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Sede Consorziata: **Centro di Ricerca sul Cancro "Cesare Maltoni"**

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EFFECTS OF EXTREMELY LOW FREQUENCY AND RADIOFREQUENCY ELECTROMAGNETIC FIELDS ON CIRCADIAN RHYTHMS OF SOME BLOOD PARAMETERS IN SPRAGUE-DAWLEY RATS

Direttore della Scuola : Ch.mo Prof. Marco Martini
Supervisore :Ch.mo Prof. Massimo Morgante

Dottorando : Laura Contalbrigo

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GENERAL AIM

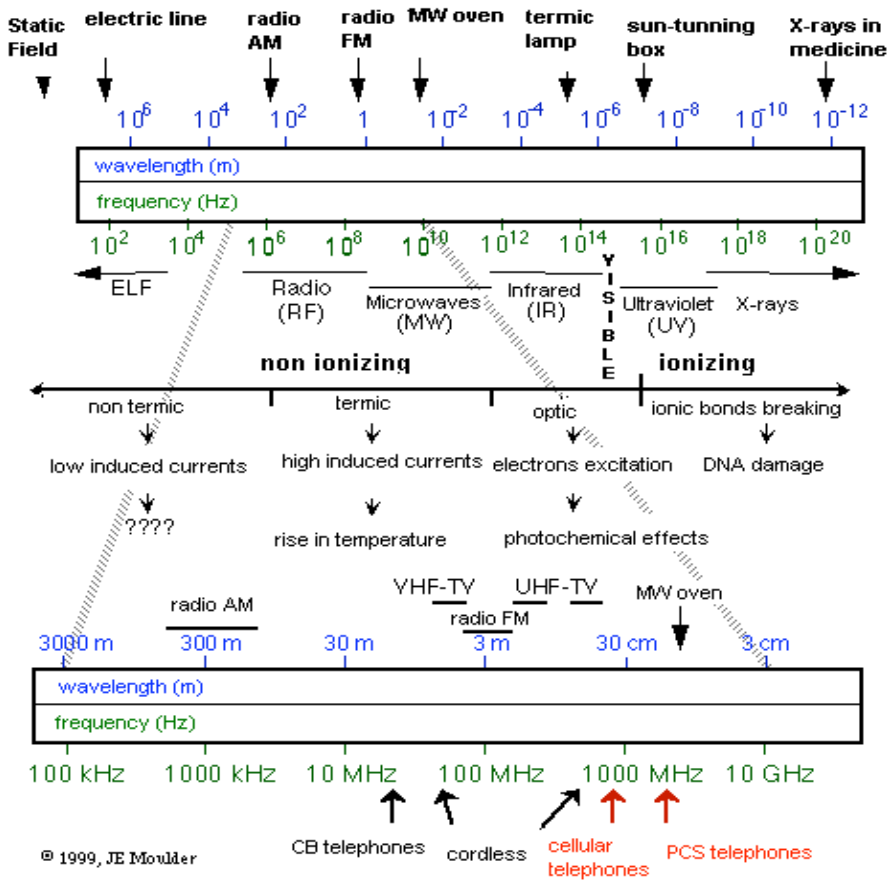
The aim of this study is to investigate the biological effects of electromagnetic fields on the circadian rhythms of three blood parameters: glycaemia, total cholesterol and triglycerides in Sprague-Dawley rats. Glycaemia, total cholesterol and triglycerides could be good homeostatic balance indicators of energetic metabolism in a living organism. In this study, it is taken under consideration the effects of extremely low frequency electromagnetic fields (ELFEMF-50 Hz) and radiofrequency electromagnetic fields (RFEMF-1.8 GHz, GSM). They are the principle source of "electro-smog" in modern society and people exposure is increasing year after year, even if there are no certainties about their health effects, especially long-term effects. Therefore this study tries to improve knowledge about interaction between electromagnetic fields and living organisms supposing EMF to alter the activity of animals' biological clock, the suprachiasmatic nucleus.

ELECTROMAGNETIC FIELDS

Physical characteristics of electromagnetic radiation

Electromagnetic radiation belongs to the wide whole of physical phenomena called radiations, apparently very different one another: i.e. light emission from a lamp, the heat generated by a flame, particles emission from a radioactive source. The common characteristics of all radiations are the presence of a source, the transport of energy through space and its interaction with matter. When a radiation interacts with matter, it can transfer such an energy to matter atoms that electrons of external orbits can leave atoms, giving rise to ions. This group of radiations are called Ionizing Radiation and it includes i.e. X-rays and γ-rays. Electromagnetic radiation has not enough energy to cause a definitive electron removing from its atomic orbit but it can excite atoms causing vibrational and rotational motions in electrons. Therefore electromagnetic radiation is called Non-Ionizing Radiation. This group includes ultraviolet radiation (UV), light (VIS), infrared radiation (IR), microwaves (MW), and the subjects of this study: radiowaves (10000 Hz-300 GHz) and extremely low frequency waves (3-3000 Hz) (Fig. 1).

Figure 1. Electromagnetic spectrum. (from Moulder JE, 1999).



Electromagnetic waves are space perturbations which carry energy. They are generated by periodic variations of an electric field and a magnetic field correlated each other in time and space. They are characterized by intensity, frequency and wavelength. Intensity depends on the intensity of the electric and the magnetic field which have a specific rate:

$$E/H=377 \text{ ohm}$$

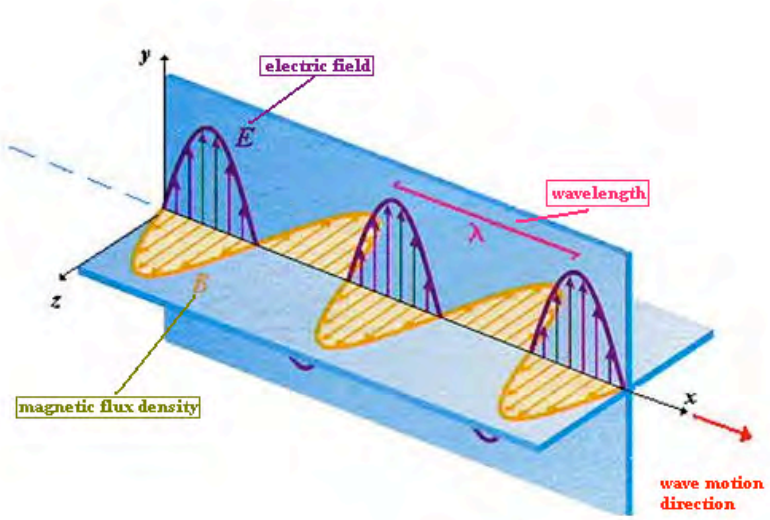
Frequency (*f*) is the number of wave oscillations (or cycles) that occur in one second; it is measured in hertz (Hz). Referring to an electromagnetic wave it is the number of times in which the same intensity of the electromagnetic field repeats itself in the time unit.
Wavelength (λ) is the distance, measured in metres, between a wave peak and the next peak of the same polarity, or the distance covered by the wave in a period (T) of oscillation ($T=1/f$). As a consequence wavelength is linked to frequency by this relationship:

$$\lambda=c/f$$

$c=10^8$m/s.

Referring to an electromagnetic wave, it is the distance between two points of the space that have the same electromagnetic field intensity (Fig. 2).

Figure 2. Electromagnetic wave motion.



Mechanism and propagation speed of all electromagnetic waves are the same, even if frequency and wavelength are different. Frequency determines gradual variations in the interaction with matter; actually waves emission, their absorption and their propagation through matter depend on the nature and physical state of the source, of the propagation medium and of the absorbent substances.

The electromagnetic field is composed by an electric field and a magnetic field linked by the specific relationship described above. They both originate from electric charges. The electric field (E) is described as "the force that an electric charge exercises in every points of the space". Therefore an electric field can exist only if a charge gives rise to an electric force (F):

$$F=q*E$$

F = electric force (vector quantity)

q = electric charge unit (Coulomb or C)

E = electric field (vector quantity)

Electric field is a vector quantity and so it is characterized by a direction and a line which are the same of a positive electric charge located in that point and embedded in the electric field. Its intensity is measured in Volts per meter (V/m):

$$E=V/d$$

E = electric field

V = potential difference between opposite charges separated by a distance (d). It is measured in Volt (V).

d = distance between charges. It is measured in meter (m).

It is represented as "force lines" which comes from positive charges and end on negative ones. It acts on a charged body causing its attraction or its repulsion by any other charged body. Electric fields are characterized by another important vector quantity electric flux density (D):

$$D=\varepsilon*E$$

ε = dielectric constant

E = electric field

Besides, thanks to the force that an electric field exercises on the electric charges, it causes an electric current. This current density (J) throughout the transversal section of a wire tissue is related to the electric field intensity (E) and to the medium electric conductivity (σ):

$$J= \sigma * E$$

Charges movement originates also the magnetic field (H) which is represented as "rings" which close around the same currents that generate them; it is always associated to the magnetic flux density (B), called also magnetic field intensity:

$$B=\mu * H$$

μ = permeability of biological tissues (generally the same as air or vacuum).

Magnetic field is measured in amperes per meter (A/m) and its intensity in Tesla (T) (1 Tesla is the intensity of a magnetic field which acts with a strength of 1 Newton on an electric charge of 1 Coulomb which is running perpendicular to the field at 1m/s); 1μT=0,8A/m.
Magnetic fields exert a force on moving electric charges, called Lorentz Force and measured in Newton (N):

$$F=q*v*B$$

F = Lorentz Force (direction perpendicular to *v* and B)
q = electric charge
v = charge speed vector
B = magnetic field vector

To characterize low frequency electromagnetic fields in the "near field" that means at distances from the source minor than $2D^2/\lambda$ (D=maximum size of the source) is necessary

to measure the electric and the magnetic field individually because in this region does not exist any relation between them. Characterization of high frequency electromagnetic fields is interesting in the "far field", that means at distances from the source major than $2D^2/\lambda$. In this case electric field and magnetic field vectors are perpendicular and their amplitudes depend on the medium impedance. Therefore to characterize the high frequency electromagnetic fields is sufficient to consider just the electric or the magnetic field or power density. Indeed power density (S), measured in Watt/m^2, is associated at the electromagnetic field by this formula:

$$S=E*H=E^2/\eta=\eta H^2$$

η=377 ohm.

Power density is an electromagnetic energy flux which goes through a unit surface set perpendicularly to its propagation direction in one second.

Finally to characterize an electromagnetic field, it is necessary to establish the polarization of the incident wave. Polarization is the time-variation of electric and magnetic field vectors in a space point:

- Linear Polarization: electric and magnetic field vectors have time-changing amplitude but constant direction in the point taken in examination. The two sinusoidal components of the field are in phase.
- Circling Polarization: electric and magnetic field vectors have time-changing direction but constant amplitude in the point taken in examination. The vector tip describes a clockwise or anticlockwise circle with constant angular velocity, equivalent to the electromagnetic wave frequency. The two sinusoidal component of the field have the same amplitude but they are out of phase (+/-90°).
- Elliptical Polarization: electric and magnetic field vectors have time-changing direction and amplitude. The vector tip describes a clockwise or anticlockwise ellipse with constant angular velocity, equivalent to the electromagnetic wave frequency.

Table 1 compares electric field and magnetic field characteristics.

Table 1. Characteristics of electric and magnetic fields.

Electric Fields	Magnetic Fields
1. Electric fields arise from voltage	1. Magnetic fields arise from current flows.
2. Their strength is measured in Volts per meter (V/m).	2. Their strength is measured in amperes per meter (A/m). Commonly EMF investigators use a related measure flux density in microtesla (μT) or millitesla (mT) instead.
3. An electric field can be present even when a device is switch off.	3. Magnetic fields exist as soon as a device is switch on and current flows.
4. Field strength decreases with distance from the source.	4. Field strength decreases with distance from the source.
5. Most building materials shield electric fields to some extent.	5. Magnetic fields are not attenuated by most materials.

Electric and Magnetic Static Fields

An electric static field is a forces field, with unchanging intensity, generated by opposite sign electric charges, separated by macroscopic distance. These charges produce a potential difference, measured in Volt, which is proportional at their number. Electric static fields are naturally present next to the Earth surface with an intensity of 130 V/m that becomes 40 kV/m during storms (Dolezalek H, 1979).

A magnetic static field originates from magnetic bodies or from steady currents; a steady current is an electric current flowing only in one direction. In any battery-powered appliance, the current flows from the battery to the appliance and then back to the battery, generating a magnetic static field which attracts or repels other electric currents or magnet set in the space where its forces act. Earth generates a magnetic static field whose vertical component is maximum at the Poles (70 μT) and it is near zero at the Equator. While the horizontal component is maximum at the Equator (30 μT) and zero at the Poles.

An electromagnetic static field does not change over time and has not polarization.

As regards artificial source, there are few devices and activities associated to electric and magnetic static fields higher than natural ones. The most important for the population exposure is railway networks (magnetic induction until 1mT in high speed train working at 30 kV) (Grandolfo M *et al.*, 1989). The other ones are medical equipment for magnetic

resonance, electrolytic processes (i.e. aluminium production), particle accelerator, magnetohydrodynamical systems, and isotope separation facilities.

Static field interaction with biological systems

An electric static field is always perpendicular to body surface and it doesn't seep into, but it induces electric charge on it. The perception threshold is about 20 kV/m, while more than 25 kV/m could be annoying (Clairmont BA *et al.*, 1989). These type of fields seem to have no biological effects in animals nor in humans (AGNR, 1994; IARC, 2002).

Magnetic static fields have three physical mechanisms to interact with living being: magnetic induction, magnetomechanical and electronic interactions (Ueno S and Iwasaka M, 1999).

Through magnetic induction, magnetic static fields act on moving ionic charges, originating electric fields and induced currents. This characteristic of magnetic static field depends on Lorentz forces (F).

Magnetomechanical interactions cause magnetoorientation and magnetomechanical translation.

Electronic interactions can have some effects on the spin of radical electron in intermediate state of certain chemical reactions (Zeeman effect).

Magnetic static fields with an intensity minor than 2 T don't have any effects on body temperature, cardiac functionality, blood pressure or mental activity of both laboratory animals and human, nevertheless it is possible that fields with an higher intensity can influence cardiovascular activity and behaviour (Tenforde TS, 1992). Besides many studies showed no effect on gestation, embryo implantation and foetal development (Kowalczuk CI *et al.*, 1991;High WB *et al.*, 2000; Tablado L *et al.*, 2000), and no carcinogenetic effects (Bellossi A and Toujas L, 1982; Bellossi A, 1984; Barregard L *et al.*, 1985; Bellossi A, 1986; Ronnenberg A *et al.*, 1999; IARC, 2002). Recently, the International EMF Project of the World Health Organization (WHO) published an Environmental Health Criteria monograph on static electric and magnetic fields. The main conclusions are that no acute effects other than transient phenomena such as vertigo and nausea have been observed with exposure to static magnetic flux densities up to 8 T. There are no reports of long term or chronic adverse effects following prolonged static magnetic field exposure, but few data are available on which to base any judgment (van Rongen E *et al.*, 2007).

International guidelines on exposure to static magnetic fields

International exposure guidelines are developed by the International Commission on Non-Ionizing Radiation Protection (ICNIRP). This independent body is officially recognized by WHO and its exposure guidelines advice is based upon the health risk assessments published by WHO and cancer reviews and classifications carried out by IARC.

Exposure guidelines serve five main function:

1. A general framework for the protection of people who may be exposed to static electric or magnetic fields whether at work, in public spaces or in the home;
2. A tool for practical safety assessment of exposures in relation to recommended exposure restrictions (compliance assessment);
3. A basis for national standards and regulations on limiting exposure;
4. A basis for the development of technical standards pertaining to equipment design, device emissions and measurement procedures;
5. A basis for operational procedures at workplaces and facilities, especially if exposure to high field strengths are required for short periods of time in occupational settings.

Exposure restrictions are recommended below which acute adverse effects will not occur. In specifying restrictions on exposure, it is important that caution is exercised to ensure the adequate protection of all members of the community even of more susceptible people like neonates.

ICNIRP published its advice on limiting public and occupational exposure to static magnetic fields in 1994 (ICNIRP, 1994). This had the objective of protecting individuals from the direct effects of fields, from indirect effects on ferromagnetic objects, and on implanted devices such as pacemakers, aneurism clips etc. ICNIRP's guidelines followed after the development of a few national exposure guidelines including those developed in the former USSR in 1978 and in the UK by the National Radiological Protection Board (NRPB, 1993). ICNIRP noted that the scientific knowledge existing at that time not suggest any detrimental effect on major developmental, behavioural and physiological parameters following transient exposure to static magnetic flux densities up to 2 T. In the absence of knowledge on possible adverse health effects from long-term exposure, ICNIRP recommended a restriction of 200 mT on time-weighted exposure. In addition, the movement of a person in a magnetic field of 200 mT was thought to result in a current density of between 10 and 100 mA/m^2, which was considered not to result in adverse effects on the function of the central nervous system at frequencies of less than 10 Hz.

ICNIRP recommended a time-weighted average exposure of 200 mT during the working day for occupational exposures, with a ceiling value of 2 T. A ceiling value of 5 T was considered acceptable for extremities, because they do not contain large blood vessels or critical organs. A continuous exposure limit of 40 mT was given for the general public. This is, in effect, a ceiling value, although "*occasional access to special facilities where magnetic flux density exceed 40 mT can be allowed under controlled conditions, provided that the appropriate occupational exposure limit is not exceeded*". ICNIRP suggested that wearers of cardiac pacemakers, ferromagnetic implants and implanted electronic devices might not be adequately protected by the exposure limits for direct effects. Therefore, ICNIRP recommended that people with cardiac pacemakers and implanted defibrillators should avoid locations where magnetic flux density exceed 0.5 mT. Finally, ICNIRP stated that precautions should be taken to prevent hazards from flying metallic objects if the magnetic flux density exceeded 3 mT (Tab. 2).

Table 2. Limits of exposure to static magnetic fields (ICNIRP, 1994).

Exposure Characteristics	Magnetic Flux Density
Occupational	
Whole work day (time-weighted average)	200 mT
Ceiling Value	2 T
Limbs	5 T
General Public	
Continuous exposure[a]	40 mT

[a]: Occasional access for members of the public to special facilities where magnetic flux densities exceed 40 mT can be allowed under appropriately controlled conditions, provided that the appropriate occupational limits is not exceeded.

The ICNIRP exposure restrictions for the general public provided the basis for a Council of the European Union Recommendation on limiting public exposure to static magnetic fields throughout the European Community (CEU, 1999).

Time-Varying Electromagnetic Fields: Extremely Low-Frequency Electromagnetic Fields (ELFEMF)

Time-varying electromagnetic fields are produced by alternating currents; this type of electric currents reverse their direction at regular intervals. Exposure levels are usually due to human activity rather then natural background. The most important source of this type of fields are generation, transmission and consumption of electricity. Therefore high-voltage line and electrical devices which are present in domestic and working environment are the principal source of time-varying electric and magnetic fields with an "industrial frequency" of 50 Hz in Europe (transmission lines voltage 380-400 kV) and 60 Hz in the USA, Canada and Japan (transmission line voltage 735 kV). That means in Europe, electricity changes direction with a frequency of 50 cycles per second; equally the associated electromagnetic field changes its orientation 50 times every second; they are called extremely low frequency electromagnetic fields (ELFEMF) with an electric field intensity between 10-100 V/m and a magnetic field intensity between 0.1-1 mT.

The electric field produced by high-voltage line depends on voltage line, on the distance from the line itself (it is maximum in the area under the line), on the wire high from ground and on wire structure: electric field is strongest close to a charge or charged conductor, and its strength rapidly diminishes with distance from it; it is vertical under the wire, while its horizontal component, perpendicular to the line, becomes appreciable only at lateral distance from the line which are twice the wire high. In this point the field intensity is very low compared to its maximum value. The horizontal component parallel to the line is slight. Conductors such as metal shield it very effectively. Other materials, such as building materials and trees, provide some shielding capabilities. Therefore, the electric fields from power lines outside the house are reduced by walls, buildings, and trees. When power lines are buried in the ground, the electric fields at the surface are hardly detectable. In table 3 are listed the electric field intensity measured in the most common exposure conditions.

Table 3. Typical electric field strengths measured near household appliances. (From: Grandolfo M and Vecchia P, 1985; Federal Office for Radiation Safety, Germany 1999).

Exposure Site	Electric Field Strength (V/m)
Under a high voltage line (380kV)	5000
Inside a house	0-10
In a city area	0-50
In the countryside	0-0,05
At 30 cm from an electric blanket	250
At 30 cm from a stereo receiver	180
At 30 cm from an electric kettle	130
At 30 cm from a fridge	120
At 30 cm from an iron	120
At 30 cm from a mixer	100
At 30 cm from a toaster	80
At 30 cm from a hairdryer	80
At 30 cm from a colour TV	60
At 30 cm from a vacuum cleaner	50
At 30 cm from an electric clock	15
At 30 cm from an electric oven	8
At 30 cm from a light bulb	5
Guideline limit value	**5000**

The magnetic field intensity, produced by high-voltage line, depends on the electric current which flows through the wire and on the distance from the wire. Both the horizontal and the vertical components of the magnetic field, orthogonal to the line, determine the intensity of the field itself, while the horizontal component, parallel to the line, is slight. Like electric fields, magnetic fields are strongest close to their origin and rapidly decrease at greater distances from the source. Magnetic fields are not blocked by common materials such as the walls of buildings.

Table 4 lists the magnetic induction measured in some common exposure conditions. It illustrates two main points: First, the magnetic field strength around all appliances rapidly decreases the further you get away from them. Secondly, most household appliances are not operated very close to the body. At a distance of 30 cm the magnetic fields surrounding most household appliances are more than 100 times lower than the given guideline limit of 100 μT at 50 Hz (83 μT at 60 Hz) for the general public.

Table 4. Typical magnetic field strength of household appliances at various distances (From: Federal Office for Radiation Safety, Germany 1999).

Electric appliance	3 cm distance (µT)	30 cm distance (µT)	1 m distance (µT)
Hair dryer	6 – 2000	0.01 – 7	0.01 – 0.03
Electric shaver	15 – 1500	0.08 – 9	0.01 – 0.03
Vacuum cleaner	200 – 800	2 – 20	0.13 – 2
Fluorescent light	40 – 400	0.5 – 2	0.02 – 0.25
Microwave oven	73 – 200	4 – 8	0.25 – 0.6
Portable radio	16 – 56	1	< 0.01
Electric oven	1 – 50	0.15 – 0.5	0.01 – 0.04
Washing machine	0.8 – 50	0.15 – 3	0.01 – 0.15
Iron	8 – 30	0.12 – 0.3	0.01 – 0.03
Dishwasher	3.5 – 20	0.6 – 3	0.07 – 0.3
Computer	0.5 – 30	< 0.01	-
Refrigerator	0.5 – 1.7	0.01 – 0.25	<0.01
Colour TV	2.5 - 50	0.04 – 2	0.01 – 0.15
With most household appliances the magnetic field strength at a distance of 30 cm is well below the guideline limit for the general public of 100 µT.			

ELFEMF interaction with biological systems

Biological systems work like dielectric wires; they undergo a polarization (a separation of their electric charges) when they are exposed to electromagnetic fields. They are characterized by:

- Permittivity (ε): the capacity of a body to store energy coming from the electric field; it depends on body water content. $\varepsilon=\varepsilon_r*\varepsilon_o$ (ε_r relative permittivity; ε_o vacuum permittivity).
- Permeability (μ): magnetization level of a body embedded in a magnetic field; for biological system it is the same of air or vacuum. $\mu=\mu_r*\mu_o$ (μ_r relative permeability; μ_o vacuum permeability).
- Conductivity: capacity of a body to be crossed by electricity.

Tiny electrical currents exist in the human body due to the chemical reactions that occur as part of the normal body functions, even in the absence of external electric fields. For example, nerves relay signals by transmitting electric impulses. Most biochemical reactions from digestion to brain activities go along with the rearrangement of charged particles. Even the heart is electrically active - an activity that can be traced with the help of an electrocardiogram.

Low-frequency electric fields influence the human body just as they influence any other material made up of charged particles. When electric fields act on conductive materials, they are upset by them because they have a different permittivity and conductivity rather than air. Electric fields influence the distribution of electric charges at the body surface. They cause current to flow through the body to the ground; these currents, called Eddy currents, have the same direction of electric field vector. The electric field intensity is bigger in the higher body areas and declines in the sloping ones. Low-frequency magnetic fields induce circulating currents within the human body; these circulating currents are always perpendicular to the magnetic field vector. Their strength depends on body dimension and on the intensity of the outside magnetic field which is not influenced by the body and it is the same into the body as well as on its surface. If sufficiently large, these currents could cause stimulation of nerves and muscles or affect other biological processes. Both electric and magnetic fields induce voltages and currents in the body but even directly beneath a high voltage transmission line, the induced currents are very small compared to thresholds for producing shock and other electrical effects (WHO, 2007). However the body, crossed by an electromagnetic wave, absorbs energy. Even if, in the case of ELFEMF, the energy absorbed is too little to cause a direct damage to DNA, it may

interfere with some metabolic processes, modifying intracellular enzymatic pathways and causing an increase in the production of free radicals which may alter or interfere with DNA reparation or replication mechanisms, protein and lipid-containing structures (IARC, 2002).

ELFEMF and cells free radicals production

Although 50/60 Hz EMF do not directly lead to genotoxic effects, it is possible that certain cellular processes altered by exposure to EMF, indirectly affect the structure of DNA, causing strand breaks and other chromosomal aberrations (IARC, 2002). The possible initial cellular event, compatible with the multitude of effects observed after exposure to ELFEMF, is an increasing levels of free radicals. Such a general activation is compatible with the diverse nature of observed effects (Ding GR *et al.*, 2003; Lupke J *et al.*, 2003; Rosenspire AJ *et al.*, 2003; Simkó M and Mattsson MO, 2004). Free radicals are intermediates in natural processes like mitochondrial metabolism and are also a key feature of phagocytosis in macrophages. Macrophages play an essential role in the body's defences and immune system: activated macrophages release free radicals as reactive oxygen species (ROS), reactive nitrogen species (RNS), and also cytokines (Dröge W, 2002). ROS are unstable reactive molecules which are produced continuously in several cells, not only in macrophages. Free radicals including superoxide anion radicals, hydroxyl radicals, and hydrogen peroxides are formed as by-products in various metabolic processes. ROS are involved in intracellular signal transduction pathways and regulation of gene expression determining the anti-inflammatory response, cell growth, differentiation, proliferation and stress response (Lassegue B *et al.*, 2003).

Enzymes such as NAD(P)H-oxidases, xanthine oxidases or arachidonic acid-metabolizing enzymes mediate the main production of ROS in macrophages. In phagocytic cells, the NADPH-oxidase is commonly associated with the "repiratory burst" activity catalyzing the reduction of oxygen to superoxide anion radical. This high level of free radical formation is a primary host defence mechanism against any invading microorganism and is connected with cell activation. Isoforms of NADPH-oxidase are present in various non-phagocytic cells which were found to have similar characteristics. NADH-oxidase has been implicated in numerous cellular processes within signal transduction cascades and regulatory processes (Grienling KK *et al.*, 2002). It seems that 50 Hz EMF stimulates the NADH-oxidase pathway (and not NADPH pathway) to produce superoxide anion radicals. Furthermore, it has been showed an oscillation (1–10 days) in superoxide anion radical

release in mouse macrophages, indicating a cyclic pattern of NADH-oxidase activity (Rollwitz J *et al.*, 2004).

Alternatively, superoxide anion radical generation occur non-enzymatically by redox reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain (Dröge W, 2002). In response to outside influence, superoxide anion radical is the primary one generated by phagocytes. It has a low bactericidal potency and are converted into other ROS that serve as mediators in many regulatory processes (Roos D *et al.*, 1992). In cells, free radical concentration is determined by the balance between their rate of production and their rate of clearance, controlled by different enzymes and antioxidant compounds. These regulatory processes are important to reset the original state of redox homeostasis after temporary production of free radicals (Rollwitz J *et al.*, 2004).

In contrast to molecules such as cytokines (large molecules signalling by docking with specific receptors and change molecular surfaces on the target cells) molecules such as ROS could react with diverse cell compounds in a non-specific mechanism. Therefore, free radicals play a decisive role in cytotoxicity and also as cellular messengers to control non-cytotoxic physiological responses.

Free radical release is inducible by ionizing radiation or phorbol ester treatment, both leading to genomic instability (Roy S *et al.*, 1995). EMF might be a stimulus to induce an " activated state" of the cell such as phagocytosis, which then enhances the release of free radicals, in turn leading to genotoxic events. EMF exposure can cause both acute and chronic effects that are mediated by increased free radical levels:

- Direct activation of macrophages (or other cells) by short-term exposure to EMF leads to phagocytosis (or other cell specific responses) and consequently, free radical production. This pathway may be utilized to positively influence certain aspects of the immune response, and could be useful for specific therapeutic applications.
- EMF-induced macrophage (cell) activation includes direct stimulation of free radical production.
- An increase in the lifetime of free radicals by EMF leads to persistently elevated free radical concentrations. In general, reactions in which radicals are involved become more frequent, increasing the possibility of DNA damage.
- Long-term EMF exposure leads to a chronically increased level of free radicals, subsequently causing an inhibition of the effects of the pineal gland hormone melatonin.

Taken together, these EMF induced reactions could lead to a higher incidence of DNA damage and therefore, to an increased risk of tumour development. While the effects on melatonin and the extension of the lifetime of radicals can explain the link between EMF exposure and the incidence of for example leukaemia, the two additional mechanisms described in mouse macrophages, can explain the possible correlation between immune cell system stimulation and EMF exposure (Rollowitz J *et al.*, 2004; Simkó M and Mattsson MO, 2004).

ELFEMF and cell membrane ions channels

In particularly, researches focused their attention on the influence of weak electromagnetic fields on the Ca^{2+} metabolism of living cells. Ca^{2+} has many important roles in all living organisms: it has a structural function in bone matrix and in stabilizing membranes but, first of all, it is essential in cellular homeostasis, most notably as an intracellular messenger (Bauréus Koch CLM *et al.*, 2003). The free Ca^{2+}concentration in the cytosol is strictly regulated and kept at 0,1-0,2 μM. Localized increases in the cytosolic free Ca^{2+} concentration in the form of waves and gradients are involved in the regulation of many processes in the cells including secretion, adhesion, motility, growth and differentiation as well as in the control of many metabolic activities of all organism like the earliest stages of reproduction, its growth and development (Clampham DE, 1995). The low cytosolic Ca^{2+} concentration is maintained by a range of active transporters; the plasma membrane Ca^{2+} pump (Ca^{2+}-ATPase) plays an important role. In most cells, it represents the main mechanism for pumping Ca^{2+} out of the cell, whereas Ca^{2+}pumps located in the intracellular Ca^{2+} stores are important for the short term control. In contrast, the movement of Ca^{2+}into the cytosol from the extracellular medium or from intracellular Ca^{2+} stores is passive and can be achieved through the opening of various Ca^{2+} permeable channels (Clapham DE, 1995). Voltage-dependent calcium channels are composed of four principal subunits: the transmembrane, pore-forming α1 subunit and three accessory subunits that modulate channel function: the glycosilated α2δ subunit, the integral membrane γ subunit and the cytoplasmatic β subunit. There are several isoforms of each of these channel subunits, and the composition of the channel complex determines its expression level, localization, kinetics and pharmacology (Dolphin AC, 2006) (Tab.5).

Table 5. Voltage-dependent calcium channels. (From: Catterall WA, 2005).

Type	Voltage	α_1 subunit (gene name)	Associated subunits
L-type calcium channel: - long-lasting - AKA - DHP receptor	HVA (high voltage activated)	$Ca_V1.1$ (*CACNA1S*) $Ca_V1.2$ (*CACNA1C*) $Ca_V1.3$ (*CACNA1D*) $Ca_V1.4$ (*CACNA1F*)	$\alpha_2\delta$, β, γ
P-type calcium channel/Q-type calcium channel	HVA (high voltage activated)	$Ca_V2.1$ (*CACNA1A*)	$\alpha_2\delta$, β, possibly γ
N-type calcium channel - Neural	HVA (high voltage activated)	$Ca_V2.2$ (*CACNA1B*)	$\alpha_2\delta/\beta_1$, β_3, β_4, possibly γ
R-type calcium channel	intermediate voltage activated	$Ca_V2.3$ (*CACNA1E*)	$\alpha_2\delta$, β, possibly γ
T-type calcium channel - Transient	low voltage activated	$Ca_V3.1$ (*CACNA1G*) $Ca_V3.2$ (*CACNA1H*) $Ca_V3.3$ (*CACNA1I*)	not yet established

Calcium channel expression changes also with normal aging. For example in hippocampal neurons, the expression of L-type channels increases with age; it is correlated with increase failure of excitatory postsynaptic potentials as well as increased susceptibility to cell death. At the whole-animal level, these changes are correlated with decreased performance on spatial learning tasks.(Audesirk G *et al.*, 2000). The activity of calcium channels is regulated by a wide variety of intracellular signalling pathways but the three most well understood are binding and activation of calmodulin, phosphorylation by several protein kinases, and binding of G protein $\beta\gamma$ subunits (Park D and Dunlap K, 1998). These pathways not only modulate calcium channels activity, all are themselves modulated in various ways by calcium influx through calcium channels, and many interact with one another (Catterall WA *et al.*, 2005).

Calmodulin mediates many of the intracellular effects of calcium influx through calcium channels, especially L-type channels. Calcium entry via the L-type channel appears to be

critical for activation of a number of different transcriptional pathways, including those mediated by CREB (cycling-AMP responsive element binding protein), NF-AT (nuclear factor of activated T-cell), and p38 MAP (mitogen activated protein) kinase (Gwack Y *et al.*, 2007; Takeda K *et al.*, 2004; Zhao R *et al.*, 2007). Besides calmodulin acts in the feedback regulation of calcium entry through L-type channels: it binds to the intracellular carboxy terminal of the α1C subunit; calcium, entering through the channel, binds to this calmodulin and reduces further calcium inflow. Thus calmodulin can act as a key molecule both in stimulation of gene transcription and other cellular events and in feedback inhibition of calcium channels (Zhan R *et al.*, 2005).

Calcium channels may be phosphorylated by many protein kinases, including calcium/calmodulin-dependent protein kinase, protein kinase A (PKA) and protein kinase C (PKC) (Wagner S *et al.*, 2006). PKA is activated by cAMP and it increases calcium channels activity. PKC activation depends on diacylglycerol (an important product of phosphoinositol cascade). It can cause a decrease or a biphasic change (increase followed by a decrease) in $Ca_V2.1$ current amplitude. In contrast, $Ca_V1.2$ channels are inhibited by PKC. Analysis of the molecular basis for this effect implicates phosphorylation of two threonine residues in the N-terminal domain of $Ca_V1.2$ (McHuge D *et al.*, 2000).

Calcium influx through both voltage- and ligand-gated channels (e.g., the N-methyl-D-aspartate receptor) contributes to alterations in calcium homeostasis, as well as intracellular organelles and calcium ATPases. These intracellular calcium stores and ATPases interact with channel mediated calcium entry to give rise to locally confined calcium increases, or responses such as calcium spikes and waves. The amplitude, time course, and intracellular distribution of calcium signals are important determinants of the cellular response (Audesirk G *et al.*, 2000): in neurones the frequency of calcium transients regulates differentiation, axon growth and growth cone turning (Spitzer NC *et al.*, 2000); in lymphocytes, they selectively activate specific transcriptional pathways (Dolmetsch RE *et al.*, 1998). Therefore local microdomains of intracellular calcium provide a further mechanism for generating specificity: in these microdomains with volumes of the order of femtolitres, $[Ca^{2+}]_I$ can accumulate to levels that are orders of magnitude greater than the global average $[Ca^{2+}]_I$ measured throughout the cell (Bootman MD *et al.*, 2001). In this way voltage-gated calcium channels in excitable cells act locally to trigger exocytosis, activate K^+ channels, elicit Ca^{2+} release through ryanodine receptors and activate gene transcription pathways. In a similar fashion, store-operated calcium channels that open in

response to Ca^{2+} store depletion are known to elicit local effects on Ca^{2+}-sensitive adenylate cyclases, nitricoxide synthase and mitochondria in non excitable cells (Bautista MD and Lewis RS, 2004).

Several studies have showed various effects of exposure to ELFEMFs upon the calcium efflux in biological systems (Bawin S *et al.*, 1975; Blackman CF *et al.*, 1982; Blackman CF *et al.*, 1988; Blackman CF *et al.*, 1989; Smith S, 1987). An attempt to explain these observations resulted in the ion cyclotron resonance (ICR) model (McLeod BR and Liboff AR, 1986): it considers the Lorentz force acting on a moving charge in magnetic and electric fields, therefore the ELFEMFs work directly on the ion transport dynamics. In other models, the cellular response constitutes a secondary effect of altered ion binding properties due to interactions with the applied magnetic fields. The primary site of interaction may be the calcium transporting proteins in the cell membrane and more specifically, the opening of calcium channels seems to be influenced by ELFEMF (Baréus Koch CLM *et al.*, 2003; Kindelskii AL *et al.*, 2003). The consequent changes in the periodic oscillation of $[Ca^{2+}]_I$ causes the activation of some proteins like calmodulin and protein-kinase C (PKC) which modulate DNA, RNA and proteins synthesis (Dibirdik I *et al.*, 1998); the linkage between DNA and the transcriptional factor CREB (cycling-AMP responsive element binding protein) (Zhou J *et al.*, 2002); the activation of some oncogenes like *c-myc*, *c-fos*, *c-jun* (Goodman R *et al.*, 1989; Gold S *et al.*, 1994; IARC, 2002), and it modifies the activity of immune system cells like lymphocytes and natural killer cells (increase interleukins production and release, alteration of cytotoxic activity) (Becherer U *et al.*, 2003; Lewis RS, 2001). Besides ELFEMFs may interact with potassium channels, causing their persistent opening and slacking the activation of leucocytes (Lewis RS, 1995; Panyi G *et al.*, 2004), and with adenosine receptors causing their up-regulation (Bautista MD *et al.*, 2002). Adenosine is an endogenous modulator which interacts with four membrane receptors coupled with protein G (A1, A2a, A2b and A3). Especially A2a receptor is expressed in neutrophils, monocytes, lymphocytes, platelets, mast cells and macrophages. It has an anti-inflammatory function reducing TNF-α, IL-6, IL-8 and elastasis production (Bautista MD and Lewis RS, 2004).

ELFEMFs seem also to influence the activation of other cell membrane receptors, especially they increase the activity of ornithine-decarboxylase (ODC) (IARC, 2002). ODC is an enzyme implicated in the S phase of the cell cycle, so it is necessary for the control of DNA replication and cell proliferation. It regulates polyamines biosynthesis,

implicated in the DNA and RNA synthesis thanks to ornithyne decarboxylation to putrescine. Therefore ODC activity always increases immediately before mRNA neo-synthesis and it is higher in rapid growing cells and in neoplastic cells. ODC is regulated by many growth factors and hormones throughout membrane cell receptors and so it may be one of the enzymatic pathways activated by ELFEMF interaction with the cell membrane (Byus CV *et al.*, 1987; Cain DC *et al.*, 1993).

Some other speculation regarding mechanisms has focused on the influences of power-frequency electromagnetic fields on the cell membrane (Adey WR, 1990; Adey WR, 1990; Kavet R, 1996; Luben RA, 1995). In this regard, some experimental work has investigated possible effects of power-frequency electromagnetic fields on gap junction. Gap junctions are pores formed by specialized proteins located in the plasma membrane which mediate the transfer of low-molecular-weight molecules and ions from cell to cell. A well-established physiological role for gap junctions has been demonstrated in electrically excitable tissues such as myocardium, nerve cells and smooth muscle (DeMello W, 1987). A variety of experimental evidence has also implicated gap junctions intercellular communication (GJIC) in the processes of embryonic development, differentiation and growth control (Loewenstein WR, 1979; Metha PP *et al.*, 1986; Guthrie SC and Gilula NB, 1989), although the exact role of GJIC in these processes has not been elucidated. Extensive data exist in the literature which support the hypothesis of a link between GJIC and regulation of cell growth. Evidence in support of this hypothesis comes from observations that GJIC is reduced or absent in transformed cells (Mikalsen SO and Sanner T, 1993), it can be diminished or abolished by treatment with tumour promoters such as phorbol esters (Mesnil M *et al.*, 1986; Miki H *et al.*, 1990) and is reduced by the expression of certain oncogenes (Trosko JE *et al.*, 1990). A variety of transfection experiments has demonstrated that introduction of gap junction protein genes (a class of protein called connexins) into transformed cells at least partially controls the growth characteristics of the transformed phenotype (Metha PP *et al.*, 1991; Zhu D *et al.*, 1991). Thus an obvious corollary hypothesis might be disruption of GJIC that could contribute to the process of carcinogenesis. Some investigators have reported that exposure to power frequency electromagnetic fields may effect GJIC demonstrating for example that magnetic field exposure interfered with and decreased GJIC (Benane SG *et al.*, 1996; Blackman CF *et al.*, 1995; Li CM *et al.*, 1999; Ubeda A *et al.*, 1995; Ubeda A *et al.*, 1995) inducing a hyperphosphorylation of 43 connectine protein (Hu GL *et al.*, 2001; Yamaguchi

DT *et al.*, 2002). On the other hand, in some studies there were no robust conclusive effect of magnetic field exposure on GJIC (Schimmelpfeng J *et al.*, 1995; Griffin GD *et al.*, 2000).

In addition to these direct effects on cells and especially on the immune system, ELFEMFs may have a very important indirect biological effect suppressing the normal nocturnal rise in melatonin (Stevens RG, 1987).

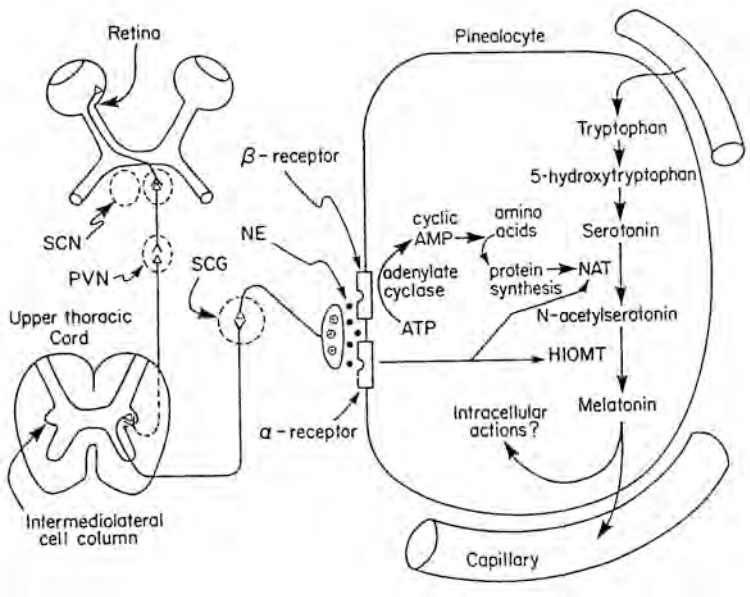
Melatonin Hypothesis

According to "melatonin hypothesis", the exposure to ELFEMFs may alter the normal function of the pineal gland, suppressing or reducing melatonin nocturnal synthesis and release increasing risk of breast cancer and other pathologies (Stevens RG, 1987; Wilson BW *et al.*, 1989; Wilson BW *et al.*, 1990). Another possible mechanism of action is that ELF fields cause an increase of free radicals production by peripheral tissues and so their consumption of melatonin which is also an antioxidant agent (Reiter RJ *et al.*, 2000). Melatonin is an indoleamine synthesized by the pineal gland as well as by a number of extrapineal organs (lens, retina, Harderian gland, gut, liver, reproductive organs, bone marrow cells, lymphoid cells, several brain regions). However pineal gland remains the organ that mainly contributes to the levels of melatonin present in the blood. The melatonin secretion rhythm in the pineal gland and in the visual system (retina and Harderian gland) complies with the light-darkness rhythm: light (10^{14}-10^{15} Hz) acts as "a visually and chronobiologically effective radiant energy for human beings"(Stevens RG and Rea MS, 2001), in fact light of sufficient intensity and suitable spectral quality (460-470 nm) suppresses pineal melatonin production (Smith KA *et al.*, 2004) whereas in the other organs and tissues its secretion probably does not depend on the degree of illumination (Kvetnoy IM, 2002). The photic information is transduced into a neural signal which is projected through the retinohypothalamic tract to the suprachiasmatic nuclei of the hypothalamus (SCN). Output signals, generated in the SCN during darkness at night, run into a complex neural pathway which includes axons of the SCN that project to the paraventricular nuclei of the hypothalamus, whose fibres descend to the upper thoracic cord where they terminate on preganglionic sympathetic cell bodies. The axons of these neurons exit from the spinal cord and synapse on postganglionic sympathetic cells in the superior cervical ganglia. Ultimately, the axons of these neurons innervate the pineal gland where they control the production of melatonin (Erren TC *et al.*, 2003). Melatonin has the essential aminoacid tryptophan as a precursor; it is hydroxylated to 5-hydroxytryptophan

and then decarboxylated to serotonin. The latter is N-acetylated by the rate-limiting enzyme in melatonin production arylalkylamine-N-acetyltransferase (AA-NAT) to N-acetylserotonin and finally converted to melatonin by the enzyme hydroxyindole-O-methyltransferase (Klein DC, 1979). The synthesis of melatonin is initiated by the release of norepinephrine (NE) into the synaptic clefts between the sympathetic nerve endings and the pinealocytes. NE is released during the dark phase and activates adenylate cyclase, which includes cyclic adenosine monophosphate (cAMP) production. This activates AA-NAT, the key enzyme in melatonin synthesis, as well as its transcription and translation (Touitou Y, 2001). Rhythmic activation of AA-NAT is based on the transcriptional regulation of the AA-NAT gene, involving two antagonist transcription factors of the cAMP signalling pathway: CREB as activator of gene expression and ICER (inducible cAMP early repressor) as an inhibitor (Stehle JH *et al.*, 2003). Therefore serotonin/melatonin transformation is enhanced through the regulation of cAMP pathway; a key element of the cAMP pathway is calcium ions (Fig. 3). Calcium ion efflux from the pinealocytes has the effect of reducing melatonin through reducing the cAMP. If ELFEMFs, as described above, can alter Ca^{2+} channels activity, they can modify also melatonin production.

There is no identified storage machinery for melatonin in the pineal gland therefore it is synthesized and immediately released into the circulation and directly into the cerebrospinal fluid of the third ventricle (Tricoire H *et al.*, 2002). Once into the blood stream, only 30% of melatonin escapes binding to plasma albumin. The active compound has a half-life of 20 minutes, then it is rapidly metabolized in the liver by microsomal hydroxylation to 6-hydroxymelatonin and, after conjugation with sulphuric or glucuronic acid, is excreted in the urine (Gram C *et al.*, 1998). Due to the cyclic activation and inhibition of melatonin production by the pinealocyte, indole plasma levels follow a well-known circadian rhythm, synchronized with the external environment, with highest values at night. The amount of melatonin produced in the pineal is genetically determined. Besides most of the daytime level of melatonin in the blood is probably derived from synthesis in the gastrointestinal tract, in which melatonin concentration exceed blood melatonin levels by 10-100 times (Bubenik GA, 2002). Circadian secretion of melatonin produced by gastrointestinal tract appears to be regulated by food intake: increase of circulating melatonin levels are observed after tryptophan administration, food intake and long-term food deprivation (Bubenik GA *et al.*, 1992).

Figure 3. The biochemical mediation system for serotonin transformation to melatonin in the pinealocytes showing the signal transduction pathways from the retina to the cell and the cell receptor, through cyclic AMP and NAT to the transformation process. (From Reiter RJ, 1994)



Melatonin Receptors

The physiological effects of melatonin are, in part, mediated by specific receptors classified into three subtypes: MT_1, MT_2, MT_3. MT_1 and MT_2 have seven transmembrane domains and belong to the G-protein coupled receptor superfamily, linked to multiple signal transduction cascades. While MT_3 belongs to quinine reductase enzyme family (Witt-Enderby PA *et al.*, 2003). MT_1 has a vast tissue distribution and it can couple to a wide variety of G-proteins. It produces inhibitory responses on the cAMP signal transduction cascade, resulting in decreases in protein kinase A (PKA) activity and in CREB phosphorilation (Witt-Enderby PA *et al.*, 1998). It stimulates phospholipase C-dependent (PLC-dependent) signal transduction cascades, directly or indirectly via a specific G-protein subunit (Gβγ); it can activates PKC (Witt-Enderby *et al.*, 2001) as well as many other kinases and modulates the formation of arachidonic acid (Godson C and Reppert SM, 1997). MT_1 melatonin receptors inhibit the neuronal firing rate in the SCN, prolactin secretion from the *pars tuberalis* of the pituitary gland and induces vasoconstriction; in fact they are expressed in cardiac vessels (Doolen S *et al.*, 1998) and involved in modulating circadian rhythms (Dubocovich ML *et al.*, 1998).

MT_2 receptors are localized in the SCN of the hypothalamus, in the cerebellum, in the retina, in the kidney, in the ovary, in the cardiac vessels and it has been found even in various cancerous cell lines (Von Gall C *et al.*, 2002). They are involved in retinal physiology, in modulating circadian rhythms in the SCN, in dilating cardiac vessels, and in the inflammatory response at the level of microcirculation (Dubocovich ML *et al.*, 1998). Activation of MT_2 phase shifts circadian rhythms within the SCN, inhibits dopamine release in the retina, induces vasodilatation, enhances splenocyte proliferation and inhibits leukocyte rolling in the microvasculature. Besides MT_2 receptors are able to determine an inhibition of cAMP formation and a stimulation of phosphoinositide hydrolysis (MacKenzie RS *et al.*, 2002). MT_3 is expressed in the liver, kidney, brain, hearth, brown adipose tissue, skeletal musculature, lung, intestine, testis and spleen of different mammalian species (Nosjean O *et al.*, 2001). This protein may be involved in the reduction of intraocular pressure in rabbits (Pintor J *et al.*, 2001) and in inflammatory responses in the microvasculature, inhibiting leukotriene B_4-induced leukocyte adhesion (Lotufo CM *et al.*, 2001).

Melatonin may also act at intracellular sites, through binding to cytosolic calmodulin, which affects calcium signalling by interacting with target enzymes such as adenylate cyclase and phosphodiesterase, as well as with structural proteins (Benitez-King G and Anton-Tav F, 1993), nuclear retinoid Z receptors (Becker-Andre M *et al.*, 1994) and other nuclear proteins (Benedetti M *et al.*, 2005).

Melatonin physiological role

Melatonin has many physiological receptor-mediated roles, taking part to the circadian organization of biological rhythms; the enhancement of immune response, specifically enhancing the T-cells, i.e. the T-helper cells and T-killer cells. When melatonin is received, a cascade of events is set in motion including stimulation of Interleukin-4 (IL-4) which then stimulates natural killer cells (NK), B-cells, IgA, phagocytes and T-Cytotoxic cells. The NK cells specialize in attacking cancer cells and virus (Reiter RJ and Robinson J, 1995); the control of tumour promotion and growth. It has also a protective effect on the cardiovascular system reducing blood cholesterol and blood pressure, and a marked anti-inflammatory and analgesic effects (Macchi MM and Bruce JN, 2004). Besides melatonin has other non-receptor-mediated functions such as its free radical scavenging and antioxidant action: it directly neutralizes a number of free radicals and oxygen and nitrogen species furthermore it stimulates enzymes involved in metabolizing reactive oxygen

intermediates (Reiter RJ and Tan DX, 2004; Rodriguez C *et al.*, 2004). Melatonin plays a vital free radical scavenging role in the brain where, because of its high iron concentration, there is a high production rate of hydroxyl radicals (OH·). Free radical damage is now known to play a formative role in most brain disorders, including Alzheimer' disease, Lou Gehrig's disease, multiple sclerosis and Parkinson's disease. While the Blood Brain Barrier (BBB) denies access to most free radical scavengers, melatonin has free access (Reiter RJ and Robinson J, 1995). Therefore it may provide protection against ageing trough the attenuation of the cell damage effects: studies in rats and mice have shown that diminished melatonin secretion may be associated with an acceleration of the aging process (Benedetti M *et al.*, 2005). On the other hand, melatonin concentrations reduce with age (Zhou J *et al.*, 2003), so it cannot be excluded that age-related reduction in night time melatonin secretion could be a consequence of the aging process, rather then its cause. Currently, many data suggest melatonin's pro-apoptotic and anti-apoptotic action; it may act as a neuroprotective agent preventing neurons from apoptosis as well as increasing cancer cells death. In this case melatonin target seems to be mitochondria, which are implicated in the intrinsic pathway of apoptosis (Leon J *et al.*, 2005). Melatonin may also affect bone metabolism, both in a direct and a non-direct way. Pineal indole induces an increase in the proliferation of human osteoblasts, and an increase in proteins that are incorporated into the bone matrix, like procollagen type I c-peptide; it also impairs osteoclast activity in bone trough its free radical scavenging and antioxidant properties (Cardinali DP *et al.*, 2003). Melatonin is also connected to thyroid growth and function: it reduces blood thyroid-stimulating hormone (TSH) and thyroid hormones concentrations (Wajs E and Lewinski A, 1992). In addition the type 2 iodothyronine deiodinase gene, which is important for thyroid hormones synthesis, is involved in the regulation of seasonal reproduction (Watanabe M *et al.*, 2004). Finally the hypothalamic-pituitary-thyroid axis and melatonin possibly interact in the control of body temperature in humans: core body temperature has a circadian rhythm with lower levels during night time, when melatonin concentrations are the highest, besides it is well known that thyroid hormones influence thermogenesis. Therefore it is possible that pineal hormone modulates hypothalamic-pituitary-thyroid axis and especially the response of anterior pituitary gland to hypothalamic thyroid-relazing hormone (TRH) and of thyroid to hypophyseal TSH (Mazzoccoli G *et al.*, 2004).

Melatonin and cancer

The biological mechanisms by which melatonin exerts its antiproliferative and oncostatic properties on some types of neoplastic cells seem to be due to its ability (Pawlikowski M *et al.*, 2002):

- to suppress cancer cell proliferation by increasing cell-to-cell interactions (in cancer cells, a defective cell adhesion and/or a deficiency in multifunctioning gap-junction contacts are present).
- to increase the degradation of calmodulin which plays an important role in the proliferation of normal and cancer cells.
- to act as an indirect antioxidant and a free radical scavenger: tumour cells at an advanced stage of carcinogenesis are characterized by a persistent oxidative stress which is insufficient to cause cell death, because of the reduced sensitivity to oxidative stress of tumour cells.
- to act on the immune system by activating the cytokine system which demonstrates growth-inhibitory properties over a wide range of tumour cells.
- to suppress the uptake and metabolism of tumour fatty acid (fatty acid are specific tumour growth signalling molecules and their high concentrations in cancer cells seem to increase tumour growth) (Blask DE *et al.*, 2002).
- to induce apoptosis and to act as an antiangiogenic molecule (Lissoni P *et al.*, 2001).

Alterations in melatonin concentrations in the blood, as well as in the excretion of its main metabolite sulphatoxymelatonin, have been demonstrated in patient suffering from different type of both endocrine-dependent (mammary, endometrial, prostate cancer) and non-endocrine-dependent cancers (lung, gastric, colorectal cancer) (Bartsch C *et al.*, 1997; Grin W and Grunberger W, 1998; Karasek M *et al.*, 1996; Mazzoccoli G *et al.*, 2003).

ELFEMF (50/60 Hz): *In Vitro* Studies Results

Exposure to power-frequency (50 or 60 Hz) electromagnetic fields (EMFs) is hypothesized risk factor for cancer in humans. The results of a number of epidemiological studies have identified brain, breast and hematopoietic tissues as possible targets for the action of EMFs (NIEHS, 1998). However, many other epidemiological studies have found no association between MF exposure and neoplasia in any site has been demonstrated (Guenel P *et al.*, Kheifets LI *et al.*, 1997; McBride ML *et al.*, 1999; Michaelis J *et al.*, 1997; Linet MS *et al.*, 1997; Rosembaum PF *et al.*, 1994; Sorahan T *et al.*,1999; Stenlund C and Floderus B,

1997; Johansen C and Olsen JH, 1998). In instances where epidemiological data do not support conclusive identification and quantification of environmental hazards, laboratory studies using relevant experimental model systems increase in importance. At present, no plausible biochemical or molecular mechanisms have been identified through which EMFs may stimulate neoplastic development. *In vitro* studies using appropriate cellular models and well-controlled EMF exposures may identify relevant biological targets for the action of EMFs and therefore may provide critical data on mechanisms that will support EMF hazard assessment. So *in vitro* studies have the target to evidence EMFs exposure effects on cellular events implicated in the neoplastic transformation like the induction of genes' expression and protein synthesis; cell membrane signal transduction and calcium role; intercellular communication (gap junction); ornithine-decarboxylase (ODC) activity; melatonin role and cellular proliferation; free radicals production and antioxidants activity as described in the previous sections.

The ELFEMF data synthesized in the 2004 final report of the European Union Programme "Quality of life and management of living resources" on risk evaluation of potential environmental hazards from low frequency electromagnetic field exposure using sensitive *in vitro* methods allow the following conclusion:

- ELFEMF had genotoxic effects on primary cell cultures of human fibroblasts and on other cell lines. ELFEMF generated DNA strand breaks at a significant level at a flux density as low as 35 μT. A strong positive correlation was observed between both the intensity and the duration of exposure to ELFEMF and the increase in single and double strand DNA breaks and micronuclei frequencies. Surprisingly this genotoxic effect was only found when cells were exposed to intermittent ELFEMF, but not to continuous exposure. Responsiveness of fibroblast to ELFEMF increased with the age of the donor and the presence of specific genetic repair defects.
- ELFEMF at a flux density of 10-100 μT increased the proliferation rate of neuroblastoma cells and at a flux density of 0,8 mT it enhanced the differentiation of mouse stem cells into cardiomyocites. In contrast to these results, no clear cut and unequivocal effects of ELF-EMF on DNA synthesis, cell cycle, cell differentiation, cell proliferation and apoptosis were found in the many other cell systems.
- ELFEMF inhibited the spontaneous apoptosis in neuroblastoma cells which was followed by an increase of the proliferation rate, when the cells were exposed for 63 hours to ELFEMF at a flux density of 50 or 100 μT.

- Neoplastic development is commonly associated with altered expression of oncogenes and/or tumour suppressor genes. On this basis, differential expression of cancer-related genes provides a plausible mechanism for the action of EMFs in human cells. Some studies have reported increases in expression of *MYC*, *FOS*, *SRC* and several housekeeping genes in human promyelocitic leukaemia (HL60) cells (Goodman R and Shirley-Henderson A, 1991; Goodman R *et al.*, 1992, Xu ZP *et al.*, 2003); other investigations have reported EMF-induced increases in immediate-early gene expression in T lymphocytes (Phillips JL *et al.*, 1992). These reports could be controversial because other laboratories have been unable to replicate this finding in HL60 cells, in Epstein-Barr virus-transformed lymphoid cells or Daudi cells (Balcer-Kubiczek EK *et al.*, 1996; Loberg LI *et al.*, 1999; Owen RD, 1998), and they support the evidence that ELFEMF exposure does not cause changes in gene expression in human cells either alone or in combination with estrogen or xenoestrogens (Dees C *et al.*, 1996; Loberg LI *et al.*, 2000). However ELFEMF at a flux density of about 2 mT seems to up-regulate the expression of genes (p21, c-jun, egr-1, p-53) and proteins in a variety of cell systems. The results of the whole genome cDNA micro-array and proteomic analyses indicate that EMF may activate several groups of genes that play a role in cell division, cell proliferation and cell differentiation.

ELFEMF (50/60 Hz): *In Vivo* Studies Results

The interaction of electromagnetic fields (EMF) with humans has raised significant public concern as to the potential of their long-term health effects. To address this issue, an examination of EMF exposures has often involved *in vivo* studies. Such studies, conducted in animals, provide integrated biological systems where experimental variables can be controlled, specific hypothesis can be explored, and EMF exposure can be precisely assessed. Given the somewhat ambiguous results and the relatively low power of EMF epidemiology to identify casual relationships, animal studies are particularly important in evaluating any potential relationship between EMF exposure and health.

Investigations in animals have included a wide range of biological end points, from behaviour and neuroendocrine responses, to reproduction and growth. The evolution of EMF studies over the last decade has moved from primarily screening and phenomenological investigations (i.e., is anything happening with exposure) to hypothesis driven and specific animal model approaches (i.e., test of specific questions and/or

potential mechanisms). Continuing advances in animal models (e.g., genetically altered animals, organ-specific targeting, sensitive populations) have dramatically improved the precision with which questions can be addressed as well as continuing improvement and understanding in exposure methodology and dosimetry has greatly enhanced the value of laboratory studies.

Studies to examine carcinogenesis in animal models are the most common type of study because of the questions arising from epidemiological drivers. In the area of cancer research, animal models have been used in a variety of ways depending largely upon the hypothesis selected for investigation: a long-term (typically two years) bioassay model is utilized to examine EMF as a possible complete carcinogen. In such an experiment, animals are exposed to EMF fields during the major portion of their lifetime and the occurrence of tumours, in number, type and time of development are the critical endpoints. This type of study usually includes several dose groups and requires a relatively large number of animals. Another approach is to test EMF as an initiator or a promoter of cancer since as carcinogenesis is a multi-step process: initiation includes the direct interaction of EMF as a genotoxic agent, thereby directly altering the DNA; promotion includes the application of EMF some number of weeks or months subsequent to initiation. Promotion is associated with subcellular events that are usually non-genotoxic and it is responsible for the conversion of initiated cells to cancerous cells. A specific initiation/promotion model is typically limited to an assessment of a specific type of cancer and may provide only general information on possible biological mechanisms of EMF exposure and cancer development.

The number of completed carcinogenesis studies examining EMF exposed animals has grown rapidly over the last decade, with most major long term studies not demonstrating increased carcinogenesis with exposure. In the initiation/promotion models examining EMF exposure in animals, there is a more mixed message comprising of both positive and negative studies with regard to influence of EMF exposure on cancer.

As far as the results of ELFEMF studies on experimental animals are concerned, the International Agency for Research on Cancer has evaluated the extremely low-frequency magnetic fields as *possibly carcinogenic to humans* (Group 2B) based on "*inadequate evidence in experimental animals for the carcinogenicity of extremely low-frequency magnetic fields"* and *"limited evidence in humans of the carcinogenicity of extremely low-frequency magnetic fields in relation to childhood leukaemia"* while there is *"inadequate*

evidence in humans for the carcinogenicity of extremely low-frequency magnetic fields in relation to all other cancer" (IARC, 2002).

At the moment the only international level institution that points out a not fully negative evaluation of ELFEMF carcinogenic activity in relation to results obtained from studies on experimental animals is US National Toxicology Program (NTP). In the NTP reports, a review of the results available from experimental studies is presented confirming that most of them have led to negative results, even if some suggestive positive results were reported in promotion assays (in particular, rat mammary gland, rat liver and mouse skin) (McCann J *et al.*, 1997; NTP, 1999). In the concluding remarks of its two-year lasting "Toxicology and carcinogenesis studies of 60 Hz magnetic fields in F344/N rats and B6C3F_1 mice", the NTP specifies that *"there was equivocal evidence of carcinogenic activity of 60 Hz magnetic fields in male F344/N rats based on increased of thyroid gland C-cell neoplasms in the 0.02 and 2 G groups"*, while "*there was no evidence of carcinogenic activity in female F344/N rats or male or female B6C3F_1 exposed to 0.02, 2 or 10 G, or 10 G intermittent 60 Hz magnetic fields"* (NTP, 1999). In this study, groups of 100 male and 100 female rats, and of 100 male and 100 female mice were exposed to 60 Hz magnetic fields at intensities of 0, 0.2, 2, or 10 G (0, 200 or 1000 μT), as well as of 10 G intermittent magnetic fields (1 hour on, 1 hour off) for 18.5 hours per day, 7 days per week, 106 weeks. Moreover, the NTP, in the conclusions relative to its three initiation/promotion studies in which female Sprague-Dawley rats (100 animals per experimental group) were initiated by DMBA (7,12-Dimethylbenz(a)anthracene) and exposed to 50 Hz magnetic fields at 1 or 5 G field intensities (100 or 500 μT) or to 1 G 60 Hz magnetic fields specifies that *"there was no evidence that magnetic fields promoted the development of mammary gland neoplasm"* (NTP, 1999).

However a joint evaluation of the two-year study data indicates a thyroid C-cell focal hyperplasia increase in female rats at the same exposures at which the neoplasm increment of the same cells has been observed only in male rats (on which the "equivocal evidence" NTP classification is based) (Zapponi GA and Marcello I, 2004).

In addition to thyroid C-cell data, other statistically significant results emerge in the NTP two-year studies. In particular, for skin tumours, a statistically significant increase of trichoepithelioma at the 1000 μT exposure level (P=0.029) is reported in male rats, together with a statistically highly significant exposure-response trend for this neoplasm (P=0.002), and with statistically significant trends (P=0.008 and P=0.018) respectively for

trichoepithelioma or basal cell adenoma, jointly considered, and for squamous cell papilloma, keratoacanthoma, tricoepithelioma, basal cell adenoma or squamous cell carcinoma, jointly considered. The significant exposure-related trichoepithelioma and trichoepithelioma plus other skin neoplasm reported for male rats in the two-year studies finds some support in the female rat data relative to trichoepithelioma reported in incidence summary of the NTP 26-week initiation/promotion study, even if the experimental designs of the two studies are different (Zapponi GA and Marcello I, 2004).

Lastly, a significant increase (P=0.032) of preputial gland carcinoma is reported for male rats. For mice, the only positive result reported is a statistically significant exposure-response trend (P=0.032) in males, for the adrenal cortex adenoma but it must be underlined an exposure-response trend (P=0.03) considering the first three doses (0, 2 and 200 μT) for Langerhans islets adenoma in female mice with a statistically significant incidence increase (P=0.101) in animals exposed at 200 μT. In rats, an indication of exposure-related increase of single mammary carcinomas emerges from the summary of neoplasm incidence. In this framework, other studies pointed out that breast cancer promotion assays have shown a more consistent effect than other assays, suggesting a breast cancer promoting activity of magnetic field exposure (substantially limited to the 10 and 100 μT intensities of 50 Hz magnetic fields) (Baum A *et al.*, 1995; Löscher W *et al.*, 1993; Mevissen M *et al.*, 1995). Besides there is a weak exposure/response relationship for myeloid and monocytic leukaemia in female rats with a limited statistic significance (P=0.1) and a linear trend with a 6% increase from 0 to 1000 μT.

The NTP study pointed out many other non-neoplastic effects with a significant increase: thyroid and pituitary (*pars distalis*) cysts, pituitary focal angioectasia, hematopoietic cells proliferation, focal hyperplasia and hypertrophy of the adrenal cortex, mammary cysts, chronic inflammation of preputial gland, thymus epithelial cells proliferation, mesenteric lymph nodes hyperplasia (2 μT) and mesenteric lymph nodes atrophy (200 μT) in mice, spleen hematopoietic cells proliferation. More recent data hypothesize also brain cells damage in rats exposed to 60 Hz magnetic fields: this exposure seems to initiate an iron-mediated process (e.g, the Fenton reaction) that increases free radical formation in brain cells, leading to DNA strand breaks and cell death (Lay H and Singh PN, 2004).

As far as ELFEMF reproductive effects are concerned, there are no definitive results. In some studies it was reported a little reduction in number and weight of the newborns (Rivas L *et al.*, 1985) and an increase of skeletal anomalies in the offspring of exposed

animals, indicating alterations in the ossification process (Huuskonen H *et al.*, 1993; Huuskonen H *et al.*, 1998; Kowalczuc CI *et al.*, 1994; Mevissen M *et al.*, 1994). However recent studies conducted both in rats and mice don't point out any toxicological effects on fertility and reproduction (Chung MK *et al.*, 2003; Elbetieha A *et al.*, 2002).

Extrapolation methods from experimental animals to human

If biological effects of ELFEMF are caused by electric and magnetic currents induced throughout tissues, the conversion factor between rats and humans is 5-7 times: a rat exposed at 100 μT is like a man exposed at 10-20 μT (Dan Bracken T, 1992; Löescher W and Mevissen M, 1994; Löescher W and Mevissen M, 1995). Rodents exposure levels have to be increased compared to human ones because the current density depends on the volume and shape of the body. Therefore, in the NTP study, the weakest field intensity (2 μT) utilized in rodents induces an exposure level which could be considered like the human residential exposure. The results of NTP study identify 2 μT as possible LOAEL (lowest adverse effect level) for many end points. In addition the exposure/response trend is not an increasing monotonic function so, to study ELFEMF, it couldn't be used the paradigms utilized in the evaluation of chemical risk.

ELFEMF (50/60 Hz): Epidemiologic Studies Results

Exposures to extremely low-frequency electric and magnetic fields emanating from the generation, transmission and use of electricity are an ubiquitous part of modern life. Concern about a possible danger has risen in the last 20 years and has initially been brought to prominence by a report in 1979 of an epidemiologic study in Denver on the relation between risk of childhood leukaemia and a proxy measure of degree of exposure to EMF radiation from electricity transmission lines (Wertheimer N and Leeper E, 1979). Since that study, the most intensive epidemiologic efforts has concerned childhood malignancy, especially leukaemia, but there are also been considerable research on possible occupational associations with cancer in adults, on cardiovascular and neurological/psychological diseases in adults, and on reproductive outcomes.

The quality of epidemiologic studies on these topics have improved over time and several of the recent studies on childhood leukaemia and on cancer associated with occupational exposure are close to the limit of what can realistically be achieved in terms of size of the study (Ahlbom A *et al.*, 2001). However exposure assessment is a particular difficulty of EMF epidemiology because exposure is imperceptible, ubiquitous, it has multiple sources,

and it can vary greatly over time and short distances; besides the exposure period of relevance is before the date at which measurements can realistically be obtained and of unknown duration and induction period. Lastly the appropriate exposure metric is not known and there are no biological data from which to impute it. In this framework, a large body of high-quality data exists, with measurements of exposure, strong methodology, and large study sizes, for childhood leukaemia and brain tumours (Green LM *et al.*, 1999; Kavet R *et al.*, 2004; McBride ML *et al.*, 1999; Söderberg KC *et al.*, 2002; Tynes T *et al.*, 1997; UKCCS, 1999). and for occupational exposure in relation to adult leukaemia and brain tumours (Harrington JM *et al.*, 1997; Johansen C and Olsen JH, 1998; Rodvall Y *et al.*, 1998). Among all the outcomes evaluated in epidemiologic studies of EMF, childhood leukaemia in relation to postnatal exposures above 0.4 μT is the one for which there is most evidence of an association: the relative risk has been estimated at 2.0 in a large pooled analysis (Green LM *et al.*, 1999; UKCCS, 1999; Schüz J *et al.*, 2007) but it may be partly due to bias and this is difficult to interpret in the absence of a known mechanism or reproducible experimental support; in addition only 0.8% of all children were exposed above 0.4 μT (Ahlbom A *et al.*, 2000). The research on the risk of adult leukaemia in relation to occupational and residential magnetic field exposure includes many studies whose results have ranged from null to rather strong associations, with relative risks in the upper exposure categories above 2.0 (Bethwaite P *et al.*, 2001; Feychting M *et al.*, 1997; Forssen UM *et al.*, 2000). Nevertheless, the evidence at present supporting a role for EMF in the etiology of adult leukaemia is weak (Ahlbom A *et al.*, 2001) as well as for the other nervous system tumours (Håkansson N *et al.*, 2002; Kheifets LI *et al.*, 1999; Sorahan T *et al.*, 2001; van Wijngaarden E *et al.*, 2001; Villeneuve PJ *et al.*, 2002; Wrensch M *et al.*, 1999). The totality of evidence linking EMFs to breast cancer, in men or women, remains weak (Davis S *et al.*, 2002; Gammon MD *et al.*, 1998; Feychting M *et al.*, 1998; Kliukiene J *et al.*, 1999;Laden F *et al.*, 2000; Zheng T *et al.*, 2000). Limited attention has been focused on other cancers like lung cancer (Armstrong B *et al.*, 1994), non-Hodgkin's lymphoma (Schroeder JC *et al.*, 1997), colon cancer (Guenel P *et al.*, 1996), melanoma (Tynes T *et al.*, 2003), prostatic tumours (Charles LE *et al.*, 2003). A particularly intriguing line of research has been the possibility of a relation between childhood cancer and parental occupational EMF exposure. However, results have been inconsistent and unconvincing (Sorahan T *et al.*, 1999; Feychting M *et al.*, 2000).

Concern about possible psychiatric or psychological effects of EMF exposure were raised by investigators from the Soviet Union in the late 1960s and early 1970s on the basis of anecdotal reports of symptoms such as insomnia, memory loss, and headache (Asanova TP, 1972), but the reports remained basically unconfirmed (Knave B *et al.*, 1979). Relatively recently, however, hypothesis relating EMF to neurodegenerative disorders and psychiatric disorders have attracted a new interest, at least partly as a consequence of the hypothesis that EMF may affect melatonin levels. As far as neurodegenerative disorders are concerned, the overwhelming focus has been on amyotrophic lateral sclerosis (ALS) (Savitz DA *et al.*, 1998; Johansen C *et al.*, 1998) and Alzheimer's disease (AD) (Savitz DA *et al.*, 1998; Savitz DA *et al.*, 1998) but the results are intriguing: they point toward a possible risk increase in subjects with EMF exposure but the studies' designs are too weak (Alhbom A *et al.*, 2000). The literature on depressive symptoms, suicide and EMF is difficult to interpret because the findings are not consistent (Baris D *et al.*, 1996; McMahan S *et al.*, 1994; Poole C *et al.*, 1993; Van Wijngaarden E *et al.*, 1999; Verkasalo PK *et al.*, 1997).

Recent investigations suggest that there may be some direct cardiac effects of EMF exposure, mostly related to heart rate. These effects, however, appear to occur only under certain conditions (Sastre A *et al.*, 2000). No known substantive changes occur in the other parameters of cardiac function, such as the shape of electrocardiogram or blood pressure, in relation to EMF exposure (Jauchem JR, 1997). Several occupational cohort studies have examined mortality from cardiovascular disease (CVD) among electric utility workers (Dekker JM *et al.*, 1997; Graham C *et al.*, 2000; Liao D *et al.*, 1997; Sastre A *et al.*, 1998; Savitz DA *et al.*, 1999; Willich SN *et al.*, 1993). In summary, evidence of cardiovascular effects due to elevated exposure to magnetic fields is weak, and whether a specific association exists between exposure and altered autonomic control of the heart remains speculative (Johansen C, 2004).

As far as reproductive effects are concerned, little evidence has been available; it does not support the hypothesis that maternal exposure through both residential exposure and through the workplace is associated with adverse pregnancy outcomes (Bracken MB*et al.*, 1995; Lee GM *et al.*, 2002; Lee GM *et al.*,2000; Li DK and Neutra RR, 2002; Savitz DA, 2002a; Savitz DA, 2002b; Savitz DA, 2003)

ELFEMF (50/60 Hz): Risk Assessment and Regulatory Aspects

It is very difficult to demonstrate the supposed health risks related to an environmental factor exposure and so to decide the measures needed to safeguard public health. As far as ELFEMF are concerned, some precaution policies have been developed in the last years because of the absence of scientific certainties:

- Precautionary Principle: "better safe than sorry". It involves the necessity to take "protective measures without having to wait until the reality or seriousness of those risks becomes apparent.". It needs a risk assessment and a costs/benefits rate evaluation. Regulatory action based on the Precautionary Principle is generally guided by the results of epidemiology studies. Even though laboratory research on electromagnetic fields (EMF) has supplied much relevant information and continues to do so, it is often overlooked. Laboratory research has shown that EMF of many frequencies stimulate many biological systems, and at low thresholds of both field strength and duration. It has also shown that EMF stimulate protein synthesis in cells and accelerate electron transfer reactions. In the last few years, important practical insights have been provided by the research on the cellular stress response, where the same specific biological response is induced in cells by both ELF (power frequency) and RF (radio frequency) fields, despite the very different energy levels. Since this protective biological response is not determined by the level of energy absorbed, safety standards based on the best available biological evidence must recognize non thermal protective responses and include cumulative exposures across the EM spectrum (Blank M, 2006).
- Prudent Avoidance: low cost measures to reduce environmental exposure without risk assessment or scientific data collection.
- ALARA (As Low As Reasonably Achievable): it minimizes well known risks, keeping exposure level as low as possible considering costs, technology, public health benefit and other social and economic factors. It is based on the "acceptable risk" concept and not on limits imposed by threshold values. Nowadays it can not be applied to non-ionizing radiation because of the absence of any risk value assessed at low exposure levels and because of the ubiquity of the exposure.

In April 1998, the International Commission on Non-Ionizing Radiation Protection (ICNIRP) published guidelines for limiting exposure to time-varying electric, magnetic and electromagnetic fields in the frequency range up to 300 GHz (ICNIRP, 1998). These guidelines replaced previous advice issued in 1988 (INIRC, 1988) and 1990 (INIRC,

1990). Restrictions on the effects of exposure to time-varying EMFs are based on biological considerations of their interactions with the body and are termed *basic restrictions*. Depending on frequency, the physical quantities used to specify the basic restrictions are: induced current density, specific energy absorption rate (SAR) and, for pulsed radiations, specific energy absorption (SA). The basic restriction on exposure to EMFs in the frequency range between 10 Hz and 1 kHz, which includes the power frequency of 50 Hz, is 10 mA/m^2 and above 1 kHz it is frequency dependent. The basic restriction for EMFs with frequencies between 100 kHz and 10 GHz is 0.4 W/kg for whole body SAR. Higher values apply for partial body exposure.

Compliance with the basic restrictions on exposure cannot be measured directly, they need mathematical models. *Investigation levels* (called also *reference levels*) are, therefore, recommended as values of electric field strength, magnetic field strength, magnetic flux density, power density and contact current. These are provided for the purpose of comparison with values of measured field quantities for investigating whether compliance with the basic restrictions is achieved. They have been developed using dosimetric models which assess the interaction of EMFs with the body. If the measured values are greater than the relevant investigation levels, it does not necessarily follow that the basic restrictions are exceeded but that further investigation is needed to assess compliance. No distinction is made between occupational exposure and that of members of the public.

For electric fields, contact current investigation levels may be used to indicate whether there is a need to take appropriate action to prevent shock and/or RF burn.

ICNIRP states that compliance with the reference levels will ensure compliance with the relevant basic restrictions. If the measured value exceeds the relevant reference level, it does not necessarily follow that the relevant basic restriction will be exceeded. However, an investigation is indicated to assess compliance with the relevant basic restriction and to determine if additional protective measures are required.

The basic restriction for occupational exposure to electric and magnetic fields with frequencies up to 1 kHz is 10 mA/m^2 and above that it is frequency dependent. The value of 10 mA/m^2 was chosen as less than one-tenth of the value of the current density above which thresholds for acute changes in excitability of the central nervous system are exceeded. In addition, however, for exposures received by members of the general public, a reduction factor of five is applied, resulting in a basic restriction of 2 mA/m^2. In its clarification, ICNIRP notes that compliance with this basic restriction may permit higher

current densities in body tissues other than the central nervous system under the same exposure conditions.

The basic restriction for occupational exposure to EMFs with frequencies between 100 kHz and 10 GHz is 0.4 W/kg for whole body SAR. For exposures of the general public, a reduction factor of five is again applied, resulting in a basic restriction on whole body SAR of 0.08 W/kg. The factor of five reduction also applies to the basic restriction on localised SAR, the values for those occupationally exposed and for the general public being 10 W/kg and 2 W/kg averaged over any 10 g of tissue. In the frequency range from 100 kHz to 10 MHz, basic restrictions on both induced current density and SAR apply.

For occupational exposure there are a few situations where ICNIRP reference levels may be exceeded. For exposures to electric fields this may occur beneath overhead power conductors and close to some transmitters. At higher frequencies, there are other situations where the levels may be exceeded – close to dielectric heaters and diathermy equipment and in some broadcast environments, for example. However, these exposures can be averaged over time. There are other situations in which occupational exposure to magnetic fields may exceed the reference levels. Exposures on trains and from induction heaters are examples. Medical, broadcast and dielectric heating exposures may exceed the reference level but, on further investigation, are often found not to result in the basic restrictions being exceeded.

Where members of the general public can gain access to the workplace they also have the potential for exposure above reference levels. Normally they will be excluded from such areas. However, electric field exposure levels under power lines and magnetic field exposure levels close to some security systems may exceed the ICNIRP general public reference levels.

Table 6 lists ICNIRP limits of exposure and reference levels for ELFEMF (50/60 Hz).

Table 6. ICNIRP basic limits and reference levels for ELFEMF (50/60 Hz) (ICNIRP, 1998).

Exposure Characteristics	50 Hz	60 Hz
Occupational		
Basic Restriction	10 mA/m	10 mA/m
Electric Field	10 kV/m	8.33 kV/m
Magnetic Field	400 A/m	333 A/m
Magnetic Flux Density	500 μT	417 μT
General Public		
Basic Restriction	2 mA/m	2 mA/m
Electric Field	5 kV/m	4.17 kV/m
Magnetic Field	80 A/m	67 A/m
Magnetic Flux Density	100 μT	83 μT

The European Union Council issued the European Union Recommendation on limiting public exposure to time-varying electric and magnetic fields in 1999. It is based on the ICNIRP exposure restrictions for the general public (CEU, 1999). As far as occupational exposure is concerned, the European Parliament and the Council of 29 April 2004 issued the European Union (EU) directive 2004/40/EC on the minimum health and safety requirements regarding the exposure of workers to the risks arising from physical agents (electromagnetic fields). It came into effect on 30 April 2004. The directive sets the maximum values for occupational exposure to electric and magnetic fields. For 50 Hz fields the directive action value is set as field strength 10 kV/m and flux density 500 μT. If the action value is exceeded, the employer has to make sure that the exposure limit value is not exceeded. The limit value is set as current density in head or trunk and shall not exceed 10 mA/m^2 (2004/40/EC, 2004). Since the exposure of the employees is relatively difficult to measure, the demands of the directive are divided into two steps. The directive gives action values that are easier to measure. On power frequencies these action values are given as electric and magnetic field strengths. Compliance with the action value ensures that electric and magnetic fields will not cause any negative health effects. If the action values are exceeded, the employer has to show that induced current density inside the body

of the employee does not exceed the limit value of the directive. This limit value must not be exceeded in any circumstances.

All country members of the European Union approved the 1999 European Union Recommendation, except Italy. In Italy, legislation on protection from ELFEMF is based on DPCM (Decree of the Ministers' Council President) 1992 (GU, 1992). This decree establishes the maximum exposure levels to electromagnetic fields (50 Hz) in the residential and open space. These limits are 5 kV/m with a magnetic flux density of 100 μT, in accordance with the ICNIRP. If the exposure is limited only at few hours a day the electric field may be accepted until 10 kV/m and the magnetic flux density until 1 mT. Besides DPCM fixed also the distance of residential buildings from high voltage lines: more than 10 m for 132 kV lines; more than 18 m for 220 kV lines and more of 28 m for 380 kV lines.

In 2001 a new outline low (n°36/2001) was passed by Parliament (GU, 2001) and in 2003 the first two decrees for the low n°36/2001 accomplishment were issued. They concern only public exposures (not occupational exposures) and they refer to European Union Recommendation for all restriction levels except for 50 Hz electromagnetic fields produced by long-distance power lines. In this case the decrees refers to the precautionary principle and it indicates not only the exposure limits but also an attention value (10 μT) for public place where people can stay for more than 4 hours a day and a quality target (3 μT) for new long-distance power lines.

Time-Varying Electromagnetic Fields: Microwaves and Radio-Frequency Electromagnetic Fields (MW/RFEMF)

RF waves have long been used for different types of wireless broadcast, such as for wireless Morse code, radio, television, and so on. The radio-wave spectrum spans the frequency range from about 0.5 MHz in the AM radio band up to about 30,000 MHz in the radar band. RF-emitting devices have become common place in homes, offices, and schools (Tab. 7).

Table 7. Typical RF sources contributing to modern-day RF waves background.

RF Source	Frequency (MHz)
AM commercial radio	0.5-1.7
FM commercial radio	88-108
VHF commercial television (analog)[a]	54-88; 174-216
UHF commercial television (analog and digital)	512-700
Radar (aviation, marine, police)	10,000-33,000
Millimeter wavelength radar (metereological; military)	100,000
Satellite stransmission (global positioning; military)	220-400
Satellite transmission (television)	4,000-6,000
Cellular telephones (analogue)	806-890
Cellular telephones GSM	890-960
Cellular telephones digital	1,850-1,990
Dispatch radio: (pagers, aviation, marine, fire, emergency, police)	900-950
Fixed microwave links (computers, television, telephone, military)	>30,000
Cordless telephones, baby monitors, wireless toys, wireless telemetry	27-60; 900; 2,400; 5,800
Computer monitors, wireless computer connectivity, RF tags (e.g. WiFi)	1,900; 2,500; 5,700
Microwave ovens, diathermy machines	2,450
Industrial scientific and medical (ISM) band data links	2,400-5,400
RF noise (lightning, solar flares, fluorescent fixtures, neon lights, spark ignition, power-line corona discharge)	Broadband

[a]: The VHF band is split into two parts, with FM radio in the middle.

The actual RF level from each source depends on the details of the exposure location (i.e., distance from the antenna), whether the source is more ubiquitous and universal or more limited and local. A vast number of communication networks interconnect societies worldwide, and cellular wireless technology networks make up an increasing fraction of this number. Nowadays cellular wireless technology is capable of delivering voice, text, images, music, and other data to consumers everywhere, and it relies on an extensive network of fixed antennas, or base stations, for relaying information using RF signals. The number of base stations required increases with greater mobile phone use (requiring extensive micro-cell or pico-cell distributed antenna systems in urban areas), with market competition (enabling more operators to provide services), and with new technological capabilities (e.g., 3G). Therefore the presence of radiofrequency (RF) waves from wireless technologies has become ubiquitous. Mobile telephony (construction and operation of telephones or telephonic systems) is relied on by > 1.4 billion people, or around 20% of the

world's population. Given that the public is frequently reminded that we are all surrounded by ever-present electromagnetic fields (EMFs), which some call "electro-smog". It is not surprising that some individuals and groups express concern about possible health effects from low-level, chronic exposure to a variety of RF sources. The public, regulators, and scientists have questioned whether there are possible health consequences of this mushrooming mobile phone technology, particularly because the handset operates in close proximity to the human body and because large numbers of base station antennas are required. Although the RF levels produced by base stations at consumer locations are much lower than those from use of the phone handset, the more continuous exposure from base stations has produced a greater public concern, even if, also for individuals in the vicinity of transmitting antennas, surveys of RF levels report results that are far below the applicable exposure guidelines both in the United States (Burch JB *et al.*, 2006; Tell RA and Mantiply ED, 1980) and in Europe (Foster K, in press).

The total electromagnetic energy available, in terms of effective radiated power from an RF source (or antenna), varies widely according to source type (Tab. 8).

Table 8. Approximate radiated-power emission strength for sources of electromagnetic waves (Valberg PA *et al.*, 2007).

RF Source	Energy (W)
Cellular telephone handset	0.6
Single ham radio antenna	1,000
Array of cellular phone base station antennas	1,200
Typical AM radio station transmitter	50,000
Typical FM radio station transmitter	100,000
Typical UHF TV transmitter	1,000,000

Among RF sources, cellular telephone base stations are at the low end when considering the strength of the source of RF power. Radiofrequency exposure is typically quantified as RF energy flux per unit area, for example, watts of RF energy crossing a square meter of area (W/m^2). Alternatively, the intensity of radiowaves can be given in terms of electric field intensity, where the units are volts per meter (V/m). These two metrics are mathematically related to each other when considering locations many wavelengths distant from the antenna (or the RF source). That is, the energy flux per unit area (S) is proportional to the square of the electric field intensity (E):

$$S\ (W/m^2) = [E(V/m)]^2/[377(V^2/W)]$$

For example, RF energy of 1 W/m^2 is equal to 19.4 V/m, and 10 W/m^2 is equal to 61.4 V/m (because of the squared dependence between S and E). The relevant RF energy flux (in terms of potential health impacts) is at exposure points where people may intercept the RF energy, and is measured in power per square meter of surface area. A comparison of energy fluxes in this regard is given in table 9, which compares both RF and non-RF sources.

Table 9. Incident energy from a broad spectrum of sources of electromagnetic energy.

Source	Energy Flux (W/m^2)	Electric Field (V/m)
Sunlight at noon[a]	1,370	
1 m from a 1,500 W electrical heater unit[b]	480	
On black body surface at 37°C (λ_{max} 10μm)[c]	520	
Microwave oven, RF leakage standard	50	140
1 m from a 100 W light bulb[d]	8	
Cell telephone (2GHz) public guideline[e]	10	61
Cell telephone (850 MHz) public guideline[e]	4.3	4
RF levels near cellular base antenna (calculated)	0.05	4.3
Average urban RF levels: TV and radio[f]	0.4	0.7
Average urban RF level: cellular telephony[f]	0.1	0.3

[a]: The average amount of solar energy reaching the earth's atmosphere is defined as the solar constant: 1,370 W/m^2.
[b]: Assuming that a reflector behind a 1-m-long heating element directs the 1,500 W of energy into the half-cylinder in front of the heater, the surface area at 1-m radius is 3.14 m^2, so 1,500 W divided by 3.14 m^2 is 477 W/m^2.
[c]: Wien's Law states that the wavelength, λ, at which most power is radiated by a body at temperature T is $\lambda = 2898/T = \lambda$ (μm), where T is degrees Kelvin and the wavelength is given in micrometers. The Stefan-Boltzmann Law states that the energy flux from a black body at temperature T is given approximately by Φ, where $\Phi = \sigma T4\ W/m^2$, where σ is the Stefan-Boltzmann constant ($5.67*10^{-8}\ W/[m^2\ K^4]$).
[d]: Assume spherical radiation, at 1 m, the surface area is $4\pi r^2 = 12.6\ m^2$. Hence, 100 W/12.6 m^2 = 8 W/m^2.
[e]: ICNIRP reference level for general public exposure (ICNIRP, 1998).
[f]: Anglesio L *et al*., 2001.

It can be seen that more energetic electromagnetic waves (visible light, infrared waves) are normally present at energy flux levels more intense than the maximum allowable RF intensities in the cell telephone band. In fact, our body surfaces radiate sufficient infrared energy that they are easily seen by "night vision" cameras. Because of their warm temperature, our bodies also emit RF energy in the microwave band (~ 30–300 GHz) at about 0.003 W/m^2. Table 9 also illustrates that the amount of electromagnetic energy that is present due to cellular telephones and cellular base stations is quite small in comparison to both electromagnetic energy sources generally and RF sources in particular.

Within the home and office environment, a variety of other sources of RF energy are used. The International Commission on Non-Ionizing Radiation Protection (ICNIRP 1998) identified allowable public exposure levels for electric field (E-field) over these frequencies ranging from approximately 30 V/m to 60 V/m.

Spot measurement data often show that, where a particular RF source is the focus of concern, other, less visually obvious sources may give greater contributions to exposure.

The data also show that exposures vary greatly, even at similar distances from base stations, illustrating the dramatic effect of the local environment on RF signals through physical processes such as reflection, diffraction, and mutual interference of signal elements travelling through multiple, different paths (Ardoino L *et al.*, 2004; Mann S *et al.*, 2006).

For assessing occupation RF exposure in the context of base station antennas, a "compliance boundary" can be used, defined so that for personnel outside the boundary, RF levels are low enough to be in compliance with relevant safety standards. The size and shape of a given compliance boundary varies with frequency, with type of antenna, and with antenna power output. For a typical base-station antenna running at 25 W, the compliance boundary has the shape of a cylinder with a diameter of 3 m, and a height corresponding to the antenna height plus about 0.5 m. The height is centered on the antenna, and the cylinder wall begins about 0.1 m behind the radiative front of the antenna and extends to about 2.9 m in front of the antenna. This virtual space encloses the volume where the RF signal may be in excess of ICNIRP occupational standards, but for all distances outside this cylinder, RF levels are low enough to be considered safe (Mild KH *et al.*, 2006). By comparison, in occupations such as "plastic sealers," RF levels can be considerably higher than in the close vicinity of base antennas (Wilen J *et al.*, 2004).

Both measurement surveys and theoretical predictions show that RF levels from base stations and wireless technologies generally decrease with distance from the device (with

focused antenna arrays, maximum ground level RF is 50-300 m from the antenna base).

That is, the greater the distance from the antenna, the lower the RF. Under conditions typical for public exposure to base stations and for wireless consumer devices, the RF energy fluxes are > 100 fold below international RF guidelines for public locations. However, in very close proximity to base-station antenna elements under occupational conditions (i.e., when performing maintenance on an operating antenna), or immediately adjacent to wireless local area network (LAN) and Bluetooth transmitters, there is the possibility that RF absorption limits for the general public may be exceeded (Kühn S *et al.*, 2006). Thus, there is a need to ensure that, under normal operating conditions, these devices comply with the international limits. In the case of non occupational exposure to RF from base stations, the most common circumstance is that the contribution of base stations to a person's total RF exposure is minimal.

Mobile phones and related technologies

Cellular telephone radio waves transmit information that is encoded into electromagnetic waves at a frequency ranging from 800 MHz to 900 MHz and from 1,600 to 2,000 MHz (GSM) or > 2,000 MHz (UMTS). Analogical telephones work with a frequency modulation system whereas digital telephones emit pulsed MW.

The Global System for Mobile Communications (GSM) is the hugely successful wireless technology. In less than ten years since the first GSM network was commercially launched, as the second generation of mobile phones, it has become the world's leading and fastest growing mobile standard. It is in use by more than one-sixth of the world's population. GSM radiation is characterized by a "high frequency" carrier wave which periodically pulsed at "low frequency". Modulation refers to the patterns of change in the frequency and/or amplitude of the RF carrier wave. The carrier wave has a specific impulse sequence: every impulse sequence lasts 120 ms and it is made up by 26 impulses, each one lasts 4.6 ms; the frequencies of these two periods are 8.3 Hz and 217 Hz. As cellular telephone technology has advanced, the modulation patterns have become increasingly complex using extremely low frequency (2 Hz-17.6 Hz). The geographical area around a GSM base station for which it provides coverage is known as a cell. Cells may be divided into sectors, in which case the base station transmits different frequencies into the different sectors. Cells vary in size depending on the number of mobile phone users and the topography of the surrounding area. The largest cells are known by the industy as macrocells; smaller cells, particulary those in urban areas, can be classified as microcells or picocells.

- A *macrocell* provides the main coverage in a mobile network. The antennas for macrocells are mounted on ground-based masts, rooftops and other existing structures. They must be positioned at a height that is not obstructed by surrounding buildings and terrain. Macrocells have a typical power output of tens of watts.
- A *microcell* provides infill radio coverage and additional capacity where there are high numbers of users within a macrocell. The antennas for microcells are mounted at street level, typically on the external walls of existing structures, lamp posts and other street fornitures. The antennas are smaller than macrocell antennas and, when mounted on existing structures, can often be disguised as building features. Typically microcells provide radio coverage across small distances and are placed 300-1000 m apart. They have lower outputs than macrocells, usually a few watts.
- A *picocell* provides more localised coverage than a microcell. They are normally found inside buildings where coverage is poor or where there are high numbers of users, such as airport terminals, train station or shopping centres. They also have lower outputs than macrocells and occupancy is generally low, although close approach to the antennas may occur.

Table 10. Some operationg characteristics of GSM signals.

Item	GSM
Access scheme	Time division multiple access (TDMA)
Modulation Scheme	Gaussian minimum shift keying (GMSK)
Frequency band for uplink	890-915 MHz (GSM 900) 1710-1785 MHz (GSM 1800)
Frequency band for downlink	935-960 MHz (GSM 900) 1805-1880 MHz (GSM 1800)
Maximum peak power of handset	2 W (GSM 900) 1 W (GSM 1800)
Maximum time-averaged power of handset	0.25 W (GSM 900) 0.125 W (GSM 1800)

Moreover, there are a range of other technological developments that result in exposure of the population to RF fields like those described below.

Third-Generation Mobile Telephone Technology (3G) provides the ability to transfer both voice data (a telephone call) and non-voice data (e.g., downloading information, exchanging e-mail, and instant messaging). Its function in Europe is based on the

Universal Mobile Telecommunications System (UMTS) standards. It operates at frequencies between 1900 and 2200 MHz; given a typical sector antenna with again of 18 dB, the maximum radiated power would be around 25 W. The UMTS standard specifies two modes, known as frequency division duplex (FDD) and time division duplex (TDD). In FDD mode, two separate radiofrequency channels are allocated: one for the uplink (mobile to base station) transmission and one for the down link (base station to mobile) transmission, similarly to the second-generation GSM standards. In TDD mode, the uplink and downlink transmissions are carried over the same frequency channel but at different times.

The peak output powers of UMTS handsets are lower than those of GSM handsets; however, the transmissions of GSM handsets are pulsed whereas UMTS handsets transmit continuously (in FDD mode). Consequently,the maximum time-averaged power is the same for UMTS hadsets as for GSM handsets operating in the 1800 MHz frequency band. Both GSM and UMTS technologies support adaptative power control, therefore time-averaged powers under typical conditions of usage may be much lower than the maximum value specified. The peak and spatial distribution of specific energy absorption rate (SAR) in the head under standard test conditions might be expected to be similar to UMTS and GSM 1800 handsets. In practice, the SAR will depend on the characteristics of individual handsets, in particular the design and location of the antennas.

It is expected that the radiated power of 3G base stations will generally be less than that of GSM base stations because 3G cell sizes are generally smaller. Nevertheless, as with 2G base stations, powers will be allocated to individual base stations based on their particular site circumstances and a range of powers up to the maximum lincensed power may be used. Exposures at particular locations will be largely determined by the local power density, which can be measured, as has been done with GSM base stations. On the assumption that the powers of 3G sites are generally no more than those of GSM sites and that mast configurations, e.g. antenna heights, antenna beam configurations and the tendency for shielding at public exposure locations due to intervening buildings etc are similar, exposures would be expected to be very much below guideline levels, as with GSM sites.

Table 11. Some operationg characteristics of UMTS (FDD) signals.

Item	UMTS (FDD)
Access scheme	Direct-sequence code division multiple access (DS-CDMA)
Modulation Scheme	Quadrature (quaternary) phase shift keying (QPSK)
Frequency band for uplink	1920-1980 MHz
Frequency band for downlink	2110-2170 MHz
Maximum peak power of handset	0.125 W
Maximum time-averaged power of handset	0.125 W

Terrestrial Trunked Radio (TETRA) is an emergency service radio standard introduced by many countries since 1997. It is not simply a replacement for the large number of old, out-of-date and incompatible analogue radio systems that the police have been using. There are operational advantages: it provides clearer and more secure and extensive coverage than the existing analogue systems; it allows for groups calls to be set up quickly; it can cope with very high peak demand, meaning that police operations will not be hindered at major incidents when many officers need to communicate at the same time. TETRA technology provides a high standard of encryption, preventing eavesdropping on police communications. In terms of data transmission, it allows police officers to use their radios to connect to facilities without needing to return to their station. Photographs for people identification can be transmitted, as can maps and instructions. TETRA network can also be used to transmit data from satellite tracking systems on the locationof both people and vehicles. It operates at frequencies around 400 MHz and it has similar architectures to mobile phone networks. In trunked mode operation (TMO) mobile terminals, ie. Hand portables or vehicle-mounted terminals, communicate with each other through fixed base stations with antennas mounted above ground level on masts or buildings. TETRA also supports direct mode operation (MDO) whereby a mobile terminal so that the radio signals do not pass through the infrastructure of base stations.

AGNIR described the maximum power radiated from TETRA base station transmitters as similar to that from mobile phone base station transmitters, ie a few tens of watts (AGNIR, 2001). NRPB had made measurements of the power density of radio signals at publicly

accessible locations in the vicinity of several TETRA base stations. The results indicated that the exposure values from base stations should be less than the ICNIRP guidelines for the general public if the exclusion zones are correctly set by the operators. The waveforms of the EMFs from base stations are continous and not pulsed as they are from mobile terminals (ICNIRP, 2002). Hand portable equipment transmits at a peak power of 1 W or 3 W, depending on the class of radio; however, a time division multiple access (TDMA) scheme is used that reduces the average power output during speech transmission to 0.25 W or 0.75 W for the two classes, respectively. For speech transmission, the signal emitted by a TETRA hand portable is pulsed with a power modulation frequency of 17.6 Hz. Since a base station could be in any direction with respect to the user, the hand portable antennas are designed to radiate equally in all directions. This means that a proportion of the radiated power is directed towards and absorbed by the part of the user's body next to the hand portable, normally the head or waist. Measurements of SAR in a phantom head at various positions of likely use for two commercially hand portables (1 W and 3 W) were reported to comply with the NRPB and ICNIRP occupational guidelines (Chadwick PJ, 2003; NRPB, 2004).

Wireless Local area networks (WLANs) provides wireless connectivity. Wireless computer networking is becoming increasingly widespread in offices, school and homes. It is also possible to access Internet services via radio from a personal computer (PC) at locations remote from the home or workplace, known as wireless hotspots. Computer terminals in WLANs are known as clients and have antennas either mounted outside their body-shell or integrated internally. The antennas may be removable if they are attached to or installed within PC cards or Personal Computer Memory Card International Association (PCMCIA). Clients communicate with fixed access points that provide an interface with conventional wired networks. WLANs operate in various frequency bands between 2.4 and 5.85 GHz providing data-transmission rates in the range of 1-50 Mbps (megabytes per second). Exposures to WLAN equipment will depend on how the transmitting antennas are located with respect to the body, the duration of any transmissions and the peak output power.

Bluetooth is a term generally designating digital wireless communication among personal-computer-associated devices i.e., "digital enhanced cordless telecommunication" between laptops, personal computers, personal digital assistants, cell phones, printers, digital cameras, etc. Bluetooth devices are classified into three power classes. The maximum output power of devices is about 100, 2.5 and 1 mW in classes 1, 2 and 3 respectively. This

technology is being increasingly used in business and in the home. It operates at a frequency of 2.45 GHz.The modulation scheme is gaussian frequency shift keying (GFSK) and frequency hopping is implemented at rates of up to 1600 s^{-1} in normal operation. The frequency hopping occurs over 79 channels spaced at 1 MHz intervals from 2402 to 2480 GHz. The technology can support small networks known as piconets, and these have a point-to-multipoint configuration.

Ultra-wideband (UWB) uses spreading techniques such as Orthogonal Frequency Division Multiplexing (OFDM) or impulse modulation that result in a broad emission spectrum usually centred at frequencies of a few gigahertz or tens of gigahertz. UWB has applications in radar, imaging and wireless communications, particularly short-range, high speed data transmissions suitable for broadband access to the Internet. The attractions of the technology are high data rates, low power, security and immunity from interference effects. Furthermore, the low power spectral density of UWB ensures that interference with other users of the radio spectrum is minimised.

Radiofrequency Identification Devices (RFID) is another area where low power wireless communication is widely used. Devices continue to be introduced utilising the benefits of modern digital signal processing for transmitting data from transponders or tags placed on a variety of goods for purposes of asset tracking and security. The radiocommunications system enables the tag devices to be interrogated and read (and in some cases programmed) remotely for purposes of identifying goods vehicles or animals. Both readers and tags have radio antennas as required for wireless communication using propagating electromagnetic waves. Frequencies up to about 2.5 GHz are used for current applications, often using bands assigned for industrial scientific and medical (ISM) use. Higher frequency bands up to 6.8 GHz have been allocated for possible use in the future. The power required depends upon the range under given conditions for the tag to respond. It will also depend on the system and whether fixed position or hand-held equipment is used to interrogate the tag and read the data.

RFEMF interaction with biological systems

As cellular telephone technology has advanced, the radiofrequency patterns used become more and more complex with lower frequencies, nearer to biological ones and so they may interfere with the sophisticated electromagnetic circuits of human body, for example in the brain.

Biological processes in living organisms include many interactions among electric charges (on ions, molecules, proteins, and membranes). Hence, it is clearly possible that exposure to RF, the electromagnetic fields of which can exert forces on fixed and moving charges, might have the potential to modulate biological function. For RF to cause or exacerbate disease in humans, the RF electric fields would have to trigger an initial transduction step, and then also begin a cascade of sequential steps that leads to a disease outcome.

Every organism has electric oscillating activities which store energy like neuronal circuits in the brain that emitted electromagnetic waves of various frequencies depending on the brain state (sleeping, waking, REM phase, non-REM phase); cardiac circuits; neuromuscular circuits; circuits regulating circadian rhythms. Besides extremely low frequency electromagnetic fields are associated to the brain electrochemistry, calcium fluxes and neurotransmitters systems as well as high frequency electromagnetic fields govern cellular processes like cellular division. An exogenous electromagnetic wave with a frequency near those of biological circuits, may influence them by non-thermal mechanisms like:

- "*Resonant Amplification*": an unbearable increase in the energy level of the biological system.
- "*Interference*": a reduction or an inhibition of some essential activities like melatonin releasing.
- "*Forcing*": of a bio-frequency to a value incompatible with the homeostasis.
- "*Lighting*": a rapid activation of processes for which the endogenous energy level is not sufficient.

Even a minimal energy interference may change the homeostasis of living organisms in a non-linear way because they are able to detect an exogenous oscillating or modulated electromagnetic field (Hyland GJ, 2000; Hyland GJ, 2001).

In conclusion MW/RF electromagnetic fields may influence ionic fluxes throughout cellular membrane altering homeostatic balance. The mechanism is based on the quantic electrodynamics. Water is the most important element in all living organisms, it is a bipolar molecule influenced by natural electromagnetic fields. These EM fields divide the water of

an organism into “coherent” spatial regions called *domains* composed by molecules oscillating in the same way surrounded by “non-coherent” regions. Circular orbits of ions dissolved in water are localized on the surface of “coherent” *domains*. The frequency of ionic orbits ,called cyclotron orbits, are proportional to a static EM field resulting from the vector addition of all EM fields present in the environment. If an oscillating EM field with a frequency similar to the cyclotron overlaps the static EM field, ionic orbits are altered and ions change their position as to the surface of the *domains*. If *domains* are not next to the cell membrane, ions stop in the “non-coherent” water region and they are replaced by other ions. If *domains* are next to the cell membrane, ions go across it, thanks to the potential difference between the two side of the cell membrane. Many physiologic oscillating electromagnetic fields exist into the animal body providing the preservation of the homeostatic balances. Nevertheless if an artificial EM field with a frequency like the cyclotron frequency of a ionic species is present in the environment, that ionic species will be influence by the EM field and non-physiologic ionic fluxes through the cell membrane can happen causing an homeostatic imbalance (Preparata G, 1995; Zhadin MN *et al.*, 1998). Another possible mechanism, based on quantic mechanics, is the dissociation of ion-protein complexes due to weak oscillating EM fields in the presence of a static electromagnetic field. In this case there is an imbalance between intra and extra-cellular ionic concentration which originates metabolic disorders and high stress levels (Binhi VN, 1998).

However some researches assert that modulation introduces a spread of frequencies into the RF signal, but the frequency bandwidth of the net RF signal generally remains a small fraction of the central, carrier frequency. This means that the most representative frequency range for modulated electromagnetic waves is that of the (high-frequency) RF carrier, not the (low-frequency) modulation pattern. Even though the power of the RF signal may vary in step with the modulation frequency, the transmitted RF spectrum contains no electromagnetic waves at the modulation frequency (Valberg PA *et al.*, 2007). Any biological interaction mechanisms capable of detecting the difference between a modulated RF signal and a non-modulated RF signal must be either be fast enough to respond to and detect changes in the central RF frequency, or sensitive to the RF power changes occurring at the modulation frequency. Scientists have not been able to identify neither biological structures capable of the necessary high-frequency RF tuning or bandwidth discrimination nor biological structures which are sensitive to the power changes and so nonlinear at low power levels (i.e., that can “rectify” the RF) (Valberg PA *et al.*, 2007).

Parameters of potential biological significance include the frequency content of the signal (ratio of modulation frequency to carrier wave frequency), the ratio of peak-to-average RF wave amplitude, the central frequency of the RF (carrier wave), the modulation frequency (typically ~ 0-10 kHz) and the Specific Absorption Rate (SAR) which is the energy absorbed by the irradiated biological system (W/kg).

SAR and energy distribution into an organisms depend on many aspects including organs and tissues composition (water content), organ and tissues size compared to MW/RF waves frequency, body shape, body structure and orientation and the distance from the RF source. Therefore the distribution of the absorbed energy into the organism is not uniform and so it can give rise at hot spots where energy is more concentrated. Actually, when energy is absorbed by a biological system, it is converted in heat causing an increase in dipolar molecules activity and so in tissues temperature, altering their biological function. However biological effects (non-thermal effects) described above seem to appear even if MW/RF intensity is not enough to cause detectable thermal effects: actually living systems have considerable thermal output, overall thermal inertia, and efficient thermal regulation.

RFEMF: *In Vitro* Study Results

Many researches demonstrated the capacity of radiofrequency electromagnetic fields to cause DNA damage, chromosomes aberrations and the forming of micronuclei in mammal cells even if temperature is kept into the physiological range (Balode Z *et al.*, 1996; Fucic A *et al.*,1992; Garaj-Vrhovac V *et al.*, 1990; Garaj-Vrhovac V *et al.*, 1991; Garaj-Vrhovac V *et al.*, 1992; Haider T *et al.*, 1994;Kundi M, 2002; Maes A *et al.*, 1993; Maes A *et al.*, 1996; Maes A *et al.*, 1997; Verschaeve L and Maes A, 1998). DNA functionality could be altered in human and hamster cells cultured *in vitro*; especially the transcription and the expression of proto-oncogenes *C-jun* and *C-fos* (Ivachuk OI *et al.*, 1997; Goswami PC *et al.*, 1999). Data on the radiofrequency activation of the expression of some ubiquitous genes without any temperature increase, are supported by an other important study on Nematoda (de Pomerai D *et al.*, 2000). These worms are completely characterized from the genetic point of view and they have a high sensibility and specificity to identify environmental stress, including electromagnetic fields. They were exposed at the frequency used in wireless telephony (750-1000 MHz) and at a lower intensity (SAR: 0.004-0.15 W/Kg) than those used for mobile phones (SAR: 0.02-1.0 W/Kg) at 25°C (28°C is the temperature at which “reporter genes” expression like *gfp* and *LacZ* is activated by temperature increase). In these conditions, the exposure to electromagnetic fields does not

cause any temperature increase but it induces the expression of reporter genes in adult worms and a rapid growth of maggots. They observed also a structural modification of cell membrane proteins. However there are also many studies funded by Motorola Inc and other wireless telephony companies which pointed out no effects on DNA and chromosomes after human leucocytes, mouse cells and bacterial cells irradiation with various frequencies (835, 837, 847,1900, 2450 MHz) (Malyapa RS *et al.*, 1997; Malyapa RS *et al.*, 1997; Phillips LP *et al.*, 1999; Vasquez MV *et al.*, 1999; Vijayalaxmi LBZ *et al.*, 2001).

As far as genetic mutations are concerned, radiofrequency waves seem to have no mutagen effects on different Salmonella stocks, on bacteria (*Escherichia coli*) and yeasts (*Saccharomyces cerevisiae*) (Léonard A *et al.*, 1983) as well as on somatic and germinal cells of both *Drosophila melanogaster* and mammals.

Besides *in vitro* carcinogenesis test demonstrates that radiofrequency electromagnetic fields and microwaves may be co-promoters and co-carcinogens (Balcer-Kubiczek EK and Harrison GH, 1991; Watson JM *et al.*, 1998); actually many studies found out an increase in the ODC activity, one of the most important enzyme implicated in the carcinogenesis promotion (Stewart Report, 2000).

The RFEMF data synthesized in the 2004 final report of the European Union Programme "Quality of life and management of living resources" on risk evaluation of potential environmental hazards from radiofrequency electromagnetic field exposure using sensitive *in vitro* methods allow the following conclusion:

- RFEMF produced genotoxic effects in fibroblasts, HL-60 cells, granulosa cells of rats and neural progenitor cells derived from mouse embryonic stem cells. Cells responded to RFEMF exposure between SAR levels of 0.3 and 2 W/Kg with a significant increase in single and double strand DNA breaks and in micronuclei frequency. Chromosomal aberrations in fibroblasts were also observed after RFEMF exposure. In HL-60 cells an increase in the intracellular generation of free radicals accompanying RFEMF exposure could clearly be demonstrated.
- No clear-cut and unequivocal effects of RFEMF on DNA synthesis, cell cycle, cell proliferation, cell differentiation and immune cell functionality were found in the cell systems under investigation. There is some indication that RFEMF may affect the growth arrest and DNA damage inducible gene GADD45 and the neuronal differentiation by inhibition of *Nurr1* in neural progenitor cells.

- No clear-cut and unequivocal effects of RFEMF on apoptosis were found in the cell systems under investigation was observed. There is some indication that RFEMF may have some influence on the *bcl-2* mediated anti-apoptotic pathway in neural progenitor cells and on the *p38MAPK/hsp27* stress response pathway in endothelial cells of human origin which may in turn exert an inhibitory effect on apoptosis.
- RFEMF at a SAR 1.5 W/Kg down-regulated the expression of neuronal genes in neuronal precursor cells and up-regulated the expression of early genes in *p53*-deficient embryonic stem cells, but not in wild-type cells. Proteomic analysis on human endothelial cell lines showed that exposure to RFEMF changed the expression and phosphorylation of numerous, largely unidentified proteins. Among these proteins is the heat shock protein *hsp27*, a marker for cellular stress responses. The results of the whole genome cDNA micro-array and proteomic analysis indicated that EMF may activate several groups of genes that play a role in cell division, cell proliferation and cell differentiation.

RFEMF: *In Vivo* Studies Results

Laboratory animals studies on RF/MW-EMF have the highest predictive role to evidence a possible health risk, especially carcinogenetic risk, for humans but they are very expensive and they require long time to give results (4-5 years). Therefore only few research centres can manage this type of experiments.

Carcinogenic studies in laboratory animals provide some insight as to the biological effects of RF modulation and of RF exposure generally. The researches fund by telephony industry found out only negative results on the carcinogenetic power of RF and MW (Adey WR *et al.*, 2000; Fritze K *et al.*, 1997; Imaida K *et al.*, 2000; McCann J *et al.*, 1997); many publications that reported carcinogenic assays in rodent species, after RF exposure ranged from 435 MHz to 9400 MHz (the frequency range applicable to mobile telephony) reveal a preponderance of null results, especially the more recent, better-designed studies were overall negative (WHO, 2006). Besides these data do not tend to support the idea that modulated RF is more potent than non-modulated RF; overall, the weight of evidence in animals exposed for extended periods, up to lifetime exposures of 2 years, at a variety of frequencies and modulations, suggests that exposure to modulated RF does not increase risk of tumour development; (Dasenbrock C, 2005). Whereas independent studies indicate a promoter effect of RF and MW on mammary and skin tumours in mice (Szmigielski A *et*

al., 1982) and on lymphoma (Repacholi MH *et al.*, 1997) but they are not conclusive and so other researches are necessary (Taioli E, 2001).

Many studies demonstrated that radiofrequency electromagnetic fields can cause structural DNA damage in many organs of exposed animals. In 1994, researchers found out DNA fragmentation in brain and testicular cells of rats exposed at 2450 MHz (1 mW/cm^2) (Sarkar S *et al.*, 1994). These observations were confirmed by other studies (Lai H and Singh NP, 1995; Lai H and Singh NP, 1996). DNA breaks may be produced by the direct action of RF/MW electromagnetic energy as well as by the inhibition of reparation mechanisms. They are the molecular base for many DNA and chromosomal aberrations. In addition other studies demonstrated that animals treated with naltrexone (an opiate antagonist), melatonin and BPN (N-t-butyl-phenyl-nitrone) (two free radicals "scavengers") do not sustain any DNA damage (Lai H, 2003; Richter ED *et al.*, 2000); so RF/MW are able to activate endogenous opiates in the brain causing many biological effects (Lai H, 1992) and they probably increase free radical production. Therefore RF/MW may be the promoters of many neurodegenerative pathologies like Alzheimer disease and Parkinson disease as well as brain tumours.

RF/MW electromagnetic fields seem also to alter hemato-encephalic barrier permeability in rodents, with albumin infiltration in young animals (12-16 weeks of age), and consequent neuronal damage (Frey AH *et al.*, 1998; Salford LG *et al.*, 1993; Salford LG *et al.*, 1994; Weinberger Z and Richter E, 2003); to modify bioelectric activity of the brain, neurotransmitter functions and the activity of some drugs in rats; actually RF/MW-EMF activate the opiates, a group of neurotransmitters produced in the brain, they disturb acetylcholine activity in the central nervous system modifying cholinergic receptor number and they induce CRH release (Lai H, 1996). So they cause a brain stress answer; actually the EMF effects on opiates and on acetylcholine are the same pointed out in stress conditions. In addition, RFEMF reduce memory and cognitive capacities of rats, modifying their normal behaviour (Lai H, 1997).

As far as reproductive effects are concerned, acute exposure at high intensity RF/MW electromagnetic fields, such that they cause a body temperature increase, produces aberrations of spermatocyte epithelium and so it reduces male fertility. Nothing is sure about the effects of chronic exposure at low intensity RF/MW electromagnetic fields, without temperature increase; it does not seem to cause any damage at spermatozoa (Verschaeve L and Maes A, 1998). Many studies evidenced also teratogen and embryotoxic effects of high intensity RF/MW electromagnetic fields, with an increase of

miscarriages and embryonic deformities linked to the maternal body temperature increase. Nowadays, researches can not exclude that also chronic exposure at low intensity RF/MW electromagnetic fields may have some embryotoxic or teratogen effects (O'Connor ME *et al.*, 1999).

RFEMF: Epidemiologic Studies Results

The RF electromagnetic fields produced by mobile communication systems and the different types of mobile phones are widespread in the living environment and have exercised their effects on public health only since very recent years compared to ELFEMF. Therefore conclusions on their health effects on humans are uncertain.

Epidemiologic studies on the health effects of RF/MW electromagnetic fields can be divided into three groups: occupational exposures, residential and environmental exposures, cordless and cellular telephones use exposures.

The results of researches on occupational exposures are not univocal and they are affected by some important restrictions: as far as dosimetry is concerned, exposure definition is often inadequate and it is difficult to know exactly the frequency and the intensity of electromagnetic fields as well as the duration of the exposure (daily and weekly temporal profiles, total duration of exposure). Besides workers may be exposed to other carcinogenic agents at the same time without any documentation in this connection. So risk indexes may be underestimated or overestimated.

Most of occupational epidemiologic studies were conducted on soldiers finding out an increase of leukaemia, lymphoma, testicular tumours, brain tumours, eyes tumours (intraocular melanoma) and mammary tumours (Davis RL and Mostofi FK, 1993; Garland FC *et al.*, 1988; Garland FC *et al.*, 1990;Grayson JK, 1996; Holly EA *et al.*, 1996; Milham SJ *et al.*, 1985; Milham SJ *et al.*, 1988; Milham SJ *et al.*, 1990; Szmigielski S, 1996; Thomas TL *et al.*, 1987). Other studies on civil workers exposed to RF/MW electromagnetic fields confirmed an increase in many type of tumours especially mammary and uterus tumours in women (Hayes RB *et al.*, 1990; Tynes T *et al.*, 1996). However there are also some occupational studies with no statistical significant cancer increase (Finkelstein MM, 1998; Lagorio S *et al.*, 1997; Muhm JM, 1992; Robinette CD *et al.*, 1980).

Some epidemiologic studies on residential exposures to RFEMF evidenced an increased incidence of leukaemia and lymphoma in adult people and children and positive trends for melanoma, bladder tumours and brain tumours (Anderson BS and Henderson AK, 1986;

Colorado Department of Public Health and Environment, 1998; Comba P *et al.*, 2002; Dolk H *et al.*, 1997; Dolk H *et al.*, 1997;Giovanazzi A, 2002; Hocking B *et al.*, 1996; Michelozzi P *et al.*, 2002; Terracini B *et al.*, 2001), whereas other studies, two of them sponsored by electronic industries (Telecom New Zeeland and Federation of the Electronic Industry), found out only negative results (Ehwood JM, 1999; McKenzie DR *et al.*, 1998; Moulder JE, 2003; Selvin S *et al.*, 1992). Besides several epidemiologic studies of potential cancer risk, using proximity to commercial broadcast transmission towers as the measure of RF exposure, have not provided sound evidence that RF exposure from the transmitters increased the risk of cancer or any other health effect (Jauchem JR, 2003). The reporting of cancer "clusters" around RF broadcast transmitters and mobile phone base stations has heightened concern among the general public, but given the random nature of the distribution of cancers in the population it may be not surprising statistically that such clusters should appear. Also, given the ubiquity of base stations, one would expect that a base station being near existing cancer clusters is a likely occurrence (Valberg PA *et al.*, 2007).

In 2003 TNO-study (Netherlands Organization for Applied Scientific Research, Physics and Electronics Laboratory) explored the effects of controlled exposure to mobile communication system radio-frequency electromagnetic fields at base station intensities on human well-being and cognitive function considering the second-generation Global System for Mobile Communication (GSM) widely used around the world, and its successor, the Universal Mobile Telecommunications System (UMTS), the third generation of mobile network (Zwamborn AMP *et al.*, 2003). TNO-study was the first to investigate the short-term effects of a base-station like exposure and to indicate a reduction in well-being and an alteration of brain functions. These results were not confirmed by a more recent study which describe only weak brain effects on sensitive and non-sensitive subjects exposed to UMTS-like EMF (Regel SJ *et al.*, 2006). However no conclusions can be drawn regarding short term effects of GSM and UMTS base-station like exposure. Other investigations on the health of people living near mobile telephone relay station do not role out effects on wellbeing, subjective symptoms, sleeping problems, headache, depression, discomfort, cognitive perturbations (Hutter HP *et al.*, 2006; Santini R *et al.*, 2002). Many studies reported health items related to "microwave sickness" or "RF syndrome". The "RF syndrome" exists in two versions:

1. The “screen dermatitis”, caused by professional use of video screens; it has various skin localizations sometimes associated to central nervous system and heart disease;
2. The “electromagnetic syndrome” is due to electric lines, cellular telephones, electronic equipment, mobile telephone and radio-base stations. It is characterized by central nervous system disease (asthenia, apathy, irritability, headache, memory loss, anxiety, sleeping disorders), heart disease (palpitations, tachycardia), muscular disease (muscular asthenia, myalgia) and skin disease (erythema, diffuse itches, burning).

The “electromagnetic syndrome” seems to affect people living near mobile telephone-relay station with a severity statistically correlated at the measured power density (Navarro EA *et al.*, 2003; Rudolph K, 2002; Santini R *et al.*, 2002; Santini R *et al.*, 2000). Reviews of the evidence on electromagnetic hypersensitivity have been conducted (Fox E, 2006): an extensive systematic search identified relevant blind or double-blind provocation studies of individuals potentially hypersensitive to the presence of EMF. A meta-analysis found no evidence of an improved ability to detect EMF in “hypersensitive” participants. That is, it was concluded that weak electromagnetic fields are not likely to be causative factors for neurological symptoms (Rubin GJ *et al.*, 2005; 2006a; 2006b). An investigation into possible differences in blood cells between patients reporting EMF hypersensitivity and normal patients did not find any differences in lymphocyte response to RF from GSM mobile telephones (Markova E *et al.*, 2005). Other investigators have likewise concluded that “based on the limited studies available, there is no valid evidence for an association between impaired well-being and exposure to mobile phone radiation” (Seitz H *et al.*, 2005). However, it is important to recognize the plight of people suffering from “hypersensitivity reactions.” The WHO recently issued a fact sheet about people reporting non-specific symptoms that they relate to RF fields from base stations and other EMF devices (WHO, 2005). Moreover, researchers are continuing to analyze possible electromagnetic hypersensitivity reactions (Eltiti S *et al.*, 2007).

Regarding the stronger but much more localized exposure by mobile phone handset, data on well-being are inconclusive (Rubin GJ *et al.*, 2006; Seitz H *et al.*, 2005), yet various studies identified subtle effects regarding changes in brain activity or influences on cognitive function such as reaction times, working memory, and attention (Curcio G *et al.*, 2005; Freude G *et al.*, 2000; Huber R *et al.*, 2005; Hyland G, 2000). They found out also alterations in the bioelectric activity of the brain (Lebedeva NN *et al.*, 2000), especially

during sleeping period. When some volunteers are exposed to an intermittent bilateral irradiation (15'on/15'off) with modulate MW, emitted by a GSM cellular phone (900 MHz; SAR=1 W/Kg) their non-REM phase electroencephalogram pattern become anomalous: its waves are altered in a specific frequency range (7-14 Hz) (Borbely AA *et al.*, 1999). Furthermore the brain blood flux is also influenced by cellular electromagnetic fields: there are a blood flux increase in the dorsolateral-prefrontal area of the brain only on the side where the cellular phone is applied and this increase persists for 30' after the end of the electromagnetic impulse (Huber R *et al.*, 2000; Huber R *et al.*, 2002).

Some of the reported changes (acceleration of response times in certain cognitive tasks, altered oscillatory activity in the electroencephalogram as a function of time and task), however, were inconsistent and could not be replicated (Haarala C *et al.*, 2003; Krause CM *et al.*, 2004; Preece AW *et al.*, 2005).

Many epidemiologic studies demonstrated a statistical significant correlation between cellular phones use and some "RF syndrome" items, especially headache, supposing that RFEMF modify the haemato-encephalic barrier or the dopamine-opiate pathway in the brain; two mechanisms involved in the headache aetiology (Chia SE, 2000; Frey AH, 1998; Hocking B, 1998). Also several laboratory studies with volunteers have investigated whether low-level exposure to RF fields associated with mobile phones can affect brain function and behavior. Reported reactions to assumed RF exposure include a wide variety of non-specific symptoms. Most commonly reported symptoms are sleeplessness, fatigue, dizziness, digestive disturbances, and concentration difficulties. Large, well-controlled and -conducted double-blind studies have shown that symptoms are not correlated with RF exposure. There are also some indications that these symptoms may be caused by pre-existing conditions such as stress reactions resulting from worrying about perceived RF health effects rather than the RF exposure per se. To date, only subtle and transient effects have been reported, and any implications for health remain unclear and unlikely (Cosquer B *et al.*, 2005).

Of special concern is the risk of brain tumours since this part of the body is highly exposed during phone calls compared with other parts: cellular telephones emit radio frequency signals during calls, SAR differs in absolute values as well as in anatomical distribution between various types of cellular phones, and information about SAR values was not available until most recent years. Therefore tumours localized in the brain, meninges, glial tissue, hematopoietic tissue in the parietal bones of the cranium, acoustic nerve, parotid gland, skin and soft tissues of the head area exposed at the use of cellular phones are

suitable to study to demonstrate health effects of a long-term exposure to RF-fields. Especially acoustic neuroma might be a "signal" tumour for an association, since it is located in an area with the highest exposure. An interesting approach that has been used is to compare temporal trends in disease rates with temporal trends in prevalence of cell phone use: however trends in acoustic neuroma incidence in England and Wales were found not to lag behind trends in cell phone use in a correlated fashion (Nelson PD *et al.*, 2006).

Furthermore, the risk would be higher for tumours on the same side of the head as the exposure to the RF-field (ipsilateral exposure).

However most of the published studies on the association between cellular telephones and brain tumours have a big shortcoming: too short latency period (Auvinen A *et al.*, 2002;Dreyer NA *et al.*, 1999; Inskip PD *et al.*, 2001; Johansen C *et al.*, 2001; Morgan RW *et al.*, 2000; Muscat JE *et al.*, 2000; Rothman KJ *et al.*, 1996) which may explicate their negative results. Even if they found a risk increase but not statistically significant, for neuroephitelioma, acoustic neuroma, testicular tumours, uterus tumours and pituitary tumours. Thus, both longer latency period and higher cumulative number of hours for use are necessary to get a more precise estimate of the risk. Researchers found an increased risk for brain tumours that was most pronounced in people with >10 year latency period (Hardell L *et al.*, 2006; Lönn S *et al.*, 2004; Lönn S *et al.*, 2005). Moreover the risk was highest for analogue and digital cellular telephones with ipsilateral exposure with a more pronounced effect for high-grade astrocytoma whereas there is no association for meningioma (Hardell L *et al.*, 2003; Hardell L *et al.*, 2005a; Hardell L *et al.*, 2005b; Hardell L *et al.*, 2005c); of special concern is the higher risk for use of digital cellular telephones and cordless phones in age groups <20 years at start (Hardell L *et al.*, 2006). Furthermore, some weak association were found also between ipsilateral skin angiosarcoma and cordless use (Hardell L *et al.*, 1999), glioma and analogue phones use (Auvinen A *et al.*, 2002), eye uveal melanoma and cellular telephones use (Stang A *et al.*, 2001); in this case the author supposed that the MW emitted by cellular phones have a carcinogenic promoting action reducing melatonin activity or increasing its consumption: actually melatonin produced both in the retina and in the ciliary bodies, stops the growth of uveal melanoma cells *in vitro*. Another important epidemiologic study is the ongoing INTERPHONE collaboration which is a multi-centre, comprehensive study on mobile phones and cancer. It is coordinated by the International Agency on Research on Cancer (IARC), a specialized cancer agency of the WHO, and researchers in 13 countries are

taking part using a common protocol. The INTERPHONE protocol is a population based, case-control study correlating head and neck tumours with mobile-phone use by persons 30-59 years of age who reside in the study regions. Exposure assessment is reliable because it is based on individual records of cell phone use. Because of pooling of data from all participating centres, the study is statistically powerful. For example, the risk of acoustic neuroma in relation to mobile phone use has been assessed via six population-based, shared-protocol, case-control studies in four Nordic countries and the United Kingdom. The authors concluded that there was no association of risk with duration of use, lifetime cumulative hours of use, or number of calls, for phone use overall or for analogue or digital phones separately (Schoemaker MJ *et al.*, 2005). Recent results from INTERPHONE have reported lack of brain tumour or acoustic neuroma risk in Japan (Takebayashi T *et al.*, 2006), Germany (Berg G *et al.*, 2006; Schuz J *et al.*, 2006), and in a meta-analysis of five Northern European countries (Lahkola A *et al.*, 2007). There have also been other studies of mobile telephone users, particularly on brain tumours (and less often on other cancers and on symptoms). Results of these studies to date give no consistent or convincing evidence of a causal relation between RF exposure and any adverse health effect (Ahlbom A, 2006; Ahlbom A *et al.*, 2004; Lonn S *et al.*, 2005). A 4-year British survey released in 2006 showed no link between regular, long-term use of cell phones and the most common type of brain tumour, glioma (Hepworth SJ *et al.*, 2006). A German study did find an elevated risk of glioma in long-term users, but the increase was not statistically significant. The authors concluded that no overall increased risk of glioma or meningioma was observed among these cellular phone users (Schuz J *et al.*, 2006).

RFEMF: Risk Assessment and Regulatory Aspects

The heath-effect guidelines of ICNIRP in the mobile telephony frequency spectrum range from about 40-60 V/m (4.3-10 W/m^2) (ICNIRP 1998). The ICNIRP guidelines have been widely accepted (> 30 countries worldwide) and, for example, are consistent with Health Canada (1999), U.S. [American National Standards Institute/Institute of Electrical and Electronics Engineers (2006), Federal Communications Commission (2006)], UK [National Radiation Protection Board/Health Protection Agency (NRPB/HPA 2004a, 2004b)], and Australian [Australian Radiation Protection and Nuclear Safety Agency (ARPANSA 2002)] standards. However, some countries and regions have adopted more stringent guidelines. In contrast to the ICNIRP levels, the following are some examples of

these more restrictive guidelines, in the mobile telephone frequency range (Baumann J, 2006; Vecchia P, 2006):

• ICNIRP Guidelines: 40-60 V/m or 4.3-10 W/m^2

• "Italian Exposure Limit": 6 V/m

• "Paris Charter": 2 V/m, 24-hr average, indoors

• "Salzburg Protection Value": 1 W/m^2

• "Swiss Regulation": 4-5 V/m at full power.

The issues that most often drive more localized RF guidelines are not established health risk per se, but rather risk perceptions (Siegrist M *et al.*, 2005). In this regard, the "Precautionary Principle" is often invoked, part of which involves taking "protective measures without having to wait until the reality or seriousness of those risks becomes apparent." One expression of how "protective measures" might be applied to RF levels is: "The proposed [RF] standard also recommends that it is generally sensible to minimize exposure which is unnecessary or incidental to achievement of service objectives or process requirements, provided this does not introduce other risks and it can be readily achieved at modest expense" (ARPANSA 2001).

The term "modest expense" implies some type of cost–benefit analysis. Appropriate application of the Precautionary Principle requires that the policies be tailored such that the time, effort, expense, and risk of any "protective measures" be commensurate with what the society expends on other public risks of similar magnitude. However, if scientific research is not able to establish "apparent risks" in a quantitative way, making such a calculation is problematic. Despite unavoidable uncertainty and other limitations of the scientific method, science remains our best source of knowledge about how the world works and how we can rely on natural laws to understand interactions between the environment and living things. Perhaps the most important element of risk communication is to assure audiences that RF standards have been and continue to be under ongoing scrutiny. Large numbers of scientists, medical doctors, and public health professionals of disparate orientations and areas of expertise sift existing data and contribute new data in an ongoing risk assessment effort. The vast majority of human cancers are likely caused by unavoidable environmental exposures (e.g., viruses, diet, lifestyle, sunlight, background ionizing radiation) or to processes inherent to life itself (e.g., genetic instability, copying errors in DNA, endogenous hormones, creation of mutagens and free radical molecules by metabolism of food, production of reactive chemicals for microbicidal defense) (Gotay CC, 2005; Henderson BE *et al.*,1991; McKean-Cowdin R *et al.*, 2000; Wogan GN *et al.*,

2004). In scientific risk assessment, one compares the ability of the exposure of interest to increase risk above these baseline, natural processes.

The conclusions of the WHO workshop on "Base Stations and Wireless Networks" indicate that there are no health consequences of base-station RF exposure, and no adverse effects are foreseen at the RF levels typical of cellular telephone technology.

Several groups in Great Britain have evaluated potential health effects of RF. The Advisory Group on Non-Ionizing Radiation (2003) updated the year 2000 report of the Independent Expert Group on Mobile Phones (2000) and concluded that "exposures due to living near to base stations are extremely low, and the overall evidence indicates that they are unlikely to pose a risk to health." (Advisory Group on Non-Ionizing Radiation 2003).

The UK Health Protection Agency (formerly the National Radiation Protection Board) (NRPB/HPA 2004a) also has concluded that RF energy can potentially cause health effects only if people are exposed to RF levels significantly exceeding international limits.

That is, they recommended that exposure to EMFs (0–300 GHz) in the UK be based on the 1998 guidelines issued by ICNIRP (NRPB/HPA 2004a).

In a specific review of cellular telephone technology (NRPB/HPA 2004b), the agency proposed that even though "there is a lack of hard information showing that the mobile phone systems in use are damaging to health," they continued to endorse a "precautionary approach" to the use of mobile phone technologies.

The Health Council of the Netherlands (Health Council 2002) has prepared a report on the potential risks of EM fields from mobile telephones. The report concluded that "the EM field of a mobile telephone does not constitute a health hazard, according to the present state of scientific knowledge." Moreover, the review committee concluded that "the scientific information concerning non-thermal effects discussed in this report provides no reason to apply the precautionary principle and lower the SAR limits for partial body exposure" (Health Council 2002). A 2005 Health Council update concluded that "the Committee therefore disagrees that a connection has been found between living in the proximity of a base station and the occurrence of cancer" (Health Council 2005).

ARPANSA prepared a fact sheet titled "What about base stations and telecommunication towers–are there any health effects?". ARPANSA concluded that "the weight of national and international scientific opinion is that there is no substantiated evidence that RF emissions associated with living near a mobile phone base station or telecommunications tower poses a health risk" (ARPANSA 2003a). ARPANSA also evaluated the potential for risk to children and concluded that "the balance of evidence does not indicate a risk to the

health of people, including children, living in the vicinity of base stations where the exposure levels are only small fractions of the ARPANSA Standard" (ARPANSA 2003b).

The Royal Society of Canada has an "Expert Panel on Potential Health Risks of Radiofrequency Fields from Wireless Telecommunication Devices," and their most recent update (2004) notes that "all of the authoritative reviews completed within the last two years have concluded that there is no clear evidence of adverse health effects associated with RF fields" (Royal Society of Canada 2004). The advice of the U.S. Health Physics Society (a professional society of specialists in radiation safety) is that there is no reason to believe that cellular base station towers could constitute a potential health hazard to nearby residents or students (Health Physics Society 2006). At present, the only established effects that can result from excessive exposure to RF energy are related to tissue heating. Although RF energy can be absorbed by living organisms to some degree at any frequency, available data do not demonstrate adverse health consequences at exposure levels below internationally accepted limits, which do not allow significant heating.

In summary, biophysical considerations indicate that there is little theoretical basis for anticipating that RF energy would have significant biological effects at the power levels used by modern mobile phones and their base station antennas. The epidemiological evidence for a causal association between cancer and RF energy is weak and limited. Animal studies have provided no consistent evidence that exposure to RF energy at non-thermal intensities causes or promotes cancer. Extensive *in vitro* studies have found no consistent evidence of genotoxic potential, but *in vitro* studies assessing the epigenetic potential of RF energy are limited. Overall, a weight-of-evidence evaluation shows that the current evidence for a causal association between cancer and exposure to RF energy is weak and unconvincing. However, the existing epidemiology is limited and the possibility of epigenetic effects has not been thoroughly evaluated, so that additional research in those areas will be required for a more thorough assessment of the possibility of a causal connection between cancer and the RF energy from mobile telecommunications (Moulder JE *et al.*, 2005). Although scientists generally assign low priority to conducting research on base stations or other wireless technologies having such weak RF signals, some gaps in knowledge still exist (Repacholi MH, 1998). Research recommended to fill these gaps can be found in the WHO RF research agenda (WHO 2006b).

CIRCADIAN RHYTHMS

Rhythmic oscillations in the environment range in period from a few femtoseconds (10^{-15} seconds) to ten of thousands of years. Of all environmental rhythms, only those that depend on the Earth's rotation around its axis cause predictable changes in the geophysical environment, thereby providing organisms with options to occupy appropriate spatio-temporal niches. Most organisms place themselves suitably in such niches using precise time-keeping mechanism(s) that can measure passage of time on an approximately 24 hour scale; they are known as *circadian clocks* (DeCoursey PJ, 2004) and they generate biological rhythms called *circadian rhythms*.

The designation *circadian* is reserved for biological rhythms endogenously generated, with a frequency of oscillation between 10 and 14 μHz and a period between 19 and 28 hours, which can be synchronized by environmental cycles with 24-hour periods. Rhythms with a longer period are called *infradian*, for example the estrous cycle, while rhythms with a shorter period are called *ultradian*, for example cardiac and respiratory cycle (Refinetti R, 2006) or the release of hormones involved in reproduction as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Knobil E, 1987) as well as cortisol (Weibel L *et al.* 1996) and insulin which are secreted at intervals of approximately 1 hour (Lefcourt AM *et al.*, 1999).

The term *clock* is used in a sense that includes timer and pacemaker because a *biological clock* has the ability to generate a self-sustaining oscillation (that is, the ability to repeat a process over and over without external input) like a pacemaker but it can also estimate the time of day, when synchronized to the alternation of day and night, like a real clock and in addition, it allows the organism to time different functions along the circadian cycle like a timer that undergoes a constant change of state over time (Refinetti R, 2006).

Circadian clocks regulate a wide variety of behavioural processing including locomotor activity, feeding, excretion, sensitivity, learning capability. They control also many autonomic processes; the most studied ones are body temperature, cardiovascular function, melatonin secretion, cortisol secretion, metabolism and sleep. Many animal studies suggested that, under constant environmental conditions (constant darkness or constant light), circadian rhythms free-run with periods slightly different from 24 hours because the base value of period is genetically determined: different species have different circadian period (Eskin A, 1971; Mrosovsky N *et al.*, 1976; Honma S *et al.*, 1988; Jilge B, 1991;

Ebihara S and Gwinner E, 1992; Refinetti R *et al.*, 1992). Single gene mutation that affect circadian period have been described in hamsters (Ralph MR and Menaker M, 1988; Mrosovsky N,1989; Menaker M and Refinetti R,1992) and mice (Vitaterna MH *et al.*, 1994; King DP *et al.* 1997; Antoch MP *et al.*, 1997; Spoelstra K *et al.*, 2002).

Circadian clocks enhance the fitness of organisms by improving their ability to efficiently anticipate periodic events in their external environments, especially periodic changes in light, temperature, humidity, food availability (Dhanashree AP and Vijay KS, 2005); therefore the circadian pacemaker must be able to synchronize to these *Zeitgeber*. They *entrain* it so that the period of the self-sustaining oscillation conforms to that of the *zeitgeber*, setting a stable phase relationship between the two of them (Refinetti R, 2006).

In mammals, the strongest *Zeitgeber* is photoperiod. Its effects on infradian and circadian rhythms have been documented in a variety of animals (Sulzman FM *et al.*, 1982; Chakraborty SC *et al.*, 1992; Basco PS *et al.*, 1996; Schilling A *et al.*, 1999; Foà A and Bertolucci C, 2001; Herrero MJ *et al.*, 2003; Rieger D *et al.*, 2003), especially rodents (Kramm KR and Kramm DA, 1980; Puchalsky W and Lynch GR, 1991; Humlova M and Illnerova H, 1992; Elliott JA and Tarmarking L,1994; Haim A *et al.*, 1994; Haim A and Zisapel N, 1995; Haim A *et al.*, 1995; Pitrosky B *et al.*, 1995; Vuillez P *et al.*, 1996; Anchordoquy HC and Lynch GR, 2000; Deboer T, 2000; Jac M, *et al.*, 2000; Mrugala M *et al.*, 2000; Sumova A *et al.*, 2000; Haim A *et al.*, 2001; Larkin JA *et al.*, 2001; Refinetti R, 2002; Oosthuizen MK *et al.*, 2003; Garidou ML *et al.*, 2003; Gorman MR, 2003; Sumova A, 2003; Refinetti R, 2004; Evans JA *et al.,* 2004). Nonphotic stimuli such as ambient temperature (TokuraH and Ashoff J, 1983; Francis AJP and Coleman GJ, 1988; Goldman BD *et al.*, 1997; Pohl H, 1998; Rajaratman SMW and Redman JR, 1998; Pálková M *et al.*, 1999; Herzog ED and Huckfeldt RM, 2003), food availability (Edmonds SC and Adler NT, 1977; Sulzman FM *et al.*, 1977; Sulzman FM *et al.*, 1977; Rusak B *et al.*, 1988; Boulos Z *et al.*, 1989; Coleman GJ and Francis AJP, 1991; Kennedy GA *et al.*, 1991; Challet E *et al.*, 1996; Kennedy GA *et al.*, 1996; Challet E *et al.*1999; Holmes MM and Mistlberger RE, 2000;Sharma VK *et al.*, 2000), physical activity (Reebs SG and Mrosovsky N, 1989; Edgar DM and Dement WC, 1991; Mistlberger RE, 1991; Laemle LK and Ottenweller JE, 1999; Hut RA *et al.*, 1999) and social contact (Greco AM *et al.*, 1989; Tornatzky W and Miczek KA, 1993; Meerlo P *et al.*, 1996; Keeney AJ *et al.*, 2001) can affect circadian rhythms but they are weaker than light. However these multiple environmental stimuli have combined effects on the circadian system, producing the behaviour of animals (including humans) in the real world (Refinetti R, 2006). Actually circadian rhythmicity is

an evolutionarily old process found in all domains of life: some organisms are diurnal, some are nocturnal and some crepuscular but their circadian system don't seem to differ and the adaptation of diurnal or nocturnal niches is determined by mechanisms located "downstream" from the pacemaker (Refinetti R, 2006).

Circadian rhythms, like many other processes must undergo *development* because animals are not born with the full anatomical and functional characteristics of adults. For example newborn human babies do not have a daily rhythm of melatonin secretion until 3 months of age (Attanasio A *et al.*, 1986; Ardura J *et al.*, 2003); in rats rhythm of body temperature attains the adult range (1.6°C) of oscillation only at 45 days of age (Kittrell EL and Satinoff E, 1986), which is the age when the rhythm of corticosterone secretion reaches the adult pattern (Allen C and Kendall JW, 1967). Besides circadian system undergoes some changes caused by aging; the best defined one is a reduction in the *amplitude* of circadian rhytms (Brock MA, 1985; Turek FW *et al.*, 2001); for example the rhythm of body temperature in a rat of 2 months of age has an amplitude of almost 2°C whereas in a rat of 29 months of age the amplitude is only of 1°C (Refinetti R *et al.*, 1990). Aging is also associated with a change in the phase and period of circadian rhythms. A small advance in the phase angle of entrainment in old age has been documented in rodents (Halberg J *et al.*, 1981; Li H and Satinoff E, 1995; Weinert D and Waterhouse J, 1999) and humans. Humans have an endogenous period longer than 24 hours; old people tend to wake up earlier just to compensate for an age-dependent lengthening of the circadian period (Nakazawa Y *et al.*, 1991; May CP *et al.*, 1993; Baehr EK *et al.*, 2000; Klerman EB *et al.*, Robiliard DL *et al.*, 2002).

Although it is thought that the ability to anticipate environmental changes imparts survival advantages to an organism, an important aspect of circadian control may also be to time and synchronize metabolic processes to optimize metabolic networks by enabling a temporal partitioning of metabolic events within and between different tissues, for example, by temporally separating chemically antagonistic reactions and by limiting the expression of certain enzymes to the time of the day they are needed (Stratmann and Schibler, 2006).

Physical Substrates

The *physical substrates* of circadian rhythms are the cellular and molecular phenomena that underlie physiological processes. An organism receives information from the environment (*input*), processes it (*processor*) and acts on it (*output*). That is the organism senses stimuli in the environment (*sensors*), the information from the stimuli affects the pacemaker's operation (*pacemaker*), and the pacemaker controls effector mechanisms such as locomotor activity and body temperature (*effectors*). The main flow of information in the circadian system may be conceptualized as the receptors-pacemaker-effectors triad, but it involves many physical components of each member of the triad, as well as multiple feedback loops among them.

Receptors

The *sensors* in the circadian system include both photic and nonphotic *receptors*. *Sensory receptors* are specialized neural structures that transduce specific forms of energy (such as light, sound, and pressure) into changes in the polarization of afferent neurons to create ionic flows which reach the brain, and especially the circadian pacemaker in a usable form. Mammals have an only photosensitive organ: the eye. Eyes have visual and non-visual functions called respectively *image-forming photoreception* and *irradiance detection*. Photic transduction takes place in the retina, in the back of the eye, thanks to retinal photoreceptor: rods and cones. They are indeed needed for vision; rods are associated with nighttime (black-and-white) vision, while cones are associated with day-time (color) vision. They use photopigments to absorb incident light. Rhodopsin, the photopigment in the rods, is made up of two substances: retinal and opsin. In darkness, retinal has an isomeric configuration called 11-*cis*. When retinal absorbs light, its isomeric configuration changes to all-*trans* which cannot bind to opsin, so that rhodopsin is depleted in the presence of light. The breakdown of rhodopsin leads to the production of many intermediate compounds which cause the closure of Na^+ channels as well as a change in the permeability of the cell membrane and in the polarization of the photoreceptor cells. Cones work in the same way but they contain other types of opsin that have a different peak sensitivity. Rods and cones make connection with bipolar cells which then connect with ganglion cells. The axon of ganglion cells form the optic nerve, which take photic information to the brain. (Ganong WF, 1996). However rods are not used by the circadian system and cones play a minor part in its photic stimulation (Foster RG *et al.*, 1991, Yoshimura T *et al.*, 1994; Freedman MS *et al.*, 1999;Panda S *et al.*, 2002; Ruby NF *et al.*, 2002; Yoshimura T *et al.*, 2002; Mrosovsky N, 2003). The photic stimulation of the

circadian system depends on some ganglion cells with sparse and wide dendritic fields ($0.1mm^2$), which depolarize in response to local photic stimulation (Berson DM *et al.*, 2002; Warren EJ *et al.*, 2003). They are capable of providing adequate photic input in the absence of rod and cones (Takahashi JS *et al.*, 1984; Nelson DE and Takahashi JS, 1991). These photoreceptive ganglion cells contain melanopsin, a photopigment found in the retina of rats, mice, monkeys and humans (Provencio I *et al.*, 2000; Gooley JJ, *et al.*, 2003; Hannibal J and Fahrenkrug J, 2004). Mice lacking rodes, cones and melanopsin are not entrained or masked by light-dark cycles and show no papillary constriction in response to photic stimulation (Hattar S *et al.*, 2003; Panda S*et al.*2003).

Little is known about the way in which the circadian pacemaker acquires the information about temperature and nutritional state required for nonphotic entrainment. Temperature signals are available from three basic types of receptors located in the skin. *Polymodal nociceptors* are pain receptors that respond to various stimuli, including heat. *Cold receptors* generally yield a tonic response to thermal stimuli below normal skin temperature (peaking at 25°C) while *warm receptors* yield a tonic response to thermal stimuli above normal skin temperature (peaking at 42°C). Information about skin temperature ascends through the lateral spinothalamic tract (or trigeminal nucleus) to the ventrobasal complex of the thalamus and then to the somatosensory cortex to generate cold and warm sensations. Afference destined to the hypothalamus to contribute to body temperature regulation seems to diverge at the cervical level and to synapse at the midbrain raphe nuclei (Brück K and Hinckel P, 1990); how the information reaches the circadian pacemaker is not known.

Thermosensitivity is present not only in the skin but also in the brain (Carlisle HJ and Laudenslager ML, 1979; Klir JJ and Heath JE, 1994; Kanosue K *et al.*, 1999), spinal cord (Wünnenberg W, 1983; Graf R *et al.*, 1987), and visceral organs (Jessen C and Feistkorn G, 1984). In the brain three areas are thermosensitive: the preoptic area and the anterior hypothalamic area (POAH) which are important for both behavioural and autonomic responses, and the posterior hypothalamic area which seems to be responsible only for the activation of behavioural thermoregulatory responses (Aidar ER, 1974; Refinetti R and Carlisle HJ, 1986; Tanaka H *et al.*, 1986). Neurons in the POAH play a double role as thermoreceptors and as integrators of thermal information received from other parts of the body (Ishiwata T *et al.*, 2001; Bratincsák A and Palkovits M, 2004). The circadian pacemaker (in the suprachiasmatic nucleus) has about 10% of warm-sensitive cells, and 2% cold-sensitive cells; it is temperature compensated, meaning that its period is not

significantly affected by variations in local temperature but these variations can phase shift the pacemaker (Derambure PS and Boulant JA, 1994). By generating the body temperature rhythm the circadian pacemaker may subject itself to these phase-shifting stimuli (Ruby NF *et al.*, 1999).

Food availability acts as a zeitgeber both for the master circadian pacemaker and for the food-entrainable oscillator but it is not known how sensory mechanisms provide the necessary information to the pacemakers. Hunger and satiety signals are available from the blood concentration of nutrients, taste and smell of the food being ingested, gastric distension, and blood levels of many hormones secreted by stomach (ghrelin), intestines (cholecystokinin), pancreas (insulin), fat cells (leptin). Each of these hormones has a specific circadian rhythm which may be conditioned by meal time, amount and rate of nutrient intake or caloric intake, composition of body mass or a physiological states of increased energy expenditure (Weller A *et al.*, 1990; Phillips RJ *et al.*, 1996; Stephan FK, 1997; Balligand JL *et al.*, 1998; Janneke GL *et al.*, 1998; Brüning JC *et al.*, 2000; Ritter S *et al.*, 2000; Tshöp M *et al.*, 2000; Sánchez-Vásquez F *et al.*, 2001; St-Pierre DH *et al.*, 2003) and they act both on the lateral and the ventro-medial hypothalamic areas and on the paraventricular and the arcuate nuclei to regulate food-intake, energy expenditure (Strubble JH, 1994; Woods SC *et al.*, 1998; Funahashi H *et al.*, 2003; Kreier F *et al.*, 2003), and many haematochemical and haematological parameters involved in the energetic metabolism (Piccione G *et al.*, 2002).

Pacemakers

The master circadian pacemaker in mammals is located in the *suprachiasmatic nucleus* (SCN) in the rostroventral hypothalamus. Lesion studies identified it in 1972 (Stephan FK and Zucker I, 1972; Moore RY and Eichler VB, 1972; Honma S *et al.*, 1988; Warren WS *et al.*, 1994;; Scheer FAJL *et al.*, 2001; Othsuka-Isoya M *et al.*, 2001), and *in vitro* and *in vivo* functional studies (Shibata S and Moore RY, 1987; Derambure PS and Boulant JA, 1994; Shibata S and Moore RY, 1994; Meijer JH *et al.*, 1997; Nakamura W *et al.*, 2001; Nunez AA *et al.*, 1999; Sumová A *et al.*, 2000; Schwarz MD *et al.*, 2004), as well as transplant studies have corroborated the finding since than (Romero MT *et al.*, 1993; LeSauter J and Silver R, 1994; Viswanathan N and Devis FC, 1995; Grosse J and Davis FC, 1998; Boer GJ *et al.*, 1999; Earnest DJ *et al.*, 1999; Zlomanczuk P *et al.*, 2002; Tousson E and Meissl H, 2004).

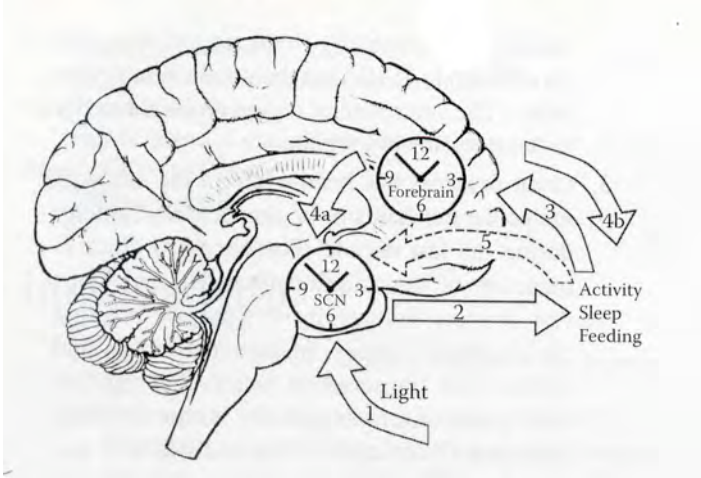
The SCN is composed of several sets of small and densely packed neurones in which different peptides are expressed. It has two main subdivision: the ventrolateral (or core)

region and the dorsomedial (or shell) region. Neurons in the ventrolateral region generally are not intrinsically rhythmic and synthesise as main neurotrasmitter vasoactive intestinal peptide (VIP), whereas neurons in the dorsomedial region express most of all arginine vasopressin (AVP); in addition SCN cells are coupled by intercellular communication via GABA-ergic signalling (Mendoza J, 2006). The SCN mechanism is cell-autonomous: this has been demonstrated by recording circadian rhythms of electrical firing from individual SCN cells, Ca^{2+} concentrations, metabolic activity and gene expression (Hastings MH *et al.*, 2004). The generation and maintenance of circadian clock function depend on clock genes and their protein products in autoregulatory transcriptional feedback loops, consisting of both positive and negative elements (Hastings MH, 2000). Positive elements are the protein products of *Clock* and *Bmal1* genes, which activate the transcription of the *Period (Per 1-3)* and *Cryptochrome (Cry1-2)* genes. PER and CRY proteins form complexes and accumulate in the nucleus where they inhibit expression of their genes acting on CLOCK/BMAL1 heterodimers (Reppert SM and Weaver DR, 2002). CLOCK/BMAL1 heterodimers activate *Rev-erbα* which produces a nuclear receptor. This is another negative regulator of *Bmal1* expression and it is expressed in phase with *Per* and *Cry* during circadian day. Disappearance of REV-ERBα during circadian night releases its inhibition on *Bmal1* transcription. Another key component of the circadian oscillator molecular mechanism that integrates the mammalian clock and energy metabolism is PGC-1α, a transcriptional coactivator that stimulates the expression of clock genes, especially *Bmal1* and *Rev-erbα*, through coactivation of the ROR family of orphan nuclear receptors. Mice lacking PGC-1α show abnormal diurnal rhythms of activity, body temperature and metabolic rate because of an aberrant expression of clock genes. Analyses of PCG-1α deficient fibroblasts and mice with liver-specific knockdown of PGC-1α indicate that it is required for cell-autonomus clock function (Liu C, *et al.*, 2007). In addition, there is a circadian expression of clock-controlled genes (CCGs) responsible for imposing temporal order given by the clock to the whole body (Preitner N *et al.*, 2002). It seems that all these molecular oscillations in individual clock cells are coordinated by calcium influx via voltage-dependent calcium channels (VDCCs) to provide for the ensemble rhythmicity of the SCN (Nahm SS *et al.*, 2005). In mammals, intracellular calcium concentration submits to a specific circadian rhythm in SCN cells (Colwell CS, 2000; Ikeda M *et al.*, 2003), indeed these cells express all major subtypes of VDCC currents (Cloues RK and Sather WA, 2003) and oscillations in the calcium corrents generated by some VDCC subtypes (Pennartz CM *et al.*, 2002; Kim DY *et al.*, 2005). VDCCs have also been implicated in

SCN intercellular communication, because treatment with cadmium, a non-selective calcium channel antagonist, disrupts the synchronization of circadian rhythms in firing rate between synaptically paired SCN neurons (Shirakawa T *et al.*, 2000, Nahm SS *et al.*, 2005).

The SCN is not the only circadian pacemaker in mammals, the presence of circadian clocks in peripheral tissues and also in other brain regions, outside the SCN, has been demonstrated (Balsalobre A, 2002; Abe M *et al.*, 2002; Schbler U *et al.*, 2003). A circadian clock gene expression has been detected in the liver, heart, muscle, kidney, pancreas, adipose tissue and lung (Lanza-Jacoby S *et al.*, 1986; Piccione G *et al.*, 2003, Muhlbauer E, 2004; Zvonic S *et al.*, 2006) and in mice self-sustained oscillations in peripheral tissues have been reported by Yoo *et al.* (2004). Recent studies show that circadian rhythmicity in firing rate and clock gene expression can be found at the central nervous system level in the striatum, the septum, the medial preoptic region, the bed nucleus of the stria terminalis, the pineal and pituitary glands, the amygdale, the olfactory bulbs, in many hypothalamic nuclei and especially in the forebrain (Green CB and Menaker M, 2003; Amir S *et al.*, 2004; Granados-Fuentes D, *et al.*, 2004; Lamont EW *et al.*, 2005). Thanks to these recent discoveries it is possible to conceptualized a circadian system in which environmental light entrains the circadian clock in the SCN, the clock in the SCN controls effector organs, the feedback from effector organs (including feeding) entrains the clock in the forebrain and the clock in the forebrain modulates SCN activity under normal circumstances or directly controls the effector organs when the SCN is damaged or weakened. Dirrect feedback from the effector organs to the SCN may also occur (Green CB and Menaker M, 2003)(Fig. 1).

Figure 1. A circadian pacemaker in the forebrain.(From Refinetti R, 2006).



Afferent Pathways

Afferences are the pathways through which the receptors send information to the master pacemaker. In mammals all photic input to the circadian system comes from the eyes. The eyes provide input to four different systems: information for the visual system crosses the body's midline at the optic chiasm, it proceeds to the lateral geniculate nucleus (LGN) of the thalamus, and from there to the visual cortex in the occipital lobe of the brain. Information used for the pupillary reflex proceeds to the pretectal area (PT). Information used for other eye reflexes (for example saccadic eye movements) proceeds to the superior colliculus (SC) in the midbrain (Ganong WF, 1996). Photic information destined for the circadian system proceeds mainly to the suprachiasmatic nucleus (SCN). Ganglion cells in the eye make monosynaptic contact with SCN through the retino-hypothalamic tract (RHT) and arrive especially in the ventral region of the SCN. The afference to the SCN are bilateral with only a small bias towards the controlateral side (Muscat L *et al.*, 2003; Abizaid A *et al.*, 2004). The SCN receives photic information through an indirect pathway called the geniculo-hypothalamic tract (GHT). Within the LGN of the thalamus lies a small structure called the intergeniculate leaflet (IGL), which receives direct projection from the retina (Vrang N *et al.*, 2003) and projects directly to the SCN (this projection is the GHT) (Moore RY *et al.*, 2000). The third structure which projects primarily to the ventral SCN is the group of raphe nuclei (Hay-Schmidt A *et al.*, 2003) (Fig. 2).

In addition the SCN receives afference from the limbic system and the hypothalamus, which project mostly to the dorsal region, from the pretectum projecting first of all to the

anterior hypothalamic area and only sparsely to the SCN itself, from the paraventricular portion of the thalamus projecting to both the dorsal and ventral section of the SCN as well as to other structures dorsally. Nonphotic information from all regions of the body ascends to the brain through a variety of nerves and it seems that the anatomical structures above and especially the GHT are the main pathways by which these inputs reach the circadian pacemaker (Moga MM and Moore RY, 1997) (Fig. 3).

Figure 2. The nervous pathways from the eye to the brain. The organization of visual circuits influencing the circadian activity of the suprachiasmatic nucleus. (From Refinetti R, 2006).

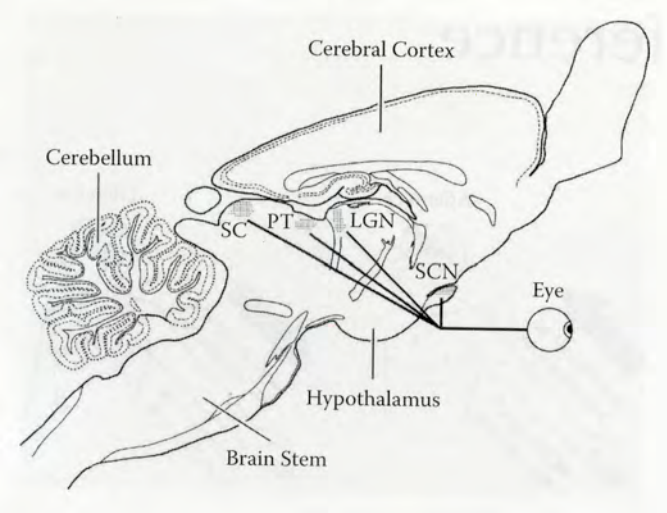
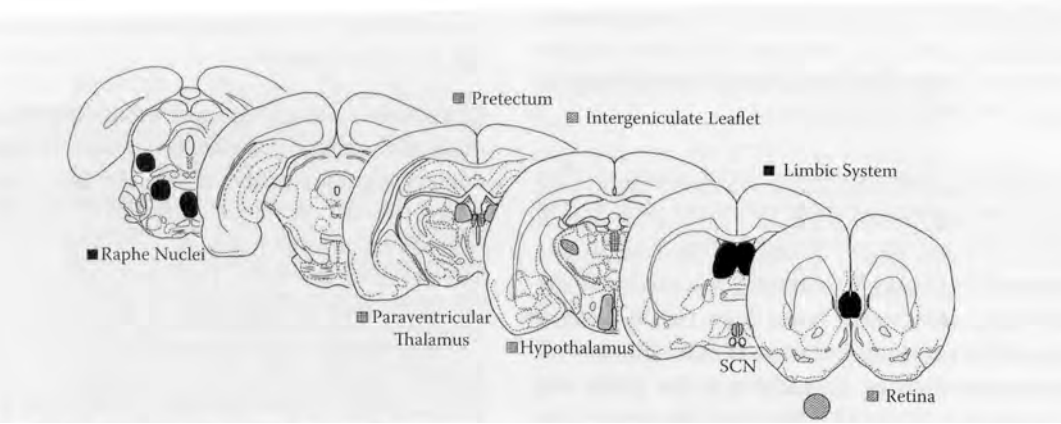


Figure 3. Brain afferents to the SCN. The diagrams identify the seven major brain structures with terminal fields in the SCN of the laboratory rat (*Rattus norvegicus*). (From Refinetti R, 2006).



Efferent Pathways

Efferences are the pathways through which the pacemaker send information to the various effector organs. Efferent organs are muscles, glands and the various sections of the brain. The circadian system communicates with all these organs through central nerves tract and peripheral nerves. In mammals peripheral nerves are either part of the somatic nervous system or of the autonomic nervous system. Fibers from the somatic motor system originate in the cerebral cortex or in the cerebellar cortex and proceed directly (pyramidal system) or indirectly (extra-pyramidal system) to the spinal cord or brain stem. There the fibers make synaptic contact with somatic motor neurons which innervate striated muscles responsible for voluntary movements of the body. Fibers from the autonomic motor system usually originate from the hypothalamus, the limbic system or the prefrontal area. They make synaptic contact with preganglionic neurons in the spinal cord which send fibers to autonomic ganglia. The postganglionic neurons innervate smooth muscles and glands.

The autonomic nervous system is subdivided in sympathetic nervous system and the parasympathetic nervous system. The first one is involved in energy-spending processes while the second one is involved in restorative processes. Most organs are innervated by both of them but some are not as the pineal gland. Both systems use acetylcholine (through nicotinic receptors) as neurotransmitter at the ganglionic synapses; at the postganglionic synapses the sympathetic system uses norepinephrine while the parasympathetic system uses acetylcholine through muscarinic receptors. The sympathetic fibers leave the spinal cord at the thoracic or lombar levels and their ganglia are arranged in a row of ganglia parallel to the spinal cord, while the parasympathetic ganglia are located close to each target organ and its fibers leave the central nervous system at the cranial or sacral levels.

The SCN projects to three main areas: the hypothalamus, the thalamus, and the septal area.

The ventrolateral SCN projects to the dorsomedial SCN and to the lateral subparaventricular zone ($_{L}$SPV) of the hypothalamus (Leak RK and Moore RY, 2001).

The dorsomedial SCN projects to the paraventricular nucleus of the thalamus (PVT), the paraventricular nucleus of the hypothalamus (PVN), the medial subparaventricular zone ($_{M}$SPV), the preoptic area (POA), the dorsomedial hypothalamic nucleus(DMH) (Leak RK and Moore RY, 2001) and the oval nucleus of the bed nucleus of the stria terminalis (Amir S *et al.*, 2004).

SCN has separate projections to the PVN for the sympathetic and parasymphatetic branches. Projections from the dorsal SCN proceed monosynaptically to the PVN; the PVN projects either to the dorsal motor nucleus of the vagus nerve (DMV), which projects

to the parasympathetic ganglia, or to the intermediolateral column of the spinal cord (IML), which projects to the sympathetic ganglia (Buijs RM *et al.*, 2003)

SCN efference seems also to projects mainly indirectly, through polysynaptic inputs coming from the DMH and the $_{V}$SPV, to two important areas of the brain involved in the control of sleep and wakefulness: the ventrolateral preoptic area (VLPO) and the nucleus of the locus coeruleus (LC) which is implicated in the control of arousal (Chen S *et al.*, 2001; Deurveilher S *et al.*, 2002) (Fig. 4). VLPO cells exhibit a daily rhythmicity which peaks during the day even in nocturnal animals like rats (Novak CM and Nunez AA, 1998). This fact suggests that VLPO is closely connected to the SCN functionally and that the VLPO may be a first-order efferent target. Besides SCN efferences mediated by DMH, reach also the lateral hypothalamic area (LHA) associated with the control of feeding and activity and PVN associated with the control of corticosteroid secretion (Chou TC *et al.*, 2003) (Fig. 5).

Figure 4. SCN efference to locus coeruleus. The diagram of a sagittal section of the rat brain illustrates the finding that the suprachiasmatic nucleus (SCN) sends signals to thelocus coeruleus (LC) through the dorsomedial hypothalamic nuclus (DMH). The locus coeruleus is involved in the control of arousal. (From Refinetti R, 2006).

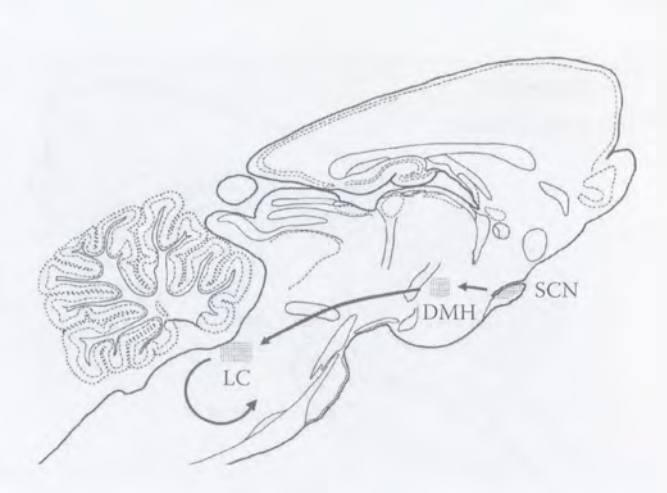
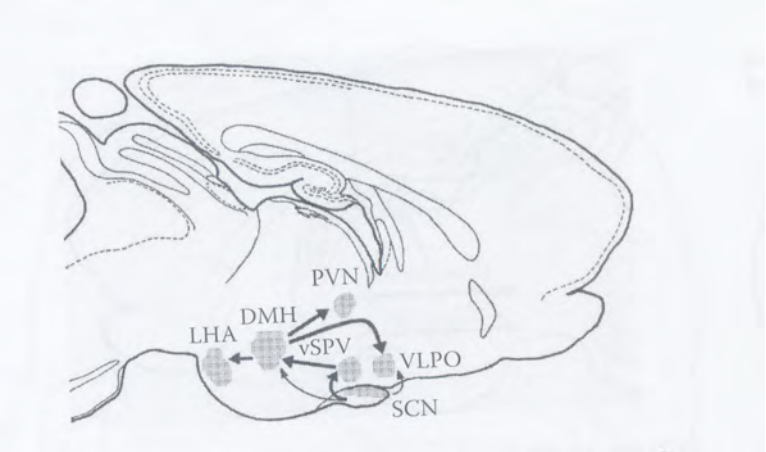


Figure 5. Functional role of dorsomedial hypothalamic nucleus. This diagram of a sagittal section of the rat brain illustrates the role of the dorsomedial hypothalamic nucleus (DMH) in the functional efferent pathways of the suprachiasmatic nucleus (SCN). The ventrolateral preoptic area (VLPO) is involved in the control of sleep, the lateral hypothalamic area (LHA) in the control of feeding and activity, and the paraventricular nucleus (PVN) in the control of corticosteroid release. $_{v}$SPV= ventral subparaventricular zone. (From Refinetti R, 2006).



Neurotransmitters

Neurotransmitters are very important component of the circadian system. The presence of neurotransmitters in the afferent and efferent projections of the SCN is equally important for the entrainment of the clock and for the control of overt rhythms: neurotransmitters are released at the inputs for entrainment, in the clock itself for integration and consolidated output ,and in efferent projections for the control of overt rhythms. Every neurotransmitter may have more than one function, it may input from various pathways and its influence may vary by itself and by way of modification of SCN function. Neurotransmitter like acetylcholine, glutamate, neuropeptide Y (NPY), serotonin, vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI), and arginine vasopressin (AVP) have been implicated in the functioning of the SCN. Glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP) as well as excitatory amino acids like L-aspartate and N-acetyl-aspartylglutamate and substance P are indicated as principal neurotransmitters of the retinohypothalamic tract (RHT) (Reghunandanan V and Reghunandanan R, 2006).

Glutamate and PACAP are the principal neurotransmitters involved in conveying photic information to the SCN by RHT. Light stimulation of the retina results in direct secretion of glutamate from the RHT into the ventral VIP-containig part of the SCN (Mikkelsen JD *et al.*, 1992; Ding JM *et al.*, 1994; Vries MJD *et al.*, 1994). The action of glutamate on the SCN may be potentiated by PACAP, which is co-localized in a subpopulation of

glutamate-containing retinal ganglion cells (Harrington ME *et al.*, 1999; Minami Y *et al.* 2002). Another neuroactive substance implicated in the light-input pathway is nitric oxide (NO) (Chen D *et al.*, 1997; Caillol M *et al.*, 2000). Nitric oxide production in the SCN has been linked to N-methyl D-aspartate (NMDA)-induced cyclic guanosine monophospahate (cGMP) production which cause phase-shift *in vitro* (Prosse RA *et al.*, 1989) and it seems to be required for phase changes of electrical activity (Watanable A *et al.*, 1994).

The RHT seems to act on the circadian system using also histamine, neurotensin (NT), neuromedin S (NMS), gastrin releasing peptide (GRP) and acetylcholine (Ach); the last one has only a minor role in the light-input pathway modulation (Reghunandanan V and Reghunandanan R, 2006).

Histamine can induce phase shifts in circadian rhythm in a manner similar to that of the light pulses, it has a direct excitatory effects mediated via H_1 receptors and inhibitory effects via H_2 receptors on the SCN (Liou SY *et al.*, 1983; Stehle J, 1991). There is a clear circadian rhythm in the histaminergic activity, with high levels during the active period and low levels during the sleep period. Maintenance of circadian rhythmicity of sleep-wakefulness cycles, food intake, motility and adrenocortical hormone release seems to be correlated with histaminergic activity (Reghunandanan V and Reghunandanan R, 2006). Actually histamine H_1 receptor signalling seems to be central to the regulation of the circadian rhythm of feeding, leptin sensitivity and obesity. In addition, H_1 receptors in the PVN may be important in regulating food intake and body mass: early disruption of H_1-receptor-mediated functions in H1KO mice may lead to hyperfagia and decreased expression of UCP-1 mRNA, a protein expresses exclusively in brown adipose tissue, which plays a central role in energy expenditure and nonshivering thermogenesis in rodents, as well as in regulating body mass (Takayuki M *et al.*, 2004).

Cells body of the ventral region of rat SCN contain the neurotensin (NT) and two NT receptor types (NTS1 and NTS2) (Francois-Bellan AM *et al.*, 1992; Boudin H *et al.*, 1996; Alexander MJ and Leeman SE, 1998). NT-binding sites receive photic and non-photic information, therefore they are involved in the synchronization of clock to these environmental stimuli (Coogan AN *et al.*, 2001) and when co-applied with NPY, NT was found to damp the profound inhibitory effect of NPY (Cutler DJ *et al.*, 1998;Gribkoff VK *et al.*, 1998).

Neuromedin S (NMS) is a potent brain-gut 36 amino acid neuropeptide located in the SCN core part. It was found to act as neurotransmitter of the circadian oscillator system with implication in the regulation of circadian rhythms thanks to a paracrine and/or autocrine

actions through its specific receptors. It has a diurnal peak under light-dark cycle (Nakahara K *et al.*, 2004; Mori K *et al.*, 2005).

Gastrin releasing peptide (GRP) and its receptor BB_2 are found to be synthesized by rodent SCN neurons (Ledenheim EE *et al.*, 1992). It is involved in photic entrainment: McArthur and co-workers (2000) used the resetting actions of GRP application on electrical activity rhythms during subjective day, early subjective night and late subjective night *in vitro* in rats and hamsters. They showed phase delay on SCN neuron firing during early subjective night, and phase advance during late subjective night with no response on application during subjective day. Phase shifts were blocked by a BB_2 receptor antagonist.

The geniculohypothalamic tract (GHT) is a second afferent photic projection from the intergeniculate leaflet (IGL) to the SCN. The IGL receives input directly from the retina via a separate branch of the RHT. The projection from IGL via GHT terminates in the areas of the SCN that overlap the direct RHT-SCN input. GHT provides a secondary, indirect photic input as well as an alternate input which has an important role in entrainment mediated by non-photic stimuli such as motor activity. GHT neurotransmitters are Neuropeptide Y (NPY), Serotonin (5HT) and GABA.

Neuropeptide Y (NPY) has been found to act presynaptically to inhibit GABA-mediated synaptic transmission through inhibition of calcium currents (Chen G, 1996) and to produce a phase shifts on pacemaker neurons of the SCN in hamster (Biello SM *et al.*, 1997).

Serotonin (5HT) is the neurotransmitter of a dense and robust projection from the midbrain raphe nuclei to the retinorecipient region of the SCN. Its major function is the modulation of the pacemaker responses to light, maybe modulating light-induced glutaminergic input. Raphe nuclei receive retinal afferents and hence the raphe-retina projection may be another indirect photic input to the biological clock (Moore RY *et al.*, 1978; Bons N *et al.*, 1983). Serotonin is also found to regulate SCN neurons by both pre- and post synaptic inhibitory mechanisms (Jiang ZG *et al.*, 2000). Considering these findings, there exists a possibility of the involvement of 5HT in tonic inhibition of the light input pathway to the SCN and so these serotonergic projection from raphe to the SCN may be the anatomical substrate for affective disorders to alter human circadian rhythms (Reghunandanan V and Reghunandanan R, 2006).

Gamma amino butyric acid (GABA) is an important neurotransmitter of the SCN for regulating SCN function. Most SCN neurons express GABA and its receptors $GABA_A$ and $GABA_B$. It produces neuronal inhibition through membrane hyperpolarization and

increased membrane conductance: it has an excitatory effect during day and an inhibitory effect at night. This has been attributed to changes in $[Cl^-]_I$ during the circadian cycle (Strecker GJ *et al.*, 1997; Wagner S *et al.*, 1997; Wagner S *et al.*, 2001). GABA is responsible of the synchronization of spiking in the SCN neurons: differential day-night modulation of GABAergic neurotransmission seen in the SCN may provide a time-dependent gating mechanism to counteract propagation of excitatory signals throughout the biological clock during the day and to promote it at night (de Jeu M and Pennartz C, 2002). Its activity is essential for circadian synchrony of the SCN neurons in the normal working of the clock which need to have a strong intra-SCN communication to produce integrated output (Otha H *et al.*, 2005).

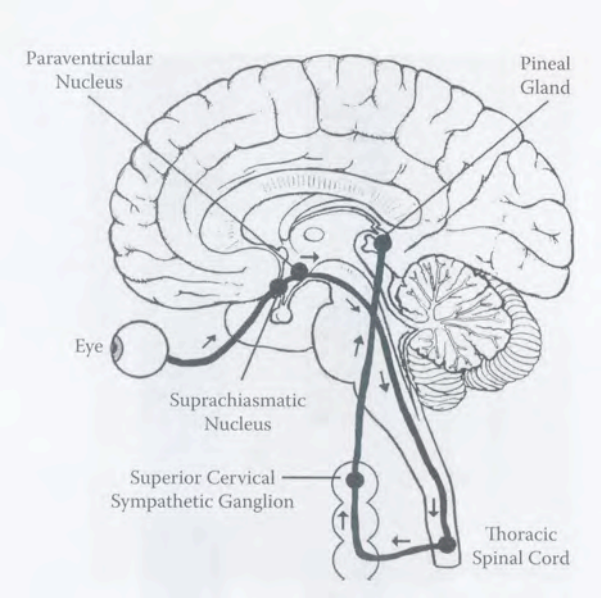
Another important synchronizer of SCN neurons is vasoactive intestinal polypeptide (VIP). VIP is the main neurotransmitter for cells of the ventrolateral region and in the dorsal projection of SCN. (Hofman MA, 2003). VIP is released from synaptic terminals at a higher rate during the night than during the day in rodents and humans maintained under a light-dark cycle, and the release remains rhythmic in animals maintained in costant darkness (Dardente H *et al.*, 2004), maybe because of delayed rhythmic signals received from the dorsomedial region. It has an inhibitory function (Reed HE *et al.*, 2002; Itri J and Colwell CS, 2003): mice lacking the gene that encodes the major VIP receptors (*vipr2*) exhibit weak circadian rhythmicity of locomotor activity in costant darkness (Hughes AT *et al.*, 2004).

Nearly one third of the SCN neurons, mostly located in the dorsomedial part of the SCN and extensively interconnected synthesize vasopressin (AVP) as main neurotransmitter (Van den Pol AN and Gorcs T, 1986; Castel M *et al.*, 1990). AVP is produced and secreted by the SCN in a circadian pattern (Ingram CD *et al.*, 1996); it has an excitatory role by activating V1 receptors to increase the amplitude of firing rates in the SCN during subjective day and enhance SCN output (Ingram CD *et al.*, 1998). Decrease in the AVP neurons and AVP content in the SCN causes a decrease amplitude in activity rhythms, increased rhythm of fragmentation, and a disruption of the normal sleep/wake cycle (Hofman MA and Swaab DF, 1994; Hofman MA and Swaab DF, 1995; Lucassen PJ *et al*., 1995). Correlation between SCN-AVP expression and circadian organization of locomotor behaviour has been shown across species including rats (Isobe Y and Nishino H, 1998) and hamsters (Van der Zee EA *et al.*, 2002).

Besides neurotransmitters also hormones are involved in intra-SCN communication, especially melatonin, the hormone from the pineal gland. The most important target of

melatonin in humans appears to be the SCN, because the SCN contains the highest density for melatonin receptors (Reppert SM *et al.*, 1988). Melatonin has a double effect in the SCN: an immediate effect suppressing neuronal SCN activity towards night time levels (Van den Top M *et al.*, 2001) and a long term effect phase shifting and amplifying circadian rhythmicity of the SCN (Dollins AB *et al.*, 1994). This hormone is also related to seasonal rhythms in mammals: The retinohypothalamic-pineal (RHP) axis is comparable in humans and animals. In both of them melatonin is secreted exclusively at night. The RHP is capable of detecting changes in night length to make proper adjustments for the duration of nocturnal melatonin secretion so that animals can use this melatonin message to trigger seasonal changes in behaviour (Wehr TA, 1997) (Fig. 6).

Figure 6. The retinal-pineal pathway. This diagram of the brain illustrates the long circadian-controlled pathway from the eyes to the pineal gland. (From Refinetti R, 2006).



The efferent projections of the SCN are primarily seen to the nearby hypothalamic and thalamic nuclei, particulary to the medial preoptic nucleus, the medial part of the paraventricular nucleus of the hypothalamus, the anterior part of the paraventricular nucleus of thalamus, the medial part of the dorsomedial nucleus of hypothalamus and the subparaventricular zone (Kalsbeek A *et al.*, 1993; Saper CB *et al.*,2005). Projections to the ventrolateral preoptic nucleus from the dorsomedial nucleus, the preoptic nucleus and the subparaventricular zone appear to serve as the anatomical basis for the control of sleep and wakefulness, as the ventrolateral preoptic nucleus is implicated in the control of sleep

states (Deurveilher S *et al.*, 2002; Deurveilher S and Semba K, 2003). Although efferent projections seem to have mainly AVP and VIP as transmitters, there is another output system in the SCN, anatomically separate, with a different daily expression profiles that use TGFα as humoral substance mediating the circadian signal to couple peripheral oscillators to the master oscillator thereby synchronizing the activity of an organ with the central clock (Oishi K *et al.*, 1998; Kramer A *et al.*, 2001; Van der Zee EA *et al.*, 2005). TGFα is found extensively n the brain and is a member of the epidermal growth factor (EGF) family produced by both neurons and astrocytes (Junier MP, 2000).

SCN output pathways in addition to influencing the hypothalamic neighbourhood can be traced to extra hypothalamic sites as far as liver, thyroid, adrenal and salivary glands in rats (Kalsbeek A, *et al.*, 2000; la Fleur SE *et al.*, 2000). Furthermore a long neural signalling pathway from the SCN regulates the pineal gland secretion of melatonin, SCN neurons also stimulate gonadotrophin releasing hormone (GnRH), synthesizing neurons of the preoptic area and thereby affect sex hormone cycles (De la Iglesia HO *et al.*, 2003).

In addition to the above neurotransmitters, the SCN also contains neurons capable of producing a number of other neurochemicals like Somatostatin (SS), Calbindin (CalB), Calretinin (CALR), Galanin (Gal), Angiotensin II (ANG II), Met-Enkephalin (mENK) and Prokineticin 2 (PK2).

SS producing neurons of the SCN are located in both the core and the shell portions and form a distinct peptidergic neuronal group (Tanaka M *et al.*, 1996) which has an inhibitory modulating role on VIP rhythmicity (Fukuhara C *et al.*, 1994).

CalB neurons are also found throughout the core and shell subdivisions of the SCN, they are densely packed and receive direct retinal synaptic input and respond to photic stimuli (Silver R *et al.*,1996; Bryant DN *et al.*, 2000). CalB cells appear to be non rhythmic, however they receive photic input from RHT and GHT, and rapid transmission of light information to CalB neurons may facilitate circadian output (Jobst EE *et al.*, 2004). Light induces clock gene expression in the SCN during the night; this gene expression is mirrored by changes in subcellular localization of CalB, which is a calcium binding protein: the cellular nucleus is devoid of CalB during the night and behavioural as well as molecular responses to nocturnal light pulses also disappear on decreasing CalB levels (Hamada T *et al.*, 2003). Thus, CalB cells in the SCN function as gates to relay photic signals when open and to block the signals when closed, which suggests a central role for CalB neurons in gating photic input. Moreover they are essential for the maintenance of circadian locomotor activity rhythm (Reghunandanan V and Reghunandanan R, 2006).

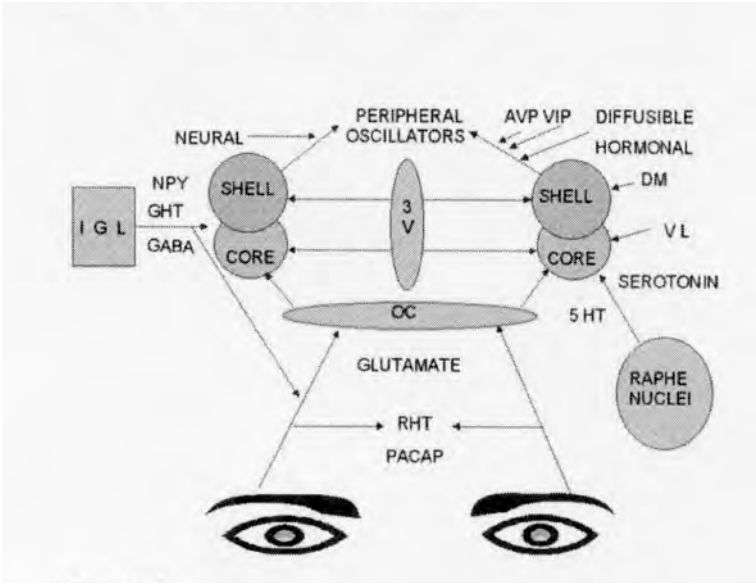
CALR is another calcium binding protein located in the core ventral part of SCN and linked to RHT activity (Ikeda M and Allen CN, 2003) as well as Gal, a neuropeptide seen in many parts of the nervous system including SCN.

SCN-derived ANG II seems to act as neuromodulator and neurotransmitter with the effects being modulated through angiotensin 1 (AT_1) receptors located on the endothelial plasma membrane of SCN parenchyma (Thomas MA *et al.*, 2004).

mENK neurons are located primarily dorsomedially in the shell of the SCN and overlap with the distribution of AVP neurons (Takatsuji K and Tohyama M, 1989). mENK is a putative neurotransmitter that act on opioids receptors (μ and δ), maybe having a modulator function in circadian pattern of food and water intake (Reghunandanan V *et al.*, 1988; Reghunandanan V *et al.*, 1992).

PK2 has been identified as an output molecule from the SCN circadian clock (Cheng MY *et al.*, 2002). It is involved in the transmission of behavioural circadian rhythm as well as in local function within the SCN to synchronize the output. PK2 was already known for its ability to stimulate intestinal smooth muscle contractility and it seems to have a major role in inhibiting locomotor activity during the day in nocturnal species (Cheng MY *et al.*, 2002). Furthermore the molecular rhythm of PK2 in the SCN is regulated by both circadian clock and light, with the clock having a predominant role and light having a modulatory role (Cheng MY *et al.*, 2005) (Fig. 7).

Figure 7. Afferent inputs and efferent pathways of the SCN. RHT: retinohypothalamic tract; GHT: geniculohypothalamic tract; OC: optic chiasm; 3V:third ventricle; IGL: intergeniculate leaflet; DM: dorsomedial SCN; VL: ventrolateral SCN; NPY: neuropeptide Y; GABA: gamma amino butyric acid; PACAP: pituitary adenylate cyclase-activating polypeptide.(From Reghunandanan V, 2006).



Circadian rhythm in plasma glucose, cholesterol and triglycerides

By possessing an internal clock mechanism, cells/organisms are able to anticipate temporal changes in the environment, optimizing biological processes so that they occur at an advantageous time in the day. This clock mechanism allows adipose and other tissues to anticipate diurnal variations in its environment, such as circulating levels of glucose, fatty acid and triglycerides, as well as various hormones, including insulin and adrenaline. In doing so, the circadian clock prepares these tissues for the anticipated stimulus, allowing an appropriately rapid response. For daily functioning, maintaining glucose homeostasis is essential. Disturbances in the circadian rhythm of glucose regulation and insulin action may have severe consequences and may even lead to disease, including metabolic syndrome, type 2 diabetes and obesity (Bartol-Munier I *et al.*, 2006). Plasma glucose concentrations fluctuate with a 24-h rhythm thanks to the regulating action of SCN. In the rat, plasma glucose concentrations increase toward the end of the light period, just before the onset of activity (la Fleur SE *et al.*, 1999). SCN plays a role also in regulating glucose tolerance due to a variation in insulin sensitivity (la Fleur SE *et al.*, 2001). The daily rhythm in glucose tolerance follows the same pattern as the daily rhythm in plasma glucose concentrations; therefore the biological clock prepares the individual for the upcoming

activity period by two separate mechanisms: increasing plasma glucose concentrations and making tissue more tolerant to glucose. There are several mechanisms via which the SCN may generate daily fluctuations in plasma glucose concentrations, glucose tolerance and insulin sensitivity. It seems that SCN uses differential signals over the light-dark cycle, ranging from the α-cells of the pancreas that synthesize glucagon, to the adrenal gland, where adrenaline and corticosterone are produced (Buijs RM *et al.*, 1999; Ueyama T *et al.*, 1999); however data obtained from both human and rat studies suggest that the rise in plasma glucose concentrations at the end of the sleep period is endogenous and caused by increased hepatic glucose production (HGP) (Bolli GB *et al.*, 1984; Boden G *et al.*, 1996; la Fleur SE, 2003). The SCN is connected to the liver through both the parasympathetic and sympathetic branches of the autonomic nervous system via preautonomic neurons located in the PVN: lesions of the SCN in rats abolish diurnal variations in whole body glucose homeostasis (Cailotto C *et al.*, 2005). Activation of preautonomic PVN neurons, either by stimulation of NMDA receptors or by relief from inhibitory GABAergic inputs, results in an increase in plasma glucose concentrations, likely attributable to an increased HGP. Therefore the daily rise in basal plasma glucose concentrations at dusk is caused by a biological clock-mediated withdrawal of the GABAergic inhibition of sympathetic preautonomic PVN neurons (Kalsbeek A *et al.*, 2004). In addition the pronounced rhythm in liver glycogen content, with its acrophase at light onset, has been shown to be attributable to daily changes in the balance between the activities of glycogen-phosphorylase and glycogen-synthase (Ishikawa K and Shimazu T, 1976; Chen C *et al.*, 1993).

In addition to glucose metabolic rhythm, energetic metabolism is influenced by plasma cholesterol and triglycerides as well. In mammals dramatic diurnal variation in adipocyte lipolysis and lipogenesis occur. When an animal sleeps, rates of lipolysis increase, resulting in increased release of non-esterified fatty acids into circulation. In contrast, when an animal is awake, rates of lipolysis decrease, with a concomitant increase in lipogenesis (Bray MS and Young ME, 2006): in rats, both total serum triglycerides and triglyceride-carrying lipoproteins show acrophases in the light period when the animals sleep, while they decrease in the dark period, when the animals are active (Mondola P *et al.*, 1995). Diurnal variation in adipose triglycerides turnover have been explained primarily in terms of reciprocal changes in neurohumoral influences promoting lipolysis (sympathetic activity) and lipogenesis (insulin) and then in terms of an intrinsic variation in adipocyte activity (Suzuki M *et al.*, 1983) which suggests that the diurnal variation in adipocyte lipid

turnover are transcriptionally mediated, potentially by the intra–adipocyte circadian clock (Bray MS and Young ME, 2006).

Cholesterol seems to have a particular daily rhythm: the rhythm of HDL, the main lipoprotein transporter of serum cholesterol in rats, increases in the dark period, in total contrast with the variations of VLDL and IDL-LDL cholesterol (Rivera-Coll A *et al.*, 1994; Mondola P *et al.*, 1995) which have the acrophase in the light period. This finding coincides with data on rat liver HMG CoA reductase, the key enzyme in the cholesterol synthesis which is minimal during the light phase and peaks at the midpoint of the dark period. The increase activity of HMG CoA reductase and the increase in overall rate of cholesterol syntesis in the liver as well as in the jejunum and in the distal ileum may be due to the ingestion of food (Edwards PA *et al.*, 1972). VLDL and IDL-LDL have acrophase during the day maybe because they are responsible of the cholesterol transport from the liver to other tissues where cholesterol is used for cellular processes during the resting phase. Actually rats accelerate fat oxidation and so fat consumption during the day while they tend toward fat accumulation by the suppression of fat oxidation in the dark phase; so HDLs need to have acrophase during the dark phase because they transport cholesterol from tissues to the liver (transforming in IDL) (Kazuhiko T *et al.*, 2002). The liver stocks cholesterol coming from peripheral tissues and from the diet and synthesizes it if necessary.

The molecular mechanism used by circadian clock to control lipid metabolism is based on many clock-genes; their expression seems to be conditioned also by the fat content of the diet (Kudo T *et al.*, 2007) but other studies are needed. Several papers have reported that many lipid metabolism-related clock-controlled genes exhibit circadian oscillations (Panda S *et al.*, 2002; Ueda HR *et al.*, 2002; Oishi K *et al.*, 2003), and nuclear receptors are involved in circadian rhythm and metabolism (Yang X *et al.*, 2006).

Data Analysis of circadian rhythmicity

Data analysis in circadian physiology consists of identifying circadian rhythmicity in data sets that naturally contain rhythmic and nonrhythmic components. The data points in a data set refer to successive observations made over time; the set is called a *time series*. The analysis of time series can be effectuated using two different types of statistical methods: methods in the *time domain* look for regularities in a time series itself, while methods in the *frequency domain* treat the time series as a composite of underlying oscillatory processes.

The rhythmic pattern can be fully described by four parameters: period, mean level, amplitude, and phase.

Period is the distance between two consecutive peaks and it is measured in seconds; this parameter can be expressed also as its reciprocal: *frequency* which is measured in Hertz (Hz). The period of a rhythm is mostly commonly determined by various methods for inspecting actograms, by Fourier analysis, by the Enright (chi square) periodogram, or by the Lomb-Scargle periodogram (Refinetti R, 1993).

Mean level is the value around which the wave undulates: the mean of a time series measures the "central tendency" of data that is the point of balance of the distribution of values. Instead of calculating the mean level, circadian physiologists use a procedure called *cosinor rhythmometry* (Nelson W *et al.*, 1979). Cosinor rhythmometry is a model based on the fact that circadian rhythms can be thought of as smooth rhythms with added noise, a cosine wave could be fitted to the data to estimate the pattern of the smooth rhythm. When the single cosinor is employed, the mean level of the fitted curve is used as the mean level of the rhythm called *mesor* (midline-estimating statistic of rhythm). This method is based on a mathematical model that allows the computation not only of mean level but also amplitude and phase at once. The best fitting cosine wave can be described by the function:

$$f(t) = M + A \cos(\omega t + \Phi)$$

Where $f(t)$ denotes the value of the function at time t, M is the mesor, A is the amplitude, ω is the angular frequency (that is 360°/24 if the cycle is 24hours long and t is measured in hours) and Φ is the acrophase in degrees. A system of three equations with three unknowns can be derived and solved in algebraic form (Ruf T, 1996).

Amplitude is half the range of oscillation of the wave, it equals the distance between the mean value and the peak (or between the mean value and the trough, as it is assumed that peak and trough are equidistant from the main level). To calculate the amplitude, it is possible to use the actual data as well as a cosine function fitted to the data.

Phase is a relative term used to indicate the displacement between a chosen point (the peak) and a reference point (the rock). The phase of a circadian rhythm could be calculated using the cosinor method. The peak of the cosine wave provides a suitable phase marker: the *acrophase*.

Two additional parameters are waveform and robustness. *Waveform* refers to the form of the wave: generally circadian rhythms are not stationary and there are no standard procedure to quantify their waveform. However the closer a rhythm is to stationary, the

greater is its *robustness* which can be estimated by periodogram analysis of filtered data sets.

Besides circadian physiologists need also to use some tests of statistical significance to distinct real rhythms from random oscillations. Generally one can be more confident about the results of the tests if one has a long time series covering several cycles of the rhythm. However significance test can be conducted on educed rhythms as well. (Refinetti R, 2006).

THE RAT AS ANIMAL MODEL IN "*IN VIVO*" RESEARCH

Kingdom	Animalia
Phylum	Chordata
Class	Mammalia
Order	Rodentia
Family	Muridae
Genus	Rattus
Species	Norvegicus

The Norway rat (*Rattus Norvegicus*) is believed to originated in Asia and spread throughout the world with modern civilization. The rat, considered to be the first animal to be domesticated for strictly scientific purposes (Richter CP, 1959), was first used experimentally in France in the study of adrenal gland function (Philipeaux JM, 1856). Early research with rat in the areas of nutrition, endocrinology, physiology, and behaviour led to discoveries such as the nutritional quality of various aminoacids in mammals, the existence of vitamins, the characterization of the hormones of the anterior pituitary, and the existence of circadian rhythms. The rat has become a species of choice for almost every area of biological and medical research because of its size, relatively docile nature, life span, gestation period, and metabolic similarities to humans. The extensive use of the rat in research has led to the development of a large historical database of its nutrition, diseases, and general biology.

General information about husbandry and physiological parameters are given in the following table, (Tab. 1). Normal values will vary based on the strain of animals, supplier, feed, and housing conditions.

Table 1. Selected Normative Data (from Gad SC, 2006).

Husbandry	
- Room Temperature(°C)	18-26
- Relative humidity (%)	30-70
- Ventilation (air change/hr)	10
- Light/dark cycle (hours)	12-14/12-10
- Minimum cage floor size:	
Housed individually (cm^2)	350
Breeding with pup (cm^2)	800
Group housed (cm^2 adult)	250
General	
- Life span (years)	2.5-3.0
- Surface area (cm^2)	0.03-0.06
- Chromosome number (diploid)	42
- Water Consumption (mL/100g/day)	10-12
- Food Consumption (g/day)	20-40
- Average body temperature (°C)	37.5
Reproduction	
- Puberty (males and females)	50±10 days
- Breeding season	All year
- Type of estrous cycle	Polyestrous
- Length of estrous cycle	4-5 days
- Duration of estrous	10-20 hr
- Mechanism of ovulation	Spontaneous
- Time of ovulation	7-10 hr after onset of oestrus
- Time of implantation	Late day 4 or 5[a]
- Length of gestation	21-23 days
- Litter size	8-16 pups
- Birth weight	5-6 g
- Eyes open	10-12 days
- Weaning age/weight	21 days/40-50 g
Cardiovasclar	
- Arterial blood pressure	
Systolic (mmHg)	116-145
Diastolic (mmHg)	76-97
- Heart rate (beats/min)	296-388
- Cardiac output (mL/min)	10-80
- Blood volume (mL/kg)	64
Pulmonary	
- Respiration (breaths/min)	100-140
- Tidal volume (mL)	1.1-2.5

Pulmonary - Compliance (mL/cm H_2O) - Resistance (cm H_2O/mL*s) - Pattern	0.3-0.9 0.1-0.55 Obligate nasal
Renal - Urine Volume - Na^+ excretion - K^+ excretion - Urine osmolarity - Urine pH - Urine specific gravity - Urine creatinine - Glomerular filtration rate	 15-30 mL/24hr 200 mmol/L/24hr 150 mmol/L/24hr 2,000 mOsm/kg H_2O 7.3-8.5 1.01-1.07 6 mol/L/24hr 1.0 mL/min/100g body weight

[a]: The estrous cycle length can vary from 4 to 5 days between strains. Time of implantation can vary based on the length of the estrous cycle and is dependent on Day 0 or the first day sperm is found in the vagina.

Table 2 and Table 3 summarize literature values for clinical chemistries and haematological values of common laboratory rats.

Table 2. Clinical Chemistry Values. (from Gad SC, 2006).

	Male		Female		
	M	**SD**	**M**	**SD**	**Range**
Bilirubin (mg/dL)	0.35	0.02	0.24	0.07	0.00-0.55
Cholesterol (mg/dL)	28.3	10.2	24.7	9.62	10-54
Creatinine (mg/dL)	0.46	0.13	0.49	0.12	0.20-0.80
Glucose (mg/dL)	78.0	14	71	16	50-135
Urea nitrogen (mg/dL)	15.5	4.44	13.8	4.15	5-29
Uric acid (mg/dL)	1.99	0.25	1.79	0.24	1.20-7.5
Sodium (mEq/L)	147	2.65	146	2.50	143-156
Potassium (mEq/L)	5.82	0.11	6.70	0.12	5.40-7
Chloride (mEq/L)	102	0.85	101	0.90	100-110
Bicarbonate (mEq/L)	24	3.80	20.8	3.60	12.6-32
Phosphorous (mg/dL)	7.56	1.51	8.26	1.14	3.11-11
Calcium (mg/dL)	12.2	0.75	10.6	0.89	7.2-13.9
Magnesium (mg/dL)	3.12	0.41	2.60	0.21	2.24-3.81

	Male		Female		
	M	SD	M	SD	Range
Amylase (Somogyi units/dL)	245	32	196	34	128-313
Alkaline phosphatase (IU/I)	81.4	14.8	93.9	17.3	56.8-128
Acid phosphatase GIULIA	39	4.30	37.5	3.70	28.9-47.6
Alanine transaminase (SGPT) (IU/I)	25.2	2.05	22.5	2.50	1.5-30.2
Aspartate transaminase (SGOT) (IU/I)	62.5	8.40	64.0	6.50	45.7-80.8
Creatin phosphokinase (CPK) (IU/I)	5.60	1.30	6.80	2.40	0.84-11.6
Lactic dehydrogenase (LDH) (IU/I)	92.5	13.9	90	14.5	61.0-121
Serum total protein (g/dL)	7.61	0.50	7.52	0.32	4.70-8.15
Albumin (g/dL)	3.73	0.53	3.62	0.52	2.70-5.10
%	49	7.10	48.1	7.40	33.3-63.8
α_1-Globulin (g/dL)	1.03	0.22	0.89	0.25	0.39-1.60
%	13.5	2.20	11.9	3.80	4.30-21.1
α_2-Globulin (g/dL)	0.71	0.14	1.40	0.32	0.20-2.10
%	9.3	1.80	8.60	2.70	3.20-14.7
β-Globulin (g/dL)	1.07	0.35	1.31	0.26	0.35-2.00
%	14.1	4.70	17.4	3.60	5.70-26.8
γ-Globulin (g/dL)	1.05	0.21	1.18	0.21	0.62-1.60
%	13.8	2.70	14	2.80	10-19.8
Albumin/globulin	0.96	0.24	0.93	0.25	0.72-1.21

Table 3. Hematology values of common rat strains. (from Gad SC, 2006).

Test	Unit	Long-Evans (Blu:LE)	Wistar/Lewis Albino	Osborne-Mendel	Fischer Inbred Strain 344/Cr
Erythrocytes (RBC)	$x10^6/mm^3$	5.98-8.30	7.20-9.60	6.26-8.96	6.68-9.15
Hemoglobin	g/dL	13.1-16.7	12-17.5	14.30-17.7	13.4-17.2
MCV	v^3	52-69	57-65	52-66	54-67.5
MCH	vvg	18.5-23.5	14.6-21.3	18.8-23.3	17-21.8
MCHC	%	32-38.5	26-38	32-42	26-35.5
Hematocrit (PCV)	mL%	39-48	42.5-49.4	39.4-46.2	46-52.5
Leukocyte (WBC)	$x10^3/mm^3$	3.30-7.90	5-8.96	6.23-12.6	5.35-11.2
Neutrophilis	%	5.50-35.5	9-34	4.5-23.5	11.5-41.6
Basophilis	%	0	0-1.50	0	0
Lymphocytes	%	60-93.5	65-84.5	72-94	43-79.5
Monocytes	%	0-5.50	0-5	0.50-3.50	0-2
Eosinophilis	%	0-1.50	0-2.50	0-1	0-4
Platelets	$x10^3/mm^3$	140-160	160-470	145-450	150-450

THE EUROPEAN FOUNDATION OF ONCOLOGY AND ENVIRONMENTAL SCIENCES "B. RAMAZZINI", THE CANCER RESEARCH CENTER "CESARE MALTONI" AND ITS SPRAGUE-DAWLEY RATS COLONY: A SENSITIVE ANIMAL MODEL FOR CARCINOGENIC STUDIES

The European Foundation of Oncology and Environmental Sciences "B. Ramazzini" was born in the seventies in Bologna to develop a research program aimed at the prevention of cancer: the most serious disease in the industrial world because of number of cases and high mortality. Cancer is responsible of the 22,8% of all deaths in the USA (American Cancer Society, 2005) and in Italy, referring to Bologna and province, in 2002, cancer deaths were 35,60% of deaths among men and 25,88% among women (Soffritti M *et al.*, 2006).

An individual's likelihood of developing cancer is expressed by three factors: heredity predisposition, age and environmental exposure. Nowadays a man out of two and a woman out of three are destined to fall ill with cancer during their life (American Cancer Society, 2005). Many efforts are addressed to find effective therapies against cancer but there are not decisive results yet, rather its incidence has been increasing in the last 50-60 years because of the lifetime lengthening (10 years for men and 15 years for women) and the great diffusion of industrial products causing chemical and physical environmental pollution which affected both working and domestic environment (Soffritti M *et al.*, 2005). Therefore a possible concrete pathway, men can go along to modify incidence and mortality of this disease, is the identification of possible carcinogenic agents and the reduction or the exclusion of the exposure to reduce the risk (Schmähl D, 1988). With this target, in the last 35 years, the Ramazzini Foundation has developed industrial carcinogenic experimental studies; chemoprevention studies, especially about natural and hormonal molecules that may be preventive cure in the mammary cancer; molecular biology studies to understand the mechanisms of onset and development of cancer and epidemiologic studies (Mortality Named Register of Bologna and province, studies about cancer incidence and mortality between environmental and occupational exposed people) (Maltoni C*et al.*, 1999).

Ramazzini Foundation's program takes place in the Cancer Research Center "Cesare Maltoni" (CRCCM) located in Bentivoglio castle near Bologna. The programm started in

1966 thanks to Cesare Maltoni, director of the Oncology Institute "F. Addari". He maintained the idea that cancer must be fought studying cancerogenic agents and reducing exposure risks. He demonstrated that carcinogenic experiments on rodents give a good prediction for human risk if they were conducted with standard procedures, acting in advance of his time by the application of Good Laboratory Practices which were codified under a specific regulations in the USA first, and then in other countries, only in the eighties (Soffritti *et al.*, 2005).

CRCCM researchers have studied 205 compounds using 160.000 rodents. Many of them were found cancerogenic in agreement with the National Toxicology Programm (NTP), planned by the National Institute of Environmental Health and Sciences (NIEHS) in the USA (Fung VA *et al.*, 1995). The most important results obtained by CRCCM researchers were the discovering of carcinogenic effects of Vinyl Chloride, a plastic monomer causing hepatic angiosarcoma (Maltoni C *et al.*, 1975; Maltoni C, 1977); the carcinogenic effects of Benzene (Maltoni C and Scarnato C, 1977; Maltoni C *et al.*, 1983; Maltoni C *et al.*, 1985; Maltoni C *et al.*, 1989); the toxicity of formaldehyde (Soffritti M*et al.*,1989) and methyl-butyl ether (MTBE) which cause lymphoma and leukaemia (Belpoggi F *et al.*, 1995; Belpoggi F *et al.*, 1997; Belpoggi F*et al.*, 1997); the carcinogenic effects of aspartame ,a large used artificial sweetener, on the hematopoietic system (Soffritti M *et al.*, 2006). These findings have been the scientific bases for primary preventive measures and specific lows (Maltoni C *et al.*, 1999). At the present time, there are three important projects running in the CRCCM: tests on artificial sweeteners; test on powder coming from the disaster of the World Trade Center of New York; and the experiment on the effects of radiofrequency electromagnetic fields (1,8GHz).

The experimental model of the Cancer Research Center "Cesare Maltoni"

The use of carcinogenesis experiments for research and safety assessment requires properly designed and well-conducted test. Therefore the carcinogenesis studies of CRCCM are managed following a standard protocol (Soffritti M *et al.*, 2002):

- Aim: to determine the toxicity and the carcinogenic effects of physical and chemical agents.
- Animal model: Sprague-Dawley rats (*Rattus norvegicus*) coming from the CRCCM colony (only in some cases Wistar rats, Swiss mice and Golden hamster).

- Experimental groups: two-three or more groups, including a control group (untreated group), depending on the diffusion of the agent and its impact on public health. Each group is composed by 50-60 animals per sex, up to 500-600 animals per sex when the agent studied is a weak carcinogen.
- Exposure routes: they mime the human exposure routes. The most used are ingestion, inoculation, inhalation and environmental exposure (for physical agents like radiation).
- Concentration/Dose/Intensity: three dose levels are studied for each agents: the maximum tolerable level (MTL), a level comparable to human exposure level and a mean one. If MTL is not known, it is defined by range-finding experiment.
- Treatment Start: it can start during foetal life (12° days of gestation), perinatal age, or at 6-8 weeks of age, only in particular cases other ages are chosen.
- Treatment Period: 104 weeks or life-span
- Experiment Period: until spontaneous animals' death
- Experiment Management: CRCCM Sprague-Dawley rats are weaned and divided by sex at 4-5 weeks of age. They are individually identified by ear punch following Jackson Laboratory standard. They are housed five by five in makrolon cages (cm 41x26x15) with stainless still covers and wood shaving bedding. Environment temperature is 21±3°C, with a humidity between 40% and 60% and 12 hours light-dark cycle. Animals are feeding *ad libitum* with a pellet standard feed "Corticella Type" produced by "Laboratorio Dottori Piccioni"; water is taken from the local aqueduct and it is *ad libitum* as well. Both feed and water are controlled every 6 months to determine the presence of chemical or microbiological contaminants. Cage water and food consumption are taken for the first 50 males and females of each experimental group weekly during the first 13 weeks and then every two weeks until 110 weeks of age. Each animal is weighed and carefully examined once a week during the first 13 weeks, every two weeks until 110 weeks of age, and then every 8 weeks until the end of the experiment. Health and behaviour controls are made three time a

day; dying or ill animals are isolated and eventually suppressed by the veterinary.

- Pathology: every animal undergoes necropsy. All organs are fixed in 70% alcohol while bones are fixed in formalin 10% and then decalcifying with formaldehyde 10% and formic acid 20%. All organs and pathologic lesions are trimmed, processed, included in paraffin and getting specimen coloured by Hematoxylin-Eosin. Then they are drown to histologic examination following an individual file.

An important aspect of CRCCM protocols is the duration of the experiments until all animals' spontaneous death rather then stopping them at 110-112 weeks of age like many other laboratories do. Ending *in vivo* carcinogenesis studies at 110-112 weeks of age means to loose data from old animals, which are the subjects developing the major number of tumours in rats as well as in humans. Indeed the highest rate of malignant tumours bearers has been observed after 112 weeks of age in historical controls: malignant tumours bearers dead after 112 weeks of age are 45% in males and more than 46% in females (Falcioni L, 2006). The sensibility of CRCCM experiments have been showed in many cases like in Xylenes, Vynil Chloride, Benzene and Mancozeb (a widespread fungicide) studies (Soffritti M *et al.*, 2002).

The animal model of the Cancer Research Center "Cesare Maltoni": Sprague-Dawley rat

In chemical carcinogenic and drug-safety testing, a carcinogen is defined as an agent that when administered by an appropriate route cause an increased incidence of tumours in experimental animals as compared to unexposed control animals. Although a carcinogen may cause the appearance of tumours in organs where tumours do not usually occur in a given strain, the usual response is to increase the types of tumours seen spontaneously and to shorten the period of latency (Hardisty JF, 1985). An animal model for "life span" carcinogenesis studies needs to be repeatable and sensitive enough to develop spontaneous tumours with a pattern and an age distribution human-equivalent, moreover there must be background data in tumour incidences of historical control animals to know spontaneous tumours variations in the strain. The animal model for CRCCM studies is the Sprague-Dawley rat, especially the strain reared in the last 30 years in the CRCCM. This colony has steady biological characteristics and so it can give many information about expected neoplastic and non-neoplastic pathologies. The spontaneous incidence of malignant and

benignant tumours is constantly evaluated on historical controls: animals constituting control groups (untreated groups) in the experiments run in the examined time. Historical controls are not homogeneous animals groups for generation, age or experimental conditions; therefore they can not be used instead of the control group in a specific experiment but they are useful to confirm data when the incidence of tumours observed in the control group is in the range of historical controls.

Considering 4539 untreated Sprague-Dawley rats (2265 males and 2274 females) of the CRCCM colony, 60,75% of males and 76,74% of females are benignant tumour bearers, while 41,24% of males and 40,65% of females are malignant tumours bearers. The most common benignant tumours in males are benign pheochromocytoma (33,86%), pituitary adenoma (24,33%), pancreatic islet cell adenoma (8,43%) and benign testicular interstitial cell tumour (7,28%); in females they are mammary fibroma and mammary fibroadenoma (44,5%), pituitary adenoma (41,91%), benign pheochromocytoma (24,98%) and uterine endometrial stromal polyps (15,57%). As regards malignant tumours, lymphoma and leukaemia are frequent in males (20,44%) as well as in females (13,10%) while mammary malignant tumours have a high incidence (10,07%) only in females. The 92,1% of them are mammary carcinomas; the other are most of all liposarcomas (4,8%) and fibrosarcomas (2,2%) (Falcioni L, 2006). These evidences are largely borne out by other studies about spontaneous tumour profiles of aging Sprague-Dawley rats (Chandra M *et al.*, 1992; McMartin DN *et al.*, 1992; Attia MA, 1996).

Comparing the CRCCM background data with a human homogeneous sample (2650 autopsies of patients died in Trieste hospital in 1984) and considering 16 weeks of age in rats equivalent to 10 years of age in humans, the distribution of tumours bearers, males and females, shared out by age, has the same trend in rats and humans. Besides the same trend was found also comparing the distributions of lymphoma and leukaemia cases in CRCCM Sprague-Dawley rats and in the American population in 2002, shared out both by age and by histological type (Falcioni L, 2006). Finally CRCCM female Sprague-Dawley rats are a model for mammary tumours because their incidence, age distribution and histologic type are similar in women (Maltoni C, 1982).

As regards the present study on the effects of ELFEMF and RFEMF on the circadian rhythm of the energetic metabolism, Sprague-Dawley rat seems to be a sensible animal model as well. Indeed most medical and chronobiological research is performed in nocturnal rodents like rats. Even if humans are diurnal mammals, research findings from nocturnal species can be apply to them (Kalsbeek A *et al.*, 2006). The circadian rhythms of

neural, metabolic, neurotransmitter, and clock gene expression in SCN neurons are similarly phased in nocturnal and diurnal animals (Smale L *et al.*, 2003). The diurnal chronotype apparently being generated downstream of the SCN. The efferent projections of the "diurnal SCN" do not show obvious differences with those of the "nocturnal SCN" (Novak CM *et al.*, 2000), and overall the gross anatomic substructures and major neuronal connections of the SCN are also conserved in humans, implying conservation of the neuroendocrine and autonomic control of rhythms by the SCN across chronotypes (Dai JP *et al.*, 1998). Therefore the nocturnal/diurnal difference does not seem to reside in the hardware of the SCN, but more likely in the composition of its output (i.e., an exchange of stimulatory and inhibitory outputs) or in the translation of the SCN output in its target areas (i.e., inhibitory or stimulatory interneurons). Actually the major amount of SCN projections is directed toward target areas that contain mainly interneurons (Saper CB *et al.*, 2005) rather than directly toward endocrine or preautonomic structures (Kalsbeek A and Buijs RM, 2002). On the other hand, the sympathetic output to the pineal is stimulated during the dark period in both nocturnal and diurnal species, but the timing of the stimulatory input of the autonomic nervous system to other physiological functions like body temperature and cardiovascular activity, is often linked to the activity state of the animals. Furthermore, night-time exposure to environmental light has an opposite influence on temperature, corticosteroid release, and cardiovascular regulation in humans compared to rats (Buijs RM *et al.*, 1999; Scheer FAJL *et al.*, 2003). This means that during the light period, and possibly during nocturnal light exposure as well, GABAergic projections from the SCN inhibit "pineal" and "cardiovascular" PVN neurons simultaneously in nocturnal species, whereas in diurnal species, the pineal and cardiovascular PVN neurons need to be inhibited separately during the light and dark period, indeed there are anatomical evidence that such a specialization exists in the SCN of mammals (Kreier F *et al.*, 2006). Although more then half of all studies dealing with circadian rhythms are conducted on human subjects, animal studies most of all on rats and mice are largely used to identify the role of the SCN in certain disease condition because they are considered reliable animal models (Refinetti R, 2006).

Ramazzini Foundation long-term *in vivo* bioassay on the biological effects, especially carcinogenic effects, of Extremely Low Frequency Electromagnetic Fields-50 Hz

Introduction

A large number of accepted studies on the effect of extremely low frequency electromagnetic fields on the human health, like those described in the preceding chapters, suggests the opportunity to extend the *in vivo* experimental activity in order to confirm or negate the largely accepted possibility that these electromagnetic fields have some health effects, in particular on more sensitive population like children or technical operators. In many countries people take particular attention to this question and solicit a precise answer. In Italy "Ramazzini Foundation" has projected a large scale experiment and financed the research activity developed for the design of expositive systems. A large *in vivo* bioassay (called mega-experiment) is supposed to be able to indicate the possible existence of direct or promoting carcinogenic effects and other health effects as well as the risk assessment and the threshold level. Such an experiment must be conducted using laboratory animals with a well-known physiologic and pathologic profile, especially their basic tumour pattern and its fluctuations. Animals have to be exposed during all phases of their development and they must be clinically controlled since spontaneous death. Necropsy and histopathologic examination must be systematic and as extended as possible. All procedures must be highly standardized and many check points must be fixed to control standardization. The animals chosen for the mega-experiment are out-breeding Sprague-Dawley rats from the CMCRC/FER colony. 12-day-pregnant rats are exposed to various intensity EMF continuously, for 19 hours a day except for the II group which is exposed in an intermittent way. Reproductive data and toxic effects on the offspring or on the adults are collected. Offspring live in the same exposure conditions since spontaneous death.

Aim

The mega-experiment was designed to build an integrate experimental project finalized to:

- Evaluate reproductive and embryo-fetal-toxicity of ELFEMF-50Hz.
- Evaluate carcinogenic effects of ELFEMF-50Hz in various exposure conditions for dose and type of exposure (continuous/discontinuous); (Experiment BT1-4CEMbr).
- Define the target organs of carcinogenic effects, types of tumours observed and other pathologic effects; (Experiment BT1CEM).
- Evaluate the ELFEMF effects on animals exposed to ionizing-radiation (gamma rays); (BT3CEM).
- Evaluate the ELFEMF effects on animals exposed to other carcinogenic chemical agents (formaldehyde, Aflatoxin B1); (BT2CEM; BT4CEM).
- Evaluate possible pathogenetic mechanisms of ELFEMF; (Experiment BT1-4CEM).

In this study is taken into consideration only the base experiment BT1CEM.

Materials and Method

1 Experimental Animals.

1.1 Sprague-Dawley Rats: Rats came from the strain, reared in the last 30 years in the CRCCM. This colony has steady biological characteristics and so it can give many information about expected neoplastic and non-neoplastic pathologies (more than 15,000 historical controls). Besides Sprague-Dawley rats are universally recognized as a good animal model in toxicological and carcinogenic studies.

1.2 Animals Identification: Animals are identified by ear punch using Jackson Laboratory system, weighed and divided according to sex and experimental group at 4-5 weeks of age.

1.3 Cages: Rats are housed in groups of 5 in makrolon cages (41x25x15 cm) with stainless-steel wire tops and a shallow layer of white wood-shavings as bedding. Wood-shaving is regularly analyzed to exclude the presence of the most common

chemical pollutant. Every cage is identified by a card which specifies experiment number, sex, experimental numbers and pedigrees of the animals in the cage.

1.4 Environment Conditions: The six experimental groups are kept in the same room (60x15x4 m), at 22 ± 3°C and 40-60% relative humidity, with a light/dark cycle of 12 hours. Temperature and humidity are daily recorded.

1.5 Food and Water: Animals are fed *ad libitum* with a pellet feedstuff called "Corticella" from "Laboratorio Dottori Piccioni". Feedstuff is analyzed every six months to verify its nutritional composition and the absence of contaminants like pesticides, metals, estrogenic compounds, nitrosamines and aflatoxins. Analyses results are registered. Feedstuff is utilized until three months after production date; production day and the last supplying day are recorded as well as packing list of every stock and labels of each batch. Drinking water from the local aqueduct is periodically analyzed to identify the presence of possible bacterial or chemical pollutant. Water is available *ad libitum*.

1.6 Clinical and Behavioural Controls: Animal behaviour and health are checked three times a day (twice on Sunday). Ill animals are isolated and taken under constant control. Generally drugs are not supplied. Infective pathologies' description and their course are recorded in a specific register. As far as animal welfare is concerned, the experiment is conducted in the respect of Dlgvo 116/92.

2 Treatment.

2.1 Characteristics of the expositive system (ELFEMF-50Hz): the expositive system is composed by 12 independent units called toroids. The toroid is designed with 24 coils made of three turns of insulated copper cable, mounted on a superstructure of aluminium composed of two insulated parts in order to avoid a closed loop subject to total field. The total copper cross section is 11x28 mm^2, and the total current used for 1 mT level is 359.6 A. The low current density and the large amount of a good thermal conducting metal avoid heating. The support structure, designed to allocate rat cages, is mounted inside the toroidal magnet. The support structure is maintained mechanically floating as to the toroidal magnet in order to avoid the

introduction of any kind of possible cage vibrations. The wooden support structure and the plastic cages do not interfere with the magnetic field as well as the non-magnetic metal of cage covers. The computation of currents induced in covers showed they can be neglected both for thermal and field distorsion effects. Each level of the support structure can contain 120 rats divided in 24 cages with 5 rats each. They are allocated in the 5 levels. The top level is about 1.6 m high from the room floor. The toroid has an external diameter of 4.7 m and it is 2.1 m high (Fig. 1). Each toroid can expose 500-600 animals at a sinusoidal electromagnetic field (50 Hz) with a maximum intensity of 1000 μT. The electromagnetic fields is linearly polarised, with a field uniformity >10%. The field lines are horizontal and parallel to the Earth magnetic field whose intensity and direction are reported. The supply currents have a maximum harmonic distortion of 3%. The field rise time at power up is at least ten periods: for 50 Hz, 200 ms. The natural field level is not more than 0,1 μT and any mutual interaction of the systems is avoided; therefore the control group can stay in the same room. Each unit is realised to make easier the access to cages and cleaning procedures during daily treatment suspension (5 hours). Obviously the current generator is noiseless, coils do not produce any noise or vibration and the joule effect on windings do not alter the environmental temperature, with a maximum variation of 2°C next to the coils (Fig. 1).

2.2 ELFEMF-50 Hz exposure monitoring system: as far as artificial electromagnetic field is concerned, exposure condition are constantly monitored using apposite software. Whereas the natural magnetic field is defined during the expositive system tests and then periodically monitored.

2.3 Exposure Conditions: animals are exposed at ELFEMF-50 Hz from the 12° day of their embryonic life until their spontaneous death, for 19 hours a day. The treatment is stopped 4 hours in the morning to allow cleaning procedures and animals health monitoring and 1 hour in the evening to have the last animals health check.

3 Experimental Plan.

3.1 Assessment of reproductive effects and embryo-toxicity on rats exposed to ELFEMF-50 Hz. (BT1CEMbr): The animals of the BT1CEM experiment are the

offspring of rats used in the experiment BT1CEMbr. 1500 in-bred Sprague-Dawley rats (750 males and 750 females) of 24 weeks of age, coming from the CMCRC colony are divided into 6 groups, each one proportioned to the number of offspring required to constitute the planned BT1CEM experimental groups. The animals of the same group are out-bred to assure offspring's homogeneity. Breeding takes 5 days. All data on reproductive parameters are collected, complete necropsy is conducted on all died pregnant female rats and their embryos, slipped feta or died newborns. The treatment starts the 12° day of embryonic life. Offspring and their mothers are exposed together to ELFEMF-50 Hz until weaning; Then mothers, fathers and exceeding nests are sacrificed whereas the other offspring continue the treatment until spontaneous death.

3.2 BT1CEM Experimental Groups: as the antenatal treatment, animals are already divided in the experimental groups. BT1CEM is composed by 6 groups;

- Group I: 523 rats (253 males and 270 females) exposed until spontaneous death to 1000 μT, in continuous, 19 hours a day, starting from 12° day of embryonic life.
- Group II: 500 rats (250males and 250 females) exposed until spontaneous death, to 1000 μT, intermittently (30'on/30'off), 19 hours a day, starting from 12° days of embryonic life.
- Group III: 1000 rats (500 males and 500 females) exposed until spontaneous death, to 100 μT, in continuous, 19 hours a day, starting from 12° day of embryonic life.
- Group IV: 1003 rats (501 males and 502 females), exposed until spontaneous death, to 20 μT, in continuous, 19 hours a day, starting from 12° day of embryonic life.
- Group V: 1002 rats (500 males and 502 females), exposed, until spontaneous death, to 2 μT, in continuous, 19 hours a day, starting from 12° day of embryonic life.
- Group VI: 1001 rats (500 males and 501 females) not exposed to any artificial electromagnetic fields (control group).

Rats implicated in this experiment are 5029 altogether.

4 Experiment Management.

4.1 Water Consumption: starting from 6 weeks of age, individual water consumption is measured on the first 100 animals of each group (50 males and 50 females), every 2 weeks for 8 weeks and then every 4 weeks until 110 weeks of age.

4.2 Food Consumption: starting from 6 weeks of age, individual food consumption is measured on the first 100 animals of each group (50 males and 50 females), every 2 weeks for 8 weeks and then every 4 weeks until 110 weeks of age.

4.3 Body Weight: starting from 6 weeks of age, weight is recorded for each animal every 2 weeks for 8 weeks and then every 4 weeks until 110 weeks of age, afterwards every 8 weeks until experiment conclusion.

4.4 Clinical Controls: animal general health conditions are checked three times a day, except on Sunday (just twice). If there are changes in the clinical control routine, they are reported. Ill or dying animals are isolated and reported to the veterinary. All died animals are autopsied in 24 hours. Besides, starting from 6 weeks of age, animals are clinically examined to diagnose pathological changes. Clinical controls are executed weekly for the first 4 weeks and then every 2 weeks until the end of the experiment.

4.5 Pathology.

4.5.1 Necropsy: all dead animals are autopsied. Survey and raising of dead animals happen 4 times a day. Dead animals are recorded and kept at 4°C until necropsy. Animals are usually autopsied not more than 16 hours after death ascertainment; (necropsy are executed from Monday to Friday, from 8.00 a.m. to 19.00 p.m. and on Saturday and Sunday from 8 a.m. to 12.00 a.m.). In the post-mortem report is indicated death date and hour, necropsy execution date and hour, animal sex and identification number (experimental number and pedigree). Necropsy provides an external physical examination followed by the examination of all internal organs *in situ*. Every pathologic lesion is described and photos are taken.

4.5.2 Sampled Organs and Tissues:

- Every macroscopic lesion.
- Skin
- Mammary Glands
- Encephalon and Cerebellum
- Pituitary Gland
- Zymbal Gland and auditory meatus
- Salivary Glands
- Harder Gland.
- Cranium (5 sections) (oral and nasal cavity, external and internal auditory meatus).
- Tongue
- Thyroid and parathyroid
- Pharynx
- Larynx
- Thymus
- Lung, trachea and main bronchi
- Heart
- Diaphragm
- Liver (2 lobes)
- Spleen
- Pancreas
- Kidneys
- Adrenal Glands
- Esophagus
- Stomach
- Intestine (duodenum, jejunum, ileum, colon, rectum)
- Urinary Bladder
- Prostate Gland
- Uterus
- Ovaries
- Testicles
- Epididymis

- Brown Fat
- Submandibular, subcutaneous, mediastinal and mesenteric lymph-nodes, moreover all lymph-nodes next to a neoplastic lesion.

All organs are fixed in alcohol 70%, except bones, fixed in formalin and then decalcified. Organs are trimmed in the usual way, exposing as more surface as possible; then they are included in paraffin. Organs sections (3-5 μm) are usually coloured by EE.

4.5.3 Histopathology: organs and tissue have a microscopic examination to identify pathologic lesions. All sections are first examined by a junior pathologist and then by a senior one. Results are then registered in a systematic and standard way.

4.5.4 Molecular Biology: tissue samples are immediately identified, frozen in liquid nitrogen and stoked at -70°C. Bio-molecular analysis will be conducted.

The experiment is managed following GLP (Good Laboratory Practices) which concern planning, management, execution, registration of all experimental phases and the redaction of intermediate and final reports.

5 Quality Control.

5.1 Internal Quality Control System: a quality control procedure is activated to verify the correct application of the experimental protocol in all its scientific, structural (equipment and facilities suitability) and operative aspects, assuring reliability of data, in compliance with GLP.

5.2 Standard Operating Procedures (SOP): in accordance with GLP, all procedures conducted in the CMCRC are executed following specific SOP which defined methodologies to apply during every phase of the experiment (exposure system, animals care, equipment and facilities management, data collection and registration, final reports redaction). All incidental non-compliances or protocol variations are justified and documented.

5.3 Archives: every sample and all original data are preserved in compliance with GLP.

Results Evaluation

1 BT1CEMbr: the following parameters will be evaluated;

- Body weight.
- Animal Survival.
- Deliveries Trend.
- Reproductive Data.
- Pathologic lesions found out at necropsy and after histological examination.

2 BT1CEM: the following parameters will be evaluated.

- Body weight.
- Food and water consumption.
- Animal Survival.
- Macroscopic and microscopic pathologic lesions.
- Tumours and pre-neoplastic lesions.
- Number and rate of tumour and pre-neoplastic lesion bearers.
- Number of benignant and malignant tumours or pre-neoplatic lesion for each bearer.
- Number of malignant tumours every 100 animals.
- Latency of benignant and malignant tumours and of pre-neoplastic lesions.
- Clinical observations (animal behaviour, general appearance, signs of toxic effects).
- Metabolic studies (circadian rhythms alterations, melatonin measuring: BT1CEMbis).

3 Statistical Analysis: data are analysed by a qualified researcher using universally recognised statistical methods: Kruskall-Wallis Test, χ^2 Test or Fisher Test. Log Rank Test.

The study started on 15th July 2002. Complete final report will be available in 2009.

Figure 1. ELFEMFs-50 Hz exposure system.

Ramazzini Foundation long-term *in vivo* bioassay on the biological effects, especially carcinogenic effects, of Radiofrequency Electromagnetic Fields-1.8 GHz

Introduction

In the last years communication systems know an expansion without precedents; the diffusion of these technologies have cause an increase of the radiofrequency electromagnetic fields emission, rising from many different sources, in the natural environment and in the working places. As described in the previous chapter, *in vitro*, *in vivo* and epidemiologic studies conducted to define the possible long-term health effects of the exposure to RF/MW-EMF are often inadequate and inconclusive so they have not given any definite results yet. Besides, even if *in vivo* studies are the best methods to evaluate health effects, especially carcinogenic effects, of a physical or chemical agent, and to give precise information about carcinogenic power, dose-response correlation, target organs and effective risk assessment, they are very few, with an insufficient number of animals for experimental groups and no proper expositive systems. Therefore in 2005, in the CMCRC facilities, Ramazzini Foundation starts a mega-experiment involving 2448 Sprague-Dawley rats exposed to RFEMF-1.8 GHz; the electromagnetic emission of the worldwide use GSM system.

Aim

The mega-experiment was designed to build an integrate experimental project finalized to:

- Evaluate thermal effects caused by environmental exposure to RF/MW electromagnetic fields (1.8 GHz). (BT1CEMRFbis)
- Evaluate, throughout a range-finding experiment, lasting 90 days, if exposure doses defined for the long-term experiment could damage animal health so that they are not compatible with a chronic exposure. (BT1CEMRFter)
- Evaluate reproductive and embryo-fetal-toxicity of RFEMF-1.8 GHz. (BT1CEMRFbr).
- Evaluate if there are carcinogenic risks due to the environmental exposure (far-field exposure) to RFEMF-1.8 GHz using large animals groups. Rats are exposed starting from the 12° day of their embryonic life unitil their spontaneous death. (BT1CEMRF).

- Define the dose-response rate, the target organs of carcinogenic effects, types of tumours observed and their pre-neoplastic lesions as well as any other pathologic effects. (BT1CEMRF).
- Evaluate thanks to bio-molecular analysis, the possible pathogenetic mechanisms causing the carcinogenic effects. (BT1CEMRFbr; BT1CEMRF).

In this study is taken into consideration only the experiment BT1CEMRF.

Materials and Method

1 Experimental Animals.

1.1 Sprague-Dawley Rats: Rats came from the strain, reared in the last 30 years in the CMCRC. This colony has steady biological characteristics and so it can give many information about expected neoplastic and non-neoplastic pathologies (more than 15,000 historical controls). Besides Sprague-Dawley rats are universally recognized as a good animal model in toxicological and carcinogenic studies.

1.2 Animals Identification: Animals are identified by ear punch using Jackson Laboratory system, weighed and divided according to sex and experimental group at 4-5 weeks of age.

1.3 Cages: Rats are housed in groups of 5 in makrolon cages (41x25x15 cm) with plastic tops and a shallow layer of white wood-shavings as bedding. Wood-shaving is regularly analyzed to exclude the presence of the most common chemical pollutant. Every cage is identified by a card which specifies experiment number, sex, experimental numbers and pedigrees of the animals in the cage.

1.4 Environment Conditions: The six experimental groups are kept in the same room (60x15x4 m), at 22 ± 3°C and 40-60% relative humidity, with a light/dark cycle of 12 hours. Temperature and humidity are daily recorded.

1.5 Food and Water: Animals are fed *ad libitum* with a pellet feedstuff called "Corticella" from "Laboratorio Dottori Piccioni". Feedstuff is analyzed every six

months to verify its nutritional composition and the absence of contaminants like pesticides, metals, estrogenic compounds, nitrosamines and aflatoxins. Analyses results are registered. Feedstuff is utilized until three months after production date; production day and the last supplying day are recorded as well as packing list of every stock and labels of each batch. Drinking water from the local aqueduct is periodically analyzed to identify the presence of possible bacterial or chemical pollutant. Water is available *ad libitum*.

1.6 Clinical and Behavioural Controls: Animal behaviour and health are checked three times a day (2 times on Sunday). Ill animals are isolated and taken under constant control. Generally drugs are not supplied. Infective pathologies' description and their course are recorded in a specific register. As far as animal welfare is concerned, the experiment is conducted in the respect of Dlgvo 116/92.

2 Treatment.

2.1 Characteristics of the Facilities: facilities have the characteristics of a total absorbent area to reproduce in the laboratory the exposure conditions of an open space. They are shielded rooms to avoid "electro-smog". Every room has metallic walls covered with expanded polyurethane cones charged with graphite like the usual shielded room utilized in electromagnetic compatibility applications. Rooms don't have any resonance frequency near working frequency so that reflections are minimized. Anechoic material utilized is very little; just the necessary to avoid residual reflections which can disturb electromagnetic field distribution. Rooms have specific connections to supply the antenna and electromagnetic field sensors. Control group is allocated in a facility next to the anechoic rooms. In all anechoic and non-anechoic rooms are guaranteed the same environmental conditions: 22°C ± 3°C; 40%-60% relative humidity, light/night cycle of 12 hours, uniform illumination.

2.2 Characteristic of the Expositive System: the expositive system simulates the real environmental emission of a radio-base station, following the principles of the "Far Field" electric signals propagation. The system can generate a transmitting pattern which completely represents the impulsive nature of GSM system, with an energy

level which is the maximum duty-cycle signal admitted by GSM protocol. To have the "Far Field" condition the distance from the emitter has to be at least 4-5 wave lengths and the electromagnetic field must be homogeneous and uniform. Considering that the frequency required is 1,850 GHz which has a wave length of about 16 cm, the distance from the emitter has to be at least 80 cm: at this distance signal propagation uses waves with a spherical phase front. Therefore "Far Field" exposure is realized only placing the emitter, or the antenna for the radio-base station, in the centre of a circular-base wooden rack of 5 levels, with a 5 m diameter where cages of each experimental group are allocated on a perimeter whose diameter is not minor of 450 cm. Like racks, cages, tops, drinking troughs and label holders are made with non-conductive materials to avoid interferences with the generated electromagnetic field. Antennas are supplied by throughout an amplifier with a signal corresponding to 4 physic channels, obtained mixing the outputs of 4 frequency converters. Their inputs come from a generator which associate a specific number of logic channels to the physic channel produced. The many modulations possibilities of the generator and the automatic management of the system, provided with a timer which controls the expositive cycle, allow to emit signals with analogue characteristics of those of a radio-base station (GSM) and with the desired field intensity (from 50 V/m to 1 V/m) for 20 hours a day. The electromagnetic field is constant in the cage area thanks to a "stacked dipoles" configuration of the antenna. It allows to concentrate more than 80% of energy emitted in the cage area and very little energy emission towards the floor and the ceiling which are not utilized in the experiment and so they have no shielding. Each unit is allocated in a separated room and it is structured to allow an easy access to technicians. The frequency utilized are near to the downlink band but the are not coincident with GSM channels to avoid any interference with the GSM traffic of the area. In summary, the expositive system is composed by the main generator, external control panels, antennas and field sensors.

2.2.1 The main generator (650x700x1100 mm; 100 kg) combines the following functions:

- Generator of GSM signals with complete channel simulation and frequency pre-selection.
- Phase of signal pre-amplification.

- Phase of final amplification, dimensioned for a total power emission +50dBm.
- Retroactive system to control the emitted power in a close loop way. The control loop is closed at the antenna connection to make stable the radiofrequency electromagnetic field levels emitted by the system.
- Supplying unit.
- Cooling unit.

It has the following indicators:

- Power on
- RF on/off indicator
- Out-Power control
- RFEMF level digital display.
- Digital timer to set the daily cycle.
- General supply disconnecting switch.
- Emergency push button
- RF-waves selector.

2.2.2 External control panels are allocated near the doors of anechoic rooms, they are connected to the principal unit thanks to a multipolar cable.

They have the following indicators:

- Emergency push button.
- Key for the temporary suspension of RFEMF emission (utilized to have extemporary access to the experimental area during active cycle phase).
- RF on/off indicator.

2.2.3 Antennas are connected to the main generator through a coaxial cable (maximum 10 m long) at low stray loss. They are collinear and allocated in the centre of the toroidal rat cages structure. They transmit an homogenous RFEMF with cylindric-cardioid signal distribution which cover all the cage area. A reflector located in the high part of the antenna reduces vertical emissions. A power splitter takes away a small fraction of the signal supplying the antenna. This fraction is utilized like an active feedback to close the control loop of the emitted power stabilization.

2.2.4 Field Sensors (TESY2001) are probes which can measure EMF intensity and record it up to 80 active monitoring days. The probes transfer data to a PC which shows constantly the field intensity of each expositive rooms, with updating every 10 seconds. Every 6 minutes data are unloaded (each sample is the mean value of the data pointed out in 6 minutes, with a sampling slot of 2 seconds that means one data every 2 seconds). The software TESY2001_WIN controls probes, downloads recorded information and produces diagrams of field intensity every days. Connection between field sensors and PC is made by an optic fibre line with a maximum length of 200 m.

2.3 Characteristics of the Exposure Monitoring System: exposure monitoring system is composed by one or more isotropic field sensors linked to a data acquisition system which is connected to a PC used to manage the system operation (field intensity, exposure duration, alarms, and exc.).

3 Experimental Plan.

3.1 Assessment of reproductive effects and embryo-toxicity on rats exposed to RFEMF-1.8 GHz. (BT1CEMRFbr): The animals of the BT1CEMRF experiment are the offspring of rats used in the experiment BT1CEMRFbr. 960 in-bred Sprague-Dawley rats (480 males and 480 females) of 22 weeks of age, coming from the CMCRC colony are divided into 4 groups, each one proportioned to the number of offspring required to constitute the planned BT1CEMRF experimental groups. The animals of the same group are out-bred to assure offspring's homogeneity. Breeding takes 7 days. All data on reproductive parameters are collected, complete necropsy is conducted on all died pregnant female rats and their embryos, slipped feta or died newborns. The treatment starts the 12° day of embryonic life. Offspring and their mothers are exposed together to RFEMF-1.8 GHz until weaning; Then mothers, fathers and exceeding nests are sacrificed whereas the other offspring continue the treatment until spontaneous death.

3.2 BT1CEMRF Experimental Groups: as the antenatal treatment, animals are already divided in the experimental groups. BT1CEMRF is composed by 4 groups;

- Group I: 409 rats (207 males and 202 females) exposed until spontaneous death to RFEMF (1.8 GHz) 50 V/m, 19 hours a day, starting from 12° day of embryonic life.
- Group II: 411 rats (209 males and 202 females) exposed until spontaneous death, to RFEMF (1.8 GHz) 25 V/m, 19 hours a day, starting from 12° days of embryonic life.
- Group III: 811 rats (401 males and 410 females) exposed until spontaneous death, to RFEMF (1.8 GHz) 5 V/m, 19 hours a day, starting from 12° day of embryonic life.
- Group IV: 817 rats (412 males and 405 females) not exposed to any RFEMF (control group).

Rats implicated in this experiment are 2448 altogether.

4 Experiment Management.

4.1 Water Consumption: starting from 6 weeks of age, individual water consumption is measured on the first 100 animals of each group (50 males and 50 females), once a week for 4 weeks and then every 4 weeks until 110 weeks of age.

4.2 Food Consumption: starting from 6 weeks of age, individual food consumption is measured on the first 100 animals of each group (50 males and 50 females), once a week for 4 weeks and then every 4 weeks until 110 weeks of age.

4.3 Body Weight: starting from 6 weeks of age, weight is recorded for each animal every week for 4 weeks and then every 4 weeks until 110 weeks of age, afterwards every 8 weeks until experiment conclusion.

4.4 Clinical Controls: animal general health conditions are checked three times a day, except on Sunday (just twice). If there are changes in the clinical control routine, they are reported. Ill or dying animals are isolated and reported to the veterinary. All died animals are autopsied in 24 hours. Besides, starting from 6 weeks of age, animals are clinically examined to diagnose pathological changes. Clinical controls are executed weekly for the first 4 weeks and then every 2 weeks until the end of the experiment.

4.5 Pathology.

4.5.1 Necropsy: all dead animals are autopsied. Survey and raising of dead animals happen 4 times a day. Dead animals are recorded and kept at 4°C until necropsy. Animals are usually autopsied not more than 16 hours after death ascertainment; (necropsy are executed from Monday to Friday, from 8.00 a.m. to 7.00 p.m. and on Saturday and Sunday from 8 a.m. to 12.00 a.m.). In the post-mortem report is indicated death date and hour, necropsy execution date and hour, animal sex and identification number (experimental number and pedigree). Necropsy provides an external physical examination followed by the examination of all internal organs *in situ*. Every pathologic lesion is described and photos are taken.

4.5.2 Sampled Organs and Tissues:

- Every macroscopic lesion.
- Skin
- Mammary Glands
- Encephalon and Cerebellum
- Pituitary Gland
- Zymbal Gland and auditory meatus
- Salivary Glands
- Harder Gland.
- eyeballs
- Cranium (5 sections) (oral and nasal cavity, external and internal auditory meatus).
- Tongue
- Thyroid and parathyroid
- Pharynx
- Larynx
- Thymus
- Lung, trachea and main bronchi
- Heart
- Diaphragm
- Liver (2 lobes)

- Spleen
- Pancreas
- Kidneys
- Adrenal Glands
- Esophagus
- Stomach
- Intestine (duodenum, jejunum, ileum, colon, rectum)
- Urinary Bladder
- Prostate Gland
- Uterus
- Ovaries
- Testicles
- Epididymis
- Brown Fat
- Sternum
- Submandibular, subcutaneous, mediastinal and mesenteric lymph-nodes, moreover all lymph-nodes next to a neoplastic lesion.

All organs are fixed in alcohol 70%, except bones, fixed in formalin, then decalcified, and eyeball fixed in Davidson solution. Organs are trimmed in the usual way, exposing as more surface as possible; then they are included in paraffin. Organs sections (3-5 μm) are usually coloured by EE.

4.5.3 Histopathology: organs and tissue have a microscopic examination to identify pathologic lesions. All sections are first examined by a junior pathologist and then by a senior one. Results are then registered in a systematic and standard way.

4.5.4 Molecular Biology: tissue samples are immediately identified, frozen in liquid nitrogen and stoked at -70°C. Bio-molecular analysis will be conducted.

The experiment is managed following GLP (Good Laboratory Practices) which concern planning, management, execution, registration of all experimental phases and the redaction of intermediate and final reports.

5 Quality Control.

5.1 Quality Control System: a quality control procedure is activated to verify the correct application of the experimental protocol in all its scientific, structural (equipment and facilities suitability) and operative aspects, assuring reliability of data, in compliance with GLP. The Quality Control Committee is composed by Italian and foreign experts nominated by European Ramazzini Foundation and ARPA-Emilia Romagna (the study sponsor). Committee's Inspections are periodical (at least every 12 months) followed by written relations which must be registered. The scientific director must give a report to the Foundation Administration Council for each problem which can interfere with a proper study conduction. Besides the final report is revised by the Committee to verify that final results agree with the original data collected during the study.

5.2 Standard Operating Procedures (SOP): in accordance with GLP, all procedures conducted in the CMCRC are executed following specific SOP which defined methodologies to apply during every phase of the experiment (exposure system, animals care, equipment and facilities management, data collection and registration, final reports redaction). All incidental non-compliances or protocol variations are justified and documented.

5.3 Archives: every sample and all original data are preserved in compliance with GLP.

Results Evaluation

1 BT1CEMRFbr: the following parameters will be evaluated;

- Body weight.
- Animal Survival.
- Deliveries Trend.
- Reproductive Data.
- Pathologic lesions found out at necropsy and after histological examination.

2 BT1CEMRF: the following parameters will be evaluated.

- Body weight.
- Food and water consumption.

- Animal Survival.
- Macroscopic and microscopic pathologic lesions.
- Tumours and pre-neoplastic lesions.
- Number and rate of tumour and pre-neoplastic lesion bearers.
- Number of benignant and malignant tumours or pre-neoplatic lesion for each bearer.
- Number of malignant tumours every 100 animals.
- Latency of benignant and malignant tumours and of pre-neoplastic lesions.
- Clinical observations (animal behaviour, general appearance, signs of toxic effects).
- Metabolic studies (circadian rhythms alterations: BT1CEMRFmet).

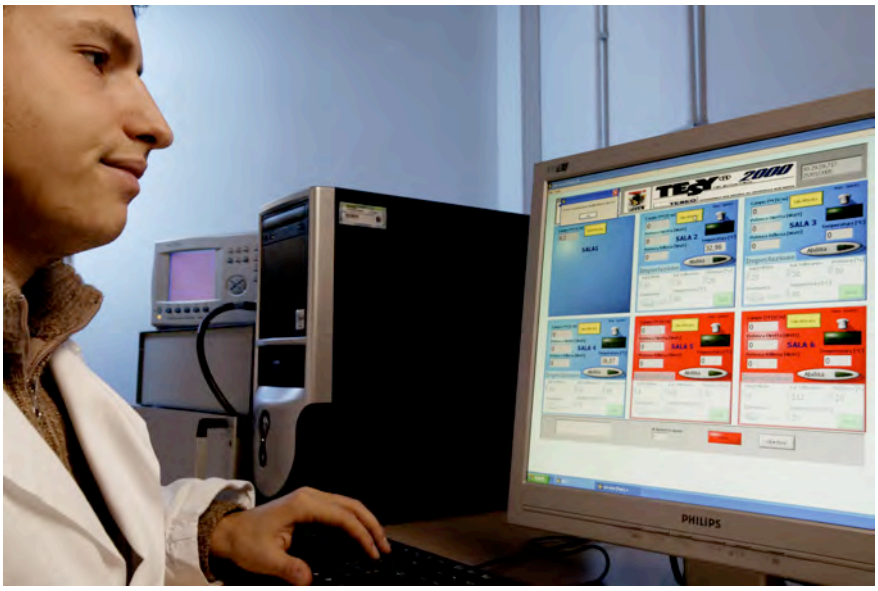
3 Statistical Analysis: data are analysed by a qualified researcher using universally recognised statistical methods: Kruskall-Wallis Test, χ^2 Test or Fisher Test. Log Rank Test.

The study started on 20th October 2005; Complete final report will be available in 2010.

Figure 1. RFEMFs-1,8 GHz expositive system.

Figure 2. RFEMFs-1.8 GHz expositive system.

Figure 3. RFEMFs-1.8 GHz expositive system.



PROJECT: EFFECTS OF ELECTRO-MAGNETIC FIELDS (ELFEMF-50 HZ AND RFEMF-1.8 GHZ) ON CIRCADIAN RHYTHMS OF SOME BLOOD PARAMETERS IN SPRAGUE-DAWLEY RATS

Abstract

Since electro-magnetic fields are supposed to influence the ionic membrane exchanges, they may also produce some metabolic changes in the normal activity of organism cells with important changes in its metabolism. In the framework of two mega-experiments aimed to evaluate the long-term effects of ELFEMF-50 Hz and RFEMF-1.8 GHz, in particular carcinogenic effects, two satellite studies are performed to determine the interactions between ELFEMF, RFEMF and circadian rhythms of some blood parameters of energetic metabolism (glycaemia, total cholesterol and triglycerides) in Sprague-Dawley rats (*Rattus norvegicus*). The results of these studies highlight that: as far as concerned 50 Hz magnetic sinusoid fields exposure, the circadian rhythm of glycaemia in female rats exposed to 1000 μT, the circadian rhythms of tryglicerides in male rats exposed to 1000 μT and 100 μT are inverted whereas the circadian rhythm of glycaemia in male rats exposed to 1000 μT and 100 μT is totally lost. As far as concerned 1.8 GHz electromagnetic field exposure, the circadian rhythms of glycaemia, trygliceridhaemia and cholesterolhaemia in female rats exposed to 50 V/m are absent as well as cholestrolhaemia in female rats exposed to 25 V/m. Lipid metabolism of the other groups, maintains its physiologic circadian rhythm.

Introduction

Nowadays everyone in the world is exposed to a complex mix of EMF frequencies in the range 0-300 GHz. EMF has become one of the most pervasive environmental influences and exposure levels at many frequencies are increasing significantly as the technological revolution continues unabated and new applications using different parts of the spectrum are found. Major sources of EMF exposure include: electric power generation, distribution and use; transportation systems; telecommunications facilities and associated devices such

as mobile telephones; medical, commercial and industrial equipment; radars; and radio and television broadcast antennas.

The extensive use of technologies based on EMF suggests that users do not in general judge them to present a significant health hazard. Rather they have welcomed the technology and brought it into use in their every day lives. Netherless, since their introduction there have been persisting concerns about the possible impact of mobile phone technologies on health. In conclusion it is not possible at present to say that exposure to EM radiation even at levels below national guidelines is totally without potential health effects, and that the gaps in knowledge are sufficient to justify a precautionary approach. The World Health Organization (WHO) takes seriously the concerns raised by reports about possible health effects from exposure to electromagnetic fields (EMF). Cancer, changes in behaviour, memory loss, Parkinson and Alzheimer's diseases, and many other diseases have been suggested as resulting from exposure to EMF. In the framework of the great impact of EMF on public health, in Italy, Ramazzini Foundation started an important study project on ELFEMF and RFEMF long-term health effects, especially carcinogenic effects, with two *in vivo* mega-experiment: the first one about ELFEMF-50 Hz exposure started in 2002 and the second one about RFEMF-1.8 GHz exposure started in 2005. Trying to investigate possible metabolic effects of EMF on living organism and revising the current literature on their possible mechanisms of action, the present study takes into consideration the possibility that EMF may cause an alteration of living organisms' biological clock changing their homeostatic balance and so compromising their general health and their welfare as well as inducing many diseases, from metabolic syndrome to cancer.

Experiment 1: Effects of extremely low frequency electromagnetic fields (50 Hz) on circadian rhythms of some blood parameters in Sprague-Dawley rats

Aim

Numerous physiological functions have rhythmic course with various frequency (ultradian, circadian, circatrigentan, circannual). In depending of these rhythmic metabolic processes some hematological parameters can show variations that have rhythmic course themselves. Biochemical evaluation of rhythmic parameters indicates the stability of organ with clocked functions and the good homeostatic condition of the organism which is the base of its health and its possibility to effectively resist to diseases.

As described in previous chapters extremely low frequency electro-magnetic fields (50 Hz) are largely investigated because of their great diffusion, their potential health effects, especially carcinogenic risk and the uncertainty about their mechanisms of action on living organisms. Among the many hypotheses, they are supposed to influence the ionic membrane exchanges, causing the opening of cationic channels (Na^+, K^+, Ca^{2+}), producing metabolic changes in the physiological activity of organism cells, in particular those of central nervous anatomic districts that are involved in the regulation of metabolic rhythms. In the framework of the Ramazzini Foundation mega-experiment aimed to evaluate ELFEMF long-term effects, in particular carcinogenic effects, a satellite experiment was performed to determine the interactions between ELFEMF-50Hz and circadian rhythms of the three most important blood parameters of energetic metabolism (glycaemia, total cholesterol and triglycerides) in *Rattus norvegicus*: Sprague-Dawley Rats.

Materials and Methods

1 Experimental Animals.

1.1 Sprague-Dawley Rats: Rats came from the strain, reared in the last 30 years in the CRCCM. This colony has steady biological characteristics and so it can give many information about expected neoplastic and non-neoplastic pathologies (more than 15,000 historical controls). Besides Sprague-Dawley rats are universally recognized

as a good animal model in toxicological and carcinogenic studies. The animals used in this experiment were born from out-breeding rats mated to obtain experimental animals of the main experiment BT1CEM. Selected rats are undergone to blood collections at 84 weeks of age.

1.2 Animals Identification: Animals are identified by ear punch using Jackson Laboratory system, weighed and divided according to sex and experimental group at 4-5 weeks of age.

1.3 Cages: Rats are housed in groups of 5 in makrolon cages (41x25x15 cm) with stainless-steel wire tops and a shallow layer of white wood-shavings as bedding. Wood-shaving is regularly analyzed to exclude the presence of the most common chemical pollutant. Every cage is identified by a label which specifies experiment number, sex, experimental numbers and pedigrees of the animals in the cage.

1.4 Environment Conditions: The three experimental groups are kept in the same room (60x15x4 m), at 22 ± 3°C and 40-60% relative humidity, with a light/dark cycle of 12 hours. Temperature and humidity are daily recorded.

1.5 Food and Water: Animals are fed *ad libitum* with a pellet feedstuff called "Corticella" from "Laboratorio Dottori Piccioni". Feedstuff is analyzed every six months to verify its nutritional composition and the absence of contaminants like pesticides, metals, estrogenic compounds, nitrosamines and aflatoxins. Analyses results are registered. Feedstuff is utilized until three months after production date; production day and the last supplying day are recorded as well as packing list of every stock and labels of each batch. Drinking water from the local aqueduct is analyzed every six months to identify the presence of possible bacterial or chemical pollutant. Water is available *ad libitum*.

1.6 Clinical and Behavioural Controls: Animal behaviour and health are checked three times a day (twice on Sunday). Ill animals are isolated and taken under constant control. Generally drugs are not supplied. Infective pathologies' description and their course are recorded in a specific register. As far as animal welfare is

concerned, the experiment is conducted in the respect of Dlgvo 116/92. Animals chosen to have blood collection are clinically healthy.

2 Treatment.

2.1 Characteristics of the expositive system (ELFEMF-50Hz): the expositive system is the same described in the experiment BT1CEM. Animals of this experiment are placed in corresponding toroids of experiment BT1CEM depending on the exposure level required.

2.2 Exposure Conditions: animals are exposed at ELFEMF-50 Hz from the 12° day of their embryonic life until their spontaneous death, for 19 hours a day. The treatment is stopped 4 hours in the morning to allow cleaning procedures and animals health monitoring and 1 hour in the evening to have the last animals health check.

3 Experimental Plan. (Table 1).

3.1 Experimental Groups: as the antenatal treatment, animals are already divided in the three experimental groups:

- Group I: 12 rats (6 males and 6 females) exposed until spontaneous death to ELFEMF (50 Hz), 1000 μT, 19 hours a day, starting from 12° day of embryonic life.
- Group II: 12 rats (6 males and 6 females) exposed until spontaneous death, to ELFEMF (50 Hz) 100 μT, 19 hours a day, starting from 12° days of embryonic life.
- Group III: 12 rats (6 males and 6 females) not exposed to any artificial electromagnetic field (control group).

4 Experiment Management.

4.1 Water Consumption: starting from 6 weeks of age, individual water consumption is measured on the first 100 animals of each group (50 males and 50 females) of the main experiment BT1CEM, every 2 weeks for 8 weeks and then every 4 weeks until 110 weeks of age.

4.2 Food Consumption: starting from 6 weeks of age, individual food consumption is measured on the first 100 animals of each group (50 males and 50 females) of the main experiment BT1CEM, every 2 weeks for 8 weeks and then every 4 weeks until 110 weeks of age.

4.3 Body Weight: starting from 6 weeks of age, weight is recorded for each animal every 2 weeks for 8 weeks and then every 4 weeks until 110weeks of age, afterwards every 8 weeks until experiment conclusion.

4.4 Clinical Controls: animal general health conditions are checked three times a day, except on Sunday (just twice). If there are changes in the clinical control routine, they are reported. Ill or dying animals are isolated and reported to the veterinary. All died animals are autopsied in 24 hours. Besides, starting from 6 weeks of age, animals are clinically examined to diagnose pathological changes. Clinical controls are executed weekly for the first 4 weeks and then every 2 weeks until the end of the experiment.

4.5 Blood Collection: Blood samples (0,5ml each) are collected from each experimental animals, every 3 hours around the clock, using 400µl test-tubes containing heparin. The retro-orbital plexus is the ideal site for periodic sampling; this methods has been shown to be reliable for the repeated collection of blood samples. Collection from this site must be conducted under anesthesia to reduce pain and stress to the animal. The animals are anesthetized with ethyl ether. Blood is collected using the fine end of a Pasteur pipette. The tube is inserted into the orbit of the eye at the anterior angle formed by the lids and the nicitating membrane. A short thrust past the eyeball will make the tube enter the slightly resistant horny membrane of the sinus. The tube can be rotated slightly as it is inserted. Once the sinus has been punctured blood will fill the tube. Once the tube contains the blood volume required, the blood can be poured out into the test-tubes containing heparin. If the flow stops, the tube can be pulled out or advanced slightly to reestablish flow (Hitzelberg R *et al.*, 1985; Johnson MD, 2007).
Low intensity red light was used during the night to avoid retina-hypothalamic tract stimulation.

4.6 Laboratory Analyses: Blood glucose level was immediately determined after the collection using a portable glucometer (Accu-Check Compact-Roche). Total plasma cholesterol and triglycerides were determined using an autoanalyzer (Hitachi 911 Plus).

4.7 Statistical Analyses: Statistical elaboration of the data was based on the average values obtained at the various time points (equidistant 3 hours) for each animal group divided by sex, since the intra-group variance is not significant. Based on these average values, all the results were expressed as mean ± SD. One-way repeated measures analysis of variance (ANOVA) was used to determine significant difference. p values < 0.05 were considered statistically significant. SNK (*Statistical Newman-Keuls)* test was applied for post-hoc comparison. Besides a trigonometric statistic model has been applied in order to analytically describe the periodic phenomenon, by individuating the main parameters that characterize it. Therefore for each variable of each animal, a cosine wave was fitted to the data points according to the function $Yt = A + M \times \cos(qt + j)$, where Yt denotes each data point in the time series, M is the mean level of the rhythm, called also MESOR (Midline Estimating Statistic of Rhythm). It represents the intermediate value between the highest and the smallest values of a function used to describe a rhythm, expressed in the unit of the relative considered parameter and with the fiduciary limit at 95%. A is the amplitude which identifies the difference between the maximum and the minimum level in a determinate period, and expressed in the same unit of the relative Mesor. qt is the trigonometric angle (in degrees) corresponding to time t, and j is the angle displacement for the acrophase. The acrophase of a rhythm (u) was determined by an iterative curve-fitting procedure based on the single cosinor procedure (Nelson W *et al.*, 1979). Acrophase and batiphase respectively represent the superior and the inferior point of a model which is in relation with a reference phase, chosen and expressed in hours, with a confidence interval at 95%. Cosine waves were then fitted to the averaged 24-h rhythm, and the time corresponding to the peak of the best-fitting cosine wave was taken as the acrophase of the rhythm. So the value of u was determined by iteration: the true value of u was considered to be the one that produced the smallest sum of squares of the deviations between iterated cosine functions and the raw data.

Table 1. Experiment Plan.

Group N.	Treatment[a] (ELFEMF-50Hz)			Experiment lasting[b]	Animals[d]			
	EMF[c] (μT)	Start	Period	(hours)	Sex	N.	Experimental	Pedigree N.
I	1000	embrionic life	life span	24	F	6	4	174057
							5	174058
							6	174060
							7	174061
							9	174067
							13	174071
I	1000	embrionic life	life span	24	M	6	55	174064
							57	174073
							59	174081
							60	174080
							61	174079
							62	174078
II	100	embrionic life	life span	24	F	6	204	178461
							206	178463
							207	178471
							209	178475
							210	178476
							212	178479
II	100	embrionic life	life span	24	M	6	253	178470
							254	178469
							255	178468
							256	178467
							258	178465
							260	178474
III	0	-	-	24	F	6	502	177347
							503	177348
							504	177349
							507	177352
							508	177353
							511	175361
III	0	-	-	24	F	6	551	177358
							552	177357
							554	177355
							555	177354
							557	177370
							559	177368

[a] Treatment starts 12° day of embrionic life (15/07/2002) exposing pregnant female rats. Animals treatment is life span for 19 hours/day
[b] Blood collections are effectuated every 3 hours around the clock on the same animals at 84 weeks of age
[c] EMF: electromagnetic field
[d] Total animal number:36 rats

Results

Statistic data analyses have utilized the mean values of blood samples executed in different time points (equidistant three hours), for each group divided by sex thanks to the non-significant intra-group variance (Fig.1-6).

ANOVA application points out a significant time effect for all hematic parameters studied on female rats. ($P<0.05$ is considered statistically significant) (Table 1).

Table 1. ANOVA Results. Females. I, II and III Group.

	Glycaemia		**Cholesterol**		**Triglycerides**	
	$F_{7,35}$	$p<$	$F_{7,35}$	$p<$	$F_{7,35}$	$p<$
I group	3.55	0.005	6.64	0.0001	53.53	0.0001
II group	5.79	0.0002	11.13	0.0001	33.89	0.0001
III group	3.65	0.005	7.70	0.0001	44.39	0.0001

Whereas on male rats, ANOVA points out a significant time effects only for triglycerides and cholesterol of the first and the second group as well as for glycaemia of the third group (Table 2).

Table 2. ANOVA Results. Males. I, II and III Group.

	Glycaemia		**Cholesterol**		**Triglycerides**	
	$F_{7,35}$	$p<$	$F_{7,35}$	$p<$	$F_{7,35}$	$p<$
I group	-	-			5.71	0.0002
II group	-	-	10.12	0.0001	19.77	0.000
III group	-	-	27.24	0.0001	24.27	0.000

"Cosinor" analyses, applied to periodic parameters, defined acrophases (expressed in hours). On female rats, glycaemia has diurnal acrophase (07.52.00AM) in the first group whereas in the second and third group it is nocturnal (01.00.00AM and 00.24.00PM respectively).

Trygliceride have diurnal acrophases in all groups (10.08.00AM for the I group, 12.08.00AM for the II group and 10.36.00AM for the III group). Cholesterol has nocturnal acrophases in all groups (01.32.00AM for the I group, 11.24.00PM for the second group and 00.44.00PM for the III group); (Table 3).

Table 3. Mesor (M), with confidence interval (CI) at 95%, Amplitude (A) and Acrophase (Φ), in hours, with confidence interval (CI) at 95% of parameters resulted periodic for the circadian rhythm, in female Sprague-Dawley rats of the three groups.

PARAMETER	I GROUP FEMALE				
	M	**C.I. 95%**	**A**	**Φ**	**C.I. 95%**
Glycaemia (mg/dL)	102.81	(96.35-109.27)	13.53	07.52.00AM	(02.32.00AM-01.12.00PM)
Cholesterol (mg/dL)	67.19	(62.25-72.13)	11.72	01.32.00AM	(09.21.00PM-05.40.00AM)
Tryglicerides (mg/dL)	64.15	(54.67-73.64)	24.87	10.08.00AM	(06.44.00AM-01.32.00PM)
	II GROUP FEMALE				
	M	**C.I. 95%**	**A**	**Φ**	**C.I. 95%**
Glycaemia (mg/dL)	138.01	(131.38-144.64)	17.37	01.00.00AM	(09.28.00PM-04.32.00AM)
Cholesterol (mg/dL)	71.35	(68.05-74.65)	13.01	11.24.00PM	(09.24.00PM-01.24.00AM)
Tryglicerides (mg/dL)	86.95	(74.75-99.15)	33.65	12.08.00PM	(08.56.00AM-03.20.00PM)
	III GROUP FEMALE				
	M	**C.I. 95%**	**A**	**Φ**	**C.I. 95%**
Glycaemia (mg/dL)	129.05	(122.22-135.88)	15.01	00.08.00AM	(07.08.00PM-05.08.00AM)
Cholesterol (mg/dL)	79.71	(74.29-85.14)	13.53	00.44.00PM	(08.44.00PM-04.44.00AM)
Tryglicerides (mg/dL)	141.76	(127.44-155.69)	40.51	10.36.00AM	(07.36.00AM-01.36.00PM)

On male rats, glycaemia has nocturnal acrophase (03.08.00AM) in the third group. Cholesterol has a nocturnal acrophase in the first and in the second group (03.48.00AM and 04.12.00AM respectively).

Tryglicerides have a nocturnal acrophase in the first and the second group (05.56.00AM and 05.48.00 AM respectively); (Table 4).

Table 4. Mesor (M), with confidence interval (CI) at 95%, Amplitude (A) and Acrophase (Φ), in hours, with confidence interval (CI) at 95% of parameters resulted periodic for the circadian rhythm, in male Sprague-Dawley rats of the three groups.

PARAMETER	I GROUP MALE				
	M	**C.I. 95%**	**A**	**Φ**	**C.I. 95%**
Cholesterol (mg/dL)	64.78	(62.13-67.42)	6.68	03.48.00AM	(00.00.00AM-07.36.00AM)
Tryglicerides (mg/dL)	75.58	(71.44-79.72)	11.10	05.56.00AM	(02.20.00AM-09.32.00AM)
	II GROUP MALE				
	M	**C.I. 95%**	**A**	**Φ**	**C.I. 95%**
Cholesterol (mg/dL)	51.46	(47.50-55.42)	9.15	04.12.00AM	(00.00.00AM-08.24.00AM)
Tryglicerides (mg/dL)	77.24	(73.12-81.36)	21.51	05.48.00AM	(04.08.00AM-07.28.00AM)
	III GROUP MALE				
	M	**C.I. 95%**	**A**	**Φ**	**C.I. 95%**
Glycaemia (mg/dL)	109.71	(105.61-113.82)	13.37	03.08.00AM	(00.28.00AM-05.48.00AM)

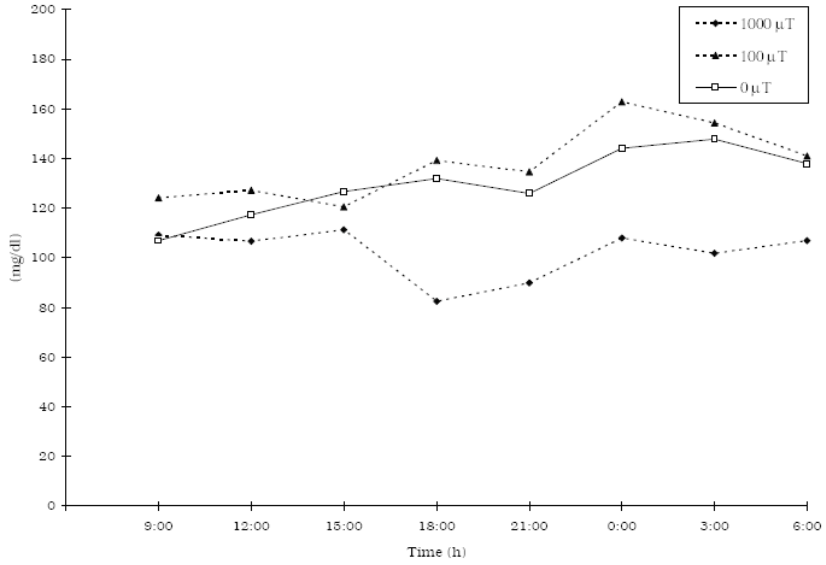


Fig. 1. Glycaemia in female rats

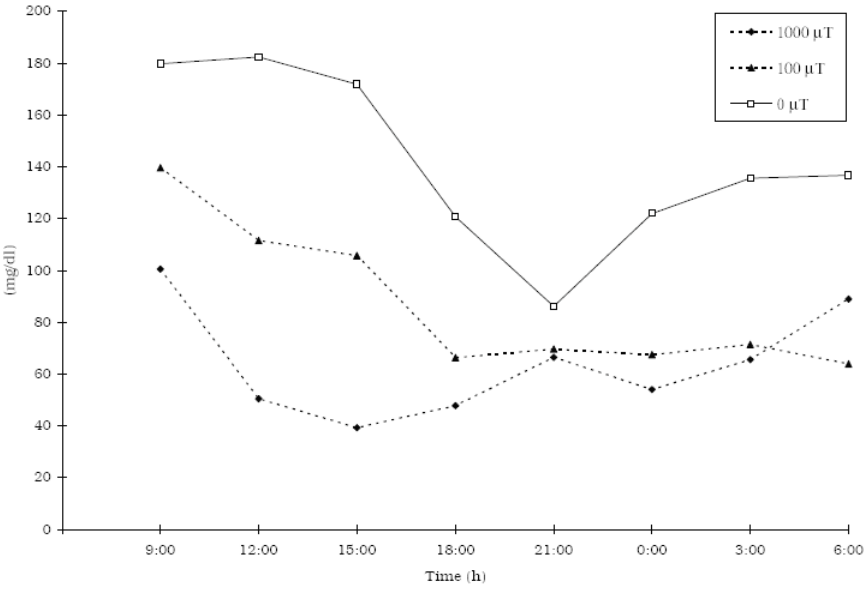


Fig. 2. Triglyceridhaemia in female rats

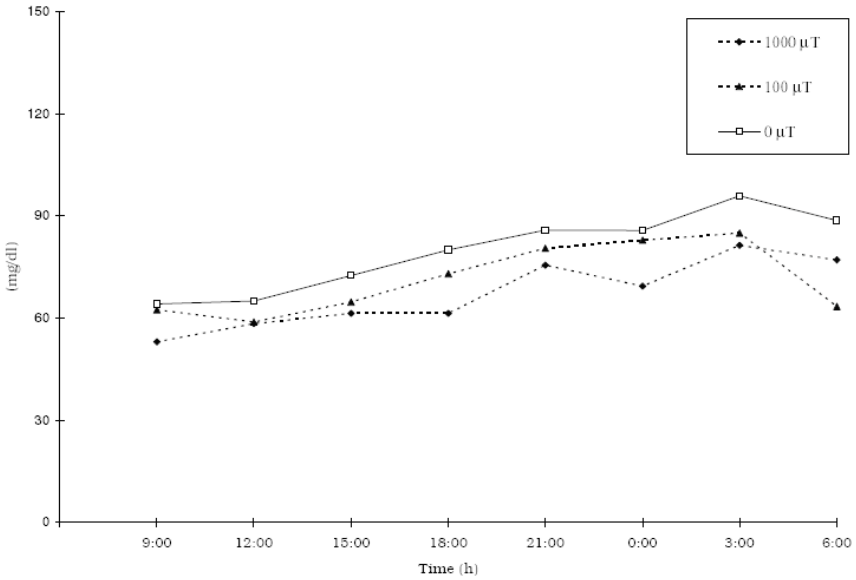


Fig. 3. Cholesterolhaemia in female rats

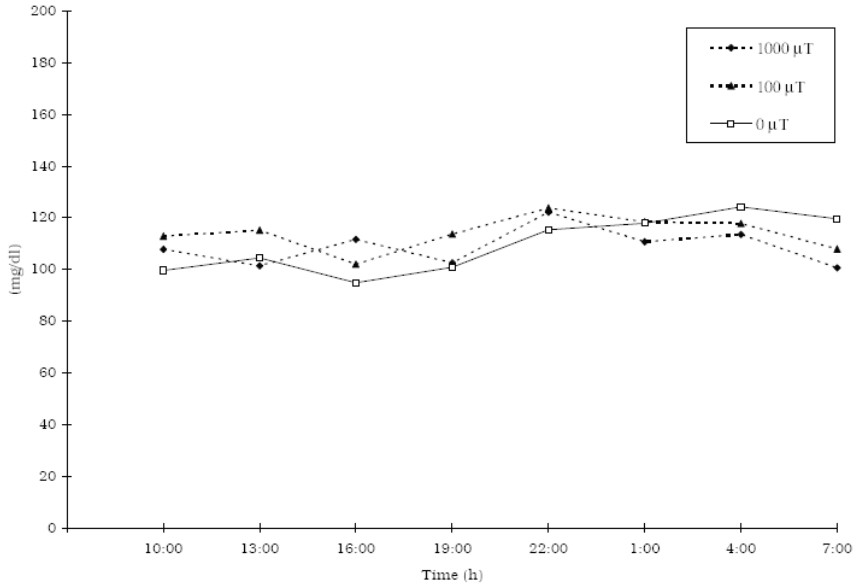


Fig. 4. Glycaemia in male rats

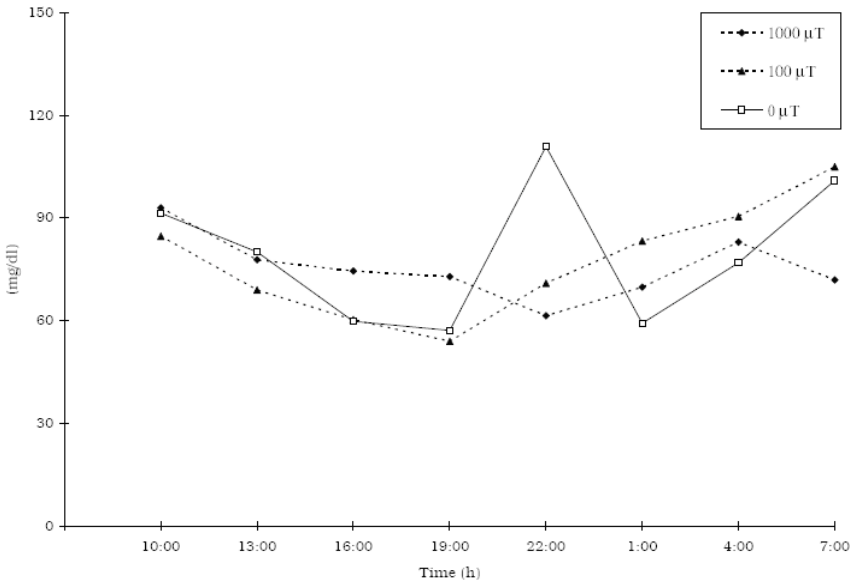


Fig. 5. Triglyceridhaemia in male rats

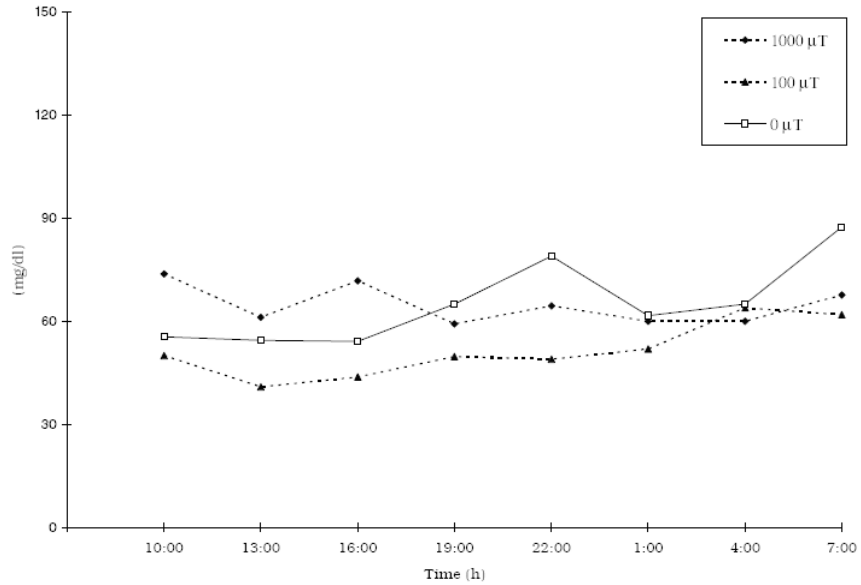


Fig. 6. Cholesterolhaemia in male rats

Discussion and Conclusions

Rats are nocturnal animals, therefore, as described in previous sections, the circadian rhythm of their energetic metabolism assures suitable responses to the energetic requirements of the organism active phase which onsets in the evening. Glucose blood concentration increases to allow appropriate rapid responses of all situations rats may run into during their activities (e.g. social contacts, dangers, hunting, feeding), whereas during resting phase which takes place during the day, lipids metabolism is activated to allow the organism to accelerate fat oxidation and so fat consumption. So in physiologic conditions glycaemia and cholesterol have acrophases during the night whereas tryglicerides blood concentration has acrophase during the day.

The results of this study highlight that the circadian rhythms of some examined energetic metabolism parameters (glycaemia, total cholesterol and triglycerides) are inverted in rats exposed to 50 Hz magnetic sinusoid fields with an intensity of 1000 μT and 100 μT.

In fact 1000 μT exposed female rats have glucose acrophase in the morning (07.52.00AM) whereas females of the second group (exposed to 100 μT), and the control group maintain a physiologic acrophase at night (01.00.00AM and 00.08.00PM). As far as concerned triglycerides and cholesterol on female rats, triglycerides maintain a physiologic acrophase during the day, (10.08.00AM, 12.08.00AM, 10.36.00AM for the first, second and control group respectively) whereas cholesterol maintains acrophase during the night (01.32.00AM, 11.24.00PM, 00.44.00PM for the first, second and control group respectively). On male rats,. the glycaemic circadian rhythm is lost in 1000 μT and 100 μT exposed animals. The control maintains glucose acrophase at night (03.08.00AM). Time has a significant effect only for triglycerides and total cholesterol in the two treated group Cholesterol have a physiologic night-time acrophase in exposed groups at 03.48.00AM (1000 μT) and 04.12.00AM (100 μT). On the contrary, triglycerides inverted their physiologic circadian rhythm in treated animals; in fact they have acrophase in the dark phase at 05.56.00AM (1000 μT) and at 05.48.00AM (100 μT).

It is hypothesized that these changes may be due to an alteration of SCN activity which controls glucose tolerance (independent of insulin) by changing the translocation of the peripheral glucose transporters. This translocation depended on central nervous output as well as on peripheral factors like membrane fluidity (Challet E *et al.*, 2004; la Fleur SE, 2003). Besides the molecular mechanism used by circadian clock to control lipid metabolism is based on many clock-genes and many lipid metabolism-related clock-

controlled genes exhibit circadian oscillations. Since some studies have reported DNA strand breaks induced by ELFEMF both *in vivo* and *in vitro* (Lai H *et al.*, 2004), it is possible to think to an incorrect feed-forwarding information for determinable parameters with temporal defined variations (rhythms) as those examinated in this study.

Moreover the effects of ELFEMF on animal and human bodies are related not only to the characteristics of the magnetic field but also to the body size. These results suggest that in humans, or in animals of larger body size than those studied in the experiment, the effects of ELFEMFs could induce changes in circadian rhythms of some energetic metabolism parameters. Potentially these variations could also result with lower intensity magnetic fields expositions. Disturbances in the circadian rhythm of glucose regulation and lipid metabolism may have severe consequences and may even lead to disease, including metabolic syndrome, type 2 diabetes and obesity (Bartol-Munier I *et al.*, 2006) that are serious public health problem strictly correlated to other serious deseases like heart failure and tumours.

However factors as sex, age and the exposition to agents affecting both central functionality and the related efferent nervous information warrant further investigations.

Experiment 2: Effects of radiofrequency electromagnetic fields (1.8 GHz) on circadian rhythms of some blood parameters in Sprague-Dawley rats

Aim

Every organism has electric oscillating activities which store energy like neuronal circuits in the brain that emitted electromagnetic waves of various frequencies depending on the brain state (sleeping, waking, REM phase, non-REM phase); cardiac circuits; neuromuscular circuits; circuits regulating circadian rhythms. In fact numerous physiological functions have rhythmic course with various frequency (ultradian, circadian, circatrigentan, circannual). In depending of these rhythmic metabolic processes some hematological parameters can show variations that have rhythmic course themselves. Biochemical evaluation of rhythmic parameters indicates the stability of organ with clocked functions and the good homeostatic condition of the organism which is the base of its health and its possibility to effectively resist to diseases. Besides extremely low frequency electromagnetic fields are associated to the brain electrochemistry, calcium fluxes and neurotransmitters systems as well as high frequency electromagnetic fields govern cellular processes like cellular division. An exogenous electromagnetic wave with a frequency near those of biological circuits could influence physiological processes in many ways. Hence, it is clearly possible that exposure to RFEMF, which can exert forces on fixed and moving charges, might have the potential to modulate biological function causing or exacerbating disease in animals and humans. The RFEMF would have to trigger an initial transduction step, and then also begin a cascade of sequential steps that leads to a disease outcome. The first step might be also alterations of circadian rhythms that could increase oxidative stress in organs with clocked function like liver, brain and so on. These alterations could be easily demonstrated analysing hematic rhythmic parameters like glycaemia, cholesterol and tryglicerides that indicate the good activity state of energetic metabolism and so of many organs involved (e.g. SCN, liver, pancreas).

As described in previous chapters, radiofrequency electgromagnetic fields (1.8 GHz) are largely investigated because of their great diffusion, their potential health effects, especially carcinogenic risk and the uncertainty about their mechanisms of action on living organisms. Among the many hypotheses, MW/RF electromagnetic fields may influence ionic fluxes throughout cellular membrane altering homeostatic balance, producing

metabolic changes in the physiological activity of organism cells, in particular those of central nervous anatomic districts that are involved in the regulation of metabolic rhythms. In the framework of the Ramazzini Foundation mega-experiment aimed to evaluate RFEMF (1.8 GHz) long-term effects, in particular carcinogenic effects, a satellite experiment was performed to determine the interactions between RFEMF (1.8 GHz) and circadian rhythms of the three most important blood parameters of energetic metabolism (glycaemia, total cholesterol and triglycerides) in *Rattus norvegicus*: Sprague-Dawley Rats.

Materials and Methods

1 Experimental Animals.

1.1 Sprague-Dawley Rats: Rats came from the strain, reared in the last 30 years in the CRCCM. This colony has steady biological characteristics and so it can give many information about expected neoplastic and non-neoplastic pathologies (more than 15,000 historical controls). Besides Sprague-Dawley rats are universally recognized as a good animal model in toxicological and carcinogenic studies. The animals used in this experiment are the out-bred rats of the experiment BT1CEMRF. BT1CEMRF selected rats are undergone to blood collections at 56 weeks of age.

1.2 Animals Identification: Animals are identified by ear punch using Jackson Laboratory system, weighed and divided according to sex and experimental group at 4-5 weeks of age.

1.3 Cages: Rats are housed in groups of 5 in makrolon cages (41x25x15 cm) with plastic tops and a shallow layer of white wood-shavings as bedding. Wood-shaving is regularly analyzed to exclude the presence of the most common chemical pollutant. Every cage is identified by a label which specifies experiment number, sex, experimental numbers and pedigrees of the animals in the cage.

1.4 Environment Conditions: The environment conditions are those of the experiment BT1CEMRF; 22 ± 3°C and 40-60% relative humidity, with a light/dark cycle of 12 hours. Temperature and humidity are daily recorded.

1.5 Food and Water: Animals are fed *ad libitum* with a pellet feedstuff called "Corticella" from "Laboratorio Dottori Piccioni". Feedstuff is analyzed every six months to verify its nutritional composition and the absence of contaminants like pesticides, metals, estrogenic compounds, nitrosamines and aflatoxins. Analyses results are registered. Feedstuff is utilized until three months after production date; production day and the last supplying day are recorded as well as packing list of every stock and labels of each batch. Drinking water from the local aqueduct is analyzed every six months to identify the presence of possible bacterial or chemical pollutant. Water is available *ad libitum*.

1.6 Clinical and Behavioural Controls: Animal behaviour and health are checked three times a day (2 times on Sunday). Ill animals are isolated and taken under constant control. Generally drugs are not supplied. Infective pathologies' description and their course are recorded in a specific register. As far as animal welfare is concerned, the experiment is conducted in the respect of Dlgvo 116/92. Animals chosen to have blood collection are clinically healthy.

2 Treatment.

2.1 Characteristics of the expositive system (RFEMF-1.8 GHz): the expositive system is the same described in the experiment BT1CEMRF.

2.2 Exposure Conditions: animals are exposed at RFEMF (1.8 GHz) from the 12° day of their embryonic life until their spontaneous death, for 19 hours a day. The treatment is stopped 4 hours in the morning to allow cleaning procedures and animals health monitoring and 1 hour in the evening to have the last animals health check.

3 Experimental Plan. (Table 1).

3.1 Experimental Groups: as the antenatal treatment and being the experimental animals of BT1CEMRF, rats are already divided in the three experimental groups:

- Group I: 12 rats (6 males and 6 females) exposed until spontaneous death to RFEMF (1.8 GHz), 50 V/m, 19 hours a day, starting from 12° day of embryonic life.
- Group II: 12 rats (6 males and 6 females) exposed until spontaneous death, to RFEMF (1.8 GHz), 25 V/m, 19 hours a day, starting from 12° days of embryonic life.
- Group IV: 12 rats (6 males and 6 females) not exposed to any artificial electromagnetic field (control group).

4 Experiment Management.

4.1 Water Consumption: starting from 6 weeks of age, individual water consumption is measured on the first 100 animals of each group (50 males and 50 females) of the main experiment BT1CEMRF, once a week for 4 weeks and then every 4 weeks until 110 weeks of age.

4.2 Food Consumption: starting from 6 weeks of age, individual food consumption is measured on the first 100 animals of each group (50 males and 50 females) of the main experiment BT1CEMRF, once a week for 4 weeks and then every 4 weeks until 110 weeks of age.

4.3 Body Weight: starting from 6 weeks of age, weight is recorded for each animal every week for 4 weeks and then every 4 weeks until 110weeks of age, afterwards every 8 weeks until experiment conclusion.

4.4 Clinical Controls: animal general health conditions are checked three times a day, except on Sunday (just twice). If there are changes in the clinical control routine, they are reported. Ill or dying animals are isolated and reported to the veterinary. All died animals are autopsied in 24 hours. Besides, starting from 6 weeks of age, animals are clinically examined to diagnose pathological changes. Clinical controls are executed weekly for the first 4 weeks and then every 2 weeks until the end of the experiment.

4.5 Blood Collection: Blood samples (0,5ml each) are collected from each experimental animals, every 3 hours around the clock, using 400µl test-tubes containing heparin. The retro-orbital plexus is the ideal site for periodic sampling; this methods has been shown to be reliable for the repeated collection of blood samples. Collection from this site must be conducted under anesthesia to reduce pain and stress to the animal. The animals are anesthetized with ethyl ether. Blood is collected using the fine end of a Pasteur pipette. The tube is inserted into the orbit of the eye at the anterior angle formed by the lids and the nicitating membrane. A short thrust past the eyeball will make the tube enter the slightly resistant horny membrane of the sinus. The tube can be rotated slightly as it is inserted. Once the sinus has been punctured blood will fill the tube. Once the tube contains the blood volume required, the blood can be poured out into the test-tubes containing heparin. If the flow stops, the tube can be pulled out or advanced slightly to reestablish flow (Hitzelberg R *et al.*, 1985; Johnson MD, 2007).
Low intensity red light was used during the night to avoid retina-hypothalamic tract stimulation.

4.6 Laboratory Analyses: Immediately after collection, blood samples are centrifuged (3000 rpm) for 10 minutes to obtain plasma (0.25 ml). Plasma is collected and stocked into cryovials to be freezed in fluid nitrogen and kept at -70 °C. Then glycaemia, total plasma cholesterol and triglycerides were determined using an autoanalyzer (Hitachi 911 Plus).

4.7 Statistical Analyses: Statistical elaboration of the data was based on the average values obtained at the various time points (equidistant 3 hours) for each animal group divided by sex, since the intra-group variance is not significant. Based on these average values, all the results were expressed as mean ± SD. One-way repeated measures analysis of variance (ANOVA) was used to determine significant difference. p values < 0.05 were considered statistically significant. SNK (*Statistical Newman-Keuls)* test was applied for post-hoc comparison. Besides a trigonometric statistic model has been applied in order to analytically describe the periodic phenomenon, by individuating the main parameters that characterize it. Therefore for each variable of each animal, a cosine wave was fitted to the data points according to the function $Yt = A + M \times \cos(qt + j)$, where Yt denotes each

data point in the time series, M is the mean level of the rhythm, called also MESOR (Midline Estimating Statistic of Rhythm). It represents the intermediate value between the highest and the smallest values of a function used to describe a rhythm, expressed in the unit of the relative considered parameter and with the fiduciary limit at 95%. A is the amplitude which identifies the difference between the maximum and the minimum level in a determinate period, and expressed in the same unit of the relative Mesor. *qt* is the trigonometric angle (in degrees) corresponding to time *t*, and *j* is the angle displacement for the acrophase. The acrophase of a rhythm (*u*) was determined by an iterative curve-fitting procedure based on the single cosinor procedure (Nelson W *et al.*, 1979). Acrophase and batiphase respectively represent the superior and the inferior point of a model which is in relation with a reference phase, chosen and expressed in hours, with a confidence interval at 95%. Cosine waves were then fitted to the averaged 24-h rhythm, and the time corresponding to the peak of the best-fitting cosine wave was taken as the acrophase of the rhythm. So the value of *u* was determined by iteration: the true value of *u* was considered to be the one that produced the smallest sum of squares of the deviations between iterated cosine functions and the raw data.

Table 1. Experiment Plan.

Group N.	Treatment[a] (ELFEMF-50Hz)			Experiment lasting[b]	Animals[d]			
	EMF[c] (V m^{-1})	Start	Period	(hours)	Sex	N.	Experimental	Pedigree N.
I	50	embrionic life	life span	24	F	6	168	192729
							179	192759
							184	192772
							187	192784
							193	192797
							201	192813
I	50	embrionic life	life span	24	M	6	317	192456
							342	192502
							407	192631
							468	192766
							498	192802
							507	192815
II	25	embrionic life	life span	24	F	6	1156	193295
							1169	193319
							1171	193324
							1182	193338
							1191	193350
							1201	193365
II	25	embrionic life	life span	24	M	6	1307	192981
							1366	193085
							1472	193290
							1493	193332
							1500	193355
							1506	193370
IV	0	-	-	24	F	6	3363	195193
							3367	195202
							3373	195213
							3382	195231
							3394	195247
							3405	195266
IV	0	-	-	24	F	6	3516	194492
							3521	194504
							3527	194514
							3534	194523
							3544	194535
							3671	194772

[a] Treatment starts 12° day of embrionic life (15/10/2005) exposing pregnant female rats. Animals treatment is life span for 19 hours/day
[b] Blood collections are effectuated every 3 hours around the clock on the same animals at 56 weeks of age
[c] EMF: electromagnetic field
[d] Total animal number:36 rats

Results

Statistic data analyses have utilized single values of blood samples executed in different time points (equidistant three hours), for each group divided by sex (Fig. 1-6).
ANOVA application points out a significant time effect only for glycaemia on female rats of the second and control group. ($P<0.05$ is considered statistically significant) (Table 1).

Table 1. ANOVA Results. Females. I, II and III Group.

	Glycaemia		**Cholesterol**		**Triglycerides**	
	$F_{7,70}$	$p<$	$F_{7,70}$	$p<$	$F_{7,70}$	$p<$
I group	-	-	-	-	-	-
II group	3.01	0.008	-	-	-	-
III group	7.07	0.001	-	-	-	-

Whereas on male rats, ANOVA points out a significant time effects only for triglycerides in all groups (Table 2).

Table 2. ANOVA Results. Males. I, II and III Group.

	Glycaemia		**Cholesterol**		**Triglycerides**	
	$F_{7,70}$	$p<$	$F_{7,70}$	$p<$	$F_{7,70}$	$p<$
I group	-	-	-	-	13.77	0.001
II group	-	-	-	-	11.67	0.0001
III group	-	-	-	-	17,83	0.0001

"Cosinor" analyses, applied to periodic parameters, defined acrophases (expressed in hours). On female rats, glycaemia has physiologic nocturnal acrophase in the second group (00.32.00AM). Its circadian rhythm is completely lost in the first group exposed at 50 V/m (Table 3).
On male rats, tryglicerides have a physiologic diurnal acrophase in all three group.

Table 3. Mesor (M), with confidence interval (CI) at 95%, Amplitude (A) and Acrophase (Φ), in hours, with confidence interval (CI) at 95% of parameters resulted periodic for the circadian rhythm, in Sprague-Dawley rats of the three groups.

PARAMETER	I GROUP MALE				
	M	**C.I. 95%**	**A**	**Φ**	**C.I. 95%**
Tryglicerides (mmol/L)	1.10	(1.07-1.13)	0.23	08.12.00AM	(07.12.00AM-09.12.00AM)
	II GROUP FEMALE				
	M	**C.I. 95%**	**A**	**Φ**	**C.I. 95%**
Glycaemia (mmol/L)	8.00	(7.74-8.28)	0.60	00.32.00AM	(08.00.00PM-05.00.00AM)
	II GROUP MALE				
	M	**C.I. 95%**	**A**	**Φ**	**C.I. 95%**
Tryglicerides (mmol/L)	0.79	(0.74-0.83)	0.22	07.24.00AM	(05.40.00AM-09.08.00AM)
	III GROUP FEMALE				
	M	**C.I. 95%**	**A**	**Φ**	**C.I. 95%**
Cholesterol (mmol/L)	2.33	(2.66-2.40)	0.24	09.48.00PM	(07.12.00PM-00.12.00AM)
	III GROUP MALE				
	M	**C.I. 95%**	**A**	**Φ**	**C.I. 95%**
Tryglicerides (mmol/L)	0.80	(0.73-0.88)	0.24	09.00.00AM	(06.24.00AM-11.36.00AM)

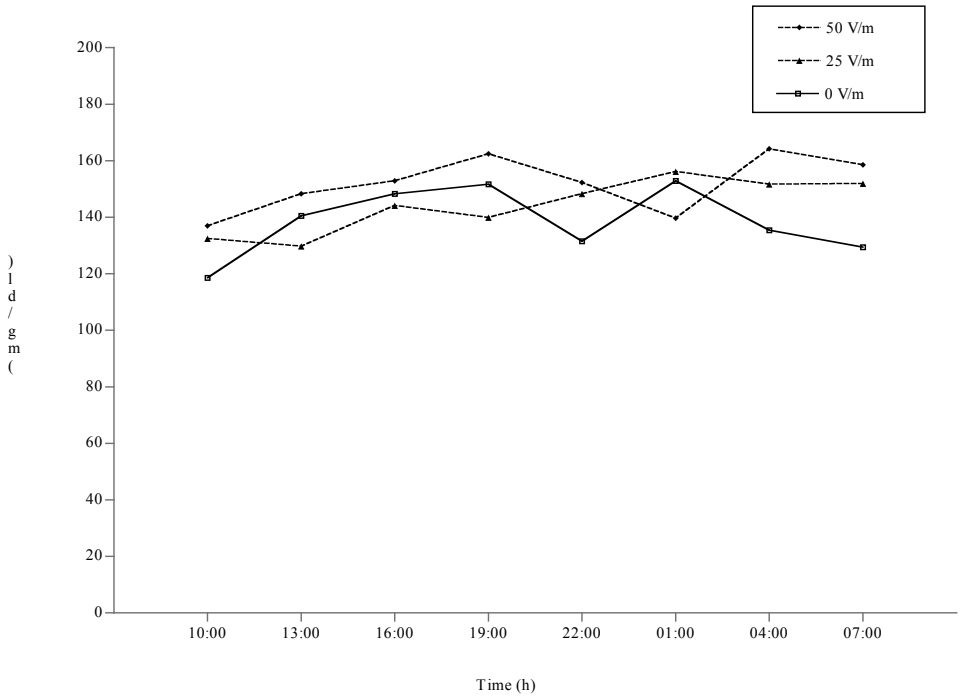


Fig. 1. Glycaemia in female rats.

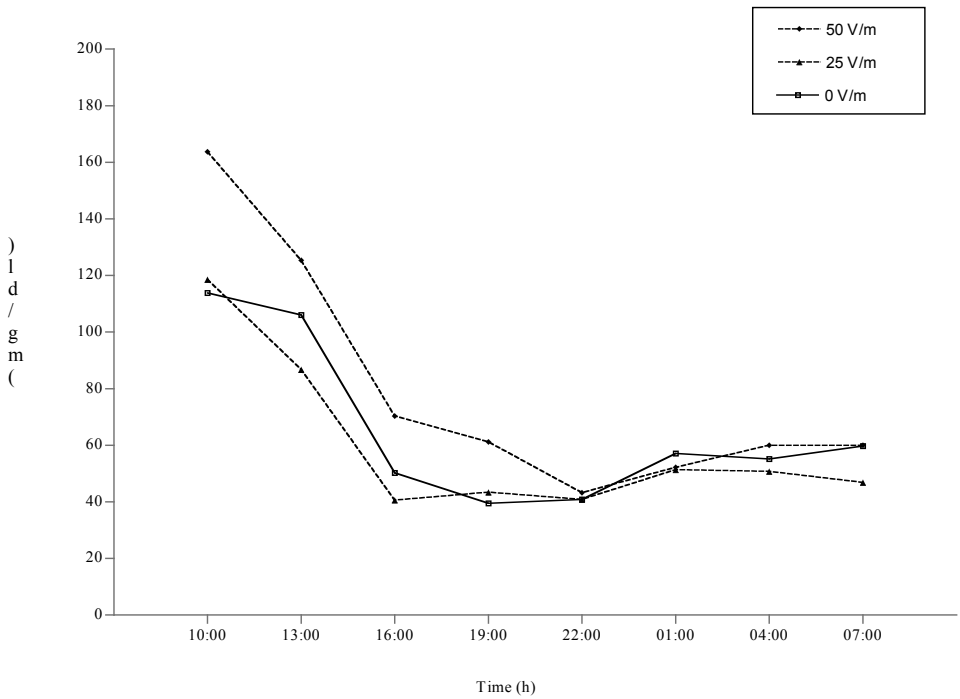


Fig. 2. Triglyceridhaemia in female rats.

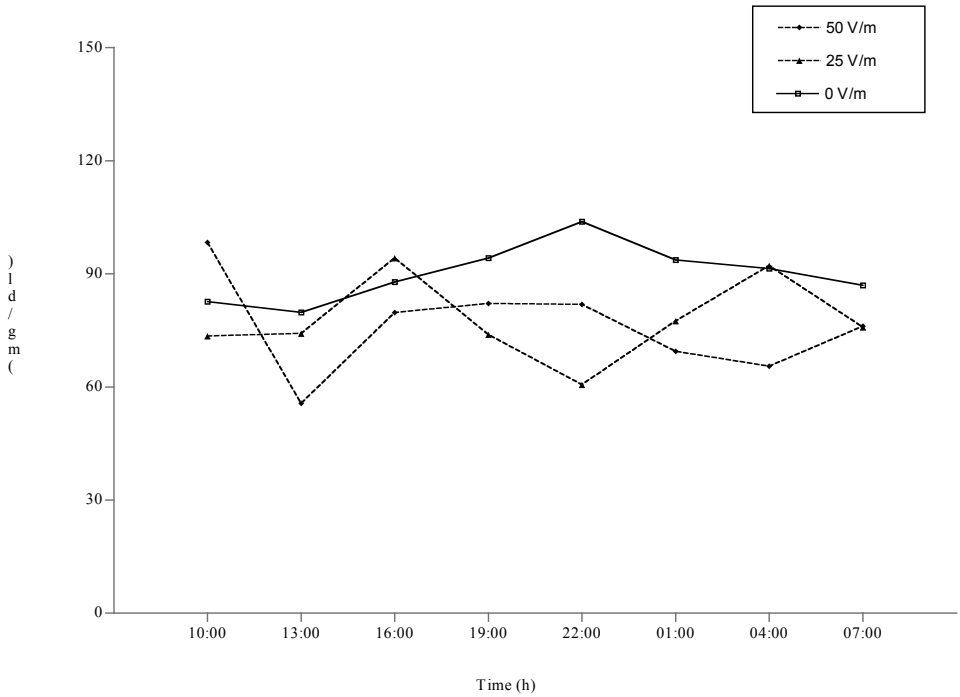


Fig. 3. Cholesterolhaemia in female rats.

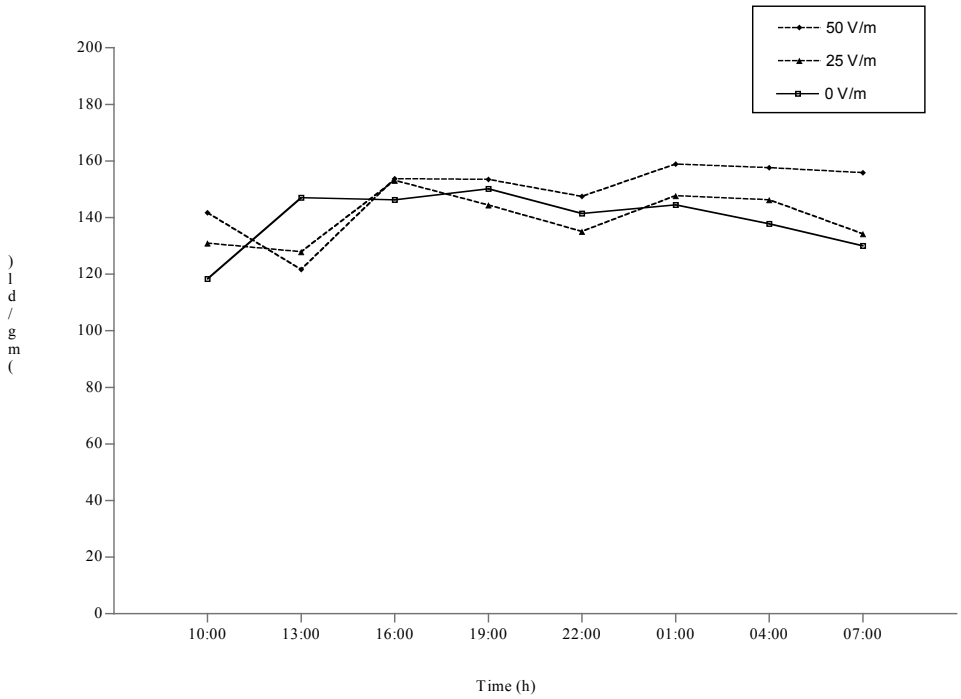


Fig. 4. Glycaemia in male rats.

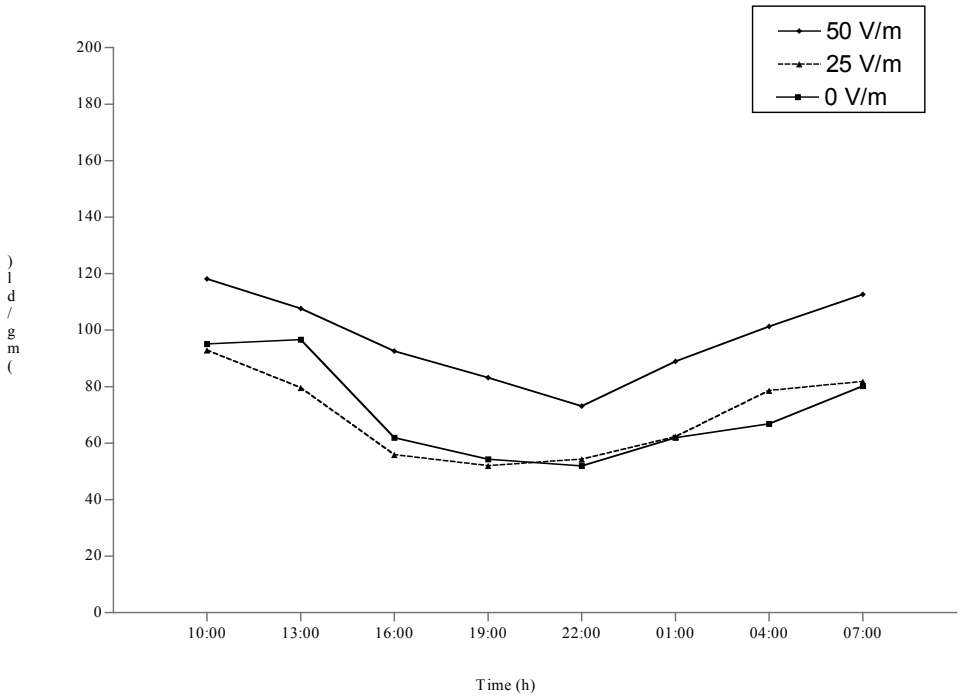


Fig. 5. Triglyceridhaemia in male rats.

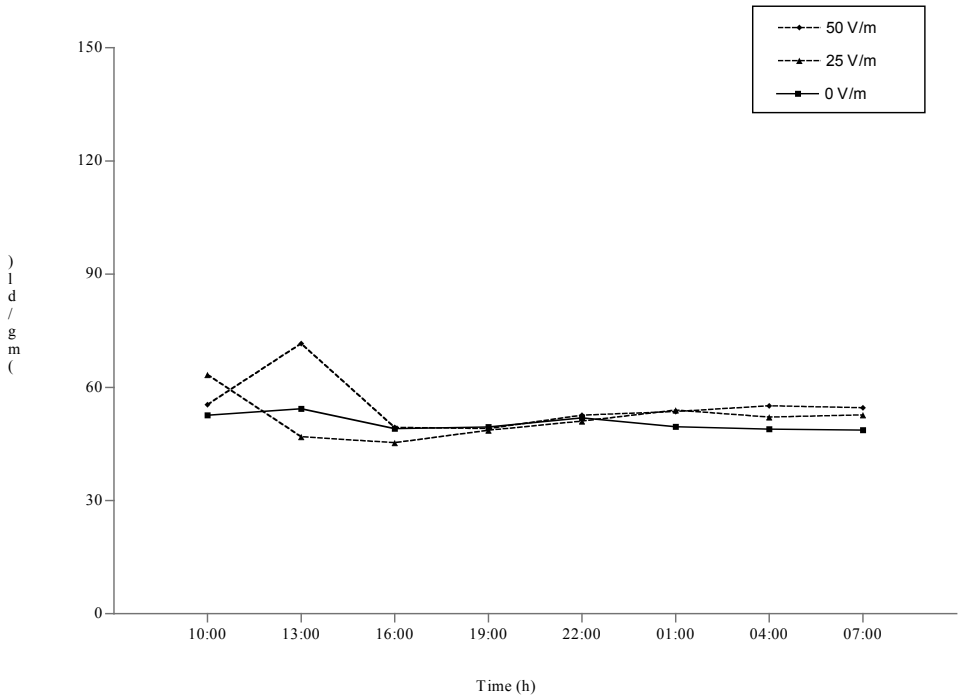


Fig. 6. Cholesterolhaemia in male rats.

Discussion and Conclusion

The results of this study highlight that only few of the examined parameters (glycaemia, total cholesterol and triglycerides) maintain a rhythmicity and are periodic. On female rats glycaemia has a physiologic circadian rhythm with acrophase during the night at 00.32.00AM in the second group. Whereas in the first group exposed to 50 V/m, glycaemia looses its circadian rhythm. As far as the lipid metabolism is concerned, triglycerides are not periodic both on treated and control female rats. Total cholesterol is not periodic in treated female rats whereas in the control group, it has a physiologic acrophase at night (09.48.00PM). Besides, considering the mean values trend of cholesterolhaemia, it seems that in female rats exposed to 25 V/m cholesterol reaches the lowest plasma concentration during the night, in contrast with the physiologic trend of the control group. On male rats only triglicerides have a physiologic circadian rhythm with acrophase during the day in the three groups, at 08.12.00AM for the first group (50 V/m), at 07.24.00AM for the second group (25 V/m) and 09.00.00AM for the control group. Glycaemia and cholesterol don't give any information because they have not periodicity in the three groups.

Thanks to these results, it seems that "Far-field" chronic exposure to RFEMF may interfere with the sophisticated electromagnetic circuits regulating circadian rhythms by non-thermal mechanisms, causing a loss of rhythmicity in periodic parameters of the energetic metabolism and so a possible loss of homeostatic balance in many organs involved (e.g. SCN, liver, pancreas).

GENERAL CONCLUSION

Nowadays extremely low frequency electromagnetic fields (ELFEMF-50 Hz) and radiofrequency electgromagnetic fields (RFEMF-1.8 GHz) are largely investigated because of their great diffusion, their potential health effects, especially carcinogenic risk and the uncertainty about their mechanisms of action on living organisms. Among the many hypotheses, ELFEMFs and RFEMFs may influence ionic fluxes throughout cellular membrane altering homeostatic balance, producing metabolic changes in the physiological activity of organism cells, in particular those of central nervous anatomic districts that are involved in the regulation of metabolic rhythms. This study demonstrate the existence of an interaction between electromagnetic fields and the circadian rhythms of some blood parameters of the energetic metabolism in Sprague-Dawley rats, especially in female rats, affecting both the glucidic and the lipidic metabolism and as a consequence, the homeostatic balance of all connected organs like brain, liver and pancreas. Our conclusions are in agreement with the results of other studies which reported that magnetic fields can affect a variety of behaviours and physiological functions in animals (Abbasi M *et al.*, 2007; Chuian OM *et al.*, 2004; Izumi R *et al.*, 2001; Selamaoui B *et al.*, 1999).

Even if other factors as sex, age and the exposition to agents affecting both central functionality and the related efferent nervous information warrant further investigations, these results suggest that chronic exposure to electromagnetic fields may have insidious health effects on animals, but potentially on humans too, and they could promote the onset of serious degenerative deseases.

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GENERAL ABSTRACT

The aim of this research was to investigate the biological effects of electromagnetic fields on the circadian rhythms of three blood parameters: glycaemia, total cholesterol and triglycerides in Sprague Dawley rats. Glycaemia, total cholesterol and triglycerides could be good homeostatic balance indicators of energetic metabolism in a living organism.
This research is conducted in the framework of two mega-experiments aimed to evaluate the long-term effects, especially carcinogenic effects, of ELFEMF-50 Hz and RFEMF-1.8 GHz.

-Experiment 1-

The study was carried on 18 male and 18 female healthy Sprague-Dawley rats of 84 weeks of age, exposed to ELFEMF-50Hz for 19 h/day, starting from 12° days of their embryonic life. They were divided in three groups of 12 rats each (6 males and 6 females). Each group was exposed to a different magnetic field intensity (1000 μT, 100 μT and 0 μT).
Blood samples (0,5 ml each) were collected by puncting the retro-orbital plexus of each experimental animals, every 3 hours around the clock. Blood was stocked in 400μl test-tubes containing heparin. Blood glucose level was immediately determined after the collection using a portable glucometer (Accu-Check Compact-Roche). Total plasma cholesterol and triglycerides were determined using an autoanalyzer (Hitachi 911 Plus). Statistical elaboration of the data was based on ANOVA, SNK (*Statistical Newman-Keuls*) and Cosinor analysis.
The results of this study highlight that the circadian rhythm of glycaemia in female rats exposed to 1000 μT and the circadian rhythm of tryglicerides in male rats exposed to 1000 μT and 100 μT are inverted compared to the physiologic ones; whereas the circadian rhythm of glycaemia in male rats exposed to 1000 μT and 100 μT is totally lost. These results suggest that in humans, or in animals of larger body size than those studied in the experiment, the effects of ELFEMFs could induce changes in circadian rhythms of some energetic metabolism parameters. Potentially these variations could also result with lower intensity magnetic fields expositions. Disturbances in the circadian rhythm of glucose regulation and lipid metabolism may have severe consequences and may even lead to degenerative diseases.

However factors as sex, age and the exposition to agents affecting both central functionality and the related efferent nervous information warrant further investigations.

-Experiment 2-

The study was carried on 18 male and 18 female healthy Sprague-Dawley rats of 56 weeks of age, exposed to RFEMF-1.8 GHz for 19 h/day, starting from 12° days of their embryonic life. They were divided in three groups of 12 rats each (6 males and 6 females). Each group was exposed to a different field intensity (50 V m^{-1}, 25 V m^{-1} and 0 V m^{-1}).

Blood samples (0,5 ml each) were collected by puncting the retro-orbital plexus of each experimental animals, every 3 hours around the clock. Immediately after collection, blood samples are centrifuged (3000 rpm) for 10 minutes to obtain plasma (0.25 ml). Then glycaemia, total plasma cholesterol and triglycerides were determined using an autoanalyzer (Hitachi 911 Plus).

Statistical elaboration of the data was based on ANOVA, SNK (*Statistical Newman-Keuls*) and Cosine analysis.

The results of this study highlight that the circadian rhythms of glycaemia, trygliceridhaemia and cholesterolhaemia in female rats exposed to 50 V/m are absent as well as cholestrolhaemia in female rats exposed to 25 V/m. Lipid metabolism of the other groups, maintains its physiologic circadian rhythm.

Thanks to these results, it seems that “Far-field” chronic exposure to RFEMF-1.8 GHz may interfere with the sophisticated electromagnetic circuits regulating circadian rhythms by non-thermal mechanisms, causing a loss of rhythmicity in periodic parameters of the energetic metabolism and so a possible loss of homeostatic balance in many organs involved (e.g. SCN, liver, pancreas).

It is concluded that ELFEMF-50 Hz and RFEMF-1.8 GHz could interact with biological systems causing an alteration of circadian rhythmicity of glycaemia, cholesterolhaemia and trygliceridhaemia in rats and potentially in humans too after a long term exposure.

SOMMARIO

L' obiettivo di questo studio è la valutazione dei possibili effetti biologici dei campi elettromagnetici sui ritmi circadiani di tre parametri ematici: glicemia, colesterolo totale e trigliceridi in ratti Sprague-Dawley. Questi tre parametri possono essere ottimi indicatori dell'equilibrio omeostatico esistente in un organismo vivente per quanto riguarda il suo metabolismo energetico.
Questa ricerca è stata condotta nell'ambito di due ampi progetti volti a valutare gli effetti biologici e specialmente cancerogeni dei campi elettromagnetici a bassissima frequenza (50 Hz) e delle radiofrequenze (1.8 GHz-GSM).

-Esperimento 1-

Lo studio è stato condotto su 18 maschi e 18 femmine di ratto Sprague-Dawley di 84 settimane di età, in buone condizioni di salute. Questi animali sono stati esposti a campi elettromagnetici a bassissima frequenza (50 Hz) per 19 ore al giorno, a partire dal 12° giorno di vita embrionale. Sono stati divisi in tre gruppi sperimentali di 12 ratti ciascuno (6 maschi e 6 femmine) esposti a diverse intensità di campo (1000 μT, 100 μT e 0 μT rispettivamente).
I campioni di sangue di 0,5 ml ciascuno,sono stati ottenuti per contusione del plesso retro-orbitale. Ogni animale è stato sottoposto a prelievo ogni 3 ore per 24 ore. Il sangue è stato raccolto in provette pediatriche contenenti eparina. La glicemia è stata rilevata immediatamente mediante glucometro portatile (Accu-Check Compact-Roche). Colesterolemia e trigliceridemia sono state analizzate successivamente in laboratorio.
L'elaborazione statisticadei dati si è basatasull'impiego dei seguenti metodi: ANOVA, SNK (*Statistical Newman-Keuls*) e Cosinor.
I risultati di questo studio evidenziano un'inversione dei ritmi circadiani della glicemia in ratti femmina esposti a 1000 μT e della trigliceridemia in ratti maschi esposti a 1000 μT e 100 μT. Inoltre i ratti maschi esposti a 1000 μT e 100 μT perdono completamente anche la ritmicità della glicemia.
Questi risultati suggeriscono che anche in animali di dimensioni maggiori rispetto a quelli qui utilizzati come modello ed eventualmente anche nell'uomo, i campi elettromagnetici a bassissime frequenze possono alterare i ritmi circadiani di parametri indicatori della buona funzionalità del metabolismo energetico. Tali alterazioni possono avere gravi conseguenze

favorendo l'insorgenza di patologie degenerative. Comunque sono necessari ulteriori studi per valutare anche l'influenza del sesso, dell'età e l'esposizione contemporanea ad altri agenti che possono influenzare l'orologio biologico.

-Esperimento 2-

Lo studio è stato condotto su 18 maschi e 18 femmine di ratto Sprague-Dawley di 56 settimane di età, in buone condizioni di salute. Questi animali sono stati esposti a campi elettromagnetici a radiofrequenza (1.8 GHz) per 19 ore al giorno, a partire dal 12° giorno di vita embrionale. Sono stati divisi in tre gruppi sperimentali di 12 ratti ciascuno (6 maschi e 6 femmine) esposti a diverse intensità di campo (50 V/m, 25 V/m e 0 V/m rispettivamente).

I campioni di sangue di 0,5 ml ciascuno,sono stati ottenuti per contusione del plesso retro-orbitale. Ogni animale è stato sottoposto a prelievo ogni 3 ore per 24 ore. Il sangue è stato raccolto in provette pediatriche contenenti eparina. I campioni sono stati immediatamente centrifugati (3000 rpm) per 10 minuti; il plasma (0,25 ml) è stato prelevato e congelato in azoto liquido in attesa delle analisi di laboratorio.

L'elaborazione statistica dei dati si è basata sull'impiego dei seguenti metodi: ANOVA, SNK (*Statistical Newman-Keuls*) e Cosinor.

I risultati di questo studio evidenziano una perdita del ritmo circadiano di glicemia e colesterolemia in ratti femmina esposti a 50 V/m e della colesterolemia in ratti femmina esposti a 25 V/m. Il metabolismo lipidico nei restanti animali non è invece influenzato dal trattamento.

Da questi risultati sembra che l'esposizione cronica "in campo lontano" a campi elettromagnetici a radiofrequenze (1.8 GHz) possa interferire con i soffisticati circuiti elettromagnetici che regolano i ritmi circadiani a livello del nucleo soprachiasmatico dell'ipotalamo, causando la perdita di ritmicita in quei parametri periodici del metabolismo energetico qui analizzati. La conseguenza può essere una perdita dell'equilibrio omeostatico in molti organi coinvolti quali fegato, pancreas e sistema nervoso centrale.

Si può concludere che i campi elettromagnetici a bassissime frequenze (50 Hz) e a radiofrequenze (1.8 GHz) possono interferire con i sistemi biologici alterando il ritmo circadiano di glicemia, colesterolemia e trigliceridemia nei ratti e potenzialmente anche nell'uomo a seguito di esposizione cronica.

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