate misurazioni della temperatura all'interno del campione nelle 2 ore di irraggiamento. L'incremento termico di 0.7 °C, osservato inizialmente, è stato ridotto a 0.25°C mediante l'utilizzo di ventole. Inoltre, al fine di mantenere le cellule in condizioni ambientali costanti (37°C, 5% CO_2), le esposizioni *in vitro* sono state effettuate all'interno dell'incubatore.
Per conferire maggiore robustezza scientifica allo studio, sul modello cellulare costituito da fibroblasti umani dermici, sono state condotte molteplici prove sperimentali utilizzando differenti metodologie.
Nello specifico, per valutare un eventuale danno cromosomico e chiarirne l'origine clastogenica o aneugenica è stato utilizzato il test dei micronuclei con metodo CREST, le rotture a doppio filamento del DNA (DSBs) sono state invece investigate con l'analisi dei foci di riparazione

γ-H2AX/53BP1. Inoltre, poiché l'arresto del ciclo cellulare è una conseguenza del danno al DNA, il potenziale effetto citotossico di tale radiazione è stato saggiato mediante analisi del ciclo cellulare.
Come atteso in base ai parametri espositivi utilizzati, in particolare il valore SAR, nel complesso i risultati di questo studio non hanno evidenziato danno genotossico, né aneugenico, né clastogenico. Inoltre non sono state osservate differenze significative tra i due tipi di segnale (CW e PW).
L'aspetto peculiare di questo studio riguarda l'analisi dell'espressione genica, effettuata per la prima volta in cellule umane esposte *in vitro* ad RF mediante l'innovativo approccio di sequenziamento dell'RNA.
L'analisi statistica dei risultati, basata sul False Discovery Rate (FDR), ha evidenziato un unico gene differenzialmente espresso nei fibroblasti esposti a CW. Tuttavia, utilizzando un approccio statistico meno stringente, sono stati individuati numerosi geni con differente profilo di espressione per entrambi i tipi di segnale valutati.
Questi geni non sembrano essere coinvolti in pathways specifici, ma è interessante notare che alcuni di essi sono correlati al citoscheletro, struttura spesso riportata negli studi sugli effetti biologici delle radiazioni non-ionizzanti. La risposta biologica di questi geni, ad ogni modo, sembra essere transitoria in quanto si evidenzia solo 2 ore dopo l'esposizione a 2.45 GHz CW, il che suggerisce che non ci sia un effetto sul processo di traduzione. Questa considerazione, concorda con i risultati dell'analisi ultrastrutturale sulle cellule esposte, in cui non sono stati osservati cambiamenti nella polimerizzazione dei filamenti di actina, suggerendo l'assenza di alterazioni del citoscheletro.
In conclusione, i risultati di questo studio rappresentano un contributo alla conoscenza scientifica sui potenziali effetti biologici non-termici associati all'esposizione a 2.45 GHz.
Inoltre, il disegno sperimentale del presente progetto di Dottorato, che ha previsto un sistema d'irraggiamento ben caratterizzato e un approccio metodologico multiparametrico, arricchito dall'innovativa tecnica di sequenziamento NGS, potrebbe costituire un modello sperimentale per future ricerche in questo settore.

ABSTRACT

The human exposure to non-ionizing electromagnetic field in particular radio frequencies (RF) (3 khz - 300 GHz), is growing over the last decades with the concomitant development of technological tools from both domestic and industrial uses, raising questions about their possible impact on human health.
The most widely accepted RF mechanism of interaction with biological system is thermal increase caused by the excitation of electrons to a higher vibrational/rotatory energy state when passing through matter.
Currently, safety standards for limiting human exposures to RF-EMF have been established on these effects.
To date, based on the limited evidence of an association between mobile phone use and cancer in both human and animal investigations, the International Agency for Research on Cancer (IARC), classified RF exposure as a possible human carcinogen (class 2B).
Several studies have been performed to investigate the biological and health non-thermal effects of RF-EMF, in particular many researchers evaluated the *in vitro* genotoxicity of these radiation, but the results are still controversial.
Results reported in literature are very heterogeneous with some studies reporting direct DNA damage, indirect genotoxic effects and other showing no effect, giving no consistent or convincing evidence of a causal relation between exposure and any adverse health effect.
An open question is related to possible different biological effects induced by signal modulation that occurs in a wide variety of RF applications (radar, wireless communications, broadcast communications and industrial processes). Few studies comparing the effects of continuous and modulated wave signals have been performed with no clear results, consequently modulation is a parameter poorly considered in most guidelines for limiting human exposure to RF-EMF.
A new interesting field of research on biological response to RF radiation is related to the identification of sensitive genes which could modulate their expression profile after exposures. To date few studies have been performed on this topic with no clear results.
Overall, the controversial outcomes from the studies reported in literature that often make difficult comparing the results obtained in the different laboratories, could mainly be related to heterogeneous exposure systems, different cellular models, frequencies, SAR, exposure times considered and insufficient number of experimental replicates.

Moreover, many of the studies evaluating gene expression modulation suffers of methodological limitation related to the approach used (microarrays) and lack of validation through RT-PCR.
In the light of these observations, the goal of this Ph.D. project was to perform a good quality *in vitro* investigation on the potential RF biological effects through a well designed experimental study and using a properly characterized exposure system.
We chose as frequency of interest 2.45 GHz, because the widespread use of Wi-Fi technologies in everyday life is leading to concerns about their possible health consequences. The selected SAR value for these experiments was 2W/kg, which is the maximum value recommended by the guidelines for limiting human exposures to RF-EMF.
Moreover, because of the growing interest on possible different biological effects related to signal modulation, the present study focused on the effects of 2.45 GHz with both continuous (CW) and pulsed (PW) signals.
Since one of the most critical point in these studies is related to inaccurate dosimetry and uncharacterized exposure source and conditions, in this project particular time and emphasis was addressed to identify the appropriate exposure system. Comparing different design solutions, the system that better satisfied our research requirements was a WPC-based exposure system. During the set-up of the system, particular attention was paid to ensure that RF-energy was deposited in a homogeneous manner within the sample, avoiding the temperature rise in some part of the Petri dishes with the formation of "hot spots". In order to evaluate the non-thermal effects of the studied frequency, before the *in vitro* experimental exposures a series of calibration measurements were performed to monitoring the temperature distribution inside the sample area using a fluoroptic thermometer, during the 2 hours of exposure. Since a temperature increase of 0.7°C was observed, an active cooling with forced air was used to reduce the temperature with a maximum thermal increase of 0.25°C. Moreover, to maintain cells under appropriate conditions (37°C, 5% CO_2) the *in vitro* exposures were performed inside the incubator.
We used as *in vitro* cellular model human dermal fibroblasts (HDF) and in order to obtain robust scientific results, several methodologies and at least three experimental replicates for end-points and type of exposures were performed.
To evaluate chromosomal damage and to identify the clastogenic or aneugenic origin, CREST micronuclei test was carried out, while DNA double strand breaks (DSBs) were identified by γ-H2AX/53BP1 foci

assay. Moreover the potential genotoxicity of the selected frequency was evaluated with cell cycle analysis, since cell cycle arrest is a consequence of DNA damage.
As expected, the results from these end-points suggest that 2.45 GHz did not induce genotoxic effects, neither aneugenic nor clastogenic at the SAR exposure limits recommended by European guidelines for limiting the exposures to RF-EMF. Moreover, no significant differences between the two type of signal tested (CW and PW) were detected.
This research is relevant especially with regards to the gene expression analysis, performed for the first time in human cells *in vitro* exposed to RF by the high-throughput RNA sequencing approach.
The results of this analysis showed no evidence of altered gene expression profile in exposed fibroblasts, except one, with FDR-adjusted statistical analysis in CW exposed samples. However, using a less stringency statistical approach several genes with different expression profiles were detected. Among these genes no pathways seems to be particularly affected, but interestingly some cytoskeleton-related genes were identified. Nevertheless their biological response seems to be transitory, appearing differentially expressed only 2 hours after exposure, suggesting no effect on protein translation process. This consideration seems to be indirectly supported by the ultrastructural analysis, that showed no morphological changes in the polymerization of actin filaments in exposed cells, suggesting that proteins involved in the cytoskeletal structure may not be affected under the exposure conditions evaluated in this study.
In conclusion the results of this Ph.D. project, based on well characterized exposure system and evaluating the potential biological effect using a multiparametric methodology approach, enhanced by the high-throughput NGS sequencing technology, aims at strengthening the scientific knowledge on the potential adverse effects of 2.45 GHz exposure. Moreover, it could be a procedure model for future researches on biological effects of non-ionizing electromagnetic radiation, which are essential for updated the guidelines for limiting RF exposures.

1. INTRODUCTION

1.1 Electromagnetic fields and non-ionizing radiation

According to the effects that radiation exerts on the matter, the electromagnetic field (EMF) spectrum can be distinguished in ionizing radiation (high-frequency radiation) and non-ionizing radiation (low to very low-frequency radiation) (Figure 1). Non-ionizing EMF, in the frequency range up to 300 GHz, are ubiquitous in our environment and over the last decades, the population exposure to EMFs has progressively increased with the concomitant development of technological tools from both domestic and industrial uses (Gajšek et al., 2016).

Non-ionizing radiation, differently from ionizing radiation, do not carry enough energy to remove an electron from an atom or molecule or to break a bond; however it has sufficient energy to cause the excitation of an electron to a higher vibrational/rotational energy state (Trosic and Pavicic, 2009; IAEA, 2010; Manna and Ghosh, 2016).

These radiations are classified into: High Frequency (HF) electromagnetic waves, also called Radiofrequency (RF), that comprises frequency ranging from 100 kHz to 300 GHz. This region lies between the low frequency (from 1 to 100 kHz) and the optical part of the spectrum that includes infrared, visible and ultraviolet radiations (ICNIRP 16/2009; IARC, 2013). Microwave (MW) radiation is located inside the RF region, with wavelength ranging from 1 mm to 1 m that corresponds to the frequency band between 30 kHz and 300 GHz (IARC, 2013).

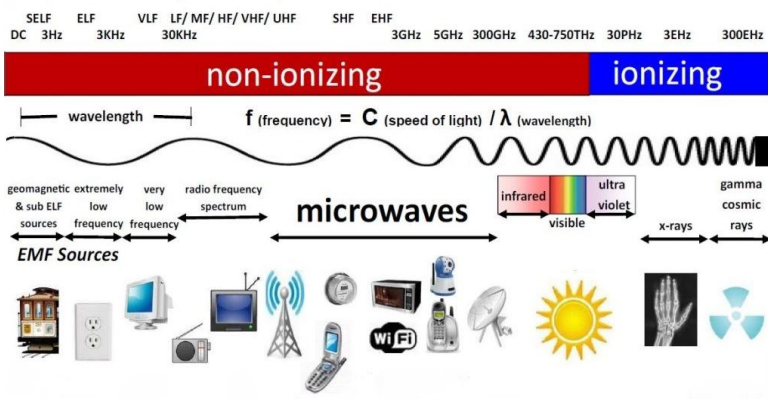

Fig. 1 Electromagnetic spectrum

1.2 Radiofrequency electromagnetic field (RF-EMF)

1.2.1 Applications

In the last decades, RF have been widely used in many applications like wireless communication devices and base stations, television and radio broadcasting facilities, radar, medical equipment, microwaves ovens and radiofrequency heaters as well as other electronic devices within our living and working environments (Foster et al., 2007; McNamee and Chauhan, 2009). During the last years, the use of telecommunication technology is growing worldwide and the use of mobile phones and related technologies like wireless local area networks (WLAN) has increased rapidly. Wireless fidelity (Wi-Fi) is the most popular wireless technology used and most of the devices operate at 2.4 –2.4835 GHz, while others operate in 5.15– 5.825 GHz (Peyman et al., 2011). These communication systems are now largely employed in a wide variety of environments including offices, schools, industrial and manufacturing facilities as well as private homes. Therefore, the increasing use of wireless devices in everyday life has led to concerns about potential health risks associated with increasing public exposure to RF field. Moreover, because the health effects of such equipment are still unclear (Tytherleigh et al., 2005; Obajuluwa et al., 2017), the Council of Europe recommends restrictions on the use of mobile phones and internet access in all schools across the continent to protect young children from potentially harmful radiation (Obajuluwa et al., 2017; Khalid et al., 2011; Peyman et al., 2011).

1.2.2 Safety Standard exposure guidelines

The risk assessment regarding the exposure to RF-EMF is performed by many national and international organizations, as the International Commission on Non-Ionizing Radiation Protection (ICNIRP), the Institute of Electrical and Electronic Engineers (IEEE) and the International Agency for Research on Cancer (IARC).
In 1998, guidelines on reference values, exposure limits, and restrictions (up to 300 GHz) were issued by the ICNIRP with the aim of protecting both occupational and general public against the possible harmful effects from the exposure to this type of radiation (ICNIRP, 1998). The current safety standards are essentially based on the evaluation of thermal effects

obtained in short-term (acute) exposures. The most important parameters used in determining the RF effects are: Specific Absorption Rate (SAR), which is the power absorbed per unit mass of human tissue (W/kg) and power density, which is the power absorbed per unit area (W/m^2). European guidelines often differentiate between occupational and general public exposure and the limits are respectively 0.4 and 0.08 W/kg for the whole body exposure and 10 and 2 W/kg for the head exposure (ICNIRP, 1998).
Recently, IARC has reviewed *in vitro* as well as *in vivo* (animals and humans) peer-reviewed scientific literature on the carcinogenic potential of RF exposure. Based on the limited evidence of an association between mobile phone use and cancer in both human and animal investigations, RF exposure is classified as a possible human carcinogen (class 2B) (IARC, 2013). Moreover, national and international expert groups of scientists have recommended a precautionary approach as well as further research (Verschaeve, 2012; Vijayalaxmi, Scarfi, 2014).

1.3 Biological effects of RF radiation

1.3.1 Thermal and non-Thermal effects

The most widely accepted RF mechanism of interaction with biological system is heating, since RF-EMF have sufficient energy only to excite an electron to a higher energy state inducing thermal increase (Trosic and Pavicic, 2009; Manna and Ghosh, 2016; Sienkiewicz et al., 2016). This heat is generated as a result of the RF energy absorption by water molecules, and since biological materials and tissue contain a considerable amount of water, they manifest strong phenomena of dispersion. As result of friction, dipolar molecule movements dissipates energy obtained from RF that leads to the conversion of electromagnetic energy into thermal energy, which is able to modify different reactions and molecules (McNamee and Chauhan, 2009; Manna and Ghosh, 2016). The specific biological responses to non-ionizing radiations are generally related to the rate of energy absorption that strongly depend on the frequency, intensity and orientation of the incident fields as well as the body size and the constitutive properties of the different tissues (dielectric constant and conductivity) (McNamee and Chauhan, 2009). Thermal effects occur with the temperature increase exceeding 1°C causing cellular and intracellular changes particularly at molecular level (Marjanovic et al., 2012).

A number of well-established biological effects and adverse health effects from acute exposure to intense RF radiation have been documented (Adair and Black, 2003; Foster and Glaser 2007; Manna and Ghosh, 2016) and they are mainly related to localized heating or stimulation of excitable tissue from intense non-ionizing radiation exposure (McNamee and Chauhan, 2009). The current safety standards guidelines are based on the thermal effects of RF-EMF (ICNIRP, 2009). Since there has been an increasing concern about chronic or long-term exposures to the low-level RF-EMF, understanding the involvement of non-thermal effects of RF has been the focus of several studies. Non-thermal effects occur when the RF intensity is sufficiently low, that the amount of energy involved would not significantly increase the temperature of a cell, a tissue or an organism, but may induce some physical or biochemical changes. Some studies reported some non-thermal effects (Behari et al., 2010; Giuliani et al., 2010; Mohammed et al., 2013), as changes in permeability of the blood brain barrier and ocular symptoms (Curcio et al., 2005; Balik et al., 2005). Even if some hypotheses about the interaction between RF and biological tissues (i.e. calcium efflux and free radical production) has been proposed, the underlying mechanism responsible for the non-thermal effects of RF-EMF is still under investigation (Mohamm et al., 2013; Consales et al., 2012).

1.3.2 Modulation-dependent biological effects

An open question in RF-EMF research field is related to possible different biological effects exerted by continuous-wave (CW) and different type of modulated signals. CW signals consist of sinusoidal oscillations at a single frequency between 0.1 MHz to 300 GHz. In order to enable it to carry information, the carrier is modulated (varied in frequency or amplitude). Different telecommunication technologies use a wide range of modulation schemes. Most of these involve amplitude modulation (AM), in which the carrier wave is modulated by a low frequency signal. The frequency modulation (FM) involves modulation of the frequency so that it varies in a narrow band around the basic frequency. Pulse modulation (PM), is a form of amplitude modulation, such as that used for radars, which emit very high-intensity, short duration pulses. While rather basic AM and FM signals have been used in radio broadcasting and in analogue telephone, modern digital telecommunication technologies are based on complex modulation

schemes that include combination of different forms of modulation (Juutilainen et al., 2011).
To date, few studies compared the effects of CW and modulated RF signals, or compared the effects of different kinds of modulation (Reviewed in Juutilainen et al., 2011) and modulation has played only a limited role in development of most guidelines for exposure of humans to RF energy (Foster and Rapacholi, 2004). Recently, the increasing human exposure to RF energy from wireless communication systems led to a growing interest of possible modulation-specific effects. Since these are kind of signals having a total different time behavior, the question of possible specific modulation dependent effects is of fundamental importance to address future research on biological and health effects of RF-EMF.

1.3.3 Studies on health and biological effects: State of art

Several studies have been performed to investigate the biological and health effects of RF-EMF and the most investigated frequencies are those used for mobile and wireless communication systems: 900 MHz, 1.8 GHz and 2.45 GHz (ICNIRP 16/2009). The results from these researches are still controversial (reviewed in Kundi et al., 2004; Verschaeve et al., 2010; Vijayalaxmi and Scarfì, 2014; Manna and Ghosh, 2016), and could be related to the heterogeneity of exposure systems, the different magnetic stimulation parameters, the inadequate dosimetry and the variability of responses depending on the different experimental models used (Paffi et al., 2010; Vijayalaxmi, 2016).
Studies to evaluate RF-EMF biological effects can be classified into human epidemiological, animal (*in vivo*), and cellular (*in vitro*).

Epidemiological Studies

These type of studies are fundamental to provide direct evidence of RF-EMF exposure health effects, in particular related to the growing use of mobile phone and wireless technologies. To date, most researches have investigated the carcinogenic potential of RF exposure, especially, radiation-induced brain cancer, since the mobile phone is usually held close to the head.
Among the epidemiological studies, cohort studies are the more expensive and show difficulties in determining the individual dose estimation. Moreover, a large number of subjects is needed to achieve a

sufficient statistical power to detect presumable small health risks (Breckenkamp et al., 2009).

Differently from the cohort studies, case-control studies generally focus on a reduced numbers of subjects and the assessment of occupational RF-EMF exposure seems to be more feasible. Hence, case-control study designs might be a more powerful approach to estimate the association between RF-EMF exposure and health risks (Breckenkamp et al., 2009).

The most important international, largely population-based case-control study performed until now, the INTERPHONE study (2010), was conducted in 13 countries using a common protocol. It focused on the relationship between mobile phone use and the risk of developing tumors in tissues that most absorb RF energy emitted by mobile phones: brain tumors (glioma and meningioma). Overall, results from this study report no increase in risk of glioma or meningioma with use of mobile phones. There were suggestions of an increased risk of glioma at the highest exposure levels, but biases and error prevent a causal interpretation. Nevertheless, the possible effects of long-term heavy use of mobile phones require further investigation.

In other epidemiological investigations on the potential effect of mobile phone use, an increased risk in developing glioma associated to short-term exposure to mobile phone use was reported in two studies (Auvinen et al., 2002; Hardell et al., 2006). All the studies evaluating meningioma gave no consistent evidence of an increased risk of this tumor among mobile phone users, but this result is questionable because of the short period of observation, since meningioma is characterized by a long latency period (30 years or more) (Ahlbom et al., 2009). Although there are currently many epidemiological investigations available on the health risks associated to the exposure to RF, the results are still inconclusive an there are no convincing evidence of a causal relation between RF exposure and any adverse health effect. These studies have also several deficiencies that make difficult to rule out a cause-effect relationship. The most common problems in all these studies are related to the assessment of exposure, the number of subjects employed, that is small and insufficient to achieve an adequate statistical relevance to detect presumable small health risks, and the follow up period is often too short (Ahlbom et al., 2004).

In vivo Studies

Studies on experimental animals are suitable for acute, short and long-term RF exposure investigations. These investigations play an essential role in evaluating the integrated response of different organ system, in

particular the nervous, endocrine and immune systems, that are largely responsible for homeostasis, which maintains the steady-state of the internal environment. Otherwise, when a living organism is challenged by external stimuli, the interdependent response of these systems cannot be fully defined through *in vitro* experiments. Moreover, animal studies provide the opportunity to test whether lifetime exposure to well characterized RF sources is related to cancer. However, the main limitation of these studies is the extrapolation of the observations in animals to humans, in fact responses observed in animal studies do not necessarily imply a health risk for humans (Vijayalaxmi 2016).

Thus far, *in vivo* investigations have examined the effect of different RF frequencies, power intensity, modulation, SAR, duration of exposure, etc. in bacteria, nematodes, normal and sensitive strains of fruit fly, normal rats, normal mice as well as transgenic and tumor-prone mice, rabbits and primates. The influence of RF exposure on behavior, blood-brain barrier, chemical and hormone levels, genetic damage, immune and neurochemistry, oxidative stress, reproduction and development, longevity and carcinogenesis, etc. have been investigated (Vijayalaxmi 2016).

In particular, genotoxic effects were assessed through comet assay, chromosomal aberrations, micronuclei and mutations. In a research performed by Trosic et al. (2002) on rats exposed to continuous 2.45 GHz for 2 hours/day, the authors reported a significantly increased MN frequency in the peripheral blood after a period of 8 days, whereas no induced MN frequency was observed after 15 and 30 days. In another investigation, performed on C3H/HeJ mice exposed to continuous 2.45 GHz for 20 hours/day, 7 days/week over 18 months, a small but statistically significant increase of MN was observed in peripheral blood lymphocytes (Vijayalaxmi et al., 1998). On the other hand, no increase In MN frequency was observed in bone marrow cells from rats exposed to continuous 2.45 GHz for 24 hours (Vijayalaxmi et al., 2001).

Among the studies evaluating single and double DNA strand breaks using the comet assay, Lay and Singh (1995, 1996) found a significant increase in DNA strand breaks in rat brain cells immediately and 4 hours after 2 hours exposure to 2.45 GHz. Conversely, in a replication study, Malyapa et al. (1998) didn't confirmed the earlier data.

Overall the *in vivo* studies, performed until now have not demonstrated convincingly direct DNA damage after acute or chronic animal exposure to RF radiations.

In vitro Studies

In vitro studies are the most common for the evaluation of RF-EMF biological effects. They are carried out on tissue or cell cultures of animal or human origin, on transformed or primary cells and cell lines.
Moreover, they are relatively simple to handle and the control of experimental condition, including exposure, is better than *in vivo* animal or human studies because they represent well-described models.
These *in vitro* investigations examined the effect of different RF radiation, power intensity, modulation, SAR, continuous or intermittent exposure, duration of exposure, combined RF exposure with known environmental agents, evaluating different cellular end-points.
In particular *in vitro* genotoxicity studies are important because one of the main concern with RF radiation is if they have the ability to induce genetic damage within cells which is a hallmark of cancer cells (Manna and Ghosh, 2016). The genotoxicity can be exerted on DNA molecule and/or chromosomes, causing structural or numerical aberrations.
DNA damage induced by RF exposure as single and double strand breaks is mainly assessed using comet assay. It is based on the principle of electrophoretic migration of damaged DNA away from the nuclei immobilized in agarose gel, forming a structure resembling a comet and the damage is evaluated by the comet tail length that reflects the frequency of DNA breaks (Ruediger et al., 2009). Some investigations using this methodology on human blood lymphocytes and on human dermal fibroblasts did not report DNA damage (Speit et al., 2007; Franchini et al., 2018) but other studies described this effect in rat brain cells (reviewed in Vijayalaxmi and Obe, 2004, and Verschaeve et al., 2010). A research group, surprisingly, observed a significant decrease in DNA single strand breaks (SSBs) following exposure of Molt-4 Tlymphoblastoid cells at cellular phone frequencies (813.56 and 836.55 MHz) at different SAR values (Phillips et al., 1998). Some authors suggested that these controversial results can be due to the use of comet assay with different details in the protocols and of different cellular models (Vijayalaxmi and Obe, 2004).
In the last years, to identify DNA double strand breaks (DSBs) a more specific and sensitive method that consist in evaluating the formation of phosphorylated H2AX histone (γ-H2AX) and P53 binding protein (53BP1) was used. Both H2AX and 53BP1 are DNA damage checkpoint proteins, rapidly phosphorylated few minutes after DNA damage and are then gathered in the vicinity of DNA DSBs. Here they form foci which represent an initial and specific step in the repair process of exogenously induced DSBs (Ruediger et al., 2009) and can be visualized as

fluorescence spot inside the nuclei by indirect immunofluorescence technique (Fernandez-Capetillo et al., 2002; 2004).
Some researches, in which the evaluation of DNA damage was performed by the identification of γ-H2AX alone or in association with 53BP1, found no induction of DNA DSBs (Danese et al 2017; Franchini et al., 2018). In a study of Markovà et al. (2005), DSBs were evaluated in lymphocytes, from healthy persons and from persons reporting hypersensitivity to RF, exposed to 905 and 915 MHz. The authors observed no difference in the number of foci between healthy and hypersensitive subjects *in vitro* exposed to 915 MHz whereas results of 905 MHz exposure showed an individual variation in the foci number, suggesting a carrier-frequency dependent effect. The same effect was hypothesized in a subsequent study (Belyaev et al., 2009), in which changes in γ-H2AX/53BP1 repair foci were observed in lymphocytes, from hypersensitive and healthy subjects, exposed to 915 MHz GSM and 1947 UMTS. Other authors, intermittently exposed to GSM 1.8 GHz Chinese hamster lung cells for 1 or 24 hours, describing an increased number of γ-H2AX foci only at 24 hours exposure (Zhang et al., 2006).
The most used approach to evaluate chromosomal damage is the cytokinesis-block micronucleus assay developed by Fenech and Morley (1985), assessing micronuclei (MN) inside binucleated cells. Micronuclei may originate from acentric chromosomes fragments (clastogenic effect) or from whole chromosomes (aneugenic effect) that are enable to interact with the spindle resulting not included in the main daughter nuclei during mitosis.
Several studies evaluating chromosomal damage reported MN induction after exposure to RF radiation. Among them, Maes et al. (1993) observed a higher frequency in chromosome aberrations and MN in human peripheral blood lymphocytes exposed to 2.45 GHz for 120 min (SAR 75 W/kg) respect to controls. An increased MN frequency was also reported in another study on human peripheral blood lymphocytes *in vitro* exposed to electromagnetic fields with different frequencies (2.45 and 7.7 GHz) and power density (10, 20 and 30 mW/cm^2) for three time of exposures (15, 30 and 60 min) (Zotti-Martelli et al., 2000). The authors observed a MN induction mainly for both high power density and long exposure time. In a subsequent study (Zotti-Martelli et al., 2005), carried out on human peripheral blood lymphocytes exposed to 1.8 GHz CW at different power density (5, 10 and 20 mW/cm^2) and time (60, 120 and 180 min), a higher MN frequency with short-time exposures to medium power density fields was reported.

On the other hand some studies did not report chromosomal damage. McNamee and colleagues (2002), performed the cytokinesis-block micronucleus assay and alkaline comet assay on blood cultures exposed for 2 hours to 1.9 GHz pulsed modulated RF fields at mean SARs ranged from 0 to 10 W/kg. The results showed no evidence in terms of primary DNA damage between sham and exposed samples to any SAR tested. No significant differences in the frequencies of chromosomal aberration and MN between exposed or sham samples were observed by Vijiyalaxmi et al. (2006), that *in vitro* exposed blood lymphocytes for 24 hours to 835.62 MHz to SAR of 4.4 or 5.0 W/kg.

One of the targets in researches evaluating the effects of RF exposures, consist in the detection of possible numerical chromosomes aberrations, defined aneuploidies, as a marker of genome instability. Some authors performed both conventional MN analysis and comet assay in order to indirectly deduce an aneugenic or clastogenic damage combining the results from these two approaches. Among these studies McNamee et al. (2002), did not observe DNA damage or increased MN frequency in blood cultures exposed to 1.9 GHz RF field for 2 hours. No effects on human lymphocytes exposed to 935 MHz for 24 hours (SAR 1-2 W/kg) were reported in another study (Stronati et al., 2006). Also Speit et al. (2007), that exposed human fibroblasts and Chinese hamster cells to 1.8 GHz (SAR 2 W/kg), reported no DNA damage and MN induction in both the cell lines.

Few studies have been performed using specific assays to directly evaluate aneuploidy. One of the mainly used method includes a variant of the conventional MN assay, based on indirect immunofluorescence using kinetochore antibodies on binucleated cells that thanks to the presence or absence of the immunofluorescence signals allows to distinguish between MN arising from acentric fragments or whole chromosome.

Another approach used is based on the fluorescence *in situ* hybridization (FISH) techniques applied to specific chromosomes on interphase nuclei, the distribution pattern of centromeric probe signals in the two daughter nuclei allows to visualize chromosomal nondisjunction events.

Among these studies, some authors reported an increased number of aneuplodies in exposed samples respect to sham samples. Schwarz et al. (2008), exposed human cultured fibroblasts to 1.95 GHz (SAR< 2 W/kg) for 8, 12 or 24 hours and evaluated the genotoxic damage by alkaline comet assay and MN frequency and their origin was determined by fluorescence labeled anticentromere antibodies. Results showed in cells exposed for 24 hours at 0.5 W/kg, an enhanced comet tail factor

response and an increased MN formation, based on the presence of acentric fragments indicating not chromosome loss but DNA damage.
In a study performed on human peripheral blood lymphocytes, *in vitro* exposed for 72 hours to 830 MHz (SAR 6-8.8W/kg) the anleuploidy of chromosome 17 was evaluated. Using FISH on interphase cells a linear increase in the aneuploidy of this chromosome was observed as a function of the SAR value (Mashevich et al., 2003). Also Mazor and colleagues (2008) evaluated aneuploidy on human lymphocytes *in vitro* exposed to continuous 800 MHz (SAR 2.9-4.1 W/kg) using an interphase FISH approach on four pairs of chromosomes (1, 10, 11 and 17). For higher SAR value an increased aneuploidy was observed for chromosome 1 and 10, while for chromosomes 11 and 17, the increases were observed only for the lower SAR. The results of this study suggest the possible existence of a non-thermal effect of RF radiation that causes increased chromosomes number alteration. Aneuploidy induction due to chromosome loss was reported in a recent study performed by Franchini et al. (2018) on human fibroblasts exposed for 20 min to 25 GHz (SAR 10 W/kg). The aneuploidy was assessed evaluating the origin of MN by fluorescence labeled anticentromere antibodies and chromosome distribution in binucleated cells by interphase FISH for chromosomes 4, 10 and 17.
Conversely, Bourthoumieu et al. (2011) in a study on human amniotic cells *in vitro* exposed for 24 hours to 900 MHz (SAR 0.25, 1, 2 and 4 W/kg) using interphase FISH on chromosomes 11 and 17, found no significant change in the rate of aneuploidy for these chromosomes.
To date few studies have been carried out on the possible different biological effects induced by RF specific type of signal (i.e continuous and pulsed wave) and the results are rather unclear.
D'Ambrosio et al. (2002), reported an induction of MN and chromosomal aberrations in lymphocyte cultures exposed to phase modulation RF-EMF at 1.740 GHz, but not after the corresponding exposure to a CW field. In another study (Campisi et al., 2010), astroglial cell cultures were *in vitro* exposed to 900 MHz continuous or amplitude modulated waves for different times. A significant increase in DNA fragmentation and a higher reactive oxygen species (ROS) levels was observed only after exposure to modulated signal for 20 min. No evident effects were detected when shorter time intervals of modulated exposures or continuous waves were used. Tice et al. (2002), evaluated the effects of continuous and modulated 837 MHz on human lymphocytes and reported that continuous signal did not induced a significant increase in DNA DSBs or MN, whereas the modulated signal

induced an increased number of MN. Other investigations didn't report modulation dependent effects as in Maes et al. (2001), which evaluated cytogenetic effects on human lymphocytes exposed for 2 hours to 900 MHz with different type of signals and SAR values between 0 and 10W/kg. No significant differences in primary DNA damage and MN induction were observed in human leucocytes *in vitro* exposed to 1.9 GHz continuous and pulsed signals (McNamee et al., 2002).

Other extensively investigated end-points, to evaluate the biological effects of RF, are related to cell proliferation and cell cycle analysis. Cell proliferation is a basic cellular process and is influenced by changes in the cell cycle distribution and rate of DNA synthesis. Cell cycle arrest is a consequence after DNA damage, it is therefore important to evaluate the RF-EMF effects on cell cycle to assess its genotoxic potential (Manna and Ghosh, 2016). Few studies reported changes in cell proliferation, as Velizarov et al. (1999), that reported a significant decrease in cell proliferation in transformed human epithelial amnion cells exposed to 960 MHz amplitude-modulated field. Total inhibition of cell proliferation was observed in human breast fibroblasts after 2.1 GHz modulated exposure (Esmekaya et al., 2013). An impaired cell cycle progression through G2-M arrest, was observed after 24 hours of exposure to 900 MHz by Buttiglione et al. (2007). A lower fraction of cell population undergoing mitosis was observed in V79 cells exposed to 2.45 GHz (Ballardin et al., 2011). Conversely, most studies performed on different cellular models evaluating the effects of various RF radiation frequencies reported no changes in cell proliferation kinetics and cell cycle distribution (Zeni et al. 2003; Miyakoshi et al., 2005; Merola et al., 2006; Sanchez et al., 2006; Gurisik et al., 2006; Lee et al., 2011; Franchini et al., 2018).

A new interesting field of research on biological response to RF radiation, is related to the identification of sensitive genes which could modulate their expression profile after exposures. To date few studies with not clear results have been performed on this topic. Gene expression is an intracellular metabolic process in which a DNA sequence (gene) is transcribed into the correspondent mRNA. Some genes are expressed quite uniformly with little variation over time, routinely producing proteins to maintain the normal functions in the cell, while expression of other genes can be induced or repressed by signals that depend on external agents or pathological conditions (Malone and Oliver, 2011).

The methodological approach mainly used to assess the mRNA expression level in a cell is microarray analysis using DNA chips, that

allows the detection of a number of preselected known genes. With the improvement of microarray technology (i.e. Affymetrix) it has been possible to evaluate the expression profile of all the known genes (Bumgarner, 2013).
Current microarrays do not always detect responsive genes accurately and have a high probability of detection of false positives, while small changes in expression may not be detectable. However, candidate responsive genes require confirmation by RT-PCR, that is considered the gold standard to evaluate gene expression profiles.
A particular focus in gene expression studies are heath shock proteins (HSPs), commonly used markers of cellular stress. Several investigations suggest that non-thermal RF radiation exposures do not determine cellular stress response characterized by altered HSP gene or protein expression (Chauhan et al., 2006; Nylund and Leszczynski, 2006 ; Chauhan et al., 2007; Hirose et al., 2007). A limited number of studies reported an over expression of HSP (Leszczynsky et al., 2002; Miyakoshi et al, 2005; Sanchez et al., 2007) with an increasing HSP production that influences signal transduction pathways of particular interest (Leszczynsky et al., 2002).
The RF exposure effects on proto-oncogenes expression profile has also been studied, evaluating in particular *c-myc*, *c-fos* and *c-jun*, which are early responses genes involved in cell proliferation.
Some studies reported that RF radiation may affect the expression of a number of proto-oncogenes in exposed cell (Morrissey et al 1999; Goswami et al., 1999), whereas others showed no effects of RF exposure on the expression of these genes (Chauhan et al., 2006; Chauhan et al., 2006; Whitehead et al., 2005; Finnie et al., 2006; Finnie et al., 2007).
Zhao et al. (2007) showed that intermittent exposure of rat neurons to 1.8 GHz increase or decrease the expression of genes involved in multiple cellular functions involving cytoskeleton, signal transduction pathways and metabolism.
Some authors reported a cell dependent effect of RF exposure on gene expression, in particular Remondini et al. (2006) *in vitro* exposed six human cell types to 900 MHz and 1.8 GHz, and Nylund and Leszczynski (2006) evaluated the effects of 900 MHz on two variant of endothelial cells. Among the large-scale studies using microarray technology, Le Quèment et al. (2012) showed that 60 GHz had no massive effect on human keratinocytes but could change the expression levels of some genes. In a subsequent study (Habauzit et al., 2014), performed on the same cellular model and frequency, whole transcriptome analysis using

microarray technology revealed a slight but specific radiation effect on gene expression in hyperthermia conditions.
An *in vivo* study, reported that chronic exposure to 2.45 GHz increased the expression of some microRNAs (miRNA) in rat brain (Dasdag et al., 2015). In another recent *in vivo* study McNamee et al. (2016), evaluated gene expression changes in several rodent brain regions exposed to 1.9 GHz (pulsed and continuous wave signal; 4 hours/day for 5 consecutive days). No consistent changes in gene expression were observed using a False Discovery Rate (FDR) statistical approach whereas a number of genes showed a differentially expressed profile using a less stringent statistical approach.
In several other studies however, no effect on gene expression was reported on different cellular models exposed to various RF radiation using microarrays (Chauhan et al., 2006; Qutob et al., 2006; Sakurai et al., 2011) and using the more advantageous affymetrix technology (Whitehead et al., 2006; Zeng et al., 2006; Hirose et al., 2007).
Overall, the results of studies about RF fields effects on gene expression, performed by microarray technology, shows that it is difficult to find a marked cellular response to RF, probably due to the heterogeneous exposure system, cell line, frequency, SAR and exposure time used in these investigations. The advent of high-throughput genome sequencing technologies (Next Generation Sequencing, NGS) that allows also the whole transcriptome analysis through RNA sequencing (RNA-seq), seems to be a powerful and promising technology in these type of studies.
To date, only one recent *in vivo* study, used this high-throughput sequencing RNA-seq (Illumina HiSeq 2000) to evaluate the genome-wide mRNA expression profile in Caernorhabditis elegans under prolonged exposure to 1.750 GHz radiation. Results showed that these exposure significantly promote gene expression changes (Gao et al., 2016).

1.4 Gene expression and whole transcriptome analysis using NGS

The tools for evaluating mRNA expression have been available for years and the mainly used approaches includes: reverse-transcription PCR (RT-PCR) and microarrays.
RT-PCR, has become one of the most widely used method to detect changes in gene expression because of its large dynamic range, high sensitivity, accuracy, specificity, reproducibility and low cost. Until

now, quantitative RT-PCR assay is considered the gold standard for measuring the number of copies of specific cDNA targets. The main problem related to the use of this methodology, is due to the fact that it allows the analysis of one gene (of known sequence) each time, being a time consuming approach (Costa et al., 2013).

The entire set of RNA molecules or transcripts (messenger RNAs, microRNA, long noncoding RNAs) produced in a population of cells or tissues represent the trancriptome. The goal of whole transcriptome analysis is to identify genes differentially expressed among different conditions, leading to a new understanding of the genes or pathways associated with different conditions (Malone and Oliver, 2011).

The rapid quantification of the transcriptome became possible only with the development of gene expression microarrays (Schena et al., 1995). Since the introduction of DNA microarrays in the 1990s, it has been the technology of choice for large-scale studies of gene expression. The ability of these arrays to simultaneously interrogate tens of thousands of transcripts has led to important advances in addressing different biological issues including identification of genes that are differentially expressed between diseased and healthy tissues, in response to different pharmacological treatment or to chemical and physical agents and in other specific conditions compared to a basal status (Hatfield et al, 2003; Passador-Gurgel et al., 2007). Currently, microarrays remain the most popular approach for transcript profiling and can be readily afforded by many laboratories. The improvement of microarrays technology (i.e. Affymetrix), permits to perform the transcriptome analysis but with the limit of evaluating the expression profile of only the known genes for which the relative probes could be designed. Other limits are the background hybridization that reduces the accuracy of expression measurements, particularly for transcripts present in low abundance and that the probes differ considerably in their hybridization properties (Zhao et al., 2014).

Recently, through advancements in the fields of molecular biology and technical engineering, impressive progress has been made in the genome sequencing methodology by Next Generation Sequencing (NGS), giving a substantial contribution to the understanding of genome expression and regulation (Maguerat and Bähler, 2010). These technologies are being exploited not only to analyse static genomes but also dynamic transcriptome in an approach termed RNA sequencing (RNA-seq). This innovative methodology is also used to analyse small RNAs, including identification of differentially expressed miRNAs, prediction of novel

miRNAs, and annotation of other small non-coding RNAs (Lee et al., 2013).

The main advantages of the high throughput RNA-seq over microarrays approaches are that it gives the possibility to identify novel transcripts, does not require a sequenced genome and overcomes background noise associated with fluorescence quantification. Furthermore, unlike microarray technology, RNA-seq allows genome-wide analysis of transcription at single nucleotide resolution, including identification of alternative splicing events and post-transcriptional RNA editing events (Costa et al., 2013). Different NGS sequencing platforms (Illumina, Applied Biosystems ABI SOLiD, 454 Pyrosequencing Roche Genome Sequencer) are available, based on different sequencing technologies. Although all NGS platforms perform sequencing of millions of small DNA fragments in parallel, they differ regarding price, throughput, read length and generation and error rate. Currently, Illumina industry offers the highest throughput of all platforms, the lowest per-base cost and allows to obtain read lengths of up to 300 bp, compatible with almost all types of application (Liu et al., 2012).

The raw data generated at the end of the sequencing process, consists of short nucleotides sequence, named reads. These data are most often supplied in FASTQ format, that contains an ID number for each read, the read sequence and a quality score, which indicates the reliability of each base call (Mutz et al., 2013).

From a RNA-seq experiment a large volume of data are generated, for example NextSeq 500 (Illumina) can produce up to 30 Gb (75-cycle high output kit) and 400 million clusters (high output kits) as maximum reads per run. Dealing with the large amount of RNA-seq data is a time-consuming and challenging step of the analysis pipeline.

The major analysis steps involves: quality control, read alignment with a reference genome or transcriptome and approaches for detecting differential gene expression.

Quality assessment of the raw data is the first step of the bioinformatics analysis, and it is also a prerequisite before analysis. This step consist of removing low-quality sequences or base adaptors, contaminations or overrepresented sequences to improve read quality to ensure a coherent final result. Once high quality data are obtained, the next step is to map the short reads to the reference genome or to assemble them into contigs and align them to the reference genome.

Read alignments can be performed among multiple genome annotation database, RefGene, Ensembl and the UCSC annotation database are the most popular ones (Han et al., 2015).

After mapping the reads to the reference genome, an important step is the quantification of reads that map to a gene, that correspond to the measurement of the gene's expression level.
One of the most crucial step in the analysis pipeline consists in the data normalization in order to eliminate variation between samples, that may be due to contamination as well as biological reasons, and to enables the comparison between different genes as well as different experiments.
One of the most commonly used normalization method is R package EDASeq (Han et al., 2015).
An important application of RNA-seq is the comparison of transcriptomes among different developmental stage, disease conditions compared to normal cells or specific experimental stimuli compared to physiological conditions. Differential gene expression analysis (DGE), requires that genes expression values should be compared among samples.
Regarding the RNA-Seq DGE analysis, some software package such as edgeR (Robinson et al., 2010) and baySeq (Hardcastle and Kelly, 2010), adopt the negative binomial model as the main approach. Some other methods are based on transcript detection, which have been developed in order to identify unknown transcripts or isoforms and can be also applied to the identification of DEGs, are EBSeq (Leng et al., 2013) and Cuffdiff2 (Trapnell et al., 2013).
To date, there is not complete agreement about which statistical approach is the most appropriate or ensures the validity of the results in terms of robustness, accuracy and reproducibility, so this topic still needs more researches.

1.5 Challenges in studies evaluating the biological effects of RF-EMF

The scientific literature on biological effects of RF-EMF show a large number of controversial results that can in part be attributed to inaccurate dosimetry and to lack of well-defined exposure conditions. In fact, several issues must be considered in planning experimental researches for the evaluation of the non ionizing radiation biological effects in order to perform high-quality *in vitro* studies. Before starting the experimental research particular emphasis should be placed on the selection of the appropriate exposure system and conditions that includes, the radiation source, frequency, modulation scheme, power stability and noise level. Moreover the determination of the measure of

the absorbed energy rate by the human body/tissue exposed (SAR W/Kg) or any other dosimetric quantity should be as precise as possible (Paffi et al., 2010).
It is known that certain RF-EMF setups induce temperature increase within the exposed cell cultures dishes and then it is crucial to avoid thermal confounding factors in the studies. For this reason an essential step is the temperature monitoring by using non-perturbing probes and active cooling with either forced air or water to avoid temperature increase (Simkò et al., 2016).
Another significant challenge that must be addressed in these type of studies, is related to ensure the homogeneity of energy absorption within the sample (SAR distribution) avoiding the formation of "hot spots" during the exposure (McNamee and Chauhan, 2009).
When conducting *in vitro* RF radiation experiments, it is important to maintain cells under appropriate conditions (37°C, 5% CO_2) and since these radiation behaves reflecting off metallic surfaces, particular attention must be paid to ensure that samples are exposed in a reverberant-free environment. This result can be achieved containing the RF radiation within a closed exposure system inside a standard incubator or designing an alternative non-RF radiation perturbing culture environment (McNamee and Chauhan, 2009).
In order to ensure that other conditions of the experiment do not cause cellular changes which might than falsely be attributed to RF field exposure it is necessary to include in each experiment also sham control samples, that consist in cells which are grown in exactly the same conditions and undergo all the manipulations of the RF-exposed cells, except for the RF exposure itself (Simkò et al., 2016).
Another quality parameter is the reproducibility of the experiments performing a sufficient number of independent experiments. In this manner also the statistical relevance is increased. Finally, in order to prevent any kind of bias in data analysis, the experiments should be performed in a blind manner, with samples coded so that exposed cells are unknown until the data are analysed (Simkò et al., 2016).

2. AIM

The increasing use of RF-EMF in different applications resulted in a growing interest on the possible adverse effects of these radiation on human health. Even if several i*n vitro* studies have been carried out evaluating the genotoxic and cytotoxic effects from RF exposures, the results are rather controversial. Much of these conflicting outcomes could be related to the heterogeneity of exposure systems, inadequate dosimetry and the variability of responses depending on the used experimental models.
In this unclear context, the aim of this Ph.D. project is to contribute in clarifying the contradictory conclusions of the previous studies performing a good quality investigation on the potential non-thermal RF biological effects through a well designed experimental study and a well characterized exposure system.
Since the widespread use of Wi-Fi technologies, we chose as frequency of interest 2.45 GHz and the related potential genotoxic effects were evaluated on human dermal fibroblasts, that represent a suitable *in vitro* model because non-ionizing radiation penetration power is limited to few hundred micrometer of skin. The selected SAR value for these experiments was 2W/kg, maximum value recommended by the European guidelines for limiting the exposures to RF-EMF (ICNIRP, 1998). In order to assess the non-thermal effects, samples were exposed for two hours to avoid a thermal increase that could occur inside the Petri dishes for longer time of exposures.
Moreover, since there is a growing interest on possible different biological effects induced by continuous respect to modulated signals, the effects of both continuous (CW) and pulsed (PW) signals were evaluated using a multi-parametric methodology approach.
In particular, the end-points evaluated include:

- Flow cytometric analysis, to assess effects on cell cycle
- Micronuclei anti-kinetochore antibody (CREST) analysis, to evaluate the potential to induce chromosome instability and to distinguish chromosome loss (aneugenic effect) from chromosome break (clastogenic effect)
- γ-H2AX/53BP1 assay, to identify DSBs
- Gene expression by transcriptome analysis using the innovative NGS technology (RNA-seq).
- Ultrastructural analysis by transmission electron microscopy, in order to identify possible morphological changes.

3. MATERIAL AND METHODS

3.1 Cell cultures

Human adult fibroblasts HDF (Human Dermal Fibroblasts, ECACC) from two lots of different donors were used in these experiments. In particular, one derived from normal facial skin of a 75 years old Caucasian man (lot A) and the other one from normal breast skin from a 54 years old Caucasian female (lot B). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Euroclone) supplemented with 10% foetal bovine serum (Euroclone), 1% 2 mM Lglutamine, 1% penicillin/streptomycin (Gibco) and 1% non essential amino acids (Euroclone). Cell cultures were grown incubated at 37°C, in humidified atmosphere, at 5% CO_2. About 24 hours before RF-EMF exposure, cells were seeded into 3.5 cm diameter polystyrene Petri dishes (Corning 3295), in 2 ml of medium at the density of 2 x 10^5 cells.

3.2 EXPOSURE SYSTEM

The *in vitro* exposures of cell cultures at the frequency of 2.45 GHz, with both continuous (CW) and pulsed (PW) wave signal, were performed using a Wire Patch Cell (WPC) based system.

3.2.1 *Description of the components*

The entire system consist of a module necessary for the signal generation, one for amplification and transmission of the signal to the WPC and a module for the control and monitoring of the generated signal (Figure 2).

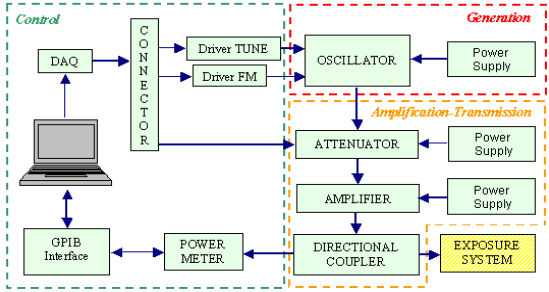

Fig. 2 Block diagram of the system for generation and control of the EM signal

A YIG oscillator (Micro Lambda, mod. MLOM-0704) was used as source for the signal generation. It is supplied by a 15 V voltage to the DC gate, works in a range of frequency between 700 MHz and 4 GHz and provide a nominal power of 14 dBm ± 3 dBm. To obtain the desired power dynamics for the output signal, the YIG oscillator is followed by a programmable attenuator and an amplifier with fixed gain. The programmable attenuator (Weinshel, mod.4228-63.75) works in a range of frequencies between 800 MHz and 2500 MHz. Its attenuation can be varied in the interval between 0 dB and 63.75 dB, with a minimum step of 0.25 dB, through a 8 bit digital control signal. The amplifier (AETHERCOMM SSPA 0.8-3.3-10) works in a range of frequencies between 800 MHz and 3200 MHz, with a nominal gain of about 32 dB. The maximum output power is equal to 9 W. Being a class A amplifier, it is characterized by a linear functioning over a wide range of powers, but it presents an high dissipation. On this subject, it has been provided with a refrigerating system, consisting in a fan placed over the refrigerating fins, in order to maintain the device at a constant temperature during the experiments. The generated signal then, passes through the bidirectional coupler (MITEQ, mod. CD2-102-402-20S) where a fraction (1%) of power transmitted to the exposure system is subtracted and measured by a power meter (Agilent E4419B).

Control signals for the oscillator and the attenuator come from a DAQ card (6715 National Instruments), connected to a notebook, and are calculated by using software specially designed in Labview 7.0 environment.

The functioning of each system component and of the complete chain for the EM signal generation was experimentally characterized by using a test bench composed of: two power supply Elind, one power meter (Hewlett Packard E4418B), one spectrum analyzer (Hewlett Packard 85047A) and a signal generator (Hewlett Packard 8350B), one Agilent E8363 network analyzer.

The WPC is the exposure chamber, that allows the contemporary exposure of four Petri dishes of 35 mm diameter (Figure 3). It is a symmetric structure, with two squared metallic patches (roof and ground) of the same size connected together through four ground wires (props) located at each corner. It is fed through a coaxial cable, with the central pin and the outer conductor connected to the ground and the roof, respectively. The WPC can be considered as a sort of tradeoff between a radiating system and a resonant one. In fact, it is based on a wire patch antenna, but when loaded with the sample, it is used as a one port

resonant structure with a locally TEM wave inside. During the *in vitro* experiments, the WPC is placed inside a standard incubator (RS Biotech Galaxy S+) to control environmental conditions in terms of temperature and CO_2 percentage. To avoid disturbances to the electronic setup of the incubator, the WPC is inserted in a metal-grid shielding cage (40x40x20 cm^3) with 3 mm thick walls. Moreover, in order to avoid field reflection on metallic walls, six blocks of Emerson & Cuming Microwave Products foam absorbing material (60 mm of thickness, 20 dB attenuation), one for each side of the cage, were used to wrap up the WPC.
The shielding effectiveness of the cage was verified by measuring the E field in the surrounding space, using a radiation meter (Wandel & Goltermann EMR-300) equipped with an isotropic probe (Wandel & Goltermann Type 8).

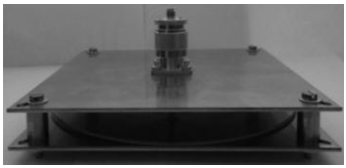

Fig. 3 Wire Patch Cell (WPC) exposure chamber

3.2.2 *Numeric and experimental characterization of the WPC*

In order to optimize the WPC for 2.45 GHz frequency band exposures, measurements of the scattering parameter S_{11} of the WPC empty and loaded with Petri dishes with 2 and 3 ml of DMEM solution, were performed using an Agilent E8363 network analyzer. The system can be employed in the first Wi-Fi channel, where the S_{11} is below -15 dB, when the whole exposed volume is equal to 8 ml (2 ml for each of the four Petri dishes).
The SAR distribution was calculated within the 2 ml DMEM solution, through the option "calculate point SAR" of CST Microwave Studio, 2008. Calculated SAR were extracted and processed through a Matlab® program to obtain statistical values (mean value, standard deviation) in the whole volume inside each Petri dish and within single layers of sample. The SAR was also experimentally evaluated by means of temperature measurement using the following relationship:
$SAR = c\ (dT / dt)$ (W/kg)

where c is the specific heat (J/kg . °C), and dT (°C) is the temperature variation induced by the exposure to a high power pulse at the microwave frequency during a short time interval dt (s). The c value is equal to 3851.86 J/kg/°C. The temperature increase was measured through a thermometer equipped with two high-impedance thermistor probes (Vitek TP100, accuracy 0,04 °C) and remotely controlled by GPIB interface, by a Labview^{TM} program running on a personal computer. The power efficiency of the system was evaluated in terms of SAR induced in the exposed biological sample per 1 W of input power ((W/kg)/W) and is equal to 3.2 (W/kg)/W.

3.2.3 *Exposure settings*

The software Labview 7.0 allows the user to select typology, frequency and power of the signal that is monitored in real time on the PC monitor. In order to expose the biological samples to a SAR value of 2 W/kg, for generating a CW signal an input power of 623.05 mW was provided. For PW 2.45 GHz field exposures a train of pulses 1 ms wide, with a duty cycle of 50% (ratio between the pulse length and period) was delivered. In this case, to obtain the same SAR value of 2 W/kg, the input power was set to 1,246.1 mW. It was double than in the case of CW exposure, since the input signal was ON only half of time. For both type of signals studied the time of exposure was 2 hours.

3.2.4 *Temperature monitoring*

In order to exclude thermal increase of cell cultures during exposure with the established parameters, a series of calibration measurements of the temperature distribution inside the exposed samples were performed before starting the experimental *in vitro* exposure. These measurements were carried out using a fluoroptic thermometer (Luxtron 710) that consists of a fiber-optic with a temperature sensor inserted inside the medium of the Petri dish. The temperature, displayed on a screen, was monitored during the 2 hours of exposure and thermal measurement were recorded every 30 sec.

3.3 Biological end-points

During each session, four Petri dishes were simultaneously exposed inside the WPC and for each exposed sample a corresponding sham control sample, placed in the same incubator, was analysed. The samples

were exposed for two hours and then processed for the required time points. Cells from the same passage were used in all the experiments and three or four experimental replicates were performed for each endpoint evaluated. In all the 2.45 GHz CW exposures HDF cells from lot A were used and in all 2.45 GHz PW exposures cells from lot B.

3.3.1 Cell Cycle Analysis

The exposure effect on cell cycle was determined by flow cytometric analysis. Two and 24 hours after irradiation, adherent and suspended cells were harvested, centrifuged at 1500 rpm for 10 min and washed twice with cold phosphate buffered saline (PBS). The assay was then performed as previously described in Masuelli et al. (2013). Cells were analysed with flow cytometry using a FACS Calibur cytometer running CellQuest software.

3.3.2 Micronuclei anti-kinetochore antibody (CREST) analysis

Cytokinesis-blocked binucleated (BN) cell preparations were obtained according to the Cytokinesis Block MicroNucleus (CBMN) technique. After exposure, cytochalasin B (3 µg/ml final, Sigma) was added to irradiated and sham cultures to block cytokinesis. After 24 h incubation at 37°C, cells were harvested, treated with hypotonic solution (KCl 0.075 M) and fixed in absolute ice-cold methanol. Methanol-fixed cells were processed for anti-kinetochore staining. Cells were washed 3 min in PBS-Tween20 (0.01%) and in PMN solution (20% phosphate buffer pH 8, 0.5% Nonidet, 0.02% sodium azite, 5% fat-free milk powder and H_2O). CREST anti-kinetochore antibody (Antibody Inc. Davis CA, USA) 1:1 in PBS-Tween20 (0.1%) was put on slides that were incubated over night in wet chamber at 37°C. Then three wash steps were performed in PBS/BSA 1% (5 min) and one in PMN. The Rabbit anti-human IgA, IgG, IgM (H+L) FITC-conjugated secondary antibody (Sigma Immunochemicals, St. Louise, USA) was then added on the slides in a 1:80 dilution in PBS/BSA 1%. Slides were let dry
45 min in wet chamber at 37°C. Three washing steps in PBS/BSA 1% were performed followed by one wash in cold PBS 1X. Slides were then counterstained with DAPI (2µg/mL) 1:1 with Vechashield antifade. MN were classified for the presence (CREST-positive, MN+) or absence (CREST-negative, MN-) of kinetochore reaction under appropriate filters for DAPI and FITC using an 40X objective. The scoring was

performed using a fluorescence microscope Axio Imager M1 (Carl Zeiss) following the scoring criteria described for MN in Fenech and Morley (1985).

3.3.3 γ-H2AX/53BP1 Immunofluorescence staining

Analysis of γ-H2AX/53BP1 colocalized foci was performed by immunofluorescence staining 30 min, 2 and 24 hours after exposure. After irradiation, cells were spotted onto coverslips and fixed using 2% formaldehyde/PBS for 5 min, permeabilised using 0.5% Triton-X/PBS for 5 min and blocked using 1% bovine serum albumin (BSA, Sigma) in PBS for 10 min. Then, cells were incubated with a combination of 1:500 mouse monoclonal anti-γH2AX antibody (Merk Millipore) and 1:1000 rabbit polyclonal anti-53BP1 antibody (Calbiochem) in 1% BSA/PBS for 45 min at room temperature in wet chamber. Subsequently, cells were washed in 1% BSA/PBS three times for 3 min and incubated in 1:500 anti-mouse Alexa Fluor 488 conjugated antibody (Molecular Probes, Life technologies), 1:500 anti-rabbit Alexa Fluor 555 Goat anti Rabbit IgG (Molecular Probes, Life technologies) for 30 min at room temperature in wet chamber in the dark. The cells were extensively washed with PBS, dried and finally slides were mounted with 4,6-diamidino-2 phenylindole (DAPI) in Vectashield (Burlingame, CA Vector Laboratories) solution, turned upside down on slide and edges sealed using nail polish.

Slides were viewed with an epifluorescence microscope (Imager Z1, Carl Zeiss) equipped with a CCD (charge-coupled device) camera. The automated image acquisition was performed using Metafer 4 software (version 3.6.9, from MetaSystems). 100-250 fields of each spot were selected and acquired by Metafer Autocapt module, using an immersion plan Apochromat oil 63X objective (Carl Zeiss). To compile all the 3-dimensionally distributed gamma-H2AX foci throughout the nuclei in one image, 26 2D-images for each field were acquired with a 0.3 µm z-axis step between two slides. The resulting fields of view (FOV) were transformed into training images (TNR) with the "Create TRN from FOV" to allow each colour channel to be exported as an individual greyscale tiff file.

The foci scoring was performed in uncompressed high-quality images using the free cell image analysis software CellProfiler (version 2.0, Broad Institute) as described by Carpenter et al., 2006.

3.3.4 Statistical analysis

Statistical analyses have been performed using different tests according to the assay. *T* test has been used for γH2AX/53BP1 and FACS analyses. The chi-squared test has been carried out for MN-crest analyses. Statistical significance has been considered for value $p<0.05$.

3.3.5 Gene expression profiling

RNA extraction

Total RNA, from exposed samples and the respective control sham samples, was isolated using QIAamp RNA Blood Mini Kit (Qiagen) immediately after, 2 and 24 hours after 2.45 GHz (CW or PW) exposure. Cells were washed ones in PBS and then detached from the Petri dishes using 0.05% trypsin (Euroclone). In order to denature RNase present in the samples cells were lysed in 350 µl RLT buffer (Qiagen) containing guanidinium isothiocyanate (GITC) and betamercaptoethanol (ß-ME). The lysed cells were then homogenized by centrifugation in a QIAshredder (Qiagen). For RNA isolation, 70% ethanol was added to the samples to bind the RNA to a silica membrane filter. To completely remove DNA contamination in RNA samples a DNase digestion step was performed incubating the samples with RDD buffer (Qiagen) for 15 min at room temperature. Impurities were removed by washing steps before finally eluting RNA in 50 µl RNase free water (Qiagen). RNA integrity was monitored using QuantiFluor RNA system (Promega) and the samples were stored at -80°C.

mRNA sequencing

For the sample preparation TruSeq Stranded mRNA (Illumina) was used and the starting amount of total RNA was of 500 ng/µl. The first step in mRNA sequencing protocol consists in obtaining mRNA library which are composed by a cDNA insert of certain size flanked by adapter sequences. The library preparation protocol start purifying poly-A containing mRNA molecules from total RNA using poly-T oligo attached magnetic beads (Illumina) using two rounds of purification. This process results in an increased depth of sequencing allowing the identification of lowly expressed mRNA transcripts. During the second elution of the poly-A mRNA, mRNA molecules were fragmented and primed for the synthesis of first strand complementary DNA (cDNA) using SuperScript II reverse Transcriptase (Invitrogen). Subsequently the

second strand cDNA was synthesized obtaining blunt-ended cDNA and a single 'A' nucleotide was added to the 3' ends of the blunt fragments, to prevent them from ligating to one another during the adapter ligation reaction. The adapter oligonucleotides were ligated to the cDNA and amplified by a PCR performed with a PCR Primer Cocktail (Illumina) that anneals to the ends of the adapters. Prior to sequencing the libraries were validated checking the size (approximately 260 bp) and purity using a DNA-specific chip (Agilent DNA 1000) on an Agilent Technologies 2100 Bioanalyzer. After validation, the libraries were normalized and pooled. The library pool to be sequenced was then denatured and diluted in the resulted optimal concentration (1.2 pM). The high-throughput next generation sequencing was performed using the Illumina sequencing technology platform (NexSeq500). The first sequencing step consist in cluster generation, performed on the flowcell containing immobilized on the surface oligonucleotides complementary to the adapters which are used as primers to form an initial copy of the individual sequencing template molecule with a reaction named "bridge amplification". After binding to the primers on the surface, the complementary sequence is produced and template strand is removed. After that, surface attached DNA strand bends over and anneals to the closest complementary primer, a new strand is synthesized and the replication is repeated. Consequently, millions of clusters consisting of clonally amplified fragments are formed on the flow cell. To perform the sequencing reaction, DNA polymerase, nucleotides containing 3' blocking group and a fluorophore, and first sequencing primers are added. DNA polymerase adds suitable nucleotide to the growing chain, unincorporated nucleotides are washed away. Following each base pair incorporation step, an image is made by laser excitation for each cluster and the signal is detected by a CCD camera. After cleavage of blocking group 3'-OH and removal of fluorescent, washing step is repeated and continue next cycle. Within every new cycle, the DNA chain is elongated and more images are recorded. The output of the sequencing consist in generating row reads (FASQ format) as starting material for the analysis of the mRNA-seq data.

- *Data analysis and statistical methodology*

The raw reads generated at the end of the sequencing process are supplied in a FASTQ file generated with the bcl2fastq software. Each FASTQ file contain an ID number for each read, the read sequence and a quality score. The quality control of the generated raw reads was performed using FastQC (v.0.11.4) in order to evaluate sequence quality,

GC content, the presence of adaptors, overrepresented k-mers and duplicated reads to detect sequencing errors, PCR artifacts or contaminations. The poor-quality bases were eliminated using Sickle software. The trimmed reads generated were subsequently mapped to the human reference genome UCSC (hg19 version) using the Subread software (v.1.6.1). The SeqMonk platform (v.1.41.0) (Babraham Bioinformatics, Cambridge, UK), was used to visualize the mapped read, quantify the reads and perform the statistical analysis. The quantification was performed at gene level by counting the number of reads which fall into exons of each gene and correct for the total number of reads in the sample. The final quantitated values are reported as $\log_2$ transformed RPM (Reads Per Million Reads of Library). In order to identify the differentially expressed genes (DEGs) DESeq2 R package R version 3.4.4was run in SeqMonk. DESeq2 performs statistical analysis (calculation of sample-to-sample distances; PCA) on raw counts to execute normalization, variance estimation and differential expression. The software uses a model based on negative binomial distribution by adjusting the obtained P-values by the Benjamini and Hochberg's procedure for controlling the false discovery rate. Stringent parameters consisting on a >2fold expression ratio and a false discovery rate (FDR) of p-values less than 0.001, were used in DESeq2 to identify differential expressed genes. DGEs analysis was also performed with the R package edger (v. 3.14.0). EdgeR implements novel statistical methods based on the negative binomial distribution as a model for count variability, including empirical Bayes methods, exact tests, and generalized linear models. It takes as input raw read counts and introduce possible bias sources into the statistical model to perform an integrated normalization as well as a differential expression analysis. The genes differentially expressed are characterized by a measure describing the observed difference from an initial to a final value (fold change) and by the statistical significance performed by the FDR analysis, to check the potential false positives introduced by multiple testing . The thresholds of false discovery rate (FDR) <0.01 and absolute log fold change < 1.5 were used to determine the significantly up-regulated or down-regulated genes between different groups. Thereafter, the above statistical analysis performed with DESeq2 and EdgeR was repeated under lower-stringency parameters, without FDR-adjustment and considering a p-value less than 0.05 and fold change of 1.5 as minimum cut-off value.

- *Functional analysis of differentially expressed genes*

The last step in a transcriptomics study is the characterization of molecular functions or pathways in which DGEs are involved. In this study PANTHER (Protein Analysis Through Evolutionary Relationships) classification system (http://www.pantherdb.org), that is part of the Gene Ontology (GO) Phylogenetic Annotation Project, was used. GO terms analysis of the screened DGEs was performed on the three ontology levels, biological processes, molecular function and pathways.

3.3.6 Transmission electron microscopy

Ultrastructural analysis was performed on samples 2 and 24 hours after exposure by cell observation with a transmission electron microscopy. Cells were fixed in 2.5% glutaraldehyde in PBS (pH 7.4) at 4°C and processed for transmission electron microscopy following routine procedure. Briefly, cells were post-fixed with 1.33% osmium tetroxide, dehydrated in graded alcohols, and then embedded in Epon 812 resin (Fisher Chemical Co., Dallas, TX, USA). The resin was allowed to polymerize in a dry oven at 60 °C, and specimens were cut on a Reichert ultra-microtome, stained with uranyl acetate and lead citrate, and observed under a Philips Morgagni 268D transmission electron microscope (Masuelli et al., 2012).

4. RESULTS

4.1 Exposure system

4.1.1 Thermal conditions

In order to verify that no thermal increase occurred inside the Petri dishes during the exposure, the temperature was measured using a fluoroptic thermometer. Since a temperature increase of 0.7°C was observed inside the samples during the calibration experiments, different conditions were tested in order to reduce the temperature inside the Petri dishes. The best result, with an increase of 0.25°C, was obtained inserting two cooling fans inside the incubator. This configuration was chosen for the subsequent exposure procedure.

4.2 Biological End-Points

The 2.45 GHz exposures with the two types of signal (CW and PW) were performed on HDF cells from two different donors, as reported in the Material and Method section. The differences observed in basal level frequency of the evaluated markers between cells exposed to CW and PW signals, could be related to the different lot used corresponding to two different donors.

4.2.1 Cell Cycle Analysis

The FACS analysis on DNA content was performed on HDF cells 2 and 24 hours after exposure to 2.45 GHz (CW or PW). Results obtained from the cells exposed to the two different type of signals were compared to those of the corresponding sham samples. No significant change in the different phases of the cell cycle was observed 2 and 24 hours after exposure to both CW and PW signals (Table 1 and Table 2).

Time points	Sample	Sub-G1[1]		G0/G1		S		G2/M	
		Mean	p	Mean	p	Mean	p	Mean	p
2 h	Sham	0.71±0.13	NS	72.84±1.12	NS	4.06±0.61	NS	22.62±1.58	NS
	Exposed	0.84±0.13	NS	75.00±0.97	NS	4.17±0.51	NS	20.23±1.43	NS
24 h	Sham	0.49±0.16	NS	84.10±0.54	NS	2.00±0.17	NS	13.53±0.41	NS
	Exposed	0.44±0.18	NS	82.43±1.47	NS	2.24±0.34	NS	15.03±1.02	NS

Tab 1. Cell cycle analysis on HDF cells exposed to 2.45 GHz CW. No differences were observed 2 and 24 hours after irradiation.1Percentage of cells in the sub-G1, G0/G1, S and G2/M phase were calculated using Cell Quest software. The data are representative of three experiments. NS= not significant (2-tailed T test).

Time points	Sample	Sub-G1[1]		G0/G1		S		G2/M	
		Mean	p	Mean	p	Mean	p	Mean	p
2 h	Sham	1.92±0.42	NS	50.92±12.97	NS	6.85±1.77	NS	40.61±11.66	NS
	Exposed	1.44±0.76	NS	50.12±12.95	NS	7.97±2.18	NS	40.81±10.13	NS
24 h	Sham	0.80±0.66	NS	86.03±2.33	NS	3.46±0.49	NS	9.85±2.35	NS
	Exposed	0.82±0.70	NS	86.31±2.61	NS	3.53±0.59	NS	9.50±2.81	NS

Tab. 2 Cell cycle analysis on HDF cells exposed to 2.45 GHz PW. No differences were observed 2 and 24 hours after irradiation.[1]Percentage of cells in the sub-G1, G0/G1, S and G2/M phase were calculated using Cell Quest software. The data are representative of three experiments. NS= not significant (2-tailed T test).

4.2.1 Micronuclei anti-kinetochore antibody (CREST) analysis

For both CW and PW signals, CREST analysis on exposed samples respect to the sham showed no significant increase in the total number of MN, corresponding to the sum of CREST positive (MN+) and CREST negative (MN-) micronuclei. Similarly, no significant increase was observed in the frequency of MN+ and MN- in the exposed samples (CW or PW) respect to the sham samples. These results are reported for CW signal in figure 4 and for PW in figure 5.

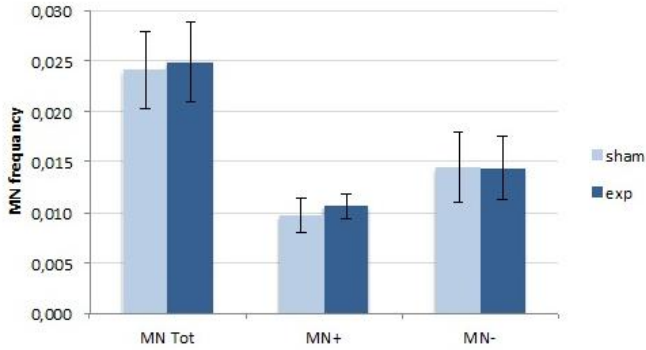

Fig. 4 CREST analysis on HDF exposed to 2.45 GHz CW. No significant increase ($\chi 2 < 0.05$) in MN tot, neither in the frequency of MN+ and MN- in exposed samples respect to sham. Bars indicate the standard error and data are representative of 4 independent experiments.

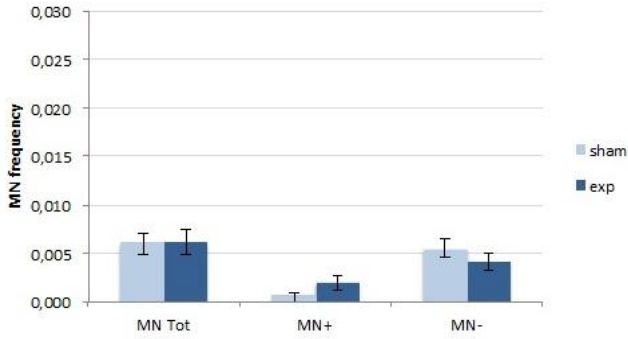

Fig. 5 CREST analysis on HDF exposed to 2.45 GHz PW. No significant increase ($\chi 2<0.05$) in MN tot, neither in the frequency of MN+ and MN- in exposed samples respect to sham. Bars indicate the standard error and data are representative of 4 independent experiments.

4.2.2 γ-H2AX/53BP1 assay

The analysis of colocalized γ-H2AX/53BP1 foci shows no significant differences between exposed and sham samples at each of the three time points evaluated and for both types of signal (CW or PW). The results are showed in figure 6 and 7.

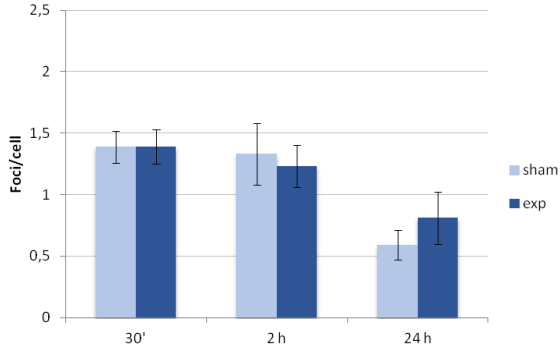

Fig. 6 γ-H2AX/53BP1 foci analysis on HDF exposed to 2.45 GHz CW. No significant differences (T test: $p > 0.05$) were observed 30 min, 2 h and 24 h after exposure to 2.45 GHz respect to sham samples. Data are representative of 4 experiments and bars denote the standard error.

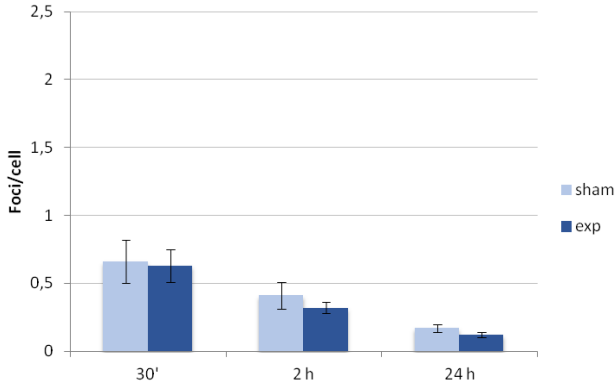

Fig. 7 γ-H2AX/53BP1 foci analysis on HDF exposed to 2.45 GHz PW. No significant differences (T test: $p > 0.05$) were observed 30 min, 2 h and 24 h after exposure to 2.45 GHz respect to sham samples. Data are representative of 4 experiments and bars denote the standard error.

4.2.3 Gene expression analysis using RNA-seq

Gene expression profiling was evaluated by transcriptome analysis using the high-throughput RNA-seq approach on Illumina NextSeq 500 platform. The analysis was performed on sham and 2.45 GHz (CW or PW) exposed samples at three different time-points: immediately after, 2 and 24 hours post exposure. For both CW and PW signals 4 experimental replicates were performed, with a total of 48 sequenced samples. For each run 8 samples were sequenced with an average of 50 million of generated reads per sample and about 8% of poor quality trimmed reads, indicating that the percentage of good quality reads was high.

In order to verify if all the sequenced samples were comparable, a QC plot was generated by SeqMonk software. In figure 8 and 9 are reported the results for the samples exposed to 2.45 GHz CW and to 2.45 GHz PW, respectively.

The metrics calculated by the plot are: percentage of reads that falls into genes, exons, rRNA, the percentage of genes measured, the percentage of reads measured in the samples, percentage of reads that falls into mitochondrion and the percentage on sense strand.

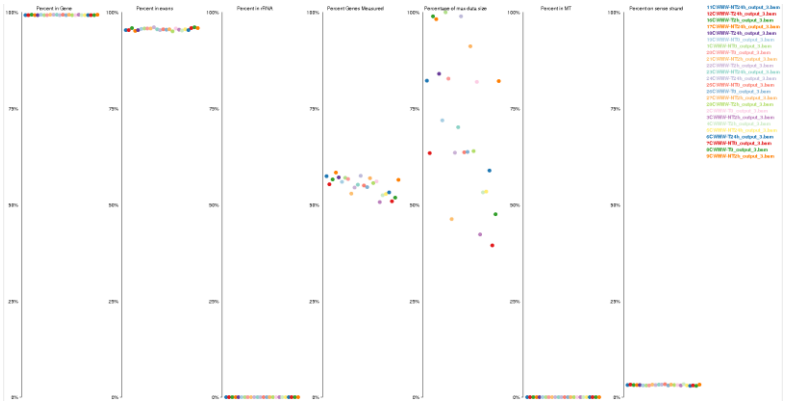

Fig. 8 RNA-seq QC plot of samples 2.45 GHz CW exposed. Data are representative of 4 independent experiments that correspond to 24 sequenced samples, each identified by a specific colour.

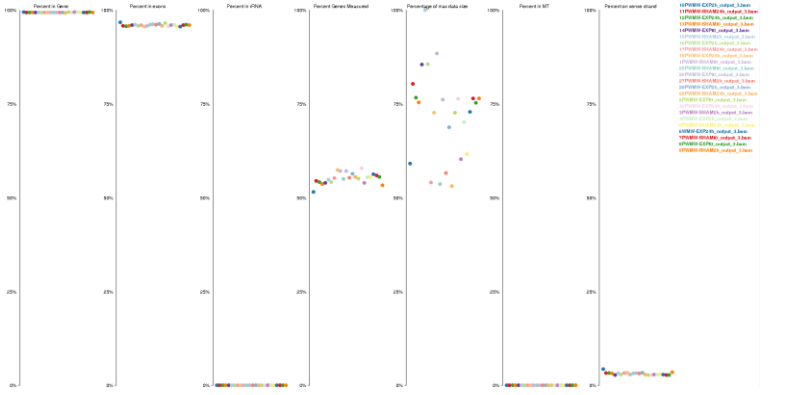

Fig. 9 RNA-seq QC plotof samples 2.45 GHz PW exposed. Data are representative of 4 independent experiments that correspond to 24 sequenced samples, each identified by a specific colour.

The results from the QC plot analysis are in both cases satisfactory, indeed no biases in the data between the different replicates were highlighted indicating that all sequenced samples follows the same trend and are comparable.

4.2.4 Differential gene expression analysis

- *CW exposure*

Differential gene expression (DGEs) between cells exposed to 2.45 GHz CW wave signal and sham-control samples, following statistical analysis using an FDR-adjusted *p*-value cut-off, evidenced only one gene target in the samples examined 2 hours after exposure. This probe is a long non-coding RNA (*RMRP*, RNA Component of Mitochondrial RNA Processing Endoribonuclease) that resulted down regulated. Since the FDR-adjusted statistical analysis may have resulted in the rejection of some "true positive" responses (type 2 error), the data were reanalysed using a similar statistical approach without FDR, considering a fold change of 1.5 as minimum cut-off value. With this statistical analysis a total of 53 genes representing a variety of cellular function were identified as differentially expressed in exposed samples (table 3). In particular the analysis showed, 6 genes (5 up-regulated; 1 down-regulated) in the samples processed immediately after exposure, 39 genes 2 hours after exposure (19 up-regulated; 20 down-regulated) and 8 genes 24 hours after exposure (5 up-regulated; 3 down-regulated). Only three genes (RN7L1, RNA, 7SL, cytoplasmic 1; RN7L2, RNA, 7SL, cytoplasmic 2; ANKRD36C, Ankyrin repeat domain-containing protein 36) were observed to be down regulated (fold change > 1.5) both 2 and 24 hours after exposure respect to the sham group.

2,45 GHz CW	Gene ID	Gene name	Description	FC	regulation
immediately after exposure	ACOT4	Acyl-coenzyme A thioesterase 4	Signaling receptor binding and palmitoyl-CoA hydrolase activity	1,6	up
	DLX5	Homeobox protein DLX-5	Transcriptional factor involved in bone development	2	up
	FAM72D	Protein FAM72D	Unknown function	2	up
	LRP2BP	LRP2-binding protein	Protein binding	2,5	down
	NIPSNAP3B	Protein NipSnap homolog 3B	Rutative roles in vesicular trafficking	2,2	up
	SUN3	SUN domain-containing protein 3	Protein binding	2,3	up
2 hours after exposure	HES4	Transcription factor HES-4	Basic helix-loop-helix transcription factor	1,6	down
	HMCN1	Hemicentin-1	Receptor binding	1,8	up
	ALMS1	Alstrom syndrome protein 1	Cytoskeletal protein binding	1,5	up
	ANKRD36C	Ankyrin repeat domain-containing protein 36	Protein Coding gene involved in ion channel inhibitor activity	1,6	down
	GRIP2	Glutamate receptor-interacting protein 2	Multi-PDZ domain scaffolding proteins required for dendrite development	1,5	down
	BSN	Protein bassoon	Scaffolding protein involved in organizing the presynaptic cytoskeleton	1,6	up
	FAM53A	Protein FAM53A	Encodes a secreted peptide hormone and member of the EGF family of protein	1,5	down
	EREG	Epiregulina	Transient receptor potential cation channel	1,5	down
	SLC9B1	Sodium/hydrogen exchanger 9B1	Transmembrane transporter activity	1,7	down
	LUCAT1	lung cancer associated transcript 1	Non-coding RNA	1,6	down
	EGR1	Early growth response protein 1	Transcriptional regulator	1,7	up
	AC005618.6	Protocadherin gamma-B3	Cell adhesion, cell-cell signaling	1,8	up
	HIST1H2AD	Histone H2A type 1-D	Histone	2	down
	HIST1H2BG	Histone H2B type 1-C/E/F/G/I	Histone	1,8	down
	IER3	Radiation-inducible immediate-early gene IEX-1	Cell proliferation and survival	2,2	down
	PRSS35	Inactive serine protease 35	Serin protease activity	1,7	down
	SAMD3	Sterile alpha motif domain-containing protein 3	Protein binding	1,8	up
	GPER1	G-protein coupled estrogen receptor 1	Protein binding	1,5	down
	RIMS2	Regulating synaptic membrane exocytosis protein 2	Protein binding	1,6	up
	ZNF462	Zinc finger protein 462	Protein binding	1,7	up
	SYNPO2L	Synaptopodin 2-like protein	Protein binding	1,8	up
	CNNM1	Metal transporter CNNM1	Protein binding	1,5	down
	C11orf96	Uncharacterized protein C11orf96		1,7	down
	BEST1	Bestrophin-1	Ion binding	1,6	up
	STYK1	Tyrosine-protein kinase STYK1	Receptor binding	1,5	up
	RPPH1	Ribonuclease P RNA Component H1	long non-coding RNA	3,9	down
	RN7SL1	RNA, 7SL, cytoplasmic 1	small cytoplasmic RNA	2,7	down
	RN7SL2	RN7SL2	small cytoplasmic RNA	4,1	down
	RHOJ	Rho-related GTP-binding protein RhoJ	small GTPase	1,5	down
	SYNE2	Nesprin-2	Actin binding	2	up
	AHNAK2	Protein AHNAK2	RNA binding	1,5	up
	FBXL22	F-box and leucine-rich protein 22	Protein ubiquitination	1,6	up
	SLC43A2	Large neutral amino acids transporter small subunit 4	Transmembrane transporter activity	1,7	up
	MYCBPAP	MYCBP-associated protein	Cell differenziation	2	up
	ZNF433	Zinc finger protein 433	DNA-binding (transcription)	1,3	up
	ZNF233	Zinc finger protein 233	Nucleic acid binding, regulation of transcription	1,8	up
	RP1-198K11.5		Non-coding RNA	1,5	down
	MXRA5	Matrix-remodeling-associated protein 5	Receptor binding	1,8	up
	RMRP	RNA Component Of Mitochondrial RNA Processing Endoribonucl	Non-coding RNA	8,8	down
24h hours after exposure	ANKRD36C	Ankyrin repeat domain-containing protein 36	Protein Coding gene, ion channel inhibitor activity	1,6	down
	MIR145	microRNA 145	Non-coding RNA	1,4	up
	KIAA0895	Uncharacterized protein KIAA0895		1,8	up
	LCNL1	Lipocalin-like 1 protein	Binding, isomerase activity	2,3	up
	RASGEF1A	Ras-GEF domain-containing family member 1A	protein binding, small GTPase regulator activity	1,5	up
	RN7SL1	RNA, 7SL, cytoplasmic 1	small cytoplasmic RNA	1,8	down
	RN7SL2	RNA, 7SL, cytoplasmic 2	small cytoplasmic RNA	1,8	down
	RGS11	Regulator of G-protein signaling 11	Regulator of G protein signaling	1,7	up

Tab. 3 List of genes differentially expressed between exposed and sham samples after exposure to 2.45 GHz CW. The table includes: gene name, gene function, fold change (FC) and regulation.

- *PW exposure*

DGEs analysis assessed in 2.45 GHz PW exposed samples showed no genes differentially expressed for each time point evaluated following statistical analysis with FDR-adjustment. When the data were reanalysed without FDR-adjustment and considering a minimum fold change of 1.5 as cut-off value, 33 genes were identified as differentially expressed in exposed cells (Table 4). Among them 5 genes (3 up-regulated; 2 down-regulated) were identified in the samples analysed immediately after exposure, 21 genes (9 up-regulated; 12 down-regulated) 2 hour after exposure and 7 genes (all up-regulated) 24 hours after exposure. Only one gene (*RMRP*, RNA Component of Mitochondrial RNA Processing Endoribonuclease), differentially expressed 2 hours after exposure, was in common with the results obtained from the samples exposed to CW.

2,45 GHz PW	Gene ID	Gene name	Description	FC	regulation
immediately after exposure	KIAA1324	UPF0577 protein KIAA1324	RNA binding	1,6	up
	KIAA1211	Uncharacterized protein KIAA1211	Unknown function	2,3	up
	CXCL3	C-X-C motif chemokine 3	Chemokine	1,6	down
	EGR3	Early growth response protein 3	Transcriptional regulator	1,5	down
	SLC16A13	Monocarboxylate transporter 13	Transmembrane transporter	1,5	up
2 hours after exposure	TMEM240	Transmembrane protein 240	Transmembrane-domain containing protein	1,54	up
	TNFRSF25	Tumor necrosis factor receptor superfamily member 25	Signaling receptor activity	1,7	up
	BEST4	Bestrophin-4	Anion channel	1,8	up
	RNF175	RING finger protein 175	Ubiquitin- protein ligase	1,7	up
	KLKB1	Plasma kallikrein	Serin-protease	1,5	down
	PTGER4	Prostaglandin E2 receptor EP4 subtype	G-protein coupled receptor	2	down
	MDFI	MyoD family inhibitor	Transcription factor binding	2	up
	PPP1R9A	Neurabin-1	Actin binding	1,9	down
	RMRP	RNA Component Of Mitochondrial RNA Processing Endoribonuclease	Non-coding RNA	5,7	down
	PRUNE2	Protein prune homolog 2	Pyrophosphatase activity	1,6	down
	ENO4	Enolase 4	Lyase activity	1,9	down
	KCNQ1OT1	KCNQ1 opposite strand/antisense transcript 1	non-coding RNA	1,7	down
	OLR1	Oxidized low-density lipoprotein receptor 1	Lipoprotein receptor	2,1	down
	HOXC11	Homeobox protein Hox-C11	Transcription factor	1,7	up
	PTPRQ	Receptor-type tyrosine-protein phosphatase R	Protein phosphatase	1,8	down
	PAPLN	Papilin	Peptidase activity	1,7	down
	TMEM121	Transmembrane protein 121		2	up
	ATF7IP2	Activating transcription factor 7-interacting protein 2	ATPase activity	2,4	down
	CNBD2	Cyclic nucleotide-binding domain-containing protein 2	cAMP binding	1,7	up
	LIF	Leukemia inhibitory factor	Cytokine activity	1,8	down
	PDZD4	PDZ domain-containing protein 4	Ubiquitin protein ligase activity	2,1	up
24 hours after exposure	PPP1R1C	Protein phosphatase 1 regulatory subunit 1C	Signaling molecule, phosphatase inhibitor	2,6	up
	ADAMTS13	A disintegrin and metalloproteinase with thrombospondin motifs 13	Metallopeptidase activity	1,5	up
	PANO	Proapoptotic Nucleolar Protein 1	Apoptosis-inducing protein	1,9	up
	NEAT1	nuclear paraspeckle assembly transcript 1	Non-coding RNA	1,5	up
	VAMP1	Vesicle-associated membrane protein 1	Transport	1,5	up
	GOLGA8B	Golgin subfamily A member 8B	Membrane traffic protein	1,5	up
	GUSBP11	Putative inactive beta-glucuronidase protein GUSBP11	Hydrolase activity	1,5	up

Tab. 4 List of genes differentially expressed between exposed and sham samples after exposure to 2.45 GHz PW. The table includes: gene name, gene function, fold change (FC) and regulation.

4.2.5 DGEs functional classification

- *CW exposure*

Gene Onthology terms analysis of the DGEs was performed on the three ontology levels: biological processes, molecular function and pathways.
The differentially expressed genes, identified in the samples analysed immediately after CW exposure, were mainly implicated in metabolic processes and most of them has binding or catalytic activity. In particular, transcriptional factors related to metabolic processes (DLX5, Homebox protein DLX-5; ACOT4, Acyl-coenzyme A thioesterase 4) and involved in cellular component organization (SUN3, SUN domain-containing protein 3) were up-regulated whereas one gene with binding activity was down regulated (LRP2BP, LRP2-binding protein).
Most of the genes differentially expressed 2 hours after exposure were involved in metabolic processes and cellular component organization. Regarding the molecular function, they have been divided in those with binding, catalytic, signal transduction and transporter activity.
More in detail, the up-regulated genes are mainly related to cytoskeletal structure (ALMS1, Alstrom syndrome protein 1; BSN, Protein bassoon; SYNPO2L, Synaptopodin 2-like protein; SYNE2, Nesprin-2). Transcriptional factors including some early response genes were both up-regulated (EGR1, Early growth response protein 1; ZNF462, Zinc finger protein 462; AHNAK2, Protein AHNAK2; ZNF433, Zinc finger protein 433; ZNF233, Zinc finger protein 233) and down-regulated (IER3, Radiation-inducible immediate-early gene IEX-1; HES4, Transcription factor HES-4). Some of the genes involved in signal transduction were up-regulated (SAMD3, Sterile alpha motif domain-containing protein 3; RIMS2, Regulating synaptic membrane exocytosis protein 2; STYK1, Tyrosine-protein kinase STYK1; MYCBPAP, MYCBP-associated protein) whereas other were down-regulated (GRIP2, Glutamate receptor-interacting protein 2; FAM53A, Protein FAM53A; GPER1, G-protein coupled estrogen receptor 1). The down regulated genes includes also those involved in ion-channel activity (ANKRD36C, Ankyrin repeat domain-containing protein 36; EREG, Epiregulina; CNNM1, Metal transporter CNNM1; BEST1, Bestrophin-1) and a non-coding RNA (LUCAT1, lung cancer associated transcript 1; RPPH1, Ribonuclease P RNA Component H1; RMRP, RNA Component Of Mitochondrial RNA Processing Endoribonuclease).
The genes differentially expressed 24 hours after exposure were involved in different biological processes with binding or catalytic activity. Among them, the up-regulated genes encodes for enzymes

(LCNL1, Lipocalin-like 1 protein; RASGEF1A, Ras-GEF domain-containing family member 1A) and proteins involved in signal transduction (RGS11, Regulator of G-protein signaling 11) whereas the down-regulated include a gene that inhibits ion channel activity (ANKRD36C, Ankyrin repeat domain-containing protein 36) and small cytoplasmic RNAs (RN7SL1, RNA, 7SL, cytoplasmic 1; RN7SL2, RNA, 7SL, cytoplasmic 2).
Summarizing the results obtained in the samples 2.45 GHz CW exposed, the genes resulted mainly involved in metabolic and cellular component organization or biogenesis processes, and the most relevant molecular function was related to binding activities. No significantly affected pathways were identified. In table 5 are reported the number of genes involved in the GO biological processes, for time points evaluated.

Biological Processes	Time-points		
	Immediatly after	2 h after	24 h after
cellular component organization or biogenesis	x	xxxxxx	
localization		xxxx	x
biological regulation		xxxxxxx	
response to stimulus		xx	
developmental process	x	xx	
biological adhesion		xxx	
locomotion		xxx	
metabolic process	xx	xxxxxx	x
immune system process		x	

Tab. 5 The table reports, for each time points after 2.45 GHz CW exposure, the number of genes involved in the different biological processes as reported in *GO term* analysis

-PW exposed
Regarding the samples exposed to 2.45 GHz PW and analysed immediately after exposure, the genes resulted involved in metabolic, cellular and localization processes with binding or transporter activity. The genes showing an up-regulated profile includes one involved in transport activity (SLC16A13, Monocarboxylate transporter 13) and a transcriptional factor (KIAA1324, UPF0577 protein KIAA1324) whereas the down-regulated includes an early response gene (EGR3, Early growth response protein 3) and a chemokine (CXCL3, C-X-C motif chemokine 3).
Most of the genes differentially expressed 2 hours after exposure were involved in metabolic, cellular and response to stimulus processes with the majority of them having binding or catalytic activity. Among them,

the transcriptional factors were all up-regulated (MDFI, MyoD family inhibitor; HOXC11, Homeobox protein Hox-C11), whereas those involved in signal transduction were both up-regulated (TNFRSF25, Tumor necrosis factor receptor superfamily member 25; TMEM121, Transmembrane protein 121; CNBD2, Cyclic nucleotide-binding domain-containing protein 2) and down-regulated (PTGER4, Prostaglandin E2 receptor EP4 subtype; LIF, Leukemia inhibitory factor).

The genes differentially expressed 24 hours after exposure were involved in different cellular processes as metabolic, biological regulation, cellular component organization or biosynthesis and response to stimulus processes. These genes were all up-regulated and among them some resulted involved in transport activities (VAMP1, Vesicle-associated membrane protein 1; GOLGA8B, Golgin subfamily A member 8B) and enzyme function (PPP1R1C, Protein phosphatase 1 regulatory subunit 1C; ADAMTS13, A disintegrin and metalloproteinase with thrombospondin motifs 13).

The results obtained for the genes differentially expressed after exposure to 2.45 GHz PW indicate that the most relevant biological processes in which they were involved were related to metabolic or response to stimulus processes with many of them having binding or catalytic activity. Even for this type of exposure no significantly affected pathways were identified. In table 6 are reported the number of genes involved in the GO biological processes, for time points evaluated.

Biological Processes	Time-points		
	Immediatly after	2 h after	24 h after
cellular component organization or biogenesis		x	x
localization	x		
biological regulation		xxx	x
response to stimulus		xxxx	x
developmental process		xx	
biological adhesion			
locomotion			
metabolic process	x	xxxxx	x
immune system process			

Tab. 6 The table reports, for each time points after 2.45 GHz PW exposure, the number of genes involved in the different biological processes as reported in *GO term* analysis

4.2.6 Ultrastructural Analysis

Ultrastructural analysis was performed on HDF cells exposed to 2.45 GHz (CW or PW) and compared to sham control cells, 2 and 24 hours after exposure. No morphological differences were observed between sham and CW and PW exposed cells, as showed in figure 10 and 11 respectively.

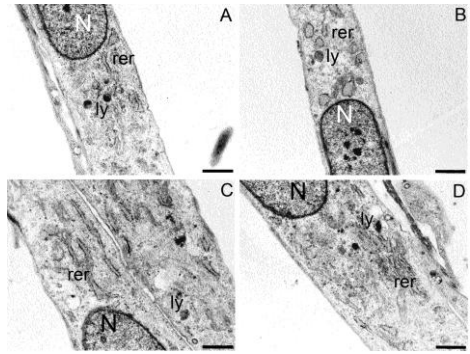

Fig. 10 Ultrastructural analysis of sham (A, C) and 2.45 GHz CW (B, D) exposed HDF. Exposed cells were examined 2 hours (B) or 24 hours (D) after exposure. N: nucleus, rer: rough endoplasmic reticulum, ly: lysosome. (Bars correspond to 1 µm)

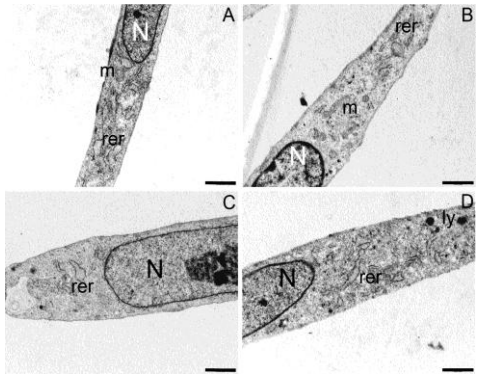

Fig. 11 Ultrastructural analysis of sham (A, C) and 2,45 GHz PW (B, D) exposed HDF. Exposed cells were examined 2 hours (B) or 24 hours (D) after exposure. N: nucleus, rer: rough endoplasmic reticulum, m: mitochondria, ly: lysosome. (Bars correspond to 1 µm)

Sham and exposed samples from both the signals (CW or PW) and for each time point evaluated, appeared as similar elongated cells with elongated centrally located nuclei, essentially formed by euchromatin with poor heterochromatin and well-organized nucleoli. Abundant rough endoplasmic reticulum sometimes dilated, few mitochondria and lysosomes were visible in the cytoplasm.

5. DISCUSSION

The exponential increase of human exposure to RF-EMF, especially due to the large use of these radiations in wireless communication devices and in diagnostic and therapeutic medicine (Consales et al., 2012; Vijayalaxmi, 2016), raises questions about their possible impact on human health. Consequently several studies have been performed to investigate the health risks and biological effects related to RF radiation but there is still a certain degree of uncertainty about the outcomes (Manna e Ghosh, 2016). These contradictory results can mainly be related to an inadequate experimental design and a lack of well-characterized exposure conditions (Vijayalaxmi, 2016).

Therefore additional studies based on high quality research methods, including well defined exposure conditions, measurable end-points, sample size with sufficient statistical power, are needed to identify possible biological effects of RF-EMF exposure (WHO, 2010). In the light of these observations, the goal of this Ph.D. project was to perform a good quality *in vitro* investigation on the potential RF biological effects through a well designed experimental study.

We chose as frequency of interest 2.45 GHz, because the widespread use of Wi-Fi technologies in everyday life is leading to concerns about their possible health consequences. The selected SAR value for these experiments was 2W/kg, which is the maximum value recommended by the European guidelines for limiting the exposures to RF-EMF (ICNIRP, 1998).

An open question is related to possible different biological effects induced by signal modulation, that occurs in a wide variety of RF applications (radar, wireless communications, broadcast communications and industrial processes). Few studies comparing the effects of continuous and modulated wave signals have been performed with no clear results, consequently modulation is a parameter poorly considered in most guidelines for limiting the exposure of humans to RF-EMF. Because of the growing interest on possible different biological effects related to signal modulation, the present study focused on the effects of 2.45 GHz with both CW and PW signals.

Since one of the most critical point in previous studies is related to inaccurate dosimetry and uncharacterized exposure source and conditions (Vijayalaxmi, 2016), in this project particular emphasis was addressed to identify the appropriate exposure system. The choice was made considering the number and dimension of samples, the specific

end-points to evaluate and problems regarding realization and cost. After the examination of different design solutions, the system that better satisfied the above mentioned requirements was a WPC-based exposure system. During the set-up of the system, particular attention was paid to ensure that RF-energy was deposited in a homogeneous manner within the sample, avoiding the temperature rise in some part of the Petri dishes with the formation of "hot spots". Since heating is the most widely accepted cause of RF biological effects, the main concern is related to evaluate possible non-thermal effects (McNamee and Chauhan, 2009; Vijayalaxmi, 2016). Therefore, before the *in vitro* experimental exposures a series of calibration measurements were performed to monitor the temperature distribution inside the sample area using a fluoroptic thermometer, during the 2 hours of exposure. Since a temperature increase of 0.7°C was observed, an active cooling with forced air was used to reduce the temperature with a maximum thermal increase of 0.25°C. Moreover, to maintain cells under appropriate conditions (37°C, 5% CO_2) the *in vitro* exposures were performed into the incubator thanks to the reduced dimension of the WPC exposure chamber.

Skin cell type represents an efficient *in vitro* model to evaluate biological effects of RF-EMF because these frequencies can penetrate the human skin only few hundred micrometer, thus in this study we used, as *in vitro* cellular model, human primary dermal fibroblasts. Moreover, in order to ensure the reproducibility and reliability of the results, cells at the same passage in all replicated experiments were employed and for each exposed sample a corresponding sham control sample was analysed to exclude that possible observed effects were due to other factors.

In order to obtain robust scientific results, the effects of 2.45 GHz, with both CW and PW signals, were investigated using a multiparameric approach. Moreover, to increase the statistical power of the data at least three experimental replicates were performed for each end-points.

Since cell cycle arrest is a consequence of DNA damage, we used flow cytometry to assess cell cycle position based on the DNA content in order to evaluate the potential genotoxicity of the selected frequency. The results of this analysis showed that 2.45 GHz with both type of signal (CW or PW) at each time point evaluated, did not affect the cell cycle. Our findings are in line with a previous study (Hansteen et al., 2009) in which the effects of 2.3 GHz with both CW and PW signals were evaluated on human lymphocytes, and with other studies investigating the effects of different RF radiation on various cellular

types (Takashima et al., 2006; Sekijima et al., 2010; Franchini et al., 2018).

The chromosomal damage and its origin was assessed by indirect immunofluorescence CREST-MN analysis that showed, as expected, no aneugenic or clastogenic effects; no significant induction in the total number of MN between sham and 2.45 GHz exposed samples, for both type of signals, was observed. These observations are in agreement with other studies evaluating chromosomal damage by the conventional MN assay, on different cellular types exposed to 2.45 GHz (Koyama et al., 2004; Figueiredo et al., 2004) or to other RF range (McNamee et al., 2002; Vijayalaxmi et al., 2006; Zeni et al., 2008).

The absence of a clastogenic effect of 2.45 GHz (CW or PW), suggested from the not significant increase in CREST-negative MN, was confirmed by γ-H2AX/53BP1 foci assay that showed no significant induction of DSBs. This finding was in agreement with a previous study, in which γ-H2AX/53BP1 was assessed on the same cellular type exposed to a different RF radiation (Franchini et al., 2018). Moreover, the same result was obtained in other investigations, performed using comet assay, on human lymphocytes after exposure to 2.45 GHz (Vijayalaxmi et al., 2006) or on different cellular types exposed to 2.1425 GHz with both continuous or modulated signals (Sakuma et al., 2006).

In recent years, there is a growing scientific interest on the identification of sensitive genes that could change their expression profile following exposure to chemical or physical environmental factors (Liu et al., 2012). Considering that no DNA damage was observed, our attention focused on the evaluation of possible genes differentially expressed after exposure to 2.45 GHz (CW or PW) at three different time points (immediately after, 2 and 24 hours after exposure). Moreover, the innovative aspect of this Ph.D. project is related to the whole transcriptome analysis by RNA sequencing, based on Next Generation Sequencing technology.

Currently, the widely used method for transcript profiling is based on microarrays technologies that permit to test only a preselected number of genes. The improvement of this technology (Affymetrix) allows evaluating whole transcriptome expression profile (Malone et al., 2011), but it still has some limitations. The main difficulties related to the arrays, are due to the possibility to investigate only known genes for which probes can be designed. Moreover, since probes differ considerably in their hybridization properties, the accuracy of the expression measurement is limited (Zhao et al., 2014).

The RNA-seq approach for whole transcriptome analysis shows considerable advantages in comparison to microarrays. In particular it allows the identification not only of all known genes but also of the unknown genes, it overcomes background noise associated with fluorescence quantification and allows genome-wide analysis of transcription at single nucleotide resolution, including identification of alternative splicing events and post-transcriptional RNA editing events (Costa et al., 2013).

From each RNA-seq experiment a large amount of reads (short nucleotides sequences) are generated. In the current study, for each time point and type of signals, four experimental replicates were performed in order to reduce the experimental error. From each sequencing experiment approximately 50 million reads for sample were obtained. These reads, subsequently undergone to the bioinformatics analysis, the most time-consuming and challenging step of the entire analysis pipeline. The quality assessment of the obtained data resulted satisfactory, indeed, no biases between the different replicates were observed indicating that all sequenced samples followed the same trend and were comparable.

The identification of DEGs, performed using a high stringency statistical analysis (FDR-adjusted), showed only one down-regulated gene for a long non-coding RNA (*RMRP*, RNA Component of Mitochondrial RNA Processing Endoribonuclease) 2 hours after 2.45 GHz CW exposure. Long non-coding RNAs (lncRNAs) are a class of non-coding RNA (ncRNA) which are implicated in a number of important events, such as epigenetic, transcriptional and post-transcriptional regulations. Although *RMRP* functions are not fully understood, it has been reported that this ribonucleoprotein complex play a role in mitochondrial DNA replication where it cleaves the RNA primer, consisting of an RNA/DNA hybrid, that initiate the mitochondrial DNA replication (Hermanns et al., 2008). Moreover, RMRP seems to be involved in the modulation of the cell cycle by regulating the expression of Cyclin D2 (Shao et al., 2016).

A second analysis of the bioinformatics data was performed without correction for multiple comparison testing, using an approach already reported in literature (McNamee et al., 2016), by which only those genes with p-values less than 0.05 and a fold-change of 1.5 were selected. As expected, a number of genes showed a differentially expressed profiles for both the type of exposures and for each time point evaluated. After Gene Onthology terms analysis these genes resulted involved in multiple functions mainly related to metabolism, signal transduction, cellular component organization. Most of these genes were either up or down-

regulated making difficult to establish a common trend. Overall, the results indicated that there is a minimal induction of genes with altered expression profile immediately after exposure, a higher number was observed 2 hours post exposure while few genes were detected 24 hours after exposures. Furthermore, the genes seems to follow a time-dependent modulation profile since they showed distinct temporal response.

Moreover, there were no genes differentially expressed in common between the two type of signals, except for the lncRNA *RMRP* that showed a down-regulated expression profile 2 hours after both CW and PW exposures.

Comparing the differentially expressed genes obtained in this study with those reported in literature is particularly challenging because of their heterogeneity, probably due to the different frequencies, biological models and methodological approaches used. In fact, among the studies reporting RF-induced gene modulation, some of them are based on a not sufficient number of experimental replicates and suffer of methodological limitation as the lack of validation through RT-PCR (Pacini et al., 2002; Gurisik et al., 2006; Lee et al., 2005, Remondini et al., 2006). Conversely, a number of studies reported no effects of RF on gene expression. Among them, some performed using microarrays (Chauhan et al., 2007; Qutob et al., 2006) evaluated the effects of 1.9 GHz pulse-modulated signals on glioma cell line at different SAR values (0.1, 1 and 10 W/kg).

Also the few studies in which whole transcriptome analysis was performed using Affymetrix technology to evaluate if RF fields could induce gene expression modulation, found no significant gene expression changes in cells exposed to different RF-EMF (Whitehead et al., 2006; Zeng et al., 2006; Hirose et al., 2007). In a study evaluating transcriptome analysis on primary human keratinocytes exposed to 60 GHz (Habauzit et al., 2014), no modification in gene expression was observed when the temperature was artificially maintained constant, while a slight but specific effect of the radiation was observed in hyperthermia conditions.

In a recent *in vivo* study, McNamee and colleagues (2016), evaluated the effects of 1.9 GHz continuous and pulsed wave signal on gene expression within a variety of mouse brain tissues performing whole transcriptome analysis. Using a FDR statistical approach no convincing evidence of consistent changes in gene expression was observed, while an altered expression for some genes was observed using a non-FDR-adjusted statistical approach. The authors concluded that even if subtle

changes could not be ruled out, pathways analysis and RT-PCR did not provide supporting evidence that the exposure conditions evaluated resulted in consistent changes in gene expression in the mouse brain regions analysed.

Few studies evaluated the effects of 2.45 GHz, frequency investigated in this research, on gene expression. Sakuraj and colleagues (2011), reported no significant gene expression modulation in human glial cells at different SAR values (1, 5 and 10 W/kg) and time of exposure (1, 2 and 24 hours) using DNA microarray. Another research performed on HL-60 cells exposed for 2 or 6 hours (SAR 10 W/kg), reported several genes differentially expressed but these results originate from a single experiment and were not confirmed by RT-PCR (Lee et al., 2005). In a recent *in vivo* study, Dasdag et al. (2015) evaluated the expression profiles of five miRNA in the brain of rats exposed to 2.4 GHz (24 hours/day for 12 months) using RT-PCR. Results showed that long-term exposures, altered the expression of two of five miRNA investigated.

Focusing on the results of the DGEs functional analysis performed in the present study, some cytoskeleton-related genes were highlighted among the genes differentially expressed 2 hours after CW exposure. These genes, all up-regulated, includes *ALMS1*, that encodes for the ALMS1 protein located in the centrosomes and basal bodies of ciliated cells. Centrosome is the major microtubule-organizing centre of animal cells and influencing the cytoskeleton can be involved in cell shape, polarity and motility. It also has a crucial function in cell division because it determines the poles of the mitotic spindle that segregates duplicated chromosomes in the daughter cells (Braune et al., 2017). The *BSN* gene encodes for a scaffolding protein that seems involved in the organization of the presynaptic cytoskeleton, binding to ERC2/CAST1 (Winter et al., 1999). The gene *SYNPO2L*, encodes for an actin-binding associated protein involved in the regulation of cell migration (Weins et al., 2001). Another cytoskeleton related gene is *SYNE2,* encoding for proteins that belong to the family of giant spectrin-repeat (nesprins). These proteins, in association with SUN proteins, play an important role in the linker of the nucleoskeleton and cytoskeleton (LINC) complex because they can bind the nuclear envelope and cytoskeletal elements (Lombardi et al., 2011). We report that also *SUN3* (up-regulated) was identified among the genes differentially expressed in the samples analysed immediately after CW exposure.

Even if not the same genes were identified, our finding seems to be in agreement with the study of Zhao and colleagues (2007) that reported an altered expression profile of genes linked to cytoskeleton, signal

transduction pathway and metabolism, in rat neuron cells exposed for 24 hours to 1.8 GHz.

These genes related to cytoskeleton resulted particularly interestingly because this structure, composed of intermediate filaments, actin filaments and microtubules, exerts a central role in spindle formation and orientation and so it is involved in the correct chromosome segregation (Kunda e Baum, 2009). Some investigations supposed that RF might cause perturbation on the cytoskeleton and spindle assembly through microtubules vibration (reviewed in Marjanovic and al., 2012). In particular, in one of these studies the authors reported an alteration in the mitotic apparatus of Chinese hamster cells exposed to 2.45 GHz (15 minutes), suggesting that the radiation possibly interacted with components of centrosomes that make the poles of the mitotic spindle. However, the researchers speculated that the observed spindle alterations were reversible allowing cells to re-enter the cell cycle (Ballardin et al., 2011). Moreover, some studies evaluating chromosome loss and malsegragation events suggested that RF-EMF exposures may results in aneuploidy induction (Mazor et a., 2003; Mashevich et al., 2003; Franchini et al., 2018) that could be related to problems in spindle microtubule formation resulting in defects in the attachment to kinetocores, that is the most common mechanism that leads to chromosome malsegregation.

Finally, considering these results obtained for gene expression, in order to evaluate cellular morphological changes as cytoskeleton organization, transport vesicle formation and organelle architecture, the ultrastructural analysis by electron microscopy was performed. These observations revealed no morphological changes related to 2.45 GHz exposures for both signals and time-points evaluated, in agreement with previous *in vitro* studies on human dermal fibroblasts (Franchini et al., 2018) or on various cellular models (Liu et al., 2015) exposed to different type of RF radiation. Moreover, this finding seems to be in agreement with the transitory over expression of cytoskeleton-related genes, only 2 hours after exposure, suggesting that the proteins involved in the cytoskeletal structure were not affected, for the exposure conditions evaluated in this study.

6. CONCLUSION

The results from the present Ph.D. project are characterized by a strong scientific robustness, since the non-thermal biological effects of 2.45 GHz with two different types of signal (CW and PW), were evaluated using a well characterized exposure system and a multiparametric methodological approach. Overall, as expected, the evidences from the different assays used suggest that 2.45 GHz did not induce non-thermal genotoxic effects, neither aneugenic nor clastogenic at the SAR exposure limits recommended European guidelines (ICNIRP, 1998). Moreover, no significant differences between the two types of signal tested (CW and PW) were detected.

This research is relevant especially with regards to the gene expression analysis because performed for the first time in human cells *in vitro* exposed to RF by the high-throughput RNA-seq approach.

The results of this analysis showed no evidence of altered gene expression profile in exposed fibroblasts, except one, with FDR-adjusted statistical analysis. But using a less stringency statistical approach several genes with different expression profiles were detected. Among these genes no pathways seems to be particularly affected, but interestingly some cytoskeleton-related genes were identified. However their biological response seems to be transitory, appearing differentially expressed only 2 hours after exposure. Moreover, the ultrastructural analysis indirectly suggests that proteins involved in the cytoskeletal structure were not affected under the exposure conditions evaluated in this study, since no morphological changes in the polymerization of actin filaments in exposed cells were observed.

Overall, NGS sequencing resulted a powerful and promising approach to identify sensitive genes in order to understand the underlying mechanism of possible biological responses and effects induced by non-ionizing radiation.

In particular, the results of this project may play a role in overcoming the unclear results on the potential adverse effects of 2.45 GHz exposure on human health. In fact, one of the main concerns with RF-EMF is whether they can induce genetic damage that is a hallmarks of cancer cells (Jackson et al., 2009). Moreover, the current study, based on a well characterized exposure system and strengthened by the high-throughput NGS sequencing technology, could be a procedure model for future researches on biological effects of non-ionizing electromagnetic radiation.

The future perspective of the present study will focus in particular on the evaluation of the non-thermal biological effects after longer time of exposures, in order to reproduce conditions more similar to the real public and work environments Wi-Fi exposure. To achieve this goal the availability of a more sophisticated exposure system will be necessary in order to avoid the potential temperature increase during longer exposure time.
Furthermore, future studies with a more complete characterization at the molecular level, as the evaluation of epigenetic modification that could be involved in the regulation of specific genes, may help highlighting the underlying mechanism of these radiation.

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8. APPENDIX

Publication during Ph.D.

- Franchini V, Ceccuzzi S, Doria A, Gallerano GP, Giovenale E, Ravera GL, De Amicis A, De Sanctis S, Di Cristofaro S, **Regalbuto E**, Coluzzi E, Marinaccio J, Sgura A, Bei R, Benvenuto M, Modesti A, Masuelli L, Lista F. "Biological effects of 25 to 100 GHz radiation after in vitro exposure of human fibroblasts: a review of experimental results" Journal of Infrared Millimeter and Terahertz Waves. 2018 39: 899-911.

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- Franchini V, De Sanctis S, Marinaccio J, De Amicis A, Coluzzi E, Di Cristofaro S, Lista F, **Regalbuto E**, Doria A, Giovenale E, Gallerano GP, Bei R, Benvenuto M, Masuelli L, Udroiu I, Sgura A. "Study of the Effects of 0.15 Terahertz Radiation on Genome Integrity of Adult Fibroblasts". Environmental and Molecular Mutagenesis. 2018 Mar 30.

Conference oral presentation

- **Regalbuto E**, Anselmo A, De Sanctis S, Franchini V, Lista F, Coluzzi E, Bei R, Benvenuto M, Masuelli L, D'Inzeo G, Paffi A, Trodella E, Sgura A. "Studio degli effetti genotossici in fibroblasti umani esposti a 2,45 GHz con segnale continuo e impulsato" XVIII Congresso nazionale della Società Italiana per le Ricerche sulle Radiazioni (SIRR), Roma (10-13 settembre 2018)

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