UNIVERSITÀ POLITECNICA DELLE MARCHE
FACOLTÀ DI MEDICINA E CHIRURGIA

Dottorato di Ricerca XXXIII CICLO

in

SALUTE DELL'UOMO

ERGONOMIC ARTIFICIAL LIGHTING: STUDY OF THE CHRONOBIOLOGICAL EFFECTS OF LIGHT RADIATIONS ON HUMAN HEALTH

Relatore: Chiar.mo
Prof. Massimo Bracci

Dottoranda:
Dr.ssa Maria Fiorella Tartaglione

Anno Accademico 2019-2020

Ad Antonio,

A tutti i passi, grandi e piccoli, fatti insieme fin qui.

Ogni mio traguardo è un nostro traguardo.

INDEX

ABSTRACT

Background: The skin is able to absorb external radiations in the range of the visible spectrum. The skin has circadian cycles dependent on both the central circadian system and peripheral clocks of each cell. During the evolution, the selective pressure of the day/night cycle has allowed the conservation of biologically advantageous circadian clocks in organisms until the mammals. The fruit fly is an excellent model organism in the study of biological clocks. This thesis work aims to study the chronobiological effects of light radiation in vitro on keratinocytes (HaCaT cells) and in vivo on *Drosophila melanogaster*.

Methods: The effects of light radiation were studied using LED lamps emitting blue and red light. The exposure to the LED lamps involved three days of light cycles repeating the following four different exposure: 12h Light-12h Dark and 1h Light-23h Dark; high and low intensity. On HaCaT cells, cell viability, the mechanism of apoptosis, the cell cycle, and the expression of circadian genes (clock genes) were investigated. An analysis of cellular proteomics expressed after light stimuli was performed. We examined the effects of light radiation on intracellular ROS and mitochondrial structure. Subsequently, an in vivo experimental model was set up with *Drosophila melanogaster*. The parameters studied were: responses to thermal stresses, effects of paraquat exposure, climbing assay, and clock gene expression analysis.

Results: LED lamps has different biological effect on keratinocytes, in relation to the wavelength. Blue light has an inhibitory effect on cell proliferation. This effect is greater with increasing exposure time and light intensity emitted by the LED source. The assay of apoptosis also confirms that with blue light there is a strong increase in the percentage of apoptotic cells. Cell cycle reveals significant differences only in cells exposed to blue light for 12h high intensity. Blue light can directly synchronize the circadian clocks within keratinocytes. The alterations caused by exposure to cycles of 12h high intensity on proteins demonstrate that the class of oxidoreductases is involved. The concentration of intracellular ROS is significantly higher when cells are irradiated with blue light. Conversely with 12h low intensity light cycles, intracellular ROS

equally increased after both blue and red light. On *Drosophila melanogaster*, we observed that locomotor capacity increased in insects when exposed to blue light. In experiments performed to evaluate the response to thermal stresses, a significant decrease in the number of insects surviving after 24h from high temperature stress was observed in the group exposed to high intensity blue light. In parallel, analysis of recovery time from cold-induced coma showed that insects exposed to blue light were able to wake up faster. Paraquat exposure increased the number of dead insects in those treated with blue light. The clock genes analysis shows an increased expression induced by blue light. This overexpression indicates how blue light may affect the synchronization ability of the central circadian clock in insects. From the evidence obtained in the two experimental models in vitro and in vivo, we conclude that blue light has chronobiological effects in both.

Conclusion: Blue light decreases keratinocyte viability and the effect depends on the exposure time and on the intensity. Keratinocytes possess autonomous regulation of their circadian cycle probably to cope with toxic light insults (blue light) from the external environment. In insects, after exposure to blue light, clock genes are overexpressed and circadian rhythmic functions are altered. LED lamps have an influence on biological systems. The choice of the best lamps for lighting must take into account the physiological circadian biological cycles, as they lead to important physiological functions.

1. INTRODUCTION

1.1 Light radiation: definition and features

The following thesis work will cover the concept of light and its historical development. In the past, humans have wondered about what light is and many great intellectuals came out with theories concerning it. In the seventeenth century a controversary arose: is light a wave or a particle flow?

At Cambridge, Sir Isaac Newton argued that Grimaldi's diffraction phenomena simply demonstrated a new form of refraction which could only be understood if the light was composed of corpuscles (particles) due to the fact that waves do not travel in straight lines. Later on, after conducting several experiments, Newton stated that light is made of corpuscles, not waves. This theory has been considered as the only reliable one until the end of 19th century when Sir Thomas Young clearly demonstrated wave interference by accepting wave theory (Young T. et al., 1807).

Physicists faced a major crisis in the early 19th century: light behaved like waves in some experiments such as interference and diffraction, but also like particles in other experiments, such as photoelectric effect.

After different scientific evidence reached some physicists have theorized that a single photon (particle of light) has a quantum energy (Q_v) that is directly proportional to the frequency (f) (sometimes symbolized by the Greek letter, ν) of the wave; where h is known as 'Planck's Constant':

$$\mathbf{Q_v = h \times f}$$

This led to the concept of 'wave–particle duality'. Physicists ended up realizing that light simultaneously can be a flow of particles and a wave so thus nowadays, we all can affirm that light has a double nature made of waves and particles. It is equally clear that the limits of actual extended visibility goes from about 310 nm in the Ultraviolet to about 1100 nm near infrared, but they depend a lot on the radiance of light source (Sliney, 2016).

Currently the International Commission on Illumination (CIE) provides a technical definition of light on the "International Vocabulary (ILV)'' pin which emphasizes that

light is: "both a characteristic of all sensations and perceptions that is specific to vision, and a radiation that is considered from the point of view of its ability to excite the human visual system".

It is well known that in today's society our lives are completely immersed in radiation of all kinds. The spectrum of visible light represents only a small part of that of electromagnetic waves and varies from 400 to 700 nm depending on the wavelength (fig. 1). The full electromagnetic range extends from X-rays to the deep UV, through the visible light spectrum, and out to the far IR and beyond into microwaves and radio waves. The solar source emits white light perceived by the human eye which unifies all the radiations of the visible spectrum.

The CIE defines two separate systems for measuring light: the photometric and the radiometric systems.

The radiometric system is based upon fundamental physical units. Radiometry, the measurement of electromagnetic radiation, is critically important for many environmental studies and is applicable to the development of illumination sources for commercial and industrial use. Advanced instruments support radiometric measurements in the UV, visible light, and the Near-Infrared from 250-2500 nm. Radiance (L): it is the ratio between the luminous flux emitted by a surface element around that point and the area of the element itself. The luminance of a point on a surface is measured in lux on white.

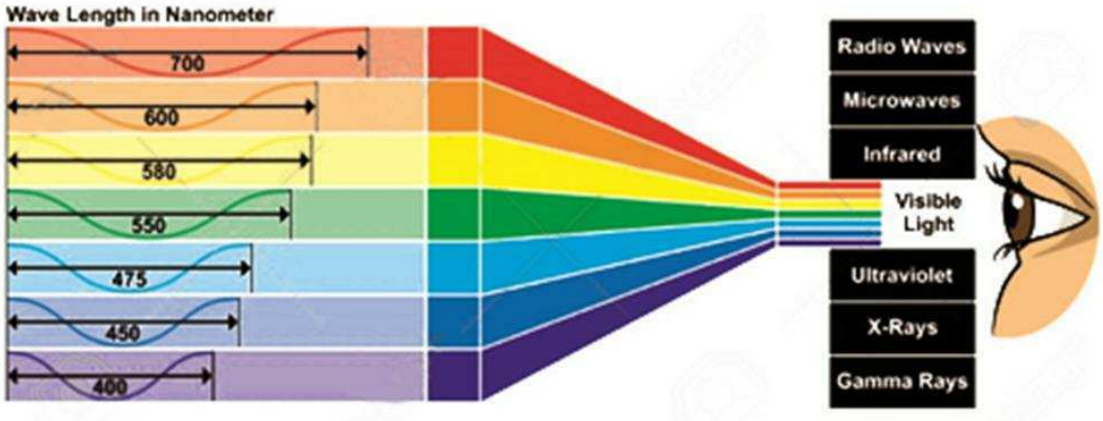

Figure 1. *Components of light radiation. Visible light (700-400 nm) shows wavelength difference between spectrum colours that have different properties. Human eye can perceive only this spectrum; white colour is composed of all colours of the rainbow. Source: https://it.123rf.com/photo_72098113_luce-visibile-con-differenza-di-lunghezza-d-onda-tra-i-colouri-di-spettro-che-danno-propriet%C3%A0-divers.html.*

Photometry concerns the measurement of radiometric sources and their interaction with the human eye (Hengstberger. F., 2006). Spectroradiometry concerns the measurement of absolute radiometric quantities over specific wavelength bands. Radiometry is the measurement of the electromagnetic radiation- or wave- that is generated by the oscillation of photons. The frequency of these waves, or the wavelength (λ), determines the amount of energy carried by the photons, and hence the "colour" of the radiation.

The photometric system is used in lighting design and illumination engineering, it is based upon a standardized (V(λ)) spectral response of daylight (photopic) vision with units of: lumens (luminous power Φv), lux (lm/m2 for illuminance Ev), candelas (lm/sr for luminous intensity Iv), and nits (cd/m2 for luminance Lv, ie, 'brightness').

So the radiometric system is used by physicists to quantify the radiant energy of light regardless of wavelength. Photometric quantities, on the other hand, are used only for visible light.

The design of the quantity and quality of light in a technical way is recent and has given rise to a new branch of the study of lighting: "lighting engineering." The development of lighting engineering came along with that of artificial light sources. The ever-increasing development of light sources has helped transform a marginal component of lighting into an autonomous discipline managed by a specialist. The basic principles and quantities involved in lighting engineering are derived from photometry. When the distance of the measured object can be estimated, photometry can provide information about the total energy emitted by the object: its size, temperature and other physical properties. In defining the amount of light, a distinction must be made between the light energy emitted by a source (intensity) and the flow of light through a given section (or reaching a given surface). For the total light emitted from a source, the 'unit of measure is the candela (cd). Of course, the farther we get from a (point) source, the less light reaches us. So it is important, while comparing different illuminations, to also establish a unit of measure for the light flux (the power passing through the unit area). The unit of measure for photometry is the lumen (lm). Photometric quantities quantify light flux in terms of the response of the human visual system, which has different sensitivities to different wavelengths.

Photometric quantities are divided into qualitative and quantitative.

The quantities are:

- ✓ **Luminous flux (lx)**: it expresses the power of the radiations emitted from a source weighed according to the spectral sensitivity of the human eye, it is therefore the translation of light output into a power. It is a scalar magnitude (φ) and its unit of measurement is the lumen (lm). A 1 cd light source emitting in an angle of 1 steradian produces a luminous flux of 1 lm.

- ✓ **Luminous intensity (I)**: is the ratio of the infinitesimal luminous flux emitted by the source in a given direction to the elementary solid angle. It is a vector magnitude of symbol I expressed analytically as: I = df/dw. Its unit of measurement is the candle (cd) defined as the luminous intensity of a monochrome source with a peak at 555 nm. The knowledge of the light intensity emitted by a source, in different directions, allows to build the photometric solid, which is that geometric figure delimited by a closed surface, formed by the location of the extreme points of segments whose length is proportional to the luminous intensity in that direction and centre of the source. If the photometric solid is known from an artificial source, the value of the luminous intensity in the various directions can be traced from it.

- ✓ **Illumination (E):** it is a scaled size given by the ratio of the luminous flux incident on a surface to the surface area itself: E = F/A. The unit of measurement is lux. A lux is the illumination produced by the flux of a lumen evenly distributed over an area of one square metre.

- ✓ **Luminance(L_v)**: it is the ratio between the luminous intensity emitted in that direction and the emitting surface projected on a plane perpendicular to the direction itself. The unit of measurement (nit) is the candle per square meter (cd/m^2). It is a vector magnitude of symbol and it is indicative of the glare that can induce a source. The right balance of luminances is of great importance in the design of indoor and outdoor lighting systems (e.g.: squares, streets, etc.).

In addition to the quantitative parameters, just analysed, light can also be characterized with other qualitative parameters, as they describe the visual sensation perceived by our eye. Such quantities are generally used to identify artificial lighting sources and they are mainly:

- ✓ **The colour temperature (CT)** of a light radiation is defined as: the absolute temperature of a black body that emits a visible radiation of equal colour to the light emitted by the source, thus describes the feeling of «hot» or «cold» light produced by the shade of light, which is one of the elements that contribute to define the quality of lighting. The chromatic effect produced on the human eye by a source can therefore be described based on the comparison of the light emitted by the analysed source and that one emitted by a black body. The red light corresponds to a low colour temperature, while the blue light corresponds to a high colour temperature.

- ✓ **The chromatic rendering** describes how much an artificial light alters the colour of the illuminated objects and indicates the fidelity with which the light provided by an artificial source is able to reproduce the real colours (from the sunlight). The ability of a light to "render" the colour is measured by comparing the colour rendering of the light in question with the colour rendering of a standard lamp that reproduces natural lighting. This rating system is defined **CRI** (Colour Rendering Index). CRI is a dimensionless number between 0 and 100 where 0 can be considered the minimum and 100 as the maximum value for colour rendering). The more the light source is characterized by a high value of CRI, the more realistic the colours appear.

The retinal exposures depends on: retinal irradiance and retinal illuminance.

- ✓ The **retinal irradiance (Er)** is directly proportional to the radiance (brightness) L of the source being viewed. The retinal irradiance Er in W/cm^2 is:

$$\mathbf{Er = 0.27 \times L \times \tau \times d_e^2}$$

  - L: radiance in $W/cm^2/sr$;
  - τ: transmittance of the ocular media;

- d_e: pupil diameter in cm.

Two persons looking at the same scene can easily have a pupil size sufficiently different to readily have a retinal irradiance differing by a factor of 2 (100%). The retinal irradiance from ambient outdoor illumination is of the order of 0.02–0.1 mW/cm^2 and these levels are just comfortable to view.

✓ The **retinal illuminance** (photometric measure) is luminous flux incident on the retina. The simplified formula is:

$$\mathbf{T = LS}$$

- L: luminance (cd/m^2) of the source viewed;
- S: pupil area in mm^2.

The retinal illuminance T is then given in trolands(td). Retinal illuminance decreases with age due to absorption of the lens and to a reduction in pupil size. (Butterworth-Heinemann, 2009). This unit has been widely used in studies of 'flash blindness' and some areas of vision research (Sliney, 2016).

1.2 Light sources

The word 'light' refers to visible radiant energy, but can also refer to different sources of illumination. A first important division to be made for lighting sources is that between natural lighting sources and artificial lighting sources. Sunlight (or natural light) is the first source of illumination that has always been present. The development of artificial light sources has developed only in the last two centuries. In fact, almost the entire evolution of mankind has been characterized by the presence of only natural light of the Sun and fire until a revolutionary light source came out: electrically powered lamps. We are today surrounded by light source and, since the 1820s, by additional light sources like gas lamps (later) all day long. Thus today electrically powered lamps are the most common source of light.

1.2.1 Natural light (Sunlight)

The earth has been bathed in Sunlight for more than 3 billion years. The Sun produces an enormous amount of energy, called solar energy. Solar energy refers to the energy emitted by the Sun and transmitted to Earth in the form of electromagnetic radiation (light and heat). Solar radiation consists of a spectrum of different electromagnetic emissions with increasing energy, higher than that of radio waves, but lower than that of x-rays (fig. 2).

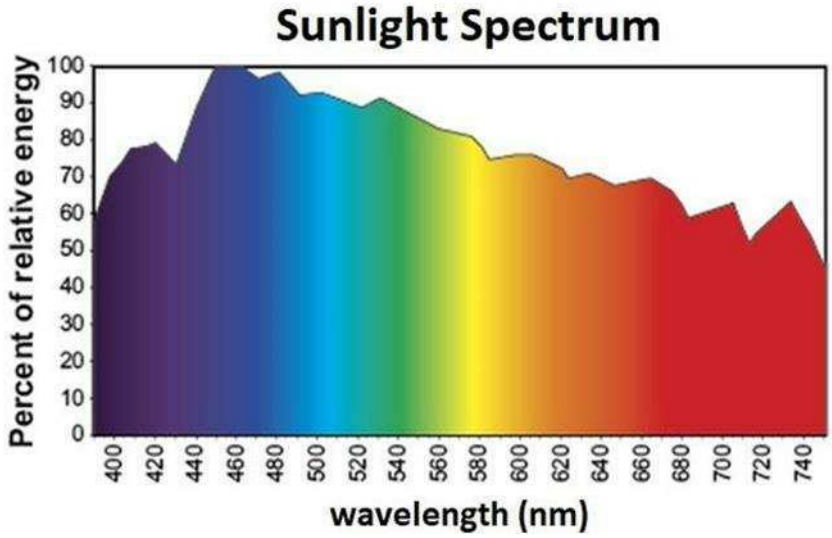

***Figure 2**. Sunlight spectrum. Included in the Sun's rays are all wavelengths of electromagnetic radiation: cosmic rays, gamma rays, X-rays, ultraviolet radiation, visible radiation, and infrared rays. Source: https://www.carnivero.com/pages/light-sources.*

What we see is the visible light, at intermediate frequency and energy, but in the rays of the Sun cosmic rays, gamma rays, X-rays, ultraviolet radiation, visible radiation and infrared rays are also included. Infrared and ultraviolet radiations are invisible to our eyes but they are able to penetrate them while high energy cosmic radiation, gamma rays and X-rays are reflected or absorbed by the atmosphere surrounding our planet.

All forms of life on earth use the energy emitted by the Sun. In the ocean, life forms are also exposed to Sunlight; in fact, energy from the Sun was effectively used by early phytoplankton to produce carbohydrates as an energy source (Holick, 2016).

In addition, Sunlight is essential for the alternation of day and night, the span of time determined by the earth's rotation around its axis. the concept of time is in fact closely

related to the concept of light. Thanks to Sunlight we can know when a day begins (dawn) and when it ends (sunset) (Figure 3).

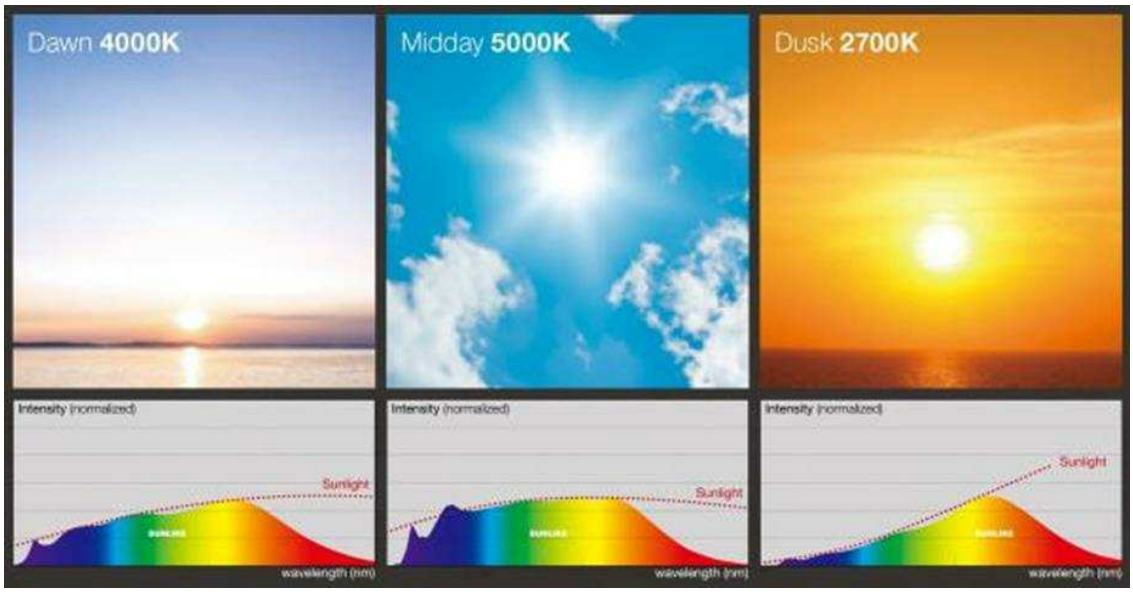

Figure 3. *Sunlight is not fixed. The colour and absolute power (irradiance) change throughout the day. Sunlight is rich in red component when it is low in the sky, while it is rich in blue component when it is high in the sky. Outdoors, light intensity (illuminance) varies between 4000K (sunrise), 5000K (midday) and 2700K (sunset). Source: https://www.eenewseurope.com/news/led-illumination-products-match-and-recreate-natural-Sunlight.*

Daylight is direct and indirect electromagnetic radiation from the Sun, which is altered by various reflections, transported and filtered through the atmosphere. The duration and availability of daylight depends on geographic latitude, seasons, and weather conditions. Daylight has a broad continuous distribution of spectral power, which changes within and between days and with weather conditions in: absolute power (irradiance), colour, scatter, polarity, and direction. Outdoors on a sunny day, the intensity of light (illuminance) varies between 20,000 and 100,000 lx, instead it is about 3,000 lx when it rains, and comes to measure 1,000 lx during twilight (fig. 3). Indoors, daylight fluxes are usually much lower and decrease exponentially with distance from windows (Wirz-Justice et al., 2020). Sunlight is red when it is low in the sky, and a significant change in the spectrum is often unnoticed due to the selective colour adaptation of our visual system. In contrast, the midday Sun is rich in shorter wavelengths with a colour temperature of about 6500 K.

Light-time signals are used to entrain the endogenous circadian rhythms of living organisms by about 24 hours (Gerber et al., 2015).

It has been argued that living organisms are subjected to daily changes in Sunlight intensity and temperature, and because of this circadian endogenous clock that allow for maximum increase in an organism's performance have been developed (Gerber et al., 2015).

Perhaps it is this "uniqueness" that implies that daylight is superior to electric light (Wirz-Justice et al., 2020). Indeed, although theoretically the two light sources (natural or solar and the artificial) are well known, technically it is extremely difficult to mimic all the properties of natural light with electric light and combine them into a “single artificial light source” (Webler et al., 2019). Sunlight is very dynamic, varying continuously throughout the day, and this contrasts with the static (almost everywhere) characteristics of electric light. Furthermore, depending on the light source (incandescent, fluorescent, or LED), the spectral power distribution of the lights is very different from each other and from daylight, even though all, to the human eye, are perceived as "white" light (Figure 4).

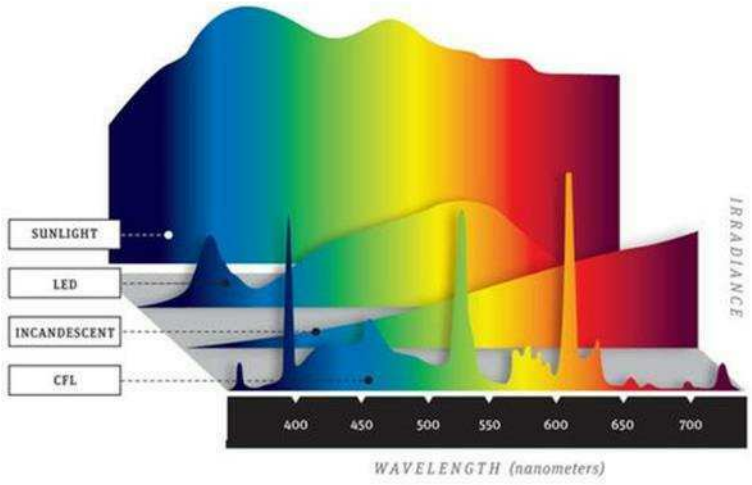

***Figure 4**. Comparison of the spectra of Sunlight with artificial electric light (incandescent lamp, fluorescent lamp or LED lamp); the spectral power distribution of the lights is very different in both wavelength and irradiance. Source: https:// caloriesproper.com/circadian-rhythms-exercise-meal-timing.*

1.2.1.1 Sunlight and human diseases

The Sun is an irreplaceable source of life: its rays provide the Earth with heat and light; they are responsible for activating the process of chlorophyll photosynthesis by which

plants produce energy and nutrients. Several researchers reported the effects of Sunlight related to human health, it has been seen that Sunlight is positive for human health in the production of vitamin D (Holick, 2003) or beneficial effect on mood. In contrast it can be harmful and promote the onset of diseases, including the induction of skin cancer (Kennedy et al., 2003). Over the years, several groups of researchers have been interested in the interaction between man and Sunlight. It has been shown that the lack of Sunlight is related to different diseases (Holick & Chen, 2008). Seasonal affective disorder, a form of depression, whose frequency is much higher in high latitude where the availability of light is decreased and thus the amplitude and phase of circadian rhythms is blunted (Kurlansik & Ibay, 2012). The absence of Sunlight has been seen to be a likely contributor to suicide, in fact, serotonin (happiness hormone) is a monoamine neurotransmitter produced in the presence of Sunlight (Vyssoki et al., 2012); (Makris et al., 2016). In addition, decreased of the Sunlight has been found to be directly correlate with age of onset of bipolar disorder (Bauer et al., 2014). However, the exposure to the Sunlight has some negative consequences, the adverse health effects of excessive Sunlight exposure have also been investigated.

1.2.1.2 Sunlight and Cancer

How Sunlight can influence lives by reducing the risk to get many deadly cancers is not deeply known yet. Human cells in the body can transform Vitamin D from 25(OH)D to 1,25(OH)2D (Adams & Hewison, 2010). During this process, 1,25(OH)2D interacts with its receptor in the cell to unlock a wide variety of genes connected to more than 80 different pathways (throughout different biological processes), including DNA repair, apoptosis, oxidative stress, and anti-inflammatory activity. These signalling pathways has been related to malignancy (Holick, 2016). Several studies reported a strong positive correlation with reduced cancer risk. In the beginning of last century a comparison between indoor workers and outdoor workers health situation was made (Hoffman F, 1915). The results have shown that indoor workers had an 8-fold increased risk of dying from cancer. In the 1980 Garland et al noticed that people living at a high latitude in the United States developed a higher risk of contracting colorectal cancer than others. This has therefore brought to the correlation between this disease and

deficiency of Vitamin D. Several studies have shown that at least 1,000 IU of vitamin D per day can reduce cancer risk (Lefkowitz & Garland, 1994);(Grant & Mohr, 2009).

Type of skin cancers, including melanoma, it can also appear on skin not normally exposed to the Sun. Melanoma-related factors involve sunburn experiences, genetic predisposition, red hair colour, increased number of moles on the body (Green et al., 1985). The theory that occupational Sun exposure reduces the chance of experience deadly skin cancer has been accepted but the creation of free radicals due to UVA radiation have to be considered too. Free radicals can also damage DNA in skin cells by increasing the risk of malignancy (Brem et al., 2017). Also, less exposure to the Sun can cause the risk of chronic and infectious diseases (Mead, 2008).
Considering that the fundamental source of Vitamin D is Sun exposure for all human beings, in order to get healthy lifestyle, Sun exposure is needed.
So we can affirm that in today's scientists have come to a similarly dichotomous recognition that exposure to the ultraviolet radiation (UVR) in Sunlight has both beneficial and deleterious effects on human health.

1.2.2 Artificial light

In the world of artificial lighting there are different types of light sources (fig. 5), which are divided into: incandescent sources, discharge sources, induction sources and Leds, list them quickly to understand their differences. They have been quickly listed below to understand their differences.

Open flames and incandescent sources are technically described as having low-colour temperatures, typically 2800 Kelvins (K) rich in longer visible (orange, red) wavelengths and infrared–near-infrared radiation. The incandescent sources were the first light sources to operate thanks to electricity; invented in 1860. They consist of a thin tungsten (W) filament placed under vacuum in a glass vial, which, run by electric current, once heated by the Joule effect, it is brought to high temperature (2000-2500 °C) emitting light.

In these sources the emission of light is no longer due to a solid filament, but to the passage of electrons brought at sufficient velocity through a gas.

Their operation is based on the synthesis of the principles of electromagnetism and electric discharge in gases. Practically it is a fluorescent lamp without electrodes and the current that passes through the gas is nothing more than an induced current due to a magnetic field generated by a coil fed at high frequency.

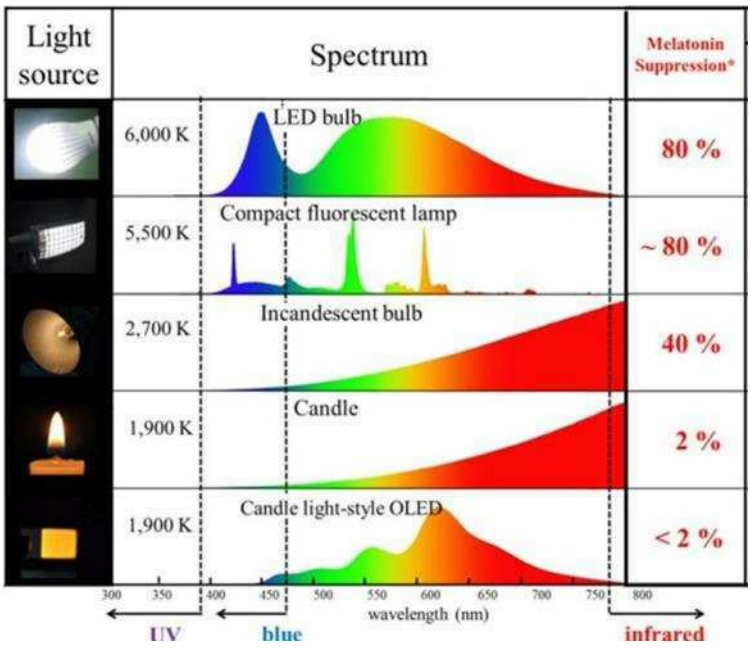

***Figure 5**. Light spectra of different types of artificial lamps: LED bulb, compact fluorescent lamp, incandescent bulb, candle and candle light-style OLED. Source: https://drbillyhealth.com/the-right-ligh.*

1.2.2.1 LED

This research will show the best performance from the fourth source: the LEDs.

The Light Emitting Diode (LED) has the same characteristics as a diode, an electronic semiconductor consisting of a P-N junction between a P-type doped semiconductor and an N-type doped semiconductor, which if polarized directly, or following a passage of current, emits a light radiation due to the effect of electroluminescence of the junction (fig. 6). This phenomenon was then perfected and applied to LED technology in 1962 through the use of special semiconductors, such as Gallium arsenurium (Gaas), by Nick Holonyak Jr that is considered, in all respects, the inventor of the LED.

***Figure 6**. The mechanism behind LED technology. (Source: https://electricalgang.com/working-principle-of-light-emitting-diode-led/). At right, example of red, blue, green, yellow and white LEDs. (Source: https://webshop.elektronikk-service.no/produkt/led-5mm/).*

Mainly used materials for the construction of these particular diodes:

- ✓ GaAs (Gallium arsenide)
- ✓ GaP (Gallium phosphide)
- ✓ GaAsP (Gallium arsenide phosphide)
- ✓ SiC (silicon carbide)
- ✓ GaInN (Gallic and Indio nitruro)

The frequency of the radiation emitted depends on the material used in the P-N junction, and as a result there is a change in the colour of the LED. Depending on the doping agent used, LEDs produce different colours including blue colour (InGaN (Nitruro di Gallio e Indio)) and red colour (GaP (Fosfuro di Gallio), AlGaAs (Arseniuro di Alluminio - Gallio), GaAsP (Arseniuro di Gallio - Fosforo), InGaAlP (Fosforuro di Allumino, Gallio e Indio), GaAs (Arseniuro di Gallio) which have been studied produce different colours.

Main parameters that characterize the LEDs:

- ✓ **Direct Current**: (If, in mA) the current value required for the LED to obtain the desired light intensity.
- ✓ **Direct Voltage**: (Vf) the voltage present between the two terminals when the LED is run by direct current; in most LEDs it is between 1.5 and 3V for normal LEDs, and from 3 to 5V for high brightness LEDs (HL).

- ✓ **Colour Temperature**: the temperature at which the black body should be brought so that it emits a light as close as possible to that of the source being examined.
- ✓ **Luminous Flux**: the amount of energy that light emits in a second in all directions, therefore represents the luminous sensation binding it to the power of the stimulus. The luminous flux of a LED is indicated in relation to its power (luminous efficiency) and in "standard" operating conditions.
- ✓ **Efficiency**: the relationship between light intensity emitted (measured in millicandelas mcd) and electric current in milliamperes (mA); values can be between 0.5 - 2 mcd at 20 mA, and reach, at high efficiency, up to 20 mcd at 10 mA.
- ✓ **Average life of the LED**: hours after which the LED source has a decay of the initial luminous flux of 30%. Generally it is around 50000h - 60000h.

The range of use of LEDs is constantly expanding as well as the increase in their performance; they are characterized by a reduced consumption and a high lifespan, resulting in both energy savings and less maintenance compared to other light sources, reducing costs by a long way. Their small size allows to direct the luminous flux in the desired area and their reduced heat emissions technology allows them to be used also in contact with other technological devices. Another advantage of LED lighting is the infinite colour variations of RGB lamps, optimal for creating special atmospheres useful for decorative lighting in public places. In addition, LED lamps have no components in the UV area and most of the radiation is emitted in yellow and red. “Natural White" LEDs are characterized by a compromise between brightness, colour temperature and colour rendering factor, representing the new frontier of lighting. From the mixing of these technologies comes the Dynamic White, which allows to obtain range of whites from a colour temperature of 3100 K up to 6500 K, this tool is ideal for commercial environments that seek the enhancement of the goods on display. The light efficiency of this technology is very high, it is between 50-100 lm/W with a lifetime of around 50000 hours. Despite their positive prerogatives, these lamps appear to have a higher purchase cost than incandescent or fluorescent lamps and, since most of them operate at low voltage, they need to be powered by the use of power supplies that further raise the value and the footprint. The most common problems to solve are related to the disposal

of the heat generated, the fidelity of the colours reproduced and the overall energy absorption.

1.2.2.1.1 General LED application

Over the decades, both indoor and outdoor lighting has undergone many changes. About half a century ago, the phenomenon of "light pollution" appeared first only found in large metropolises, then spread to almost all of Europe. The night sky of Western Europe seen from space shows the enormous impact of electric lighting. Today, inside the house (interior lighting) in addition to the presence of LED lamps that illuminate the room, there are more and more electronic devices that are used constantly such as smartphones, tablets, computers, etc. The utilisation of LEDs has changed accordingly to its own development, in a very first phase, LEDs unique function was: the production of very narrow wavelength bands (single-colour visible LEDs). Later, multi-chip LEDs and fluorescent LEDs took place with their revolutionary ability to emit “white” light (Sliney, 2016) and today, we can affirm that, products with LED backlit displays are an essential component of our everyday life: game consoles for children, office computers for adults, e-readers are normally employed everywhere by anyone. Most of all, innovative devices with LED backlit displays can modify colour temperature (Chaopu et al., 2018) according to the time of day by reducing it at night. Nowadays, awareness has developed in the technical-scientific community of a possible biological effect of BLUE light, which has allowed the development of special settings (practically now present in all smartphones) and apps that can adjust the colour temperature and exclude or eliminate the presence of blue (blue light filter).

1.2.2.1.2 LED in Human Health

Light plays an important role in the therapy industry, it is the oldest method used to recover from several health problems, and its recognized benefits are dated back to Egyptians, Chinese, and Indian populations (Daniell & Hill, 1991). Lately, during the last few decades, the advantageous effects of low level light therapy (LLLT) (LASER) to treat various pathologies have been approved. Some of these favourable effects include improvement of peripheral nerve regeneration, reduction of inflammatory reactions, enhancement of bone formation wound healing, angiogenesis subjects

(Rohringer et al., 2017). Examining lasers' effects on endothelial cell morphology, two different theories must be taken into consideration (Ricci et al., 2009). The first one supports that, LLLT affects the structure of endothelial cytoskeletons, Li Y. et al. in the year 2020 (Li et al., 2020) supports that, by using laser light, a dose-dependent increase of human umbilical vein endothelial cell proliferation occurs. Thanks to advantages like broad beam and cost-efficiency, light emitting diodes (LED) have stimulated a huge interest in several researchers' studies where LED are described as a valid alternative to LLLT. At the beginning of the twentieth century, Oscar Raab and Herman von Tappeiner gave birth to the term "photodynamic therapy (PDT)" denoting the chemical reaction in which oxygen is consumed following induction by a photosensitization process. In 1903, for the first time ever, artificial irradiation in phototherapy was used by a Danish physician, Niels Ryberg Finsen (winner of the Nobel Prize in Physiology or Medicine) and a combination of light and a topical substance, eosin, to treat skin tumours was found out by von Tappeiner and Jesionek (Jesionek-Kupnicka et al., 2013). From its discovery till now, PDT is growing fast and significant progress has been made so far in light-based treatment of different disorders such as lung disease (Sutedja & Postmus, 1996), different kinds of solid tumours (Breskey et al., 2013), age-related degenerative processes of macula (Silva et al., 2008), and urology (Jichlinski, 2006).

The primary beneficial effects of human health-oriented LED application were discovered by the National Aeronautics and Space Administration dealing with the enhancement of LEDs that are able to generate a narrow spectrum of light in a non-coherent manner delivering the proper and requested wavelength and intensity. LED technology has been progressively optimized during the last 10 years and over (Oh & Jeong, 2019).

The advancement of a thorough understanding of the characteristics of LEDs makes their use in phototherapy viable for both the clinic and the laboratory. Some of the most important relevant features of LED practice are price and versatility. Comparing LEDs to the other sources described so far, LEDs appear to be quite inexpensive, plus, when arranged in a certain arrangement, they are capable of irradiating large areas. Since they can be powered by batteries, it is very easy to take them wherever they are needed (Brancaleon & Moseley, 2002).

Also referring to their characteristics, last but not less important, they can be arranged in different geometric combinations to balance/recompensate for difficult anatomical areas (non-melanoma skin cancer for example tends to occur in the face and head where large curvatures can reduce the effectiveness of other light delivery systems). For all the reasons just explained, interest in the use of LED in phototherapy is increasing.

In the cosmetic field, LED therapy is gaining momentum after approval by the US Food and Drug Administration due to its ability to increase ATP and transcription factor production, regulate oxidative stress, and modulate collagen synthesis (Sorbellini et al., 2018).

Speaking of the link between light and acne vulgaris, many encouraging results of the use of light in the treatment of acne vulgaris (multifactorial skin disorder associated with pilosebaceous inflammation of the unit) have been shown (Alba et al., 2017). Promising results have been obtained using both red light (630 nm) and blue light (415 nm). Some *in vitro* studies have shown a significant reduction in sebum production regarding the effect of red light while, the effects of blue light can be basically described as a reduction in the proliferation of human sebocytes.

The therapeutic approach of PDT is also becoming increasingly common in the field of skin rejuvenation due to its safety and efficiency. In fact, many *in vitro* and *in vivo* studies have demonstrated the ability of LED therapy to activate skin collagen synthesis (Brondon et al., 2009); (Lee et al., 2007); (Jagdeo et al., 2018). One of PDT fields of application is the treatment of hair loss. After demonstrating PDT success, in 2007 it got the approval from FDA and it was finally released as the first LLLT device to treat hair loss problem, androgenetic alopecia especially (Tzung et al., 2009). Very recently, LED therapy has been applied in psoriatic subjects and from the results obtained, a reduction of the erythematous plaque of 33.9% and 26.7% can be observed.

Psoriasis is a skin disorder and precisely: it is an immune-mediated inflammatory skin disease. The percentage of people suffering from this disorder is around 2-3%. Protoporphyrin IX (Ppix) is present in psoriatic conditions and is a potential target for photodynamic treatment (Zhang & Wu, 2018).

1.2.2.1.3 LED and Cancer

Improvements in the therapeutic application of LED have made possible its current use in the treatment of numerous clinical conditions, for example, several types of solid tumours (Vreman et al., 1998). Researchers and physicians have begun to be attracted to the idea of using LED for cancer treatment. In fact, LED light therapy combined with taking chemical drugs has shown high therapeutic efficacy (Tartaglione et al., 2021). This breakthrough discovery will definitely reduce the dose of drugs, leading to a reduction in adverse side effects, without compromising the effectiveness of the treatment (Hwang et al., 2013).

These different uses of LED have stimulated the interest of many researchers in the last twenty years. However, all its possible applications have yet to be examined and this evaluation takes quite a long time because for each light treatment 3 important points must be carefully selected:

- ✓ the precise wavelength to be used (and consequently the colour) of the light radiation;
- ✓ the intensity and brightness of the light radiation;
- ✓ the exposure time (duration and frequency) of the light radiation.

1.2.2.1.4 LED BLUE

Improvements in people's living standards and display technology make humans more aware of photobiological safety. In fact people's demands for display devices were no longer only dedicated to image quality and energy conservation. Concerning photobiological safety, blue light hazard and circadian rhythm have to be considered. Blue light hazard refers to the harm to the retina of human eye provoked by the photochemical effect of blue light in the range of wavelength 400-500 nm (Shen et al., 2014); while the circadian rhythm, also called the non-visual effect, refers to that blue light component in the visible light, through inhibiting pineal gland to secret melatonin and stimulating the adrenal glands to secrete cortisol, it is able to modify body's circadian rhythm and adapt alertness and biological clocks (Stevens & Zhu, 2015). Starting from 1966, many researchers and scholars found out and realized how blue light can badly affect human body. In 1966, Noell W. K. (Noell et al., 1966) shown that

blue light can damage rod cells, and in 2006, Peep V.Algvere (Algvere et al., 2006) described the impact of blue light hazard on age-related maculopathy. In 2011, P. N. Youssef et al. (Youssef et al., 2011) introduced a blue light hazard mechanism. In the following period, Rao Feng and Ancheng et al. conducted numerous studies on the interaction of blue light hazard and circadian rhythm of LED backlight displayer and LED lighting. The effects induced by the blue light are still not well understood, although many researchers are studying them. It has been observed that, in the skin and retina, blue light induced suspected mediators of skin aging and age-related macular degeneration. Several *in vitro* and *in vivo* investigations on skin cancer have revealed that blue LED induced apoptosis in cancer cells (Tartaglione et al., 2021); (Oh et al., 2015); (Arthaut et al., 2017). In addition, it has been demonstrated that blue LED irradiation triggers apoptotic cell death through the mitochondria-mediated intrinsic pathway and shortens the early stage of tumour growth in melanoma cells (Oh et al., 2015). On the other hand comparing different wavelengths effects in an excision model in rats, Adamskaya N. et al (Adamskaya et al., 2011) has verified and confirmed blue light high power in terms of reducing wound size and development of epithelialisation**.**
In conclusion, blue light radiation in LEDs can be considered as a powerful tool in numerous therapeutic fields (under control), on the contrary, it has been recognized as dangerous in everyday life. The United Nations proclaimed the International Year of Light and Light Technologies recognition of how light “revolutionized medicine, open international communication via Internet continues to be essential for cultural, economic connection, and political aspects of the global economy" (http://www.light2015.org/Home/About.html).

1.2.3 Ergonomic Artificial Lighting

To fully understand what ergonomic artificial lighting is, it is necessary to start from its etymology.

The word ergonomics (from the Greek "ergo", meaning work, and "nomos" meaning regulation, laws) represents the science that studies performance and their well-being, in relation to the purpose of their activity, the work equipment and the work environment.

This translates into the design of products and/or processes that utilize an individual's capabilities, considering their physiological and intellectual needs.

Ergonomics therefore seeks to identify the most important parameters for a correct man/work relationship, to eliminate any negative factors that may be present and thus make the use of work objects more natural.

Ergonomic research means the environmental, structural and procedural study of work organization in order to identify the requirements and criteria for their best acceptability by the operators.

So ergonomics, according to the IEA (International Ergonomics Association), is the science that deals with the interaction between the elements of a system and the function for which they are designed, in order to improve user satisfaction and the performance of the system as a whole

Basically, it is the science that deals with the study of the interaction between individuals and technologies, in our case to lighting technology. The effectiveness of a lighting project is the result obtained from the development of two different analyses:

- ✓ **quantitative**, given by the determination of the number of sources quantitative, given by the determination of the number of light sources and their positioning;
- ✓ **qualitative**, given by the choice of the most suitable type of light to perform a given activity and its distribution in space.

The innovative invention of electric light, only about 130 years ago, has brought about numerous developments and improvements in a society where people now need to receive, 24 hours a day, 7 days a week, certain goods and services, in any time of day or night, while working, sleeping, eating or playing. Under these circumstances, they are more or less exposed to electric light based on their lifestyle (e.g., use of electronic devices at night), the location of their homes (e.g., urban light pollution) and night work or as a shift worker (e.g., a worker whose schedule changes between morning, day, evening and night). While electric light has clearly benefited humanity, exposure to the elements, unnatural electric light such as night light (LAN) or differences in the amount and spectrum of electric light to which one is exposed, has inevitably led to some problems (Gaston et al., 2015).

Unlike many other anthropogenic changes that have been made to the environment (e.g. in CO2, temperature, habitat change), those resulting from LAN are completely unprecedented. There have been no natural analogues, on any time scale, to the nature, extent, distribution, timing, or rate of spread of LAN (Gaston et al., 2015). Only in recent years there has been an explosion of research interest in the biological and human health impact of LAN (Khan et al., 2020). An important outcome of the workshop was to increase the visibility of these pervasive and often unavoidable exposures and their associations with potential adverse health effects (Gaston et al., 2015).

1.3 Light–Human Interaction

1.3.1 The biological clock

The strong selective pressure of the day-night cycle makes it so that organisms evolutionarily very distant could develop and preserve similar and highly beneficial endogenous molecular mechanisms: circadian biological clocks (Hall, 1995). The existence of these biological clocks has in fact been identified at various levels of organization and complexity, from unicellular organisms to pluricellular ones, both vegetal and animal, suggesting their early appearance in the phylogenetic tree. Rhythmic oscillations of biological parameters and physiological processes are in fact found in all living matter and at all levels of organization, from subcellular structures, to cells, tissues, systems and apparatuses (Pittendrigh, 1993).

The ability to anticipate these oscillations is considered a possible adaptive advantage and explains the evolution of endogenous molecular mechanisms able to temporally organize numerous metabolic and behavioural activities (Dunlap, 1999);(Hall & Sassone-Corsi, 1998). The circadian biological clock generates cyclic oscillations of biological functions with a period approaching the duration of an astronomical day. The duration of daylight illumination and the intensity of radiation (photoperiod) varies with

latitude according to the seasonal rhythm and affects the physiology of plants and the activities of some animals.

The term photoperiodism indicates that complex reactions that organisms present at the daily and seasonal environmental rhythm of periods of light (photophase) and darkness (scotophase). The ability of animal or plant organisms, substances or devices, to react to light stimuli allows us to distinguish organisms in photosensitive and non-photosensitive. In photosensitive organisms, circadian clocks coordinate behaviour and physiology in advance, the temporal regulation of physiology by circadian clocks works together with precise signal paths (Dibner et al., 2010).

The endogenous clock therefore maintains an internal estimate of the passage of time and program that physiological processes take place at an appropriate time of day.

This property of perceiving and anticipating any environmental variations and temporarily modifying biological parameters and functions in order to adequately respond to the changed external conditions guarantees a selective advantage and explains gene expression with rhythmic circadian of about 2-10% of the mammalian genome (Kochan & Kovalchuk, 2015). Molecular endogenous clocks have been described everywhere in the living: bacteria (Golden et al., 1997), single eukaryotic cells (Hastings & Sweeney, 1957), insects (Helfrich-Förster, 2005), birds (Wikelski et al., 2008) and mammals such as rats (Lee et al., 2010), monkeys (Honjo et al., 1963) and man (Pilorz et al., 2018).

We can distinguish environmental factors that have an indirect influence by changing the speed of the activity of the circadian system, and factors, called Zeitgeber (from German, Zeit = time, Geber = giving) that cause substantial changes in the molecular clock and cause it to be directly resynchronized. One of the privileged ways with which the environment communicates with endogenous molecular mechanisms is light.

Light is the main Zeitgeber that helps to set the rhythm of the central circadian clock through the retina and can act having only one transient effect, for example modifying locomotor activity in response to a rapid light change (fig. 7). Alternatively, it can act as a regulator, by turning specific molecular pathways involving the photoreceptors of the visual system (phenomenon of entrainment). Light is not the only Zeitgeber, even meals

can cause time lag and plasma glucose levels that vary after meals can affect the expression of the clock gene in the tissues (fig. 7).

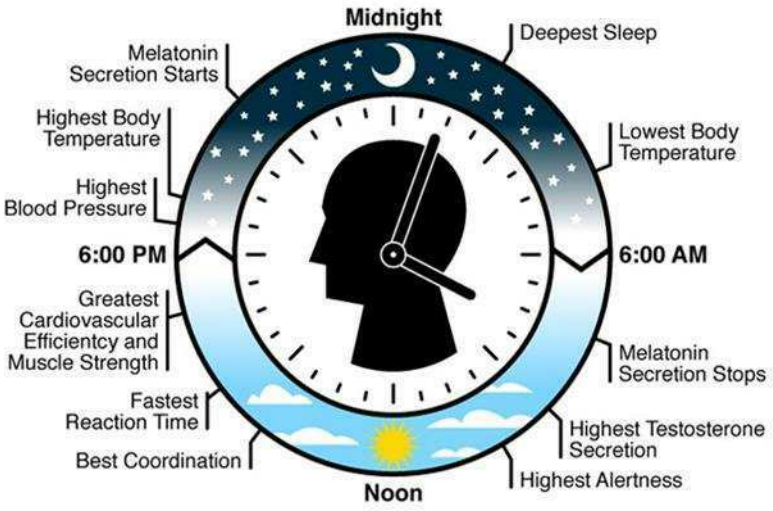

Figure 7*. Overview of rhythmic functions that are critically controlled by the human circadian pacemaker. Source: https://www.morfeoildiodelsonno.it/il-caffe-di-sera-fa-sballare-lorologio-biologico/.*

However, it is possible to distinguish two types of light effects, the first is a transient effect that does not affect the endogenous circadian clock but results in an increase or a decrease in locomotor activity in response to a rapid change bright (positive or negative masking effect). The second is the capacity of the light acting by Zeitgeber adjusting the endogenous clock through specific molecular pathways involving the photoreceptors of the visual system (phenomenon of entrainment) (Tosini et al., 2016). The environment can mediate the mechanism molecular endogenous clock also through temperature. In any case, in the presence of external stimuli the watch follows a "Zeitgeber time", ZT.

The circadian clock can regulate itself, following the "circadian time" (CT) under constant or free running conditions, in the absence of Zeitgeber (Copertaro & Bracci, 2019). In this modality, the length of a cycle is dictated exclusively by the endogenous clock. Therefore, under natural conditions, the daily circadian rhythms are within a 24-hour cycle of light-dark (Roenneberg et al., 2003).

The mechanism of the system is based on chemical reactions, related to temperature, light and the other external stimuli (Kinmonth-Schultz et al., 2013). In fact, there is also a mechanism of temperature compensation (even through a variation of temperatures

the internal clocks manage to ensure a constant period of oscillation). The importance of this statement is extremely important because the endogenous clock is based on chemical reactions whose speed is conditioned by temperature. The internal circadian oscillators in addition to having periodically internal and external events, also have two other roles: the moderation of chemical reactions that can be harmful but necessary at certain times and the temporal separation of chemically incompatible reactions (Dibner et al., 2010); (Chapman et al., 2020); (Dantas et al., 2019). In such a situation, the length of a cycle reflects only the functioning of the endogenous clock and may be different from that measured under cyclically changing environmental conditions (Green, 1998).

Importantly, adaptations to environmental changes are regulated by a complex molecular organization, generically referred to as the biological clock.

The biological clock consists of articulated oscillating self-driven endogenous circuits that are able to scan the passage of time and synchronize with external stimuli by generating metabolic, physiological, and rhythmic behavioural phenotypes to cope with periodic environmental changes (Gerber et al., 2015).

These cyclical oscillations of biological functions that are repeated over time are called biological rhythms. The biological rhythms observed in nature are classified according to their period (τ), which is the time required for a complete cycle of oscillation to be completed (Goldbeter, 2017).

Within the human physiology can be schematically divided into three groups based on their duration: ultradians (τ < 20 hours), such as electroencephalographic waves, the cardiac cycle, the respiratory act, the sleep cycle; circadians (τ =20-28 hours), such as body temperature, sleep-wake cycle, blood pressure, hormonal secretion; infradians (τ >28 hours), such as menstrual cycle, cell-mediated immunological response (Smolensky et al., 2016). Biological phenomena that relay these mechanisms are hibernation, migration, the reproduction and the instinct to search for food. The first systematic observations of cyclical activities in the living world can be traced back to Carl von Linne (1707-1778), which described the opening and rhythmic closing of the petals in various species of flowers.

The biological rhythm with periodicity of about 24 hours, termed circadian rhythms (from Latin about "around", dies "day"), is endogenously generated by the complex circadian biological clock. The main clock rhythm is probably genetically determined and is also autonomous and self-sustaining. This is related to the ability of external factors to provide cues so that circadian rhythms are most appropriately positioned to meet the demands of life, but they do not create the rhythm by influencing its periodicity. The experience conducted in a cave in Frasassi by Maurizio Montalbini, in fact, showed that the central pacemaker, while not receiving any reference from the outside Zeitgeber, continued to maintain a circadian rhythm throughout the body in conditions of total absence of light, even if with a frequency greater than 24 h. we can say that the circadian clock carries out its activity even in the absence of external signals (Copertaro, 2013).

Therefore, external factors provide only reference signals, so that circadian rhythms adapt as much as possible to the daily needs of the individual, but do not create rhythm. The existence of a circadian biological clock in all taxa, starting from invertebrates up to vertebrates, suggests that it is a molecular structure that appeared very early in the evolutionary path (Hazlerigg & Tyler, 2019). It is nowadays generally accepted that a clock has evolved in independent manner at least four times during the evolution from the realm of prokaryotes to that of animal organisms (Dunlap & Loros, 2017).
The central clock and the set of peripheral clocks allow both the single cell and the organism as a whole to synchronize in relation to the different needs of the day-night cycle and to respond with extreme efficiency to environmental variations (Copertaro, 2013).

The biological function for many peripheral clocks is not known, but it is known that some of them control local rhythms. Unlike the peripheral clocks of insects, which are able to perceive and respond to light stimuli independently, those of mammals are strictly dependent on the central one (Stanewsky, 2002).

Briefly we can affirm that the existence and the conservation of a circadian rhythmic is due to the presence of specialized mechanisms, endogenous, able to self-regenerate during the 24 h, defined biological circadian clocks.

1.3.2 The Circadian System

A circadian biological system consists of three basic components:

- ✓ **Input routes**: endogenous signals that self-regulate the rhythm;
- ✓ **Biological clock**: endogenous oscillator autonomous, located within each cell;
- ✓ **Output routes**: external environment.

The "core" of the circadian endogenous clock is capable of "measuring" time and dictating a whole series of molecular transformations (output) which in turn regulate circadian phenotypes. Examples of output are: the hormonal cyclic secretion, rhythm activity/rest, rhythm of intake of food, daily change in body temperature and expression of genes controlled downstream of circadian core (clock controlled genes or ccgs) (Honma, 2018). Ccgs are involved in the regulation of various cellular processes (e.g. apoptosis, cycle cellular, DNA damage protection systems).
The biological clock proper, or the endogenous oscillator autonomous, is the main nervous centre lies in the suprachiasmatic nuclei of the hypothalamus (NSC), consisting of neurons capable of:

- ✓ originate to circadian oscillations due to their electrical and secretion activity;
- ✓ synchronize neural activity;
- ✓ regulate its rhythms with the ambient light/dark cycle through the retinal-hypothalamic tract.

This central clock is activated by photopic pulses from retinal ganglion cells containing a photopigment, melanopsin, which is activated with a blue light wavelength between 460-484 nm. This pigment makes the ganglion cells of the retina intrinsically photosensitive (ipRGC), thus able to detect changes in light during 24 hours, analysing the incident light and distinguishing the day from the night. This represents the crucial element of the system of synchronization of circadian rhythms (regulated by the NSC) with the day/night rhythm (Paul et al., 2009). The ipRGC are connected to the suprachiasmatic nucleus (NSC) by means of the retino-hypothalamic beam. In turn, the NSC is connected to the sympathetic nervous system, and more precisely to the intermedial thoracic nucleus, and to the superior cervical ganglion from which originate the sympathetic post-ganglion fibers which, terminating near the membrane of the

pinealocytes, release norepinephrine (Lazzerini Ospri et al., 2017). Norepinephrine released in the synapses stimulates the production of melatonin by the pinealocytes contained in the epiphysis (Fan et al., 2018). Activation of the circadian system occurs in the presence of daylight; the NSC is stimulated by photopic pulses, blocking the transmission of nerve impulses from sympathetic post ganglion fibers. This inhibits the release of norepinephrine and the production of melatonin. The light stimulus coming from the retina is transmitted through the retino-hypothalamic tract to the NSC, inducing in the neurons present in it the activation of a series of transduction pathways of the signal responsible for the synchronization of the same with the light-dark cycle. The NSCs, in turn, synchronize peripheral oscillators and modulate circadian a number of biological functions, including body temperature, sleep-wake cycle, blood pressure and the release of hormones by the pituitary gland and melatonin by the pineal gland. Several experimental data have shown that in mammals the NSC represents the primary circadian clock on which the control of circadian clocks depends; but the mechanism by which the NSC modulates the action of peripheral pacemakers has not yet been fully clarified (Mouret et al., 1978); (Ralph et al., 1990). Neuro-anatomical and functional studies indicate the existence of nerve pathways between the NSC and the heart, pancreas, liver, thyroid and pineal gland (Buijs et al., 2003); (Buijs & Kalsbeek, 2001); (la Fleur et al., 2000). Using this means of communication, the NSC can activate or mute different tissues, depending on their function, at different times of the day. Thus, the circadian system functions as an orchestra, in which the NSC acts as the conductor and the peripheral oscillators are the different instrumental groups (Bonmati-Carrion et al., 2014). The biological function for many peripheral clocks is not known, but it is known that some of them control local rhythms. Peripheral oscillators regulate, in turn, the circadian rhythmic transcription of genes (clock genes) that vary depending on the specific tissue function and that represent about 10% of the entire genome (Storch et al., 2002); (Takahashi et al., 2008). In fact, the analysis of transcriptomes of different tissues has highlighted how the messenger RNA fraction transcribed with circadian kinetics represents up to 10% of the total share of messenger RNA. Therefore different peripheral oscillators contribute to the regulation of rhythmic tissue-specific phenotypes. Therefore, each multicellular organism is regulated by a set of biological circadian clocks, these include both peripheral clocks contained in each individual cell

of different tissue districts than the more complex ones present in the central nervous system; the result of their interactions leads to a circadian modulation of physiology of the body. From this it follows that the output ways influence and guide activity of genes related to clock genes (clock controller genes, ccg) and are responsible for the coordination of circadian rhythms between different functions and parts of an organism.

1.3.3 Biological circadian clock functions

The human circadian system consists of a multiplicity of oscillators which are self-regenerating and which in turn can be synchronised by environmental factors that act as Zeitgeber. As already mentioned in the previous paragraph, the temporal organization of biological functions of an individual is ensured by the interaction of these factors (Aschoff & Pohl, 1978). In humans, some examples of biological functions with circadian patterns are: body temperature, secretion of various hormones, sleep/wake cycle, pressure arteriosa, the heart rate (Hall & Sassone-Corsi, 1998). The circadian trend of body's temperature provides a rise at morning and a decrease before sleep (Baker et al., 2001); the morning rise in temperature is also evident in the absence of light and the entire rhythm is maintained even in prolonged resting subjects. The centre of thermoregulation is located at the level of the hypothalamus that acts, therefore, as a circadian clock specific for temperature control (Cedernaes et al., 2019).

Another important example is the variations in hormones, such as cortisol and melatonin. In particular, the circadian regulation of the axis hypothalamus-ipophysic-adrenal may be observed in changes in cortisol levels fluctuating between maximum values, observable in the early morning hours, and minimum levels present around midnight (Selmaoui & Touitou, 2003). It is interesting to note that these fluctuations are abolished in pathological conditions, such as in Cushing syndrome (characterized by high levels of cortisol) (Haritou et al., 2008). The peak of secretion of melatonin is reached during the night with subsequent decrease during daytime hours due to inhibitory effect exercised by light (Dijk & Cajochen, 1997). In dark conditions, the absence of photopic stimuli, on the other hand, disables the inhibitory control of the NSC on the upper cervical ganglion, with release of norepinephrine at the level of

pinealocytes and production of melatonin; the entire system retina-NSC-epiphysis is called photo neuroendocrine system (SFNE) (Cardinali et al., 1983).
Other synchronisers, such as power, exercise times, rhythm of intake of food and social activities, are also connected to the central pacemaker and peripheral oscillators (Van Someren & Riemersma-Van Der Lek, 2007).

1.3.4 Molecular Circadian Clock

In order to understand how circadian biological clocks are capable of self-regenerate and maintain their periodicity of 24 hours, it was necessary the identification of the molecular mechanisms responsible for their final functions. As already mentioned, the high selective pressure of the day/night cycle has probably ensured that evolutionary organisms far away could develop and preserve similar mechanisms and highly advantageous biological circadian clocks (Hall, 1995). In fact, the existence of these clocks has been identified in evolutionary species very distant, such as bacteria, fungi, insects and mammals. At the molecular level, the endogenous circadian oscillator (core), located within each cell, consists of the clock genes that encode for the corresponding proteins, which interact in complex self-regulatory circuits with negative feedback, producing cyclical variations in the levels of mRNA and proteins of the clock itself (Ko & Takahashi, 2006). Therefore, clock genes generate an endogenous rhythm of intracellular protein expression, which regulates biological processes and functions with periodicity close to 24 h (Reppert & Weaver, 2002). The modulation of the periodic oscillation of the clock genes expression and the corresponding protein products is carried out by activating and suppressing the transcription and translation processes in self-regulating circuits (loops) by positive and negative elements respectively (Lowrey & Takahashi, 2004). The isolation and the characterisation of some circadian rhythm mutants in organisms such as Cyanobacteria, Neurospora, Drosophila, mouse and human, has allowed the identification of some genes responsible for the operation of the circadian biological clock. Despite the evolutionary heterogeneity circadian endogenous clocks of different organisms, all have a common mechanism operation based on negative cyclical feedback system (Golden et al., 1997);

(Pittendrigh et al., 1959); (Deguchi, 1979). Circadian clocks are ubiquitous and are at various levels of organisation and complexity.

In the following paragraphs we report the mechanism of action of clock genes of the two experimental models studied in this thesis work: the fruit insect, the *Drosophila melanogaster* (used as *in vivo* model) and human (as the cells studied for the *in vitro* model are HaCaT: human keratinocytes).

1.3.4.1 Clock Genes in *Drosophila melanogaster*

The central circadian system of *Drosophila melanogaster* is located in the head, at the level of the visual system. It possesses two compound eyes, three ocelli and two organs Hofbauer-Buchner extraocular organs capable of perceiving light. In the compound eyes the photoreceptors responsible for the perception of the light stimulus are the rhodopsins that activated form a complex with G-protein able to activate phospholipase C (PLC), a protein, encoded by the gene norpA, which in turn acts on calcium channels calcium channels called Transient Receptor Potential (TRP) up to involve in the cascade of the clock neurons. These types of organs are important for clock synchronization (Hardie & Raghu, 2001); (Pak & Leung, 2003).

The two researchers isolated three several mutations at this locus: one was able to shorten the period up to 19 hours, a second mutation lengthened the period up to 29 hours, and the third one finally rendered the arithmetic flies (Konopka & Benzer, 1971). The molecular endogenous clock in *Drosophila melanogaster* is constituted of "core" that is the oscillator real centre and just regulated from a negative feedback loop mechanism (fig. 8).

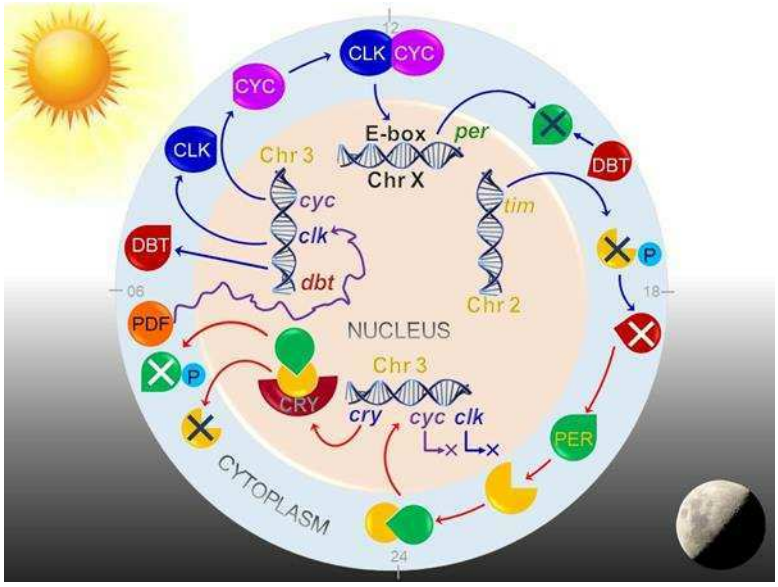

***Figure 8**. Molecular interactions of clock genes and proteins during Drosophila circadian rhythm. Source: https://upload.wikimedia.org/wikipedia/commons/6/64/Drosophila_circadian_rhythm.jpg*

This system involves the positive elements CLOCK (CLK) and CYCLE (CYC) (Allada et al., 1998), transcriptional factors containing DNA interaction domains (basic-helix-loop-helix, bHLH) and PAS dimerization domains, negative elements PERIOD (*PER*) and TIMELESS (*TIM*), proteins that can inhibit their own transcription (Dunlap, 1999). The Clock gene (*ELK*) is rhythmically transcribed with a peak of expression around at dawn, while Cycle maintains constant transcription levels. In the middle of the day *CLK* and *CYC* dimerize in a complex able to bind the particular DNA sequences (called E-box, CACGTG) (Kyriacou & Rosato, 2000) present in the promoters of the genes *PER* and *TIM* activating the transcription. The activation of the expression of *PER* and *TIM* by the heterodimer *CLK/CYC* leads to a progressive increase of their mRNA which reaches a maximum at the beginning of the night (Hardin et al., 1990); (Zerr et al., 1990). Peak protein translated from these last two mRNA occurs at the end of the night. Protein *PER* is regulated at post-translational level through a series of phosphorylations and dephosphorylations by kinases (DOUBLETIME, DBT) and phosphatase (protein phosphatase 2, PP2A). During the night *TIM* accumulates and can thus bind the *PER/DBT* complex, stabilizing it and allowing its accumulation. The *PER/TIM/DBT* complex thus formed tends to enter the core where they act on *CLK* leading to a progressive reduction of the activating effect of the *CLK/CYC* dimer on *PER* and *TIM* promoters (Darlington et al., 1998). This inhibition leads to a decrease in the protein

PER, which returns to be quickly degraded as a result of the effect of light on *TIM* whose levels drop rapidly due to light degradation dependent mediated by CRYPTOCHROME (*CRY*), blue light photoreceptor (Lin et al., 2001). At this point, the effect of *PER* and *TIM* on *CLK* ceases and can restart the cycle.

In the study of circadian rhythms, an important date is 1971. S. Benzer and R. Konopka published a study in which pupal hatching and locomotor activity of fruit flies were defined as rhythmic behaviours (about 24 hours) (Konopka & Benzer, 1971); because they were linked to the expression of a single gene, which is called Period. Today, fruit flies are one of the most advanced animals in the study of biological clocks. The fruit fly (*Drosophila melanogaster*) is an excellent model organism because it has a life cycle of only 10-14 days, is easy to handle in the laboratory, and can be reproduced in large numbers.

It is a highly differentiated organism with a complex morphology and developmental cycles characterized by different stages. From the perspective of circadian rhythm research, there are now mainly two recognized rhythmic phenotypes: pupal hatching and adult exercise capacity, the same ones discovered by S. Benzer and R. Konopka.

Pupal incubation occurs rhythmically, reaching its peak at dawn. The ecological significance of this rhythm is that in the early hours of the day, the environment is usually cooler and more humid, which minimizes the risk of dryness in infants (Hardin et al., 1990).

Athletic capacity is an index that measures the amount of activity of adults at different times of the day. In the LD 12:12 condition at a constant temperature of 23°C, motor activity is bimodal rhythmic, with two peaks where movement is maximal, interspersed with a pause. Also in Drosophila, one of the fundamental characteristics of the endogenous biological clock is that it is not isolated from the external environment, but can receive from it important signals to adjust its phase according to changes in the environment itself: such as temperature and light. The temperature of the day, for example favours more movement when it is cooler (dawn and dusk) to avoid the high temperatures of the day.

Light is a preferred way for the environment to communicate with endogenous molecular mechanisms. Two types of light effects can be distinguished. The first is the transient effect, which does not affect the endogenous circadian rhythm, but responds to rapid changes in light to increase or decrease motor activity (positive or negative masking effect). The second is the ability of light to act as a zeitgeber by regulating the endogenous clock through a specific molecular pathway involving the photoreceptors of the visual system (entrainment phenomenon) (Tataroglu & Emery, 2014).

1.3.4.2 Clock Genes in Human

In Human circadian clock are composed of genes and proteins that created two interlocking feedback loops (fig. 9). The first circuit involves *CLOCK* (Circadian Locomotor Output Cycle Kaput or NPAS2 in nervous tissue) and *BMAL1* (Brain and Muscle Arnt-Like protein 1). Their belonging to the family of transcription factors that contain bHLH-PAS domains (basic Helix-Loop-Helix Period-Arnt-Single-minded). *CLOCK* and *BMAL1* heterodimerize to form an active transcriptional complex that bind the E-box sequences in the promoter of the clock Period (*PER1, PER2* and *PER3*) and Cryptochrome (*CRY1* and *CRY2*) genes, promoting their transcription (Gekakis et al., 1998); (Kume et al., 1999). *PER* and *CRY* form a complex and inhibit their own expression by repressing the activity of the transcription factor heterodimer CLOCK/*BMAL1* (Kume et al., 1999); (Shearman et al., 2000); (Sato et al., 2006). During the night the decrease in inhibitor levels (*PERs, CRYs*) allows the activation of the CLOCK/*BMAL1* dimer allowing the start of a new transcription cycle. The balance between the stability and degradation of *PER* and *CRY* proteins, fundamental for the regulation of the molecular clock period, is controlled by the activity of specific enzymes belonging to the phosphotransferase family (Lowrey & Takahashi, 2000). The second loop involves two nuclear receptor subfamilies: REVERB α, β (Reverse transcript of erythroblastosis gene) and ROR α, β and ɣ (Retinoic acid related orphan nuclear receptor) (Akashi & Takumi, 2005). Their transcription are activated by the heterodimer *CLOCK/BMAL1* and act as transcriptional factors and promote respectively inhibition and activation of *BMAL1* transcription through competitive interaction for the orphan receptor binding sites related to retinoic acid, ROREs (ROR response elements) which are present in the *BMAL1* gene promoter (Guillaumond et al., 2005). This self-

regulating circuits with negative feedback are completed in about 24 h (Reppert & Weaver, 2002). There are other two proteins (DEC1 and DEC2) which can inhibit the formation of the *BMAL1-CLOCK* dimer, regulated in turn by the dimer itself in a third negative feedback loop (Noshiro et al., 2004). Recent studies showed *CLOCK* may function as histone acetyltransferase (HAT), allowing the activation of the *"clock controller genes"* and by regulating the transcription of *PER* and *CRY* (Doi et al., 2006).

Associated with the main oscillator system there are numerous proteins that undergo circadian cyclicity. Among these: nicotinamide phosphoribosyltransferase (NAMPT) (Ramsey et al., 2009); peroxisome proliferator activated receptor-γ (PPARγ) (Eckel-Mahan et al., 2013); Sirtuin 1 (SIRT1) (Grimaldi et al., 2009) and Protein kinase Cα (PKCα) (Robles et al., 2010).

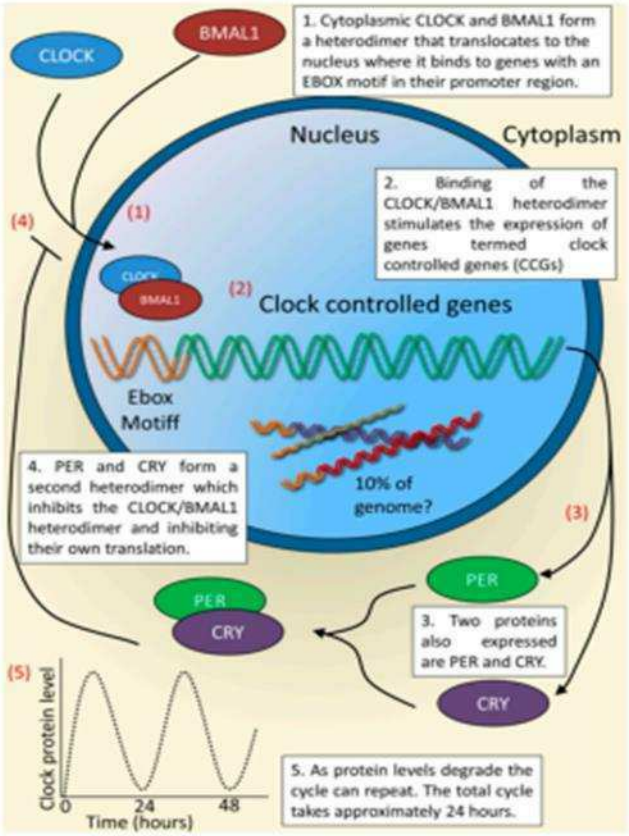

Figure 9. *The core molecular clock system in mammalians.*
Source https://pubmed.ncbi.nlm.nih.gov/27231897/.

1.3.5 Biological Clock Desynchronization

Satellite images of Earth at night revealed how LAN exposures have become ubiquitous in major cities and surrounding areas over the past decades, as the LAN now covers 80% of the world (Falchi et al., 2016). Further study of the health effects of inadequate light exposure, as well as the characterization of various exposures and endpoints (biomarkers and health outcomes), could be helpful in understanding how, in those areas with higher LAN levels, there is a decrease in the duration of healthy sleep ranging from seven or more hours in a 24-hour period, based on self-reported sleep duration among adults (Lunn et al., 2017).

1.3.5.1 Shift Work and disorders

Circadian control of physiology and behaviour is a fundamental prerequisite to a state of psychological and physical well-being (Antoch & Kondratov, 2013). Alterations in the circadian cycle may be the cause or have predictive value of some disease states (Gimble et al., 2011); (Paschos & FitzGerald, 2010). In particular, the desynchronization of circadian rhythms is considered the main cause of sleep-associated disorders such as: sleep phase advancement syndrome (ASPS), the syndrome of delayed sleep phase (DSPS), the irregular alternation of the sleep/wake cycle (Reid & Zee, 2009). Biological rhythms have the primary function of synchronizing biological functions with environmental conditions in order to ensure the best performance of the organism (Buhr & Takahashi, 2013). In humans, night time hours compared to daytime hours are characterized by low levels of attention and performance, high propensity to sleep and rest, low body temperature and sleep and relax, low body temperature and high peak in melatonin levels. In recent years, the progressive transition to the "24-hour society", which imposes on the labour market an increasing competitiveness on a global scale, has led to substantial changes in the organization of work until a few decades ago, shift and night work was adopted almost exclusively to ensure essential social services (transports, hospitals, telecommunications, public security). Over the last few years, it has become an important factor in the organization of work, aimed at increasing productivity and sustaining the competitiveness of companies and has therefore gone

extending in all the sectors (textile industry, mechanics, food, commerce, banking and free time).

By "shift work" is meant, in general, any form of organization of working time, different from the normal "daily work" in which the operating hours of the company is extended beyond the usual 8-9 hours during the day (generally between 8:00 and 17:00-18:00), to cover 24 hours, by means of the alternation of various groups of workers.

Shift work, and particularly that which includes night shifts, requires the worker to reverse the normal work cycle. The worker, in order to reverse the normal "sleep-wake" cycle, is forced to work during the period usually devoted to sleep and to rest during the usual period of wakefulness. This "adaptation" results in a progressive mismatch of biological rhythms, which increases as the number of successive night shifts increases (Copertaro, 2013). The subject is therefore exposed to a continuous stress in an attempt to adapt as quickly as possible to different schedules of work, which is invariably hampered by their continuous rotation with risks to health in the short and long term (low quality of sleep, decreased performance, increased risk of injury, gastrointestinal disease, cardiovascular disease) (Herichova, 2013).

Several studies have recently revealed a prevalence of metabolic disorders in night shift workers; especially overweight, obesity (both general abdominal), hypercholesterolemia (with reduced HFL fraction) and type 2 diabetes (increased insulin resistance) (De Bacquer et al., 2009); (Pietroiusti et al., 2010); (Karatsoreos et al., 2011). The onset of metabolic disorders is certainly favoured by changes in circadian rhythms of digestive and metabolic functions and changes in normal eating habits, conditioned by time and quality of food, and it is more evident in the case of night workers (Biggi et al., 2008); (Guerrero-Vargas et al., 2018). This, in the long run, may encourage or increase problems and disorders of the digestive system. The importance of metabolic response in relationship to the time at which food intake occurs was further demonstrated in another study where mice fed a high fat diet during the light phase showed a higher weight gain than the control group fed in the same way during the dark phase (Arble et al., 2009). Some studies (Kohsaka et al., 2007) clearly show that metabolism is in a position to control the peripheral clocks. The connection between the circadian clock and the homeostasis has been further investigated by mutation experiments against of

the main clock genes involved in the circadian circuit core (Hsieh et al., 2010). The deletion of genes CLOCK and *BMAL1*, involves the onset of abnormalities in the metabolic system Mutant mice for the Clock gene are arrhythmic and become obese developing the classic symptoms of metabolic syndrome such as hyperglycemia, (Turek et al., 2005).

Recently epidemiological studies have reported a possible association between shift work and cancer, in particular for the breast, the endometrium, the prostate, the colon-rectum, non-hodgkin lymphoma (Costa et al., 2010). Based on these studies, in 2007 the International Agency for Cancer Research (International Agency for Research on Cancer, IARC) defined that "shift work a cause of the disturbance of circadian rhythms" and thus as "probably carcinogenic" (2A) to man (Straif et al., 2007). The pathophysiological mechanisms through which circadian desynchronization can encourage the induction and promotion of malignant tumours are complex and multifactorial. In the case of breast cancer, for which there is more evidence, it is believed that, in the presence or absence of genetic predisposition, repeated desynchronization of circadian rhythms and sleep deprivations can lead to deficiencies in regulation of circadian cell cycle and consequent carcinogenic effects on breast estrogen-sensitive cells, as well as depressing immunological surveillance (Schernhammer et al., 2006).

In particular Stevens in 1987 formulated the "melatonin hypothesis" according to which the reduced production of melatonin that occurs during the night shift due to exposure to artificial light would result in increased oestrogen, recognized risk factors for the induction of breast cancer. Melatonin regulates directly the production and activity of oestrogen by interaction with estrogenic receptor and indirectly neutralizes the effects of estradiol on proliferation and invasiveness of breast cancer cells by virtue of its antioxidant properties (Stevens, 1987); (Schernhammer & Schulmeister, 2004); (Cos et al., 2008). Numerous works have highlighted the involvement of some clock genes in important processes responsible for the early stages of neoplastic transformation (e.g. DNA damage shelter, cell cycle control and apoptosis) (Collis & Boulton, 2007); (Shostak, 2017); (Soták et al., 2014). This evidence suggests that deregulation of the molecular circadian clock may be implicated in the carcinogenicity process (Bracci et al., 2014).

1.3.6 Skin Chronobiology

Studies of peripheral circadian clocks have frequently focused on organs and tissues concerning metabolism. But in recent years the peripheral circadian clock of the epidermis has attracted the attention of several research groups as it has been demonstrated that different skin functions, such as protection against stressogenic environmental factors, vary during 24h (fig. 10). The above factors can be for example the risk of physical damage, exposure to ultraviolet radiation (UV) or the risk of toxins. Where the influence of the central clock on the circadian rhythms in the epidermis is known (Tanioka et al., 2009), new evidence says that the skin does not only suffer the effects of the superchiasmatic nucleus clock, but self-regulates (Geyfman et al., 2012). The ontogenesis of the circadian clock with regard to the epidermis has yet to be deepened (Lin et al., 2009) but the skin as tissue has been framed as a model for the study of circadian clock during cell proliferation, tissue regeneration, aging and carcinogenesis.

The circadian clock almost certainly affects different gene groups and physiological functions in different types of skin cells (Sherratt et al., 2019). Circadian oscillators have been identified over the years in several known cell types, including epidermis and hair bulb keratinocytes. There is a strong circadian clock in progenitor stem cells of the basal epidermal layer (Plikus et al., 2015). Deletion of clock genes from the germ line and selectively in keratinocytes allows us to show that in this segment of the epidermis, there is a modulation of the diurnal DNA replication repair cycles given by the clock (Gaddameedhi et al., 2011). The circadian clock influences in the diversification functions of the suprabasal epidermal segments. Diurnal variations in the various physiological parameters that depend on the functional state of the supra-basal epidermal layer, include skin pH, water loss and corneal layer capacity (Yosipovitch et al., 1998); (Le Fur et al., 2001). In addition, a study on the cultures of human keratinocytes has been a support for clock regulation of the epidermal differentiation program (Janich et al., 2013). Considering that the stratum corneum is composed of dead cells, the mechanisms influenced by the daily variations of the epidermal parameters mentioned above are probably guided by the outputs of circadian genes in the epidermal layer *in vivo*, basal and/or immediately basal layers What has been found

so far is that circadian genes are expressed at least in a subset of dermal cells and skin adipose tissue (Lin et al., 2009). Cell cycle movement studies suggest that coordination between them could be treated by the effect on the duration of phase S (Clausen et al., 1979). Gaddameedhi et al in 2011 proved that this daily variation depends on a circadian clock inside the keratinocytes (Gaddameedhi et al., 2011). Melanocytes are skin cells specialized in the production of pigment and serve for protection against UV radiation. The melanocytes condense and actively displace melanin granules, which assimilate UV, to keratinocytes giving it Sunlight**.** For example, in mammals, it is known that melanocytes have an active circadian clock (Zanello et al., 2000); (Lengyel et al., 2013), but their circadian biology is not yet well understood. The control of the circadian cell cycle in the skin proposes an interfollicular epidermal strategy. However, the skin contains a variety of macromolecules including RNA, DNA and proteins that efficiently absorb UVB photons and therefore almost all of the UVB photons are absorbed by the macromolecules in the epidermis (Holick, 2016). Absorption of UVA radiation in the epidermis and dermis can cause the formation of free radicals which can damage proteins, DNA and RNA in the cells (Halliday & Byrne, 2014). Besides being an extremely efficient sunscreen, melanin, also acts as an antioxidant and free radical scavenger, thereby reducing free radical damage to the cells (Pathak & Fanselow, 1983).

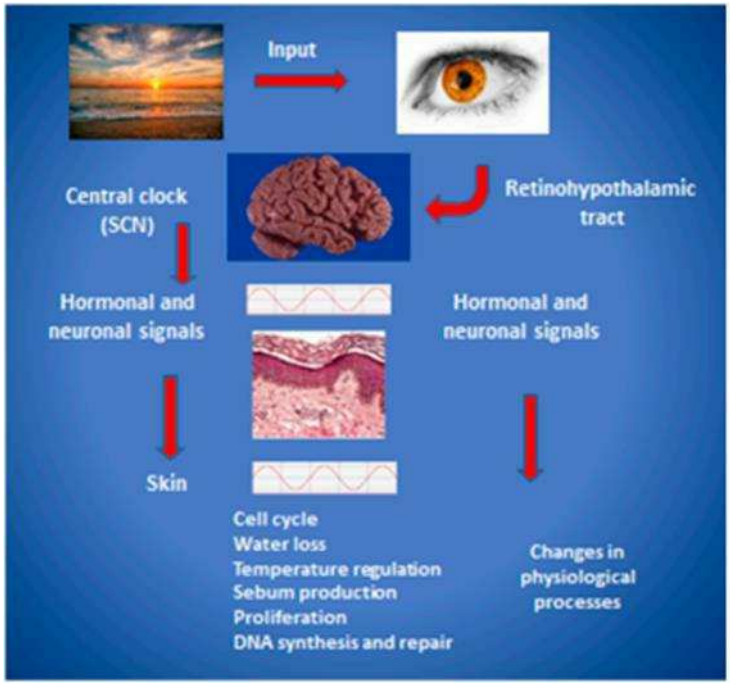

***Figure 10**. A model of hierarchical regulation and organization of the mammalian circadian clock. Hormonal and neuronal intermediaries then induce modulations of responses that ultimately produce behavioral, metabolic, and physiologic output. The skin and its appendages are the focus in this figure. (Source: Mary S. Matsui et al, Review Biological Rhythms in the Skin, 2016).*

2. PURPOSE OF STUDY

The skin is capable of absorbing external radiation belonging to the visible spectrum. Mammalian peripheral clocks are present in all nucleated cells in our bodies. Like other essential organs in the body, the skin has cycles that are informed by master regulator. In addition, skin cells have peripheral clocks that can function independently.

This thesis work aims to study circadian aspects of skin rhythms, including clock mechanisms, morphological and functional manifestations. To help learn more about the specific relationships between LED lamps and skin health consequences, the photobiological effects of blue and red light treatments on human skin cells and insects were studied to determine the biological effects resulting from different light treatments. These results will be used to hypothesize future applications in the field of artificial lighting, particularly in workplace lighting, affecting the health of workers.

3. MATERIALS AND METHODS

3.1 Study Design

In our model study, to better understand the mechanisms activated by light, we performed the experiments in two phases (fig. 11). In the first phase we used HaCaT cells, in the second phase (or *in vivo* phase) we studied the effects of light radiation on *Drosophila melanogaster*. HaCaT cells were exposed to four different exposure modes (see materials and methods section). High intensity (comparable to the energy transmitted by outdoor Sunlight) and low intensity (comparable to the energy transmitted by indoor artificial lamps) were evaluated for light intensity. The duration of exposure was fixed at 1h or 12h. With 1h of exposure, we investigated whether the circadian system and its dependent biological functions in keratinocytes were perturbed already in the presence of a minimal stimulus. With 12h of exposure, we assessed whether the circadian system and its dependent biological functions in keratinocytes were disturbed with the two different light radiations. We also emphasize that the illumination cycles had a total duration of three days, because from previous studies (Di Rosa et al., 2015) it has been seen that perturbations on the circadian cycle *in vivo* occur after a minimum duration of three days. Cells were analyzed on the fourth day (exactly at the end of 24 hours of the third lighting cycle) to assess the adaptations induced by the light stimulus. Constant darkness, already used in other studies, was chosen as control (T. Wang et al., 2020); (Di Rosa et al., 2015). After obtaining the first *in vitro* evidence we decided to expose *Drosophila melanogaster* to only two different exposure modes (see section materials and methods): high and low intensity 12L:12D cycle. The tests used provided greater clarity on the photobiological mechanisms triggered in the two experimental models.

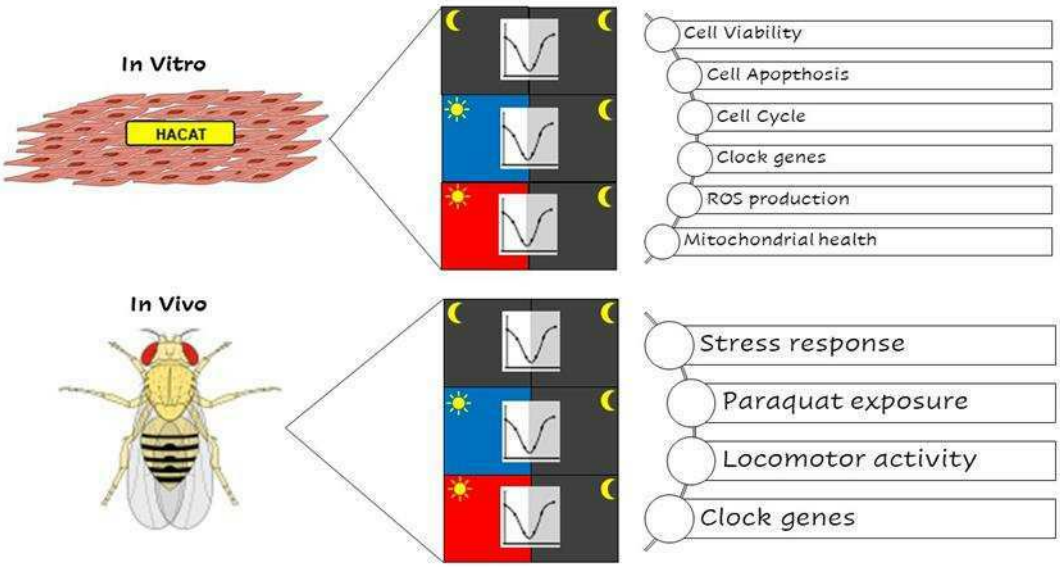

Figure 11. *Study design*

3.2 *In vitro* study

3.2.1 Cell Line and Culture Conditions

Immortalized aneuploid human keratinocytes (HaCaT) were provided by Experimental Zooprophylactic Institute of Lombardy and Emilia Romagna (Brescia, Italy). The HaCaT grown in T75 flasks (Corning, Durham, NC, USA) with 10 ml of medium Dulbecco's Modified Eagle's Medium (DMEM; Euroclone, Pero, Italy), containing 10% foetal bovine serum (FBS, Euroclone, Pero, Italy) and 1% penicillin / streptomycin (Euroclone, Pero, Italy). HaCaT cells was sub-cultured in a humidified incubator at 37 ° C in presence of 5% CO_2. Cell line was routinely tested negative for mycoplasma using the PCR Mycoplasma Test; these cells have been cultured not more than 10-12 passages. The line of non-cancerous keratinocytes HaCaT, is considered a reliable model for the *in vitro* keratinocytes of human skin (Fusenig & Boukamp, 1998); (Colombo et al., 2017).

3.2.2 Before the light exposure - starvation phase

The cells, before each treatment with the light radiation, were seeded and starved according to the following procedure. After seeding, the cells were maintained in DMEM HIGH GLUCOSE RED PHENOL (Euroclone, Pero, Italy) added with 10% of FBS + 1% pen / strep for 24 h. Before light exposure, cells were starved to synchronize the cell cycle. Cells were starved using DMEM LOW GLUCOSE (Euroclone, SPA, Pero, Italy) with 0.1% FBS + 1% pen/strep and kept in the dark for 24 h (Manzella et al., 2015; Manzella et al., 2013). Cell lines were first cultured with low-glucose DMEM (Euroclone, Pero, Italy) added with 0.1% FBS and 1% penicillin/streptomycin for 24 h. During the light exposition, cells were cultured with DMEM without red phenol (Lonza Group, Basel, Switzerland), complemented with 0.1% FBS and 1% penicillin/streptomycin for 3 days.

3.2.3 Light exposure

Samples were exposed to sham, blue, or red single-colour LEDs in a specific incubator at 37°C and 5% CO_2 for 3 days. Constant darkness was considered the sham light source, while light exposures were performed in a 12-h light/dark cycle (12L:12D) and a 1-h light/dark cycle (1L:23D) at high and low intensity. High-power blue and red LEDs (LD W5AM and LH W5AM Golden DRAGON® Plus, respectively; Osram) were used as the light sources. The LED viewing angle was 170° and the cells were placed at 14 cm above the light sources. The homogenous distribution of light and the spectrum of emission of each monochromatic LED were previously verified using an illuminance meter (CL-70; Konica Minolta Sensing, Inc.). The dominant wavelength was 465 nm for blue and 658 nm for red LEDs. Irradiance at peak wavelength at the cell surface was 0.84 W/m_2 for blue and 1.10 W/m_2 for red LEDs for high intensity and 0.01 W/m_2 for both blue and red LEDs for low intensity. Irradiance corresponded to the same total spectrum irradiance of 28.50 W/m_2 and 0.28 W/m_2 for both light sources at high and low intensity, respectively. The light energy transferred to cells every day was 1.23 J/mm_2 for high intensity and 0.01 J/mm_2 for low intensity.

The light exposure was set to reproduce the solar radiation and the indoor light. Blue and red lights were specifically chosen to test the opposite sides of the spectrum of visible light. Interference between light sources was prevented by using black curtains; sham exposure was additionally ensured by wrapping the plate with aluminum foil. LEDs in the incubator were fixed on an aluminum tank by thermal conductive paste, and the water circulation inside the tank (Amersham Multitemp III; GE Healthcare) was extracting the heat generated by the LEDs, so they could work at a constant temperature. These conditions assured a constant electric current and therefore a constant emitted energy. Air circulation inside the incubator was ensured using a fan. To exclude any thermal effects, the temperature at the cell level was verified and constantly measured during experiments with Thermochron iButton DS1922L (Maxim Integrated).

3.2.4 Cell viability assay

To assess the effect of LED light on cell viability, an XTT assay was performed, following the manufacturer's instructions (Cell Proliferation Kit II XTT; Merck KGaA, Darmstadt, Germany). HaCaT cells were seeded in 96-well plates (Costar, Corning incorporated, Corning, NY, USA) at a final concentration of $1.6x10^4$ cells/well and exposed to four different cycles of light for 3 days; constant darkness was used as control. At 24 h, 50 µl XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) solution was added to each well, followed by incubation for 2 h at 37°C and 5 % CO_2. The absorbance at 450 nm, with 650 nm as the reference wavelength, was measured using an absorbance microplate ELISA plate reader (Sunrise; Tecan Group Ltd., Männedorf, Switzerland Tecan). The proliferation index was expressed as relative change with respect to the controls set as 100%. Each assay was done in triplicate. Images of the cells were acquired on an inverted microscope (Leitz Fluovert; Leica Microsystems, Inc.) equipped with a digital camera (Canon EOS M50; Canon Inc.) at 100X and 200X magnification.

3.2.5 Apoptosis analysis

The apoptotic rate was evaluated using an Annexin V/Propidium Iodide (PI) apoptosis detection kit (eBioscience™ Annexin V-FITC Apoptosis Detection Kit; Thermo Fisher Scientific, Carlsbad, CA, USA), according to the manufacturer's instructions. Cells were seeded in a 6-well plate (Costar, Corning incorporated, Corning, NY, USA) at $2x10^5$ cells/ml for each well. After light treatments, cells were harvested, centrifuged (Eppendorf centrifuge 5415r, Merck KGaA, Darmstadt, Germany) at 770 *xg* for 5 minutes at 4°C, and washed with phosphate-buffered saline (PBS; Euroclone, Pero, Italy). Next, cells were resuspended in binding buffer plus Annexin V for 10 minutes, at room temperature, in the dark. Cells were stained with PI and analysed using FACSCalibur flow cytometer was used (BD Biosciences, San Jose, CA, USA). Cell Quest software, set on a logarithmic scale, was used for data evaluation (BD Pharmingen, Franklin Lakes, NJ). A minimum of 20,000 cells was acquired for each sample. Data were analysed using FlowJo™ Software (FlowJo™ Software for

Windows. Version 7.6.1. Ashland, OR, BD Company, USA). The analyses are done in triplicate.

3.2.6 Cell cycle analysis

HaCaT cells were seeded in a six-well plate (Costar, Corning incorporated, Corning, NY, USA) at a concentration of $2x10^5$ cells/ml. After light treatment, cells were harvested and centrifuged at 300 *xg* for 6 minutes at room temperature. Cells were fixed with 4.5 ml cold ethanol solution (70% in PBS) and kept in ice at 4°C for at least 2 hours. Subsequently, cells were washed twice with PBS and resuspended in PI staining solution with 0.1% TritonX-100 (Santa Cruz Biotechnology, Inc.; Dallas, USA), RNAse 0.2 mg/mL (Merck KGaA, Darmstadt, Germany) and PI 2 mg/ml (Merck KGaA, Darmstadt, Germany). Cells were incubated at 37°C for 15 minutes. Flow cytometric analysis was performed using FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with CellQuest software set on a linear scale (BD Pharmingen, Franklin Lakes, NJ). Data were analyzed using FlowJo™ Software (FlowJo™ Software for Windows. Version 7.6.1. Ashland, OR, BD Company, USA). A minimum of 20,000 cells was acquired for each sample. The analyses were performed in triplicate.

3.2.7 Real-Time qPCR

The cells were seeded with a density of $1x10^5$ cells / ml in six-well plates (Costar, Corning incorporated, Corning, NY, USA). After light treatments, total RNA was obtained from cells using the RNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. The total RNA first-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). Real-Time qPCR analysis was performed using the TaqMan Gene Expression Master Mix (Euroclone, Pero, Italy). *BMAL1*, *PER2* and *CRY2* gene expression levels (Thermo Fisher Scientific, Carlsbad, CA, USA) were analysed. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene expression levels were used for data normalization. The Forward (F) and Reverse (R) primers with 5'-3 'sequence for *BMAL1*, *PER2*, *CRY2* and *GAPDH* were reported in Table 1.

Genes	Primer Forward (5'->3')	Primer Reverse (5'->3')
BMAL1	CTGTTCATTTTATCCCGACGC	TCCACTGACTACCAAGAAAGC
PER2	TGTTCCACAGTTTCACCTCC	TGGTAGCGGATTTCATTCTCG
CRY2	GTCATATTCAAAGGTCAAGCGG	CATGGTTCCTACTTCAGTCTCTG
GAPDH	GGCCATCCACAGTCTTCTG	CAGCCTCAAGATCATCAGCAA

Table 1. Oligonucleotide sequences designed for target and reference gene.

The Ct values were extrapolated from the instrument software and normalized (ΔCt) with respect to the relative housekeeping (*GAPDH*). Relative expression levels were calculated according to the following formula: $2^{-\Delta Ct}$. The analyses were performed in triplicate.

3.2.8 Proteomics analysis

3.2.8.1 Protein extraction

Proteins were extracted following the protocol suggested by Ferrara company, using urea 8M-tris 100 mM HCl lysis buffer at pH = 8, scraping on ice cells with the extraction buffer. 2 x 10^5 cell/well HaCaT cells were seeded in six wells and exposed to high intensity light radiation for 12h for three days. After light, cells were washed with 1X PBS and the urea buffer 8m-tris 100mM HCL (150 µl / well in a six well plate) was added. The proteins were detached and collected using the scraper. Leave 30 minutes on ice by vortexing every 10 minutes. Cells were centrifuged (Eppendorf® Microcentrifuge 5415; Merck KGaA, Darmstadt, Germany) at 16000 *xg* for 20 minutes at 4ºC and protein concentrations were measured using a Bradford assay (Merck KGaA, Darmstadt, Germany). The sample's expedition was on dry ice. The resulting peptides were desalted on a home-made C18 StageTip. Peptides were resuspended in 20 µl of Solvent A (2% acetonitrile, 0.1% formic acid) and 3 µl was injected for every technical replica.

3.2.8.2 Mass spectrometry analysis and proteins quantification

A total of 4µl of each sample were analyzed as technical replicate on a LC–ESI–MS-MS quadrupole OrbitrapQExactive-HF mass spectrometer (Thermo Fisher Scientific, Carlsbad, CA, USA). Peptides separation was achieved on a linear gradient from 95% solvent A (2% ACN, 0.1% formic acid) to 30% solvent B (80% acetonitrile, 0.1% formic acid) over 210 minutes, from 30% from solvent B in 20 minutes and to 100% solvent B in 2 minutes at a constant flow rate of 0.25 µl/min on UHPLC Easy-nLC 1000 (Thermo Scientific) connected to a 23-cm fused-silica emitter of 75 µm inner diameter (New Objective, Inc. Woburn, MA, USA), packed in-house with ReproSil-Pur C18-AQ 1.9 µm beads (DrMaischGmbh, Ammerbuch, Germany) using a high-pressure bomb loader (Proxeon, Odense, Denmark). MS data were acquired using a data-dependent top 20 method for HCD fragmentation. Full scan MS spectra (300–1650 Th) were acquired in the Orbitrap with 60000 resolution, AGC target 3e6, IT 20 ms. For HCD spectra, resolution was set to 15000 at m/z 200, AGC target 1e5, IT 80 ms; NCE 28% and isolation width 2.0 m/z.

3.2.8.3 MS data acquisition

Two technical replicas of each sample were carried out. Raw data were processed with MaxQuant version 1.5.2.8. Peptides were identified from the MS–MS spectra searched against theuniprot_cp_Homo Sapiens (98036 entries) using the Andromeda search engine, in which trypsin specificity was used with up to two missed cleavages allowed.

Cysteine carbamidomethylation was used as a fixed modification, methionine oxidation and protein amino-terminal acetylation as variable modifications. The mass deviation for MS and MS–MS peaks was set at 5 and 20 ppm respectively. The peptide and protein false discovery rates (FDRs) were set to 0.01; the minimal length required for a peptide was six amino acids; a minimum of two peptides and at least one unique peptide were required for high-confidence protein identification. The lists of identified proteins were filtered to eliminate reverse hits and known contaminants.

Label-free analysis was carried out, including a ‘match between runs’ option (time window of 5 minutes). A minimum ratio count of 2 was considered and the ‘LFQ

intensities', which are the intensity values normalized across the entire data set, were used.

Statistical analyses were done using the Perseus program (Version 1.5.1.6) in the MaxQuant environment with a p-value 0.05. Missing values were replaced by random numbers drawn from a normal distribution by the function 'imputation' (width 0.3, down shift 1.8, separately for each column). Hierarchical clustering was set with the following parameters: distance, Euclidean, linkage, average, number of clusters, 300, for both row and column tree dendrograms generated by clustering in the Perseus environment.

3.2.8.4 Bioinformatics analysis. Functional enrichment analyses

Each differentially expressed protein that we identified has been assigned to the respective human official NCBI Gene Symbol identifier. These gene symbols were subsequently submitted to Enrichr (http://amp.pharm.mssm.edu/Enrichr) [27141961] web tool. This tool has been used to perform the gene list enrichment analysis, in order to elucidate the underlying molecular mechanisms altered by light radiations. In particular, statistical enrichment of Gene Ontology (GO) terms (sections "Biological Process", "Molecular Function", "Cellular Component", release 2018), Kyoto Encyclopedia of Genes and Genomes (KEGG, 2019) pathway database and WikiPathways 2019 were assessed by submitting the gene lists to Enrichr tool Up- and down-regulated genes were separately submitted to the web tools. Although the analysis of all differentially expressed genes/proteins together is possible, many recent studies suggested to analyse separately the up- and down-regulated gene lists, since it is more accurate [24352673, 29150651]. Statistically significant terms were selected for further analyses if reached adjusted P-value < 0.05, according to the Benjamini-Hochberg (BH) method for correction for multiple hypotheses testing.

3.2.9 Western blotting

Western Blotting is an extensively used technique for protein analysis. Proteins were extracted using RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.1% SDS, 150 mM NaCl, and 2 mM EDTA). Cells were maintained in ice for 30 minutes and vortexing

every 10 minutes. Next, the cells were centrifuged (Eppendorf centrifuge 5415r, Merck KGaA, Darmstadt, Germany) at 16,000 *xg* for 20 minutes at 4˚C. Supernatants were collected and protein concentrations were measured using a Bradford assay (Merck KGaA, Darmstadt, Germany). Proteins (30 µg) were resuspended in LDS Sample Buffer (Novex Life-Technologies, Carlsbad, CA, USA) and in Sample Reducing Agent (Novex Life-Technologies, Carlsbad, CA, USA). Denaturation step was performed boiling them at 100˚C for 5 minutes. Equal amounts of protein (30 µg) were resolved on precast 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bolt 4-12% Bis-Tris Plus; Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA) in MES SDS running buffer (Novex Life-Technologies, Carlsbad, CA, USA). The gel run is done at 100 V and 35 mA for staking gel and at 165 V and 60 mA for separating gel. A protein ladder (SeeBlue® Plus2 Prestained Invitrogen by ThermoFisher Scientific, Carlsbad, CA, USA) was used as a reference for protein size (3-198 kDa). After electrophoresis proteins were transferred onto a nitrocellulose membrane (NC; Nitrocellulose Blotting Membrane GE Healthcare Life Sciences, Amersham™ Protran™) using a semi-dry method in transfer buffer (Tris 25 mM, Glycine 0.2 M, 20% Methanol). The transfer was performed at 30 V and an amperage calculated based on the membrane's measurements (w x h x 0.8). The protein transfer was verified using red Ponceau (Ponceau S solution for electrophoresis (0,2%); SERVA Electrophoresis GmbH, Heidelberg, Germany), and then washed three times for 5 minutes with PBS-Tween-20 (PBST) 0.1%. The membrane was blocked with 5% non-fat dried milk-PBST (Euroclone, Pero, Italy) for 1 h at room temperature and then incubated overnight at 4˚C with specific primary antibodies: rabbit polyclonal anti-human anti-GAPDH antibody (dilution 1:1,000; Bethyl Laboratoires Inc. Montgomery, TX, USA), mouse monoclonal anti-human anti- Aldo-Keto Reductase family 1 member C3 (anti-AKR1C3, diluition 1:5000; Merck KGaA, Darmstadt, Germany), rabbit polyclonal anti-human anti-Thioredoxin reductase 1 (anti- TXNRD1, diluition 1:1000; Merck KGaA, Darmstadt, Germany) and rabbit polyclonal anti-human anti-superoxide dismutase 1 (anti-SOD1, diluition 1:1000; Merck KGaA, Darmstadt, Germany). Membrane was washed three times with PBST 0.1% and incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 3 h. Goat anti-Rabbit IgG (H+I) peroxidase/HRP-conjugated (dilution 1:3,000; Elabscience®

Biotechnology, Huston, TX, USA) or anti-mouse IgG (dilution 1:10000) (Goat anti-mouse IgG (H+I) FITC conjugated; Bethyl Laboratoires Inc. Montgomery, TX, USA). For signal chemiluminescent detection, the membrane was incubated for a few minutes with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific Carlsbad, CA, USA) and then developed in Alliance Mini (UVITEC Cambridge, Cambridge, UK) equipped with NineAlliance Software (UVITEC Cambridge, Cambridge, UK). Band quantification was carried out using the same software.

3.2.10 Intracellular ROS generation

Intracellular ROS levels were measured using the fluorescent dye 2'7'-dichlorofluorescein diacetate (DCFDA; oxidized by hydrogen peroxide to DCF, Merck KGaA, Darmstadt, Germany). HaCaT cells were seeded on a cover glass, in six-well plates (Costar, Corning incorporated, Corning, NY, USA) at a concentration of $2x10^5$ cells/ml. After light treatments, the cells were washed twice in with phosphate-buffered saline (PBS; Euroclone, Pero, Italy) and DCFH-DA solution in serum-free medium was added at a concentration of 10 µM for well for 45 minutes. The slides were washed 3 times, and DCF fluorescence was measured over the entire field of vision using a fluorescence microscope (Inverted Microscope Nikon Eclipse Ti2 (Nikon, Tokyo, Japan)). The mean fluorescence intensity (MFI) from 4 random fields was analyezd using ImageJ 1.41 software (National Institutes of Health, USA) according to the LINK's instructions (Lan et al., 2012). The MFI (Mean Fluorescence Intensity) of DCF was used to indicate the amount of ROS. The analyses were performed in triplicate.

3.2.11 Staining of mitochondria and nuclei

Mitochondria were staining using Mitotracker greenfluorescent probe conjugated with FITC (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA). HaCaT cells were seeded on $5x\ 10^4$ cells / ml chamber slides (Thermo Fisher Scientific, Carlsbad, CA, USA). After light treatments, cells were washed with DMEM without red phenol (Lonza Group, Basel, Switzerland) w/o FBS, and 500 ul / chamber of Mitotracker green diluted to 100 nM and prewarmed to 37 °C was added, then incubated at 37 °C in the incubator for 45 minutes. Subsequently, the cells were stained with Hoechst (Merck

KGaA, Darmstadt, Germany) at room temperature for 10 minutes. Cells were observed with a fluorescence microscope (Inverted Microscope Nikon Eclipse Ti2 (Nikon, Tokyo, Japan). The staining reactions were performed in triplicate.

3.2.12 Transmission Electron Microscope

Cells were plated on ACLAR and cultured at 1×10^5 cells/cm^2 (Ted Pella CA, USA); chips were trimmed to fit into the multiwell plates. The ACLAR insets were sterilized just before use. Cells were grown with control and neural medium, and medium was changed every 3 days. After light treatments, cells were fixed with glutaraldehyde 2%, 0.7 % tannic acid and 30 mM sucrose, in 0.1 M cacodylate buffer pH 7.0, for 1 h at room temperature and subsequently overnight at 4 °C. For TEM, cells were post-fixed in 0.5% osmium tetroxide for one additional hour, then dehydrated with acetone series and flat embedded in Luft epoxy resin using a polystyrene capsule. After hardening of the resin, ACLAR was peeled off and ultrathin sections (50 nm) obtained. The sections were stained with uranyl acetate alcoholic solution and lead citrate then imaged at 80 K volts in a Philips CM-12 electron microscope. Images are representative of three independent experiments.

3.3 *In vivo* study on *Drosophila melanogaster*

3.3.1 Flies maintenance

Drosophila melanogaster was maintained in nutrifly media (Bloomington Formulation, Genesee Scientific, San Diego, USA) added with propionic acid. Culturing of flies were kept in an incubator (Orbegonzo, Murcia, Spain) modified to maintain the constant temperature of 25.0 ± 0.2 °C in constant darkness. The genotypes used in this study are Wild-type (Canton-S), obtained from Bloomington Drosophila Stock Center, Indiana, USA. Newly hatched flies were collected and kept in vials (28.5x95 mm, Biosigma S.p.A., Cona, Italy) containing 10-mL food medium changed 2 times weekly. Unless stated otherwise; flies were killed, after necessary treatments, by instant freezing with dry ice.

3.3.2 Light treatments

High-power blue and red LEDs (LD W5AM and LH W5AM Golden DRAGON® Plus, respectively; Osram) were used as the light sources. Light emitted was measured at the level where the flies were held using an illuminance meter (CL-70; Konica Minolta Sensing, Inc.). The dominant wavelength was 465 nm for blue and 658 nm for red LEDs. Light treatments were the same used for cells treatment: irradiance at peak wavelength at the flyes level was 0.84 W/m^2 for blue and 1.10 W/m^2 for red LEDs for high intensity and 0.01 W/m^2 for both blue and red LEDs for low intensity. Irradiance corresponded to the same total spectrum irradiance of 28.50 W/m^2 and 0.28 W/m^2 for both light sources at high and low intensity, respectively. The light energy transferred to flies every day was 1.23 J/mm^2 for high intensity and 0.01 J/mm^2 for low intensity. Vials containing flies were exposed to 12-h light/dark cycle (12L:12D) of blue or red light at low or high intensity for 3 days and then tested. Control flies were kept in constant darkness. Each vial contained a total of 20 1-week-old flies. An equal number of males and females were included in each vial to avoid sex-related differences in results.

3.3.3 Responses to stress

Two methods were used to measure the response of flies to physiological stress: heat sensitivity and cold coma recovery time. For heat sensitivity, flies were exposed to heat stress. After light radiation treatment, flies were placed in new vials and placed in a water bath at 38.5°C for 45 minutes. They were then transferred to vials containing fresh food. Live flies (% survival) were recorded 24 hours later. Measurements were in triplicate.

In addition, flies were evaluated for cold coma. After light exposure flies were placed in empty vials (20 flies per vial) and immersed for 24 hours in a 4 °C water bath. Subsequently, they were left at ambient temperature ~20 °C, and spontaneous recovery time from cold coma was measured every 6 minutes for 90 minutes. Measurements were in triplicate.

3.3.4 Paraquat Exposure

In a preliminary study, flies were exposed to Paraquat (PQ, Merck KGaA, Darmstadt, Germany) at various concentrations such as 10, 20, 100, and 200 mM for 24 h to determine lethality (data not shown). However, we used only 20 mM since with this dose the lethality was around 50% after 24 h. Flies, after light exposure, were deprived of gruel and transferred to vials containing filter paper discs (Whatman, Life Sciences, UK) soaked with water containing 5% sucrose (Merck KGaA, Darmstadt, Germany) and 20 mM PQ. After 24 h, the number of dead insects was observed. Measurements were in triplicate.

3.3.5 Climbing Assay

As a behavioural biomarker, we tested the climbing ability of *Drosophila melanogaster* flies. After flies were exposed to different types of light, they were placed in transparent cylinders (without diet) with a mark placed on the side for each cm from the bottom (0-8 cm). Flies were tapped gently on the bottom (groups to be compared were tapped simultaneously); the upward movement of the fly was recorded, and images were captured after 4 seconds. These images were analyzed to calculate the average distance travelled for each fly in each vial. The measurements were in triplicate.

3.3.6 RNA extraction and Real-Time qPCR

Flies were kept on dry ice until extraction actually begins. Flies heads were separated from bodies by vortexing flies in 15 ml tubes (EuroClone, Pero, Italy) for 15 seconds (for tree times) previously submerged in liquid nitrogen. Each sample of 30 heads for tubes was homogenized in 400 µl TRIzol (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA) with a pellet pestle. The homogenate was incubated at room temperature for 5 minutes. Then was added 80 µL of chloroform (Merck KGaA, Darmstadt, Germany) shaking vigorously for 15 s, and incubated for 3 minutes at room temperature. The samples were then centrifuged in the microcentrifuge 12,000g for 15 minutes at 4°C; after differential separation, we transferred the aqueous phase to a fresh microcentrifuge tube (Biosigma S.p.A., Dominique Dutsher Company, VE, Italy) and

added 200 µL of isopropanol (Merck KGaA, Darmstadt, Germany). After 10 minutes of incubation at room temperature, a 15-minute microcentrifuge (12,000g) at 4°C was performed again to separate down the RNA. Finally 500 µl 75% ethanol (Carlo Erba, Reagents S.r.l., MI, Italy) was added to the pellet and microfuge again for 5 min. To remove the ethanol, the supernatant was removed and the RNA pellet was left to air dry for 30-45 min. Ultimately, it was diluted in 30 µl of ultrapure RNase-free filtered water. The total RNA first-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). Real-Time qPCR was performed using the TaqMan Gene Expression Master Mix (Euroclone, Pero, Italy). *TIM* (Timeless) and *PER* (Period) gene expression levels (ID: Dm01814246_g1 and ID: Dm01843684_g1 respectively; Thermo Fisher Scientific, Carlsbad, CA, USA) were analyzed. *RPL2* (ribosomal protein L2, ID: Dm02151827_g1; Thermo Fisher Scientific, Carlsbad, CA, USA) housekeeping gene expression levels was used for data normalization.

The Ct values were extrapolated from the instrument software and normalized (ΔCt) with respect to the relative housekeeping (RPL2). Relative expression levels were calculated according to the following formula: $2^{-\Delta Ct}$. The analyses were performed in triplicate. Each group consisted of 3 replicates containing 30 flyes each. Measurements were in triplicate.

3.4 Statistical analysis

Three independent experiments were conducted, and the results were reported as the mean ± standard deviation (SD). Graphpad Prism software (GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis. One-way ANOVA and Dunnett's multiple comparison test as a post-hoc test were used to evaluate statistical significance for each treatment. $P<0.05$ was considered statistically significant.

4. RESULTS

4.1 *In vitro* study

4.1.1 High Intensity exposure: effects of 1L:23D and 12L:12D Light/Dark cycle

The 12-hour high-intensity blue light treatment is more effective in reducing cell viability.

The HaCaT cells exposed to light 1h high intensity (fig. 12A) displayed a decrease in viability with blue light (BLUE) and red light (RED) compared to the control group (CTRL) ($P<0.001$ e $P<0.01$ respectively). The highest percentage of inhibition of viable cells was obtained in BLUE group for 12h (12L:12D) at high intensity, compared with the CTRL ($P<0.0001$, fig. 12B). HaCaT cells exposed to 12 hours of high-intensity blue light decreased by approximately 80% whereas for cells exposed to red light and there was approximately 30% reduction in cell viability compared with CTRL ($P<0.05$).

A B

C

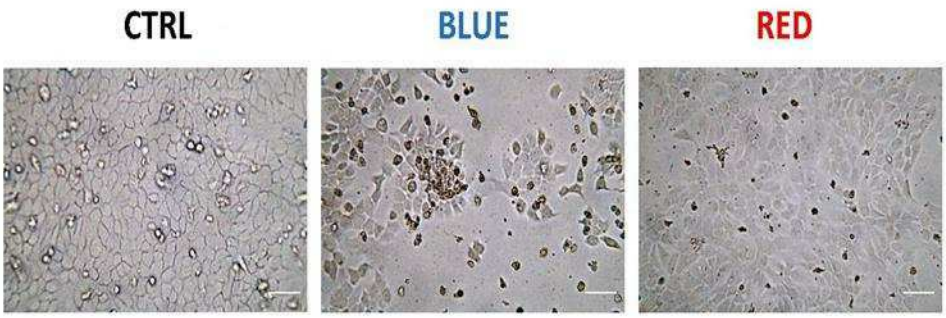

Figure 12. *Analysis of cell viability in HaCaT cell lines not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 1h high intensity 1L:23D cycles (A) and 12h high intensity 12L:12D cycles (B). Columns represent mean values and bars represent ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. CTRL. Bottom, confluence of HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 12h high intensity 12L:12D cycles (C). Representative images captured at magnifications of x100 magnification and scale bar 50 µm.*

The 12-hour high-intensity blue light treatment produces a high percentage of apoptotic cells.

Flow cytometry revealed that blue light treatment triggered a significant increase in apoptotic rate, compared with control group (Fig. 13) when cells were exposed to both 1h and 12h high-intensity light. HaCaT exposed to 1h high-intensity blue light showed an increase of apoptotic rate (21.15 %; P<0.05), as compared the CTRL (14.65 %). However, high intensity blue light treatment for 12h was more effective in increasing apoptosis compared to CTRL (80.16 % and 22.24 % respectively, P<0. 0001). The RED showed an apoptotic rate similar to the CTRL, both after 1h and 12h light treatments.

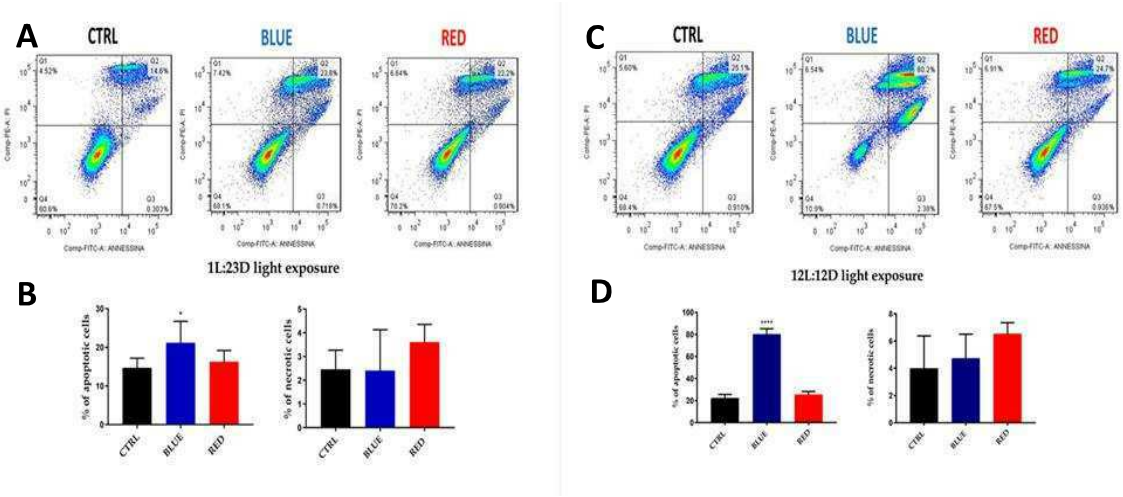

Figure 13. *Apoptotic and necrotic effects of exposure of HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 1h high intensity 1L:23D cycles and 12h high intensity 12L:12D cycles. Data indicate the percentage of apoptotic and necrotic cells of HaCaT cell lines not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 1h high intensity 1L:23D cycles (A-B) and 12h high intensity 12L:12D cycles (C-D). Columns represent mean values and bars represent ± SD (n=3). *P<0.05 and ****P<0.0001 vs. CTRL.*

The 12-hour high-intensity blue light treatment provides cell cycle shift in G_0/G_1 and G_2/M phases.

HaCaT cells, exposed to blue light for 12h at high intensity, showed a decrease in G_0/G_1 phase and an increase in cells in G_2/M phase compared to control group ($P<0.01$ and $P<0.001$ respectively; fig. 14B). HaCaT cells, exposed to 1h high-intensity blue or red light, displayed not changes in the cell cycle compared with the control group.

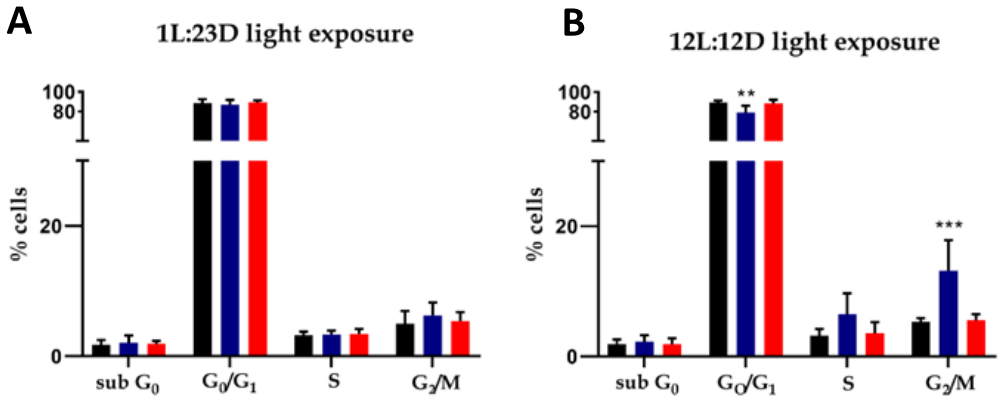

Figure 14. *Effect on cell cycle progression in HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 1h high intensity 1L:23D cycles and 12h high intensity 12L:12D cycles. Data indicate the effect of treatments on the different cell cycle phases of the HaCaT cell line exposed to LED blue light (BLUE) or LED red light (RED) at 1h high intensity 1L:23D cycles (A) and 12h high intensity 12L:12D cycles (B). Columns represent mean values and bars represent ± SD (n=3). ** P<0.01 and ***P<0.001 vs. CTRL.*

The 12-hour high-intensity blue light treatment altered the clock genes expression at mRNA levels.

Quantitative real-time polymerase chain reaction (RT-qPCR) showed a significant downregulation of mRNA levels of *BMAL1* and *PER2* after exposure to blue light for 12h at high intensity compared to the control group ($p<0.01$; fig. 15D and fig. 15E). For *CRY2* gene RNA expression levels, a non-significant decrease was observed for cells exposed to both blue light and red light 12h high intensity compared to the control group. With 1h high intensity exposure, only mRNA levels of *BMAL1* were significantly downregulated ($P<0.05$ fig.15A). In contrast *PER2* and *CRY2* mRNA expression levels were not significantly changed (fig 15B and fig 15C).

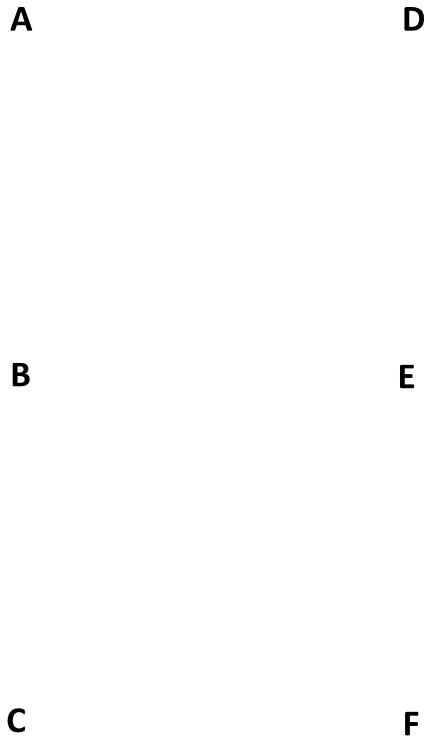

Figure 15. *Expression mRNA levels of BMAL1, PER2, CRY2 genes in HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 1h high intensity 1L:23D cycles (A-C) and 12h high intensity 12L:12D cycles (D-F). Columns represent mean values and bars represent ± SD (n=3). * P<0.05 and **P<0.01 vs. CTRL.*

Proteomic analysis revealed that 12-hour high-intensity blue light treatment provides a significant variation of protein involved in oxidative stress.

We report here the proteomic profile of HaCaT cells exposed to blue and red lights only for 12 hours at high intensity (CTRL, BLUE and RED). Hierarchical cluster analysis of protein expressions in all sample groups demonstrated a clear separation of treated samples from controls and reveals that HaCaT cells exposed to blue or red lights, compared with unexposed cells, modified their protein profile and altered the expression levels of many proteins (fig. 16.1).

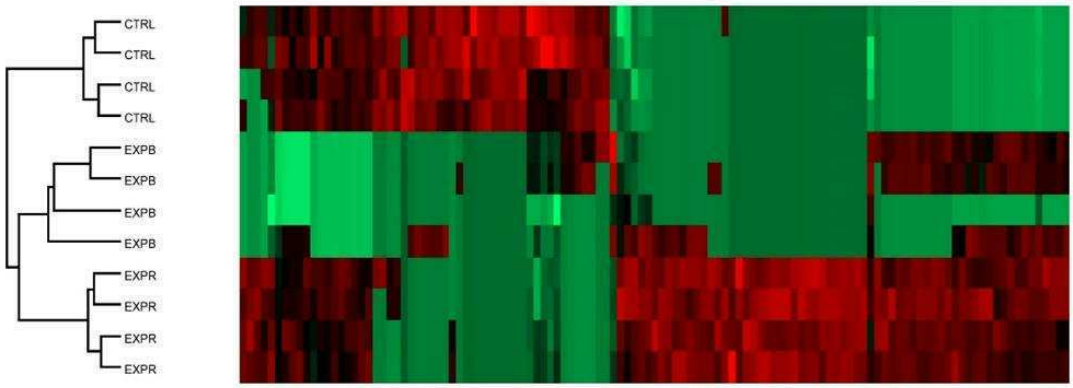

Figure 16.1 *Heat map show differential protein levels of HaCaT cells not exposed (CTRL) and exposed to LED blue light (EXPB) or LED red light (EXPR) at 12h high intensity 12L:12D cycles.*

Data analysis showed that 31, 25, 62 and 36 elements were expressed only in "Up Blue", "Down Blue", "Up Red" and "Down Red", respectively. Additionally, 21 common genes expressed in "Up Blue" and "Up Red" and 16 common elements in Down Blue" and "Down Red" were identified. Only one element was shared between "Up Blue" and "Down Red", i.e. the TNC (Tenascin C) gene. Instead, no protein was evident in common between "Up Red" and "Down Blue" (fig. 16.2).

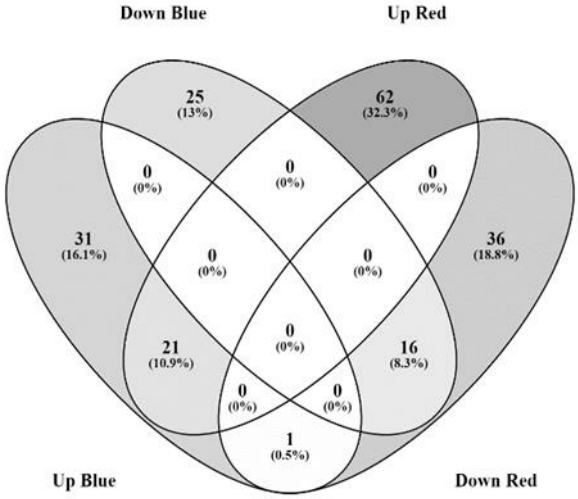

Figure 16.2 *- Venn Diagram of HaCaT cells not exposed (CTRL) and exposed to LED blue light (EXPB) or LED red light (EXPR) at 12h high intensity 12L:12D cycles.*

The Gene Enrichment Analysis, performed by using Enrichr tool, showed that the effects of both blue and red lights results in up-regulation of genes significantly (adjusted P-values < 0.05) enriched in "Oxidative stress" (WP408, WikiPathways 2019 Human) and "Oxidative Damage" (WP3941, WikiPathways 2019 Human). Interestingly, the genes AKR1C3 (Aldo-keto reductase family 1 member C3 (3-alpha hydroxysteroid dehydrogenase, type II)) and TXNRD1 (Cytoplasmic thioredoxin reductase 1) belongs to the 21 common genes expressed in "Up Blue" and "Up Red" and are included in the signalling pathways involved in oxidative stress and cellular response to stress. Moreover, SOD1 (Superoxide dismutase [Cu-Zn]), a key player in the mechanism of cellular response to oxidative stress, was the most differentially expressed between blue and red.

The 12-hour high-intensity blue light treatment modified the protein expression levels of AKR1C3, TXNRD1 and SOD1.

Western blot was then used to validate the expression levels of proteins influenced by light radiation: TXNRD1: Cytoplasmic thioredoxin reductase 1, AKR1C3: Aldo-keto reductase family 1 member C3 (3-alpha hydroxysteroid dehydrogenase, type II) and SOD1: Superoxide dismutase [Cu-Zn].

TXNRD1 and AKR1C3 were highly expressed in cells exposed to blue light compared with control group ($P<0.05$ and $P<0.01$, respectively; fig. 17A). About SOD1, western blot showed a significant increasing in their expression level under red light (RED) compared to the control group (CTRL) ($P<0.05$; fig.17A).

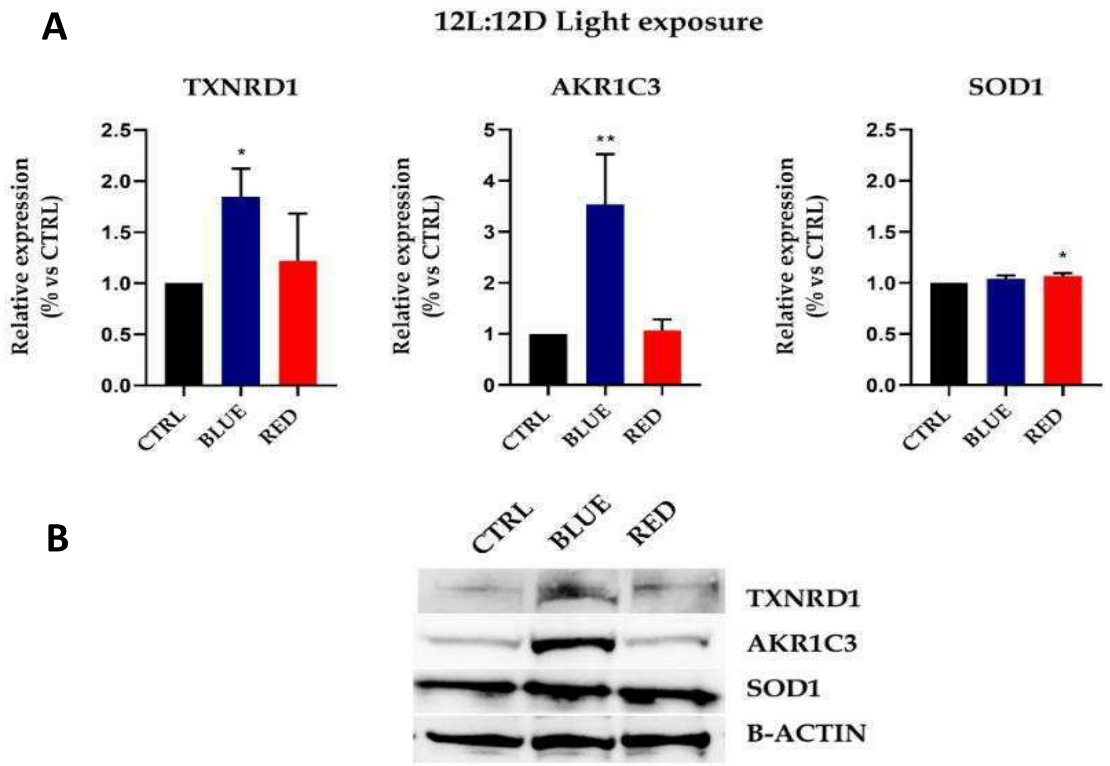

Figure 17. *(A) Analysis of protein expression levels of TXNRD1, AKR1C3 and SOD1 in HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 12h high intensity 12L:12D cycle. Values were normalized to β-ACTIN. Columns represent mean values and bars represent ± SD (n=3). *P<0.05 and **P<0.01 vs. CTRL. (B) Representative blot bands are presented.*

The 12-hour high-intensity blue light treatment increase Intracellular ROS.

Only cells exposed to blue light for 12h at high intensity showed a higher level of intracellular ROS compared to the control group ($P<0.05$; fig.18B). No significant change in ROS level was found in HaCaT exposed to red light respect to control group. The mitochondrial density of HaCaT cells appears to be influenced by high-intensity blue light for 12h (fig. 18C).

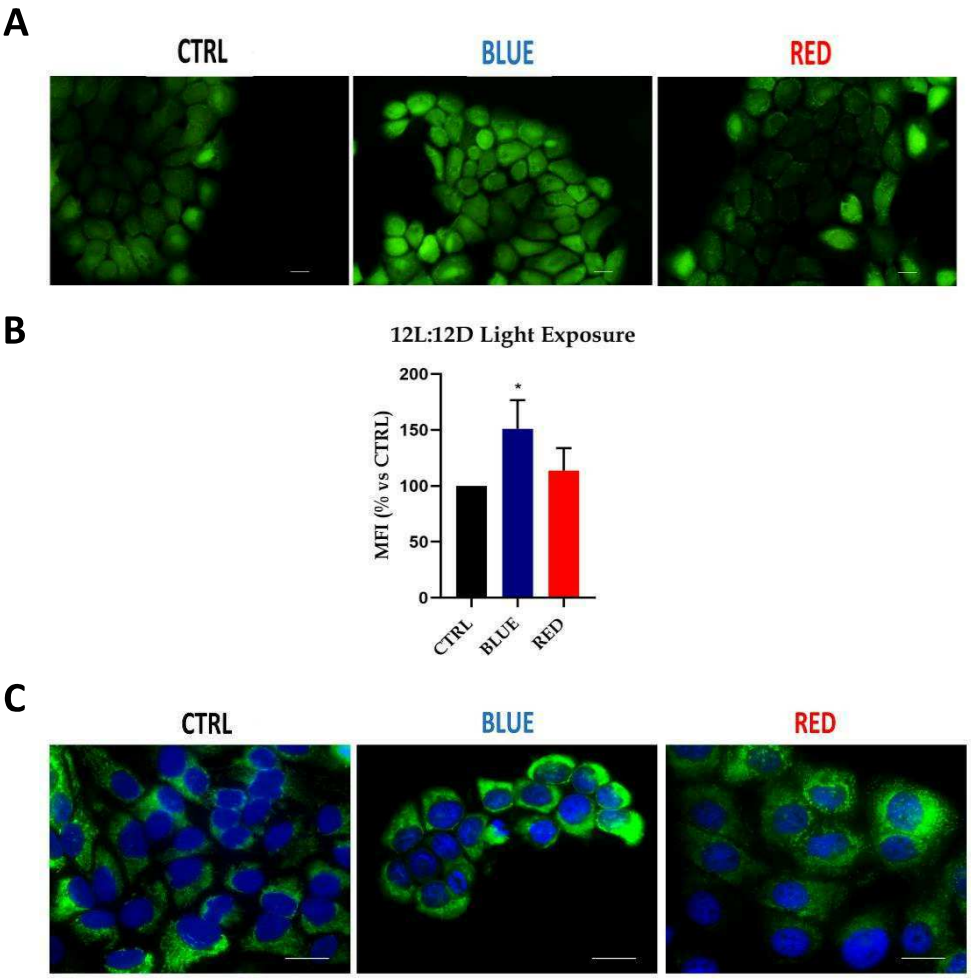

Figure 18. *(A) Representative fluorescence images of ROS levels with DCF probe (green colour) in HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 12h high intensity 12L:12D cycles; magnification x600 and scale. (B) Columns represent mean values and bars represent ± SD (n=3). *P<0.05 vs. CTRL. MFI= Mean Fluorescence Intensity. (C) Bottom, fluorescence confocal images of HaCaT cells double-stained with Mitotracker green (for mitochondria) and Hoecst (for nucleus, blue colour) not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 12h high intensity 12L:12D cycles; magnification x600 and scale bar 20 µm.*

The intracellular system and some organelles of HaCaT cells appears to be affected by high-intensity blue and red lights for 12 hours.

There are very few published descriptions of HaCaT ultrastructure. Furthermore, the batch used, and the techniques used to study the ultrastructure of this cell line may introduce morphological determinant that must be addressed. Therefore, we provide initially a brief description of the HaCaT cell line used in this research kept in dark and used as control reference for the study. The cells appear to be scarcely differentiated, the nucleus/cytoplasm ratio is around 1.5:1, the chromatin is dispersed in the nucleus, one or more nucleoli are present. Cells grow in clumps and are tightly connected via the numerous microvilli; however, no desmosomes can be seen. Within the clumps two differently contrasted cells are present (fig. 19.1). The higher contrast seems to be due to an increased protein content diffused in the cytoplasm. The darker cells seem to have more keratohyalines deposits (Yellow arrow, fig. 19.1) than the lighter ones, however this is a qualitative observation that has not been quantified. Lysosome and multivesicular bodies are present at a low concentration. Mitochondria are long branched and hyperfused. In almost all cells is present a particular kind of intracellular vesicles that shows translucent content surrounded by a rim of denser material (Red arrow; fig. 19.1). This kind of vesicles are not present in normal keratinocytes in tissues and are not reported in any previous literature. Tonofilaments are frequently seen.

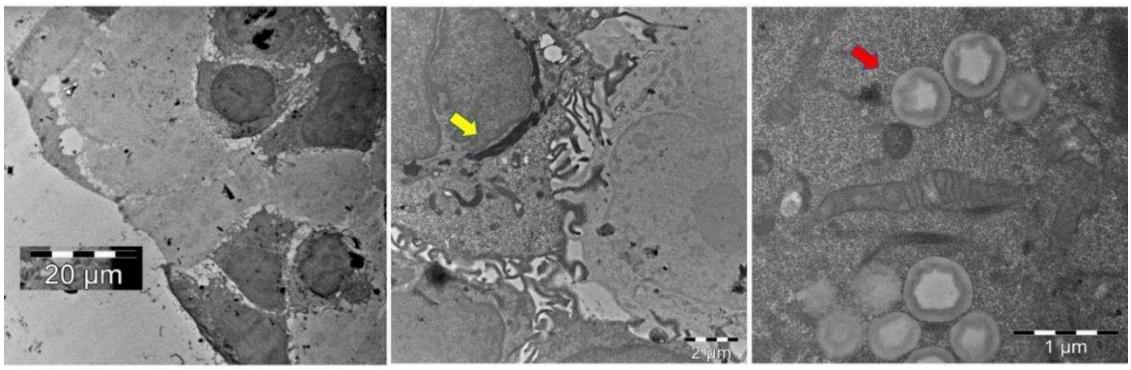

Figure 19.1. *Electron microscopy. TEM images of HaCaT not exposed to LED light (CTRL). Yellow arrow indicates keratohyalines deposits, red arrow indicates intracellular vesicles.*

Cells exposed to blue light (BLUE) show signs of damage: many cells are vacuolated (fig. 19.2), have damaged mitochondria (red arrow) and a dilated Golgi (yellow arrow).

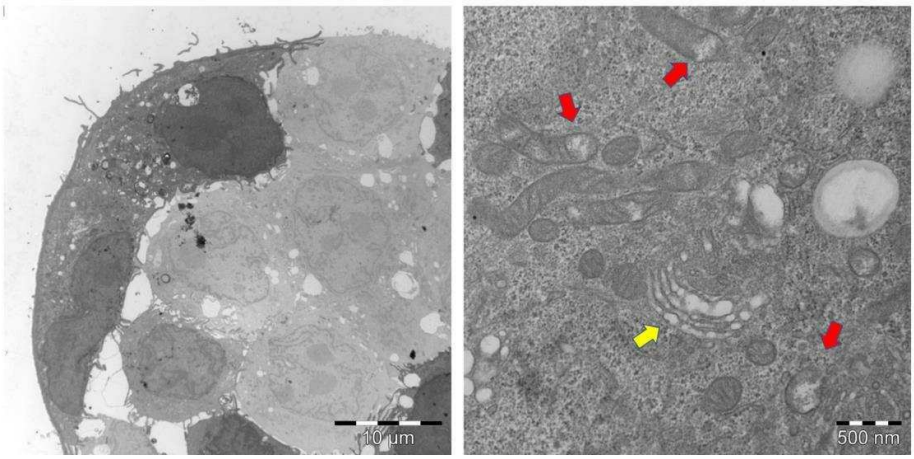

Figure 19.2 *Electron microscopy. TEM images of HaCaT exposed to LED blue light (BLUE) at 12h high intensity 12L:12D cycle. Yellow arrow indicates Golgi, red arrow indicates mitochondria.*

Cells exposed to red light (RED) contain a large amount of tonofilaments, (Red arrow; fig. 19.3) and show a higher lysosomal content respect BLUE and CTRL condition (Red arrow). Green arrow point to a possible melanin granule. Yellow arrow indicate tonofilaments.

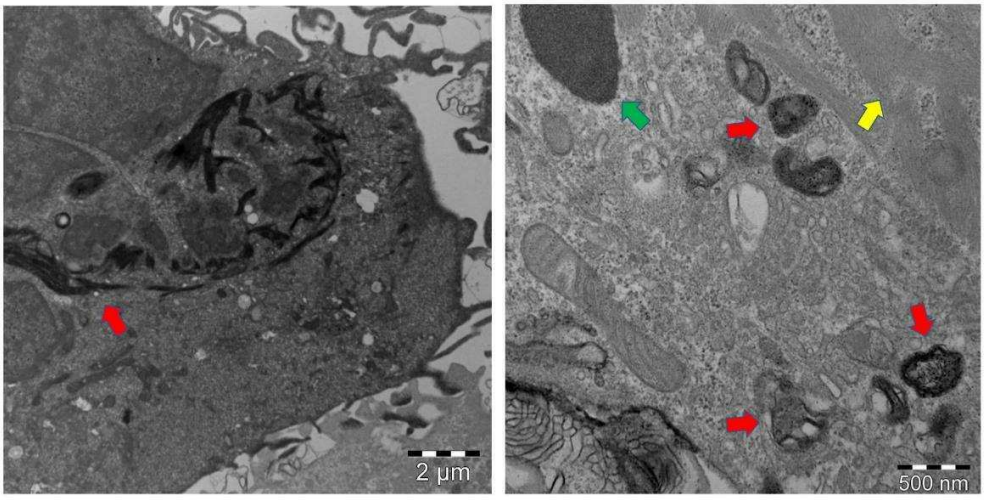

Figure 19.3 *Electron microscopy. TEM images of HaCaT exposed to LED red light (RED) at 12h high intensity 12L:12D cycle. Yellow arrow indicates tonofilaments, red arrow indicates lysosomal and green arrow indicates melanin granule.*

4.1.2 Low Intensity exposure: effects of 1L:23D and 12L:12D Light/Dark cycle

Low-intensity blue light treatment of 1h and 12h did not significantly decrease cell viability.

After 12h and 1h low-intensity light treatments, HaCaT cells showed no significant changes in cell viability compared with control group (fig. 20).

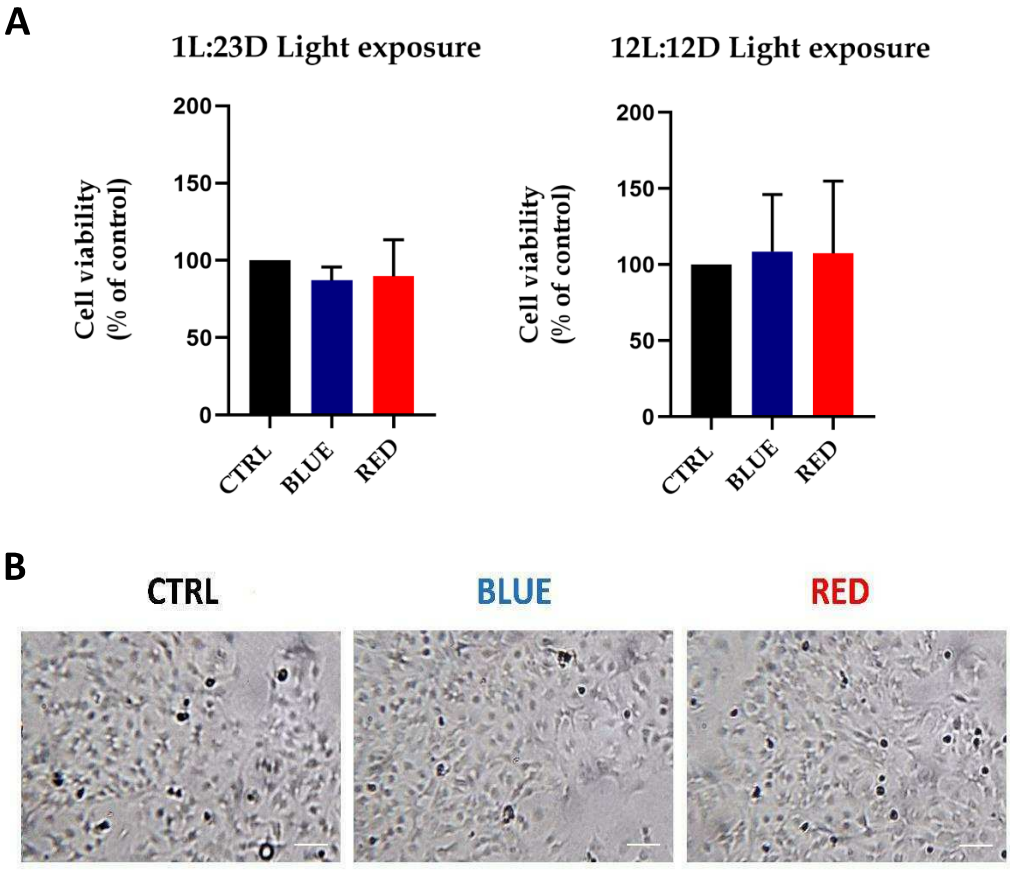

Figure 20. *Analysis of cell viability in HaCaT cell lines not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 1h low intensity 1L:23D cycles (A) and 12h low intensity 12L:12D cycles (B). Columns represent mean values and bars represent ± SD (n=3). Bottom, confluence of HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 12h low intensity 12L:12D cycles (C). Representative images captured at magnifications of x100 magnification and scale bar 50 µm.*

Low-intensity 1-hour and 12-hour blue light and red light treatments induced apoptosis in HaCaT cells.

Annexin V stanning showed that low intensity light treatments triggered, in HaCaT cells, a significant increase in apoptotic rate, compared with control (fig. 21), for both 1h and 12h of exposure period. HaCaT exposed to 1-h low-intensity light revealed that the BLUE and RED showed a significant increased number of apoptotic cells (9.85 % and 10.8 % respectively; fig. 21B), compared with the CTRL (5.37 %; $P<0.01$). However, there was an increase in apoptotic rate only in HaCaT cells exposed at 12h low intensity red light (17 %; fig. 21D) compared to CTRL ($P<0.01$). HaCaT cells showed no significant changes in percentage of necrotic cells compared with control group at both 1h and 12h low intensity (fig. 21B and fig. 21D).

Figure 21. *Apoptotic and necrotic effects of exposure of HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 1h low intensity 1L:23D cycles and 12h low intensity 12L:12D cycles. Data indicate the percentage of apoptotic and necrotic cells of HaCaT cell lines not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 1h high intensity 1L:23D cycles (A-B) and 12h high intensity 12L:12D cycles (C-D). Columns represent mean values and bars represent ± SD (n=3). **P<0.01 vs. CTRL.*

Low-intensity 12-hour blue light treatment did not affect the normal HaCaT cell cycle.

From the analysis of PI-positive cells, no change was found in HaCaT cells exposed to light for 12h at low intensity at any stage of the cell cycle compared with control cells (fig.22B).

Cells exposed to the low-intensity light cycle for 1h significantly increase from the CTRL group after exposure to red light in the G_2/M phase ($P<0.05$; fig. 22A).

Figure 22. *Effect on cell cycle in HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 1h low intensity 1L:23D cycles and 12h low intensity 12L:12D cycles. Data indicate the effect of treatments on the different cell cycle phases of the HaCaT cell line exposed to LED blue light (BLUE) or LED red light (RED) at 1h low intensity 1L:23D cycles (A) and 12h low intensity 12L:12D cycles (B). Columns represent mean values and bars represent ± SD (n=3). *P<0.05 vs. CTRL.*

The 12 hours low-intensity blue light treatment altered clock gene mRNA expression levels.

After exposure of cells to low intensity light radiation for 1h and 12h, HaCaT cells showed a significant deregulation of the *CRY* mRNA expression levels. Blue light 12h low intensity was able to induce a downregulation of *CRY2* gene expression level respect to the CTRL ($P<0.001$; fig. 23F). Differently there was significant increase of the *CRY2* mRNA expression level in cells exposed to red light compared with CTRL ($P<0.05$; fig. 23F).

HaCaT cells exposed for 1h at low intensity blue light, showed a significant downregulation of *PER2* compared with control group ($P<0.05$; fig.23B). For *CRY2* mRNA expression level, a decrease was observed for cells exposed to blue light compared to the control group ($P<0.05$; fig.23C).

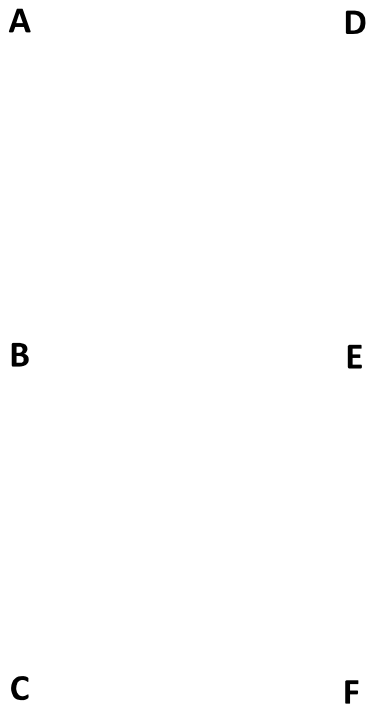

Figure 23. *Expression profiles of BMAL1, PER2, CRY2 genes in HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 1h low intensity 1L:23D cycles (A-C) and 12h low intensity 12L:12D cycles (D-F). Columns represent mean values and bars represent ± SD (n=3). * P<0.05 and ***P<0.001 vs. CTRL.*

Treatments of 12h at low intensity with blue light and red light altered AKR1C3 and SOD1 protein expression levels.

AKR1C3 was significant overexpressed in cells exposed to blue light compared with the control group (P<0.01; fig. 24A). SOD1 protein, western blot showed an increase in their expression levels only after RED treatment compared to the CTRL (P<0.05; fig. 24A).

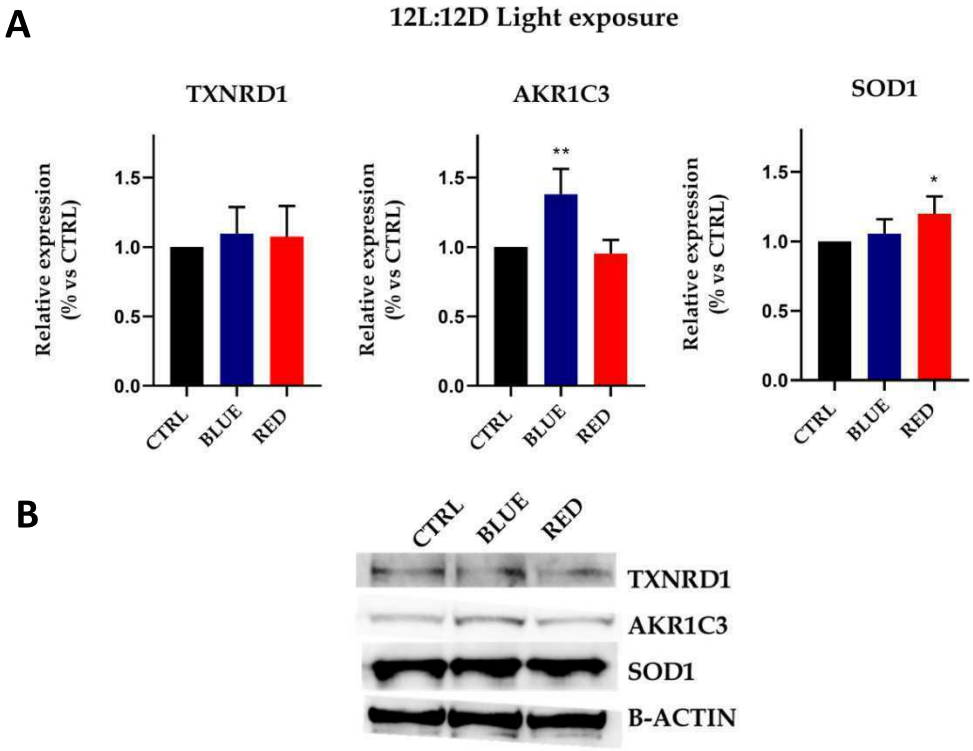

Figure 24. *(A) Analysis of proteins TXNRD1, AKR1C3 and SOD1 expression levels in cell lysates from HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 12h low intensity 12L:12D cycles. Values were normalized to β-ACTIN. (B) Columns represent mean values and bars represent ± SD (n=3). *P<0.05 and **P<0.01 vs. CTRL. Representative blot bands are presented.*

Intracellular ROS displays a significantly increased after treatment with blue and red low intensity light for 12 hours.

HaCaT cells exposed to low intensity blue and red light for 12 hours, showed a high level of intracellular ROS compared to control group ($P<0.05$; fig. 25B). The HaCaT cells show altered mitochondrial density by blue light and red light at low intensity for 12 hours (fig. 25C).

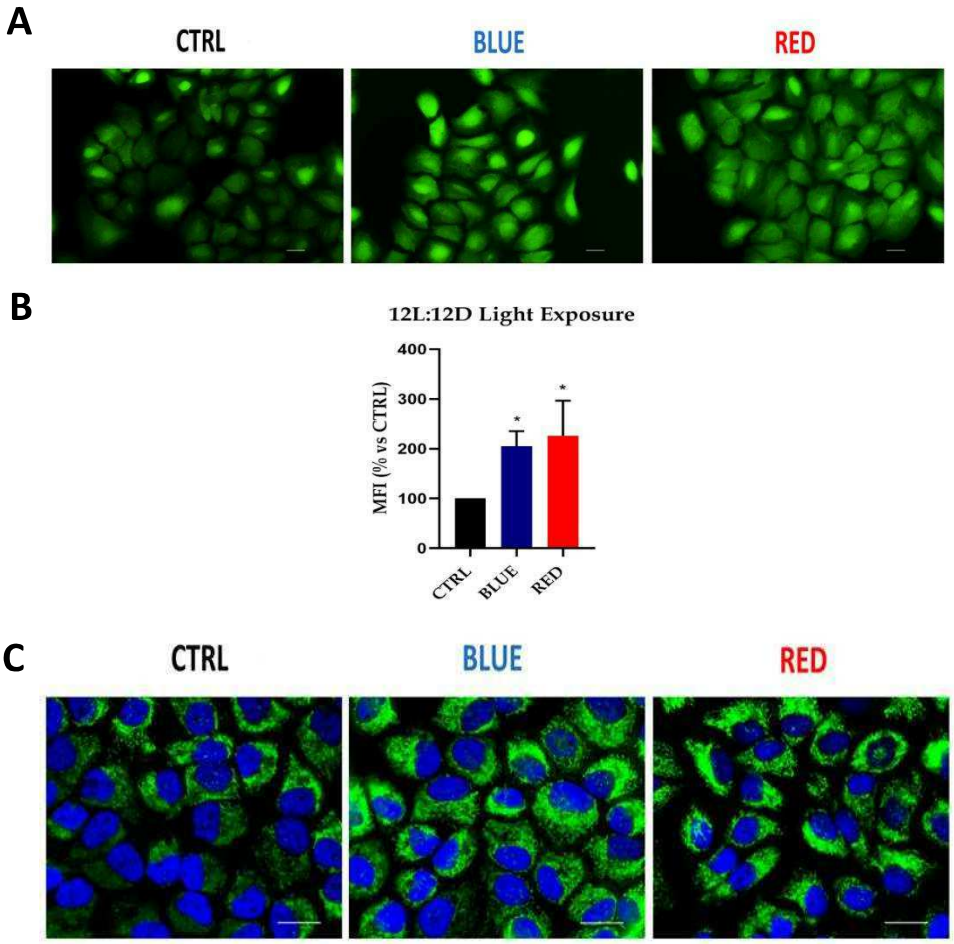

Figure 25. *(A) Representative fluorescence images of ROS levels with DCF probe (green colour) in HaCaT cells not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 12h low intensity 12L:12D cycles. Magnification x60 and scale bar 20 µm. (B) Columns represent mean values and bars represent ± SD (n=3). MFI= Mean Fluorescence Intensity. (C) Bottom, Fluorescence confocal images of HaCaT cells double-stained with Mitotracker green (for mitochondria) and Hoecst (for nucleous, blue colour) not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 12h low intensity 12L:12D cycles. Magnification x60 and scale bar 20 µm.*

4.2 *In vivo* study

4.2.1 High Intensity and Low Intensity: effects of 12L:12D Light/Dark cycle

Insects exposed to 12h low and high intensity blue light show less heat sensitivity and more ability to recover from cold coma.

Two different assays were used to analyse the response of flies to physiological stress: heat sensitivity and recovery time from cold coma.

For heat sensitivity he results showed a significant decrease in the number of surviving insects in the group exposed to high intensity blue light compared to the control group ($P<0.01$; fig 26B). Significant decrease was also observed in the *Drosophila melanogaster* exposed to low intensity blue light than control group ($P<0.05$; fig 26A).

Analysis of recovery time from cold coma showed that insects exposed to 12h low (fig. 27A and B) and high (fig. 27C and D) intensity blue light were able to wake up faster than the control group ($P<0.05$).

A **B**

Figure 26. *Heat-induced stress response capacity of Drosophila melanogaster not exposed to light (CTRL) and exposed to blue (BLUE) or red LED light (RED) for 12L:12D cycles at low intensity (A) and high intensity (B). Columns represent mean values and bars represent ± SD (n=3). * P<0.05 and **P<0.01 vs. CTRL.*

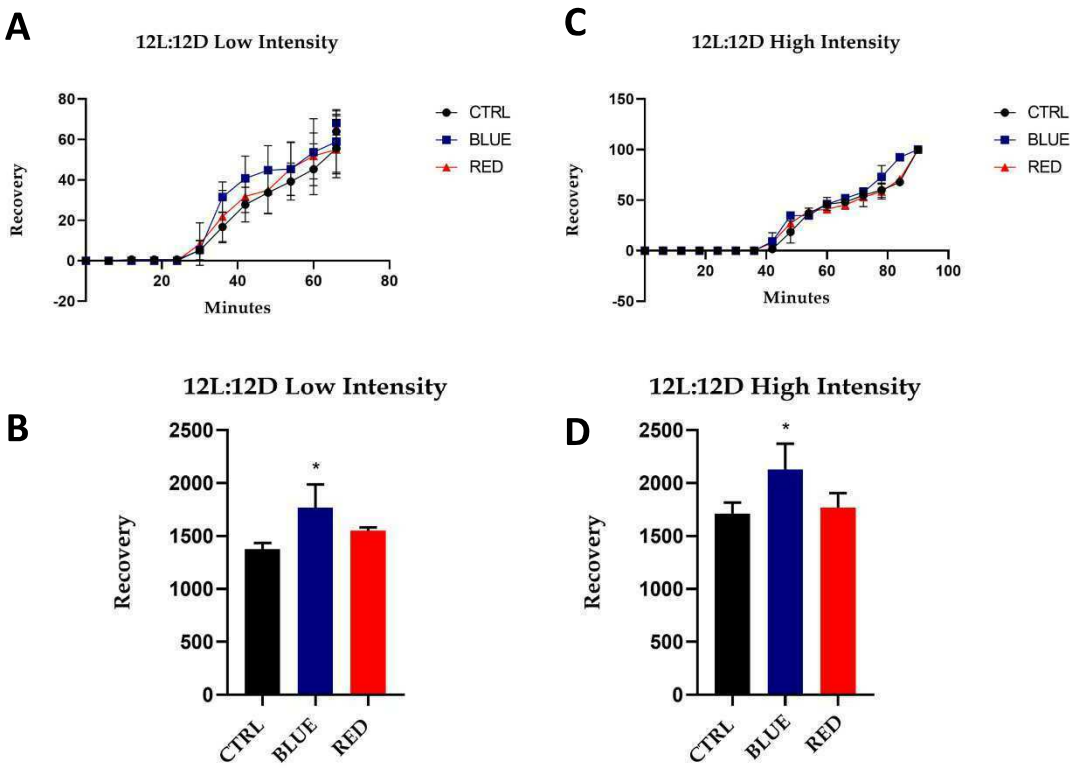

***Figure 27**. Representation of the insect awakening curve after cold coma not exposed to light (CTRL) and exposed to blue (BLUE) or red LED light (RED) for12L:12D cycles at low intensity (A) and high intensity (C). Recovery ability from cold coma of Drosophila melanogaster not exposed to light (CTRL) and exposed to blue (BLUE) or red LED light (RED) for 12L:12D cycles at low intensity (B) and high intensity (D). Columns represent mean values and bars represent ± SD (n=3). * P<0.05 vs. CTRL.*

Exposure to PQ and 12 hours low and high intensity blue light treatment increases the number of dead insects.

PQ exposure caused a significant increase in the number of dead insects after 12h high intensity blue light treatment compared to insects exposed to the control group ($P<0.05$; fig. 28B). Significant change was observed in flies exposed to 12h low intensity blue light than the control group ($P<0.05$; fig. 28A).

Figure 28. *Resistance to PQ of Drosophila melanogaster not exposed to light (CTRL) and exposed to blue (BLUE) or red LED light (RED) for 12L:12D cycles at low intensity (A) and high intensity (B). Columns represent mean values and bars represent ± SD (n=3). * P<0.05 vs. CTRL.*

Locomotor activity is increases in insects exposed to 12 hours high intensity blue light.

To investigate whether light influences the fruit fly *Drosophila melanogaster*, we compared locomotor activity, stress responsiveness, and clock gene expression between insects treated with 12h high-intensity and low-intensity blue light and red light cycles for 3 days and the control group (constant darkness). Initially, we measured vertical locomotion by climbing assay. Insects exposed to high-intensity blue light had significantly higher mean climbing ability than insects kept in constant darkness ($P<0.05$; Fig. 29C). Insects exposed to low intensity blue light did not appear to have altered mean climbing ability compared to insects kept in control group (Fig. 29B).

Figure 29. *(A) Representative photo of the method to measure climbing ability in insects. Average climbing ability of flies not exposed to light (CTRL) and exposed to blue LED light (BLUE) or red LED light (RED) for 12L:12D cycles at low intensity (B) and high intensity (C). Columns represent mean values and bars represent ± SD (n=3). * P<0.05 vs. CTRL.*

Treatment of 12 hours low and high intensity produces a significant change in clock genes.

Flies exposed to blue light and red light 12h high intensity showed an increase *PER* gene expression level compared to control group ($P<0.05$; fig. 30C). The *TIM* gene was found to be significantly increased only after blue light exposure compared with control group ($P<0.0001$; fig 30D).

Regarding the expression of the clock genes in *Drosophila melanogaster* exposed to 12h low intensity showed increased *PER* mRNA levels after blue light than the control group ($P<0.05$; fig 30A). *TIM* also showed increased mRNA levels after 12h low intensity blue light than the control ($P<0.05$; fig 30B)

Figure 30. *Expression profiles of PER and TIM genes in the heads of Drosophila melanogaster not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 12L:12D cycles at low intensity (A-B) and high intensity (C-D). Columns represent mean values and bars represent ± SD (n=3). * P<0.05 and ****P<0.0001 vs. CTRL.*

5. DISCUSSION

Skin cells are constantly exposed to the light, the blue light region of the visual spectrum is the most energetic. In the eye, blue light damage is thought to be predominantly photochemical in origin and to derive largely from eye-specific chromophores (e.g. retinoids, melanin, and lipofuscin) (Roehlecke et al., 2011). What happen in the skin is less clear, but is likely to involve chromophores associated with mitochondria (e.g. cytochromes and flavin) that absorb in the blue region of the visible spectrum (Godley et al., 2005).

In this thesis, we are going to set out the effects of LED lamps, both *in vitro* (HaCaT cells) and *in vivo* (fruit fly, *Drosophila melanogaster*), in order to try to fill the gap of knowledge related to this specific study.

Epidermal keratinocytes have several rhythmic functions that follow circadian, infradian, and ultradian cycles while responding to systemic and environmental insights (Le Fur et al., 2001). One of the main chronobiological functions of the interfollicular epidermal clock, is to regulate cell differentiation and proliferation for optimal function allowing it to respond to predictable changes in environmental stressors (Plikus et al., 2015), above all artificial light. In particular, the impact of LED light on human skin will be discussed here focusing on the implications found in circadian rhythms and skin health at cellular and molecular levels.

When the groups were compared in the study, the only treatment difference was in the characteristics of light exposure (1L:23D and 12L:12D low and high intensity), to exclude the effects of other external inputs (temperature, humidity) that equally affect the normal functions of the circadian cycle (Y. Wang et al., 2020).

The results obtained from exposure to 12h high intensity light have aroused more interest in our research group.

The epidermis undergoes a constant process of self-renewal in which epidermal stem cells engage in a complex program of terminal differentiation. Precise spatial and temporal control of keratinocyte proliferation and cell cycle withdrawal is critical for this phenomenon (Plikus et al., 2015).

Therefore, we decided to investigate whether blue and red led light interfere in the physiological process of keratinocyte proliferation.

In the *in vitro* tests, it was found that the viability of HaCaT cells decreased greatly after blue and red light exposure. Cell viability decreased by approximately 80% in blue-exposed cells and 30% in HaCaT when exposed to red light (fig. 12B), suggesting that narrowband blue light has worst impact on the physiological grow capacity of keratinocytes.

Moreover, blue light induces cellular damage that has also been associated with an increased percentage of cells in apoptosis, cell cycle blockade, and a rapid increase in intracellular ROS concentration that occurred after exposure to high-dose blue light for 12 hours cycles.

It has long been known that exposure to blue light causes retinal degeneration with subsequent development of cellular apoptosis (Donovan et al., 2001). Although in HaCaT skin cells this effect has not yet been fully elucidated, our results suggest that blue light induces apoptosis. In fact, with concomitant a marked decrease in viability, HaCaT cells exposed to blue light were found to be in a statistically greater apoptotic state than control (about 80%, fig. 13E).

We also observed changes in the cell cycle progression in HaCaT cells exposed to blue light. In fact, DNA cycle analysis revealed a significant increase in the G_2/M phases and then a decrease in the G_0/G_1 phase after blue light treatment than CTRL group (fig. 14B).

Here, blue light irradiation caused a delay in cell cycle completion. Data on DNA content, showed that cell cycle arrest occur in the G_2/M phase. In this phase cells probably did not pass the G_2/M checkpoint, starting the apoptosis process. This idea could explain the higher rate of apoptosis, the lower cell viability and the DNA content seen in HaCaT cells after blue light irradiation. It is conceivable that the cell cycle arrest pathway is directly related to the production of intracellular ROS. Indeed, several studies have shown that a sudden increase in ROS production contributes to inhibiting the normal progression of cell cycle in several experimental models (S. Sun et al., 2018; Sun et al., 2020). In the skin, cell division and DNA repair have a high correlation with

the circadian cycle (diurnal period). These rhythms impact both acute (erythema, DNA damage, and immune suppression) and long-term (skin cancers and photoaging) skin conditions. The circadian clock in epidermal keratinocytes, under physiological conditions, temporally segregates endogenous oxidative phosphorylation derived from keratinocyte proliferation, thereby it protects the genome from ROS-mediated DNA damage (Geyfman et al., 2012). This may result in increased vulnerability in humans having desynchronized circadian rhythms (such as shift workers) to light-induced skin cancers (Feller et al., 2016); (Yousef et al., 2020).

Several studies have shown that the circadian molecular clock mechanism oscillates in a cyclic manner not only in the central nervous system, but also in peripheral organs, such as the skin. In mammals, CLOCK and *BMAL1* act as positive regulators. They bind to the E-box enhancer to activate the transcription of *PER2* and *CRY2* genes (negative regulators). *PER2* and *CRY2* in turn repress *BMAL1* transcription. We studied the expressions of the circadian clock genes *BMAL1*, *PER2*, and *CRY2* in HaCaT cells to determine whether a change in circadian rhythm occurs after bright LED light exposures. Significant changes in the circadian gene expression of *BMAL1* and *PER2* in the blue light treatment group were found when compared with the control group (fig. 15). It is possible that the decrease in both *BMAL1* and *PER2* genes, although belonging to opposite phases of the circadian gene loop, is the result of an altered dark-light environment due to intensive light exposure after blue light treatment. Biological and molecular functions in keratinocytes after light exposure might have changed, the alterations in peripheral biological oscillators obtained may confirm it.

The relationship between non-ocular light exposure and circadian alteration has always been a controversial issue in circadian rhythm studies. While some studies highlight a possible direct relationship between skin and visible light (S. Sun et al., 2018), others insist that dark-light stimulation to the circadian system could not occur through non-ocular pathways (Grimm et al., 2000). Regarding the effect of blue LED light in altering the circadian rhythm, it has been reported the damage of retinal photoreceptor cells (Roehlecke et al., 2011), while in skin cells it is not fully understood.

Some *in vivo* studies have shown that peripheral light directed on the skin can affect melatonin secretion in neonates (Chen et al., 2005). Our results reveal complex

interactions between the external light regime and peripheral clock rhythms, further supporting the notion that non-ocular light exposure can alter the expression of circadian genes in the periphery of humans. Thus, even under the absence of the SCN clock, the peripheral skin clock in the *in vitro* model underwent light-dependent alterations, showing a responsiveness to external light stimuli. Currently the best explanation for our results is a tissue-dependent combination of direct light responsiveness. While our study supports the existence of a non-canonical pathway of light entry to the periphery, the nature of the signals that reset peripheral clocks in the absence of the SCN pacemaker remains to be determined.

Based on these evidences, we performed the proteome profile analysis of HaCaT cells exposed to blue or red light. Proteomic analysis revealed that 12-hour high-intensity blue light treatment provides a significant variation of proteins involved in oxidative stress.

Moreover, HaCaT cells showed an increase in some stress-related proteins when exposed to blue light: AKR1C3, TXNRD1 and SOD1 (fig. 17).

AKR1C3 belongs to the AKR1C (Aldo-keto reductase family 1) family of proteins that play a key role in maintaining steroid hormone homeostasis and regulating prostaglandin metabolism (Penning et al., 2006). Moreover, it is already known in the literature that AKR1C3 expression is upregulated by ROS (Burczynski et al., 1999).

Therefore, it is likely that the increased expression of AKR1C3 by blue light is associated with ROS production.

The adaptive response to light stress occurs through regulation of genome-protective machinery activity at cell cycle checkpoints, apoptosis, metabolism, and the oxidative stress response (Hofseth, 2004) that initially protect cells from genomic insults caused by toxic agents. Blue light stress has been proposed to induce such mechanisms.
Because AKR1C isoforms catalyze the oxidation and/or reduction of a broad spectrum of substrates, the molecular mechanisms behind the altered expression of AKR1C3 support the hypothesis that the expression of AKR1C3 upregulation is indeed a cellular defence response of keratinocytes against cellular stress induced by blue light.

Also TXNRD1 (Thioredoxin Reductase 1) was activated by blue LED light, in fact its expression is higher in HaCaT cells exposed to blue light compared to the control group.

The TXNRD1 protein reduces thioredoxins as well as other substrates, and plays a key role in selenium metabolism and protection against oxidative stress. The functional enzyme is thought to be a homodimer which uses FAD as a cofactor. Each subunit contains a selenocysteine (Sec) residue which is required for catalytic activity (Kiermayer et al., 2007).

Importantly, a thioredoxin-mediated reducing environment is required for efficient DNA binding of redox-sensitive transcription factors, including p53 and NF-κB (Yodoi et al., 2002). Thioredoxin binds ROS before they can damage cells and thus protects cells from oxidative stress. It also possesses a critical role in the regulation of cellular redox homeostasis. Studies have suggested that several human selenoproteins play an important role in tumour initiation and progression, including melanoma (Ecker et al., 2020).

The thioredoxin redox system has recently been suggested as a therapeutic target for cancer therapy (Biaglow & Miller, 2005), based on the observation that thioredoxin is overexpressed in many aggressive cancers**.** Moreover the inverse association of TXNRD1 and TXNIP with MFI found in this study supports the hypothesis that the maintenance of an active thioredoxin system is beneficial to cancer cells because it limits oxidative damage and allows them to survive.

The high levels of TXNRD1 observed after blue light exposure could be due to ROS accumulation at this later time point. We must also point out that the significant increase in the levels of this protein has been observed only after exposure to blue light 12h high intensity, when the light stimulus has represented the greatest damage to cells. In fact in the other treatments we have not observed significant differences.

Since it is known that 4-hydroxynonenal, the major product of lipid peroxidation, can increase TXNRD1 mRNA levels and that the TXNRD1 promoter contains an antioxidant response element (Gao et al., 2020), it is plausible that the observed

accumulation of ROS in our cells the day after blue light exposure is contributing to the increased expression of TXNRD1.

While AKR1C3 and TXNRD1 proteins are overexpressed after exposure to blue light, overexpression of SOD1 protein occurs after exposure to red light.

In previous studies SOD has been used as potential therapeutic agents for ROS-mediated diseases (Gu et al., 2013).

In the present study, we demonstrated that SOD1 reduced skin ROS levels induced by red light radiation. The protective function of SOD1 on human keratinocytes *in vitro* was likely due to its characteristic ROS scavenging ability (Kim et al., 2019). Catalase and SOD1 are well-known antioxidant enzymes that neutralize excess reactive oxygen species in cells. SOD1 is a direct ROS quencher and its promoter is known to contain Nrf2-ARE binding site (Dreger et al., 2009). Nrf2 is an important regulator of antioxidant responses at the cellular level through transcriptional regulation-mediated antioxidant response (ARE) (Huang et al., 2000); (Zhu et al., 2008). We demonstrated that exposure to red light increased SOD1 in HaCaT cells, suggesting an important role of this protein in the antioxidant pathway.

HaCaT cells exposed to red light appear to produce injury although to a lesser extent than the effects produced by blue light. It is likely that SOD1 produced by cells exposed to red light produces a reduction in increasing intracellular ROS levels. Thus, SOD1 is likely to mitigate mitochondrial dysfunction and subsequent apoptosis by safeguarding mitochondrial integrity, ROS levels, and the apoptosis process after red light irradiation in HaCaT cells. However, it is unclear why this does not occur in HaCaT cells exposed to blue light, in which we did not observe significant changes in SOD1 protein levels. These results suggest that SOD1 offers a novel pharmacological target to protect the skin from longer wavelength light radiation (such as red LEDs).

To confirm the rapid generation of ROS in cells exposed to blue light, the fluorescent probe dichlorofluorescein was used, which showed a significant increase in the intensity of emitted fluorescence compared with the control group (fig. 18A).

Effective suppression of radiation-induced skin damage is advantageous because several studies showed that even radiation emitted in the visible spectrum, similarly to ionizing

radiation, generates cellular ROS, which have high potential to damage cellular macromolecules, including proteins, lipids, and nucleic acids (Godley et al., 2005). Various evidence suggests that ROS play an important role in cell death and signal transduction after ionizing radiation (Azzam et al., 2012). The level of total ROS after exposure to blue light was also significantly higher than the one of the control. These results suggest that blue light produced the greatest effects observed in HaCaT cells partially due to the elevated ROS production.

In this study, we demonstrated that blue light can photo-generate ROS and increase mitochondrial density in the cytoplasm of human keratinocytes. This supports the hypothesis that mitochondria possess blue light-sensitive chromophores (Tao et al., 2019). From our results, it was revealed that ROS levels were significantly overexpressed after exposure to shorter wavelength light (blue light), whereas after exposure to longer wavelength light (red light), no significant difference was found, although a trend increase is noted.

These light-generated ROS are in addition to the low levels of ROS generated as a product of oxidative respiration. Previous studies have shown that mitochondrial DNA lesions are maximal at 3 hours after light exposure with evidence of repair at later time points, suggesting that there is an adaptive response in place to cope with the oxidative load (Godley et al., 2005). These results indicate that the short wavelength LED light may cause cell death from oxidative stress induced by the rapid increase in ROS that remains visible over time. In addition, the association between increased ROS and mitochondria induced by blue light has been reported**.** Mitochondria are thought to include blue light-sensitive chromophores. Photoreceptor cell death may be associated with oxidative stress, induced by ROS generation, after exposure to blue light. more severe ROS damage to mitochondria triggers the apoptosis pathway (Jou et al., 2004). ROS generation from photo-irradiation can lead to mitochondrial swelling, increased mitochondrial calcium levels**,** and activation of caspases-2 and -9 in the mitochondrial matrix (Takahashi et al., 2004); (Peng & Jou, 2004)**.**

In the present study, red LED light did not induce cellular damage. This may be due to the slight increase in ROS production after exposure to red LED light. Thus, it is plausible that the mitochondrial response to oxidative stress, dependent on the degree of oxidative insult, may result in sub-lethal damage or the initiation of cell death pathways.

These results indicate that cellular damage is induced by ROS generated by short-wavelength LED light. Furthermore, it was suggested that the rapid increase in ROS caused the increase in mitochondrial damage.

Beginning from these evidence we decided to observe by fluorescent microscope the mitochondria of HaCaT cells exposed to light to assess their morphology, distribution, and density. The mitochondrial density of HaCaT cells appears to be influenced by blue light (fig. 18C).

Mitochondria are vulnerable to oxidants because they are the main source of free radicals in cells and are limited in their ability to cope with oxidative stress (Godley et al., 2005). Mitochondria are closely linked to cell apoptosis, and destruction of the transmembrane potential is considered one of the earliest events in the cascade process of apoptosis and occurs before characterizing apoptosis in the nucleus, which instead occurs by chromatin condensation and DNA fragmentation (Shibayama-Imazu et al., 2008).

It has been postulated that blue light-induced retinal damage is mediated by mitochondrial respiratory enzymes (Putting et al., 1993), and it has been shown that inhibition of the mitochondrial respiratory chain blocks ROS generation (Kang & Hamasaki, 2003). In addition, it has been shown that the content of cytochrome c oxidase (which has an absorption peak at 440 nm in the reduced form) was reduced in RPE cells after exposure to blue light; and again, mitochondria contain a number of chromophores that absorb light in the blue light region (e.g., flavin and cytochrome) (Chen et al., 1992). These data implicate the involvement of mitochondrial respiratory enzymes in phototoxicity. We have shown that damage to mitochondria increases after exposure to blue light (fig. 31).

Thus, these results provide further evidence that the severity of the insult confers the shape and extent of the apoptotic process, guided by increasing intracellular concentration of ROS (Donovan et al., 2001).

To confirm these evidences, we observed HaCaT cells exposed to the 12h high intensity light cycle by transmission electron microscopy (TEM). The images collected reveal substantial cytoplasmic differences both between control cells (CTRL) and the two different light radiations (BLUE and RED), and between those two light radiations. Therefore it is conceivable that the radiation emitted by LED lamps causes alterations directly in the cytoplasm of keratinocytes *in vitro* according to the irradiated wavelength. In particular we have confirmed that in HaCaT cells exposed to blue light show some morphological features of cell damage: some mitochondria are swollen and show rarefaction in the cristae, the Golgi network is dilated and many empty vesicles are present. HaCaT cells not exposed to light shows hyperfused mitochondria network that may relate with high energy demand or starvation stress (Westermann, 2012). It is interesting to note that instead in HaCaT cells exposed to red light mitochondria seem to revert to a normal morphology, probably because they are engaged in the response to light stimulus.

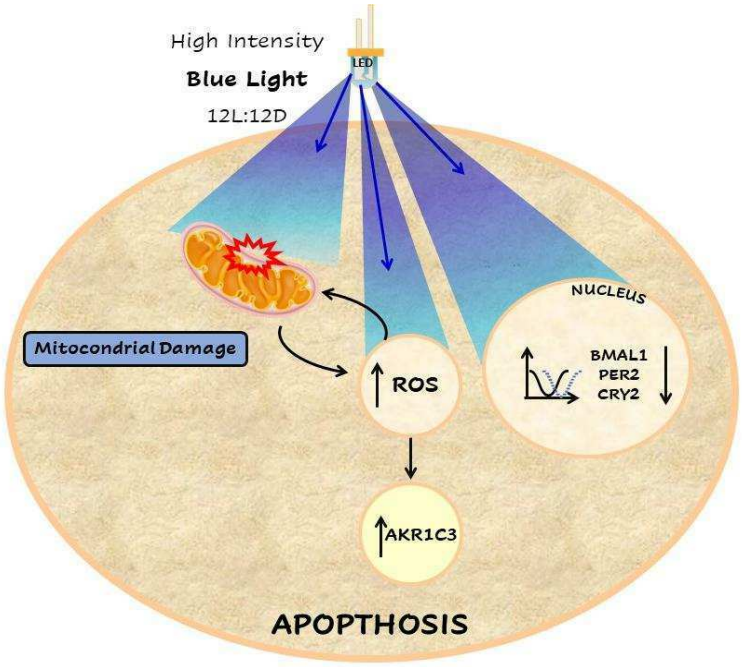

Figure 31. *The pathway of blue LED light induced HaCaT cells damage. In HaCaT cells, blue LED light induces ROS production. The rapid ROS increase leads to mitochondrial damage with oxidative stress and drives the HaCaT cells into the apoptosis pathway.*

The second mode of exposure to blue and red light radiation was 1 hour cycles at high intensity. In this case we obtained less significant evidence on the analyzed parameters.

Starting with the analysis of cell viability, the results showed a decrease in cell viability of approximately 40 % for HaCaT cells exposed to blue light and approximately 30 % for HaCaT cells exposed to red light (fig. 12).

Moreover blue light still results in a significant increase in the percentage of cells in apoptosis even though the effect is smaller (fig. 13).

From cell cycle analyses, we observed no changes in HaCaT cells exposed to light radiation compared to control (fig. 14A). In the case of HaCaT cells exposed to 1h cycles of high-intensity blue light, it is likely that after light damage, the cells still managed to complete the cell cycle and adapt to the blue light.

However, at the time point of 24 hours after blue light irradiation, the clock genes *BMAL1* were found to be downregulated (fig. 15). It is possible that the trending (although not significant) increase in *CRY2* after blue light treatment could be the result of an altered dark-light environment or light exposure, and high-level *CRY2* expression along with other negative regulators in transcriptional-translational feedback loops inhibited *BMAL1* expression. *CRY* has been considered as a blue light receptor for plants and mammals (Wang et al., 2014); (Emery et al., 1998). This explains the presence of a decrease in cell proliferation with blue light and red light; in fact, it is likely that a minor light insult is able to alter the expression of key clock genes, and that these changes are then manifested in the proliferative rate and in the induction of apoptosis. So even with only 1h cycles of exposure skin cells are photosensitive, the effects found are less severe on cellular physiological functions, but still present.
The third and fourth modes of exposure to light radiation (12L:12D and 1L:23D cycles low intensity, respectively) showed varying results.

The 12h low intensity blue light treatment did not significantly decrease cell viability, which was strongly decreased in the previous two exposures (fig. 20). This suggests that the light insult decreases keratinocyte viability and proliferation in a manner wich is proportional to the intensity of light emitted by the artificial light source. In the analysis of apoptosis, treatment with red light produced a higher percentage of apoptotic cells

(18% RED vs 5% CTRL; fig. 21) than the control group. From these results, it is possible that even in keratinocytes exposed to low-intensity red light, apoptosis processes were induced in minimal levels.

LEDs can exert biological effects at specific wavelengths, and studies have suggested that red LED radiation (620-770 nm) can penetrate deeper into soft tissues than other wavelengths, which is important for ROS scavenging and anti-inflammatory activity (Li et al., 2010). On the other hand, no significant change was observed in keratinocytes at various steps of the cell cycle after treatment with the lights compared with the control group (fig. 22).

Low-intensity light treatment produces a change in *CRY2* clock gene expressions (fig. 23). *CRY2* is downregulated after blue light treatment and upregulated after red light treatment; for the first time, significant upregulation in clock gene expression of keratinocytes exposed to red light was observed compared with the control group. Analysis of clock genes revealed different results in keratinocytes for different light exposures.

About the expression levels of proteins involved in the anti-oxidant response we observed that exposure of human HaCaT cells to even low doses of blue light induced the expression of AKR1C3 (12L:12D low and high intensity; fig. 24 and fig. 17). In contrast, the SOD1 protein was over-expressed after low dose red light exposure. As discussed previously, increased SOD1 levels are a response to a concomitant increase in intracellular ROS production.

Indeed, intracellular ROS show a significantly increased signal after red light treatment. HaCaT cells also show altered mitochondrial density by both blue and red light. These results are in agreement with a study by Q. Suna et al, 2018 it was seen that LEDs at 625 nm produced ROS scavenging and anti-inflammatory effects while LEDs at 425 nm had no effect on ROS scavenging in HaCaT cells (Q. Sun et al., 2018).

Thus, HaCaT exposed to both blue and red light produce more intracellular ROS and mitochondrial alterations with both light radiations (both short and long wavelength, fig. 24) but, activation of defense systems (alterations in clock genes and antioxidant response) still determines their cell survival.

In our model, we found that ROS-induced damage with this mode of exposure caused the triggering of light insult response reactions but did not lead to cell death (fig. 32).

In this study, we identified ROS-dependent circadian control in mammals, also observed in a 2013 study by Teruya Tamaru et al. in which resetting the circadian clock by near-lethal doses of ROS at the branch point of life and death. This research group verified that the resetting process is accompanied by simultaneous activation of cell survival systems. This adaptive response leads to biological homeostasis and cell survival. Also in our results, we noticed a direct correlation between circadian adaptation and survival phenomena in human keratinocytes exposed to red light. Thus, we hypothesized that the alterations found in clock genes could be an adaptation to ROS critical stress for cell survival (Tamaru et al., 2013).

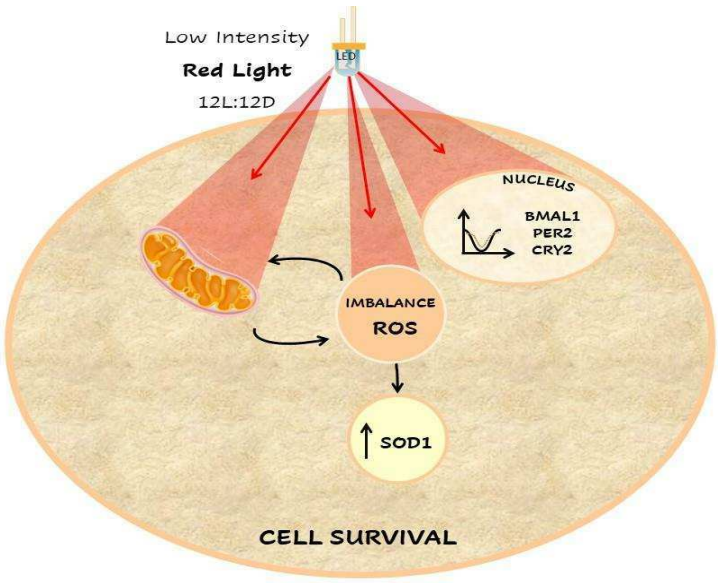

***Figure 32**. The pathway of red LED light induced to the ROS scavenging in HaCaT cells. In HaCaT cells, red LED light induces ROS imbalance. The imbalance ROS leads to mitochondrial effects and drives the HaCaT cells to survival.*

In the last light exposure mode (1L:23D low intensity), few significant results were obtained. In fact, treatment with blue light did not significantly decrease cell viability (fig.20), although it produced a higher percentage of apoptotic cells (fig. 21). Above all, red light was found to stimulate the induction of apoptosis in human keratinocytes. Cell cycle analysis showed no significant evidence (fig. 22).

Regarding the analysis of clock genes we observed a significant change in clock gene mRNA levels following the trend seen for the other exposures with a downregulation of *BMAL1, PER2 AND CRY2* after blue light treatment and upregulation after red light treatment (fig. 23). The only difference found to be significant was the deregulation of *PER2* and *CRY2* genes after blue light treatment. Thus, blue light appears to influence skin biological clock genes even with only 1 hour of low-intensity exposure.

After the evidence obtained in the *in vitro* experimental model, we investigated the mechanisms and effects induced by the light radiation of monochromatic blue and red LED lamps on *Drosophila melanogaster*. Importantly, there is evidence at the output level of the circadian clock that suggests direct homology between mechanisms in flies and mammals (Flourakis & Allada, 2015); (Barber et al., 2016).

In *Drosophila melanogaster*, the effects of light exposure were studied by testing behavior, responses to environmentally induced stress and key clock molecules.

Blue light is able to modify the response to extreme heat and cold stimuli. In fact, after 45 minutes at 38.5 °C insects exposed to both low and high intensity blue light showed a decrease in survival when compared to Drosophila exposed to red light or to the control group (fig. 26). Specularly after 24h at 4°C the insects exposed to blue light at both low and high intensity appear to wake up faster (fig. 27). Evidence for heat sensitivity and recovery time from cold-induced coma would confirm the oxidative stress induced by blue light observed in *in vitro* studies.

The mechanisms underlying chronic Paraquat (PQ) toxicity are fairly well understood in the mammalian models. Previous studies have reported that PQ is transported through the dopamine transporter (DAT) (Shimizu et al., 2003) and inhibits mitochondrial complex I (Dawson & Dawson, 2003). Mitochondrial swelling is one of the earliest ultrastructural changes upon PQ exposure *in vivo* (Magwere et al., 2006). However,

such findings have been reported predominantly under chronic exposure conditions. In the Drosophila system, the PQ resistance assay routinely employed involves exposure of flies to an acute dose of PQ (for 24 h) and evaluation of the incidence of lethality.

In addition, the negative PQ-induced effect on survival is mainly attributed to elevated oxidative stress. PQ-induced ROS production in brain tissue is primarily by mitochondria (Hajji et al., 2019). It regulates the production of H_2O_2 and lipid peroxidation (LPO) and lowers the GSH/GSSG (reduced glutathione/oxidized glutathione) (Castello et al., 2007). PQ exposure induces redox imbalance in flies, similar to previous studies reported (McCormack et al., 2005). In addition, elevated oxidative stress and high mortality due to PQ toxicity point toward increased apoptosis.

Our results revealed that with exposure to blue light at both low and high intensity, PQ causes a significant increase in the number of dead insects compared to the control group after 24 h of incubation among adult flies and this clearly suggests the presence of ongoing oxidative stress (fig. 28). The results shown by the stress response (cold, hot and PQ tests) in insects, support the evidence obtained from *in vitro* studies of a possible action of light on mitochondria and related apoptotic mechanisms activated in HaCaT by blue light.

Several *in vivo* studies have determined that blue light exposure promotes the generation of reactive oxygen species (ROS) in flies (Arthaut et al., 2017); (Mondal & Huix-Rotllant, 2019).

In addition, blue light can cause damage to the central circadian nervous system, which may be involved in the temporary acceleration of climbing ability and increased mortality after stress signals (cold, hot test and PQ test). Infact today studies using animal models exposed to various light regimes often use locomotor activity as a biomarker of circadian disruption (Alaasam et al., 2021).

The key molecules of the biological clock produce 24-hour gene expression cycles and rhythms. The biological clock is conserved in mammals and drives rhythms in behaviour, metabolic function and other physiological processes, making it a powerful regulator of any process in which regulating the time of day is beneficial to the whole organism.

It has been shown that in insects the rhythms of locomotor activity are managed by a heterogeneous network of central clock neurons that ensure such activity at the appropriate time of day in a variety of different environmental contexts (Nash et al., 2019). In our results, exposure to blue light modifies the locomotor capacity of *Drosophila melanogaster*. We have seen how exposure to blue light 12h high intensity caused a greater distance in the treatment group insects than insects at control group (fig. 29).

From our results, the main players involved in the insect biological clock (*PER* and *TIM*) were upregulated after exposure to blue light at both high and low intensity (fig. 30). This suggests that blue light constitutes an important stimulus of biological rhythms in the central circadian system (insect brain). This experimental model was useful to study how signal within the central clock cell network allows other clocks to synchronize with signals from the environment, maintaining synchronized molecular rhythms. The relative simplicity and ease of manipulation of Drosophila makes it a valuable tool for the circadian community in discovering new mechanisms that control circadian processes. Furthermore, several studies have confirmed a functional homology between Drosophila and mammalian clocks that consist of a heterogeneous network of neurons producing behavioural outputs (Vosko et al., 2007); (Welsh et al., 2010).

Several studies have reported that blue light causes damage to fruit flies (Boomgarden et al., 2019). While these studies employed a 'single acute exposure to strong light, we show that Drosophila degenerates in response to 12 hours of daily exposure, for three days, to moderate or intense blue light. Our study offers a new key to understanding the molecular mechanisms of the increasingly evident harmful side of blue light from LEDs.

6. CONCLUSION

Summarizing all the results obtained, we can affirm that blue light and red light have a biological effect in keratinocytes and act through similar mechanisms of action.

Keratinocytes exposed to red light are probably able to counterbalance the action of the light. After exposure to blue light the insult is probably greater, the cell is not able to deal with it and therefore triggered mechanisms are lead to programmed cell death.

In *Drosophila melanogaster* the results are in line with those obtained on keratinocytes. Insects exposed to blue light showed significant differences in the rhythmic phenotypes studied, demonstrating that such light radiation on the one hand can activate the metabolism of the insect that responds more efficiently to external signals, on the other hand is harmful when oxidative stress is enhanced. In insects exposed to red light we observed no difference compared to the control suggesting that either the red light has no effect or that the organisms can elaborate a defence response to such light exposure.

About the clock genes the responses in the two models seem to be influenced especially in blue light exposure. Blue light seems to downregulate clock genes in human keratinocytes, whereas in insects clock genes are overexpressed. The different phenomenon may be explained by a different response of the peripheral circadian clock of keratinocytes isolated in culture; compared to insects in which the expression levels of central biological clock genes (mRNA extracted from heads) were analyzed.

These evidences suggest that blue light is an external signal that perturbs biological systems in a variety of ways and in a time- and dose-dependent manner. Indeed, it was found that at low intensity exposures blue light did not induce cell damage and induced less damage in Drosophila (although insect clock genes were altered in the central circadian clock). Regarding the red light we noted different responses that varied with exposure time and light intensity. Collectively, our results suggest that proper use of blue and red LED lamps could be an effective approach to maintain skin homeostasis and central circadian clock. Thus, the choice of better lamps for artificial lighting should take into account the characteristic variability of sunlight during the hours of the day with predominantly red component in the hours of sunrise and sunset reserving the blue

component for the middle hours of the day so as to alter as little as possible the natural circadian biological cycles that determine several important physiological functions.

7. BIBLIOGRAPHY

Adams, J. S., & Hewison, M. (2010). Update in vitamin D. *J Clin Endocrinol Metab*, *95*(2), 471-478. https://doi.org/10.1210/jc.2009-1773

Adamskaya, N., Dungel, P., Mittermayr, R., Hartinger, J., Feichtinger, G., Wassermann, K., Redl, H., & van Griensven, M. (2011). Light therapy by blue LED improves wound healing in an excision model in rats. *Injury*, *42*(9), 917-921. https://doi.org/10.1016/j.injury.2010.03.023

Akashi, M., Soma, H., Yamamoto, T., Tsugitomi, A., Yamashita, S., Yamamoto, T., Nishida, E., Yasuda, A., Liao, J. K., & Node, K. (2010). Noninvasive method for assessing the human circadian clock using hair follicle cells. *Proc Natl Acad Sci U S A*, *107*(35), 15643-15648. https://doi.org/10.1073/pnas.1003878107

Akashi, M., & Takumi, T. (2005). The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock Bmal1. *Nat Struct Mol Biol*, *12*(5), 441-448. https://doi.org/10.1038/nsmb925

Alaasam, V. J., Liu, X., Niu, Y., Habibian, J. S., Pieraut, S., Ferguson, B. S., Zhang, Y., & Ouyang, J. Q. (2021). Effects of dim artificial light at night on locomotor activity, cardiovascular physiology, and circadian clock genes in a diurnal songbird. *Environ Pollut*, *282*, 117036. https://doi.org/10.1016/j.envpol.2021.117036

Alba, M. N., Gerenutti, M., Yoshida, V. M., & Grotto, D. (2017). Clinical comparison of salicylic acid peel and LED-Laser phototherapy for the treatment of Acne vulgaris in teenagers. *J Cosmet Laser Ther*, *19*(1), 49-53. https://doi.org/10.1080/14764172.2016.1247961

Algvere, P. V., Marshall, J., & Seregard, S. (2006). Age-related maculopathy and the impact of blue light hazard. *Acta Ophthalmol Scand*, *84*(1), 4-15. https://doi.org/10.1111/j.1600-0420.2005.00627.x

Allada, R., White, N. E., So, W. V., Hall, J. C., & Rosbash, M. (1998). A mutant Drosophila homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. *Cell*, *93*(5), 791-804. https://doi.org/10.1016/s0092-8674(00)81440-3

Antoch, M. P., & Kondratov, R. V. (2013). Pharmacological modulators of the circadian clock as potential therapeutic drugs: focus on genotoxic/anticancer therapy. *Handb Exp Pharmacol*(217), 289-309. https://doi.org/10.1007/978-3-642-25950-0_12

Arble, D. M., Bass, J., Laposky, A. D., Vitaterna, M. H., & Turek, F. W. (2009). Circadian timing of food intake contributes to weight gain. *Obesity (Silver Spring)*, *17*(11), 2100-2102. https://doi.org/10.1038/oby.2009.264

Arthaut, L. D., Jourdan, N., Mteyrek, A., Procopio, M., El-Esawi, M., d'Harlingue, A., Bouchet, P. E., Witczak, J., Ritz, T., Klarsfeld, A., Birman, S., Usselman, R. J., Hoecker, U., Martino, C. F., & Ahmad, M. (2017). Blue-light induced accumulation of reactive oxygen species is a consequence of the Drosophila cryptochrome photocycle. *PLoS One*, *12*(3), e0171836. https://doi.org/10.1371/journal.pone.0171836

Aschoff, J., & Pohl, H. (1978). Phase relations between a circadian rhythm and its zeitgeber within the range of entrainment. *Naturwissenschaften*, *65*(2), 80-84. https://doi.org/10.1007/bf00440545

Azzam, E. I., Jay-Gerin, J. P., & Pain, D. (2012). Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. *Cancer Lett*, *327*(1-2), 48-60. https://doi.org/10.1016/j.canlet.2011.12.012

Baker, F. C., Waner, J. I., Vieira, E. F., Taylor, S. R., Driver, H. S., & Mitchell, D. (2001). Sleep and 24 hour body temperatures: a comparison in young men, naturally cycling women and women taking hormonal contraceptives. *J Physiol*, *530*(Pt 3), 565-574. https://doi.org/10.1111/j.1469-7793.2001.0565k.x

Barber, A. F., Erion, R., Holmes, T. C., & Sehgal, A. (2016). Circadian and feeding cues integrate to drive rhythms of physiology in Drosophila insulin-producing cells. *Genes Dev*, *30*(23), 2596-2606. https://doi.org/10.1101/gad.288258.116

Bauer, M., Glenn, T., Alda, M., Andreassen, O. A., Angelopoulos, E., Ardau, R., Baethge, C., Bauer, R., Bellivier, F., Belmaker, R. H., Berk, M., Bjella, T. D., Bossini, L., Bersudsky, Y., Cheung, E. Y., Conell, J., Del Zompo, M., Dodd, S., Etain, B., Fagiolini, A., Frye, M. A., Fountoulakis, K. N., Garneau-Fournier, J., González-Pinto, A., Harima, H., Hassel, S., Henry, C., Iacovides, A., Isometsä, E. T., Kapczinski, F., Kliwicki, S., König, B., Krogh, R., Kunz, M., Lafer, B., Larsen, E. R., Lewitzka, U., Lopez-Jaramillo, C., MacQueen, G., Manchia, M., Marsh, W., Martinez-Cengotitabengoa, M., Melle, I., Monteith, S., Morken, G., Munoz, R., Nery, F. G., O'Donovan, C., Osher, Y., Pfennig, A., Quiroz, D., Ramesar, R., Rasgon, N., Reif, A., Ritter, P., Rybakowski, J. K., Sagduyu, K., Scippa Â, M., Severus, E., Simhandl, C., Stein, D. J., Strejilevich, S., Sulaiman, A. H., Suominen, K., Tagata, H., Tatebayashi, Y., Torrent, C., Vieta, E., Viswanath, B., Wanchoo, M. J., Zetin, M., & Whybrow, P. C. (2014). Relationship between sunlight and the age of onset of bipolar disorder: an international multisite study. *J Affect Disord*, *167*, 104-111. https://doi.org/10.1016/j.jad.2014.05.032

Biaglow, J. E., & Miller, R. A. (2005). The thioredoxin reductase/thioredoxin system: novel redox targets for cancer therapy. *Cancer Biol Ther*, *4*(1), 6-13. https://doi.org/10.4161/cbt.4.1.1434

Biggi, N., Consonni, D., Galluzzo, V., Sogliani, M., & Costa, G. (2008). Metabolic syndrome in permanent night workers. *Chronobiol Int*, *25*(2), 443-454. https://doi.org/10.1080/07420520802114193

Bonmati-Carrion, M. A., Arguelles-Prieto, R., Martinez-Madrid, M. J., Reiter, R., Hardeland, R., Rol, M. A., & Madrid, J. A. (2014). Protecting the melatonin rhythm through circadian healthy light exposure. *Int J Mol Sci*, *15*(12), 23448-23500. https://doi.org/10.3390/ijms151223448

Boomgarden, A. C., Sagewalker, G. D., Shah, A. C., Haider, S. D., Patel, P., Wheeler, H. E., Dubowy, C. M., & Cavanaugh, D. J. (2019). Chronic circadian misalignment results in reduced longevity and large-scale changes in gene expression in Drosophila. *BMC Genomics*, *20*(1), 14. https://doi.org/10.1186/s12864-018-5401-7

Bracci, M., Ciarapica, V., Copertaro, A., Barbaresi, M., Manzella, N., Tomasetti, M., Gaetani, S., Monaco, F., Amati, M., Valentino, M., Rapisarda, V., & Santarelli, L. (2016). Peripheral Skin Temperature and Circadian Biological Clock in Shift Nurses after a Day off. *Int J Mol Sci*, *17*(5). https://doi.org/10.3390/ijms17050623

Bracci, M., Manzella, N., Copertaro, A., Staffolani, S., Strafella, E., Barbaresi, M., Copertaro, B., Rapisarda, V., Valentino, M., & Santarelli, L. (2014). Rotating-shift nurses after a day off: peripheral clock gene expression, urinary melatonin, and serum 17-β-estradiol levels. *Scand J Work Environ Health*, *40*(3), 295-304. https://doi.org/10.5271/sjweh.3414

Brancaleon, L., & Moseley, H. (2002). Laser and non-laser light sources for photodynamic therapy. *Lasers Med Sci*, *17*(3), 173-186. https://doi.org/10.1007/s101030200027

Brem, R., Guven, M., & Karran, P. (2017). Oxidatively-generated damage to DNA and proteins mediated by photosensitized UVA. *Free Radic Biol Med*, *107*, 101-109. https://doi.org/10.1016/j.freeradbiomed.2016.10.488

Breskey, J. D., Lacey, S. E., Vesper, B. J., Paradise, W. A., Radosevich, J. A., & Colvard, M. D. (2013). Photodynamic therapy: occupational hazards and preventative recommendations for clinical administration by healthcare providers. *Photomed Laser Surg*, *31*(8), 398-407. https://doi.org/10.1089/pho.2013.3496

Brondon, P., Stadler, I., & Lanzafame, R. J. (2009). Pulsing influences photoradiation outcomes in cell culture. *Lasers Surg Med*, *41*(3), 222-226. https://doi.org/10.1002/lsm.20740

Buhr, E. D., & Takahashi, J. S. (2013). Molecular components of the Mammalian circadian clock. *Handb Exp Pharmacol*(217), 3-27. https://doi.org/10.1007/978-3-642-25950-0_1

Buijs, R. M., & Kalsbeek, A. (2001). Hypothalamic integration of central and peripheral clocks. *Nat Rev Neurosci*, *2*(7), 521-526. https://doi.org/10.1038/35081582

Buijs, R. M., van Eden, C. G., Goncharuk, V. D., & Kalsbeek, A. (2003). The biological clock tunes the organs of the body: timing by hormones and the autonomic nervous system. *J Endocrinol*, *177*(1), 17-26. https://doi.org/10.1677/joe.0.1770017

Burczynski, M. E., Lin, H. K., & Penning, T. M. (1999). Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase. *Cancer Res*, *59*(3), 607-614.

Butterworth-Heinemann. (2009) Dictionary of Optometry and Visual Science, 7th edition

Cardinali, D. P., Vacas, M. I., Gejman, P. V., Pisarev, M. A., Barontini, M., Boado, R. J., & Juvenal, G. J. (1983). The sympathetic superior cervical ganglia as "little neuroendocrine brains". *Acta Physiol Lat Am*, *33*(3), 205-221.

Castello, P. R., Drechsel, D. A., & Patel, M. (2007). Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. *J Biol Chem*, *282*(19), 14186-14193. https://doi.org/10.1074/jbc.M700827200

Cedernaes, J., Waldeck, N., & Bass, J. (2019). Neurogenetic basis for circadian regulation of metabolism by the hypothalamus. *Genes Dev*, *33*(17-18), 1136-1158. https://doi.org/10.1101/gad.328633.119

Chaopu, Y., Wenqing, F., Jiancheng, T., Fan, Y., Yanfeng, L., & Chun, L. (2018). Change of blue light hazard and circadian effect of LED backlight displayer with color temperature and age. *Opt Express*, *26*(21), 27021-27032. https://doi.org/10.1364/oe.26.027021

Chapman, E. C., Bonsor, B. J., Parsons, D. R., & Rotchell, J. M. (2020). Influence of light and temperature cycles on the expression of circadian clock genes in the mussel Mytilus edulis. *Mar Environ Res*, *159*, 104960. https://doi.org/10.1016/j.marenvres.2020.104960

Chen, A., Du, L., Xu, Y., Chen, L., & Wu, Y. (2005). The effect of blue light exposure on the expression of circadian genes: bmal1 and cryptochrome 1 in peripheral blood mononuclear cells of jaundiced neonates. *Pediatr Res*, *58*(6), 1180-1184. https://doi.org/10.1203/01.pdr.0000183663.98446.05

Chen, E., Söderberg, P. G., & Lindström, B. (1992). Cytochrome oxidase activity in rat retina after exposure to 404 nm blue light. *Curr Eye Res*, *11*(9), 825-831. https://doi.org/10.3109/02713689209033480

Clausen, O. P., Thorud, E., Bjerknes, R., & Elgjo, K. (1979). Circadian rhythms in mouse epidermal basal cell proliferation. Variations in compartment size, flux and phase duration. *Cell Tissue Kinet*, *12*(3), 319-337. https://doi.org/10.1111/j.1365-2184.1979.tb00154.x

Collis, S. J., & Boulton, S. J. (2007). Emerging links between the biological clock and the DNA damage response. *Chromosoma*, *116*(4), 331-339. https://doi.org/10.1007/s00412-007-0108-6

Colombo, I., Sangiovanni, E., Maggio, R., Mattozzi, C., Zava, S., Corbett, Y., Fumagalli, M., Carlino, C., Corsetto, P. A., Scaccabarozzi, D., Calvieri, S., Gismondi, A., Taramelli, D., & Dell'Agli, M. (2017). HaCaT Cells as a Reliable In Vitro Differentiation Model to Dissect the Inflammatory/Repair Response of Human Keratinocytes. *Mediators Inflamm*, *2017*, 7435621. https://doi.org/10.1155/2017/7435621

Copertaro A. (2013) Lavoro a turni notturno. Valutazione del rischio e sorveglianza sanitaria. Editore: Booksprint

Copertaro, A., & Bracci, M. (2019). Working against the biological clock: a review for the Occupational Physician. *Ind Health*, *57*(5), 557-569. https://doi.org/10.2486/indhealth.2018-0173

Cos, S., González, A., Martínez-Campa, C., Mediavilla, M. D., Alonso-González, C., & Sánchez-Barceló, E. J. (2008). Melatonin as a selective estrogen enzyme modulator.

Curr Cancer Drug Targets, *8*(8), 691-702. https://doi.org/10.2174/156800908786733469

Costa, G., Haus, E., & Stevens, R. (2010). Shift work and cancer - considerations on rationale, mechanisms, and epidemiology. *Scand J Work Environ Health*, *36*(2), 163-179. https://doi.org/10.5271/sjweh.2899

Daniell, M. D., & Hill, J. S. (1991). A history of photodynamic therapy. *Aust N Z J Surg*, *61*(5), 340-348. https://doi.org/10.1111/j.1445-2197.1991.tb00230.x

Dantas, L. L. B., Calixto, C. P. G., Dourado, M. M., Carneiro, M. S., Brown, J. W. S., & Hotta, C. T. (2019). Alternative Splicing of Circadian Clock Genes Correlates With Temperature in Field-Grown Sugarcane. *Front Plant Sci*, *10*, 1614. https://doi.org/10.3389/fpls.2019.01614

Darlington, T. K., Wager-Smith, K., Ceriani, M. F., Staknis, D., Gekakis, N., Steeves, T. D., Weitz, C. J., Takahashi, J. S., & Kay, S. A. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. *Science*, *280*(5369), 1599-1603. https://doi.org/10.1126/science.280.5369.1599

Dawson, T. M., & Dawson, V. L. (2003). Molecular pathways of neurodegeneration in Parkinson's disease. *Science*, *302*(5646), 819-822. https://doi.org/10.1126/science.1087753

De Bacquer, D., Van Risseghem, M., Clays, E., Kittel, F., De Backer, G., & Braeckman, L. (2009). Rotating shift work and the metabolic syndrome: a prospective study. *Int J Epidemiol*, *38*(3), 848-854. https://doi.org/10.1093/ije/dyn360

Deguchi, T. (1979). Circadian rhythm of serotonin N-acetyltransferase activity in organ culture of chicken pineal gland. *Science*, *203*(4386), 1245-1247. https://doi.org/10.1126/science.424750

Di Rosa, V., Frigato, E., López-Olmeda, J. F., Sánchez-Vázquez, F. J., & Bertolucci, C. (2015). The Light Wavelength Affects the Ontogeny of Clock Gene Expression and Activity Rhythms in Zebrafish Larvae. *PLoS One*, *10*(7), e0132235. https://doi.org/10.1371/journal.pone.0132235

Dibner, C., Schibler, U., & Albrecht, U. (2010). The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol*, *72*, 517-549. https://doi.org/10.1146/annurev-physiol-021909-135821

Dijk, D. J., & Cajochen, C. (1997). Melatonin and the circadian regulation of sleep initiation, consolidation, structure, and the sleep EEG. *J Biol Rhythms*, *12*(6), 627-635. https://doi.org/10.1177/074873049701200618

Doi, M., Hirayama, J., & Sassone-Corsi, P. (2006). Circadian regulator CLOCK is a histone acetyltransferase. *Cell*, *125*(3), 497-508. https://doi.org/10.1016/j.cell.2006.03.033

Donovan, M., Carmody, R. J., & Cotter, T. G. (2001). Light-induced photoreceptor apoptosis in vivo requires neuronal nitric-oxide synthase and guanylate cyclase activity and is caspase-3-independent. *J Biol Chem*, *276*(25), 23000-23008. https://doi.org/10.1074/jbc.M005359200

Dreger, H., Westphal, K., Weller, A., Baumann, G., Stangl, V., Meiners, S., & Stangl, K. (2009). Nrf2-dependent upregulation of antioxidative enzymes: a novel pathway for proteasome inhibitor-mediated cardioprotection. *Cardiovasc Res*, *83*(2), 354-361. https://doi.org/10.1093/cvr/cvp107

Dunlap, J. C. (1999). Molecular bases for circadian clocks. *Cell*, *96*(2), 271-290. https://doi.org/10.1016/s0092-8674(00)80566-8

Dunlap, J. C., & Loros, J. J. (2017). Making Time: Conservation of Biological Clocks from Fungi to Animals. *Microbiol Spectr*, *5*(3). https://doi.org/10.1128/microbiolspec.FUNK-0039-2016

Eckel-Mahan, K. L., Patel, V. R., de Mateo, S., Orozco-Solis, R., Ceglia, N. J., Sahar, S., Dilag-Penilla, S. A., Dyar, K. A., Baldi, P., & Sassone-Corsi, P. (2013). Reprogramming of the circadian clock by nutritional challenge. *Cell*, *155*(7), 1464-1478. https://doi.org/10.1016/j.cell.2013.11.034

Ecker, A., Barbosa, N. V., & Ardisson-Araujo, D. (2020). Accessing the transcriptional status of selenoproteins in skin cancer-derived cell lines. *J Trace Elem Med Biol*, *60*, 126476. https://doi.org/10.1016/j.jtemb.2020.126476

Emery, P., So, W. V., Kaneko, M., Hall, J. C., & Rosbash, M. (1998). CRY, a Drosophila clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell*, *95*(5), 669-679. https://doi.org/10.1016/s0092-8674(00)81637-2

Falchi, F., Cinzano, P., Duriscoe, D., Kyba, C. C., Elvidge, C. D., Baugh, K., Portnov, B. A., Rybnikova, N. A., & Furgoni, R. (2016). The new world atlas of artificial night sky brightness. *Sci Adv*, *2*(6), e1600377. https://doi.org/10.1126/sciadv.1600377

Fan, S. M., Chang, Y. T., Chen, C. L., Wang, W. H., Pan, M. K., Chen, W. P., Huang, W. Y., Xu, Z., Huang, H. E., Chen, T., Plikus, M. V., Chen, S. K., & Lin, S. J. (2018). External light activates hair follicle stem cells through eyes via an ipRGC-SCN-sympathetic neural pathway. *Proc Natl Acad Sci U S A*, *115*(29), E6880-e6889. https://doi.org/10.1073/pnas.1719548115

Feller, L., Khammissa, R. A. G., Kramer, B., Altini, M., & Lemmer, J. (2016). Basal cell carcinoma, squamous cell carcinoma and melanoma of the head and face. *Head Face Med*, *12*, 11. https://doi.org/10.1186/s13005-016-0106-0

Flourakis, M., & Allada, R. (2015). Patch-clamp electrophysiology in Drosophila circadian pacemaker neurons. *Methods Enzymol*, *552*, 23-44. https://doi.org/10.1016/bs.mie.2014.10.005

Fusenig, N. E., & Boukamp, P. (1998). Multiple stages and genetic alterations in immortalization, malignant transformation, and tumor progression of human skin keratinocytes. *Mol Carcinog*, *23*(3), 144-158. https://doi.org/10.1002/(sici)1098-2744(199811)23:3<144::aid-mc3>3.0.co;2-u

Gaddameedhi, S., Selby, C. P., Kaufmann, W. K., Smart, R. C., & Sancar, A. (2011). Control of skin cancer by the circadian rhythm. *Proc Natl Acad Sci U S A*, *108*(46), 18790-18795. https://doi.org/10.1073/pnas.1115249108

Gao, Q., Zhang, G., Zheng, Y., Yang, Y., Chen, C., Xia, J., Liang, L., Lei, C., Hu, Y., Cai, X., Zhang, W., Tang, H., Chen, Y., Huang, A., Wang, K., & Tang, N. (2020). SLC27A5 deficiency activates NRF2/TXNRD1 pathway by increased lipid peroxidation in HCC. *Cell Death Differ*, *27*(3), 1086-1104. https://doi.org/10.1038/s41418-019-0399-1

Gaston, K. J., Visser, M. E., & Hölker, F. (2015). The biological impacts of artificial light at night: the research challenge. *Philos Trans R Soc Lond B Biol Sci*, *370*(1667). https://doi.org/10.1098/rstb.2014.0133

Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S., & Weitz, C. J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. *Science*, *280*(5369), 1564-1569. https://doi.org/10.1126/science.280.5369.1564

Gerber, A., Saini, C., Curie, T., Emmenegger, Y., Rando, G., Gosselin, P., Gotic, I., Gos, P., Franken, P., & Schibler, U. (2015). The systemic control of circadian gene expression. *Diabetes Obes Metab*, *17 Suppl 1*, 23-32. https://doi.org/10.1111/dom.12512

Geyfman, M., Kumar, V., Liu, Q., Ruiz, R., Gordon, W., Espitia, F., Cam, E., Millar, S. E., Smyth, P., Ihler, A., Takahashi, J. S., & Andersen, B. (2012). Brain and muscle Arnt-like protein-1 (BMAL1) controls circadian cell proliferation and susceptibility to UVB-induced DNA damage in the epidermis. *Proc Natl Acad Sci U S A*, *109*(29), 11758-11763. https://doi.org/10.1073/pnas.1209592109

Gimble, J. M., Sutton, G. M., Ptitsyn, A. A., Floyd, Z. E., & Bunnell, B. A. (2011). Circadian rhythms in adipose tissue: an update. *Curr Opin Clin Nutr Metab Care*, *14*(6), 554-561. https://doi.org/10.1097/MCO.0b013e32834ad94b

Godley, B. F., Shamsi, F. A., Liang, F. Q., Jarrett, S. G., Davies, S., & Boulton, M. (2005). Blue light induces mitochondrial DNA damage and free radical production in epithelial cells. *J Biol Chem*, *280*(22), 21061-21066. https://doi.org/10.1074/jbc.M502194200

Goldbeter, A. (2017). Dissipative structures and biological rhythms. *Chaos*, *27*(10), 104612. https://doi.org/10.1063/1.4990783

Golden, S. S., Ishiura, M., Johnson, C. H., & Kondo, T. (1997). CYANOBACTERIAL CIRCADIAN RHYTHMS. *Annu Rev Plant Physiol Plant Mol Biol*, *48*, 327-354. https://doi.org/10.1146/annurev.arplant.48.1.327

Grant, W. B., & Mohr, S. B. (2009). Ecological studies of ultraviolet B, vitamin D and cancer since 2000. *Ann Epidemiol*, *19*(7), 446-454. https://doi.org/10.1016/j.annepidem.2008.12.014

Green, A., Siskind, V., Bain, C., & Alexander, J. (1985). Sunburn and malignant melanoma. *Br J Cancer*, *51*(3), 393-397. https://doi.org/10.1038/bjc.1985.53

Green, C. B. (1998). How cells tell time. *Trends Cell Biol*, *8*(6), 224-230. https://doi.org/10.1016/s0962-8924(98)01269-0

Grimaldi, B., Nakahata, Y., Kaluzova, M., Masubuchi, S., & Sassone-Corsi, P. (2009). Chromatin remodeling, metabolism and circadian clocks: the interplay of CLOCK and SIRT1. *Int J Biochem Cell Biol*, *41*(1), 81-86. https://doi.org/10.1016/j.biocel.2008.08.035

Grimm, C., Remé, C. E., Rol, P. O., & Williams, T. P. (2000). Blue light's effects on rhodopsin: photoreversal of bleaching in living rat eyes. *Invest Ophthalmol Vis Sci*, *41*(12), 3984-3990.

Gu, Q., Feng, T., Cao, H., Tang, Y., Ge, X., Luo, J., Xue, J., Wu, J., Yang, H., Zhang, S., & Cao, J. (2013). HIV-TAT mediated protein transduction of Cu/Zn-superoxide dismutase-1 (SOD1) protects skin cells from ionizing radiation. *Radiat Oncol*, *8*, 253. https://doi.org/10.1186/1748-717x-8-253

Guerrero-Vargas, N. N., Espitia-Bautista, E., Buijs, R. M., & Escobar, C. (2018). Shift-work: is time of eating determining metabolic health? Evidence from animal models. *Proc Nutr Soc*, *77*(3), 199-215. https://doi.org/10.1017/s0029665117004128

Guillaumond, F., Dardente, H., Giguère, V., & Cermakian, N. (2005). Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *J Biol Rhythms*, *20*(5), 391-403. https://doi.org/10.1177/0748730405277232

Hajji, K., Mteyrek, A., Sun, J., Cassar, M., Mezghani, S., Leprince, J., Vaudry, D., Masmoudi-Kouki, O., & Birman, S. (2019). Neuroprotective effects of PACAP against paraquat-induced oxidative stress in the Drosophila central nervous system. *Hum Mol Genet*, *28*(11), 1905-1918. https://doi.org/10.1093/hmg/ddz031

Hall, J. C. (1995). Tripping along the trail to the molecular mechanisms of biological clocks. *Trends Neurosci*, *18*(5), 230-240. https://doi.org/10.1016/0166-2236(95)93908-g

Hall, J. C., & Sassone-Corsi, P. (1998). Molecular clocks. *Curr Opin Neurobiol*, *8*(5), 633-634. https://doi.org/10.1016/s0959-4388(98)80091-3

Halliday, G. M., & Byrne, S. N. (2014). An unexpected role: UVA-induced release of nitric oxide from skin may have unexpected health benefits. *J Invest Dermatol*, *134*(7), 1791-1794. https://doi.org/10.1038/jid.2014.33

Hardie, R. C., & Raghu, P. (2001). Visual transduction in Drosophila. *Nature*, *413*(6852), 186-193. https://doi.org/10.1038/35093002

Hardin, P. E., Hall, J. C., & Rosbash, M. (1990). Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. *Nature*, *343*(6258), 536-540. https://doi.org/10.1038/343536a0

Haritou, S. J., Zylstra, R., Ralli, C., Turner, S., & Tortonese, D. J. (2008). Seasonal changes in circadian peripheral plasma concentrations of melatonin, serotonin, dopamine and cortisol in aged horses with Cushing's disease under natural photoperiod. *J Neuroendocrinol*, *20*(8), 988-996. https://doi.org/10.1111/j.1365-2826.2008.01751.x

Hastings, J. W., & Sweeney, B. M. (1957). ON THE MECHANISM OF TEMPERATURE INDEPENDENCE IN A BIOLOGICAL CLOCK. *Proc Natl Acad Sci U S A*, *43*(9), 804-811. https://doi.org/10.1073/pnas.43.9.804

Hazlerigg, D. G., & Tyler, N. J. C. (2019). Activity patterns in mammals: Circadian dominance challenged. *PLoS Biol*, *17*(7), e3000360. https://doi.org/10.1371/journal.pbio.3000360
Helfrich-Förster, C. (2005). Organization of endogenous clocks in insects. *Biochem Soc Trans*, *33*(Pt 5), 957-961. https://doi.org/10.1042/bst20050957
Hengstberger F. (2006) New Developments In Photometry And Radiometry, CSIR – National Metrology Laboratory. Simposio de Metrología
Herichova, I. (2013). Changes of physiological functions induced by shift work. *Endocr Regul*, *47*(3), 159-170. https://doi.org/10.4149/endo_2013_03_159
Hoffman F. (1915) The Mortality of Cancer throughout the World. Appendix E, Prudential Press
Hofseth, L. J. (2004). The adaptive imbalance to genotoxic stress: genome guardians rear their ugly heads. *Carcinogenesis*, *25*(10), 1787-1793. https://doi.org/10.1093/carcin/bgh196
Holick, M. F. (2003). Vitamin D: A millenium perspective. *J Cell Biochem*, *88*(2), 296-307. https://doi.org/10.1002/jcb.10338
Holick, M. F. (2016). Biological Effects of Sunlight, Ultraviolet Radiation, Visible Light, Infrared Radiation and Vitamin D for Health. *Anticancer Res*, *36*(3), 1345-1356.
Holick, M. F., & Chen, T. C. (2008). Vitamin D deficiency: a worldwide problem with health consequences. *Am J Clin Nutr*, *87*(4), 1080s-1086s. https://doi.org/10.1093/ajcn/87.4.1080S
Holonyak Jr. N. (1962); COHERENT (VISIBLE) LIGHT EMISSION FROM Ga(As1−Xpx) JUNCTIONS Appl. Phys. Lett. 1, 82 Https://Doi.Org/10.1063/1.1753706
Honjo, S., Fujiwara, T., Takasaka, M., Suzuki, Y., & Imaizumi, K. (1963). OBSERVATIONS ON THE DIURNAL TEMPERATURE VARIATION OF CYNOMOLGUS MONKEYS (MACACA IRUS) AND ON THE EFFECT OF CHANGES IN THE ROUTINE LIGHTING UPON THIS VARIATION. *Jpn J Med Sci Biol*, *16*, 189-198. https://doi.org/10.7883/yoken1952.16.189
Honma, S. (2018). The mammalian circadian system: a hierarchical multi-oscillator structure for generating circadian rhythm. *J Physiol Sci*, *68*(3), 207-219. https://doi.org/10.1007/s12576-018-0597-5
Hsieh, M. C., Yang, S. C., Tseng, H. L., Hwang, L. L., Chen, C. T., & Shieh, K. R. (2010). Abnormal expressions of circadian-clock and circadian clock-controlled genes in the livers and kidneys of long-term, high-fat-diet-treated mice. *Int J Obes (Lond)*, *34*(2), 227-239. https://doi.org/10.1038/ijo.2009.228
Huang, H. C., Nguyen, T., & Pickett, C. B. (2000). Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. *Proc Natl Acad Sci U S A*, *97*(23), 12475-12480. https://doi.org/10.1073/pnas.220418997
Hwang, H., Biswas, R., Chung, P. S., & Ahn, J. C. (2013). Modulation of EGFR and ROS induced cytochrome c release by combination of photodynamic therapy and carboplatin in human cultured head and neck cancer cells and tumor xenograft in nude mice. *J Photochem Photobiol B*, *128*, 70-77. https://doi.org/10.1016/j.jphotobiol.2013.08.001
Jagdeo, J., Austin, E., Mamalis, A., Wong, C., Ho, D., & Siegel, D. M. (2018). Light-emitting diodes in dermatology: A systematic review of randomized controlled trials. *Lasers Surg Med*, *50*(6), 613-628. https://doi.org/10.1002/lsm.22791
Janich, P., Toufighi, K., Solanas, G., Luis, N. M., Minkwitz, S., Serrano, L., Lehner, B., & Benitah, S. A. (2013). Human epidermal stem cell function is regulated by circadian oscillations. *Cell Stem Cell*, *13*(6), 745-753. https://doi.org/10.1016/j.stem.2013.09.004
Jesionek-Kupnicka, D., Chomiczewska-Skóra, D., & Rotsztejn, H. (2013). Influence of phototherapy in psoriasis on Ki-67 antigen expression: a preliminary study. *Pol J Pathol*, *64*(2), 96-103. https://doi.org/10.5114/pjp.2013.36006
Jichlinski, P. (2006). Photodynamic applications in superficial bladder cancer: facts and hopes! *J Environ Pathol Toxicol Oncol*, *25*(1-2), 441-451. https://doi.org/10.1615/jenvironpatholtoxicoloncol.v25.i1-2.280

Jou, M. J., Peng, T. I., Reiter, R. J., Jou, S. B., Wu, H. Y., & Wen, S. T. (2004). Visualization of the antioxidative effects of melatonin at the mitochondrial level during oxidative stress-induced apoptosis of rat brain astrocytes. *J Pineal Res*, *37*(1), 55-70. https://doi.org/10.1111/j.1600-079X.2004.00140.x

Kang, D., & Hamasaki, N. (2003). Mitochondrial oxidative stress and mitochondrial DNA. *Clin Chem Lab Med*, *41*(10), 1281-1288. https://doi.org/10.1515/cclm.2003.195

Karatsoreos, I. N., Bhagat, S., Bloss, E. B., Morrison, J. H., & McEwen, B. S. (2011). Disruption of circadian clocks has ramifications for metabolism, brain, and behavior. *Proc Natl Acad Sci U S A*, *108*(4), 1657-1662. https://doi.org/10.1073/pnas.1018375108

Kennedy, C., Bajdik, C. D., Willemze, R., De Gruijl, F. R., & Bouwes Bavinck, J. N. (2003). The influence of painful sunburns and lifetime sun exposure on the risk of actinic keratoses, seborrheic warts, melanocytic nevi, atypical nevi, and skin cancer. *J Invest Dermatol*, *120*(6), 1087-1093. https://doi.org/10.1046/j.1523-1747.2003.12246.x

Khan, Z. A., Yumnamcha, T., Mondal, G., Devi, S. D., Rajiv, C., Labala, R. K., Sanjita Devi, H., & Chattoraj, A. (2020). Artificial Light at Night (ALAN): A Potential Anthropogenic Component for the COVID-19 and HCoVs Outbreak. *Front Endocrinol (Lausanne)*, *11*, 622. https://doi.org/10.3389/fendo.2020.00622

Kiermayer, C., Michalke, B., Schmidt, J., & Brielmeier, M. (2007). Effect of selenium on thioredoxin reductase activity in Txnrd1 or Txnrd2 hemizygous mice. *Biol Chem*, *388*(10), 1091-1097. https://doi.org/10.1515/bc.2007.133

Kim, Y. H., Cho, A., Kwon, S. A., Kim, M., Song, M., Han, H. W., Shin, E. J., Park, E., & Lee, S. M. (2019). Potential Photoprotective Effect of Dietary Corn Silk Extract on Ultraviolet B-Induced Skin Damage. *Molecules*, *24*(14). https://doi.org/10.3390/molecules24142587

Kinmonth-Schultz, H. A., Golembeski, G. S., & Imaizumi, T. (2013). Circadian clock-regulated physiological outputs: dynamic responses in nature. *Semin Cell Dev Biol*, *24*(5), 407-413. https://doi.org/10.1016/j.semcdb.2013.02.006

Ko, C. H., & Takahashi, J. S. (2006). Molecular components of the mammalian circadian clock. *Hum Mol Genet*, *15 Spec No 2*, R271-277. https://doi.org/10.1093/hmg/ddl207

Kochan, D. Z., & Kovalchuk, O. (2015). Circadian disruption and breast cancer: an epigenetic link? *Oncotarget*, *6*(19), 16866-16882. https://doi.org/10.18632/oncotarget.4343

Kohsaka, A., Laposky, A. D., Ramsey, K. M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F. W., & Bass, J. (2007). High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab*, *6*(5), 414-421. https://doi.org/10.1016/j.cmet.2007.09.006

Konopka, R. J., & Benzer, S. (1971). Clock mutants of Drosophila melanogaster. *Proc Natl Acad Sci U S A*, *68*(9), 2112-2116. https://doi.org/10.1073/pnas.68.9.2112

Kume, K., Zylka, M. J., Sriram, S., Shearman, L. P., Weaver, D. R., Jin, X., Maywood, E. S., Hastings, M. H., & Reppert, S. M. (1999). mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell*, *98*(2), 193-205. https://doi.org/10.1016/s0092-8674(00)81014-4

Kurlansik, S. L., & Ibay, A. D. (2012). Seasonal affective disorder. *Am Fam Physician*, *86*(11), 1037-1041.

Kyriacou, C. P., & Rosato, E. (2000). Squaring up the E-box. *J Biol Rhythms*, *15*(6), 483-490. https://doi.org/10.1177/074873040001500605

la Fleur, S. E., Kalsbeek, A., Wortel, J., & Buijs, R. M. (2000). Polysynaptic neural pathways between the hypothalamus, including the suprachiasmatic nucleus, and the liver. *Brain Res*, *871*(1), 50-56. https://doi.org/10.1016/s0006-8993(00)02423-9

Lan, A. P., Xiao, L. C., Yang, Z. L., Yang, C. T., Wang, X. Y., Chen, P. X., Gu, M. F., & Feng, J. Q. (2012). Interaction between ROS and p38MAPK contributes to chemical hypoxia-induced injuries in PC12 cells. *Mol Med Rep*, *5*(1), 250-255. https://doi.org/10.3892/mmr.2011.623

Lazzerini Ospri, L., Prusky, G., & Hattar, S. (2017). Mood, the Circadian System, and Melanopsin Retinal Ganglion Cells. *Annu Rev Neurosci*, *40*, 539-556. https://doi.org/10.1146/annurev-neuro-072116-031324

Le Fur, I., Reinberg, A., Lopez, S., Morizot, F., Mechkouri, M., & Tschachler, E. (2001). Analysis of circadian and ultradian rhythms of skin surface properties of face and forearm of healthy women. *J Invest Dermatol*, *117*(3), 718-724. https://doi.org/10.1046/j.0022-202x.2001.01433.x

Lee, J. E., Atkins, N., Jr., Hatcher, N. G., Zamdborg, L., Gillette, M. U., Sweedler, J. V., & Kelleher, N. L. (2010). Endogenous peptide discovery of the rat circadian clock: a focused study of the suprachiasmatic nucleus by ultrahigh performance tandem mass spectrometry. *Mol Cell Proteomics*, *9*(2), 285-297. https://doi.org/10.1074/mcp.M900362-MCP200

Lee, S. Y., Park, K. H., Choi, J. W., Kwon, J. K., Lee, D. R., Shin, M. S., Lee, J. S., You, C. E., & Park, M. Y. (2007). A prospective, randomized, placebo-controlled, double-blinded, and split-face clinical study on LED phototherapy for skin rejuvenation: clinical, profilometric, histologic, ultrastructural, and biochemical evaluations and comparison of three different treatment settings. *J Photochem Photobiol B*, *88*(1), 51-67. https://doi.org/10.1016/j.jphotobiol.2007.04.008

Lefkowitz, E. S., & Garland, C. F. (1994). Sunlight, vitamin D, and ovarian cancer mortality rates in US women. *Int J Epidemiol*, *23*(6), 1133-1136. https://doi.org/10.1093/ije/23.6.1133

Lengyel, Z., Battyáni, Z., Szekeres, G., Csernus, V., & Nagy, A. D. (2013). Circadian clocks and tumor biology: what is to learn from human skin biopsies? *Gen Comp Endocrinol*, *188*, 67-74. https://doi.org/10.1016/j.ygcen.2013.03.033

Li, W. T., Leu, Y. C., & Wu, J. L. (2010). Red-light light-emitting diode irradiation increases the proliferation and osteogenic differentiation of rat bone marrow mesenchymal stem cells. *Photomed Laser Surg*, *28 Suppl 1*, S157-165. https://doi.org/10.1089/pho.2009.2540

Li, Y., Xu, Q., Shi, M., Gan, P., Huang, Q., Wang, A., Tan, G., Fang, Y., & Liao, H. (2020). Low-level laser therapy induces human umbilical vascular endothelial cell proliferation, migration and tube formation through activating the PI3K/Akt signaling pathway. *Microvasc Res*, *129*, 103959. https://doi.org/10.1016/j.mvr.2019.103959

Lin, K. K., Kumar, V., Geyfman, M., Chudova, D., Ihler, A. T., Smyth, P., Paus, R., Takahashi, J. S., & Andersen, B. (2009). Circadian clock genes contribute to the regulation of hair follicle cycling. *PLoS Genet*, *5*(7), e1000573. https://doi.org/10.1371/journal.pgen.1000573

Lowrey, P. L., & Takahashi, J. S. (2000). Genetics of the mammalian circadian system: Photic entrainment, circadian pacemaker mechanisms, and posttranslational regulation. *Annu Rev Genet*, *34*, 533-562. https://doi.org/10.1146/annurev.genet.34.1.533

Lowrey, P. L., & Takahashi, J. S. (2004). Mammalian circadian biology: elucidating genome-wide levels of temporal organization. *Annu Rev Genomics Hum Genet*, *5*, 407-441. https://doi.org/10.1146/annurev.genom.5.061903.175925

Lunn, R. M., Blask, D. E., Coogan, A. N., Figueiro, M. G., Gorman, M. R., Hall, J. E., Hansen, J., Nelson, R. J., Panda, S., Smolensky, M. H., Stevens, R. G., Turek, F. W., Vermeulen, R., Carreón, T., Caruso, C. C., Lawson, C. C., Thayer, K. A., Twery, M. J., Ewens, A. D., Garner, S. C., Schwingl, P. J., & Boyd, W. A. (2017). Health consequences of electric lighting practices in the modern world: A report on the National Toxicology Program's workshop on shift work at night, artificial light at night, and circadian disruption. *Sci Total Environ*, *607-608*, 1073-1084. https://doi.org/10.1016/j.scitotenv.2017.07.056

Magwere, T., West, M., Riyahi, K., Murphy, M. P., Smith, R. A., & Partridge, L. (2006). The effects of exogenous antioxidants on lifespan and oxidative stress resistance in

Drosophila melanogaster. *Mech Ageing Dev*, *127*(4), 356-370. https://doi.org/10.1016/j.mad.2005.12.009

Makris, G. D., Reutfors, J., Larsson, R., Isacsson, G., Ösby, U., Ekbom, A., Ekselius, L., & Papadopoulos, F. C. (2016). Serotonergic medication enhances the association between suicide and sunshine. *J Affect Disord*, *189*, 276-281. https://doi.org/10.1016/j.jad.2015.09.056

Manzella, N., Bracci, M., Ciarapica, V., Staffolani, S., Strafella, E., Rapisarda, V., Valentino, M., Amati, M., Copertaro, A., & Santarelli, L. (2015). Circadian gene expression and extremely low-frequency magnetic fields: an in vitro study. *Bioelectromagnetics*, *36*(4), 294-301. https://doi.org/10.1002/bem.21915

Manzella, N., Bracci, M., Staffolani, S., Strafella, E., Rapisarda, V., Valentino, M., Amati, M., Copertaro, A., & Santarelli, L. (2013). Styrene altered clock gene expression in serum-shocked cultured human fibroblasts. *Biosci Biotechnol Biochem*, *77*(6), 1296-1298. https://doi.org/10.1271/bbb.120944

McCormack, A. L., Atienza, J. G., Johnston, L. C., Andersen, J. K., Vu, S., & Di Monte, D. A. (2005). Role of oxidative stress in paraquat-induced dopaminergic cell degeneration. *J Neurochem*, *93*(4), 1030-1037. https://doi.org/10.1111/j.1471-4159.2005.03088.x

Mead, M. N. (2008). Benefits of sunlight: a bright spot for human health. *Environ Health Perspect*, *116*(4), A160-167. https://doi.org/10.1289/ehp.116-a160

Mondal, P., & Huix-Rotllant, M. (2019). Theoretical insights into the formation and stability of radical oxygen species in cryptochromes. *Phys Chem Chem Phys*, *21*(17), 8874-8882. https://doi.org/10.1039/c9cp00782b

Mouret, J., Coindet, J., Debilly, G., & Chouvet, G. (1978). Suprachiasmatic nuclei lesions in the rat: alterations in sleep circadian rhythms. *Electroencephalogr Clin Neurophysiol*, *45*(3), 402-408. https://doi.org/10.1016/0013-4694(78)90191-8

Nash, T. R., Chow, E. S., Law, A. D., Fu, S. D., Fuszara, E., Bilska, A., Bebas, P., Kretzschmar, D., & Giebultowicz, J. M. (2019). Daily blue-light exposure shortens lifespan and causes brain neurodegeneration in Drosophila. *NPJ Aging Mech Dis*, *5*, 8. https://doi.org/10.1038/s41514-019-0038-6

Noell, W. K., Walker, V. S., Kang, B. S., & Berman, S. (1966). Retinal damage by light in rats. *Invest Ophthalmol*, *5*(5), 450-473.

Noshiro, M., Kawamoto, T., Furukawa, M., Fujimoto, K., Yoshida, Y., Sasabe, E., Tsutsumi, S., Hamada, T., Honma, S., Honma, K., & Kato, Y. (2004). Rhythmic expression of DEC1 and DEC2 in peripheral tissues: DEC2 is a potent suppressor for hepatic cytochrome P450s opposing DBP. *Genes Cells*, *9*(4), 317-329. https://doi.org/10.1111/j.1356-9597.2004.00722.x

Oh, P. S., & Jeong, H. J. (2019). Therapeutic application of light emitting diode: Photo-oncomic approach. *J Photochem Photobiol B*, *192*, 1-7. https://doi.org/10.1016/j.jphotobiol.2019.01.003

Oh, P. S., Na, K. S., Hwang, H., Jeong, H. S., Lim, S., Sohn, M. H., & Jeong, H. J. (2015). Effect of blue light emitting diodes on melanoma cells: involvement of apoptotic signaling. *J Photochem Photobiol B*, *142*, 197-203. https://doi.org/10.1016/j.jphotobiol.2014.12.006

Pak, W. L., & Leung, H. T. (2003). Genetic approaches to visual transduction in Drosophila melanogaster. *Recept Channels*, *9*(3), 149-167.

Paschos, G. K., & FitzGerald, G. A. (2010). Circadian clocks and vascular function. *Circ Res*, *106*(5), 833-841. https://doi.org/10.1161/circresaha.109.211706

Pathak, M. A., & Fanselow, D. L. (1983). Photobiology of melanin pigmentation: dose/response of skin to sunlight and its contents. *J Am Acad Dermatol*, *9*(5), 724-733. https://doi.org/10.1016/s0190-9622(83)70186-6

Paul, K. N., Saafir, T. B., & Tosini, G. (2009). The role of retinal photoreceptors in the regulation of circadian rhythms. *Rev Endocr Metab Disord*, *10*(4), 271-278. https://doi.org/10.1007/s11154-009-9120-x

Peng, T. I., & Jou, M. J. (2004). Mitochondrial swelling and generation of reactive oxygen species induced by photoirradiation are heterogeneously distributed. *Ann N Y Acad Sci*, *1011*, 112-122. https://doi.org/10.1007/978-3-662-41088-2_12

Penning, T. M., Steckelbroeck, S., Bauman, D. R., Miller, M. W., Jin, Y., Peehl, D. M., Fung, K. M., & Lin, H. K. (2006). Aldo-keto reductase (AKR) 1C3: role in prostate disease and the development of specific inhibitors. *Mol Cell Endocrinol*, *248*(1-2), 182-191. https://doi.org/10.1016/j.mce.2005.12.009

Pietroiusti, A., Neri, A., Somma, G., Coppeta, L., Iavicoli, I., Bergamaschi, A., & Magrini, A. (2010). Incidence of metabolic syndrome among night-shift healthcare workers. *Occup Environ Med*, *67*(1), 54-57. https://doi.org/10.1136/oem.2009.046797

Pilorz, V., Helfrich-Förster, C., & Oster, H. (2018). The role of the circadian clock system in physiology. *Pflugers Arch*, *470*(2), 227-239. https://doi.org/10.1007/s00424-017-2103-y

Pittendrigh et al., 1959. Growth Patterns In Neurospora: A Biological Clock in Neurospora.

Pittendrigh, C. S. (1993). Temporal organization: reflections of a Darwinian clock-watcher. *Annu Rev Physiol*, *55*, 16-54. https://doi.org/10.1146/annurev.ph.55.030193.000313

Plikus, M. V., Van Spyk, E. N., Pham, K., Geyfman, M., Kumar, V., Takahashi, J. S., & Andersen, B. (2015). The circadian clock in skin: implications for adult stem cells, tissue regeneration, cancer, aging, and immunity. *J Biol Rhythms*, *30*(3), 163-182. https://doi.org/10.1177/0748730414563537

Putting, B. J., van Best, J. A., Zweypfenning, R. C., Vrensen, G. F., & Oosterhuis, J. A. (1993). Spectral sensitivity of the blood-retinal barrier at the pigment epithelium for blue light in the 400-500 nm range. *Graefes Arch Clin Exp Ophthalmol*, *231*(10), 600-606. https://doi.org/10.1007/bf00936526

Ralph, M. R., Foster, R. G., Davis, F. C., & Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. *Science*, *247*(4945), 975-978. https://doi.org/10.1126/science.2305266

Ramsey, K. M., Yoshino, J., Brace, C. S., Abrassart, D., Kobayashi, Y., Marcheva, B., Hong, H. K., Chong, J. L., Buhr, E. D., Lee, C., Takahashi, J. S., Imai, S., & Bass, J. (2009). Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. *Science*, *324*(5927), 651-654. https://doi.org/10.1126/science.1171641

Reid, K. J., & Zee, P. C. (2009). Circadian rhythm disorders. *Semin Neurol*, *29*(4), 393-405. https://doi.org/10.1055/s-0029-1237120

Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature*, *418*(6901), 935-941. https://doi.org/10.1038/nature00965

Ricci, R., Pazos, M. C., Borges, R. E., & Pacheco-Soares, C. (2009). Biomodulation with low-level laser radiation induces changes in endothelial cell actin filaments and cytoskeletal organization. *J Photochem Photobiol B*, *95*(1), 6-8. https://doi.org/10.1016/j.jphotobiol.2008.11.007

Robles, M. S., Boyault, C., Knutti, D., Padmanabhan, K., & Weitz, C. J. (2010). Identification of RACK1 and protein kinase Calpha as integral components of the mammalian circadian clock. *Science*, *327*(5964), 463-466. https://doi.org/10.1126/science.1180067

Roehlecke, C., Schumann, U., Ader, M., Knels, L., & Funk, R. H. (2011). Influence of blue light on photoreceptors in a live retinal explant system. *Mol Vis*, *17*, 876-884.

Roenneberg, T., Wirz-Justice, A., & Merrow, M. (2003). Life between clocks: daily temporal patterns of human chronotypes. *J Biol Rhythms*, *18*(1), 80-90. https://doi.org/10.1177/0748730402239679

Rohringer, S., Holnthoner, W., Chaudary, S., Slezak, P., Priglinger, E., Strassl, M., Pill, K., Mühleder, S., Redl, H., & Dungel, P. (2017). The impact of wavelengths of LED light-therapy on endothelial cells. *Sci Rep*, *7*(1), 10700. https://doi.org/10.1038/s41598-017-11061-y

Sato, T. K., Yamada, R. G., Ukai, H., Baggs, J. E., Miraglia, L. J., Kobayashi, T. J., Welsh, D. K., Kay, S. A., Ueda, H. R., & Hogenesch, J. B. (2006). Feedback repression is required

for mammalian circadian clock function. *Nat Genet*, *38*(3), 312-319. https://doi.org/10.1038/ng1745

Schernhammer, E., Chen, H., & Ritz, B. (2006). Circulating melatonin levels: possible link between Parkinson's disease and cancer risk? *Cancer Causes Control*, *17*(4), 577-582. https://doi.org/10.1007/s10552-005-9002-9

Schernhammer, E., & Schulmeister, K. (2004). Light at night and cancer risk. *Photochem Photobiol*, *79*(4), 316-318. https://doi.org/10.1562/sa-03-28.1

Selmaoui, B., & Touitou, Y. (2003). Reproducibility of the circadian rhythms of serum cortisol and melatonin in healthy subjects: a study of three different 24-h cycles over six weeks. *Life Sci*, *73*(26), 3339-3349. https://doi.org/10.1016/j.lfs.2003.05.007

Shearman, L. P., Sriram, S., Weaver, D. R., Maywood, E. S., Chaves, I., Zheng, B., Kume, K., Lee, C. C., van der Horst, G. T., Hastings, M. H., & Reppert, S. M. (2000). Interacting molecular loops in the mammalian circadian clock. *Science*, *288*(5468), 1013-1019. https://doi.org/10.1126/science.288.5468.1013

Shen, C. Y., Xu, Z., Zhao, S. L., & Huang, Q. Y. (2014). [Study on the safety of blue light leak of LED]. *Guang Pu Xue Yu Guang Pu Fen Xi*, *34*(2), 316-321.

Sherratt, M. J., Hopkinson, L., Naven, M., Hibbert, S. A., Ozols, M., Eckersley, A., Newton, V. L., Bell, M., & Meng, Q. J. (2019). Circadian rhythms in skin and other elastic tissues. *Matrix Biol*, *84*, 97-110. https://doi.org/10.1016/j.matbio.2019.08.004

Shibayama-Imazu, T., Aiuchi, T., & Nakaya, K. (2008). Vitamin K2-mediated apoptosis in cancer cells: role of mitochondrial transmembrane potential. *Vitam Horm*, *78*, 211-226. https://doi.org/10.1016/s0083-6729(07)00010-6

Shimizu, K., Matsubara, K., Ohtaki, K., Fujimaru, S., Saito, O., & Shiono, H. (2003). Paraquat induces long-lasting dopamine overflow through the excitotoxic pathway in the striatum of freely moving rats. *Brain Res*, *976*(2), 243-252. https://doi.org/10.1016/s0006-8993(03)02750-1

Shostak, A. (2017). Circadian Clock, Cell Division, and Cancer: From Molecules to Organism. *Int J Mol Sci*, *18*(4). https://doi.org/10.3390/ijms18040873

Silva, J. N., Filipe, P., Morlière, P., Mazière, J. C., Freitas, J. P., Gomes, M. M., & Santus, R. (2008). Photodynamic therapy: Dermatology and ophthalmology as main fields of current applications in clinic. *Biomed Mater Eng*, *18*(4-5), 319-327.

Sliney, D. H. (2016). What is light? The visible spectrum and beyond. *Eye (Lond)*, *30*(2), 222-229. https://doi.org/10.1038/eye.2015.252

Smolensky, M. H., Hermida, R. C., Reinberg, A., Sackett-Lundeen, L., & Portaluppi, F. (2016). Circadian disruption: New clinical perspective of disease pathology and basis for chronotherapeutic intervention. *Chronobiol Int*, *33*(8), 1101-1119. https://doi.org/10.1080/07420528.2016.1184678

Sorbellini, E., Rucco, M., & Rinaldi, F. (2018). Photodynamic and photobiological effects of light-emitting diode (LED) therapy in dermatological disease: an update. *Lasers Med Sci*, *33*(7), 1431-1439. https://doi.org/10.1007/s10103-018-2584-8

Soták, M., Sumová, A., & Pácha, J. (2014). Cross-talk between the circadian clock and the cell cycle in cancer. *Ann Med*, *46*(4), 221-232. https://doi.org/10.3109/07853890.2014.892296

Stevens, R. G. (1987). Electric power use and breast cancer: a hypothesis. *Am J Epidemiol*, *125*(4), 556-561. https://doi.org/10.1093/oxfordjournals.aje.a114569

Stevens, R. G., & Zhu, Y. (2015). Electric light, particularly at night, disrupts human circadian rhythmicity: is that a problem? *Philos Trans R Soc Lond B Biol Sci*, *370*(1667). https://doi.org/10.1098/rstb.2014.0120

Storch, K. F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F. C., Wong, W. H., & Weitz, C. J. (2002). Extensive and divergent circadian gene expression in liver and heart. *Nature*, *417*(6884), 78-83. https://doi.org/10.1038/nature744

Straif, K., Baan, R., Grosse, Y., Secretan, B., El Ghissassi, F., Bouvard, V., Altieri, A., Benbrahim-Tallaa, L., & Cogliano, V. (2007). Carcinogenicity of shift-work, painting,

and fire-fighting. *Lancet Oncol*, *8*(12), 1065-1066. https://doi.org/10.1016/s1470-2045(07)70373-x

Sun, Q., Kim, H. E., Cho, H., Shi, S., Kim, B., & Kim, O. (2018). Red light-emitting diode irradiation regulates oxidative stress and inflammation through SPHK1/NF-κB activation in human keratinocytes. *J Photochem Photobiol B*, *186*, 31-40. https://doi.org/10.1016/j.jphotobiol.2018.05.015

Sun, S., Xie, F., Xu, X., Cai, Q., Zhang, Q., Cui, Z., Zheng, Y., & Zhou, J. (2018). Advanced oxidation protein products induce S-phase arrest of hepatocytes via the ROS-dependent, β-catenin-CDK2-mediated pathway. *Redox Biol*, *14*, 338-353. https://doi.org/10.1016/j.redox.2017.09.011

Sun, X. L., Zhang, X. W., Zhai, H. J., Zhang, D., & Ma, S. Y. (2020). Magnoflorine inhibits human gastric cancer progression by inducing autophagy, apoptosis and cell cycle arrest by JNK activation regulated by ROS. *Biomed Pharmacother*, *125*, 109118. https://doi.org/10.1016/j.biopha.2019.109118

Sutedja, T. G., & Postmus, P. E. (1996). Photodynamic therapy in lung cancer. A review. *J Photochem Photobiol B*, *36*(2), 199-204. https://doi.org/10.1016/s1011-1344(96)07372-1

Takahashi, A., Masuda, A., Sun, M., Centonze, V. E., & Herman, B. (2004). Oxidative stress-induced apoptosis is associated with alterations in mitochondrial caspase activity and Bcl-2-dependent alterations in mitochondrial pH (pHm). *Brain Res Bull*, *62*(6), 497-504. https://doi.org/10.1016/j.brainresbull.2003.07.009

Takahashi, J. S., Hong, H. K., Ko, C. H., & McDearmon, E. L. (2008). The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet*, *9*(10), 764-775. https://doi.org/10.1038/nrg2430

Tamaru, T., Hattori, M., Ninomiya, Y., Kawamura, G., Varès, G., Honda, K., Mishra, D. P., Wang, B., Benjamin, I., Sassone-Corsi, P., Ozawa, T., & Takamatsu, K. (2013). ROS stress resets circadian clocks to coordinate pro-survival signals. *PLoS One*, *8*(12), e82006. https://doi.org/10.1371/journal.pone.0082006

Tanioka, M., Yamada, H., Doi, M., Bando, H., Yamaguchi, Y., Nishigori, C., & Okamura, H. (2009). Molecular clocks in mouse skin. *J Invest Dermatol*, *129*(5), 1225-1231. https://doi.org/10.1038/jid.2008.345

Tao, J. X., Zhou, W. C., & Zhu, X. G. (2019). Mitochondria as Potential Targets and Initiators of the Blue Light Hazard to the Retina. *Oxid Med Cell Longev*, *2019*, 6435364. https://doi.org/10.1155/2019/6435364

Tartaglione, M. F., Eléxpuru Zabaleta, M., Lazzarini, R., Piva, F., Busilacchi, E. M., Poloni, A., Ledda, C., Rapisarda, V., Santarelli, L., & Bracci, M. (2021). Apoptotic mechanism activated by blue light and cisplatinum in cutaneous squamous cell carcinoma cells. *Int J Mol Med*, *47*(4). https://doi.org/10.3892/ijmm.2021.4881

Tataroglu, O., & Emery, P. (2014). Studying circadian rhythms in Drosophila melanogaster. *Methods*, *68*(1), 140-150. https://doi.org/10.1016/j.ymeth.2014.01.001

Tosini, G., Ferguson, I., & Tsubota, K. (2016). Effects of blue light on the circadian system and eye physiology. *Mol Vis*, *22*, 61-72.

Turek, F. W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olson, S., Easton, A., Jensen, D. R., Eckel, R. H., Takahashi, J. S., & Bass, J. (2005). Obesity and metabolic syndrome in circadian Clock mutant mice. *Science*, *308*(5724), 1043-1045. https://doi.org/10.1126/science.1108750

Tzung, T. Y., Chen, C. Y., Tzung, T. Y., Kao, F. J., & Chen, W. C. (2009). Infrared irradiation as an adjuvant therapy in recalcitrant alopecia areata. *Dermatol Surg*, *35*(4), 721-723. https://doi.org/10.1111/j.1524-4725.2009.01120.x

Van Someren, E. J., & Riemersma-Van Der Lek, R. F. (2007). Live to the rhythm, slave to the rhythm. *Sleep Med Rev*, *11*(6), 465-484. https://doi.org/10.1016/j.smrv.2007.07.003

Vosko, A. M., Schroeder, A., Loh, D. H., & Colwell, C. S. (2007). Vasoactive intestinal peptide and the mammalian circadian system. *Gen Comp Endocrinol*, *152*(2-3), 165-175. https://doi.org/10.1016/j.ygcen.2007.04.018

Vreman, H. J., Wong, R. J., Stevenson, D. K., Route, R. K., Reader, S. D., Fejer, M. M., Gale, R., & Seidman, D. S. (1998). Light-emitting diodes: a novel light source for phototherapy. *Pediatr Res*, *44*(5), 804-809. https://doi.org/10.1203/00006450-199811000-00027

Vyssoki, B., Praschak-Rieder, N., Sonneck, G., Blüml, V., Willeit, M., Kasper, S., & Kapusta, N. D. (2012). Effects of sunshine on suicide rates. *Compr Psychiatry*, *53*(5), 535-539. https://doi.org/10.1016/j.comppsych.2011.06.003

Wang, T., Dong, J., Yin, H., & Zhang, G. (2020). Blue light therapy to treat candida vaginitis with comparisons of three wavelengths: an in vitro study. *Lasers Med Sci*, *35*(6), 1329-1339. https://doi.org/10.1007/s10103-019-02928-9

Wang, X., Wang, Q., Nguyen, P., & Lin, C. (2014). Cryptochrome-mediated light responses in plants. *Enzymes*, *35*, 167-189. https://doi.org/10.1016/b978-0-12-801922-1.00007-5

Wang, Y., Yuan, L., Su, T., Wang, Q., Gao, Y., Zhang, S., Jia, Q., Yu, G., Fu, Y., Cheng, Q., Liu, B., Kong, F., Zhang, X., Song, C. P., Xu, X., & Xie, Q. (2020). Light- and temperature-entrainable circadian clock in soybean development. *Plant Cell Environ*, *43*(3), 637-648. https://doi.org/10.1111/pce.13678

Webler, F. S., Spitschan, M., Foster, R. G., Andersen, M., & Peirson, S. N. (2019). What is the 'spectral diet' of humans? *Curr Opin Behav Sci*, *30*, 80-86. https://doi.org/10.1016/j.cobeha.2019.06.006

Welsh, D. K., Takahashi, J. S., & Kay, S. A. (2010). Suprachiasmatic nucleus: cell autonomy and network properties. *Annu Rev Physiol*, *72*, 551-577. https://doi.org/10.1146/annurev-physiol-021909-135919

Westermann, B. (2012). Bioenergetic role of mitochondrial fusion and fission. *Biochim Biophys Acta*, *1817*(10), 1833-1838. https://doi.org/10.1016/j.bbabio.2012.02.033

Wikelski, M., Martin, L. B., Scheuerlein, A., Robinson, M. T., Robinson, N. D., Helm, B., Hau, M., & Gwinner, E. (2008). Avian circannual clocks: adaptive significance and possible involvement of energy turnover in their proximate control. *Philos Trans R Soc Lond B Biol Sci*, *363*(1490), 411-423. https://doi.org/10.1098/rstb.2007.2147

Wirz-Justice, A., Skene, D. J., & Münch, M. (2020). The relevance of daylight for humans. *Biochem Pharmacol*, 114304. https://doi.org/10.1016/j.bcp.2020.114304

Yodoi, J., Nakamura, H., & Masutani, H. (2002). Redox regulation of stress signals: possible roles of dendritic stellate TRX producer cells (DST cell types). *Biol Chem*, *383*(3-4), 585-590. https://doi.org/10.1515/bc.2002.060

Yosipovitch, G., Xiong, G. L., Haus, E., Sackett-Lundeen, L., Ashkenazi, I., & Maibach, H. I. (1998). Time-dependent variations of the skin barrier function in humans: transepidermal water loss, stratum corneum hydration, skin surface pH, and skin temperature. *J Invest Dermatol*, *110*(1), 20-23. https://doi.org/10.1046/j.1523-1747.1998.00069.x

Young T. A. (1807) Course of Lectures on Natural Philosophy and the Mechanical Arts, vol. 1. William Savage: Bedford, UK, , pp 463–464

Yousef, E., Mitwally, N., Noufal, N., & Tahir, M. R. (2020). Shift work and risk of skin cancer: A systematic review and meta-analysis. *Sci Rep*, *10*(1), 2012. https://doi.org/10.1038/s41598-020-59035-x

Youssef, P. N., Sheibani, N., & Albert, D. M. (2011). Retinal light toxicity. *Eye (Lond)*, *25*(1), 1-14. https://doi.org/10.1038/eye.2010.149

Zanello, S. B., Jackson, D. M., & Holick, M. F. (2000). Expression of the circadian clock genes clock and period1 in human skin. *J Invest Dermatol*, *115*(4), 757-760. https://doi.org/10.1046/j.1523-1747.2000.00121.x

Zerr, D. M., Hall, J. C., Rosbash, M., & Siwicki, K. K. (1990). Circadian fluctuations of period protein immunoreactivity in the CNS and the visual system of Drosophila. *J Neurosci*, *10*(8), 2749-2762. https://doi.org/10.1523/jneurosci.10-08-02749.1990

Zhang, P., & Wu, M. X. (2018). A clinical review of phototherapy for psoriasis. *Lasers Med Sci*, *33*(1), 173-180. https://doi.org/10.1007/s10103-017-2360-1

Zhu, H., Jia, Z., Zhang, L., Yamamoto, M., Misra, H. P., Trush, M. A., & Li, Y. (2008). Antioxidants and phase 2 enzymes in macrophages: regulation by Nrf2 signaling and protection against oxidative and electrophilic stress. *Exp Biol Med (Maywood)*, *233*(4), 463-474. https://doi.org/10.3181/0711-rm-304

8. APPENDIX 1

In vivo study: Human Study

All human skin cell types have a functional circadian mechanism and exhibit specific periods and phase relationships in gene expression, suggesting regulatory mechanisms that are particular to each cell type. Furthermore, these oscillations in different cell types appear to act in concert to drive rhythmic functions in the skin. A limitation for studying clock gene expression in human skin has been the need for live skin biopsy in subjects. Akashi et al. have described a non-invasive method using hair follicle cells to monitor gene expression, and it is already used to demonstrate that rotating shift workers suffer perturbations in their circadian gene expression (Bracci et al., 2016). For these reasons circadian clock genes in skin of volunteers were investigated in follicle hair cells.

RNA extraction and Real-Time qPCR

To study the effects of light radiation with 12h high intensity cycle on the health of human skin was started a preliminary study on 3 subjects (interrupted due to covid). Was put in place a device capable of exposing the skin to constant dark (control), blue and red light for 3 days, with the same amount of energy of high dose given to the cells.

After exposure to light was asked to subjects to take the hairs inside the area of skin exposed, the hairs were stored in 1.5 ml tubes (Merck KGaA, Darmstadt, Germany) with RNA Later (Merck KGaA, Darmstadt, Germany) at 4°C. RNA extraction from the hair follicles was performed under chemical hood; using pestles the bulbs were homogenized and subjected to extraction following the RNeasy Mini Kit (QIAGEN, Hilden, Germany). Retrotrascription and RT-qPCR were performed as described in paragraph 4.

RESULTS and DISCUSSION

In the hair bulbs the expression levels of the clock genes were found to be modified after exposure to light.

Preliminary analyses, performed on the hair bulbs of the three subjects, showed that the expression of mRNA levels of the *BMAL1* gene is more highly expressed after exposure to red light. *PER2* gene expression analysis results in the same changes (fig. 1.1).

The circadian loop is conserved in mammals and drives rhythms in behaviour, metabolism, and other physiological processes, making it a powerful regulator of virtually any process in which time-of-day regulation is useful with profound implications for human health (Akashi et al., 2010). Although we know the identities of the molecules involved in driving intracellular rhythms, it remains unclear exactly how the rhythm of this negative feedback loop is set from light directly in the skin. The circadian cycle in the nucleus provides an elegant mechanism for time maintenance in both central clock neurons and peripheral tissues, but the study of circadian clock outputs has revealed that regulation of physiology by the intracellular clock is not straightforward. Indeed, from our initial results, there appears to be deregulation of clock genes in skin cells resulting from a direct response to light stimulation (both blue and red light). These preliminary studies advance the increasingly accepted hypothesis of an autonomous, self-controlled rhythmicity in the peripheral circadian system of human skin (*in vivo*) with perturbation also determined by visible radiation.

Figure 1.1. *Expression profiles of BMAL1 e PER2 genes in hair follicles in human not exposed (CTRL) and exposed to LED blue light (BLUE) or LED red light (RED) at 12h high intensity. Columns represent mean values and bars represent ± SD (n=3).*

Our study was unfortunately discontinued after the collection of samples from the first three subjects due to the onset of the COVID-19 pandemic. We would like to continue this trial in the near future.

9. APPENDIX 2

Apoptotic mechanism activated by blue light and cisplatinum in cutaneous squamous cell carcinoma cells

MARIA FIORELLA TARTAGLIONE[1*], MARÍA ELÉXPURU ZABALETA[1*], RAFFAELLA LAZZARINI[1], FRANCESCO PIVA[2], ELENA MARINELLI BUSILACCHI[3], ANTONELLA POLONI[3], CATERINA LEDDA[4], VENERANDO RAPISARDA[4], LORY SANTARELLI[1] and MASSIMO BRACCI[1]

[1]Section of Occupational Medicine, Department of Clinical and Molecular Sciences, Polytechnic University of Marche, I-60126 Ancona; [2]Department of Specialistic Clinical and Odontostomatological Sciences, Polytechnic University of Marche, I-60131 Ancona; [3]Section of Hematology, Department of Clinical and Molecular Science, Polytechnic University of Marche, I-60126 Ancona; [4]Section of Occupational Medicine, Department of Clinical and Experimental Medicine, University of Catania, I-95124 Catania, Italy

Received May 18, 2020; Accepted January 18, 2021

DOI: 10.3892/ijmm.2021.4881

Abstract. New approaches are being studied for the treatment of skin cancer. It has been reported that light combined with cisplatinum may be effective against skin cancer. In the present study, the effects of specific light radiations and cisplatinum on A431 cutaneous squamous cell carcinoma (cSCC) and HaCaT non-tumorigenic cell lines were investigated. Both cell lines were exposed to blue and red light sources for 3 days prior to cisplatinum treatment. Viability, apoptosis, cell cycle progression and apoptotic-related protein expression levels were investigated. The present results highlighted that combined treatment with blue light and cisplatinum was more effective in reducing cell viability compared with single treatments. Specifically, an increase in the apoptotic rate was observed when the cells were treated with blue light and cisplatinum, as compared to treatment with blue light or cisplatinum alone. Combined treatment with blue light and cisplatinum also caused cell cycle arrest at the S phase. Treatment with cisplatinum following light exposure induced the expression of apoptotic proteins in the A431 and HaCaT cell lines, which tended to follow different apoptotic mechanisms. On the whole, these data indicate that blue light combined with cisplatinum may be a promising treatment for cSCC.

Correspondence to: Professor Lory Santarelli or Professor Massimo Bracci, Section of Occupational Medicine, Department of Clinical and Molecular Sciences, Polytechnic University of Marche, Via Tronto 10/A, I-60126 Ancona, Italy
E-mail: l.santarelli@univpm.it
E-mail: m.bracci@univpm.it

*Contributed equally

Key words: skin cancer, blue light, cisplatinum, A431 cells, HaCaT cells, LED light, light therapy, cSCC, apoptosis, cytotoxicity

Introduction

The skin is a complex tissue composed of two different compartments: The epidermis, comprised mostly of keratinocytes, and the underlying dermal matrix, with fibroblasts as its major cellular component (1). Cutaneous squamous cell carcinoma (cSCC) is characterized by the abnormal proliferation of keratinocytes, which leads to the development of tumors, principally of the scalp, face and the back of the hand (2). It is the most widespread type of skin cancer and is followed by basal cell carcinoma (3). Over the last decades, the incidence of cSCC has increased by 10% per year (4), mostly among the young population (5). Although this type of skin cancer is characterized by an intricate etiology, which often leads to misdiagnosis, exposure to ultraviolet (UV) radiation may be the primary cause of cSCC, resulting in DNA damage (6). cSCC is associated with a high degree of malignancy and an elevated level of invasion (7). Indeed, recurrent cSCC is associated with a higher risk of aesthetic co-morbidity, the development of distant metastasis and mortality (8).

Radiation therapy, chemotherapy, biological therapies and surgery are conventional treatments for cSCC (9,10). The primary treatment regimen selected by oncologists is early surgical intervention. However, the invasiveness of the procedure causes marked esthetical changes that often cause high psychological distress to the patients (11). In addition, several side-effects of long-term chemotherapy have been reported, including chemoresistance, fibrosis, necrosis and secondary tumor development (12). On the other hand, the radiotherapy regimen can cause skin complications, such as exudation, dermatitis, peeling and ulcers resulting from the inflammation caused by this type of treatment (13). Therefore, the development of potential alternative therapies is necessary for cSCC.

Cisplatinum [cis-diammine-dichloroplatinum (II)] is a potent chemotherapeutic agent widely used in the treatment of skin cancer (14-16), among a wide variety of tumor types, including testicular (17,18), ovarian (19,20), cervical (21-24), head and neck (25-27) and lung (28,29) cancer. It has been

observed that cisplatinum induces DNA damage and blocks cell cycle progression at the specific G_2/M checkpoint (30), leading to the induction of apoptosis of proliferating cells (31). However, it has a relatively high cytotoxic activity and, consequently, its application must be limited to the prevention of side-effects, such as ototoxicity and neurotoxicity (32), which are considered dose-limiting effects (33). Clinicians have therefore developed a strategy with which to restrict the cytotoxicity of cisplatinum, which includes its combination with non-platinum-based drugs, such as topoisomerase inhibitors, antimetabolites and taxanes (34,35). However, numerous pre-clinical and clinical studies conducted on these combined therapies have yielded contradictory results, which seemed to depend on the treatment regimen and tumor cell features (12,34,35).

The primary beneficial effects of light-emitting diode (LED) application on human health were found by the National Aeronautics and Space Administration, with the development of LEDs that produce a narrow spectrum of light in a non-coherent manner, delivering the appropriate and required wavelength and intensity (36). Over the past 15 years, LED technology has continuously improved (36,37). Current LED therapy has the great advantage of high flexibility and adaptability that allows for the treatment of a wide variety of skin conditions exhibiting different biological effects. Its mechanism of action, namely photo-biomodulation, consists of the collective effects of 3 elements (a photosensitizing agent, a light source and oxygen) interacting contemporaneously (38). The source emits a light whose energy and wavelength emission activate the photosensitizer (38). In addition to the amount of energy, photo-biomodulation also depends on the irradiation time (36). LED therapy has been approved by the US Food and Drug Administration for its use by aestheticians, due to its capacity to increase ATP and transcription factor production, regulate oxidative stress and modulate collagen synthesis (37).

Therapeutic LED application has been improved and its use has increased in the treatment of several clinical conditions, such as lung-related diseases (39,40), age-associated macula deterioration (41) and different types of solid tumors (37,42). LEDs can generate different wavelengths, which lead to different biological activities. However, the effects induced by the blue light are not yet fully understood. It has been observed that, in the skin and retina, blue light induces suspected mediators of skin aging and age-related macular degeneration (36). The disruption of key cellular processes, such as mitosis and mitochondrial activity as a consequence of blue-light application (43) and the maintenance of DNA integrity (44) have been widely reported. The latest analyses focused on the modulation of cell signaling pathways by light energy (45,46), which includes UV wavelengths or energies belonging to the visible light range (37,47,48). Numerous studies have suggested that UVA light (320-400 nm) may stimulate anti-inflammatory and antioxidative cytoprotective pathways (49,50), playing a key anti-tumorigenic role (51). Previous *in vitro* and *in vivo* investigations on skin cancer have revealed that blue LED induces the apoptosis of cancer cells (52) and a reduction of tumor growth in mice (51). In addition, it has been demonstrated that blue LED irradiation triggers apoptotic cell death through the mitochondria-mediated intrinsic pathway and shortens the early stage of tumor growth in melanoma cells (36). Accordingly, the expression of numerous genes associated with tumorigenesis and cell metastasis is inhibited following exposure to blue LED (36).

The combination of standard therapeutic approaches has been recently attracting the attention of researchers and physicians for cancer treatment (53). On the other hand, light therapy and its combination with chemical drugs have also been shown to have a high therapeutic efficacy (12,54). This is an important advantage, since the dose of single drugs can be reduced, leading to a reduction in adverse side-effects, while also maintaining treatment efficacy (55).

In the present study, the photobiological effects of blue and red light associated with the cisplatinum treatment of A431 and HaCaT skin cell lines were investigated to determine whether a combined treatment can increase the apoptotic rate, as compared with single cisplatinum or light treatment. If that is found to be the case, combination treatment could be proven to be a promising treatment for skin cancer.

Materials and methods

Cells lines and culture conditions. The A431 epidermoid carcinoma cell line (ECACC 85090402) was purchased from Merck Life Sciences (Merck Life Science). The HaCaT non-tumorigenic keratinocyte cell line was used as a control and was acquired from the Experimental Zooprophylactic Institute of Lombardia and Emilia Romagna (Brescia, Italy). The HaCaT cell line can be used as non-tumor control of the A431 cells (56-60) and it is considered a reliable model for skin diseases and for *in vitro* carcinogenesis of human skin keratinocytes (61,62). The HaCaT and A431 cell lines were cultured under the same culture conditions to prevent differences in phototoxicity related to different growth mediums. The cell lines were cultured in high-glucose DMEM (Euroclone S.p.A.) supplemented with 10% fetal bovine serum (FBS; Euroclone S.p.A.) and 1% penicillin/streptomycin antibiotics (Euroclone S.p.A.) at 37˚C in an atmosphere containing 5% CO_2. Both cell lines were sub-cultured every 3-4 days. Both cell lines were mycoplasma-free.

Light exposure. Prior to light exposure, the cells were gradually starved to synchronize the cell cycle. Both cell lines were first cultured with low-glucose DMEM (Euroclone S.p.A.) supplemented with 0.1% FBS and 1% penicillin/streptomycin for 24 h and then DMEM without phenol red (Lonza Group, Ltd.), also complemented with 0.1% FBS and 1% penicillin/streptomycin for a further 24 h.

Samples were exposed to sham, blue, or red single-color LEDs in a specific incubator at 37˚C and 5% CO_2 for 3 days. Constant darkness was considered the sham light source, while light exposures were performed in a 12-h light/dark cycle (12L:12D). High-power blue and red LEDs (LD W5AM and LH W5AM Golden DRAGON® Plus, respectively; Osram) were used as the light sources. The LED viewing angle was 170˚ and the cells were placed at 14 cm above the light sources. The homogenous distribution of light and the spectrum of emission of each monochromatic LED were previously verified using an illuminance meter (CL-70; Konica Minolta Sensing, Inc.). The dominant wavelength was 465 nm for blue and 658 nm for red LEDs. Irradiance at peak wavelength at the cell surface

was 0.84 W/m^2 for blue and 1.10 W/m^2 for red LEDs, corresponding to the same total spectrum irradiance of 28.50 W/m^2 for both light sources. The light energy transferred each day to cells was 1.23 J/mm^2. The light exposure was set to reproduce the solar radiation as precisely as possible (63). Blue and red lights were specifically selected to test the opposite sides of the spectrum of visible light. Interference between light sources was prevented by using black curtains; sham exposure was additionally ensured by wrapping the plate with aluminum foil. LEDs in the incubator were fixed on an aluminum tank by thermal conductive paste, and the water circulation inside the tank (Amersham Multitemp III; GE Healthcare) extracted the heat generated by the LEDs, so they could work at a constant temperature. These conditions assured a constant electric current and therefore a constant emitted energy. Air circulation inside the incubator was ensured using a fan. To exclude any thermal effects, the temperature at the cell level was verified and constantly measured during experiments with Thermochron iButton DS1922L (Maxim Integrated). On the fourth day from the time of the start of sham or light exposure, the cells were transferred to the previous incubator and started the cisplatinum treatment.

Cisplatinum treatment. Cisplatinum (Merck KGaA) was dissolved in physiological solution at a final stock concentration of 3 mM and stored at -20˚C until use. The appropriate concentration of cisplatinum in distilled water was assessed for each cell line through the estimation of the half-maximal inhibitory concentration (IC_{50}) resulting after 24 h in 18 μM for A431 cells and 30 μM for HaCaT cells (data not shown). Each group, following light exposure, was divided into 2 subgroups, treated or not treated with the half-maximal inhibitory concentration (IC_{50}) of cisplatinum for 24 h at 37˚C and 5% of CO_2, and were then analyzed. Images of the cells were acquired on an inverted microscope (Leitz Fluovert; Leica Microsystems, Inc.) equipped with a digital camera (Canon EOS M50; Canon, Inc.).

5-Fluorouracil (5-FU) treatment. 5-FU (Merck KGaA), which was used for the data shown in the supplementary figures, was dissolved in physiological solution at a final stock concentration of 3.84 mM. The appropriate concentration of 5-FU in distilled water was assessed for each cell line by estimating the IC_{50} after 72 h in 75 μM for A431 cells and 100 μM for HaCaT cells. Each group, following light exposure, was divided into 2 subgroups, treated or not treated with the IC_{50} of 5-FU for 72 h at 37˚C and 5% of CO_2; cell viability assay was then performed.

Cell viability assay. To assess the effects of cisplatinum and light on cell viability, an XTT assay was performed, following the manufacturer's instructions (Cell Proliferation Kit II XTT; Merck KGaA). Briefly, the A431 and HaCaT cells were seeded in 96-well plates (Corning Inc.) at a final concentration of 1.6x10^4 cells/well and exposed to a cycle of 12L:12D (blue or red light) for 3 days; constant darkness was used as control. Subsequently, the appropriate concentration of cisplatinum for each cell line was added. At 24 h, 50 μl XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) solution was added to each well, followed by incubation for 2 h at 37˚C and 5% CO_2. Finally, the absorbance at 450 nm with 650 nm as the reference wavelength was measured using an absorbance microplate ELISA plate reader (Sunrise™ Absorbance Reader; Tecan Group Ltd.).

Apoptosis analysis. The apoptotic rate of both cell lines was evaluated using an Annexin V/Propidium Iodide (PI) apoptosis detection kit (eBioscience™ Annexin V-FITC Apoptosis Detection kit; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Cells were seeded in a 6-well plate (Corning Inc.) at a concentration of 2x10^5 cells/ml. Following treatment with light and cisplatinum, cells were harvested, centrifuged (770 x g for 5 min at 4˚C), and washed with phosphate-buffered saline (PBS; Euroclone). The cells were then resuspended in binding buffer plus Annexin V following 10 min of incubation at room temperature in the dark. Finally, cells were stained with PI and assessed using the FACSCalibur flow cytometer (BD Biosciences) equipped with Cell Quest software set on a logarithmic scale (BD Biosciences). A minimum of 20,000 cells was acquired for each sample. Data were analyzed using FlowJo™ Software (FlowJo™ Software for Windows version 7.6.1.; FlowJo LLC).

Cell cycle analysis. The A431 and HaCaT cells were seeded in a 6-well plate (Corning Inc.) at a concentration of 2x10^5 cells/ml. Following treatments, the cells were harvested and centrifuged at 300 x g for 6 min at room temperature, fixed with 4.5 ml cold ethanol solution (70% in PBS) and kept in ice at 4˚C for at least 2 h. The cells were then washed twice with PBS before being resuspended in PI staining solution with 0.1% Triton X-100 (Santa Cruz Biotechnology, Inc.), RNAse 0.2 mg/ml (Merck KGaA) and PI 2 mg/ml (Merck KGaA) and incubated at 37˚C for 15 min. Flow cytometric analysis was performed using a FACSCalibur™ flow cytometer (BD Biosciences) equipped with CellQuest software set on a linear scale (BD Pharmingen). A minimum of 20,000 cells was acquired for each sample. Data were analyzed using FlowJo™ Software.

Western blot analysis. To analyze protein expression, western blot analysis was performed. Proteins were extracted with RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.1% SDS, 150 mM NaCl, and 2 mM EDTA + protease inhibitors) as follows: Cells were lysed and maintained in ice for 30 min, with vortexing every 10 min. The cells were then centrifuged (Eppendorf® Microcentrifuge 5415; Merck KGaA) at 16,000 x g for 20 min at 4˚C. Supernatants were collected and protein concentrations were measured using a Bradford assay (Merck KGaA). Protein (30 μg) was treated with LDS Sample Buffer (Thermo Fisher Scientific, Inc.) and a Sample Reducing Agent (Thermo Fisher Scientific, Inc.). The tubes were then boiled at 100˚C for 5 min. Finally, equal amounts of protein (30 μg) were resolved on precast 4-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bolt 4-12% Bis-Tris Plus; Thermo Fisher Scientific, Inc.) in MES SDS running buffer (Thermo Fisher Scientific, Inc.) at 100 V and 35 mA, before entering the gel, and at 165 V and 60 mA after entering the gel. A protein ladder (SeeBlue™ Plus2 Prestained Standard; Thermo Fisher Scientific, Inc.) was used as a reference for protein size. Following running, proteins were transferred to a nitrocellulose membrane (NC; Amersham™ Protran™ Nitrocellulose Blotting Membrane; GE Healthcare) using a semi-dry method

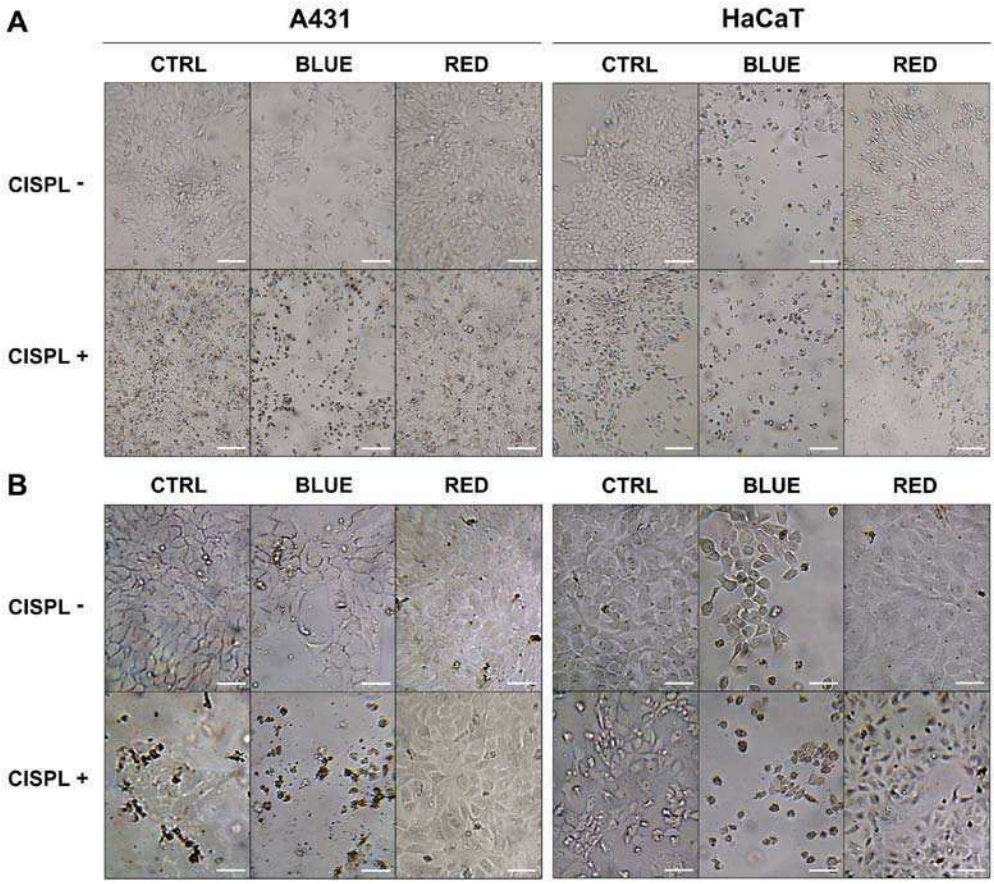

Figure 1. Morphology of A431 and HaCaT cells exposed to LED, cisplatinum, and LED and cisplatinum combined. Representative images captured at magnifications of (A) x100 and (B) x200 magnification in A431 and HaCaT cell lines. Scale bars, (A) 60 μm and (B) 30 μm. LED, light-emitting diode.

in transfer buffer (Tris 25 mM, 0.2 M glycine, 20% methanol). The transfer was performed at 30 V and an amperage calculated based on the membrane's measurements (w x h x 0.8). The protein transfer was verified by immersing the membrane in red Ponceau [Ponceau S solution for electrophoresis (0.2%); SERVA Electrophoresis GmbH] and then washed 3 times for 5 min with PBS-Tween-20 (PBST) 0.1%. Subsequently, the membrane was blocked with 5% milk-PBST (non-fat dried milk; Euroclone S.p.A.) for 1 h at room temperature and then incubated overnight at 4˚C with pertinent primary antibodies: Rabbit polyclonal anti-human anti-Gapdh antibody (dilution, 1:1,000; cat. no. A300-639A-M; Bethyl Laboratories Inc.), rabbit polyclonal anti-human anti-caspase-9 (Casp-9; dilution, 1:1,000; cat. no. 9502; Cell Signaling Technology, Inc.), mouse monoclonal anti-human anti-caspase-8 (Casp-8; dilution, 1:1,000; cat. no. 9746; Cell Signaling Technology, Inc.), mouse monoclonal anti-human anti-caspase-3 (Casp-3; dilution, 1:500; cat. no. sc-7272; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-human anti-BH3 interacting domain death agonist (Bid; dilution, 1:1,000; cat. no. 2002; Cell Signaling Technology, Inc.), rabbit polyclonal anti-human anti-Bcl-2-associated X protein (Bax; dilution, 1:1,000; cat. no. 2772; Cell Signaling Technology, Inc.), mouse monoclonal anti-human anti-cytochrome *c* (Cyt *c*; dilution, 1:1,000; cat. no. sc-13156; Santa Cruz Biotechnology, Inc.), rabbit monoclonal anti-human anti-p53 (dilution, 1:1,000; cat. no. 2527; Cell Signaling Technology, Inc.) and rabbit polyclonal anti-human anti-apoptosis independent factor (Aif; dilution, 1:1,000; cat. no. 4642; Cell Signaling Technology, Inc.). The following day, the NC membrane was washed 3 times with PBST 0.1% and incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 3 h: Goat anti-rabbit IgG (H+I) peroxidase/HRP-conjugated (dilution, 1:3,000; cat. no. E-AB-K1813; Elabscience Biotechnology, Inc.) or goat anti-mouse IgG (H+I) FITC conjugated (dilution, 1:10,000; cat. no. A90-116F; Bethyl Laboratories, Inc.). Finally, another 3 5-min washes with PBST were performed prior to membrane development. For signal chemiluminescent detection, the membrane was incubated for 2 min in the dark at room temperature with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.) and then developed in Alliance Mini (UVITEC Cambridge) equipped with NineAlliance Software (UVITEC Cambridge). Band quantification was carried out using the same software. Membrane stripping was performed prior to the addition of Gapdh where similar molecular weight proteins were previously assessed.

Statistical analysis. Experiments were conducted in triplicate, and the results were reported as the means ± standard deviation (SD) from 3 independent experiments. Graphpad Prism software (version 7.00 for Windows; GraphPad Software, Inc.) was used for all statistical analysis. One-way ANOVA and Tukey's HSD post hoc test was used to evaluate statistical significance. $P<0.05$ was considered to indicate a statistically significant difference.

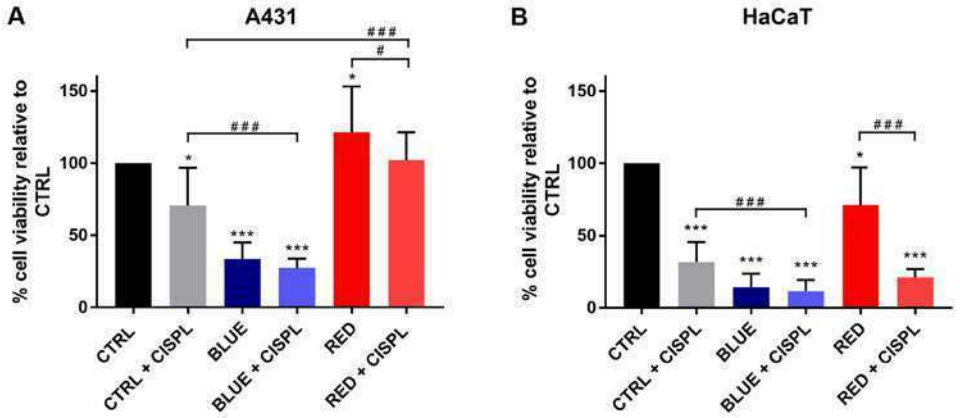

Figure 2. Analysis of cell viability in (A) A431 and (B) HaCaT cell lines exposed to LED, cisplatinum, and LED and cisplatinum combined. Columns represent mean values and bars represent ± SD (n=3). *P<0.05 and ***P<0.001 vs. CTRL. $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001. LED, light-emitting diode; SD, standard deviation.

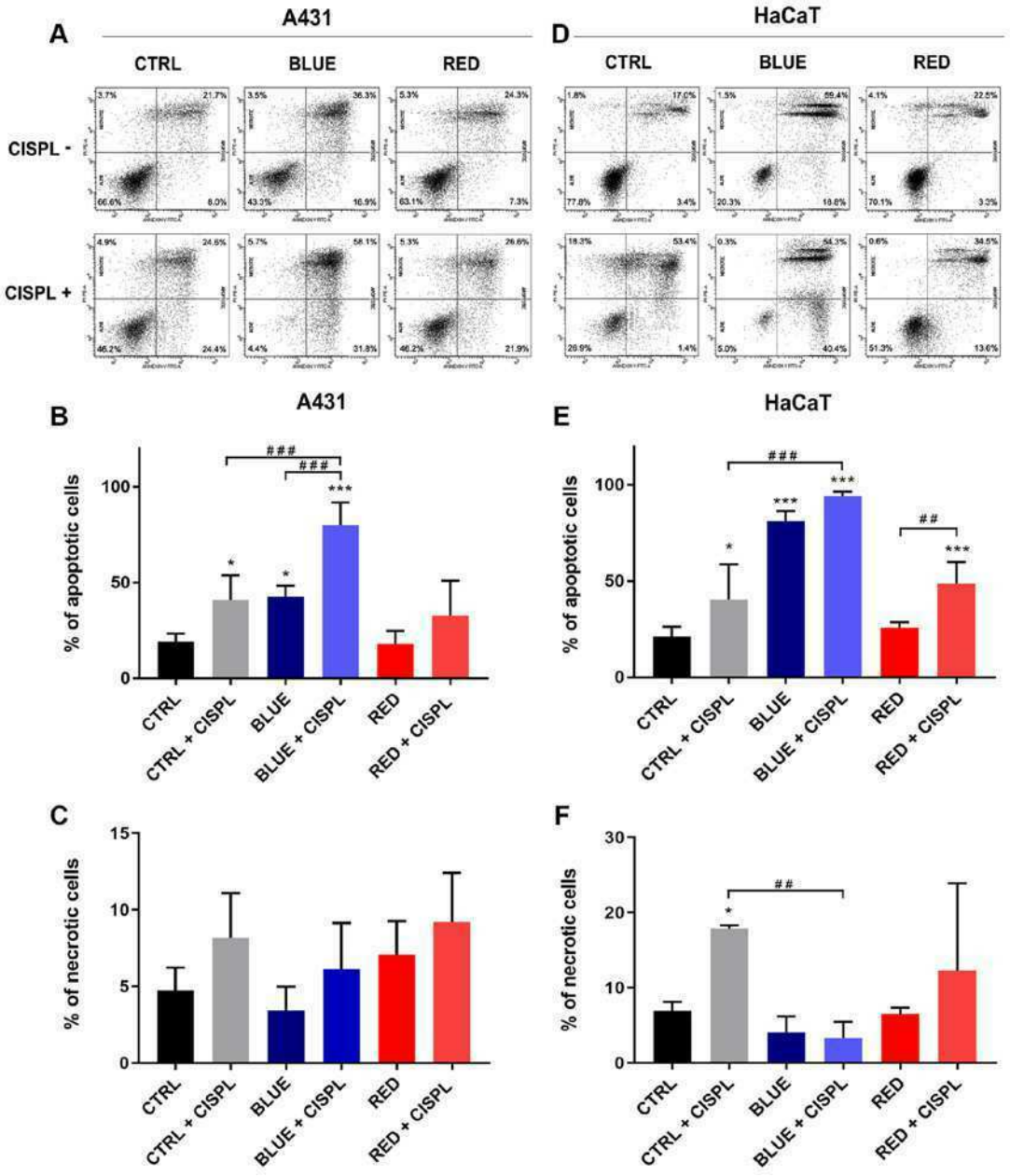

Figure 3. Apoptotic and necrotic effects of exposure of A431 and HaCaT cells to LED, cisplatinum, and LED and cisplatinum combined. Data indicate the percentage of apoptotic (Annexin V+) and necrotic (Annexin V^{-}/PI^{+}) cells of the (A-C) A431 and (D-F) HaCaT cell lines. Columns represent mean values and bars represent ± SD (n=3). *P<0.05, and ***P<0.001 vs. CTRL. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001. LED, light-emitting diode; SD, standard deviation.

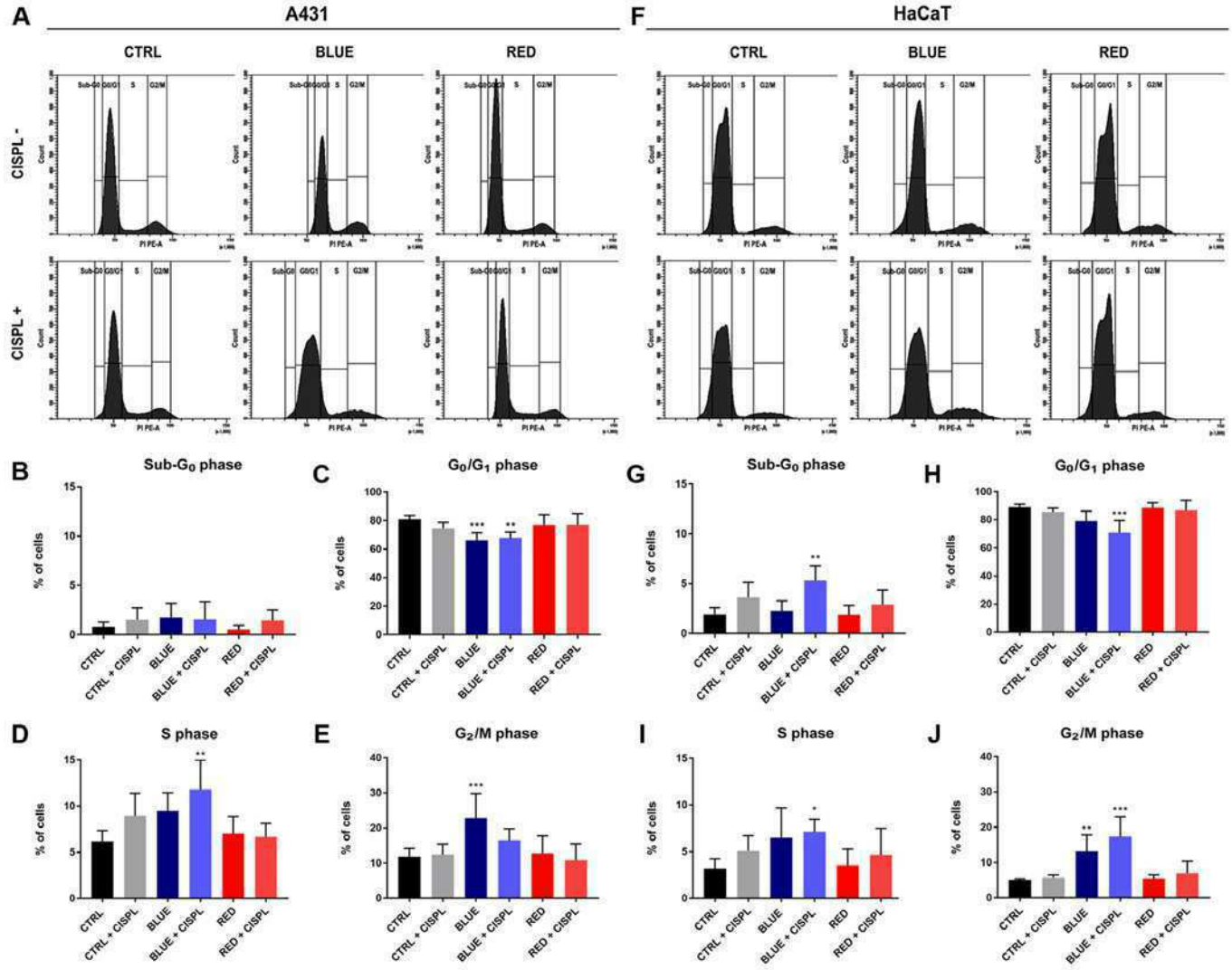

Figure 4. Effect of exposure to LED, cisplatinum, and LED and cisplatinum combined on cell cycle progression in A431 and HaCaT cells. Data indicate the effect of every treatment on the different cell cycle phases of the (A-E) A431 and (F-J) HaCaT cell lines. Columns represent mean values and bars represent ± SD (n=3). *P<0.01, **P<0.001 and ***P<0.05 vs. CTRL. SD, standard deviation.

Results

Combined treatment with blue light and cisplatinum is more effective in reducing cell viability than single treatments. The A431 and HaCaT cells were treated to assess whether the combination of cisplatinum and different light spectra decreased cell viability, as compared to the single treatments. The viability of the tumor cells was markedly inhibited following exposure to blue light (BLUE), as compared to the untreated cells (CTRL) (P<0.001; Figs. 1 and 2A). The higher percentage of inhibition in viable cells was obtained with blue light and cisplatinum (BLUE + CISPL), as compared to the CTRL group and CTRL + CISPL group (P<0.001). On the contrary, neither irradiation with red light alone (RED) nor treatment with red light and cisplatinum (RED + CISPL) led to a marked reduction in tumor cell viability, when compared with the untreated cells. Treatment with red light and cisplatinum increased A431 cell viability, as compared to the CTRL + CISPL group (P<0.001). The results obtained for the RED + CISPL group were similar to those of the CTRL, but lower than those of the RED group (P<0.05).

The HaCaT cells (Figs. 1 and 2B) displayed a notable decrease in viability in the BLUE and BLUE + CISPL groups compared to the CTRL group (P<0.001). In addition, cell viability was reduced in the BLUE + CISPL group, as compared to CTRL + CISPL group (P<0.001). With regards to the RED group, the results were different than those obtained from the A431 cells, since the RED + CISPL exhibited a lower cell viability (P<0.001 vs. CTRL; P<0.001 vs. RED).

Combined treatment with blue light and cisplatinum yields a higher percentage of apoptotic cells than single treatments. Flow cytometry confirmed that CTRL + CISPL, BLUE and BLUE + CISPL caused a significant increase in the apoptotic rate (Annexin V-positive cells), as compared to the CTRL in both cell lines (Fig. 3A, B and E). These results revealed that the BLUE + CISPL group exhibited an increase in the number of apoptotic A431 cells (mean, 80.1%), as compared to the CTRL + CISPL (mean, 40.5%; P<0.001) or BLUE (mean, 42.6%; P<0.001) groups. The HaCaT cell line displayed an increased apoptotic rate in the BLUE + CISPL group, as compared to the CTRL group (P<0.001) (Fig. 3D and E). On

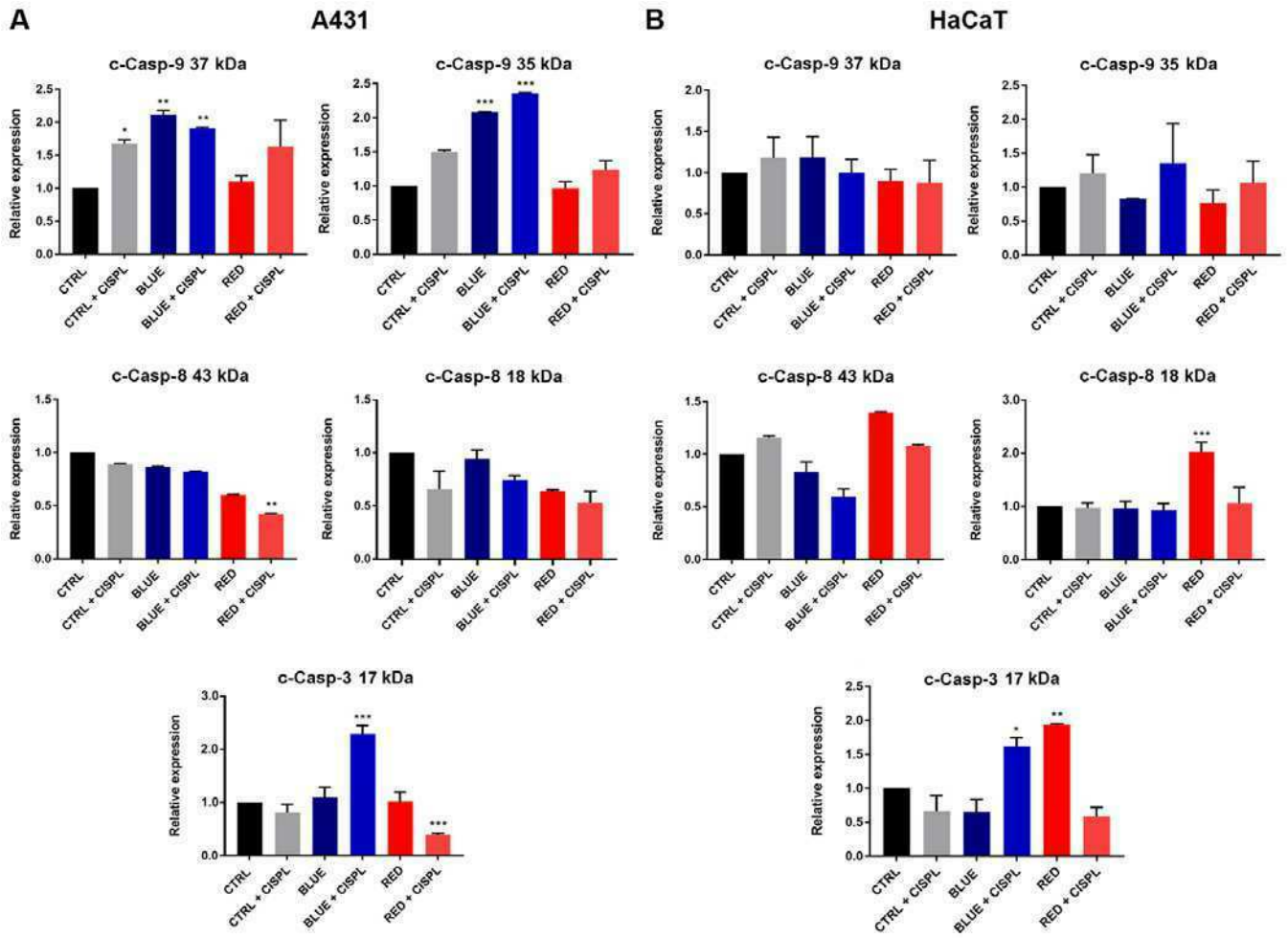

Figure 5. Analysis of apoptosis-related proteins Casp-9, Casp-8 and Casp-3 expression levels in cell lysates from (A) A431 and (B) HaCaT cells. Values were normalized to GAPDH. Columns represent mean values and bars represent ± SD (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. CTRL. Representative blot bands are presented in Fig. S1. SD, standard deviation; Casp, caspase.

the other hand, a high quantity of HaCaT cells undergoing apoptosis was also observed, even when treated with blue light alone (P<0.001 vs. CTRL) (Fig. 3D and E). The RED and RED + CISPL groups exhibited a percentage of apoptotic and necrotic A431 cells similar to the CTRL (Fig. 3A, B and C). With regards to the HaCaT cells, the RED + CISPL group exhibited a significant increase in the percentage of apoptotic cells with respect to the CTRL and RED groups (P<0.001 and P<0.01 respectively; Fig. 3D and E).

Combined treatment with blue light and cisplatinum leads to S and G2/M cell cycle arrest. Cell cycle analysis indicated that the A431 cells treated with BLUE + CISPL were arrested at the S phase (P<0.01; Fig. 4A and D), while the percentage of cells in the G0/G1 checkpoint was significantly lower, as compared to the CTRL (P<0.01; Fig. 4A and C). The BLUE group exhibited lower percentages of cells in the G_0/G_1 and higher ones in the G_2/M phase, as compared to the CTRL (P<0.001; Fig. 4A, C and E). With regards to the HaCaT cell line, cell cycle analysis indicated that the number of cells in the BLUE + CISPL group was significantly higher in the S phase and in the G_2/M phase (P<0.05 and P<0.001; Fig. 4F, I and J) and lower in the G_0/G_1 phase (P<0.001; Fig. 4A and H) compared with the CTRL. Furthermore, by observing the sub-G0 phase (Fig. 4A and G), the cells in the BLUE + CISPL exhibited a higher percentage of cells in this phase, as compared to the CTRL group (P<0.01).

Crucial role of apoptotic key factors in the fate of A431 and HaCaT cells following treatment with light and cisplatinum. The results described above led to the investigation of the molecular mechanisms underlying the effects of the treatments used. Hence, the expression levels of cell death-related proteins, such as Casp-9, Casp-8, Casp-3, Bid, Bax, Cyt c, p53 and Aif were analyzed by western blot analysis (representative protein bands are shown in Fig. S1).

The protein expression of cleaved Casp-9 in the A431 cells was in line with the results obtained from the apoptosis and cell cycle analysis, since the two cleaved forms of the protein (cleaved at 37 and 35 kDa) increased upon treatment with BLUE and BLUE + CISPL, as compared to the CTRL (Fig. 5A). With regards to the HaCaT cell line, on the other hand, no significant changes in either 37 KD or in 35 kDa cleaved Casp-9 were identified (Fig. 5B). Casp-8 expression was also analyzed. Pro-caspase 8 expression was not altered among the treatments (data not shown), while the results on the Casp-8 cleaved fragments exhibited a decreasing trend in 43 and 18 kDa protein expression in the A431 tumor cells in all treatments, as compared

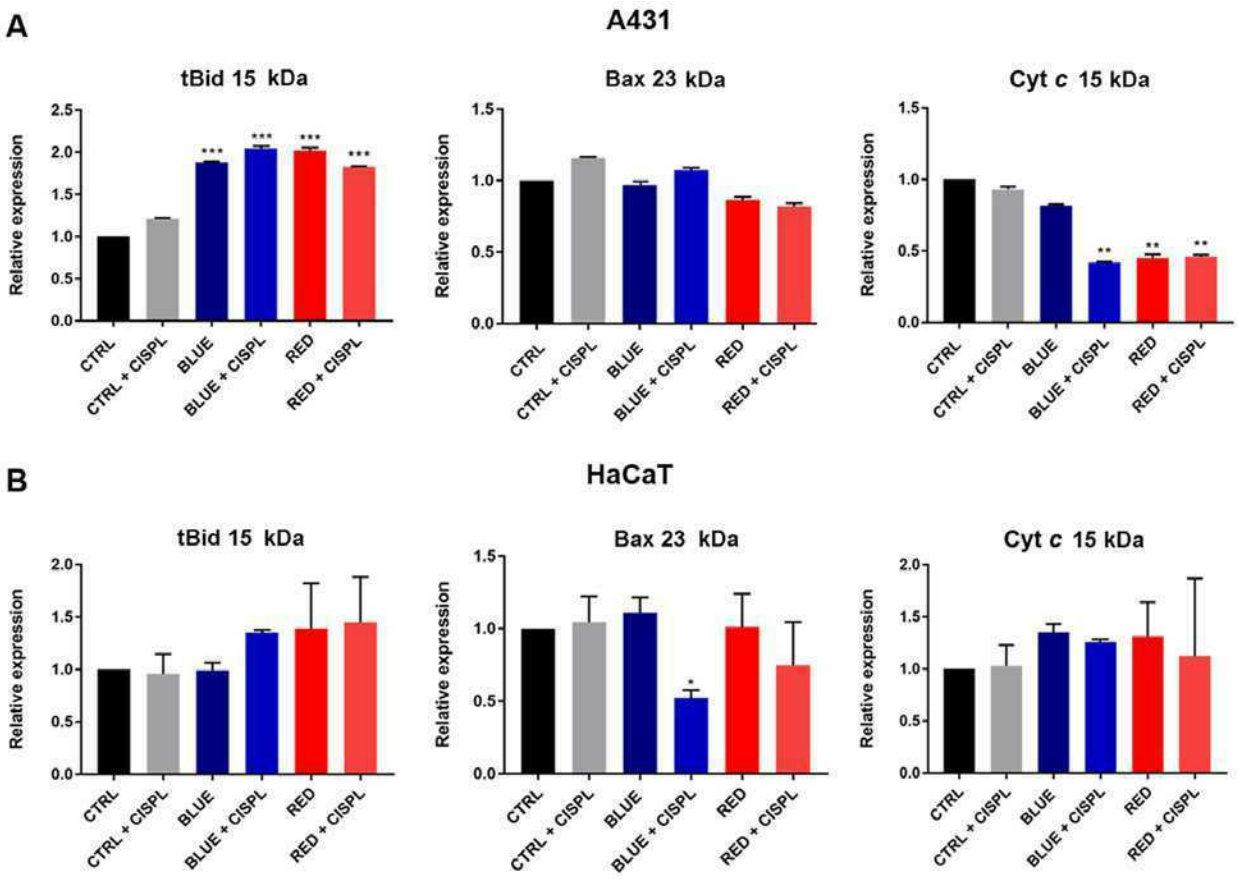

Figure 6. Western blot analysis of apoptosis-related proteins Cyt *c*, Bax and tBid in (A) A431 and (B) HaCaT cells. Values were normalized to GAPDH. Columns represent mean values and bars represent ± SD (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. CTRL. Representative blot bands are presented in Fig. S1. tBid, truncated BH3 interacting domain death agonist; Bax, Bcl-2-associated X protein; Cyt *c*, cytochrome *c*; SD, standard deviation.

to the CTRL group (Fig. 5A). However, it is important to note the higher reduction of Casp-8 43 kDa upon RED + CISPL treatment (P<0.01 vs. CTRL). In the HaCaT cells, the cleaved Casp-8 fragment (18 kDa) was significantly increased only in the RED group (P<0.05 vs. CTRL; Fig. 5B).

The key apoptotic factor, Casp-3, in the A431 cell line at the 17 kDa subunit level exhibited a significant increase in its expression in the BLUE + CISPL group as compared to the CTRL group (P<0.001; Fig. 5A). The same cell line instead exhibited a decrease in the expression of Casp-3 17 kDa in the RED + CISPL group (P<0.001). In the HaCaT cells, the level of Casp-3 17 kDa in the BLUE + CISPL and RED groups was increased (P<0.05 and P<0.01, respectively; Fig. 5B).

Bid protein was assessed by analyzing the expression of its active cleaved form with a molecular weight of 15 kDa (tBid). A significant increase was observed in its expression in the A431 cells in all groups treated with light alone and light combined with cisplatinum, as compared to the CTRL group (P<0.001; Fig. 6A). No significant differences in tBID expression were observed among the groups in the HaCaT cells (Fig. 6B).

Bax protein expression in the A431 cells was not significantly altered among the groups (Fig. 6A), while in the HaCaT cells, its expression was markedly decreased in the BLUE + CISPL group (P<0.05 vs. CTRL; Fig. 6B).

Furthermore, lower values of cytosolic Cyt *c* were observed in the A431 cells treated with blue light combined with cisplatinum, as compared to the CTRL group (P<0.01; Fig. 6A).

These results demonstrated that combined treatment with blue light and cisplatinum (BLUE + CISPL) effectively induced apoptosis, which was associated with a decrease in the p53 protein expression level in the A431 (P<0.05 vs. CTRL; Fig. 7A) and HaCaT cell lines (P<0.01 vs. CTRL; Fig. 7B). In the HaCaT cells, all treatments led to a significant decrease in p53 expression (Fig. 7B).

With regards to the analysis of expression of the necroptosis-related protein, Aif, the data revealed a marked increase in its cleaved form (57 kDa) in the A431 cells following exposure to blue light (P<0.05 BLUE vs. CTRL; Fig. 7A) and blue light combined with cisplatinum (P<0.05 BLUE + CISPL vs. CTRL). Conversely, the HaCaT cells exhibited a significant decrease in the 57 kDa-cleaved protein expression following exposure to light alone (P<0.05 BLUE vs. CTRL and RED vs. CTRL; Fig. 7B) and to light followed by the addition of cisplatinum (P<0.05 BLUE + CISPL vs. CTRL; P<0.01 RED + CISPL vs. CTRL).

Discussion

CSCC is a type of malignant skin neoplasia with a high morbidity risk (10); hence, it is fundamental for the development of novel alternative therapies to increase tumor vulnerability. Alternative therapies using LEDs (phototherapy)

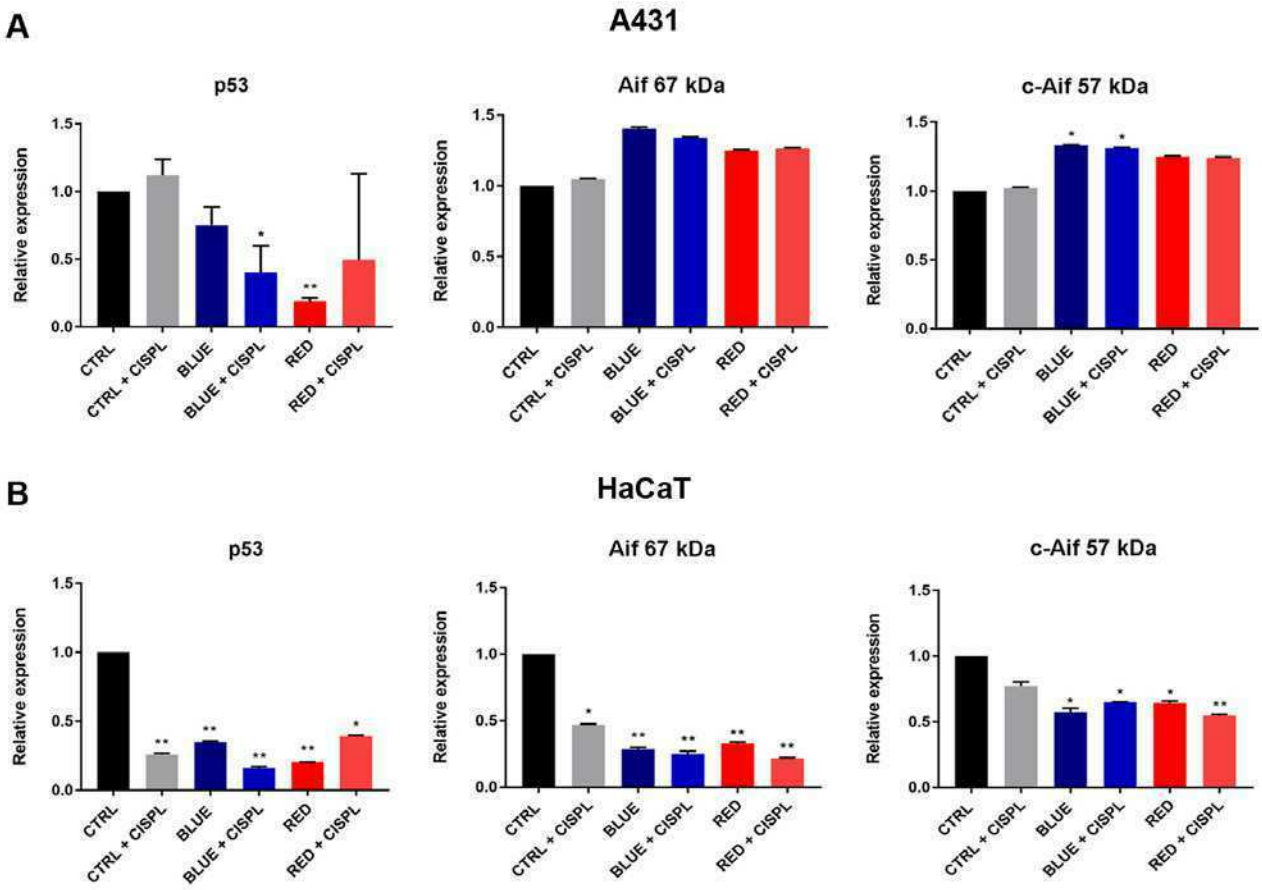

Figure 7. Western blot analysis of apoptosis-related proteins Aif and p53 in (A) A431 and (B) HaCaT cells. Values were normalized to GAPDH. Columns represent mean values and bars ± SD (n=3). *P<0.05 and **P<0.01 vs. CTRL. Representative blot bands are presented in Fig. S1. Aif, anti-apoptosis independent; SD, standard deviation.

in combination with chemotherapeutic agents, including cisplatinum, have recently been proposed as a different approach to tumor treatment (12,16,32). In the present study, the effects of LED spectra and cisplatinum, used alone or in combination, were assessed on normal keratinocytes (HaCaT) and cSCC cancer cells (A431).

The results revealed that combined treatment was more effective on the A431 cells than single treatments, significantly decreasing cell viability and increasing the apoptotic rate. A higher effectiveness of BLUE + CISPL combined treatment was observed when A431 cells were exposed to both agents, which induced a significant increase in the percentage of apoptotic A431 cells (~2-fold the effect of blue light as used alone). The apoptotic effects of blue light were observed in other *in vitro* and *in vivo* studies, where blue irradiation caused a decrease in cellular growth and induced cell death in skin cancer cell lines and animal models (51). A higher efficacy of blue light combined with cisplatinum on inducing apoptosis was observed in some cancer cell lines and xenograft mouse models (32,36,51). The present data suggested that blue light can enhance the cytotoxic effects of cisplatinum on A431 cells, exerting a more prominent effect on apoptosis when used in combination. It was also observed that cells were more sensitive to blue light exposure alone, as compared to red light exposure alone, suggesting that the blue light is more cytotoxic than the red one. Indeed, the A431 cells displayed a high viability rate when exposed to the red light and showed no changes in the number of apoptotic cells, even under combined treatment with cisplatinum. It was thus hypothesized that somehow, red light confers to A431 cells a type of resistance to cisplatinum, which was not observed when the cells were treated with blue light and cisplatinum. This could be related to the tumorigenic features of A431 cells as HaCaT cells were otherwise sensitive to red light combined with cisplatinum.

Given current insights into the association between apoptosis and cell cycle progression, the present study then sought to determine whether there were any differences in cell cycle progression upon single or combined light and cisplatinum treatment. Focusing on BLUE + CISPL treatment, a significant increase in the percentage of cells in the S and/or G_2/M phases was observed in both normal keratinocytes and skin cancer cells, suggesting that this treatment also exerted a cytostatic effect. This was in agreement with studies describing that HaCaT cells have a defective G_1 checkpoint, responding to UVB radiation by arresting cells in the S and G_2 phases (64). The same effect was reported when HaCaT cells were exposed to blue and red LEDs together with curcumin (64).

Apoptosis is the main programmed-cell death pathway that cells form under any cytotoxic stimulus (52). There are two controlled pathways involved in apoptosis: The death receptor-regulated or extrinsic pathway that activates Casp-8, and the mitochondria-controlled or intrinsic pathway that activates Casp-9 (16,65). There are other apoptosis-related proteins associated with the different pathways (intrinsic, extrinsic, or

both), including Bax (pro-apoptotic), tBid (anti-apoptotic) and Cyt *c*, which are associated with the intrinsic pathway (52,66), and p53 and Casp-3 proteins. Apart from apoptosis, other cell death mechanisms have been described, including necroptosis, in which the Aif protein plays a central role (67). To further elucidate the molecular mechanisms underlying the apoptotic process that A431 and HaCaT cells undergo following different treatments, the expression of diverse cell death-related proteins was analyzed.

The data obtained revealed several outcomes. The cytofluorimetric analysis indicated that cells underwent an apoptotic process since Annexin V was bound to phosphatidylserine molecules expressed in the outer membrane of apoptotic cells. It was therefore then evaluated whether the intrinsic or extrinsic apoptotic pathway was activated.

The activation of Casp-9, rather than Casp-8, in the A431 cells confirmed that cells treated with blue light and blue light combined with cisplatinum were involved in the intrinsic apoptotic pathway. The blue light activated the initiator Casp-9 but not the effector Casp-3; these results were associated with the low apoptotic rate of this treatment The advantage of using combined treatment (BLUE + CISPL) was associated with an increased expression of active effector Casp-3 subunit. However, following the further evaluation of Cyt *c* activated by cleaved Casp-9 (52,65,66), low expression values were observed. In addition, the tBid protein (associated with the extrinsic pathway) exhibited high levels in all treatments with light alone and light combined with cisplatinum. These contradictory outcomes were also observed in a study on cervical carcinoma cells, which demonstrated that Casp-8 formed a complex with Bid, leading to its cleavage into t-Bid and to the subsequent activation of type II extrinsic apoptosis (65). However, that study also reported the formation of the Casp-8/Bid complex in cells that were undergoing mitochondrial-independent apoptosis (65). In a previous study, p53 expression, a key apoptosis-related protein, was analyzed (68). A decrease in the expression of the total protein was found in A431 cells that had undergone BLUE + CISPL combined treatment and red light exposure alone. Previous studies on the behavior of p53 against different stresses have revealed a high temporal dynamism in its expression levels. In particular, it has been observed that cells exposed to UV (69,70) and cisplatinum (71) exhibited important variations of p53 protein expression. It was hypothesized that the low p53 expression identified in the cells in the present study was linked to the blue light and cisplatinum treatment, which differently modulated the p53 expression profile over time. Based on these conflicting data, it was hypothesized that cells were undergoing another type of cell death. Therefore, the expression levels of the necroptosis-related protein, Aif, were analyzed. Increased expression levels of cleaved (c-Aif) proteins were observed in the A431 cells treated with blue light alone and blue light combined with cisplatinum. These data suggest that tumor cells were undergoing necroptosis triggered by the light, given that single treatment with cisplatinum did not yield any significant increase in c-Aif expression values. In light of these results, it cannot be affirmed that A431 cells have one particular cell death mechanism, since protein expression analysis showed that, indeed, tumor cells also follow the intrinsic and extrinsic apoptotic pathways, and the necroptotic cell death mechanism. This could be explained by a possible phosphatidylserine re-localization, which has been described in other skin cancer cells under pro-apoptotic stimuli and has been speculated to induce smaller cross-reactive proteins that might lead to different cell death mechanisms working simultaneously (72).

In the HaCaT cell line, expression analysis of the same proteins did not yield well-defined results, with regards to the cell death mechanism. In fact, caspase analysis suggested the activation of the extrinsic apoptotic pathway, since cleaved-Casp-9 (37 and 35 kDa) expression levels were not significant at all. However, the expression of the active 18 kDa subunit of Casp-8 and the active 17 kDa subunit of Casp-3 were increased following red light treatment. It is therefore likely that the HaCaT cells underwent extrinsic apoptosis cell death only following red light treatment. Treatment with blue light combined with cisplatinum caused only the increase of the active fragment of Casp-3; further investigations of the mechanism of cell death in HaCaT treated are necessary. Furthermore, p53 expression levels were low in all groups, potentially due to the expression dynamism displayed by p53, depending on the type and intensity of the stress (69-72). The results of the expression analysis of necroptosis-related protein Aif ruled out this cell death mechanism, since both full-length proteins and cleaved fragments were very lowly expressed in all groups.

The mechanisms leading to cell death differed in each of the analyzed cell lines. However, it can be affirmed that combined treatment, particularly BLUE + CISPL, promoted the activation of programmed cell death in both cell lines, although the exact mechanisms were not clarified. The fact that both apoptotic pathways were active in both cell lines has been supported by a recent study by Laubach *et al* (73) on HaCaT and A431 cells undergoing treatment with curcumin and light irradiation. That study theorized a switch in the apoptotic mechanism related to changes in cellular redox balance and a mechanism of apoptotic stimulation independent from classical death receptors. Keratinocytes and skin tumor cells were also reported to undergo different apoptotic processes, due to the different expression of apoptotic regulators and apoptotic responses involved in reaching the diverse functional needs of the skin (73).

The combination of different treatments for the targeting of cSCC has several advantages: Targeting of different key signal transduction pathways; more efficient damage caused in tumor cells; increased therapeutic efficacy; additive or even synergistic effects that allow for the dose of the most toxic component can be reduced to eliminate or lessen noxious side-effects (32). The results of the present study indicated that the combination of blue light radiation at 465 nm followed by cisplatinum could be used as a potential treatment for cSCC and recommended further *in vivo* tests and clinical trials. In particular, the use of light combined with a local cisplatinum-based treatment could be investigated in non-metastatic cSCC, or when the conventional surgical approach is not an option. In addition, given the availability of local treatments containing 5-FU, the effect of blue radiation combined with 5-FU was investigated. Preliminary viability data (Fig. S2) revealed an effect of blue light radiation combined with 5-FU

similar to that obtained with cisplatinum, but less significant. Since novel therapeutic strategies are being developed for cSCC (74-77), the apoptotic effect of blue-light treatment can also be investigated in combination with the new treatments for cSCC, such as immune checkpoint agents or electrochemotherapy. A limitation of the present study is the use of only one skin cancer cell line making results about A431 referred only to cSCC and not to other types of skin cancer. The use of HaCaT cells instead of primary human keratinocytes is another limitation, although the selection was based on the use of the same culture conditions. As regards experiments with light, it is known that the culture conditions, in particular the medium utilized, may influence the results (78,79). The use of normal human epidermal keratinocytes-adult cells (NHEK cells) was attempted (data not shown). In the experiments in the present study, the NHEK cells exposed to light in the KGM-Gold medium showed a change in morphology, while in the same mediums used for A431, the NHEK cells showed extremely slow growth and a clear change in morphology (data not shown). Rather, the HaCaT cells growing in the same conditions of A431 cells made the results more comparable.

In conclusion, the data of the present study suggested that the combination of blue light and cisplatinum reduced the survival rate of A431 cells and triggered the apoptotic death of A431 cells. Further studies are required to fully elucidate the exact molecular mechanisms through which combined treatments of blue light and cisplatinum mediate apoptotic cell death, and to understand its overall mechanism in skin cancer.

Acknowledgements

Not applicable.

Funding

The present study was supported by a generous donation from iGuzzini illuminazione S.p.A. (Recanati, Italy). The funder had no role in the design of the study, the collection, analysis or interpretation of data, the writing of the manuscript, or the decision to publish the results.

Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

MB, MFT, MEZ and LS conceived and designed the study. MB, MFT, MEZ, RL and EMB performed the experiments. FP, CL, VR and AP were responsible for the methodology. MFT and MEZ organized and wrote the manuscript. RL, CL, VR and MB reviewed and edited the manuscript. MB and LS supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Barolet D, Roberge CJ, Auger FA, Boucher A and Germain L: Regulation of skin collagen metabolism in vitro using a pulsed 660 nm LED light source: Clinical correlation with a single-blinded study. J Invest Dermatol 129: 2751-2759, 2009.
2. Mateus C: Cutaneous squamous cell carcinoma. Rev Prat 64: 45-52, 2014.
3. Parekh V and Seykora JT: Cutaneous squamous cell carcinoma. Clin Lab Med 37: 503-525, 2017.
4. Smoller BR: Squamous cell carcinoma: From precursor lesions to high-risk variants. Mod Pathol 19 (Suppl 2): S88-S92, 2006.
5. Palme CE, MacKay SG, Kalnins I, Morgan GJ and Veness MJ: The need for a better prognostic staging system in patients with metastatic cutaneous squamous cell carcinoma of the head and neck. Curr Opin Otolaryngol Head Neck Surg 15: 103-106, 2007.
6. Garcia-Zuazaga J and Olbricht SM: Cutaneous squamous cell carcinoma. Adv Dermatol 24: 33-57, 2008.
7. Kyrgidis A, Tzellos TG, Kechagias N, Patrikidou A, Xirou P, Kitikidou K, Bourlidou E, Vahtsevanos K and Antoniades K: Cutaneous squamous cell carcinoma (SCC) of the head and neck: Risk factors of overall and recurrence-free survival. Eur J Cancer 46: 1563-1572, 2010.
8. Clayman GL, Lee JJ, Holsinger FC, Zhou X, Duvic M, El-Naggar AK, Prieto VG, Altamirano E, Tucker SL, Strom SS, *et al*: Mortality risk from squamous cell skin cancer. J Clin Oncol 23: 759-765, 2005.
9. Sreedhar A, Li J and Zhao Y: Next-gen therapeutics for skin cancer: Nutraceuticals. Nutr Cancer 70: 697-709, 2018.
10. Burton KA, Ashack KA and Khachemoune A: Cutaneous squamous cell carcinoma: A review of high-risk and metastatic disease. Am J Clin Dermatol 17: 491-508, 2016.
11. Que SKT, Zwald FO and Schmults CD: Cutaneous squamous cell carcinoma: Management of advanced and high-stage tumors. J Am Acad Dermatol 78: 249-261, 2018.
12. Compagnin C, Mognato M, Celotti L, Canti G, Palumbo G and Reddi E: Cell proliferation and cell cycle alterations in oesophageal p53-mutated cancer cells treated with cisplatin in combination with photodynamic therapy. Cell Prolif 43: 262-274, 2010.
13. Bray FN, Simmons BJ, Wolfson AH and Nouri K: Acute and chronic cutaneous reactions to ionizing radiation therapy. Dermatol Ther (Heidelb) 6: 185-206, 2016.
14. Ando N, Kato H, Igaki H, Shinoda M, Ozawa S, Shimizu H, Nakamura T, Yabusaki H, Aoyama N, Kurita A, *et al*: A randomized trial comparing postoperative adjuvant chemotherapy with cisplatin and 5-fluorouracil versus preoperative chemotherapy for localized advanced squamous cell carcinoma of the thoracic esophagus (JCOG9907). Ann Surg Oncol 19: 68-74, 2012.
15. Salman M and Naseem I: Riboflavin as adjuvant with cisplatin: Study in mouse skin cancer model. Front Biosci (Elite Ed) 7: 242-254, 2015.
16. Dasari S and Tchounwou PB: Cisplatin in cancer therapy: Molecular mechanisms of action. Eur J Pharmacol 740: 364-378, 2014.
17. Mego M, Svetlovska D, Miskovska V, Obertova J, Palacka P, Rajec J, Sycova-Mila Z, Chovanec M, Rejlekova K, Zuzák P, *et al*: Phase II study of everolimus in refractory testicular germ cell tumors. Urol Oncol 34: 122.e17-122.e22, 2016.
18. Terenziani M, De Pasquale MD, Bisogno G, Biasoni D, Boldrini R, Collini P, Conte M, Dall'Igna P, Inserra A, Melchionda F, *et al*: Malignant testicular germ cell tumors in children and adolescents: The AIEOP (Associazione Italiana Ematologia Oncologia Pediatrica) protocol. Urol Oncol 36: 502.e7-502.e13, 2018.
19. Shi T, Jiang R, Yu J, Yang H, Tu D, Dai Z, Shen Y, Zhang Y, Cheng X, Jia H, *et al*: Addition of intraperitoneal cisplatin and etoposide to first-line chemotherapy for advanced ovarian cancer: A randomised, phase 2 trial. Br J Cancer 119: 12-18, 2018.

20. Tempfer CB, Giger-Pabst U, Seebacher V, Petersen M, Dogan A and Rezniczek GA: A phase I, single-arm, open-label, dose escalation study of intraperitoneal cisplatin and doxorubicin in patients with recurrent ovarian cancer and peritoneal carcinomatosis. Gynecol Oncol 150: 23-30, 2018.
21. Gupta S, Maheshwari A, Parab P, Mahantshetty U, Hawaldar R, Sastri Chopra S, Kerkar R, Engineer R, Tongaonkar H, Ghosh J, *et al*: Neoadjuvant chemotherapy followed by radical surgery versus concomitant chemotherapy and radiotherapy in patients with stage IB2, IIA, or IIB squamous cervical cancer: A randomized controlled trial. J Clin Oncol 36: 1548-1555, 2018.
22. Kitagawa R, Katsumata N, Shibata T, Kamura T, Kasamatsu T, Nakanishi T, Nishimura S, Ushijima K, Takano M, Satoh T and Yoshikawa H: Paclitaxel Plus carboplatin versus paclitaxel plus cisplatin in metastatic or recurrent cervical cancer: The open-label randomized phase III trial JCOG0505. J Clin Oncol 33: 2129-2135, 2015.
23. Rosen VM, Guerra I, McCormack M, Nogueira-Rodrigues A, Sasse A, Munk VC and Shang A: Systematic review and network meta-analysis of bevacizumab plus first-line topotecan-paclitaxel or cisplatin-paclitaxel versus non-bevacizumab-containing therapies in persistent, recurrent, or metastatic cervical cancer. Int J Gynecol Cancer 27: 1237-1246, 2017.
24. Small W Jr, Bacon MA, Bajaj A, Chuang LT, Fisher BJ, Harkenrider MM, Jhingran A, Kitchener HC, Mileshkin LR, Viswanathan AN and Gaffney DK: Cervical cancer: A global health crisis. Cancer 123: 2404-2412, 2017.
25. Noronha V, Joshi A, Patil VM, Agarwal J, Ghosh-Laskar S, Budrukkar A, Murthy V, Gupta T, D'Cruz AK, Banavali S, *et al*: Once-a-week versus once-every-3-weeks cisplatin chemoradiation for locally advanced head and neck cancer: A phase III randomized noninferiority trial. J Clin Oncol 36: 1064-1072, 2018.
26. Strojan P, Vermorken JB, Beitler JJ, Saba NF, Haigentz M Jr, Bossi P, Worden FP, Langendijk JA, Eisbruch A, Mendenhall WM, *et al*: Cumulative cisplatin dose in concurrent chemoradiotherapy for head and neck cancer: A systematic review. Head Neck 38 (Suppl 1): E2151-E2158, 2016.
27. Szturz P, Wouters K, Kiyota N, Tahara M, Prabhash K, Noronha V, Castro A, Licitra L, Adelstein D and Vermorken JB: Weekly low-dose versus three-weekly high-dose cisplatin for concurrent chemoradiation in locoregionally advanced non-nasopharyngeal head and neck cancer: A systematic review and meta-analysis of aggregate data. Oncologist 22: 1056-1066, 2017.
28. Gridelli C, Morabito A, Cavanna L, Luciani A, Maione P, Bonanno L, Filipazzi V, Leo S, Cinieri S, Ciardiello F, *et al*: Cisplatin-based first-line treatment of elderly patients with advanced non-small-cell lung cancer: Joint analysis of MILES-3 and MILES-4 phase III trials. J Clin Oncol 36: 2585-2592, 2018.
29. Rossi A and Di Maio M: Platinum-based chemotherapy in advanced non-small-cell lung cancer: Optimal number of treatment cycles. Expert Rev Anticancer Ther 16: 653-660, 2016.
30. Sarin N, Engel F, Kalayda GV, Mannewitz M, Cinatl J Jr, Rothweiler F, Michaelis M, Saafan H, Ritter CA, Jaehde U and Frötschl R: Cisplatin resistance in non-small cell lung cancer cells is associated with an abrogation of cisplatin-induced G2/M cell cycle arrest. PLoS One 12: e0181081, 2017.
31. Liu L, Fan J, Ai G, Liu J, Luo N, Li C and Cheng Z: Berberine in combination with cisplatin induces necroptosis and apoptosis in ovarian cancer cells. Biol Res 52: 37, 2019.
32. de Freitas LM, Soares CP and Fontana CR: Synergistic effect of photodynamic therapy and cisplatin: A novel approach for cervical cancer. J Photochem Photobiol B 140: 365-373, 2014.
33. Oun R, Moussa YE and Wheate NJ: The side effects of platinum-based chemotherapy drugs: A review for chemists. Dalton Trans 47: 6645-6653, 2018.
34. Crul M, van Waardenburg RC, Beijnen JH and Schellens JH: DNA-based drug interactions of cisplatin. Cancer Treat Rev 28: 291-303, 2002.
35. Fuertes MA, Alonso C and Pérez JM: Biochemical modulation of cisplatin mechanisms of action: Enhancement of antitumor activity and circumvention of drug resistance. Chem Rev 103: 645-662, 2003.
36. Oh PS and Jeong HJ: Therapeutic application of light emitting diode: Photo-oncomic approach. J Photochem Photobiol B 192: 1-7, 2019.
37. Sorbellini E, Rucco M and Rinaldi F: Photodynamic and photobiological effects of light-emitting diode (LED) therapy in dermatological disease: An update. Lasers Med Sci 33: 1431-1439, 2018.
38. Brancaleon L and Moseley H: Laser and non-laser light sources for photodynamic therapy. Lasers Med Sci 17: 173-186, 2002.
39. Ost D: Photodynamic therapy in lung cancer. A review. Methods Mol Med 75: 507-526, 2003.
40. Sutedja TG and Postmus PE: Photodynamic therapy in lung cancer. A review. J Photochem Photobiol B 36: 199-204, 1996.
41. Silva JN, Filipe P, Morlière P, Mazière JC, Freitas JP, Gomes MM and Santus R: Photodynamic therapy: Dermatology and ophthalmology as main fields of current applications in clinic. Biomed Mater Eng 18: 319-327, 2008.
42. Breskey JD, Lacey SE, Vesper BJ, Paradise WA, Radosevich JA and Colvard MD: Photodynamic therapy: Occupational hazards and preventative recommendations for clinical administration by healthcare providers. Photomed Laser Surg 31: 398-407, 2013.
43. del Olmo-Aguado S, Manso AG and Osborne NN: Light might directly affect retinal ganglion cell mitochondria to potentially influence function. Photochem Photobiol 88: 1346-1355, 2012.
44. Chui C, Hiratsuka K, Aoki A, Takeuchi Y, Abiko Y and Izumi Y: Blue LED inhibits the growth of Porphyromonas gingivalis by suppressing the expression of genes associated with DNA replication and cell division. Lasers Surg Med 44: 856-864, 2012.
45. Rybchyn MS, De Silva WGM, Sequeira VB, McCarthy BY, Dilley AV, Dixon KM, Halliday GM and Mason RS: Enhanced repair of UV-induced DNA damage by 1,25-dihydroxyvitamin D3 in skin is linked to pathways that control cellular energy. J Invest Dermatol 138: 1146-1156, 2018.
46. Suh SS, Lee SG, Youn UJ, Han SJ, Kim IC and Kim S: Comprehensive expression profiling and functional network analysis of porphyra-334, one mycosporine-like amino acid (MAA), in human keratinocyte exposed with UV-radiation. Mar Drugs 15: 196, 2017.
47. Muhammad S, Qasid SH, Rehman S and Rai AB: Visible light communication applications in healthcare. Technol Health Care 24: 135-138, 2016.
48. Tsibadze A, Chikvaidze E, Katsitadze A, Kvachadze I, Tskhvediani N and Chikviladze A: Visible light and human skin (Review). Georgian Med News: 46-53, 2015 (In Russian).
49. Gegotek A, Atalay S, Domingues P and Skrzydlewska E: The differences in the proteome profile of cannabidiol-treated skin fibroblasts following UVA or UVB irradiation in 2D and 3D cell cultures. Cells 8: 995, 2019.
50. Zhang C, Yuchi H, Sun L, Zhou X and Lin J: Human amnion-derived mesenchymal stem cells protect against UVA irradiation-induced human dermal fibroblast senescence, in vitro. Mol Med Rep 16: 2016-2022, 2017.
51. Patel AD, Rotenberg S, Messer RL, Wataha JC, Ogbureke KU, McCloud VV, Lockwood P, Hsu S and Lewis JB: Blue light activates phase 2 response proteins and slows growth of a431 epidermoid carcinoma xenografts. Anticancer Res 34: 6305-6313, 2014.
52. Niu T, Tian Y, Wang G, Guo G, Tong Y and Shi Y: Inhibition of ROS-NF-κB-dependent autophagy enhances hypocrellin A united LED red light-induced apoptosis in squamous carcinoma A431 cells. Cell Signal 69: 109550, 2020.
53. Meulemans J, Delaere P and Vander Poorten V: Photodynamic therapy in head and neck cancer: Indications, outcomes, and future prospects. Curr Opin Otolaryngol Head Neck Surg 27: 136-141, 2019.
54. Kerr C, Adhikary G, Grun D, George N and Eckert RL: Combination cisplatin and sulforaphane treatment reduces proliferation, invasion, and tumor formation in epidermal squamous cell carcinoma. Mol Carcinog 57: 3-11, 2018.
55. Hwang H, Biswas R, Chung PS and Ahn JC: Modulation of EGFR and ROS induced cytochrome c release by combination of photodynamic therapy and carboplatin in human cultured head and neck cancer cells and tumor xenograft in nude mice. J Photochem Photobiol B 128: 70-77, 2013.
56. Zhang X, Liu X, Kang S, Liu C and Hao Y: Resveratrol enhances the effects of ALA-PDT on skin squamous cells A431 through p38/MAPK signaling pathway. Cancer Biomark 21: 797-803, 2018.
57. Tampucci S, Carpi S, Digiacomo M, Polini B, Fogli S, Burgalassi S, Macchia M, Nieri P, Manera C and Monti D: Diclofenac-derived hybrids for treatment of actinic keratosis and squamous cell carcinoma. Molecules 24: 1793, 2019.
58. Carbone C, Martins-Gomes C, Pepe V, Silva AM, Musumeci T, Puglisi G, Furneri PM and Souto EB: Repurposing itraconazole to the benefit of skin cancer treatment: A combined azole-DDAB nanoencapsulation strategy. Colloids Surf B Biointerfaces 167: 337-344, 2018.

59. Chen H, Pan J, Zhang L, Chen L, Qi H, Zhong M, Shi X, Du J and Li Q: Downregulation of estrogen-related receptor alpha inhibits human cutaneous squamous cell carcinoma cell proliferation and migration by regulating EMT via fibronectin and STAT3 signaling pathways. Eur J Pharmacol 825: 133-142, 2018.
60. Ou C, Liu H, Ding Z and Zhou L: Chloroquine promotes gefitinib-induced apoptosis by inhibiting protective autophagy in cutaneous squamous cell carcinoma. Mol Med Rep 20: 4855-4866, 2019.
61. Fusenig NE and Boukamp P: Multiple stages and genetic alterations in immortalization, malignant transformation, and tumor progression of human skin keratinocytes. Mol Carcinog 23: 144-158, 1998.
62. Colombo I, Sangiovanni E, Maggio R, Mattozzi C, Zava S, Corbett Y, Fumagalli M, Carlino C, Corsetto PA, Scaccabarozzi D, *et al*: HaCaT cells as a reliable in vitro differentiation model to dissect the inflammatory/repair response of human keratinocytes. Mediators Inflamm 2017:7435621, 2017.
63. Cordero RR, Damiani A, Seckmeyer G, Jorquera J, Caballero M, Rowe P, Ferrer J, Mubarak R, Carrasco J, Rondanelli R, *et al*: The solar spectrum in the atacama desert. Sci Rep 6: 22457, 2016.
64. Faurschou A, Gniadecki R, Calay D and Wulf HC: TNF-alpha impairs the S-G2/M cell cycle checkpoint and cyclobutane pyrimidine dimer repair in premalignant skin cells: Role of the PI3K-Akt pathway. J Invest Dermatol 128: 2069-2077, 2008.
65. Schug ZT, Gonzalvez F, Houtkooper RH, Vaz FM and Gottlieb E: BID is cleaved by caspase-8 within a native complex on the mitochondrial membrane. Cell Death Differ 18: 538-548, 2011.
66. Huang C and Yu Y: Synergistic cytotoxicity of β-elemene and cisplatin in gingival squamous cell carcinoma by inhibition of STAT3 signaling pathway. Med Sci Monit 23: 1507-1513, 2017.
67. Tao JX, Zhou WC and Zhu XG: Mitochondria as potential targets and initiators of the blue light hazard to the retina. Oxid Med Cell Longev 2019: 6435364, 2019.
68. Hientz K, Mohr A, Bhakta-Guha D and Efferth T: The role of p53 in cancer drug resistance and targeted chemotherapy. Oncotarget 8: 8921-8946, 2017.
69. Batchelor E and Loewer A: Recent progress and open challenges in modeling p53 dynamics in single cells. Curr Opin Syst Biol 3: 54-59, 2017.
70. Batchelor E, Loewer A, Mock C and Lahav G: Stimulus-dependent dynamics of p53 in single cells. Mol Syst Biol 7: 488, 2011.
71. Paek AL, Liu JC, Loewer A, Forrester WC and Lahav G: Cell-to-cell variation in p53 dynamics leads to fractional killing. Cell 165: 631-642, 2016.
72. Bowen AR, Hanks AN, Allen SM, Alexander A, Diedrich MJ and Grossman D: Apoptosis regulators and responses in human melanocytic and keratinocytic cells. J Invest Dermatol 120: 48-55, 2003.
73. Laubach V, Kaufmann R, Bernd A, Kippenberger S and Zöller N: Extrinsic or intrinsic apoptosis by curcumin and light: Still a mystery. Int J Mol Sci 20: 905. 2019.
74. Cives M, Mannavola F, Lospalluti L, Sergi MC, Cazzato G, Filoni E, Cavallo F, Giudice G, Stucci LS, Porta C and Tucci M: Non-melanoma skin cancers: Biological and clinical features. Int J Mol Sci 21: 5394, 2020.
75. Gargiulo M, Papa A, Capasso P, Moio M, Cubicciotti E and Parascandolo S: Electrochemotherapy for non-melanoma head and neck cancers: Clinical outcomes in 25 patients. Ann Surg 255: 1158-1164, 2012.
76. Goggins CA and Khachemoune A: The use of electrochemotherapy in combination with immunotherapy in the treatment of metastatic melanoma: A focused review. Int J Dermatol 58: 865-870, 2019.
77. Montuori M, Santurro L, Feliziani A, DE Sanctis F, Ricciardi E, Gaudio D, Campione E, Bianchi L, Silvi MB and Rossi P: Electrochemotherapy for basocellular and squamocellular head and neck cancer: preliminary experience in day surgery unit. G Ital Dermatol Venereol 153: 19-25, 2018.
78. Grzelak A, Rychlik B and Bartosz G: Light-dependent generation of reactive oxygen species in cell culture media. Free Radic Biol Med 30:1418-1425, 2001.
79. Stockley JH, Evans K, Matthey M, Volbracht K, Agathou S, Mukanowa J, Burrone J and Káradóttir RT: Surpassing light-induced cell damage in vitro with novel cell culture media. Sci Rep 7: 849, 2017.

10. THANKSGIVING

Alla fine di un percorso importante come questo, i ringraziamenti sono doverosi.

In primis mi sento di dover ringraziare il prof. Massimo Bracci, che in questi anni mi ha insegnato molto. Fin dall' inizio mi ha incoraggiata a crescere, riponendo in me la massima fiducia, condividendo i suoi ambiziosi progetti e le sue passioni. E' stato molto stimolante sapere che confidasse nelle mie capacità. Sento di poter dire che in questi anni abbiamo condiviso molto, non solo nel portare avanti gli stessi obiettivi nella ricerca, ma anche, e soprattutto, nello scambio reciproco di idee sulla vita e sul futuro. Grazie.

Un grazie anche alle mie compagne di laboratorio Veronica, Simona, Federica, Maria e Raffaella. In particolare ci tengo molto a ringraziare Maria e Raffaella che mi hanno insegnato i " segreti del mestiere" e si sono rivelate bellissime persone. Ringrazio inoltre tutto il gruppo di Medicina del Lavoro: la prof. Lory Santarelli, la prof. Monica Amati, il dott. Marco Tommasetti e la dott.ssa Ernesta Pieragostini per avermi accolta nel gruppo ed avermi accompagnato nel mio percorso di crescita.

Il grazie alla mia famiglia non può mancare: sapervi orgogliosi di me è la cosa più bella e importante.

Un grazie ai miei amici più cari: grazie a voi non mi sento mai sola. Il vostro sostegno e il vostro affetto mi dà la forza di andare avanti sempre.

Il grazie finale va a te, amore mio: Antonio. Scriverti la dedica è stato molto più faticoso del previsto. Se riguardo indietro, all' inizio di questi tre anni e mezzo, penso a quante cose sono cambiate per noi.. solo una cosa rimane immutata, e anzi aumenta ogni giorno: il mio amore per te. Ti amo.