Tampere University

VEERA NIKKOLA

Interactions Between Ultraviolet B (UVB) Radiation and Circadian Clock in the Skin

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ACADEMIC DISSERTATION
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to Jussi, Noora and Niilo

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Veera Nikkola

ABSTRACT

In everyday life, ultraviolet (UV) radiation from the sun is a significant carcinogen. It induces deoxyribonucleic acid (DNA) mutations and, at the same time, is immunosuppressive. From the whole solar UV radiation spectrum, the wavelengths of UVB are the most biologically active part.

There are indications that the timing of UVB exposure may affect sensitivity to sunburn, but these findings have not been confirmed in human studies. However, almost all functions of the human skin are rhythmic in a circadian (approximately a day of 24 hours) manner, and most skin cell types have their own intrinsic circadian clock. Similar to the central circadian clock in the brain, the human skin expresses clock proteins, e.g., cryptochromes which are essential to circadian function, and melatonin, which is well known for its ability to influence human chronobiology. So far, little is known about the link between skin clock proteins, melatonin, and erythema.

In the skin, UVB induced erythema is a consequence of vasodilatation after DNA damage. In this thesis, we aimed to investigate, if there were connections between skin erythema and the circadian clock.

In order to investigate the impact of the hour of the day on UVB induced erythema, we performed photosensitivity testing of the skin of 19 healthy volunteers twice, first in the morning and second in the evening. Using immunohistochemistry we also analysed skin biopsies taken at a different hour of the day, the expression of the core clock proteins. Erythema was found to be more pronounced after evening irradiations than after morning irradiations. Cryptochrome-2 (CRY2) was identified as a possible protecting factor.

The effect of UVB irradiation on circadian time in the skin was studied by performing mRNA expression analyses on skin samples obtained from 12 healthy volunteers. We compared expression of clock proteins in non-irradiated and UVB irradiated skin samples and in corresponding samples of subcutaneous adipose tissue. UVB exposures seemed to modify the expression of *CRY2* in epidermal and dermal skin, and that of Cryptochrome-1 (*CRY1*) and Circadian-associated transcriptional repressor (*CIART*) in subcutaneous adipose tissue. Irradiation also altered the *CRY1*/*CRY2* ratio.

In a further study, 39 healthy volunteers were analyzed using immunohistochemistry for the presence of melatonin in the non-UVB exposed buttocks skin at different times of the day. The photosensitivity testing was performed on the skin of all our volunteers. We discovered melatonin content of the non-irradiated skin to vary diurnally. However melatonin expression in the skin detected by immunohistochemistry did not correlate with the circadian change in erythema sensitivity.

In conclusion, the hour of the day of UVB irradiation seems to influence the erythema formation in healthy human skin. The connection is also reverse, because UVB irradiation changes the mRNA expression of clock driving proteins. It is possible that UVB serves as a cue for circadian time entrainment through the skin. Even though the melatonin content of the skin varies diurnally this does not appear to be the reason why UVB induces more erythema in the evening than in the morning. Understanding factors influencing erythema sensitivity of the skin and its interactions with the circadian clock could promote the development of skin cancer prevention strategies as well as dermatological treatments. An extensive understanding of circadian clock entrainment is an important part of overall human health.

TIIVISTELMÄ

Auringon ultraviolettisäteily on merkittävä karsinogeeni jokapäiväisessä elämässämme. Sen vaikutuksesta ihon solujen perimään kertyy mutaatioita ja samanaikaisesti se lamaa immuunipuolustusta. Biologisesti aktiivisin osa auringon ultraviolettisäteilyn spektriä on ultravioletti B (UVB) aallonpituusalue.

Eläinkokeissa on saatu viitteitä siitä, että herkkyys UVB-säteilyn aiheuttamalle punoitukselle riippuisi vuorokaudenajasta, kun taas ihmisten suhteen, on tästä esitetty vasta arvailuja. Kuitenkin monet ihmisihon toiminnot ovat tutkimuksissa osoittautuneet vuorokaudenajan suhteen rytmisiksi, ja useilla ihon solutyypeilläkin on oma sisäinen kellonsa. Kuten aivoissa sijaitsevassa vuorokausirytmin keskuksessa, myös ihmisihossa syntetisoidaan kelloproteiineja, joista keskeisimpiä ovat kryptokromit, sekä melatoniinia, joka on tärkeä vuorokausirytmiä säätelevä hormoni. Ihon kelloproteiinien ja melatoniinin yhteydestä ihon palamiseen ei toistaiseksi ole juuri lainkaan tietoa.

UVB-säteily aiheuttaa ihon palamista eli punoituksen, joka on seurausta verisuonten laajenemisesta deoksiribonukleiinihapon (DNA) vaurioitumisen yhteydessä. Tutkimuksella haluttiin selvittää tämän punoituksen ja ihon vuorokausirytmin välisiä yhteyksiä.

Vuorokaudenajan vaikutusta UVB-säteilyn aiheuttamaan punoitukseen tutkittiin mittaamalla 19 terveen vapaaehtoisen henkilön ihon punoitusta valotestissä sekä aamulla että illalla. Ihosta eri vuorokaudenaikoina otetuista koepaloista analysoitiin lisäksi kelloproteiinien määrää immunohistokemiallisilla värjäyksillä. Punoitus oli intensiivisempää illalla verrattuna aamuun, ja Cryptochrome-2 (CRY2) kelloproteiini tunnistettiin mahdolliseksi punoitukselta suojaavaksi tekijäksi.

UVB-säteilyn vaikutusta ihon vuorokausirytmiin tutkittiin lähettiribonukleiinihapon (mRNA) analyysien avulla 12 terveen vapaaehtoisen koehenkilön ihonäytteistä. Valottamattoman ja UVB-valotetun ihon ja ihonalaisrasvan kelloproteiinien mRNAn ilmenemistä verrattiin toisiinsa. UVB-säteily vaikutti muokkaavan vuorokausirytmiä säätelevien *CRY2* kellogeenin ilmenemistä epidermaalisessa ja dermaalisessa ihossa sekä *CRY1* ja Circadian-associated transcriptional repressor (*CIART*) kellogeenien ilmenemistä ihonalaisrasvassa. Säteily myös muutti kellogeenien *CRY1*/*CRY2* suhdetta.

Yhteensä 39 terveen vapaaehtoisen koepaloista analysoitiin immunohistokemiallisilla värjäyksillä melatoniinin ilmenemistä ihossa eri vuorokaudenaikoina. Kaikkien tutkittavien ihoille tehtiin valotesti. Tutkimuksessa selvitettiin, oliko melatoniinin määrä ihossa kytköksissä UVB-säteilyn aiheuttaman punoituksen voimakkuuteen ja sen vuorokausivaihteluun. Melatoniinin määrä ihossa oli erilainen aamulla ja illalla. Ihossa havaitun melatoniinin määrä ei kuitenkaan selittänyt punoitusherkkyyden vaihtelua UVB-säteilytyksen seurauksena eri vuorokaudenaikoina.

Vuorokaudenaika näyttäisi vaikuttavan UVB-säteilyn aiheuttaman punoituksen intensiteettiin terveiden henkilöiden iholla. Vuorovaikutus toimii toiseenkin suuntaan, sillä UVB-säteily muuttaa vuorokausirytmiä säätelevien kellogeenien ilmenemistä ihossa. On mahdollista, että UVB-säteily toimii sisäistä kelloa tahdistavana tekijänä. Vaikka melatoniinin määrä ihossa on erilainen eri vuorokaudenaikoina, ei ilmiö selittäne UVB-säteilyn aiheuttaman ihon punoituksen intensiteetin vaihtelua. Se, että ymmärrys ihon punoitusherkkyyteen vaikuttavista tekijöistä ja niiden yhteydestä sisäiseen kelloon lisääntyy, voi avata uusia kehityssuuntia ihosyöpien ehkäisyssä ja ihosairauksien hoidossa. Laaja ymmärrys sisäistä kelloa tahdittavista tekijöistä on myös tärkeää ihmisen kokonaisvaltaisen hyvinvoinnin kannalta.

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ABBREVIATIONS

β-END	Beta-endorphin
β-LPH	β-Lipotropin
α-MSH	α-Melanocyte-stimulating hormone
5-HT	5-hydroxytryptophan
5-MT	5-methoxytryptamine
6-4PP	6-4 photoproduct
AANAT	Arylalkylamine N-asetyltransferase
ACTH	Adrenocorticotropic hormone
AE	Atopic eczema
AFMK	N^1-acetyl- N^2-formyl-5-methoxykynuramine
AK	Actinic keratosis
AMK	N^1-acetyl-5-methoxykynuramine
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like protein 1
CCG	Clock controlled gene
CIART	Circadian-associated transcriptional repressor
CIE	Commission Internationale de l´Eclairage
CLOCK	Circadian Locomotor Output Cycles Kaput
CPD	Cyclobutene pyrimidine dimer
CRH	Corticotropin-releasing hormone
CRY1	Cryptochrome 1
CRY2	Cryptochrome 2
DLMO	Dim light melatonin onset
DNA	Deoxyribonucleic acid
GEE	Generalized Estimating Equations
HIOMT	Hydroxylindole-O-methyltransferase
HPA	Hypothalamic-pituitary-adrenal
MAS	Melatonergic antioxidative system
MC1R	Melanocortin 1 receptor
MED	Minimal erythema dose

MEQ	Horne & Östberg's Morningness-Eveningness Questionnaire
MEQ-6	Shortened Horne & Östberg's Morningness-Eveningness Questionnaire
MES	Morningness-Eveningness Score
MM	Malignant melanoma
mRNA	Messenger ribonucleic acid
MTNR1A	Melatonin receptor 1A
MTNR1B	Melatonin receptor 1B
NAS	N-acetylserotonin
NB-UVB	Narrow-band ultraviolet B
NER	Nucleotide excision repair
NMSC	Non-melanoma skin cancer
NPAS2	Neuronal PAS domain protein 2
NQO2	Quinone reductase 2
NRF2	Nuclear factor erythroid 2-related factor 2
OPN3	Opsin 3
OPN5	Opsin 5
PCR	Polymerase chain reaction
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
qPCR	Quantitative polymerase chain reaction
RORα	RAR-related orphan receptor alpha
ROS	Reactive oxygen species
SAD	Seasonal affective disorder
SCN	Suprachiasmatic nucleus
SED	Standard erythemal dose
SIRT1	Silent information regulator 1
TPH	Tryptophan hydroxylase
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C
UVR	Ultraviolet radiation

Human genes and mRNA are presented in upper case letters in italics (*PER*) and human proteins as normal upper case letters (PER) in contrast to mouse genes, which are presented after the first letter as lower case letters in italics (*Per*) and mouse proteins as normal upper case letters (PER) (Sundberg and Schofield 2010).

ORIGINAL PUBLICATIONS

I	Nikkola V, Grönroos M, Huotari-Orava R, Kautiainen H, Ylianttila L, Karppinen T, Partonen T, Snellman E (2018). Circadian time effects on NB-UVB-induced erythema in human skin *in vivo*. Journal of Investigative Dermatology 138(2):464-467. DOI: 10.1016/j.jid.2017.08.016
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1 INTRODUCTION

In the human body there is an internal circadian clock setting the pace of its functions. The first irrefutable evidence of an independent self-sustained clock was shown on volunteers who spent several weeks in isolation from external time cues such as light: their physiology still followed daily variation in an approximately 24h cycle (Aschoff 1965). In addition to internal autonomy, the timekeeping circadian clock was also found to have another important feature, flexibility. It is able to re-adjust, for example, after long flights between different time zones. In that case, symptoms of temporary desynchronization between intrinsic and environmental time cause jetlag (Rajaratnam and Arendt 2001). Based on a similar chaotic relationship between intrinsic clock and external requirements, shift work is comparable to permanent jetlag. As various epidemiological studies from the 1990s to 21th century connected shift work to health problems (Rafnsson and Gunnarsdottir 1990; Bøggild and Knutsson 1999; Schernhammer et al. 2003, 2011; Megdal et al. 2005) the assumption that circadian clock desynchronization is not a healthy condition in general, began to take shape.

Fortunately, the circadian clock in the human body can be controlled. The first clock controlling external factor, bright light, was found in 1980 to influence the circadian clock by suppressing melatonin expression (Lewy et al. 1980). Decades later other types of stimuli capable of clock entrainment have been identified (Tahara et al. 2017). However, controlling the circadian clock using bright light has become popular especially in northern latitudes, paying less attention to other types of clock synchronization possibilities.

Dermatologists regularly meet patients, who report, in addition to skin improvement, gaining energized and improved mood from solar UV radiation. In fact, artificial narrow-band UVB (NB-UVB) radiation has been reported to have mood improving related effects in human volunteers (Toledo et al. 2019). However, to this end, connections detected between UV radiation and the circadian clock have, for the most part, been based on animal experiments and cell culture studies (Slominski et al. 2018b).

As bright visible light entrains the circadian clock through the eyes, we believe that UV radiation affects the clock via the skin. Because the strength of clock entrainment by visible light depends on external and internal time (Roenneberg and Foster 1997), the impact of UV radiation could also be dependent on time of the day. The main purpose of the circadian clock is scheduling the functions of the body in optimal time windows. Skin functions are no exception (Geyfman and Andersen 2009). This leads us to the hypothesis that e.g. erythema response would differ during the day. The main aims of the present study were to examine how time of the day affects UVB-induced erythema, and the effects of UVB on circadian clock gene expression in the skin. A further aim was to examine the proposed link between circadian hormone melatonin and skin sensitivity to UVB-induced erythema.

2 REVIEW OF THE LITERATURE

2.1 UV radiation and the skin

Solar UV radiation falls between the wavelengths of X-rays and visible light in the spectrum of electromagnetic radiation. According to its electrophysical features, solar UVR is subdivided into the following three sections: UVA (315-400 nm) with lowest energy and longest wavelength, UVB (280-315 nm), and UVC (100-280 nm) by the Commission Internationale de l´Eclairage (CIE 2011). Another widely used definition for UVB is 280-320 nm, which is mostly used in photobiology. Solar UV radiation below 290 nm, including UVC, is totally absorbed by the atmosphere.

The rays reaching the earth's surface penetrate the exposed skin in a wavelength-dependent manner. As long-wave UVA radiation with lowest energy may reach the dermis, the shorter UVB wave lengths involving higher energy are mostly absorbed into the epidermal tissue (Bruls et al. 1984; Meinhardt et al. 2009; D'Orazio et al. 2013) (Figure 2). UVB has a greater biological effect on the skin than does UVA and the acute effects of solar UV radiation are mediated principally through UVB (Freeman et al. 1989). In recent years artificial narrow-band ultraviolet B (NB-UVB) consisting mainly from 309 to 313nm wave lengths and peaking at 311nm wave length has become the most used form of radiation in phototherapy. Irradiance spectra from both sun and NB-UVB phototherapy devices are shown in Figure 1.

Solar UV radiation has both adverse and beneficial effects on human health. Many of these occur as acute or subacute reactions, but some may appear years or even decades later. As the acute effect, UV radiation causes vitamin D synthesis, immunosuppression, induction of antimicrobial peptides, erythema, and tanning, and as a chronic effect solar elastosis, skin thickening and cancers (Reichrath 2006; Bernard et al. 2019). These effects are wavelength dependent because of different penetration of different UV subtypes in the skin as seen in Figure 2, so that UVA, for example causes solar elastosis and other dermal changes whereas UVB is mainly responsible for epidermal carcinomas of the skin (D'Orazio et al. 2013). From NB-UVB, depending on the thickness of the epidermis at different skin sites, less than 10% reaches the dermis (Bruls et al. 1984; Meinhardt et al. 2009).

The skin's location between external and internal environment requires it to maintain protective mechanisms against UV radiation and other external stimuli. To understand the mechanisms and their circadian function it is necessary to further review some actions between UV radiation, skin, and internal homeostasis.

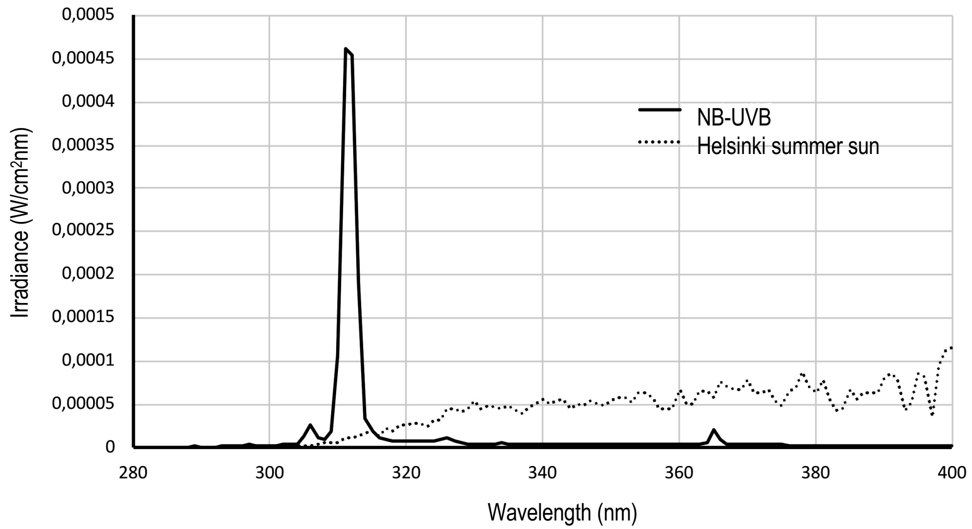


Figure 1. Irradiance of summer sun and narrow-band ultraviolet B (NB-UVB) phototherapy device

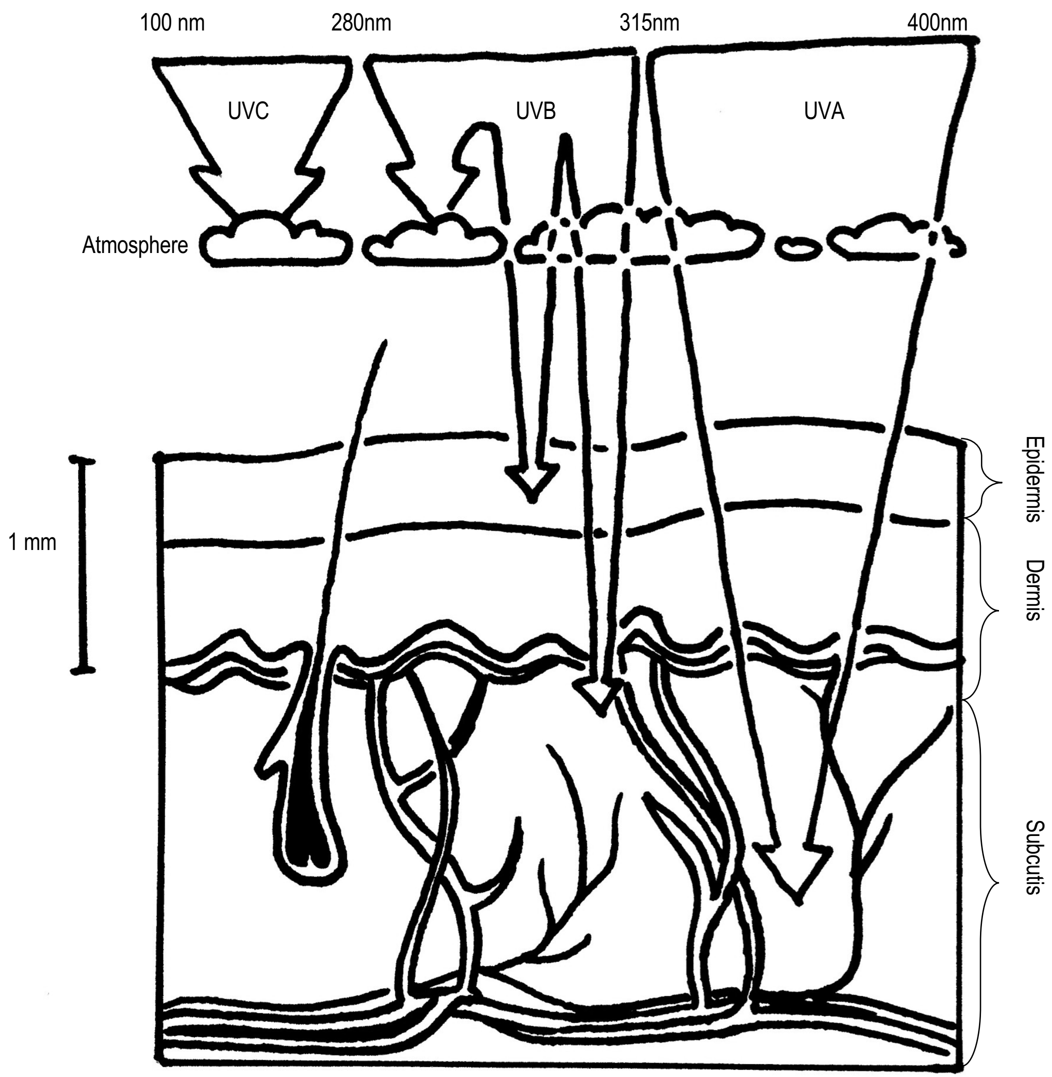


Figure 2. Solar UV radiation penetration depth in the skin

2.1.1 Acute effects

Acute effects of UVB on the skin are vitamin D synthesis, immunosuppression, induction of antimicrobial peptides, inflammation detected as erythema and tanning (Young 2006; Bernard et al. 2019). Previtamin D3 (cholecalciferol) formation in the skin due to UVB irradiation, accounting for about 90% of the requisite total 25-Hydroxyvitamin D serum concentration, is an important action for overall human health (Reichrath 2006). UVB causes immunosuppression as an acute effect after a single radiation dose. Response starts from the skin and can spread throughout the whole body (Hart and Norval 2018). The most visible of the acute effects, skin erythema, is an indicator of DNA damage that can be easily quantified. It also varies due to the properties of both the exposed skin and the source of radiation.

2.1.1.1 Erythema and pigmentation

The intensity of UV-induced erythema depends on several factors such as wavelength, UVR dose, genetic skin sensitivity to UV radiation, site of exposure, and previous light exposure. The erythema sensitivity of the skin of an individual can be presented as a Minimal erythema dose (MED), a value that indicates the smallest radiation dose required to induce barely perceptible erythema. Erythemally weighted UV radiation doses can also be presented as Standard Erythemal Doses (SED) or CIE erythema weighted doses as J m^{-2} or mJ cm^{-2} (CIE 2019) that are not related to individual.

The time course of UV-induced erythema is biphasic; it first peaks, mainly by UVA, immediately after irradiation and the second peak, mainly by UVB, follows from several hours to 24h after irradiation (Logan and Wilhelm 1963; Farr and Diffey 1986; Hönigsmann 2002). Erythema indicates several adverse effects of UV irradiation: skin inflammation, damaged DNA and oxidative stress (D'Orazio et al. 2013). At the molecular level DNA damage caused by UV radiation results mainly from the formation of highly mutagenic cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs) (Shah and Yu-Ying 2015). These photoproducts cause the so-called "UV signature mutations" characteristic of UV-induced DNA damage. Another important part of skin carcinogenesis occurring after UV exposure as independent action at the same time as erythema and p53 formation is the accumulation of reactive oxygen species (ROS) (Kulms et al. 2002).

UVB is highly erythematogenic, but does not, to a significant extent, penetrate to the level of the vascular dermis, where erythema takes place. Therefore some

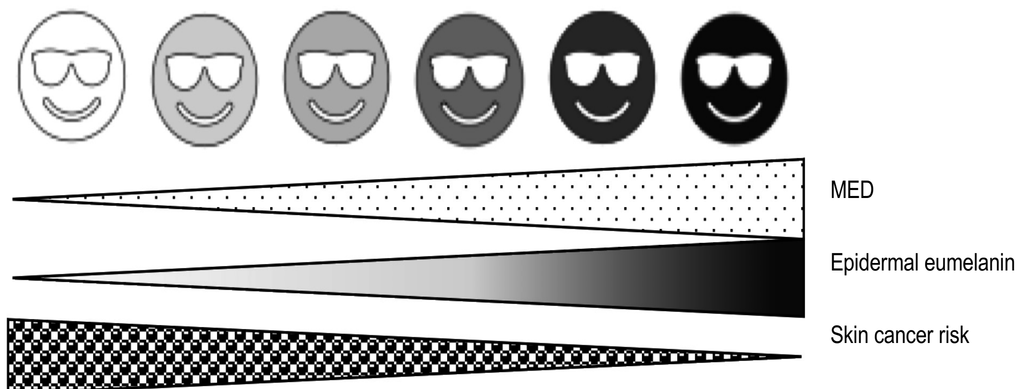
mediators must be present. These have emerged over the time one by one. At the very beginning of research on erythema, it was observed that skin erythema reaction closely resembles other inflammatory skin reactions, with increased vascular permeability by leukocyte actions. The reaction was reduced by antihistamines (Logan and Wilhelm 1963). As early as 1970 it was observed that, histamine and serotonin had only a minor role, and an unidentified smooth muscle contracting agent had a major role in mediating the erythema reaction (Greaves and Sondergaard 1970). The agent turned out to consist of prostaglandins and arachidonic acid that were present immediately and 24 hours after UVB irradiation (Black et al. 1978a). Topical and peroral prostaglandin inhibitor indomethacin suppressed UVB-induced erythema, but only partially, which suggested that not even prostaglandins alone were in responsible for erythema formation (Black et al. 1978b). It was later established, that UVB releases nitric oxide in human skin, which is assumed to be part of the erythema reaction (Deliconstantinos et al. 1995). The UVR absorbing part of the skin i.e., chromophore was clearly DNA, hence its absorption spectrum to format pyrimidine dimers and 4-6 photoproducts was found to match the absorption spectrum of erythema (Freeman et al. 1989). This fact was later confirmed *in vivo* and revealed that DNA damage likely triggers UV-induced erythema (Young et al. 1998). The action spectra for erythema formation and DNA photodamage are similar (Young et al. 1998) and they also have similar protective factors (Sheehan et al. 2002).

Fortunately, skin has ways to protect itself. By damaging DNA, UV radiation activates tumor suppressors such as p53, which leads to the recruitment of Nucleotide excision repair (NER) (Ziegler et al. 1994; Decraene et al. 2001), a major mechanism to delete large UVR damaged DNA lesions (Sancar 2004; D'Orazio et al. 2013). Even with doses of radiation when no erythema visible to the naked eye is present, acute effects of UVB radiation can be detected as an increased amount of p53 tumor suppressor protein (Healy et al. 1994). To inactivate ROS and to repair DNA damage due to its actions, the skin maintains a robust antioxidative system consisting of various enzymes such as catalase, superoxide dismutase, glutathione peroxidase, peroxiredoxin, and heme oxygenase (Hernandez et al. 2019). The final mechanism to protect the skin against cancer formation is apoptosis, a controlled cell death, that can be detected in immunohistochemistry as apoptotic keratinocytes i.e., sun burn cells (Sheehan and Young 2002)

I	II	III	IV	V	VI
Always burns, does not tan	Burns easily, rarely tans	Sometimes burns, tans gradually	Rarely burns, tans easily	Very rarely burns, darkens in the sun	Never burns

Fitzpatrick phototype

Figure 3. Fitzpatrick phototype, erythema, melanin and skin cancer risk



Skin phototypes related to photosensitivity were first presented in 1988 by Thomas B. Fitzpatrick (Fitzpatrick 1988). This classification, however, is subjective and not very reliable (Snellman et al. 1995). As an objective measurement, the MED test, the erythema response to UVB of unexposed skin, has been used to depict photosensitivity and consequently indirectly the relative risk of non-melanoma skin cancer (NMSC) development (Preston and Stern 1992). Main genetic factors determining the UV sensitivity of the skin of an individual are the type and amount of melanin in the epidermis. There are two main chemical forms of melanin, dark UV-blocking eumelanin and light UV-permeable pheomelanin (Simon et al. 2009). At a molecular level eumelanin serves as a free radical scavenger while via UV-dependent pathways pheomelanin can generate ROS (Bustamante et al. 1993; Napolitano et al. 2014). Low MED correlates with sun-sensitive Fitzpatrick phototype and low eumelanin content of the skin whereas high MEDs indicate great amounts of eumelanin and a phototolerant phototype that rarely or never burns (Fitzpatrick 1988; Lu et al. 1996). Taken together, high eumelanin content of the epidermis is one factor protecting the skin against UV radiation-induced erythema and cancer development (Lu et al. 1996; Urbach 1997).

Acute tanning results from UVA exposure without previous erythema whereas delayed tanning results from UVB after erythema formation (Hönigsmann, 2002). Tanning due to UVB radiation is in fact skin darkening by increased melanin

synthesis and peaks from days to a week after irradiation (Park et al. 2002). Like erythema, the tanning response is triggered by the DNA damage (D'Orazio et al. 2013). In tanning response, stabilization of p53 leads to the transcription of Pro-opiomelanocortin (POMC) peptides. Of these, upregulation of α-Melanocyte-stimulating-hormone (α-MSH) leads to an increase in the melanin content of epidermis via Melanocortin 1 receptor (MC1R), and Beta-endorphin (β-END) may lead to tanning dependency (Nguyen and Fisher 2018). Among the other protective mechanisms mentioned, this melanogenesis, is another pathway in the human skin to react to the DNA damaging properties of UVB.

2.1.2 Chronic effects

The chronic effects of UVB include skin thickening, carcinogenesis, and immunosuppression as a broader phenomenon. Of these, local effects occurring in the irradiated skin, are thickening of the epidermis (Lee et al. 2002) and skin cancer development (Feehan and Shantz 2016).

Repeated UVB exposure accumulates DNA mutations. At some point the risk that not all mutations are efficiently repaired by NER begins to increase. Skin cancers can be classified as malignant melanomas (MMs) and NMSCs. MMs are associated with frequent sunburns during the life course (Dennis et al. 2008) whereas NMSCs are associated with high cumulative life-time doses of UVB radiation (Urbach 1997). Despite a small increase in the incidence of NMSCs detected in patients treated with NB-UVB in a few studies (Man et al. 2005; Raone et al. 2018) to date, NB-UVB phototherapy has not been defined as carcinogenic when treating skin diseases such as psoriasis or atopic eczema (Lee et al. 2005; Nast et al. 2015; Wollenberg et al. 2018).

In addition to acute immunosuppressive effect, UVB is capable of causing immunosuppression as a chronic effect after various even suberythemal irradiation doses (Hart and Norval 2018). This was first discovered in the early 1980s when Drs. Michael S. Fisher and Margaret L. Kripke demonstrated that UV irradiation promoted the growth of skin cancers via activation of regulatory T lymphocytes (Fisher and Kripke 1982). Decades later a mediator of skin cancer promoting actions of UVB-induced immunosuppression was proven to be IL-10 (Loser et al. 2007). DNA and *trans*-urocanic acid represent the chromophores of local immunosuppressive actions (Hart and Norval 2018). UV radiation has an impact on

the human immune system at both the local and systemic levels (Bernard et al. 2019). The chronic systemic effects of UVR are reviewed below.

2.1.3 Systemic effects

Although UVB radiation penetration depth in the human skin is only millimeters, its implications extend to homeostasis of the entire body. According to animal studies, it has an impact on regulatory pathways related to immune balance (Skobowiat and Slominski 2015) and via β-endorphin (β-END) production to mood and even UV addiction (Fell et al. 2014).

The fact that UV radiation influences the human immune system was remarkable at the time of its discovery (Fisher and Kripke 1982) and by the 1990s the effect was already well established (Kripke 1994). At cellular level, it was found that in the skin exposed to UVB, *trans*-urocanic acid transforms into *cis*-urocanic acid that can act as a potent immunomodulator (Slominski and Wortsman 2000). It was also shown that in human skin UVB stimulates the production of corticotropin-releasing hormone (CRH) (Slominski et al. 1996) and POMC peptides such as adrenocorticotropic hormone (ACTH) (Zbytek et al. 2006), a-MSH, β-END, β-Lipotropin (β-LPH), and corresponding receptors possibly triggered by oxidative stress (Chakraborty et al. 1999). UVB also stimulates the production of IL-1, IL- 6 and TNFα, the cytokines that can stimulate the CRH-POMC pathway even more (Slominski and Wortsman 2000). The stress response pathway behind many systemic effects of UVB was fully described less than ten years ago; irradiated skin activates both local and central hypothalamic-pituitary-adrenal (HPA) axes to protect the human body from the adverse effects of UV radiation (Slominski et al. 2013). Nowadays it is known that UV-radiation has an impact on the human immune system both locally and systemically so that the net effect is immunosuppressive (Slominski and Wortsman 2000; Nishisgori 2015).

Mediators of systemic effects of UVB seem to be produced simultaneously with erythema. The production of POMC peptides both in skin and in serum peaks significantly about 24 hours after UV exposure (Skobowiat et al. 2011). The cytokines mentioned can also be found in the circulatory system from 12 to 72 hours after UVB irradiation simultaneously with the erythema reaction (Urbanski et al. 1990). It has also been found out that UVB can induce the respective immunosuppressive cytokines systemically even faster possibly by neural stimulation. This may occur less than one hour from irradiation but has not been

confirmed in humans *in vivo* (Skobowiat et al. 2017). Parallel occurrences of erythema and systemic changes could also be due to vasodilation as it is known that CRH acts as a vasodilator in the skin (Slominski et al. 2000; Bernard et al. 2019).

2.1.4 Population-level effects

Solar UVR is the main factor inducing NMSCs and MMs as well as premalignant skin lesions such as Actinic keratosis (AKs) and Morbus Bowens (D'Orazio et al. 2013; Reinehr and Bakos 2019). Prevalence rates of all skin cancers have increased by 43% in women and by 64% in men in Finland from 2008 to 2018 (Finnish Cancer Registry). This is a global phenomenon, and in the USA, for example, the number of skin cancers is also rising and the annual costs were over 8 billion dollars already ten years ago (Guy et al. 2015). Also the number of AKs, premalignant lesions which need to be treated in order to reduce the risk for skin cancer development, is rising, which has a significant financial impact (Siegel et al. 2017). In the future costs are expected to rise even more (Gordon and Rowell 2015).

Artificial UVR also has some population-level effects. Although solarium exposure is associated with increased risks for NMSCs and MM, tanning beds are still used for cosmetic purposes (Boniol et al. 2012; Wehner et al. 2012, 2014). The prevalence of indoor tanning ever exposure in the USA, Europe and Australia ranges from 19.3% to 55.0% with the highest prevalence in Northern and Western Europe (Wehner et al. 2014).

UV Phototherapy is a common treatment of eczema and psoriasis patients attending dermatology clinics. Consensus-based European guidelines recommend phototherapy, especially NB-UVB, as a treatment for moderate to severe psoriasis and atopic eczema (AE) (Pathirana et al. 2009; Wollenberg et al. 2018). Both are common skin diseases, and in a Finnish birth cohort study the prevalence of eczema in total was as high as 27.4% and that of psoriasis was 2.1% among adults (Sinikumpu et al. 2014). AE is the type of eczema that affects approximately 2 to 8% of adult population in most countries (Wollenberg et al. 2018). The prevalence of AE in Finland was found to be 4.8% (Sinikumpu et al. 2014). Also other types of eczemas and skin diseases can be and are successfully treated with phototherapy (Muekusch et al. 2007).

2.2 The circadian clock

2.2.1 The core circadian clock and Zeitgebers

The inbuilt circadian clock helps multicellular organisms to adjust their actions in physiology to 24 hours a day. The circadian clock's independence of the light-dark cycle was discovered almost 300 years ago, when the French astronomist Jean-Jacques d'Ortous de Mairan reported that the *Mimosa pudica* plant opened and closed its leaves in the constant darkness (De Mairan 1729). The actions of the human circadian pacemaker were described by Jürgen Aschoff in 1965 (Aschoff 1965). He proposed that without external time cues, also known as Zeitgebers, the human circadian clock adheres to a period close to but usually a little longer than 24 hours (Aschoff 1965). This observation was later proved and measured in more controlled lighting conditions under clock desynchronization protocols by Charles A. Czeisler and colleagues, who determined the average core circadian clock period to be 24.18 hours (Czeisler et al. 1999). Anatomically the central clock is located within the hypothalamus in the suprachiasmatic nucleus (SCN) in mammalian species. It was first located using lesion and transplant studies (Moore and Eichler 1972; Ralph et al. 1990). In cell culture, the finding that the SCN can initiate and maintain circadian rhythm confirmed its importance for clock machinery (Welsh et al. 1995). Later the SCN has been a rational place to discover the molecular basis of circadian pacemaker genes and proteins (King et al. 1997).

When cycle of the central clock is shorter than 24h in the absence of Zeitgebers, a phase advance occurs, in contrast to a phase delay occurring when the inbuilt clock period is longer than 24h. Individual relationships between core circadian clock periods and the external 24h environment create different chronotypes: extreme morning larks begin their days when extreme night owls goes to sleep (Roenneberg et al. 2003b). In other words, chronotype reflects the individual's timing preference in daily activities and has been recognized since 1970s (Horne and Östberg 1976). Chronotype is affected by genetic factors (Maukonen et al. 2020). In fact, the heritability of chronotype among adults in Finnish population is approximately 50% (Koskenvuo et al. 2007). Chronotype also depends on age and sex so that prevalence of morning type increases by age and, in twin cohorts, is more common in women than in men (Vink et al. 2001; Koskenvuo et al. 2007). However, in general population, morning types are overall more frequent among men (51%) than women (44%), whereas evening types are more frequent among women (13%) than men

(11%), and in both men and women the proportion of evening types decreases by age (Merikanto et al. 2012).

Chronotype is moreover affected by environmental factors such as lighting condition (photoperiod) and social jet lag, which refers to the discrepancy between sleeping schedules on working days and days off (Allebrandt et al. 2014). In a process called entrainment, Zeitgebers such as visible light, temperature, eating, and exercise (Tahara et al. 2017) are synchronized with the inbuilt circadian time (Roenneberg et al. 2003a). One example of this is bright light therapy, which can reset the clock in persons suffering from seasonal affective disorder (SAD) (Golden et al. 2005). The effect of light as well as that of other Zeitgebers, however, varies depending on the current hour of the day and among SAD patients seems to be most effective when provided in the morning hours (Colwell 2011).

The core circadian clock influences the whole body homeostasis including temperature, nutrition state, sleep-wake cycle, and production of melatonin and other hormones (Bass and Takahashi 2010). It also synchronizes clocks in the peripheral tissues (Buhr et al. 2010). Of the Zeitgebers, light has been traditionally the one believed to have its effect on the circadian clock via the SCN, while eating (Schibler et al. 2003), exercise, stress, (Tahara et al. 2017) and temperature (Buhr et al. 2010) have direct effects on peripheral clocks.

2.2.2 Clock genes and proteins

The investigation of the genetics behind inborn circadian rhythms has led scientists to discover large numbers of new genes, proteins, and feedback loops. The first clock gene *per* was discovered in the 1970s by Ronald J. Konopka and Seymour Benzer when they investigated rhythm-mutant *Drosophila melanogaster* flies (Konopka and Benzer 1971). Their studies of the genetics behind the circadian clock and other individual differences marked the beginning of a new field of science: behavioral genetics. Almost two decades later Paul E. Hardin and colleagues were the first to describe the circadian feedback loop that is still the principal mechanism behind clock protein action. In a simple clock feedback loop, a protein, for example Per in *Drosophila melanogaster* inhibits *per* gene expression that leads to a decrease in the amount of Per protein and eventually decreases the inhibition of gene expression that starts the feedback loop from the beginning (Hardin et al. 1990).

The ultimate control behind clock proteins lies in the clock genes and their cyclic expression. They form feedback loops lasting approximately 24h, which is why even

in constant darkness plants and animals, as well as humans, function in a circadian manner. The first mammalian clock gene *Clock* was found in mouse (Antoch et al. 1997; King et al. 1997) and the second clock gene, *Per* (*Drosophila melanogaster* homolog) in mouse, and as *PER* in human (Sun et al. 1997; Tei et al. 1997). Thereafter, in the early 2000s the frames of the molecular circadian clock in mammals began to emerge. The partner clock protein of CLOCK, ARNTL (Gekakis et al. 1998; Bunger et al. 2000), and both PER1 and PER2 were also identified as part of the mammalian clock protein feedback loop (Bae et al. 2001). In addition, clock proteins RAR-related orphan receptor alpha (RORα) and REV-ERBα were found to represent a part of an accessory feedback loop which, similar to PER expression, inhibits ARNTL activity (Lowrey and Takahashi 2011) (Figure 4).

Cryptochrome 1 and 2 (CRY1, CRY2) clock proteins were first discovered when *Drosophila* (6-4) photolyase homolog was cloned in humans and showed great similarity to plant blue light photoreceptors (Hsu et al. 1996; Todo et al. 1996; Van Der Spek et al. 1996). Light can suppress pineal melatonin release even in some blind humans. In fact, the circadian rhythm may be entirely normal despite of blindness (Czeisler et al. 1995; Partonen et al. 1995). This fact led scientists to posit the existence of yet unidentified circadian photoreceptors in the eyes. In the late 1990s Yasuhide Miyamoto and Aziz Sancar discovered CRY1 and CRY2 in mouse retina and proposed these to be responsible for photoreception (Miyamoto and Sancar 1998). In addition, cryptochromes were identified as essential clock proteins, as mice lacking both of them lost their autonomic circadian rhythms. The lack of either CRY1 or CRY2 led to clock function faster or slower than normal. In other words, CRY1 makes the clock tick slower whereas CRY2 makes it tick faster. (Van Der Horst et al. 1999). Cryptochromes have also been shown to regulate genotoxic stress responses to DNA damage, possibly protecting genomic integrity (Papp et al. 2015). All these different functions of mammalian cryptochromes make them an interesting part of clock machinery.

The last core clock protein so far identified has been the Circadian Associated Repressor of Transcription (CIART) that negatively regulates the transcription of ARNTL (Anafi et al. 2014; Goriki et al. 2014). Taken together, as seen in Figure 4, the basic mechanism of the clock protein feedback loop is CLOCK/ARNTL heterodimer complex activating *CRY* and *PER* gene expression via E-boxes and then respectively PER/CRY and CIART proteins inhibiting CLOCK and ARNTL formation. The accessory feedback consists of RORα/REV-ERBα and CIART that inhibits CLOCK/ARNTL. These loops affect the whole homeostasis by activating various clock controlled genes (CCGs) in a tissue specific manner (Takahashi 2017).

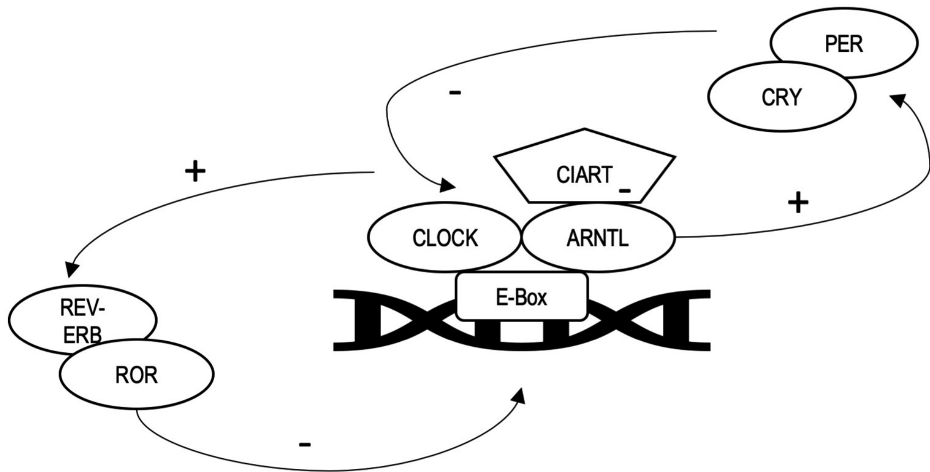


Figure 4. Core circadian clock proteins and feedback loops

2.2.3 Melatonin

Melatonin is a tryptophan derivative (N-acetyl-5-methoxytryptamine) first isolated by Aaron B. Lerner and colleagues from the bovine pineal gland in 1958. The discovery was made on the basis of observations that pineal gland extraction lightens the skin of animals by decreasing the melanocyte stimulating hormone (αMSH) (Lerner et al. 1958). However, as oral melatonin intake does not promote human skin pigmentation, the role of melatonin in skin color changes remains unclear (McElhinney et al. 1994).

Classical chronobiology associates melatonin with a circadian rhythm by focusing its secretion from the pineal gland (Reiter 1991). Pineal secretion of melatonin is controlled by the circadian clock. In daytime levels of circulating melatonin remain low because of inhibitory signals from the SCN. Respectively, in the absence of these signals, levels of circulating melatonin rise during the night (Moore 1996). In addition to these properties melatonin is involved in numerous endogenous and immunological processes in the human body (Reiter 1991). For over two decades has been clear that melatonin can serve as an antioxidant by directly scavenging reactive oxygen and nitrogen species or indirectly by stimulating other antioxidants (Reiter 1996).

Most melatonin is synthesized in the pineal gland where L-tryptophan from the blood stream is converted into melatonin in a four-step process induced by the darkness-triggered pathway from the SCN through the paraventricular nucleus (PVN) via the retinohypothalmic tract (Reiter 1991). L-tryptophan is hydroxylated to 5-hydroxytryptophan that is decarboxylated to serotonin that is acetylated to N-acetylserotonin (NAS) that is converted to melatonin. The rate limiting steps of this synthesis are mediated by tryptophan hydroxylase (TPH), arylalkylamine N-acetyltransferase (AANAT), and hydroxylindole-O-methyltransferase (HIOMT).

In general, the metabolism of melatonin in humans can occur via indolic or kynuric pathways (Hardeland et al. 2006; Kim et al. 2013). The indolic (main) pathway involves the formation of 5-methoxytryptamine (5-MT) (Grace et al. 1991) and predominantly in the liver the formation of 6- hydroxymelatonin (6-OHM) that is a main circulating form of melatonin (Reiter 1991; Hardeland et al. 2006). The kynuric pathway leads to the formation of the primary cleavage product of melatonin, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) that is deformulated to N^1-acetyl-5-methoxykynuramine (AMK) (Semak et al. 2005; Hardeland et al. 2006). In humans the actions of melatonin can be mediated through G-protein-coupled receptors MTNR1A (also known as MT1) (Reppert et al. 1994) and MTNR1B (also known as MT2) (Reppert et al. 1995) and NQO2 (formerly known as MT3) that is an enzyme (Duncan et al. 1988; Nosjean et al. 2000; Boutin and Ferry 2019). In humans, contrary to previous assumptions, RORα is not a nuclear melatonin receptor (Slominski et al. 2016).

Melatonin secretion from the pineal gland is a classic way to reset the main circadian clock. Light signals from the retina pass through the retinohypothalmic tract to the SCN and keep the circadian clock in the 24h-day rhythm by means of melatonin secretion. Without light stimulus melatonin secretion runs free in a rhythm typically a little over 24h generated by the main circadian clock in the SCN by the feedback loops of clock proteins (Reiter et al. 1971).

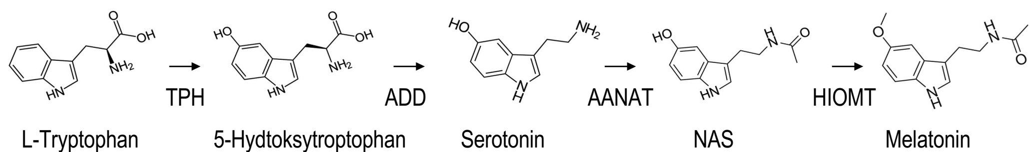


Figure 5. Synthesis of melatonin

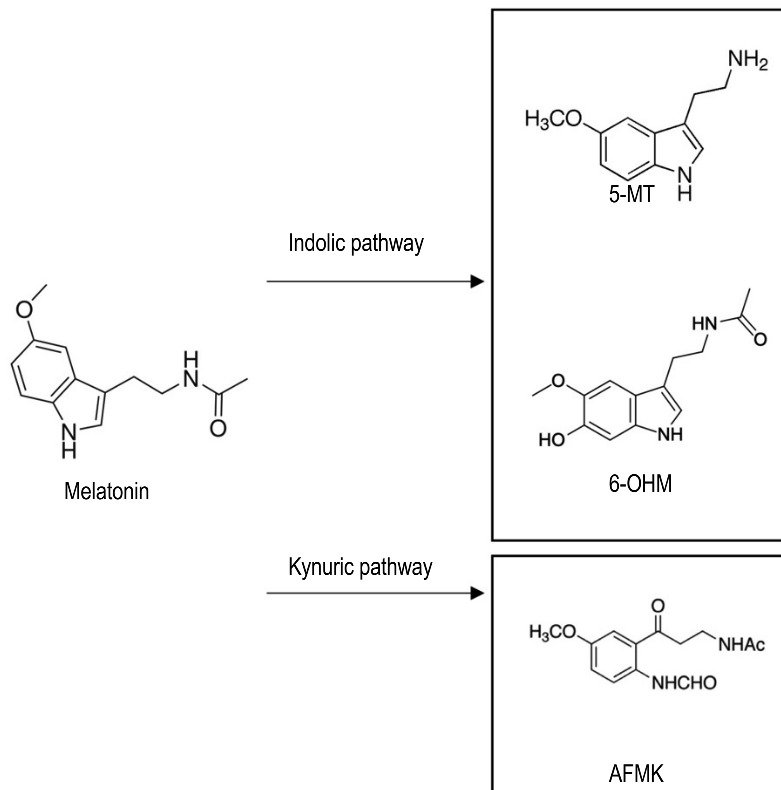


Figure 6. The basic metabolism of melatonin

2.2.4 The clock in the peripheral organs

While the core circadian clock is clearly affected by light, clocks in the peripheral tissues are entrained by various external stimuli (Sherratt et al. 2019). When these Zeitgebers were investigated in the early 2000s, the nature of the partial autonomy of circadian clocks in different organs was just emerging. The liver, for example, seemed to receive signals from both the SCN and external Zeitgebers (Brown et al. 2002). However, peripheral clocks continue, in normal circumstances, to be under the control of SCN. This synchronizes multiple peripheral clocks to follow the time together, as was first demonstrated using temperature as entrainment (Buhr et al. 2010). Taken together, the SCN senses the time of the day via external signals and synchronizes peripheral clocks by its influence in releasing e.g. melatonin (Pevet and Challet 2011), glucocorticoids (Tahara et al. 2017) or temperature changes of the body (Brown et al. 2002).

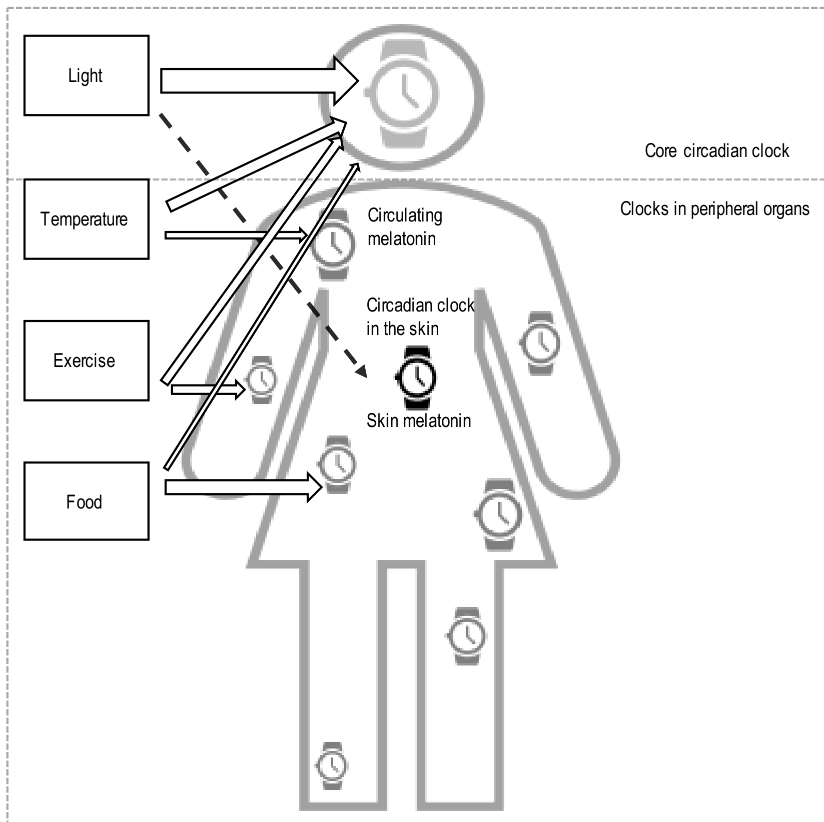


Figure 7. Clock entrainment

2.3 The circadian clock in the skin

2.3.1 Circadian function and clock genes in the skin

The skin is a very accessible and interesting link between the core circadian clock and the Zeitgebers. From the surface inwards it consists of three layers: the epidermis, the dermis, and subcutaneous fat and includes various cell types such as keratinocytes, fibroblasts, and fat cells as major components of the three layers mentioned.

The first indications of a circadian clock in the skin were obtained as early as in 1948, when William S. Bullough discovered that the mitotic activity of the epidermal cells of mouse skin were under the control of an approximately 24h-clock (Bullough 1948). Human skin circadian features were well documented by Isabelle Le Fur and colleagues in 2001 on eight healthy volunteers and circadian variation of transepidermal water loss, sebaceous gland function and surface temperature were obtained (Le Fur et al. 2001). Circadian functions of the skin have been explored even in recent years: as keratinocytes and fibroblast contains their own circadian machinery (Sandu et al. 2012), it is no surprise that circadian time significantly affects wound healing. Burns incurred in daytime heal faster than those incurred at night (Hoyle et al. 2017). One reason for this could be enhanced epidermal proliferation; Florian Spörl and colleagues showed in human skin suction blisters and punch biopsies a transcription factor that promotes epidermal proliferation to peak at noon (Spörl et al. 2012). Human skin pigmentation has also been proven to be mediated by the circadian clock (Hardman et al. 2015).

In the same year that the clinical features of the human skin circadian clock were explored (Le Fur et al. 2001) the first study that proved the circadian variation of clock genes in the human skin was published (Bjarnason et al. 2001). The first comprehensive gene expression analysis of the human skin was presented in 2012 using epidermal skin samples; 294 genes showed daytime dependent variation. The core circadian genes of the skin seemed to be *ARNTL, NPAS2, PER1-3, CRY2, REV-ERBα,* and *REV-ERBβ.* That particular study also demonstrated the circadian variation in the proliferation of keratinocytes (Spörl et al. 2012). NPAS2 is a protein very similar to CLOCK and acts as a core circadian protein, for example in brain tissue (Reick et al. 2001). The functions of other genes mentioned above were presented in detail in section 2.2.2. A study on 298 epidermis samples recently reported 110 genes fluctuating in a rhythmic manner at population level and

according to the genomics the skin seemed to be a better indicator for circadian time than blood (Wu et al. 2018).

Clocks in the skin can be affected by many external stimuli. Marc Cuesta and colleagues proved that bright light and timing of sleep can entrain the skin circadian clock (Cuesta et al. 2017). Hong Wang and colleagues demonstrated that feeding time modulates circadian clock in the skin. Clock entrainment by food intake seems to be independent of the core circadian clock (Wang et al. 2017). There is no consensus on whether entrainment by light can be mediated through tracts other than those which start from the eyes. More than twenty years ago Scott S. Campbell and Patricia J.Murphy showed that light administration to the skin of the lower limbs could delay the clock of the whole body (Campbell and Murphy 1998), and consequently entrainment directly through the skin surface seemed plausible. However, this finding could not be replicated, and the mechanism behind extraocular phototransduction in humans is so far not completely understood.

Both CRY2 and opsins have been suggested to detect light and UV radiation. Opsins are chromophore containing proteins that can detect light (Terakita 2005). In animals and humans they act as g-protein coupled receptors (Poletini et al. 2015). In humans opsins have been detected in the eyes, brain and skin (Tarttelin et al. 2003; Terakita 2005; Haltaufderhyde et al. 2015). OPN1SW, OPN2, OPN3 and OPN5 have been found in human epidermal skin (Haltaufderhyde et al. 2015). Recently it has been reported that opsin 3 (OPN3) receptor in melanocytes can react to blue light (Regazzetti et al. 2018), although another very similar study immediately thereafter failed to show any UV radiation or blue light absorption of OPN3 (Ozdeslik et al. 2019). Another possible receptor, opsin 5 (OPN5), recently studied in the rodent skin *ex vivo*, could synchronize the skin clock with UV or visible light (Kojima et al. 2011; Buhr et al. 2019; Calligaro et al. 2019). Moreover, cryptochromes in the skin, particularly CRY2, may act as photoreceptors for UV radiation, especially when binding with flavine adenine nucleotide (FAD) (Liu et al. 2010; Vanderstraeten et al. 2020).

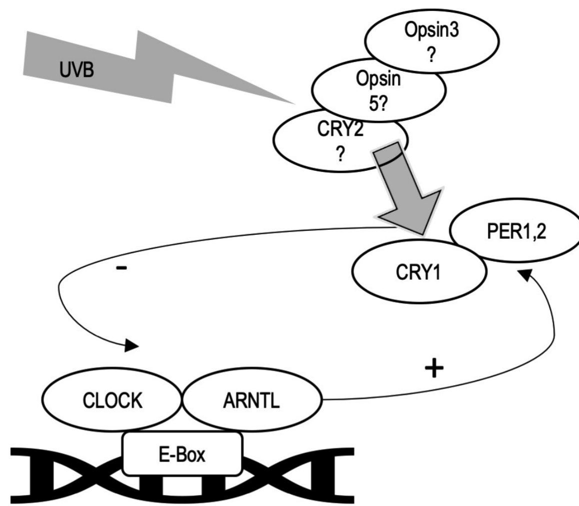


Figure 8. Opsins and CRY2 as photoreceptors

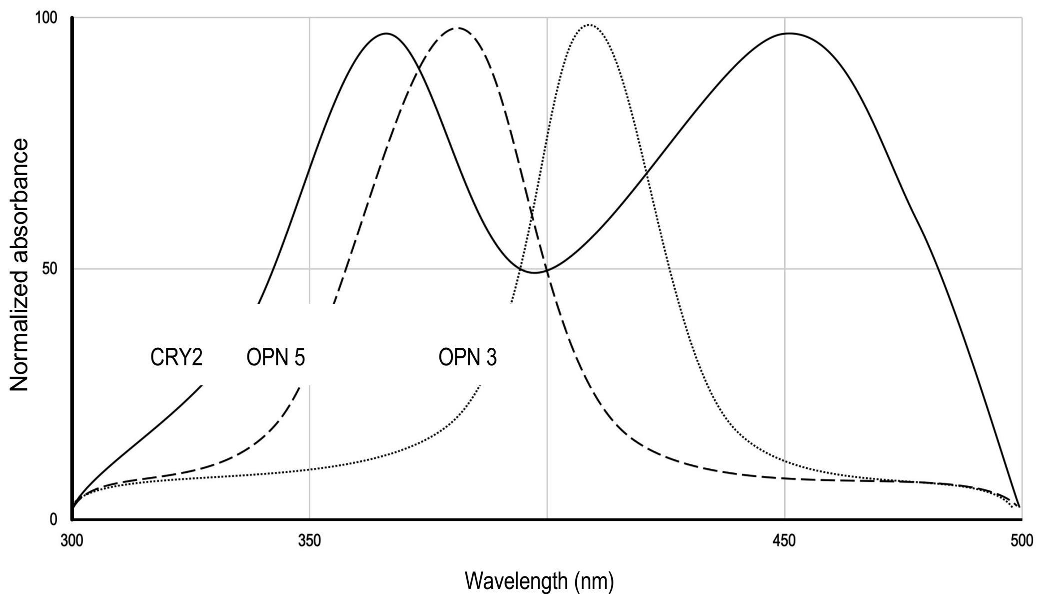


Figure 9. Absorption spectrum of CRY2 when bound with FAD in its oxidized state, opsin 3 and opsin 5 based on cell culture and tissue studies (Liu et al. 2010; Kojima et al. 2011; Regazzetti et al. 2018)

2.3.2 Melatonin in the skin

The first clue of extrapineal melatonin was that after pinealectomy melatonin continued to circulate in rats (Ozaki and Lynch 1976). Thereafter, in addition to the skin, melatonin has been found at other extrapineal sites such as retina, bone marrow, and placenta, first in animals and then in human tissues (Conti et al. 2000; Lanoix et al. 2008; Kim et al. 2013; Acuña-Castroviejo et al. 2014). The fact that pinealectomy often led to undetectable low circulating melatonin levels in rats strongly suggested that the effects of peripheral melatonin were limited to where the synthesis took place (Lewy et al. 1979). On the other hand, the impact of pinealectomy on the amount of circulating melatonin varied in different species (Huether et al. 1992; Yaga et al. 1993).

A melatonergic composition was first detected as part of the total neuroendocrine system of the skin by Slominski and colleagues in 2000. As reviewed earlier, there is a complex local neuroendocrine system in the human skin that reacts on external stressors such as UVB through the CRH–POMC mediated system resulting in pigmentation and other protective mechanisms (Slominski and Wortsman 2000; Slominski et al. 2000). As in the systemic level the CRH-POMC stress response system is complemented with activity of other neurotransmitters such as serotonin (Pacák and Palkovits 2001), Slominski and colleagues started to study the production of melatonin and serotonin in the skin in the early 2000s. They found the enzymes needed in the process (TPH, AANAT and HIOMT) to be active in the production of serotonin and melatonin in the skin and concluded that the human skin was capable of producing melatonin (Slominski et al. 2002). Later evidence from cell culture studies suggested that UVB induces local synthesis of melatonin and its derivatives through indolic and kynuric pathways in the human skin (Fischer et al. 2006a). Production of melatonin precursors NAS and serotonin was confirmed to be possible in several parts of the skin including epidermal keratinocytes (Slominski et al. 2020).

In addition to presumptive melatonin production, the human skin is able to metabolize melatonin further to its derivatives. This was first demonstrated by Tae-Kang Kim and colleagues by quantifying the production of 6-OHM, AFMK, and 5MT by liquid mass spectrometry and chromatography of healthy volunteers (Kim et al. 2015b). They also confirmed the findings of earlier *in vitro* studies that the production on 6-OHM is significantly higher than that of other derivatives, making it the major metabolite of skin melatonin (Kim et al. 2013, 2015b). Of the melatonin

receptors, MT1 and NQO2 are expressed in epidermal keratinocytes (Fischer et al. 2008b) and MT2 in adnexal structures (Fischer et al. 2008a).

The overall impact of melatonin on the human skin biology and functions seems to be extensive. It can affect hair growth (Fischer et al. 2004), take part in thermoregulation of skin blood flow (Aoki et al. 2006), promote wound healing (Romić et al. 2016) and even suppress the growth of MM cells (Fischer et al. 2006c). In other mammals even more features have been described (Fischer et al. 2008b).

Melatonin increases cell survival from UVB damage (Fischer et al. 2006b). These protective properties of melatonin may be mediated not only by melatonin itself but also by its derivatives and its properties to induce other antioxidants (Reiter et al. 2007; Rusanova et al. 2019). In addition to melatonin, its derivatives AFMK (Onuki et al. 2005) and 4OHM (Pérez-González et al. 2017) are strong antioxidants. In the human skin antioxidative actions are mainly mediated in at least four different ways: through direct free radical scavenging, through activating other antioxidants, through the NQO2 receptor, and through reducing mitochondrial damage (Reiter et al. 2016).

2.4 The circadian clock and UV- induced erythema

Circadian time is known to affect UV radiation-induced erythema in mice (Gaddameedhi et al. 2015). As predicted, Gang Wu and colleagues recently showed that the core circadian machinery is very similar in mice and humans (Wu et al. 2018). Mice repeatedly irradiated with UVB in the morning developed five times more squamous cell carcinomas than did those irradiated in the evening (Gaddameedhi et al. 2011). Whether human skin UVB induced carcinogenesis depends on time of the day has not previously been studied.

2.4.1 Clock proteins and erythema

In addition to their role as core clock proteins and photoreceptors, cryptochromes have been shown to have functions important in protecting cells by influencing inflammation, apoptosis, and DNA damage. However, the relationship between clock proteins and cancer formation is controversial.

In mouse studies, loss of cryptochromes led to increased inflammation (Narasimamurthy et al. 2012), but did not increase the risk of cancer formation

(Gauger and Sancar 2005). Loss of cryptochromes could in fact protect against cancer in case of p53 mutation by inducing increased sensitivity to apoptosis, suggesting that cryptochrome inhibition could even act as chemotherapy (Ozturk et al. 2009). Cryptochromes were also found to modulate response to DNA damage (Papp et al. 2015). This and some other data from animal studies suggest that cryptochromes were involved in clock controlled apoptosis, cell cycle regulation, and NER activity (Sancar et al. 2015). Of the other clock proteins neither loss of PER1 or PER2 (Antoch et al. 2013) nor CLOCK (Antoch et al. 2008) increased the risk of cancer development.

Binding of human CRY2 to DNA was shown to be stimulated in the presence of 6-4-photoproducts that led scientists suspect interactions between UVB induced damage and CRY2 (Özgur and Sancar 2003). In line with this is the fact that oxidative stress in human skin due to UV radiation is under the control of clock protein functions (Geyfman et al. 2012). Although it is known that UV erythema in the human skin occurs in the same context as oxidative stress and the recruitment of protective mechanisms (D'Orazio et al. 2013), knowledge of the circadian actions of erythema is lacking.

2.4.2 Melatonin and erythema

Melatonin has been assumed to be a major human skin protectant against external stressors, due to its free radical scavenging and DNA protecting properties (Fischer et al. 2008b; Slominski et al. 2018a). Used topically on the skin, it has reduced erythema reactions caused by UVR, specifically from the UVB wavelengths (Dreher et al. 1998; Scheuer et al. 2016b).

UVB-induced erythema in the skin results from inflammation. Melatonin has various anti-inflammatory effects (Reiter et al. 2016; Jahanban-Esfahlan et al. 2018). Although melatonin receptors MT1 and NQO2 can be detected in normal epidermal keratinocytes, the main actions of melatonin towards UVR in the skin seem to be mediated via receptor independent pathways (Fischer et al. 2013). The effects of melatonin against UVB-induced damage are mediated through melatonergic antioxidative system (MAS) (Fischer et al. 2006a). Tobias W. Fischer and colleagues showed melatonin to act as an antioxidant against UVB radiation in the human skin. In their study immunohistochemical staining and real-time PCR showed higher content of antioxidative enzymes and lower content of 8-OHdG, a marker of DNA

photodamage by reactive oxygen species, in melatonin-incubated skin compared to normal skin 24h after irradiation (Fischer et al. 2013).

Zorica Janjetovic and colleagues later proved in immunofluorescent staining that treating keratinocytes with melatonin and its derivatives (5-MT, 6-OHM, AFMK and NAS) significantly decreased amounts of UVB irradiation induced CPD and 4-6 photoproducts. The finding strongly suggested that melatonin enhances DNA repair capacity (Janjetovic et al. 2014). This was confirmed using a comet assay that showed less DNA damage even in non-irradiated but specifically in the UVB irradiated keratinocytes compared to cells without melatonin treatment (Janjetovic et al. 2014). Their study using cultured human melanocytes demonstrated the involvement of Nuclear factor erythroid 2-related factor 2 (NRF2) in a pathway where melatonin protects cells against UVB-induced damage (Janjetovic et al. 2017). Silent information regulator 1 (SIRT1) pathway, triggered by melatonin, has also been shown to prevent oxidative stress mediated damage in cultured human keratinocytes (Lee et al. 2016). Although melatonin as a small ambiphilic molecule reaches all parts of the cell, its notable antioxidative actions take place in mitochondria (Martin et al. 2000). It has even been suggested that in the skin DNA repair, stimulation of NRF2 and reduction of oxidative damage by melatonin would be secondary to its mitochondrial actions (Slominski et al. 2017b).

Endogenous production of melatonin in the human skin differs depending on age, gender, and race or melanin pigmentation of the skin (Kim et al. 2015a, 2015b), suggesting that there is special regulation of melatonin synthesis based on the demand for its actions. Therefore endogenous rather than exogenous melatonin might afford better for protection against UVB-induced erythema. When looking at erythema protection, the circulating melatonin seems to be irrelevant compared to melatonin produced in the human skin (Slominski et al. 2018a).

3 AIMS OF THE STUDY

The principal aim of the studies was to evaluate the associations between the effects of narrow-band UVB and the circadian clock in human skin *in vivo*. The detailed aims were:

1. To investigate the impact of time of the day on narrow-band UVB-induced erythema.
2. To assess if narrow-band UVB exposure influences the expression of circadian clock genes in the skin.
3. To investigate the relationship between melatonin and skin sensitivity to narrow-band UVB-induced erythema.

4 MATERIALS AND METHODS

4.1 Study protocols and volunteers

4.1.1 Protocols

Studies I and II were carried out from January to April in 2016 at the Päijät-Häme Joint Authority for Health and Wellbeing, Department of Dermatology and Allergology, Lahti, Finland. The protocol consisted of two visits on consequent days (day 1 and day 2) for each volunteer. Some of the volunteers also attended the clinic one day earlier (day 0) to ensure that they fulfilled the inclusion and exclusion criteria. We collected demographic data on the participants and they completed questionnaires during their first research visits. The first NB-UVB radiation exposure was carried out in the morning of day one, between 7 and 9 a.m. and the second irradiation on the same day approximately 12 hours later. The results of irradiations in the skin were assessed next day (day 2). All the skin biopsies were taken 24 hours after each irradiation on day 2.

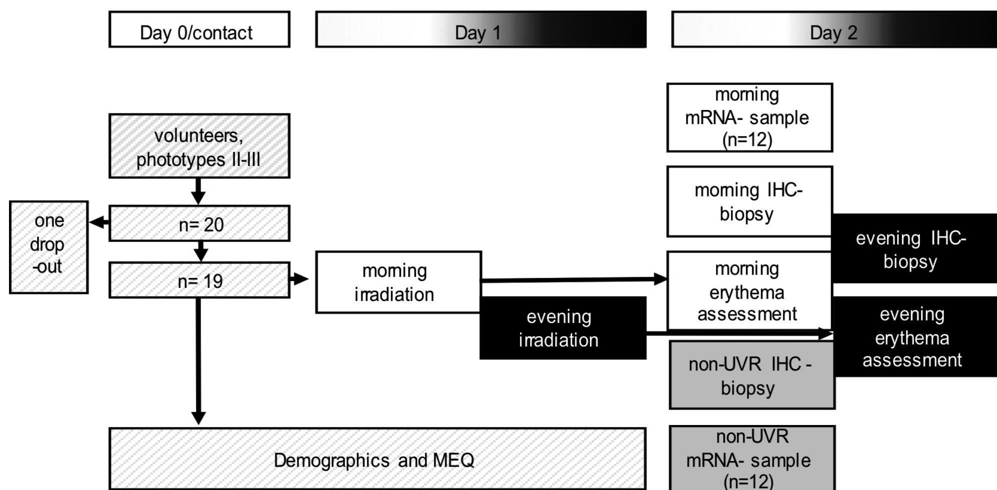


Figure 10. Study protocol in Studies I and II

Study III comprised two groups of volunteers (Groups A+B). As regards the volunteers in Group A, the protocol and target group were equal as in Studies I and II, implemented in 2016. The other Group B was recruited later in winter 2018-2019 with the intention of increasing the size of the total cohort, and in order to enable comparison of the non-irradiated biopsies at different times of the day namelys morning vs. evening. The photosensitivity was assessed equally in both Groups A and B, separately in the morning and in the evening, by performing a photo-test series. As regards the skin biopsy samplings, the groups were handled differentially, in Group A one biopsy from each volunteer was obtained in the morning from the non-irradiated skin site, whereas in Group B a total of two non-irradiated site biopsies were obtained, one in the morning and one in the evening.

4.1.2 Volunteers

In total of 20 healthy adult volunteers representing the most common skin phototypes in Finland (Fitzpatrick II and III) participated in 2016 (Studies I and II). They responded to our appeal for volunteers placed in the hospital weekly newsletter. The sample size (Studies I and II) was estimated using our best guess referring to earlier published *in vitro* and animal studies in the literature. Of the volunteers one was excluded at an early stage as the research protocol was reviewed and finally 19 volunteers completed the study. The exclusion criteria were use of photosensitizing, immunomodulatory or psychiatric medication. Individuals with pregnancy, lactation, photosensitivity, skin cancer or hypertrophic scars in their medical histories, or with marked UVR exposure in the past three months were also excluded. The anamnestic skin phototype was assessed according to a clinical interview. During the study and for at least 24 hours before no medication, was allowed.

As regards Study III Groups A+B, a total of 39 volunteers participated. Of these 19 belonged to Group A recruited in 2016, and 20 to Group B, who participated in winter 2018-2019. The same appeal and inclusion criteria were used, the only exception being that in Group B the anamnestic skin phototype could range from II to IV (Fitzpatrick 1988). In Group B the exclusion criteria were the same as in year 2016. During the study, birth control pills, thyroxin, hypercholesterolemia and hypertension medications were allowed but no other medication.

4.2 Methods

4.2.1 UVB irradiation (I-III)

UVB irradiation was performed in the form of MED testing on the skin of the buttocks. On day 1 the test area was exposed to NB-UVB in 1 cm^2 test square series, one series in the morning and the other in the evening. The NB-UVB MED testing series consisted of 1, 1.4, 2, 2.8, and 4 SED doses. The first irradiation series was carried out between 7 and 9 a.m. and the second 12 hours later on the skin of the other buttock. The rest of the skin was protected with impermeable plastic and fabric.

Waldman UV 801KL phototherapy device equipped with 4 TL20W/01 NB-UVB tubes, measured using an Ocean Optics S2000 spectroradiometer by the Nuclear Safety Authority of Finland, were used for the irradiations. Uncertainty (2σ) of the measurement of such spectroradiometer was estimated to be approximately 14% (95% confidence interval) (Ylianttila et al. 2005). The measurements can be traced to the National Institute of Standards and Technology (Gaithersburg, Maryland, USA). The time to irradiate each radiation dose was calculated based on irradiance measurements using SED units. One SED is equal to an erythemally weighted effective irradiation of 10 mJ/cm^2 (CIE, Comission Internationale de l'Eclairage, Vienna Austria), and is equivalent to a non-weighted physical irradiation dose of 172 mJ/cm^2 emitted from the NB-UVB lamps according to the measurements. According to the measurements, time for 1 SED irradiation was 97 seconds. The irradiation times of the MED series were calculated based on that time. To stabilize the irradiance the radiation tubes were preheated for seven minutes before the irradiations.

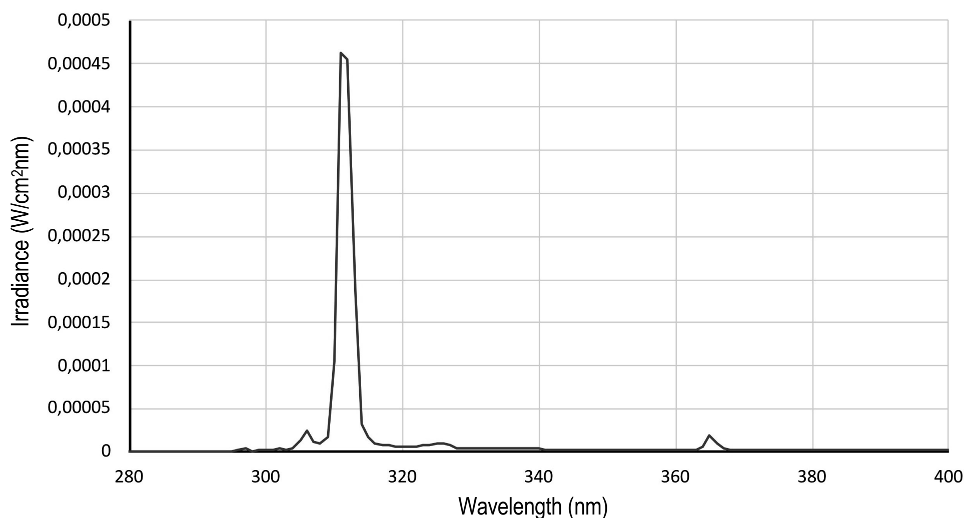


Figure 11. Spectrum of Waldmann UV 801KL Phototherapy device

4.2.2 Assessment of erythema (I-III)

Erythema was evaluated 24 hours after each UVB exposure. We initially determined the smallest UVR dose to induce barely perceptible erythema in the skin (MED) (Faurschou and Wulf 2009). If MED appeared to be higher than the maximum dose of 4 SED in our series, for subsequent analyses we defined the MED to be 5.6 SED i.e., the next dose of geometric UVR dose series where the incremental factor is √2. After defining the MED, we read out all the test squares (1, 1.4, 2, 2.8, and 4 SED) in both series using a DermaSpectrometer® (Cortex Technology, Handsund, Denmark). The DermaSpectrometer® is a reflectance spectrometer that illuminates an area of approximately 10 mm^2. The arithmetical mean values of the three read-outs were used in the analyses. Before the measurements, the device was repeatedly calibrated against controls as suggested in the manual. The DermaSpectrometer® reports the result in an erythema index (EI) that represents the intensity of the reflected red and green lights (Ly et al. 2020). It even detects erythema at UV irradiation doses below MED and has been widely used to evaluate erythema induced by UVR as well as other skin reactions (Diffey et al. 1984; Farr and Diffey 1984; Jemec and Johansen 1995; Wengström et al. 2004; Tejasvi et al. 2007; Qian et al. 2015). Each area was also scored according to the appearance of redness to the eye as “no erythema” to “barely perceptible erythema” and “intense erythema”. Both test series were read equally 12 hours apart, i.e., 24 from the NB-UVB exposures.

4.2.3 Assessment of chronotype (III)

The chronotype of the volunteer was assessed on the first days of the study, when participants completed a shortened version of Horne and Östberg's Morningness-Eveningness Questionnaire (MEQ) (Horne and Östberg 1976). The questionnaire includes six items (4,7,9,15,17, and 19 from the original MEQ, thus MEQ-6) (Hätönen et al. 2008). The sum of the scores on this questionnaire yields a Morningness-Eveningness Score (MES), ranging from 5 to 27 (Horne and Östberg 1976; Hätönen et al. 2008). The score corresponds to the original questionnaire, where higher sum scores indicate a trend towards morningness while lower scores indicate a trend towards eveningness.

4.2.4 Skin samples (I-III)

To take the 6 mm punch biopsies Lidocaine 1% was used for analgesia. All the biopsy samplings were performed 24 hours after irradiations equally in the morning and in the evening after having first read the MED. In Studies I and II, altogether two biopsies were obtained from the 4 SED irradiated skin sites: one from each volunteer in the morning (between 7 and 9 a.m.) and one in the evening (between 7 and 9 p.m.). We also took one non-irradiated control biopsy in the morning from each volunteer (between 7 and 9 a.m.) at a minimum distance of 10 cm from the irradiated photo-test sites (Figure 10). During irradiations the non-irradiated biopsy sampling site was protected against UV exposure with black impermeable plastic and fabric. The biopsy samples obtained were cut into two pieces with a scalpel each 3 mm in diameter to perform separate analyses as described later.

In Study I, all three samples (two irradiated and one control sample) of the 19 volunteers were used in analyses.

In Study II only the morning skin biopsy samples of 12 volunteers were used, these were the non-irradiated morning control samples and the NB-UVB irradiated (4 SED) morning biopsy samples.

In Study III, the aim was to study expression of melatonin and its receptors in non-UV-exposed healthy skin. As regards Group A (19 volunteers) the punch biopsy was taken from the non-irradiated skin a minimum of 10 cm away from the MED test skin area in the morning (between 7 and 9 a.m.). The samples were pooled with respective the non-irradiated skin control biopsies of Group B (20 volunteers) to form a larger study cohort Group A+B (n = 39) to increase power in analyses. In Study III Group B, we took an additional evening control non-irradiated punch

biopsy (between 7 and 9 p.m.) from volunteers (n=20) with the aim to assess the impact of different times of day on the outcome. These were obtained respectively a minimum of 10 cm away from the MED testing area. Biopsy protocols were identical for all biopsies.

4.2.4.1 Immunohistochemistry (I, III)

Immunohistochemical (IHC) staining was performed on all three biopsies of 19 participants in 2016 (I,III Group A) and on the morning and evening non-irradiated control biopsies of 20 participants in winter 2018-2019 (III Group B). The first halves of the 6 mm skin punch biopsy samples were fixed in 4% formalin, embedded in paraffin, and for further processing sectioned at 4 μm thickness. IHC staining was carried out according to the manufacturer's protocol with cryptochromes 1 and 2, p53, melatonin, and melatonin receptor MTNR1A and NQO2 antibody dilutions (Table 1.)

Table 1. Antibody dilutions in immunohistochemistry

Protein / receptor	Antibody dilution	Manufacturer
Melatonin	1:50	Abbexa, Cambridge, UK
MTNR1A	1:100	Abcam, Cambridge, UK
NQO2	1:200	Abbexa, Cambridge, UK
CRY1	1:100	Genetex, Irvine, USA
CRY2	1:50	Genetex, Irvine, USA
P53	1:1000	Leica Biosystems, Newcastle, UK

The specimens were evaluated by both the board-certified dermatopathologist Riitta Huotari-Orava M.D. and by the author (V.N.) of this thesis. There was a consensus among the evaluators. Each slide was assessed under microscopy (Olympus BX51, Tokyo, Japan) at 100 and 200 magnifications. The expression of proteins and receptors described above was evaluated using three grades: negligible (+), positive (++) and strongly positive (+++) to the naked eye. Positive findings (++ and +++) were combined and the level of expression was finally presented as two grades: low, consisting of negligible findings, and high, consisting of positive and strongly positive findings. The settings of the microscope and camera were held constant and the color balance of the pictures taken from the ICH findings was not modified.

4.2.4.2 RNA analyses (II)

We also studied gene expressions of the clock proteins, cytokines, pro-opiomelanocortin and melanocortin receptors via mRNA analysis from the non-irradiated morning control samples and 4 SED irradiated morning samples of a total of 12 healthy volunteers. The epidermis/dermis and the subcutaneous fat were cut apart from each other with a scalpel from the remaining half left over from the 6mm skin punch biopsies obtained 24 hours after the UVB irradiation on day 2. The specimens were placed in a separate Eppendorf test tubes in an RNAlater (ThermoFischer Scientific, Massachusetts, USA) for one day at +4°C, and were thereafter frozen and stored at temperatures from -80°C to -20°C for further mRNA extraction. All tissue samples were mechanically homogenized using Ultra-turrax homogenizer (IKA-Analysentechnik) in RLT buffer (Qiagen, Hilden, Germany). The centrifuging of the homogenates was performed twice at 12 000 x g for 10 minutes at +4°C temperature.

Gene expressions were analyzed using real time quantitative polymerase chain reaction (qPCR). AllPrep kit (Qiagen, Venlo, The Netherlands) was used for the isolation of total RNA from epidermis and dermis. High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) was used to reverse transcribe 400 μg of RNA to cDNA as described earlier (Wang et al. 2007). For the subcutaneous adipose tissue, VILO kit (Thermo Fisher Scientific/Invitrogen, Carlsbad, USA) was used to reverse transcribe 80 μg of RNA as instructed by the manufacturer. The expression of mRNA of the cytokines and circadian clock genes was studied by TagMan 7500 Fast System (Applied Biosystems) with PerfeCTa qPCR FastMIx (Quanta Biosciences, Gaithersburg, MD, USA). Ribosomal 18S gene was used as a housekeeping gene. polymerase chain reaction (PCR) primers and probes were received as predeveloped assay reagents from Applied Biosystems.

Table 2. Primers used for qPCR in Study II.

Primers used for qPCR		
Gene	Assay ID	Chromosome location
CRY1	Hs00172734_m1	Chr.12: 106991364 - 107093872
CRY2	Hs00323654_m1	Chr.11: 45847118 - 45883248
CIART	Hs00328968_m1	Chr.1: 150282533 - 150287093
IL-1β	Hs01555410_m1	Chr.2: 112829758 - 112836842
IL-6	Hs00985639_m1	Chr.7: 22725889 - 22732002
TNF	Hs99999043_m1	Chr.6: 31575567 - 31578336
POMC	Hs01596743_m1	Chr.2: 25160853 - 25168851
MC1R	Hs00267167_s1	Chr.16: 89917879 - 8992097
MC2R	Hs00300820_s1	Chr.18: 13882042 - 13915707

4.2.5 Statistical analysis

In Study I, the AUC used was computed with the trapezoidal method in terms of all UVB radiation doses. To model the effect of NB-UVB doses on the EI, we used Generalized Estimating Equations (GEE) analyses. The bootstrap method was used to estimate the standard errors. Differences within groups were analyzed by applying a permutation test or the McNemar test. Differences between groups were analyzed using a permutation test.

In Study II, for comparing of gene expressions in the biopsies with and without NB-UVB irradiation the regular one-way analysis of variance was used. The association between the expression of two different cryptochrome genes in UV-irradiated and the control the skin was examined using the Pearson's correlation method. In addition we used the Mann-Whitney test for the statistical analysis of target gene expression.

In Study III, statistical comparison between the groups was performed using permutation test and the Fisher-Freeman-Halton test. Repeated measures were analyzed using a permutation approach for analyzing repeated measures analysis of variance for continuous variables and exact symmetry test for categorical variables.

The descriptive statistics are presented as means with SDs or as counts with percentages. Statistical testing was performed using the statistical package of Stata 14.1 or 16.0, StataCorp LP (College Station, Texas, USA) (I), GraphPad Prism 7 Software (GraphPad Software Inc., San Diego, USA) (II).

4.2.6 Ethical aspects

The study protocol of this thesis was approved by the Regional Ethics Committee of Tampere University Hospital District (R16001) and the participating volunteers gave their written informed consent to participation.

5 RESULTS

5.1 Time of the day effects on UVB- induced erythema (I)

In Study I we compared skin erythema response and cryptochrome and p53 protein expressions by immunohistochemical (IHC) staining between morning and evening irradiation. The study population consisted of 19 volunteers and their skin samples as well as background demographics, which are presented in Table 3. We found skin erythema response but not clock protein expressions to differ in a circadian manner.

Using a reflectance spectrometer erythema was quantified to be more intense in the irradiation series conducted in the evening than in the morning ($p=0.015$) (Figure 13a). The difference was pronounced in small UVB doses and was not seen in the highest dose because the study population consisted of fair-skinned volunteers who showed strong erythema in 4 SED (Fitzpatrick 1988). MED was evaluated based on the physical appearance of redness as "no erythema" to "barely perceptible erythema" and "intense erythema". The difference in erythema was undetectable as assessed by the naked eye.

In the ICH of the skin, staining of both cryptochromes was cytoplasmic but CRY2 was seen more superficially in the epidermis than CRY1. In the ICH of the skin, we detected nuclear staining of p53.

We classified subjects according to their cryptochrome expression levels in the ICH of the non-irradiated control biopsy as seen in Table 5. We found that the erythema reactions measured using a reflectance spectrometer of subjects with high expression of CRY2 differed from those of subjects with low expression of CRY2 (Figure 13b). In subjects with low CRY2, according to reflectance spectrometer measurements of erythema, the skin reacted to UVB radiation so that in the evening it was more susceptible to radiation than in the morning. The amount of CRY1 in the control biopsy did not seem to have a similar effect.

No difference in protein expression was observed when comparing the levels of expression of cryptochromes after morning versus evening irradiations. However the amount of p53 in irradiated skin differed so that there was more p53 in the morning samples than in the evening samples ($p=0.039$).

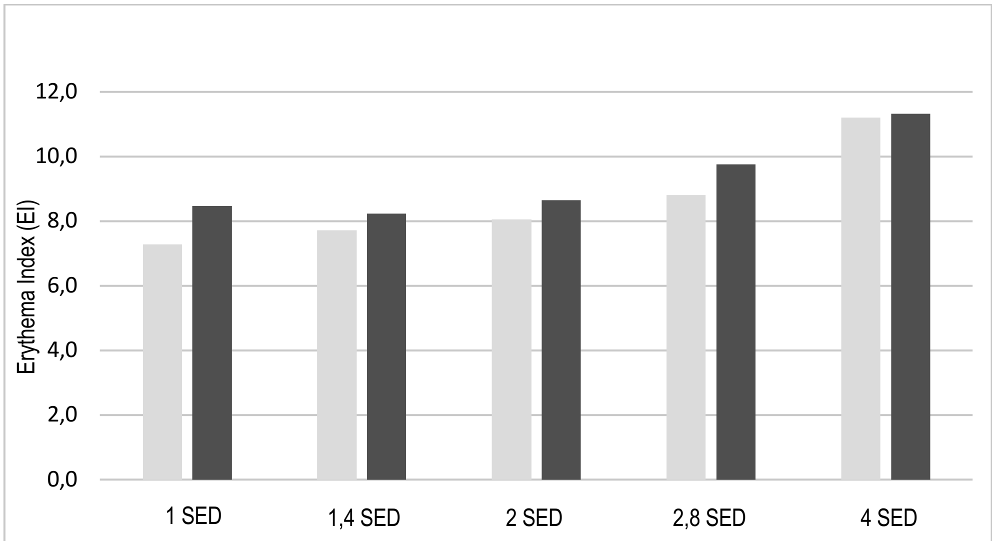


Figure 12. Mean Erythema Index of all doses of the MED test series. Morning data is shown in light and evening data in dark color.

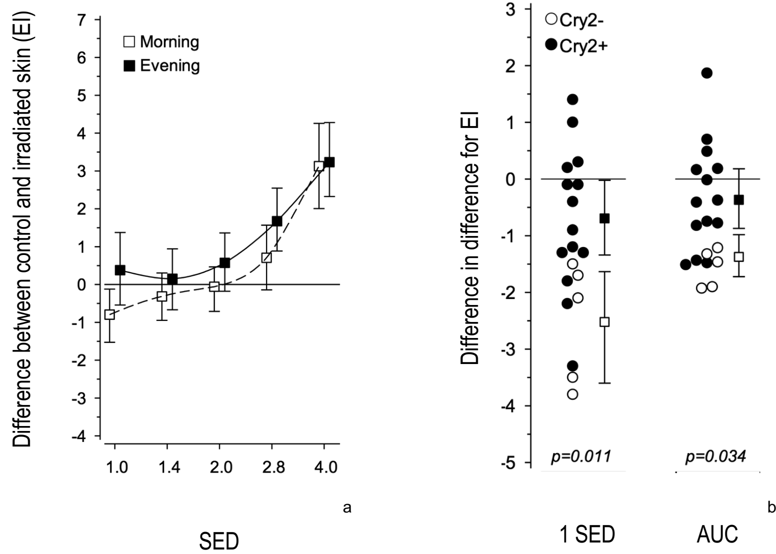


Figure 13. Impact of hour of the day and NB-UVB dose on erythema (a) and the effect of CRY2 (b). AUC = area under curve of all irradiation doses. (Nikkola et al. 2018)

Table 3. Demographic characteristics of the population in Studies I and III (Group A). Data are mean (range) or the number of subjects. BMI = body mass index; SD = standard deviation.

Population in Studies I and III (Group A)	
	n=19
Male/Female	3/16
Age (mean, range)	42 (22-64)
BMI ± SD	23,8 ± 3,7
Smokers	
Present	1
Earlier	3
Alcohol consumption	
< Once a week	15
≥ Once a week	4
Exercise	
≤ Once a week	4
> Once a week	15
Fitzpatrick's skin type II/III	8/11
Chronotype	
morning type	10
intermediate type	6
evening type	3

5.2 Effects of UVB radiation on clock genes (II)

In Study II, we investigated the possible effect of UVB on clock genes *CRY1, CRY2*, and *CIART* mRNA in epidermis/dermis and subcutaneous adipose tissue. We also investigated the effect of UVB on mRNA of cytokines *Interleukin-1β* (*IL-1β*), *Interleukin* 6 (*IL-6*) and *TNF* and of members of melanocortin system *POMC* and *MC1R*, *MC2R* also in both tissue samples. The study population of this report consisted of samples from 12 randomly selected volunteers from the population investigated in 2016 (n=19). Their demographics are presented in Table 4. We found that UVB changes mRNA expression in epidermis/dermis and even in adipose tissue.

As compared to non-irradiated skin, in the UVB irradiated skin the amount of *CRY2* mRNA expression was significantly diminished in epidermal/dermal skin (p=0.0492) while *CIART* and *CRY1* were increased in subcutaneous adipose tissue (p=0.0411, p=0.0263)(Table 5). UVB did not show a statistically significant effect on *CRY1* or *CIART* in epidermal/dermal skin or on *CRY2* in the subcutis. We found no variation in clock entrainment at mRNA level among chronotypes. A positive linear correlation between *CRY1* and *CRY2* mRNA was detected in epidermal/dermal skin (r=0.8436) and the ratio between *CRY1* and *CRY2* decreased after irradiation (p=0.0011). In other words, UVB clearly changed the *CRY1*/*CRY2* ratio. In subcutaneous adipose tissue, no such changes were seen.

As we assessed the amount of mRNA expression of proinflammatory cytokines the amount of *Interleukin-1β* (*IL-1β*) and *TNF*, but not of Interleukin 6 (*IL-6*) showed an increase after UVB irradiation at the level of epidermis/dermis. In subcutaneous adipose tissue no clear changes were seen. However, after UVB irradiation small changes were detected in *POMC* and *MC1R* mRNA expressions in all skin layers. The amount of *MC2R* mRNA did not differ.

Table 4. Demographic characteristics of the population in Study II. Data are mean (range) or the number of subjects. BMI = body mass index; SD = standard deviation.

Population in Study II	
	n=12
Male/Female	2/10
Age (mean, range)	41 (26-64)
BMI ± SD	23.5 ± 3.4
Smokers	
Present	1
Earlier	3
Alcohol consumption	
< Once a week	9
≥ Once a week	3
Exercise	
≤ Once a week	2
> Once a week	10
Fitzpatrick's skin type II/III	4/8
Chronotype	
morning type	7
intermediate type	3
evening type	2

Table 5. Classification of the population of Study I by immunohistochemical staining of cryptochromes in control (non-irradiated) biopsy and the population of Study II by changes of mRNA expressions of *CRY1*, *CRY2* and *CIART* genes after irradiation.

	Non-irradiated Skin N=19	**Irradiated Epidermis/dermis N=12**	**Irradiated Adipose tissue N=12**
	Numbers of subjects	*Change*	*Change*
CRY1/*CRY1*		No change	Increased
High	16		
Low	3		
CRY2/*CRY2*		Diminished	No change
High	14		
Low	5		
CIART/*CIART*	Not measured	No change	Increased

5.3 Effects of skin melatonin on UVB-induced erythema (III)

In Study III we investigated the association between epidermal melatonin and its receptors' expression and NB-UVB induced skin erythema, and if the level of these differed between morning and evening. Altogether 39 healthy volunteers completed Study III. In Group A we investigated the same 19 volunteers as in Study I and their demographics are presented in Table 3. In Group B we investigated 20 volunteers whose demographics are presented in Table 6.

In the ICH of the skin, the staining of melatonin was nuclear, and there was also some cytoplasmic positivity. Staining of NQO2 receptor was cytoplasmic with some nuclear positivity. Staining of MTNRIA receptor was membranous and slightly cytoplasmic. We classified the total population of this study (Groups A+B) according to their protein expression in the ICH of non-irradiated control biopsies as seen in Table 7.

There was more melatonin in the evening samples (Group B) than in the morning samples (Group A+B), when the amount in the IHC staining was graded in three, negligible (+), positive (++) or strongly positive (+++) ($p<0.001$). The erythema measured using a reflectance spectrometer was more pronounced in the evening than in the morning. The mean difference between the evening and morning erythema utilizing the whole test series was 1.4 EI units ($n=39$, 95% CI: 0.9 to 1.9; $p<0.001$).

Using a reflectance spectrometer the erythema measured for all the participants ($n=39$) classified by their melatonin expression, did not differ significantly ($p=0.08$). The graded amount (low vs. high) of melatonin in the samples did not explain the difference in erythema between morning and evening.

The expression of MTNR1A and NQO2 was rather similar in the morning and in the evening ($n=39$, $p=1.0$ and $p=0.50$ respectively). There was no correlation between the number of NQO2 or MTNR1A receptors vs. the NB-UVB induced erythema. Neither did the amount of MTNR1A nor NQO2 explain the difference in erythema in the morning vs. evening samples.

We also studied if the chronotype was associated with epidermal melatonin expression. Of the 39 participants, 21 displayed morning, eight evening and 10 intermediate chronotype (Horne and Östberg 1976). Their total Morningness-Eveningness score (MES) ranged from 9 to 25 points. There was no association of the IHC staining readouts (melatonin, MTNR1A or NQO2) or erythema with the MES or with the chronotype.

Table 6. Demographic characteristics of Group B in Study III. Data are mean (range) or the number of subjects. BMI = body mass index; SD = standard deviation.

	Group B in Study III
	n=20
Male/Female	0/20
Age (mean, range)	43 (22-65)
BMI ± SD	25.1 ± 5.1
Fitzpatrick's skin type II/III/IV	5/12/3
Chronotype	
morning type	11
intermediate type	4
evening type	5

Table 7. Immunoreactivity of melatonin, MTNR1A and NQO2

Antibody	Immunoreactivity	Morning (n=38-39)	Evening (n=20)
Melatonin	Negligible	8	0
	Positive	26	5
	Strongly positive	4	15
MTNR1A	Negligible	4	0
	Positive	30	20
	Strongly positive	5	0
NQO2	Negligible	9	1
	Positive	19	12
	Strongly positive	11	7

6 DISCUSSION

The skin is a major organ protecting the human body against potentially hazardous electromagnetic exposures from the sun and other sources. In general, the optimal operation of the circadian clock seems important for human health. The present dissertation study is among the first studies to evaluate the interactions between biologically active electromagnetic irradiation and the circadian clock in the human skin *in vivo*.

NB-UVB was chosen for the irradiations because its electromagnetic spectrum contains negligible amounts of visible light or UVA, as it is known that various wavelengths differ in their mode of action and responses. The focus was thus also on the impacts of NB-UVB because it is currently the most used UV source in phototherapy in dermatology clinics. We also wanted to alleviate the impact of visible light on the results.

We created a study protocol proposing that UVB irradiation applied to the skin is a potential pacemaker of the circadian clock of the epidermal cells, and that UVB may interact through the skin with the internal circadian clock too. In other words, we proposed that UVB could produce an interaction with the circadian clock rather similar to that of visible light which produces its main effect through the eyes (Roenneberg et al. 2003a; Golden et al. 2005). We also anticipated that the physiological response of the skin to UVR would vary according to the time of day, and that an individual's circadian profile may influence this. The knowledge from this study could be used, for example in optimizing the timing of phototherapy, in other treatments for skin diseases, treatments of skin cancers, and even more in the assessment of the dangers of sunbathing.

6.1 Circadian time and erythema (I)

In Study I we demonstrated that UVB irradiation induces less erythema in the morning than in the evening. According to our findings, UV radiation may be better tolerated in the morning hours. The circadian clock exists so that each function of the human body has its own optimal schedule. For example, because ancient humans lacking artificial light had an optimal time for physical activities in daylight, our ability for physical and mental performance is still at its best in daytime jobs (Rajaratnam and Arendt 2001). By the same token, it is not a surprise that the optimal timing for UVB exposure and the best protection against DNA damage according our Study I seems to be morning hours. This is the time, when the body has just rested during the night and the blood cortisol levels are at their highest to supply energy for daily activities in the sun.

We detected in human volunteers UVB-induced erythema to be dependent on circadian time. Studies on mice have clearly shown the same: UVB-induced erythema and susceptibility to DNA damage signals varied during the day and peaked in the morning (Geyfman et al. 2012; Gaddameedhi et al. 2015). Although we found erythema to peak in the evening, our finding is in line with previous findings, because mice and humans express opposite circadian phases (Spörl et al. 2012). Despite opposite phases, human epidermal clock genomics correlate well with that from mouse tissue (Wu et al. 2018). No other *in vivo* studies comparing circadian clock related NB-UVB induced erythema in humans have so far been presented. For comparison, in cultured human keratinocytes UVB-induced damage has also been shown to be circadian clock related. Loss of normal circadian function due to depletion of clock proteins caused suppression of DNA protective mechanisms (Sun et al. 2018). As erythema reaction after NB-UVB likely indicates DNA damage, the results of the former cellular study support our main findings (Freeman et al. 1989; Young et al. 1998; D'Orazio et al. 2013). The mechanisms behind this intermittent erythema and DNA damage response are not clear, but likely have something to do with photoprotection.

We proposed that cryptochromes may act as photoprotectors in human skin. In our study we found that the amount of CRY2 in the control biopsies taken in the morning, at the same time as the morning irradiation correlated with the circadian erythema response: less CRY2 was related to more erythema (Study I). Interestingly, one *in vitro* study has also shown a correlation between lack of CRY2 and damage response in cultured fibroblasts; the cells without CRY2 accumulated DNA damage (Papp et al. 2015). Cryptochrome homologs in lower organisms serve as protective

agents when UVB radiation causes DNA damage (Todo et al. 1996). However, it has been proven in the early stages of human circadian clock research, that CRY1 and 2 cannot directly serve as enzymes of DNA repair when UVR related damage occurs in humans (Hsu et al. 1996). One possible explanation for our finding is that a certain phase of the circadian clock protects against erythema because the amount of CRY2 reflects the phase of the skin clock (Sandu et al. 2012). In fact several damage responses, including the actions of NER, to UV radiation are controlled by the circadian clock and they likely are more efficient at certain times of the day (Dakup and Gaddameedhi 2017). This theory of a particular phase of the clock protecting against the adverse effects of UVB could be further investigated by monitoring the circadian phases of individuals while exposing them to NB-UVB in controlled conditions. As the core circadian machinery varies between individuals, such research would entail planning of personalized optimal timing of UVB exposure.

In Study I we detected more p53 protein in irradiated morning samples than in evening samples and Study II showed *CRY2* mRNA to decrease in the top layers of the skin due to irradiation. In mouse cell cultures it has been shown that UVB-induced p53 is circadian time-dependent (Kawamura et al. 2018) and both cryptochromes affect sensitivity of p53 to stress (Papp et al. 2015). Our finding could be connected to circadian clock time shift activation by UVB and simultaneous erythema protecting activation of the DNA damage response pathway. Could a different circadian time shift in the morning versus evening affect erythema response? We unfortunately do not know if UVB would initiate less *CRY2* mRNA production in the evening when p53 cumulated epidermis in smaller amounts, because we did not detect *CRY2* mRNA changes due UVB in the evening. However, suboptimal function of the circadian clock generally leads to susceptibility to UVR-induced stress (Kawamura et al. 2018), and the entrainment of the skin clock by UVB is possible according our findings in Study II.

We detected less erythema but more tumor suppressor p53 protein in the morning in Study I. Others have shown that UVB can be immunosuppressive even in suberythemal doses (Hart and Norval 2018). There is mounting evidence to show that the timing of medication and non-pharmacological treatment influences the outcome. Some of these effects have even been connected to certain core circadian gene activities (Dallmann et al. 2016; Montaigne et al. 2018). In light of these facts, NB-UVB phototherapy could be more effective with less adverse effects when administered in the morning hours. In the future, as best advantage and minimal adverse effects are warranted in the field of dermatological treatments, chronopharmacology should not be discounted.

6.2 UVB- radiation and clock proteins (II)

Our study suggests that UVB radiation can act as an entrainment for circadian clock in both epidermal/dermal skin and in the subcutis. To fully understand the entity of human circadian machinery it is important to investigate circadian clock entrainment. Visible light is the most powerful Zeitgeber, but some others, such as UVR, are involved. While the mechanism behind this kind of circadian clock regulation is not yet fully understood, some earlier research on skin cell cultures and the fact that stress in general entrains peripheral circadian clocks predicted that clock proteins would be effected by NB-UVB (Kawara et al. 2002; Tahara et al. 2017; Sun et al. 2018). However, the results from cell culture studies cannot be generalized to the level of living skin, because the local environment may affect clock proteins (Noguchi et al. 2012; Williams et al. 2018). This stresses the importance of our *in vivo* study and there is a demand for even more studies with human volunteers.

In Study II we detected changes in *CRY1*, *CRY2* and *CIART* mRNAs both in epidermal/dermal skin, and also in subcutaneous fat due to UVB irradiation. In Study I less CRY2 protein was related to more erythema. No other studies measuring clock proteins or their mRNA in skin samples after UV irradiation have been presented. In cultured human keratinocytes, the principal cells of the epidermis, UVB downregulated *ARNTL*, *CLOCK,* and *PER1* mRNA expression (Kawara et al. 2002), but when it comes to fat cells not even cell culture studies on clock gene expression have been presented. However, in general, clock in adipocytes can be regulated by some external factors such as food intake, disrupted sleep patterns and sleep deprivation (Shostak et al. 2013).

One important question concerns how the change detected in clock proteins actually occurs. The changes in clock proteins may be mediated directly through supposed circadian photoreceptors, such as opsins and/or CRY2. A link has been detected between some opsins and clock proteins in mouse melanocytes (de Assis et al. 2016, 2019). Moreover, research over ten years ago already suggested an opsin mediated system is present in human skin to detect UVR and visible light (Tsutsumi et al. 2009). In recent years, however, there have been contradictory results on whether OPN3, formerly a potential photoreceptor candidate, can sense UV radiation in different cells of the skin (Regazzetti et al. 2018; Ozdeslik et al. 2019; Lan et al. 2020). A comprehensive review still summarized that irradiation by UV wavelengths can mediate skin functions, especially melanogenesis via OPN3 (Olinski et al. 2020). New studies connecting UVB-induced core circadian clock mRNA changes and OPN3 in human skin are needed to resolve this issue.

In Study II, we detected *CRY1* and *CRY2* gene expression to be in approximately same the proportion to that detected earlier in human retina (Thompson et al. 2003). Based on the cryptochrome signaling mechanisms (Vieira et al. 2012), it is possible that even they themselves serve as UVB sensing agents in the skin. This hypothesis is also supported by the absorption spectrum of CRY2 binding with FAD (Figure 9) that matches the UVB radiation wavelength (Liu et al. 2010; Vanderstraeten et al. 2020). In the light of current evidence, UVB mediated change in the cryptochromes of the skin could be mediated by CRY2 in epidermal keratinocytes or even by opsins in melanocytes (Olinski et al. 2020; Vanderstraeten et al. 2020).

We detected different changes in clock genes *CRY1*, *CRY2* and *CIART* in two separate skin layers; epidermal/dermal skin samples versus subcutaneous adipose tissue (Study II). According to our measurements of cytokine *IL-1, IL-6,* and *TNF* mRNA levels in the same study there occurred an inflammation in the top layers of the skin but not in the adipose tissue. The fact that UVB can induce production of these cytokines in the outer layers of the skin is not a new finding (Slominski and Wortsman 2000). In addition, Eun Ju Kim and colleagues demonstrated earlier that cytokines can transmit the influence of UV irradiation into the subcutis (Kim et al. 2011). NB-UVB only penetrates the epidermal and dermal skin and none of it directly reaches the subcutis (Bruls et al. 1984; Meinhardt et al. 2009). We therefore think that the inflammation was mediated by cytokines on the circadian clock in the subcutaneous fat.

We found the amounts of *CIART* and *CRY1* mRNA to increase in subcutaneous adipose tissue due to NB-UVB irradiation. Although others have shown core circadian clock gene activity in adipose tissue, our study was the first to show change in clock gene expression in human adipose tissue due to UV radiation exposure. In fact, when CIART was first identified as an important part of clock protein machinery, Akihiro Goriki and colleagues supposed that it played an important role in regulating the metabolic pathways triggered by stress response (Goriki et al. 2014) and indeed we found the amount of *CIART* gene expression to change after significant stress response, UV irradiation. Our study supports the theory that CIART mediates the stress response to core circadian homeostasis through the skin. When studying adipose tissue, the relationship of the circadian clock to obesity is an interesting aspect. Differences in the amount of *CRY1* but not in *CIART* mRNA in muscle tissue have been detected in obese vs. non-obese individuals (Puig et al. 2020). Our study together with the research by Laura Sardon Puig and colleagues suggests that *CRY1* may be involved in the metabolism of subcutaneous fat even via

UVB irradiation, but more studies confirming our findings and linking *CRY1* with adipose tissue problems are needed.

We found no variation in clock entrainment at mRNA level among chronotypes. Aspects influencing the entrainment of the clock include among others individual differences in the efficacy of different Zeitgebers, free-running circadian period length and synchronization (entrainment) of Zeitgebers with intrinsic time (Roenneberg et al. 2003a). Because individual relationships of clock period length and dynamics with the environment create different phases of entrainment or chronotypes (Roenneberg et al. 2003b), we would expect to detect some variation in clock entrainment among the chronotypes. Our result may be due to the relatively small number of volunteers and variation in chronotype or the simple way to classify individual differences in the circadian clock. The various aspects mentioned, among individual chronotypes, should be taken into account in future studies on UVR-induced clock entrainment by measuring volunteers' individual circadian features, for example by using body temperature or dim light melatonin onset (DLMO).

According to our findings in Study II, altered gene expressions in the skin after UVB irradiation suggest that UVB can act as an external time cue to the circadian clock in the skin and adipose tissue. Earlier studies have shown that local circadian rhythm influences the susceptibility to damage by oxidative stress and radiation of various skin cell types (Sherratt et al. 2019). Suboptimal function of the circadian machinery can lead to increased cell proliferation and tumor formation in epidermal and dermal skin as mice exposed to UVB at an unfavorable time of the day expressed more tumors (Gaddameedhi et al. 2011). Human adipose tissue seems to contain a circadian clock that controls its metabolism (Christou et al. 2019). Changes in the subcutaneous adipose tissue clock can even predispose an individual to metabolic syndrome (Hernandez-Morante et al. 2012). Hence we believe that some of the unwanted effects on human health mediated by UVB radiation may occur at least in part via the circadian machinery. Considering that UVB also has other systemic effects (immune balance, mood and addiction (Fell et al. 2014; Skobowiat and Slominski 2015)) it seems possible that UVB irradiation has role in maintaining circadian homeostasis through skin clock proteins.

6.3 Skin melatonin and erythema (III)

The results from Studies I and III showed that Erythema index (EI), i.e. redness of the skin, was significantly lower in skin irradiated in the morning than in skin irradiated in the evening. Our results from Study III suggest that endogenous skin melatonin seems not to be the reason behind this variation because melatonin expression in the skin (IHC) did not correlate with the variation in erythema sensitivity. A new interesting finding in our Study III was that expression of epidermal melatonin in the non-UV-irradiated skin on the buttocks is different in the morning from that in the evening. The outcome adds information to explain the physiology of epidermal melatonin in human skin *in vivo*.

We found that melatonin expression in the skin (IHC) did not correlate with the change in erythema sensitivity (Study III). In our IHC staining, melatonin was located mostly in the epidermis. Contradicting our finding, erythema preventing actions of melatonin were earlier detected when studying the properties of topical melatonin treatment. Application of melatonin gel before UVB irradiation was first shown to reduce erythema in 1996 (Bangha et al. 1996). Two decades later, when a better dosage form for radical scavenging was investigated, melatonin strongly protected cultured human keratinocytes against UVB radiation (Marto et al. 2016). In fact several studies have shown that topical melatonin significantly prevents both UVB and natural sunlight induced erythema when applied before UV exposure (Scheuer et al. 2014, 2016a). Topically applied melatonin seems to work specifically in the stratum corneum, the topmost layer of the skin, rather than in deeper in the epidermis (Marto et al. 2016). In Study III we did not detect melatonin in the stratum corneum. Accordingly, the contradictory findings with Study III and previously published topical melatonin administration studies may be due to different location of melatonin in the skin. Melatonin absorbs radiation in wavelengths of UVB (280–320 nm) (Nickel and Wohlrab 2000). Melatonin thus involves UVB absorption capacity, which can protect the skin from sunburn when applied thickly enough on the skin. However, according to our new results, the naturally expressed melatonin detected in the deeper layers of the epidermis, may be located too deep in the skin as regards the penetration capacity of the UVB waves in the skin. Furthermore, the natural melatonin may be present in too low concentrations to act as a definite sun screen, but this could be examined in greater detail in our future studies with different UV wavelengths including UVA and broad-band UVB.

We found that melatonin content of the non-irradiated skin of the buttocks varied between morning and evening. There was more melatonin in the evening than

in the morning. This fluctuation resembles the pattern of pineal expression of melatonin that begins in the evening and peaks at night (Reiter 1991). According to earlier studies extra-pineal melatonin expression may be steady, oscillating or fluctuate in a circadian manner (Acuña-Castroviejo et al. 2014). As regards the extra-pineal localizations, rhythmic melatonin expression has been detected in retina and thymus, both in melatonin content peaking at night (Naranjo et al. 2007; Tosini et al. 2007). However, extra-pineal sites can express melatonin oscillation that does not vary in a circadian manner (Venegas et al. 2012) and pineal melatonin has only limited access from blood to the skin due to its degradation in the liver (Slominski et al. 2017a). According to our findings, the melatonin content of the skin may follow the fluctuation pattern of circulating melatonin. To learn more, in future studies we could study the melatonin content of the skin at several time points on consecutive days using a quantitative method like mass spectrometry to confirm whether epidermal daily fluctuation of melatonin is circadian or not. Blood melatonin levels should be measured in parallel.

Using ICH staining, we demonstrated that melatonin was mainly nuclear and to a small extent cytoplasmic. In an earlier human study, melatonin was found in keratinocytes and to be cytoplasmic in the skin of the scalp (Slominski 2005). Serotonin, a precursor of melatonin was likewise cytoplasmic and membranous in the same anatomical location (Slominski et al. 2020). Melatonin has a lipophilic structure, which enables it to cross the biological barriers and exert its influence on the mitochondria, cytosol and nucleus in the cell. Distribution of melatonin in different parts of the cell has been shown to differ depending on the tissue investigated. For example in the cerebral cortex the concentration of melatonin is highest in mitochondria, whereas in the liver it is highest in the cell membranes. In both tissues there was a marked amount of melatonin in the nuclei (Venegas et al. 2012). In the skin of the scalp melatonin may have actions related to hair growth via mitochondria (Rusanova et al. 2019). This may explain the dominating cytoplasmic location. Different immunolocalizations of melatonin as found in our study versus the earlier findings of Slominski and colleagues (Slominski 2005) may thus be due e.g. to the different anatomical locations of the skin biopsies taken. We posit that the melatonin responsible for cellular functions in human buttocks skin seems to be located mainly in the nucleus.

We detected no association between melatonin receptors (MTNR1A, NQO2) and erythema formation in Study III. We found melatonin in different parts of keratinocytes compared to its receptors. According to cell culture studies in the literature, ROS scavenging may be the mechanism behind the UVB protecting

actions of melatonin (Marto et al. 2016; Reiter et al. 2016). In human skin cell cultures (Janjetovic et al. 2014, 2017) as well as in animal studies (Slominski et al. 2014), the anti-inflammatory mechanism behind diminished erythema has been reported to be mediated independently of melatonin receptors, by reduction in ROS. In line with earlier research, we assume that, as melatonin and its receptors are detected in different parts of the cell, it seems that the actions of epidermal melatonin are not mediated, at least to a large extent, by MTNR1A and NQO2 receptors. Our finding that melatonin expression of epidermal skin was not significantly connected to erythema sensitivity does not rule out other types of UV protective, but receptor-free actions of melatonin. Finally, there are diverse mechanisms of action to prevent erythema, some of which may work more effectively in the morning hours. These phenomena need further detailed studies, including e.g. mRNA transcriptome sequencing in the morning compared to corresponding analysis in the evening and by measuring the number of reactive oxygen and nitrogen species.

In Study I higher level of p53 was detected after UVB irradiation in the morning than in the evening. Interestingly we did not detect melatonin amounts to be elevated at the same time of day as p53. Earlier studies have shown tumour suppressor protein 53 triggered by melatonin to be involved in UVB-induced radical scavenging action in human melanocytes (Denat et al. 2014; Janjetovic et al. 2017) and keratinocytes (Janjetovic et al. 2014). However, *in vivo* many simultaneous processes protect the skin from UVB induced damage.

6.4 Chronotype and erythema (III)

In Study III, we detected no connection between sensitivity to UVB-induced erythema and chronotype. Previous studies have found that skin temperature, as well as β-END and IL-6 detected in epidermis using IHC as a response to NB-UVB exposures were associated with individual chronotypes (Toledo et al. 2019; Weidenauer et al. 2019). Overall skin temperature is higher among morning chronotype individuals than among the evening types (Weidenauer, Vollmer, Scheiter, & Randler, 2019). The mood state related properties of NB-UVB and IL-6 responses to NB-UVB exposure also seem to depend on chronotype (Toledo et al. 2019). According to earlier studies, we expected to find a connection between chronotype and erythema. The reason why we did not find that connection may be due to our simple way of classifying chronotype.

In Study III, we found no correlation between epidermal melatonin fluctuation and chronotype. Xianchen Liu and his colleagues have earlier shown that a late-timed peak of melatonin in the circulation was associated with eveningness (Liu et al. 2000). Taking into account that melatonin is endogenously produced in keratinocytes and has limited access from the circulation to the skin (Slominski et al. 2017a), the melatonin we detected in keratinocytes may be a mixture of both origins, the blood and the skin. Unfortunately, in our present study, we did not take blood samples, but this could be done in future. Based on our finding that chronotype did not correlate with the melatonin level detected in the epidermal skin biopsies, expression of melatonin in the skin seems different from pineal melatonin expression. Further studies on the relationship between the circulating and epidermal levels of melatonin are needed to ascertain if they are connected with each other.

Evening chronotype individuals rather schedule their daily activities for the evening than for the midday hours (Adan et al. 2012). They may spend time in the sun later than morning type individuals. As in Studies I and III we detected more intense erythema in the evening than in the morning, such habits may make UVB erythema protection more important to evening type individuals than it is for others.

6.5 Limitations and strengths

Due to the invasive nature of the *in vivo* study on healthy volunteers, where biopsies were taken, the number of volunteers was limited. The focus of studies was on earlier unstudied aspects, which made it difficult to predict the outcomes.

The chronotype was characterized by a short MEQ-6 questionnaire (Hätönen et al. 2008). In future studies further quantitative and more objective assessment methods, such as dim light melatonin onset (DLMO) or body temperature recording, could be implemented to confirm the chronotype (Lewy et al. 1999; Bonmati-Carrion et al. 2014).

One limitation of Studies I and II was the lack of control biopsy in the evening. This was due to the need to reduce the invasiveness of the study. Therefore, only the morning results had a non-irradiated control sample. This inhibited conclusions on the impacts of NB-UVB exposures in different time windows in Study II. Therefore, in Study III, we recruited a further cohort of 20 volunteers, in addition to the former 19 volunteers, and from these 20 volunteers (Group B) both morning and evening non-irradiated samples were taken. In future studies determining mRNAs using an increasing erythema intensity series might also increase validity, but since the number of samples from one volunteer is limited, one way to improve the replicability of the outcome is to increase the number of volunteers.

One limitation of present study is also that there may be unknown factors influencing circadian clock in the skin that could not be controlled for during the study. Diet, exercise, stress and temperature seem to have direct effects on peripheral clocks in general, but their influence on clock proteins and circadian time in the skin is unknown (Schibler et al. 2003; Buhr et al. 2010; Tahara et al. 2017).

Quantifying the clock protein, p53, and melatonin levels of the skin using ICH is not the most accurate method. To improve validity all samples were evaluated by the same scientists including a board-certified dermatopathologist and we studied clock protein activity with two different methods, protein expression with IHC and mRNA expression, which complement each other.

Some of the strengths of this thesis can also be highlighted. Firstly, as a topic circadian clock in the skin is new and not enough is known about its actions. Another strength is the use of healthy human volunteers. Earlier studies have mostly been conducted using cell cultures and animal samples. Moreover, measuring erythema not only with the naked eye but also quantifying it by reflectance spectrometer proved to be a strength of the study design. As regards the dermatology field, putting

more emphasis in future on various chronobiology aspects seems urgent. The present studies increase our understanding that timing of therapy according to time of day may be of significance for the outcome. The study also shows that the skin is truly an interactive discussing organ with an incredible capacity to produce biological responses linked to the whole body. There remain numerous ideas for further study in the future.

7 CONCLUSIONS AND FUTURE PROSPECTS

The aim of this thesis was to investigate a possible association between the effects of UVB exposure and the human circadian clock. The connections were addressed by studying erythemal responses to UVB exposures at different times of the day, by measuring NB-UVB induced mRNA expression changes of main circadian genes, and by detecting epidermal melatonin content in the light of erythema reactions.

The results showed that, due to the functions of circadian clock in the skin, the degree of erythema induced by NB-UVB exposures depends on the time of the day. We investigated melatonin as a possible erythema protecting factor, but it turned out not to be the reason behind different erythema reactions between morning and evening. For the first time we showed difference in the expression of epidermal melatonin to vary depending on the time of the day. Like the circadian clock in general, the clock in the skin and in the subcutis also were adjustable. We showed that NB-UVB has the capacity to influence the clock machinery.

The information from the present study is relevant from two perspectives. First, the understanding of the internal clock machinery as a part of the erythema control of the skin can be of advantage when optimizing the benefits and diminishing the side-effects of dermatological NB-UVB phototherapy or even sun exposure. However, this needs further study with patients in need of phototherapy.

The result that NB-UVB exposure may pace the circadian clock should be taken into account when studying circadian functions of the skin and even the whole body. To refine the effect of UV radiation of circadian parameters, all other pacemakers should be controlled, which remains a huge challenge for future studies. One interesting confounding factor is the interindividual variation between chronotype and natural circadian period length among individuals. According to earlier findings, these differences should be taken into account when evaluating the effect of a defined pacemaker such as UV radiation on internal circadian clock.

The tree studies of this doctoral thesis support earlier observations from animal and cell culture studies suggesting that the skin maintains its own circadian machinery to regulate e.g. the acute effects of UVB radiation. The underlying mechanisms are complex but may also involve entrainment of the clock by UVB

among other clock protein functions and the anti-inflammatory properties of melatonin.

The information provided by this thesis could be used to initiate future dermatological studies targeting to prevention of skin cancer. The risk of erythema and skin cancer development may be increased when the skin is repeatedly exposed in the evening to erythematous amounts of UVB. Conversely, NB-UVB phototherapy may cause fewer side effects when used in the morning. Keeping the circadian homeostasis optimal seems to be beneficial in the prevention of various diseases. As we showed that NB-UVB radiation can have an impact on the molecular circadian clock of the skin and adipose tissue, the preventive functions may be more extensive than merely the prevention of erythema mediated actions.

REFERENCES

Acuña-Castroviejo D, Escames G, Venegas C, Díaz-Casado ME, Lima-Cabello E, López LC, et al. Extrapineal melatonin: Sources, regulation, and potential functions. Cell Mol Life Sci. 2014;71:2997–3025.

Adan A, Archer SN, Hidalgo MP, Milia L Di, Natale V, Randler C. Circadian typology: A comprehensive review. J Chronobiol Int. 2012;29:1153–75.

Allebrandt K V., Teder-Laving M, Kantermann T, Peters A, Campbell H, Rudan I, et al. Chronotype and sleep duration: The influence of season of assessment. Chronobiol Int. 2014;31:731–40.

Anafi RC, Lee Y, Sato TK, Venkataraman A, Ramanathan C, Kavakli IH, et al. Machine Learning Helps Identify CHRONO as a Circadian Clock Component. PLoS Biol. 2014;12:e1001840.

Antoch MP, Gorbacheva VY, Vykhovanets O, Toshkov IA, Kondratov R V., Kondratova AA, et al. Disruption of the circadian clock due to the Clock mutation has discrete effects on aging and carcinogenesis. Cell Cycle. 2008;7:1197–204.

Antoch MP, Song E-J, Chang A-M, Vitaterna MH, Zhao Y, Wilsbacher LD, et al. Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. Cell. 1997;89:655–67.

Antoch MP, Toshkov I, Kuropatwinski KK, Jackson M. Deficiency in PER proteins has no effect on the rate of spontaneous and radiation-induced carcinogenesis. Cell Cycle. 2013;12:3673–80.

Aoki K, Stephens DP, Zhao K, Kosiba WA, Johnson JM. Modification of cutaneous vasodilator response to heat stress by daytime exogenous melatonin administration. Am J Physiol - Regul Integr Comp Physiol. 2006;291:619–24.

Aschoff J. Circadian rhythms in man. Science. 1965;148:1427–32.

de Assis LVM, Moraes MN, de Lauro Castrucci AM. The molecular clock in the skin, its functionality, and how it is disrupted in cutaneous melanoma: a new pharmacological target? Cell Mol Life Sci. 2019;76:3801–26.

de Assis LVM, Moraes MN, da Silveira Cruz-Machado S, Castrucci AML. The effect of white light on normal and malignant murine melanocytes: A link between opsins, clock genes, and melanogenesis. Biochim Biophys Acta - Mol Cell Res. 2016;1863:1119–33.

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron. 2001;30:525–36.

Bangha E, Elsner P, Kistler GS. Suppression of UV-lnduced erythema by topical treatment with melatonin (N-acetyl-5-methoxytryptamine). A dose response study. Arch Dermatol Res. 1996;288:522–6.

Bass J, Takahashi JS. Circadian integration of metabolism and energetics. Science. 2010;330:1349–54.

Bernard JJ, Gallo RL, Krutmann J. Photoimmunology: how ultraviolet radiation affects the immune system. Nat Rev Immunol. 2019;19:688–701.

Bjarnason GA, Jordan RCK, Wood PA, Li Q, Lincoln DW, Sothern RB, et al. Circadian Expression of Clock Genes in Human Oral Mucosa and Skin Association with Specific Cell-Cycle Phases. Am J Pathol. 2001;158:1793–801.

Black A, Greaves M, Hensby C, Plummer N. Increased prostaglandins E2 and F2alpha in human skin at 6 and 24 h after ultraviolet B irradiation (290−320 nm). Br J Clin Pharmacol. 1978a;5:431–6.

Black AK, Greaves MW, Hensby CN, Plummer NA, Warin AP. The effects of indomethacin on arachidonic acid and prostaglandins E2 and F2alpha levels in human skin 24 h after u.v.B and u.v.C irradiation. Br J Clin Pharmacol. 1978b;6:261–6.

Bøggild H, Knutsson A. Shift work, risk factors and cardiovascular disease. Scand J Work Environ Heal. 1999;25:85–99.

Boniol M, Autier P, Boyle P, Gandini S. Cutaneous melanoma attributable to sunbed use: systematic review and meta-analysis. BMJ. 2012;345:e4757.

Bonmati-Carrion MA, Middleton B, Revell V, Skene DJ, Rol MA, Madrid JA. Circadian phase asessment by ambulatory monitoring in humans: Correlation with dim light melatonin onset. Chronobiol Int. 2014;31:37–51.

Boutin JA, Ferry G. Is there sufficient evidence that the melatonin binding site MT3 is quinone reductase 2? J Pharmacol Exp Ther. 2019;368:59–65.

Brown SA, Zumbrunn G, Fleury-Olela F, Preitner N, Schibler U. Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Curr Biol. 2002 Sep;12:1574–83.

Bruls W, Slaper H, van der Leun J, Berrens L. Transmission of human epidermis and stratum corneum as a function of thikness in the ultraviolet and visible wavelengths. Photochem Photobiol. 1984;40:485–94.

Buhr ED, Vemaraju S, Diaz N, Lang RA, Van Gelder RN. Neuropsin (opn5) mediates local light-dependent induction of circadian clock genes and circadian photoentrainment in exposed murine skin. Curr Biol. 2019;29:3478–87.

Buhr ED, Yoo S-H, Takahashi JS. Temperature as a universal resetting cue for mammalian circadian oscillators. Sciense. 2010;330:379–85.

Bullough WS. Mitotic activity in the adult male mouse, Mus musculus L. The diurnal cycles and their relation to waking and sleeping. Proc R Soc L B Biol Sci. 1948;135:212–33.

Bunger MK, Wilsbacher LD, Moran SM, Clendenin C, Laurel A, Hogenesch JB, et al. Mop3 is an essential component of the master circadian pacemaker in mammals. Cell. 2000;103:1009–17.

Bustamante J, Bredenston L, Malanga G, Mordoh J. Role of melanin as scavenger of active oxygen species. Pigment Cell Res. 1993;6:348–53.

Calligaro H, Coutanson C, Najjar RP, Mazzaro N, Cooper HM, Haddjeri N, et al. Rods contribute to the light-induced phase shift of the retinal clock

in mammals. PLoS Biol. 2019;17:e2006211.

Campbell SS, Murphy PJ. Extraocular circadian phototransduction in humans. Science. 1998;279:396–9.

Chakraborty AK, Funasaka Y, Slominski A, Bolognia J, Sodi S, Ichihashi M, et al. UV Light and MSH Receptors. Ann N Y Acad Sci. 1999;885:100–16.

Christou S, Wehrens SMT, Isherwood C, Möller-Levet CS, Wu H, Revell VL, et al. Circadian regulation in human white adipose tissue revealed by transcriptome and metabolic network analysis. Sci Rep. 2019;9:2641.

CIE. International Lightning Vocabulary CIE S 017/E:2011. 2011.

CIE. Erythema reference action spectrum and standard erythema dose ISO/CIE 17166:2019. 2019.

Colwell CS. Linking neural activity and molecular oscillations in the SCN. Nat Rev Neurosci. 2011;12:533–69.

Conti A, Conconi S, Hertens E, Skwarlo-Sonta K, Markowska M, Maestroni GJM. Evidence for melatonin synthesis in mouse and human bone marrow cells. J Pineal Res. 2000;28:193–202.

Cuesta M, Boudreau P, Cermakian N, Boivin DB. Skin temperature rhythms in humans respond to changes in the timing of sleep and light. J Biol Rhythms. 2017;32:257–73.

Czeisler CA, Duffy JF, Shanahan TL, Brown EN, Mitchell JF, Rimmer DW, et al. Stability, precision, and near-24-hour period of the human circadian pacemaker. Science. 1999;284:2177–81.

Czeisler CA, Shanahan TL, Klerman EB, Martens H, Brotman DJ, Emens JS, et al. Suppression of melatonin secretion in some blind patients by exposure to bright light. N Engl J Med. 1995;332:6–11.

D’Orazio J, Jarrett S, Amaro-Ortiz A, Scott T. UV radiation and the skin. Int J Mol Sci. 2013;14:12222–48.

Dakup P, Gaddameedhi S. Impact of the circadian clock on UV-Induced DNA damage response and photocarcinogenesis. Photochem Photobiol. 2017;93:296–303.

Dallmann R, Okyar A, Lévi F. Dosing-time makes the poison: circadian regulation and pharmacotherapy. Trends Mol Med. 2016;22:430–45.

Decraene D, Agostinis P, Pupe A, de Haes P, Garmyn M. Acute response of human skin to solar radiation: regulation and function of the p53 protein. J Photochem Photobiol B. 2001;63:78–83.

Deliconstantinos G, Villiotou V, Stravrides JC. Release by ultraviolet B (u.v.B) radiation of nitric oxide (NO) from human keratinocytes: a potential role for nitric oxide in erythema production. Br J Pharmacol. 1995;114:1257–65.

Denat L, Kadekaro AL, Marrot L, Leachman S, Abdel-Malek ZA. Melanocytes as instigators and victims of oxidative stress. J Invest Dermatol 2014. 2014;134:1512–18.

Dennis LK, VanBeek MJ, Freeman LEB, Smith BJ, Dawson D V, Coughlin JA. Sunburns and risk of cutaneous melanoma, does age matter: a comprehensive meta-analysis. Ann Epidemiol. 2008;18:614–27.

Diffey BL, Oliver RJ, Farr PM. A portable instrument for quantifying erythema induced by ultraviolet radiation. Br J Dermatol. 1984 Dec;111:663–72.

Dreher F, Gabard B, Schwindt DA, Maibach HI. Topical melatonin in combination with vitamins E and C protects skin from ultraviolet-induced erythema : a human study in vivo. Br J Dermatol. 1998;139:332–9.

Duncan MJ, Takahashi JS, Dubocovich ML. 2-[125i]iodomelatonin binding sites in hamster brain membranes: Pharmacological characteristics and regional distribution. Endocrinology. 1988;122:1825–33.

Farr PM, Diffey BL. Quantitative studies on cutaneous erythema induced by ultraviolet radiation. Br J Dermatol. 1984;111:673–82.

Farr PM, Diffey BL. The vascular response of human skin to ultraviolet radiation. Pltowchemistry Photobiol. 1986;44:515–7.

Faurschou A, Wulf HC. European Dermatology Guideline for the photodermatoses, 2. Phototesting [Internet]. EDF guidelines for dermatology in Europe. 2009. Available from: https://www.edf.one/dam/jcr:76d1122e-c1aa-4d5d-af4f-8b800f2edf4a/guideline_phototesting.pdf

Feehan RP, Shantz LM. Molecular signaling cascades involved in nonmelanoma skin carcinogenesis. Biochem J. 2016;473:2973–94.

Fell GL, Robinson KC, Mao J, Woolf CJ, Fisher DE. Skin β-Endorphin Mediates Addiction to UV Light. Cell. 2014;157:1527–34.

Finnish Cancer Registry. https://cancerregistry.fi/statistics/cancer-statistics.

Fischer TW, Burmeister G, Schmid HW, Elsner P. Melatonin increases anagen hair rate in women with androgenetic alopecia or diffuse alopecia: Results of a pilot randomized controlled trial. Br J Dermatol. 2004;150:341–5.

Fischer TW, Kleszczyński K, Hardkop LH, Kruse N, Zillikens D. Melatonin enhances antioxidative enzyme gene expression (CAT, GPx, SOD), prevents their UVR-induced depletion, and protects against the formation of DNA damage (8-hydroxy-2’-deoxyguanosine) in ex vivo human skin. J Pineal Res. 2013;54:303–12.

Fischer TW, Slominski A, Tobin DJ, Paus R. Melatonin and the hair follicle. J Pineal Res. 2008a;44:1–15.

Fischer TW, Slominski A, Zmijewski MA, Reiter RJ, Paus R. Melatonin as a major skin protectant: from free radical scavenging to DNA damage repair. Exp Dermatol. 2008b;17:713–30.

Fischer TW, Sweatman TW, Semak I, Sayre RM, Wortsman J, Slominski A. Constitutive and UV-induced metabolism of melatonin in keratinocytes and cell-free systems. FASEB J. 2006a;20:1564–6.

Fischer TW, Zbytek B, Sayre RM, Apostolov EO, Basnakian AG, Sweatman

TW, et al. Melatonin increases survival of HaCaT keratinocytes by suppressing UV-induced apoptosis. J Pineal Res. 2006b;40:18–26.

Fischer TW, Zmijewski MA, Zbytek B, Sweatman TW, Slominski RM, Wortsman J, et al. Oncostatic effects of the indole melatonin and expression of its cytosolic and nuclear receptors in cultured human melanoma cell lines. Int J Oncol. 2006c;29:665–72.

Fisher MS, Kripke ML. Suppressor T lymphocytes control the development of primary skin cancers in ultraviolet-irradiated mice. Science. 1982;216:1133–4.

Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. Arch Dermatol. 1988;124:869–871.

Freeman S, Hacham H, Gange R, Maytum D, Sutherland J, Sutherland B. Wavelength dependence of pyrimidine dimer formation in DNA of human skin irradiated in situ with ultraviolet light. Proc Natl Acad Sci USA. 1989;86:5605–9.

Le Fur I, Reinberg A, Lopez S, Morizot F, Mechkouri M, Tschachler E. Analysis of circadian and ultradian rhythms of skin surface properties of face and forearm of healthy women. J Invest Dermatol. 2001;117:718–24.

Gaddameedhi S, Selby CP, Kaufmann WK, Smart RC, Sancar A. Control of skin cancer by the circadian rhythm. Proc Natl Acad Sci U S A. 2011;108:18790–5.

Gaddameedhi S, Selby CP, Kemp MG, Ye R, Sancar A. The circadian clock controls sunburn apoptosis and erythema in mouse skin. J Invest Dermatol. 2015;135:1119–27.

Gauger MA, Sancar A. Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer. Cancer Res. 2005;65:6828–34.

Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacner LD, King DP, et al. Role of the CLOCK protein in the mammalian circadian mechanism. Science. 1998;280:1564–9.

Geyfman M, Andersen B. How the skin can tell time. J Invest Dermatol. 2009;129:1063–6.

Geyfman M, Kumar V, Liu Q, Ruiz R, Gordon W, Espitia F, et al. 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 USA. 2012;109:11758–63.

Golden RN, Gaynes BN, Ekstrom RD, Hamer RM, Jacobsen FM, Suppes T, et al. The efficacy of light therapy in the treatment of mood disorders: a review and meta-analysis of the evidence. Am J Psychiatry. 2005;162:656–62.

Gordon LG, Rowell D. Health system costs of skin cancer and cost-effectiveness of skin cancer prevention and screening: A systematic review. Eur J Cancer Prev. 2015;24:141–9.

Goriki A, Hatanaka F, Myung J, Kim JK, Yoritaka T, Matsubara A, et al. A novel protein, chrono, functions as a core component of the mammalian circadian clock. PLOS Biol. 2014;12:e1001839.

Grace MS, Cahi GM, Besharse JC. Melatonin deacetylation: retinal vertebrate class distribution and Xenopus laevis tissue distribution. Brain Res. 1991;559:56–63.

Greaves MW, Sondergaard J. Pharmacologic agents released in ultraviolet inflammation studied by continuous skin pefusion. J Invest Dermatol. 1970;54:365–7.

Guy GPJ, Machlin SR, Ekwueme DU, Yabroff R. Prevalence and Costs of Skin Cancer Treatment in the U.S., 2002–2006 and 2007–2011 Gery. Am J Prev Med. 2015;48:183–7.

Haltaufderhyde K, Ozdeslik RN, Wicks NL, Najera JA, Oancea E. Opsin expression in human epidermal skin. Photochem Photobiol. 2015;91:117–23.

Hardeland R, Pandi-Perumal SR, Cardinali DP. Melatonin. Int J Biochem Cell Biol. 2006;38:313–6.

Hardin PE, Hall JC, Rosbash M. Feedback of the Drosophila period gene product on circadian cycling of its messenger RNA levels. Nature. 1990;343:536–40.

Hardman JA, Tobin DJ, Haslam IS, Farjo N, Farjo B, Al-Nuaimi Y, et al. The peripheral clock regulates human pigmentation. J Invest Dermatol. 2015;135:1053–64.

Hart PH, Norval M. Ultraviolet radiation-induced immunosuppression and its relevance for skin carcinogenesis. Photochem Photobiol Sci. 2018;17:1872–84.

Hätönen T, Forsblom S, Kieseppä T, Lönnqvist J, Partonen T. Circadian phenotype in patients with the co-morbid alcohol use and bipolar disorders. Alcohol Alcohol. 2008;43:564–8.

Healy E, Reynolds NJ, Smith MD, Campbell C, Farr PM, Rees JL. Dissociation of erythema and p53 protein expression in human skin following UVB irradiation, and induction of p53 protein and mRNA following application of skin irritants. J Invest Dermatol. 1994;103:493–9.

Hernandez-Morante JJ, Gomez-santos C, Margareto J, Martínez-augustín O, Madrid JA, Ordovas JM, et al. Influence of menopause on adipose tissue clock gene genotype and its relationship with metabolic syndrome in morbidly obese women. Age (Omaha). 2012;34:1369–80.

Hernandez RA, Vallejo B, Ruzgas T, Björklund S. The effect of uvb irradiation and oxidative stress on the skin barrier — a new method to evaluate sun. Sensors. 2019;19:2376.

Hönigsmann H. Erythema and pigmentation. Photodermatol Photoimmunol Photomed. 2002;18:75–81.

Horne J, Östberg O. A self-assessment questionnaire to determine morningness–eveningness in human circadian rhythms. Int J Chronobiol. 1976;4:97–100.

Van Der Horst GTJ, Muijtjens M, Kobayashi K, Takano R, Kanno S-I, Takao M, et al. Mammalian Cry1 and Cry2 are essential for maintenance of

circadian rhythms. Nature. 1999;398:627–30.

Hoyle NP, Seinkmane E, Putker M, Feeney KA, Krogager TP, Chesham JE, et al. Circadian actin dynamics drive rhythmic fibroblast mobilization during wound healing. Sci Transl Med. 2017;9:eaal2774.

Hsu DS, Zhao X, Zhao S, Kazantsev A, Wang R, Todo T, et al. Putative human blue-light photoreceptors hCRY1 and hCRY2 are flavoproteins. Biochemistry. 1996;35:13871–7.

Huether G, Poeggeler B, Reimer A, George A. Effect of tryptophan administration on circulating melatonin levels in chicks and rats: evidence for stimulation of melatonin synthesis and release in the gastrointestinal tract. Life Sci. 1992;51:945–53.

Jahanban-Esfahlan R, Mehrzadi S, Reiter RJ, Seidi K, Majidinia M, Baghi HB, et al. Melatonin in regulation of inflammatory pathways in rheumatoid arthritis and osteoarthritis: involvement of circadian clock genes. Br J Pharmacol. 2018;175:3230–8.

Janjetovic Z, Jarrett SG, Lee EF, Duprey C, Reiter RJ, Slominski AT. Melatonin and its metabolites protect human melanocytes against UVB-induced damage: Involvement of NRF2-mediated pathways. Sci Rep. 2017;7:1274.

Janjetovic Z, Nahmias ZP, Hanna S, Jarrett SG, Kim T-K, Reiter RJ, et al. Melatonin and its metabolites ameliorate UVB-induced damages in human epidermal keratinocytes. J Pineal Res. 2014;57:90–102.

Jemec GBE, Johansen JD. Erythema-index of clinical patch test reactions. Ski Res Technol. 1995;1:26–9.

Kawamura G, Hattori M, Takamatsu K, Tsukada T, Ninomiya Y, Benjamin I, et al. Cooperative interaction among BMAL1, HSF1, and p53 protects mammalian cells from UV stress. Commun Biol. 2018;1:204.

Kawara S, Mydlarski R, Mamelak AJ, Freed I, Wang B, Watanabe H, et al. Low-dose ultraviolet B rays alter the mRNA expression of the circadian clock genes in cultured human keratinocytes. J Invest Dermatol. 2002;119:1220–3.

Kim EJ, Kim YK, Kim JE, Kim S, Kim M, Park C, et al. UV modulation of subcutaneous fat metabolism. J Invest Dermatol. 2011;131:1720–6.

Kim T-K, Kleszczynski K, Janjetovic Z, Sweatman T, Lin Z, Li W, et al. Metabolism of melatonin and biological activity of intermediates of melatoninergic pathway in human skin cells. FASEB J. 2013;27:2742–55.

Kim T-K, Lin Z, Li W, Reiter RJ, Slominski AT. N1-acetyl-5-methoxykynuramine (AMK) is produced in the human epidermis and shows antiproliferative effects. Endocrinology. 2015a;156:1630–6.

Kim T-K, Lin Z, Tidwell WJ, Li W, Slominski AT. Melatonin and its metabolites accumulate in the human epidermis in vivo and inhibit proliferation and tyrosinase activity in epidermal melanocytes in vitro. Mol Cell Endocrinol. 2015b;404:1–8.

King DP, Zhao Y, Sangoram AM, Wilsbacher LD, Tanaka M, Antoch MP, et al. Positional cloning of the mouse circadian Clock gene. Cell. 1997;89:641–53.

Kojima D, Mori S, Torii M, Wada A, Morishita R, Fukada Y. UV-sensitive photoreceptor protein OPN5 in humans and mice. PLoS One. 2011;6:e26388.

Konopka RJ, Benzer S. Clock mutants of Drosophila melanogaster. Proc Natl Acad Sci U S A. 1971;68:2112–6.

Koskenvuo M, Hublin C, Partinen M, Heikkilä K, Kaprio J. Heritability of diurnal type: A nationwide study of 8753 adult twin pairs. J Sleep Res. 2007;16:156–62.

Kripke ML. Ultraviolet radiation and immunology: something new under the sun- presidential address. Cancer Res. 1994;54:6102–5.

Kulms D, Zeise E, Po B, Schwarz T. DNA damage , death receptor activation and reactive oxygen species contribute to ultraviolet radiation-induced apoptosis in an essential and independent way. Oncogene. 2002;21:5844–51.

Lan Y, Wang Y, Lu H. Opsin 3 is a key regulator of ultraviolet A-induced photoageing in human dermal fibroblast cells. Br J Dermatol. 2020;182:1228–44.

Lanoix D, Beghdadi H, Lafond J, Vaillancourt C. Human placental trophoblasts synthesize melatonin and express its receptors. J Pineal Res. 2008;45:50–60.

Lee E, Koo J, Berger T. UVB phototherapy and skin cancer risk: a review of the literature. Int J Dermatol. 2005;44:355–60.

Lee J-H, Moon J-H, Nazim UMD, Lee Y-J, Seol J-W, Eo S-K, et al. Melatonin protects skin keratinocyte from hydrogen peroxide-mediated cell death via the SIRT1 pathway. Oncotarget. 2016;7:12075–88.

Lee JH, An HT, Chung JH, Kim KH, Eun HC, Cho KH. Acute effects of UVB radiation on the proliferation and differentiation of keratinocytes. Photodermatol Photoimmunol Photomed. 2002;18:253–61.

Lerner AB, Case JD, Takahashi Y, Lee TH, Mori W. Isolation of melatonin, the pineal gland factor that lightens melanocytes. J Am Chem Soc. 1958;80:2587.

Lewy AJ, Cutler NL, Sack RL. The endogenous melatonin profile as a marker for circadian phase position. J Biol Rhythms. 1999;14:227–36.

Lewy AJ, Tetsuo M, S.P. M, Goodwin FK, Kopin IJ. Pinealotomy abolishes plasma melatonin in the rat. J Clin Endocrinol Metab. 1979;50:204–5.

Lewy AJ, Wehr TA, Goodwin FK, Newsome DA, Markey SP. Light suppresses melatonin secretion in humans. Science. 1980;210:1267–9.

Liu H, Zhong D, Lin C. Searching for a photocycle of the cryptochrome photoreceptors. Curr Opin Plant Biol. 2010;13:578–86.

Liu X, Uchiyama M, Shibui K, Kim K, Kudo Y, Tagaya H, et al. Diurnal preference, sleep habits, circadian sleep propensity and melatonin rhythm in healthy human subjects. Neurosci Lett. 2000;280:199–202.

Logan G, Wilhelm DL. Ultra-violet injury as an experimental model of the

inflammatory reaction. Nature. 1963;198:968–9.

Loser K, Apelt J, Voskort M, Mohaupt M, Balkow S, Schwarz T, et al. IL-10 controls ultraviolet-induced carcinogenesis in mice. J Immunol. 2007;179:365–71.

Lowrey PL, Takahashi JS. Genetics of circadian rhythms in mammalian model organisms. Adv Genet. 2011;75:173–230.

Lu H, Edwards C, Gaskell S, Pearse A, Marks R. Melanin content and distribution in the surface corneocyte with skin phototypes. Br J Dermatol. 1996;135:263–7.

Ly BCK, Dyer EB, Feig JL, Chien AL, Del Bino S. Research Techniques Made Simple: Cutaneous Colorimetry: A Reliable Technique for Objective Skin Color Measurement. J Invest Dermatol. 2020;140:3–12.

De Mairan J. Observation botanique. Hist Acad Roy Sci Paris. 1729;35–6.

Man I, Crombie IK, Dawe RS, Ibbotson SH, Ferguson J. The photocarcinogenic risk of narrowband UVB (TL-01) phototherapy: early follow-up data. Br J Dermatol. 2005;152:755–7.

Martin M, Macias M, Escames G, Leon J, Acuna-Castroviejo D. Melatonin but not vitamins C and E maintains glutathione homeostasis in t-butyl hydroperoxide-induced mitochondrial oxidative stress. FASEB J. 2000;14:1677–9.

Marto J, Ascenso A, Gonçalves LM, Gouveia LF, Manteigas P, Pinto P, et al. Melatonin-based pickering emulsion for skin's photoprotection. Drug Deliv. 2016;23:1594–607.

Maukonen M, Havulinna AS, Männistö S, Kanerva N, Salomaa V, Partonen T. Genetic associations of chronotype in the finnish general population. J Biol Rhythms. 2020;35:501–11.

McElhinney DB, Hoffman SJ, Robinson WA, Ferguson J. Effect of melatonin on human skin color. J Invest Dermatol. 1994;102:258–9.

Megdal SP, Kroenke CH, Laden F, Pukkala E, Schernhammer ES. Night work

and breast cancer risk: A systematic review and meta-analysis. Eur J Cancer. 2005;41:2023–32.

Meinhardt M, Krebs R, Anders A, Heinrich U, Tronnier H. Absorption spectra of human skin in vivo in the ultraviolet wavelength range measured by optoacoustics. Photochem Photobiol. 2009;85:70–7.

Merikanto I, Kronholm E, Peltonen M, Laatikainen T, Lahti T, Partonen T. Relation of chronotype to sleep complaints in the general finnish population. Chronobiol Int. 2012;29:311–7.

Miyamoto Y, Sancar A. Vitamin B 2 -based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments. Proc Natl Acad Sci USA. 1998;95:6097–102.

Montaigne D, Marechal X, Modine T, Coisne A, Mouton S, Fayad G, et al. Daytime variation of perioperative myocardial injury in cardiac surgery and its prevention by Rev-Erbα antagonism: a single-centre propensity-matched cohort study and a randomised study. Lancet. 2018;391:59–69.

Moore RY. Neural control of the pineal gland. Behav Brain Res. 1996;73:125–30.

Moore RY, Eichler VB. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res. 1972;42:201–6.

Muekusch G, Pitman J, Smiljanic D. Photoresponsive diseases. Dermatology Nurs. 2007;19:43–7.

Napolitano A, Panzella L, Monfrecola G, D'Ischia M. Pheomelanin-induced oxidative stress: Bright and dark chemistry bridging red hair phenotype and melanoma. Pigment Cell Melanoma Res. 2014;27:721–33.

Naranjo MC, Guerrero JM, Rubio A, Lardone PJ, Carrillo-Vico A, Carrascosa-Salmoral MP, et al. Melatonin biosynthesis in the thymus of humans and rats. Cell Mol Life Sci. 2007;64:781–90.

Narasimamurthy R, Hatori M, Nayak SK, Liu F, Panda S, Verma IM. Circadian clock protein cryptochrome regulates the expression of proinflammatory cytokines. Proc Natl Acad Sci U S A. 2012;109:12662–

7.

Nast A, Gisondi P, Ormerod AD, Saiag P, Smith C, Spuls PI, et al. European S3-Guidelines on the systemic treatment of psoriasis vulgaris - Update 2015 - Short version - EDF in cooperation with EADV and IPC. J Eur Acad Dermatology Venereol. 2015;29:2277–94.

Nguyen NT, Fisher DE. MITF and UV responses in skin: From pigmentation to addiction. Pigment Cell Melanoma Res. 2018;32:224–36.

Nickel A, Wohlrab W. Melatonin protects human keratinocytes from UVB irradiation by light absorption. Arch Dermatol Res. 2000;292:366–8.

Nikkola V, Grönroos M, Huotari-Orava R, Kautiainen H, Ylianttila L, Karppinen T, et al. Circadian Time Effects on NB-UVB-Induced Erythema in Human Skin In Vivo. J Invest Dermatol. 2018;138:464–7.

Nishisgori C. Current concept of photocarcinogenesis. Photochem Photobiol Sci. 2015;14:1713–21.

Noguchi T, Wang CW, Pan H, Welsh DK. Fibroblast circadian rhythms of PER2 expression depend on membrane potential and intracellular calcium. Chronobiol Int. 2012;29:653–64.

Nosjean O, Ferro M, Cogé F, Beauverger P, Henlin J-M, Lefoulon F, et al. Identification of the Melatonin-binding Site MT3 as the Quinone Reductase 2. J Biol Chem. 2000;275:31311–7.

Olinski LE, Lin EM, Oancea E. Illuminating insights into opsin 3 function in the skin. Adv Biol Regul. 2020;75:100668.

Onuki J, Almeida EA, Medeiros MHG, Di Mascio P. Inhibition of 5-aminolevulinic acid-induced DNA damage by melatonin, N1-acetyl-N2-formyl-5-methoxykynuramine, quercetin or resveratrol. J Pineal Res. 2005;38:107–15.

Ozaki Y, Lynch HJ. Presence of мelatonin in plasma and urine of pinealectomized rats. Endocrinology. 1976;99:641–4.

Ozdeslik RN, Olinski LE, Trieu MM, Oprian DD, Oancea E. Human

nonvisual opsin 3 regulates pigmentation of epidermal melanocytes through functional interaction with melanocortin 1 receptor. Proc Natl Acad Sci U S A. 2019;116:11508–17.

Özgur S, Sancar A. Purification and properties of human blue-light photoreceptor cryptochrome 2. Biochemistry. 2003;42:2926–32.

Ozturk N, Lee JH, Gaddameedhi S, Sancar A. Loss of cryptochrome reduces cancer risk in p53 mutant mice. Proc Natl Acad Sci USA. 2009;106:2841–6.

Pacák K, Palkovits M. Stressor specificity of central neuroendocrine responses: Implications for stress-related disorders. Endocr Rev. 2001;22:502–48.

Papp SJ, Huber A, Jordan SD, Kriebs A, Nguyen M, Moresco JJ, et al. DNA damage shifts circadian clock time via Hausp-dependent Cry1 stabilization. Elife. 2015;4:e04883.

Park S-B, Huh C-H, Choe Y-B, Youn J-I. Time course of ultraviolet-induced skin reactions evaluated by two different reflectance spectrophotometers: DermaSpectrophotometer and Minolta spectrophotometer CM-2002. Photodermatol Photoimmunol Photomed. 2002;18:23–8.

Partonen T, Vakkuri O, Lamberg-Allardt C. Effects of exposure to morning bright light in the blind and sighted controls. Clin Physiol. 1995;15:637–46.

Pathirana D, Ormerod AD, Saiag P, Smith C, Spuls PI, Nast A, et al. European S3-guidelines on the systemic treatment of psoriasis vulgaris. J Eur Acad Dermatology Venereol. 2009;23:1–70.

Pérez-González A, Galano A, Alvarez-Idaboy JR, Tan DX, Reiter RJ. Radical-trapping and preventive antioxidant effects of 2-hydroxymelatonin and 4-hydroxymelatonin: Contributions to the melatonin protection against oxidative stress. Biochim Biophys Acta - Gen Subj. 2017;1861:2206–17.

Pevet P, Challet E. Melatonin: Both master clock output and internal time-giver in the circadian clocks network. J Physiol Paris. 2011;105:170–82.

Poletini MO, Ramos BC, Moraes MN, Castrucci AML. Nonvisual opsins and the regulation of peripheral clocks by light and hormones. Photochem Photobiol. 2015;91:1046–55.

Preston DS, Stern RS. Nonmelanoma cancers of the skin. N Engl J Med. 1992;326:653–7.

Puig LS, Pillon NJ, Näslund E, Krook A, Zierath JR. Influence of obesity, weight loss, and free fatty acids on skeletal muscle clock gene expression. Am J Physiol - Endocrinol Metab. 2020;318:E1–10.

Qian CY, Yuan C, Tan YM, Liu XP, Dong YQ, Yang LJ, et al. Comparing performance of Chromameter, Mexameter and full-field laser perfusion imaging for measurement of ultraviolet B light-induced erythema. Clin Exp Dermatol. 2015;40:438–40.

Rafnsson V, Gunnarsdottir H. Mortality study of fertiliser manufacturers in Iceland. Br J Ind Med. 1990;47:721–5.

Rajaratnam SMW, Arendt J. Health in a 24-h society. Lancet. 2001;358:999–1006.

Ralph MR, Foster RG, Davis FC, Menaker M. Transplanted suprachiasmatic nucleus determines circadian period. Science. 1990;247:975–8.

Raone B, Patrizi A, Gurioli C, Gazzola A, Ravaioli GM. Cutaneous carcinogenic risk evaluation in 375 patients treated with narrowband-UVB phototherapy: A 15-year experience from our Institute. Photodermatol Photoimmunol Photomed. 2018;34:302–6.

Regazzetti C, Sormani L, Debayle D, Bernerd F, Tulic MK, De Donatis GM, et al. Melanocytes sense blue light and regulate pigmentation through opsin-3. J Invest Dermatol. 2018;138:171–8.

Reichrath J. The challenge resulting from positive and negative effects of sunlight: How much solar UV exposure is appropriate to balance between risks of vitamin D deficiency and skin cancer? Prog Biophys Mol Biol. 2006;92:9–16.

Reick M, Garcia JA, Dudley C, McKnight SL. NPAS2: An analog of clock

operative in the mammalian forebrain. Science. 2001;293:506–9.

Reinehr CPH, Bakos RM. Actinic keratoses: review of clinical, dermoscopic, and therapeutic aspects. An Bras Dermatol. 2019;94:637–57.

Reiter RJ. Pineal melatonin: Cell biology of its synthesis and of its physiological interactions. Endocr Rev. 1991;12:151–80.

Reiter RJ. Functional diversity of the pineal hormone melatonin: Its role as an antioxidant. Exp Clin Endocrinol. 1996;104:10–6.

Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre-Jimenez M, Qin L. Melatonin as an antioxidant: under promises but over delivers. J Pineal Res. 2016;61:253–78.

Reiter RJ, Sorrentino S, Ralph CL, Lynch HJ, Mull D, Jarrow E. Some endocrine effects of blinding and anosmia in adult male rats with observations on pineal melatonin. Endocrinology. 1971;88:895–900.

Reiter RJ, Tan DX, Terron MP, Flores LJ, Czarnocki Z. Melatonin and its metabolites: New findings regarding their production and their radical scavenging actions. Acta Biochim Pol. 2007;54:1–9.

Reppert SM, Godson C, Mahle CD, Weaver DR, Slaugenhaupt SA, Gusella JF. Molecular characterization of a second melatonin receptor expressed in human retina and brain: The Mel(1b) melatonin receptor. Proc Natl Acad Sci USA. 1995;92:8734–8.

Reppert SM, Weaver DR, Ebisawa T. Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron. 1994;13:1177–85.

Roenneberg T, Daan S, Merrow M. The Art of Entrainment. J Biol Rhythms. 2003a;18:183–94.

Roenneberg T, Foster RG. Twilight times: Light and the circadian system. Photochem Photobiol. 1997;66:549–61.

Roenneberg T, Wirz-Justice A, Merrow M. Life between clocks: Daily temporal patterns of human chronotypes. J Biol Rhythms. 2003b;18:80–

90.

Romić MD, Klarić MŠ, Lovrić J, Pepić I, Cetina-Čižmek B, Filipović-Grčić J, et al. Melatonin-loaded chitosan/Pluronic® F127 microspheres as in situ forming hydrogel: An innovative antimicrobial wound dressing. Eur J Pharm Biopharm. 2016;107:67–79.

Rusanova I, Martínez-Ruiz L, Florido J, Rodríguez-Santana C, Guerra-Librero A, Acuña-Castroviejo D, et al. Protective effects of melatonin on the skin: Future perspectives. Int J Mol Sci. 2019;20:4948.

Sancar A. Regulation of the mammalian circadian clock by cryptochrome. J Biol Chem. 2004;279:34079–82.

Sancar A, Lindsey-Boltz LA, Gaddameedhi S, Selby CP, Ye R, Chiou Y-Y, et al. Circadian Clock, cancer, and chemotherapy. Biochemistry. 2015;54:110–23.

Sandu C, Dumas M, Malan A, Sambakhe D, Marteau C, Nizard C, et al. Human skin keratinocytes, melanocytes, and fibroblasts contain distinct circadian clock machineries. Cell Mol Life Sci. 2012;69:3329–39.

Schernhammer ES, Laden F, Speizer FE, Willet WC, Hunter DJ, Kawachi I, et al. Night-shift work and risk of colorectal cancer in the nurses' health study. J Natl Cancer Inst. 2003;95:825–8.

Schernhammer ES, Razavi P, Li TY, Qureshi AA, Han J. Rotating night shifts and risk of skin cancer in the nurses' health study. J Natl Cancer Inst. 2011;103:602–6.

Scheuer C, Pommergaard H-C, Rosenberg J, Gögenur I. Melatonin' s protective effect against UV radiation: a systematic review of clinical and experimental studies. Photodermatol Photoimmunol Photomed. 2014;30:180–8.

Scheuer C, Pommergaard H-C, Rosenberg J, Gögenur I. Dose dependent sun protective effect of topical melatonin: A randomized, placebo-controlled, double-blind study. J Dermatol Sci. 2016a;84:178–85.

Scheuer C, Pommergaard H-C, Rosenberg J, Gögenur I. Effect of topical

application of melatonin cream 12.5% on cognitive parameters: A randomized, placebo-controlled, double-blind crossover study in healthy volunteers. J Dermatolog Treat. 2016b;27:488–94.

Schibler U, Ripperger J, Brown SA. Peripheral circadian oscillators in mammals: Time and food. J Biol Rhythms. 2003;18:250–60.

Semak I, Naumova M, Korik E, Terekhovich V, Wortsman J, Slominski A. A novel metabolic pathway of melatonin: Oxidation by cytochrome. Biochemistry. 2005;44:9300–7.

Shah P, Yu-Ying H. Molecular regulation of UV-induced DNA repair. Photochem Photobiol. 2015;91:254–64.

Sheehan JM, Cragg N, Chadwick CA, Potten CS, Young AR. Repeated ultraviolet exposure affords the same protection against DNA photodamage and erythema in human skin types II and IV but is associated with faster DNA repair in skin type IV. J Invest Dermatol. 2002;118:825–9.

Sheehan JM, Young AR. The sunburn cell revisited: An update on mechanistic aspects. Photochem Photobiol Sci. 2002;1:365–77.

Sherratt MJ, Hopkinson L, Naven M, Hibbert SA, Ozolsa M, Eckersleya A, et al. Circadian rhythms in skin and other elastic tissues. Matrix Biol. 2019;84:97–110.

Shostak A, Husse J, Oster H. Circadian regulation of adipose function. Adipocyte. 2013;2:201–6.

Siegel JA, Korgavkar K, Weinstock MA. Current perspective on actinic keratosis: a review. Br J Dermatol. 2017;177:350–8.

Simon JD, Peles D, Wakamatsu K, Ito S. Current challenges in understanding melanogenesis: Bridging chemistry, biological control, morphology, and function. Pigment Cell Melanoma Res. 2009;22:563–79.

Sinikumpu SP, Huilaja L, Jokelainen J, Koiranen M, Auvinen J, Hägg PM, et al. High prevalence of skin diseases and need for treatment in a middle-aged population. A Northern Finland Birth Cohort 1966 study. PLoS

One. 2014;9:e99533.

Skobowiat C, Dowdy JC, Sayre RM, Tuckey RC, Slominski A. Cutaneous hypothalamic-pituitary-adrenal axis homolog: regulation by ultraviolet radiation. Am J Physiol Endocrinol Metab. 2011;301:E484–93.

Skobowiat C, Postlethwaite AE, Slominski AT. Skin exposure to ultraviolet B rapidly activates systemic neuroendocrine and immunosuppressive responses. Photochem Photobiol. 2017;93:1008–15.

Skobowiat C, Slominski AT. Ultraviolet B (UVB) activates hypothalamic-pituitary-adrenal (HPA) axis in C57BL/6 mice. Physiol Behav. 2015;135:1638–48.

Slominski A. The cutaneous serotoninergic/melatoninergic system: securing a place under the sun. FASEB J. 2005;19:176–94.

Slominski A, Baker J, Ermak G, Chakraborty A, Pawelek J. Ultraviolet B stimulus production of corticotropin releasing factor (CRF) by human melanocytes. FEBS Lett. 1996;399:175–6.

Slominski A, Pisarchik A, Semak I, Sweatman T, Szczesniewski A, Slugocki G, et al. Serotoninergic and melatoninergic systems are fully expressed in human skin. FASEB J. 2002;16:896–8.

Slominski A, Wortsman J. Neuroendocrinology of the Skin. Endocr Rev. 2000;21:457–87.

Slominski A, Wortsman J, Luger T, Paus R, Solomon S. Corticotropinreleasing hormone and proopiomelanocortin involvement in the cutaneous response to stress. Physiol Rev. 2000;80:979–1020.

Slominski AT, Hardeland R, Zmijewski MA, Slominski RM, Reiter RJ, Paus R. Melatonin: A cutaneous perspective on its production, metabolism, and functions. J Invest Dermatol. 2018a;138:490–9.

Slominski AT, Kim TK, Kleszczyński K, Semak I, Janjetovic Z, Sweatman T, et al. Characterization of serotonin and N-acetylserotonin systems in the human epidermis and skin cells. J Pineal Res. 2020;68:e12626.

Slominski AT, Kleszczyński K, Semak I, Janjetovic Z, Zmijewski MA, Kim TK, et al. Local melatoninergic system as the protector of skin integrity. Int J Mol Sci. 2014;15:17705–32.

Slominski AT, Semak I, Fischer TW, Kim TK, Kleszczyński K, Hardeland R, et al. Metabolism of melatonin in the skin: Why is it important? Exp Dermatol. 2017a;26:563–8.

Slominski AT, Zmijewski MA, Jetten AM. RORa is not a receptor for melatonin. Bioessays. 2016;38:1193–4.

Slominski AT, Zmijewski MA, Plonka PM, Szaflarski JP, Paus R. How UV light touches the brain and endocrine system through skin, and why. Endocrinology. 2018b;159:1992–2007.

Slominski AT, Zmijewski MA, Semak I, Kim TK, Janjetovic Z, Slominski RM, et al. Melatonin, mitochondria, and the skin. Cell Mol Life Sci. 2017b;74:3913–25.

Slominski AT, Zmijewski MA, Zbytek B, Tobin DJ, Theoharides TC, Rivier J. Key role of CRF in the skin stress response system. Endocr Rev. 2013;34:827–84.

Snellman E, Jansen C, Leszczynski K, Visuri R, Milan T, Jokela K. Ultraviolet erythema sensitivity in anamnestic (I-IV) and phototested (1-4) Caucasian skin phototypes: the need for a new classification system. Photochem Photobiol. 1995;62:769–72.

Van Der Spek PJ, Kobayashi K, Bootsma D, Takao M, Eker APM, Yasui A. Cloning, tissue expression, and mapping of a human photolyase homolog with similarity to plant blue-light receptors. Genomics. 1996;37:177–82.

Spörl F, Korge S, Jürchott K, Wunderskirchner M, Schellenberg K, Heins S, et al. Kruppel-like factor 9 is a circadian transcription factor in human epidermis that controls proliferation of keratinocytes. Proc Natl Acad Sci. 2012;109:10903–8.

Sun Y, Wang P, Li H, Dai J. BMAL1 and CLOCK proteins in regulating UVB-induced apoptosis and DNA damage responses in human keratinocytes. J Cell Physiol. 2018;233:9563–74.

Sun ZS, Albrecht U, Zhuchenko O, Bailey J, Eichele G, Lee CC. RIGUI, a putative mammalian ortholog of the Drosophila period gene. Cell. 1997;90:1003–11.

Sundberg JP, Schofield PN. Commentary: Mouse genetic nomenclature: standardization of strain, gene, and protein symbols. Vet Pathol. 2010;47:1100–4.

Tahara Y, Aoyama S, Shibata S. The mammalian circadian clock and its entrainment by stress and exercise. J Physiol Sci. 2017;67:1–10.

Takahashi JS. Transcriptional architecture of the mammalian circadian clock. Nat Rev Genet. 2017;18:164–79.

Tarttelin EE, Bellingham J, Hankins MW, Foster RG, Lucas RJ. Neuropsin (Opn5): A novel opsin identified in mammalian neural tissue. FEBS Lett. 2003;554:410–6.

Tei H, Okamura H, Shigeyoshi Y, Fukuhara C, Ozawa R, Hirose M, et al. Circadian oscillation of a mammalian homologue of the Drosophila period gene. Nature. 1997;389:512–6.

Tejasvi T, Sharma VK, Kaur J. Determination of minimal erythemal dose for narrow band-ultraviolet B radiation in north Indian patients: Comparison of visual and Dermaspectrometer readings. indian J Dermatol Venereol Leprol. 2007;73:97–9.

Terakita A. The opsins. Genome Biol. 2005;6:213.

Thompson CL, Rickman CB, Shaw SJ, Ebright JN, Kelly U, Sancar A, et al. Expression of the blue-light receptor cryptochrome in the human retina. Invest Ophthalmol Vis Sci. 2003;44:4515–21.

Todo T, Ryo H, Yamamoto K, Toh H, Inui T, Ayaki H, et al. Similarity among the Drosophila (6-4)photolyase, a human photolyase homolog, and the DNA photolyase-blue-light photoreceptor family. Science. 1996;272:109–12.

Toledo A, Karppinen T, Miettinen ME, Leppäluoto J, Vuolteenaho O, Ylianttila L, et al. Narrow-band ultraviolet B (NB UV-B) exposures

improve mood in healthy individuals differently depending on chronotype. Chronobiol Int. 2019;36:1570–80.

Tosini G, Davidson AJ, Fukuhara C, Kasamatsu M, Castanon-Cervantes O. Localization of a circadian clock in mammalian photoreceptors. FASEB J. 2007;21:3866–3871.

Tsutsumi M, Ikeyama K, Denda S, Nakanishi J, Fuziwara S, Aoki H, et al. Expressions of rod and cone photoreceptor-like proteins in human epidermis. Exp Dermatol. 2009;18:567–70.

Urbach F. Ultraviolet radiation and skin cancer of humans. J Photochem Photobiol B Biol. 1997;40:3–7.

Urbanski A, Schwarz T, Neuner P, Kurtmann J, Kirnbauer R, Köck A, et al. Ultraviolet light induces increased circulating interleukin-6 in humans. J Invest Dermatol. 1990;94:808–11.

Vanderstraeten J, Gailly P, Malkemper EP. Light entrainment of retinal biorhythms : Cryptochrome 2 as candidate photoreceptor in mammals. Cell Mol Life Sci. 2020;77:875–84.

Venegas C, García JA, Escames G, Ortiz F, López A, Doerrier C, et al. Extrapineal melatonin: Analysis of its subcellular distribution and daily fluctuations. J Pineal Res. 2012;52:217–27.

Vieira J, Jones AR, Danon A, Sakuma M, Hoang N, Robles D, et al. Human cryptochrome-1 confers light independent biological activity in transgenic drosophila correlated with flavin radical stability. PLoS One. 2012;7:e31867.

Vink JM, Groot AS, Kerkhof GA, Boomsma DI. Genetic analysis of morningness and eveningness. Chronobiol Int. 2001;18:809–22.

Wang G, Savinko T, Wolff H, Dieu-Nosjean MC, Kemeny L, Homey B, et al. Repeated epicutaneous exposures to ovalbumin progressively induce atopic dermatitis-like skin lesions in mice. Clin Exp Allergy. 2007;37:151–61.

Wang H, van Spyk E, Liu Q, Geyfman M, Salmans ML, Kumar V, et al. Time-

restricted feeding shifts the skin circadian clock and alters UVB-induced DNA damage. Cell Rep. 2017;20:1061–72.

Wehner MR, Chren M-M, Nameth D, Choudhry A, Gaskins M, Nead KT, et al. International Prevalence of Indoor Tanning A Systematic Review and Meta-analysis. JAMA Dermatology. 2014;150:390–400.

Wehner MR, Shive ML, Chren M-M, Han J, Qureshi AA, Linos E. Indoor tanning and non-melanoma skin cancer: systematic review and meta-analysis. BMJ. 2012;345:e5909.

Weidenauer C, Vollmer C, Scheiter K, Randler C. Weak Associations of Morningness-Eveningness and Stability with Skin Temperature and Cortisol Levels. J Circadian Rhythms. 2019;17:1–12.

Welsh DK, Logothetis DE, Meister M, Reppert SM. Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron. 1995;14:697–706.

Wengström Y, Forsberg C, Näslund I, Bergh J. Quantitative assessment of skin erythema due to radiotherapy—evaluation of different measurements. Radiother Oncol. 2004;72:191–7.

Williams J, Yang N, Wood A, Zindy E, Meng Q, Streuli C. Epithelial and stromal circadian clocks are inversely regulated by their mechano-matrix environment. J Cell Sci. 2018;131:jcs208223.

Wollenberg A, Barbarot S, Bieber T, Christen-Zaech S, Deleuran M, Fink-Wagner A, et al. Consensus-based European guidelines for treatment of atopic eczema (atopic dermatitis) in adults and children: part I. J Eur Acad Dermatology Venereol. 2018;32:657–82.

Wu G, Ruben MD, Schmidt RE, Francey LJ, Smith DF, Anafi RC, et al. Population-level rhythms in human skin with implications for circadian medicine. Proc Natl Acad Sci U S A. 2018;115:12313–8.

Yaga K, Reiter RJ, Richardson BA. Tryptophan loading increases daytime serum melatonin levels in intact and pinealectomized rats. Life Sci. 1993;52:1231–8.

Ylianttila L, Visuri R, Huurto L, Jokela K. Evaluation of a single-monochromator diode array spectroradiometer for sunbed UV-radiation measurements. Photochem Photobiol. 2005;81:333.

Young AR. Acute effects of UVR on human eyes and skin. Prog Biophys Mol Biol. 2006;92:80–5.

Young AR, Chadwick CA, Harrison GI, Nikaido O, Ramsden J, Potten CS. The similarity of action spectra for thymine dimers in human epidermis and erythema suggests that DNA is the chromophore for erythema. J Invest Dermatol. 1998;111:982–8.

Zbytek B, Wortsman J, Slominski A. Characterization of a ultraviolet B-induced corticotropin-releasing hormone-proopiomelanocortin system in human melanocytes. Mol Endocrinol. 2006;20:2539–47.

Ziegler A, Jonason AS, Leffellt DJ, Simon JA, Sharma HW, Kimmelman J, et al. Sunburn and p53 in the onset of skin cancer. Nature. 1994;372:773–6.

MEQ6

Olettaen sopivat ympäristön olosuhteet, kuinka helppoa sinulle on aamuisin vuoteesta nouseminen?

- ☐ ei lainkaan helppoa
- ☐ ei kovin helppoa
- ☐ melko helppoa
- ☐ hyvin helppoa

Kuinka virkeäksi tunnet itsesi aamuisin ensimmäisen ½ tunnin aikana?

- ☐ hyvin väsyneeksi
- ☐ melko väsyneeksi
- ☐ melko levänneeksi
- ☐ hyvin levänneeksi

Oletetaan, että olet päättänyt ruveta harrastamaan jotakin urheilulajia. Ystäväsi suosittelee sinulle harjoitusohjelmaksi 2 kertaa viikossa tunti kerrallaan. Paras aika hänelle on aamuisin kello 7.00–8.00. Pitäen mielessäsi vain oman ’parhaalta tuntuu’ -rytmisi, kuinka luulisit suoriutuvasi?

- ☐ olisin hyvässä vireessä
- ☐ olisin kohtuullisessa vireessä
- ☐ tuntuisi melko vaikealta
- ☐ tuntuisi hyvin vaikealta

Oletetaan, että sinun täytyy osallistua 2 tunnin rasittavaan fyysiseen työhön. Voit täysin vapaasti suunnitella aikataulusi. Ottaen huomioon vain oma ’parhaalta tuntuu’ -rytmisi, minkä vaihtoehdon valitsisit?

- ☐ kello 8.00-10.00
- ☐ kello 11.00-13.00
- ☐ kello 15.00-17.00
- ☐ kello 19.00-21.00

Oletetaan, että voit valita työaikasi. Oletetaan, että työpäivä on 5 tunnin mittainen, työ on mielenkiintoista ja palkkaa maksetaan tulosten mukaan. Mitkä viisi PERÄKKÄISTÄ tuntia valitsisit? Rastita valitsemasi viisi PERÄKKÄISTÄ kellonaikaa.

☐ *1-2*	☐ *2-3*	☐ *3-4*	☐ *4-5*	☐ *5-6*	☐ *6-7*	☐ *7-8*	☐ *8-9*
☐ *9-10*	☐ *10-11*	☐ *11-12*	☐ *12-13*	☐ *13-14*	☐ *14-15*	☐ *15-16*	☐ *16-17*
☐ *17-18*	☐ *18-19*	☐ *19-20*	☐ *20-21*	☐ *21-22*	☐ *22-23*	☐ *23-24*	☐ *24-01*

On niin sanottuja ”aamuihmisiä” (aamunvirkku, illantorkku) ja ”iltaihmisiä” (illanvirkku, aamuntorkku). Kumpaan ryhmään sinä kuulut? Valitse sopivin seuraavista vaihtoehdoista.

- ☐ Ehdottomasti "aamuihmisiin"
- ☐ Enemmän "aamu-" kuin "iltaihmisiin"
- ☐ Enemmän "ilta-" kuin "aamuihmisiin"
- ☐ Ehdottomasti "iltaihmisiin"

MEQ-19, original (E) 1976, J.A. Horne & O. Östberg, Int J Chronobiol 1976;4:97-110.
MEQ-6, v.1.2 (FI) 2014, Timo Partonen, THL, Helsinki.

PUBLICATIONS

PUBLICATION
I

Circadian Time Effects on NB-UVB-Induced Erythema in Human Skin In Vivo

Veera Nikkola, Mari Grönroos, Riitta Huotari-Orava, Hannu Kautiainen, Lasse Ylianttila, Toni Karppinen, Timo Partonen and Erna Snellman

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study with histamine-induced itch. PLoS One 2011;6: e17786.

Reddy VB, Iuga AO, Shimada SG, LaMotte RH, Lerner EA. Cowhage-evoked itch is mediated by a novel cysteine protease: a ligand of protease-activated receptors. J Neurosci 2008;28:4331–5.

Reddy VB, Lerner EA. Plant cysteine proteases that evoke itch activate protease-activated receptors. Br J Dermatol 2010;163:532–5.

Reddy VB, Shimada SG, Sikand P, LaMotte RH, Lerner EA. Cathepsin S elicits itch and signals via protease-activated receptors. J Invest Dermatol 2010;130:1468–70.

Reddy VB, Sun S, Azimi E, Elmariah SB, Dong X, Lerner EA. Redefining the concept of protease-activated receptors: cathepsin S evokes itch via activation of Mrgprs. Nat Commun 2015;6: 7864.

Robas N, Mead E, Fidock M. MrgX2 is a high potency cortistatin receptor expressed in dorsal root ganglion. J Biol Chem 2003;278: 44400–4.

Schmelz M, Michael K, Weidner C, Schmidt R, Torebjork HE, Handwerker HO. Which nerve fibers mediate the axon reflex flare in human skin? Neuroreport 2000;11:645–8.

Schonefuss A, Wendt W, Schattling B, Schulten R, Hoffmann K, Stuecker M, et al. Upregulation of cathepsin S in psoriatic keratinocytes. Exp Dermatol 2010;19:e80–8.

Shelley WB, Arthur RP. Mucunain, the active pruritogenic proteinase of cowhage. Science 1955;122:469–70.

Sikand P, Dong X, LaMotte RH. BAM8-22 peptide produces itch and nociceptive sensations in humans independent of histamine release. J Neurosci 2011;31:7563–7.

Sikand P, Shimada SG, Green BG, LaMotte RH. Similar itch and nociceptive sensations evoked by punctate cutaneous application of capsaicin, histamine and cowhage. Pain 2009;144: 66–75.

Steinhoff M, Neisius U, Ikoma A, Fartasch M, Heyer G, Skov PS, et al. Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. J Neurosci 2003;23:6176–80.

Tatemoto K, Nozaki Y, Tsuda R, Konno S, Tomura K, Furuno M, et al. Immunoglobulin E-independent activation of mast cell is mediated by Mrg receptors. Biochem Biophys Res Commun 2006;349:1322–8.

Tsujii K, Andoh T, Lee JB, Kuraishi Y. Activation of proteinase-activated receptors induces itch-associated response through histamine-dependent and -independent pathways in mice. J Pharmacol Sci 2008;108:385–8.

Zhang L, Taylor N, Xie Y, Ford R, Johnson J, Paulsen JE, et al. Cloning and expression of MRG receptors in macaque, mouse, and human. Brain Res Mol Brain Res 2005;133:187–97.

See related commentary on pg 248

Circadian Time Effects on NB-UVB–Induced Erythema in Human Skin In Vivo

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TO THE EDITOR

UVR-induced skin erythema occurs after DNA damage, a consequence of the cell signaling cascades and synthesis of various cytokines and inflammatory mediators detected as vasodilation, inflammation, and apoptosis (Sklar et al., 2013). UVB-induced carcinogenesis has been linked to circadian time in mice, because those subjected to chronic UVB radiation in the morning had a 5-fold higher number of invasive squamous cell carcinomas compared with the evening-treated group (Gaddameedhi et al., 2011).

The time of day of UVR exposure influenced the erythema response, sunburn-induced apoptosis, p53 formation, and repair of DNA photoproducts and suggested that the circadian clock plays a role in UVR-induced responses (Gaddameedhi et al., 2015). The human skin is characterized by rhythms in almost all its functions (Dakup and Gaddameedhi, 2017), and there is a functional circadian clock in most cell types in the human skin (Plikus et al., 2015). It is the site of melatonin synthesis and metabolism (Slominski, 2005), but the interaction of melatonin produced in the skin with the suprachiasmatic nucleus as a feedback has not been proved (Vriend and Reiter, 2015).

To assess the interactions of UVR exposure with circadian time in human skin, minimal erythema dose (MED) testing for narrow-band UVB (NB-UVB) radiation was performed on 19 volunteers with skin phototypes II and III (Fitzpatrick, 1988) once in the morning and once in the evening (see Supplementary Materials and Methods online). The Regional Ethics Committee of Tampere University Hospital District approved the study protocol (R16001), and all volunteers gave their written informed consent. There was no significant difference in the classification of erythema (scored as −, +, +, ++, or +++) and corresponding MED (+) either in the morning or in the evening as assessed by the naked eye. To quantify erythema by erythema index (EI), all test squares and nonirradiated control sites were read three times using the DermaSpectrometer (Cortex Technology, Hadsund, Denmark). The EI scores of all test squares appeared higher in the dose series irradiated in the evening than in that irradiated in the morning. Figure 1a shows the average dose-response curves for the morning and evening irradiations delineating the average net changes in EI scores. The EI of the summarized morning and evening values differed ($P = 0.015$), suggesting that human skin is more vulnerable in the evening.

CRY1 and CRY2 proteins maintain circadian rhythms (Van Der Horst et al., 1999), and tumor suppressor protein P53 initiates protective mechanisms against permanent DNA damage

Abbreviations: AUC, area under the curve; EI, erythema index; IHC, immunohistochemistry; MED, minimal erythema dose; NB-UVB, narrow-band UVB; SED, standard erythemal dose

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(Decraene et al., 2001). To study erythema response and these proteins, we took biopsy samples from non-irradiated buttock skin for immunohistochemistry (IHC) at the beginning (see Supplementary Materials and Methods). We assessed with IHC whether the basal expression of CRY2 associated with UVB induced erythema response for 1 standard erythemal dose (SED) and found that those initially classified as $CRY2^+$ differed in their erythema response from subjects classified as $CRY2^-$ ($P = 0.011$) (Figure 1b, left panel). $CRY2^+$ and $CRY2^-$ subjects differed from each other in their erythema sensitivity in all UVB test doses using the area under the curve (AUC) of UVB doses ($P = 0.034$) (Figure 1b, right panel). All non-irradiated specimens showed negligible staining for P53. Expression of CRY1 in the non-irradiated skin was not associated with erythema response.

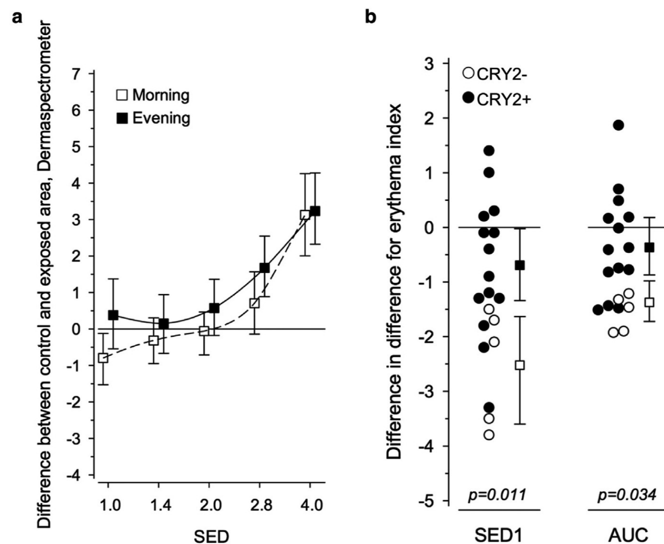

Figure 1. Impact of hour of the day and UVB dose on erythema and the effect of CRY2. (**a**) Difference in the EI of the irradiated skin and the control skin (= EI_{UVB} minus EI_{CON}) as a function of UVB radiation in SEDs. Summarized morning and evening values differed statistically significantly ($P = 0.015$). The difference was not detectable after 4 SEDs. (**b**) Difference in morning EI versus evening EI ([morning EI_{UVB} − morning EI_{CON}] − [evening EI_{UVB} − evening EI_{CON}]) calculated for the 1 SED and for the AUC of all doses in individuals with large amounts of CRY2 ($CRY2^+$) and negligible amounts of CRY2 ($CRY2^-$) in the skin without any UVB exposure. The difference in difference used in **b** is the result of morning minus evening values showed in **a**. The whisker shows 95% confidence interval. AUC, area under the curve; CON, control; EI, erythema index; SED, standard erythemal dose.

To evaluate the influence of UVB we took biopsy samples from irradiated skin at 24 hours after exposure from both the squares irradiated with 4 SEDs either in the morning or in the evening. It was the highest dose of the MED test series, and regardless of the time of day, all specimens showed positive staining for P53 using IHC. There was more P53 staining in the morning samples than in the evening samples ($P = 0.039$) (Figure 2 a−g). In mice, p53 also accumulated to a greater extent after irradiation in the early morning than in afternoon exposure when the amount of p53 was evaluated 2−12 hours after irradiation (Gaddameedhi et al., 2015). In our study, the biopsy samples were taken at 24 hours (i.e., later), and thus more functions of P53 could have been activated. In human skin, UVB-induced P53 elevation can be dissociated from erythema (Healy et al., 1994). The intensity of staining of CRY1 and CRY2 either in the morning or evening irradiations did not differ.

Our results contradict findings on increased erythema response after exposure to UVR in the morning compared with the evening in seven volunteers irradiated at 8-hour intervals (Guan et al., 2016). Different study protocols (e.g., light source resembling natural sunlight vs. NB-UVB) may influence the results. The basal expression of CRY2 protein correlated with a difference in erythema response, which is an interesting finding, because as far as we know this has not been reported earlier in humans. CRY2 may protect the skin against UVR in the morning hours. In mice, the transcriptional response to genotoxic stress was impaired in cells without Cry2, and Cry2-null cells accumulated damaged DNA, suggesting that Cry2 is particularly important for genomic integrity (Papp et al., 2015). Surprisingly, in some subjects the EI after doses of 1.0, 1.4, 2.0, and 2.8 SEDs showed even smaller values compared with the non-irradiated skin, which may be due to DNA-protective mechanisms or constriction of the capillaries. Daily periodicity characterized by low morning rates has been reported in capillary blood flow in human skin (Yosipovitch et al., 2004). Our spectrophotometric instrument, the DermaSpectrometer, even detects erythema at doses of UVR below the MED (Diffey et al., 1984). It might have been useful also to measure blood circulation using a Doppler device to detect the suspected vasoconstriction after the smallest UVR doses, but this was not determined in our protocol.

In agreement with animal studies (Gaddameedhi et al., 2015), our findings on humans suggest that circadian time protects the skin against sunburn erythema and might have implications for UVB-induced skin carcinogenesis, likely through nucleotide excision repair and P53 signaling, as previously shown by Gaddameedhi et al. (2015, 2011).

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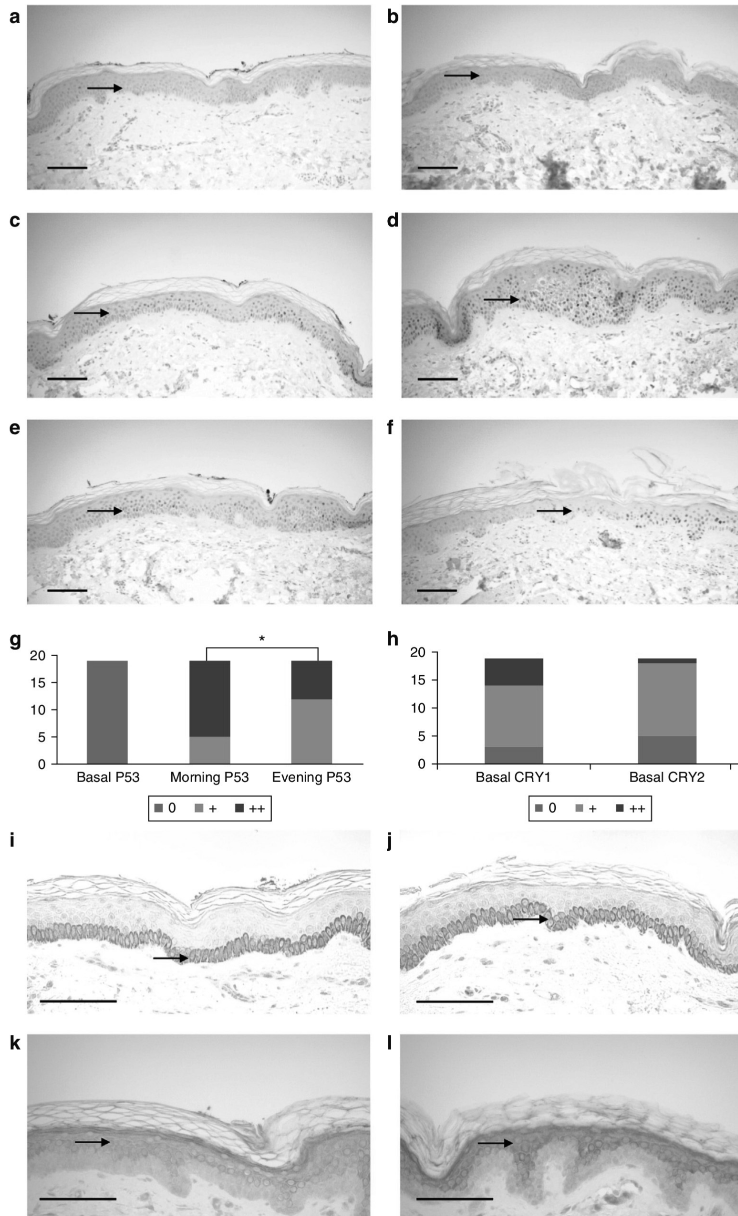

Figure 2. Basal protein expression in IHC and p35 expression after UVB exposure in both morning and evening of subjects 7 and 10 and semiquantitative analysis of all subjects. For subject 7, the left panel shows staining of basal P53, which was defined as (**a**) negligible, (**c**) strongly positive morning P53, and (**e**) positive evening P53. For subject 10 in the right panel, staining of basal P53 was defined as (**b**) negligible, (**d**) morning P53 strongly positive, and (**f**) evening P53 positive. (**g**) P53 staining in non-irradiated skin, morning and evening (n = 19). (**h**) Basal CRY1 and CRY2 of non-irradiated skin (n = 19). For subject 7, the left panel shows (**i**) staining of basal positive CRY1 ($CRY1^+$) and (**k**) negligible CRY2 ($CRY2^-$). For subject 10, the right panel shows (**j**) positive CRY1 ($CRY1^+$) and strongly positive CRY2 ($CRY2^+$). Scale bar = 0.1 mm. *P = 0.039. IHC, immunohistochemistry.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
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REFERENCES
Dakup P, Gaddameedhi S. Impact of the circadian clock on UV-Induced DNA damage response and photocarcinogenesis. Photochem Photobiol 2017;93:296—303.

Decraene D, Agostinis P, Pupe A, de Haes P, Garmyn M. Acute response of human skin to solar radiation: regulation and function of the p53 protein. J Photochem Photobiol B 2001;63:78—83.

Diffey BL, Oliver RJ, Farr PM. A portable instrument for quantifying erythema induced by ultraviolet radiation. Br J Dermatol 1984;111:663—72.

Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. Arch Dermatol 1988;124:869—71.

Gaddameedhi S, Selby CP, Kaufmann WK, Smart RC, Sancar A. Control of skin cancer by the circadian rhythm. Proc Natl Acad Sci USA 2011;108:18790—5.

Gaddameedhi S, Selby CP, Kemp MG, Ye R, Sancar A. The circadian clock controls sunburn apoptosis and erythema in mouse skin. J Invest Dermatol 2015;135:1119—27.

Guan L, Snuggs A, Ahsanuddin S, Tarrillion M, Selph J, Lam M, et al. 2016 arte poster competition first place winner: circadian rhythm and UV-Induced skin damage: an in vivo study. J Drugs Dermatol 2016;15:1124—30.

Healy E, Reynolds NJ, Smith MD, Campbell C, Farr PM, Rees JL. Dissociation of erythema and p53 protein expression in human skin following UVB irradiation, and induction of p53 protein and mRNA following application of skin irritants. J Invest Dermatol 1994;103:493—9.

Papp SJ, Huber A, Jordan SD, Kriebs A, Nguyen M, Moresco JJ, et al. DNA damage shifts circadian clock time via Hausp-dependent Cry1 stabilization. Elife 2015;4:1—19.

Plikus MV, Van Spyk EN, Pham K, Geyfman M, Kumar V, Takahashi JS, et al. The circadian clock in skin: implications for adult stem cells, tissue regeneration, cancer, aging, and immunity. J Biol Rhythms 2015;30:163—82.

Sklar LR, Almutawa F, Lim HW, Hamzavi I. Effects of ultraviolet radiation, visible light, and infrared radiation on erythema and pigmentation: a review. Photochem Photobiol Sci 2013:54—64.

Slominski A. The cutaneous serotoninergic/melatoninergic system: securing a place under the sun. FASEB J 2005;19:176—94.

Van Der Horst GTJ, Muijtjens M, Kobayashi K, Takano R, Kanno S-I, Takao M, et al. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 1999;3495:627—30.

Vriend J, Reiter RJ. Melatonin feedback on clock genes: A theory involving the proteasome. J Pineal Res 2015;58:1—11.

Yosipovitch G, Sackett-Lundeen L, Goon A, Huak CY, Goh CL, Haus E. Circadian and ultradian (12 h) variations of skin blood flow and barrier function in non-irritated and irritated skin—effect of topical corticosteroids. J Invest Dermatol 2004;122:824—9.

Xeroderma Pigmentosum Diagnosis Using a Flow Cytometry-Based Nucleotide Excision Repair Assay

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TO THE EDITOR
UVR induces dipyrimidine photoproducts such as cyclobutane pyrimidine dimer (CPD) and 6-4 pyrimidine—pyrimidone photoproduct (6-4PP), which cause distortions in the double helix (Lagerwerf et al., 2011). These dipyrimidine photoproducts are repaired through the nucleotide excision repair (NER) pathway. Xeroderma pigmentosum (XP) is a rare, autosomal recessive, hereditary disease characterized by hypersensitivity to sunlight and is associated with a high incidence of skin cancer; some patients also experience neurological symptoms. XP is classified into eight subclinical types: seven genetic complementation groups (A through G) deficient in an NER pathway and the XP variant (XP-V) type, which is deficient in translesion synthesis (DiGiovanna and Kraemer, 2012; Masutani et al., 1999). Because of NER or translesion synthesis deficiency, patients with XP cannot remove or overcome the dipyrimidine photoproducts. Therefore, to diagnose XP, the DNA repair ability and host cell reactivation have been assayed in clinical settings (Moriwaki and Kraemer, 2001). The unscheduled DNA synthesis (UDS) assay measures the cellular ability of NER (Lehmann et al., 1975). However, conventional

Abbreviations: 6-4PP, 6-4 pyrimidine-pyrimidone photoproduct; CPD, cyclobutane pyrimidine dimer; CS, Cockayne syndrome; NER, nucleotide excision repair; UDS, unscheduled DNA synthesis; XP, xeroderma pigmentosum; XP-V, xeroderma pigmentosum variant

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PUBLICATION
II

Ultraviolet B radiation modifies circadian time in epidermal skin and in subcutaneous adipose tissue

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ULTRAVIOLET B RADIATION MODIFIES CIRCADIAN TIME IN EPIDERMAL SKIN AND IN SUBCUTANEOUS ADIPOSE TISSUE

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Short title: UVB modifies circadian time in human skin

The research was conducted in Päijät-Häme Social and Health Care Group, Department of Dermatology and Allergology, Lahti, Finland.

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Background

Recent findings suggest that circadian time regulates cellular functions in the skin and may affect protection against ultraviolet radiation (UVR). It is not known, however, whether UVR through skin directly affects the expression of circadian genes. We investigated the effect of ultraviolet B (UVB) exposure on cryptochrome circadian clock 1 (*CRY1*), cryptochrome circadian clock 2 (*CRY2*), and circadian associated repressor of transcription (*CIART*) genes.

Methods

Healthy volunteers (n=12) were exposed to narrow-band UVB radiation of 4 standard erythemal dose (SED). Epidermal/dermal and subcutaneous adipose tissue samples were obtained by punch biopsies from irradiated and non-irradiated skin 10 cm away from the irradiated site 24 hours after UVB exposure. Gene expression of *CRY1*, *CRY2,* and *CIART* was measured using RT-PCR (TaqMan).

Results

Ultraviolet B radiation affected mRNA expression in the epidermal/dermal skin and in the subcutaneous adipose tissue. It down-regulated expression of *CRY2* gene in the epidermal/dermal skin, whereas it up-regulated expression of *CRY1* and *CIART* genes in the subcutaneous adipose tissue.

Conclusion

We showed for the first time that UVB radiation affects expression of circadian genes in the subcutaneous adipose tissue. Further studies are warranted to understand the mechanisms in detail.

INTRODUCTION

Ultraviolet radiation (UVR) reaching the skin is a carcinogen, inducing immunosuppression and DNA mutations (1) and the wavelengths of ultraviolet light B (UVB) represent a biologically very active part of it (2). The chromophores transduct the electromagnetic energy of UVB into neural, chemical and hormonal signals to produce rapid (neural) or slow (humoral or immune) responses at the local and systemic levels (3,4). Skin irradiated by UVB can activate both the central and local hypothalamic-pituitary-adrenal (HPA) axis to turn on homeostatic responses and deactivate local and systemic damage (4,5). Locally UVB irradiation of the skin leads to activation of pro-opiomelanocortin (POMC) and corticotropin releasing hormone (CRH) in epidermal and dermal skin, produces cutaneous POMC peptides and activates the corresponding receptors that are important regulators on the pigmentary and the inflammatory reactions. (6-8) In addition keratinocytes stimulated by UVB can, as a rapid effect, secrete e.g., interleukin-1β (IL-1β), IL-6 and tumor necrosis factor (TNF) (9). The transmission of UV radiation to the skin depends decisively on the individual skin type and UV adaptation status. UV wavelegths below 305 nm are almost totally absorbed into the stratum corneum and epidermis. For UV wavelengths above 305 nm about 10% reach the dermis. No UV radiation penetrates through the dermis to the subcutaneous adipose tissue. (1,10,11).

The skin epithelium is one of the most actively proliferating tissues in the human body and characterized by an approximately 24-hour rhythm in almost all its functions (12,13) These circadian rhythms are regulated by an endogenous time-keeping system, the circadian clock. The central pacemaker of human circadian rhythms is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus in the brain, while peripheral tissues follow the pace at transcriptional levels using a transcriptional-translational feedback loop (14). The genes involved in the regulation and maintenance of the circadian rhythms are called clock genes.

Canonical clock genes, such as cryptochrome circadian clocks (*CRY1*, *CRY2*), encode proteins which interact with each other to build up an autoregulatory feedback loop (14). The CRY1/CRY2 ratio alters the circadian period, an increasing ratio being associated with a longer circadian period (16). CRY1 or CRY2 as well as the period circadian clock (PER1 or PER2) as dimers are transcriptional repressors in the core transcription-translation feedback loop (15,16). Aryl hydrocarbon receptor nuclear translocator-like (ARNTL) protein is the key circadian clock protein and a transcription factor of *CRY1* and *CRY2* genes (19).

There is a daily rhythm in the expression of circadian associated repressor of transcription (*CIART*) gene, and it is also regulated by stress responses (20). CIART protein has been shown to have a role in the transcriptional regulation of clock-controlled genes, as it can repress circadian locomotor output cycles kaput (CLOCK) and ARNTL protein activity in different time-windows from CRY proteins and PER proteins (16,21). In a diurnal primate, the baboon, the most frequent cycling gene was *Ciart*, as it was cycling in 52 of the 64 tissues sampled every two hours over a period of 24 hours, and the expressions of the seven main clock genes in the diurnal baboon and in the nocturnal mouse were in antiphase to each other (22). Circadian clock proteins and genes have been shown to regulate cellular responses after UVB exposure: in mice, sensitivity to UVB-induced DNA damage in the epidermal skin is a process that is dependent on ARNTL as a function of the time of day (23,24). Earlier we showed the effect on circadian timing in UVB-induced erythema response in human epidermal/dermal skin (25). However, the effect of UVB radiation on clock genes is not fully understood.

Human skin cells are known to express circadian clock proteins (26), but very little is so far known about the regulation of their expression and activity. In adipose tissue *CIART* gene oscillates with a circadian frequency (27). In addition, human adipocytes express other clock genes (28,29), but as far as we know there is no earlier data on whether the clock genes are

expressed in the subcutaneous adipose tissue after UVB exposure. The effects of UVR on subcutaneous adipose tissue are transmitted through cytokines such as IL-6, IL-8, monocyte chemoattractant protein–3 (MCP-3) and placenta growth factor (PlGF) (30) and signal transduction can occur through melanocortin receptors (MC1R and MC2R) (9).

Our aim was to explore the effects of UVB on mRNA levels of *CRY1*, *CRY2,* and *CIART* genes in the skin of healthy volunteers. We also investigated for the first time whether the effects of UVB on circadian gene expression extend from epidermal/dermal skin to the subcutaneous adipose tissue and measured amounts of mRNA of the *IL-1β, IL-6, TNF, POMC*, and melanocortin receptors (*MCR1/MCR2*) in the both tissues to confirm earlier findings and find possible link between UVB and clock proteins below the skin surface.

MATERIALS AND METHODS

Healthy adult volunteers (n=12) presenting with anamnestic skin phototypes II or III (31) were eligible to participate. The skin phototype was confirmed in a clinical interview. Subjects with photosensitizing, immune modulating or psychiatric medication, photosensitivity, pregnancy, lactation, history of skin cancer or extensive scarring, or marked UVR exposure in the three preceding months were not eligible to participate. During the study no medication was allowed. Three subjects had recently taken some medication and to meet the inclusion criteria this had to be discontinued for a minimum of 48 hours before the beginning of the study.

Ethics

This study was carried out in the Department of Dermatology, Päijät-Häme Central Hospital, Lahti, Finland, from January to April 2016. Written informed consent was obtained from the participants and the ethics committee of Hospital District of Pirkanmaa approved the study protocol (#R16001)

Ultraviolet B radiation

One square cm area of the buttock skin was exposed to 4 standard erythemal dose (SED), while the rest of the skin was protected. A Waldmann UV 801KL phototherapy device equipped with 4 TL20W/01 narrowband UVB (NB-UVB) tubes was used for UVB irradiations. The bulbs were preheated for seven minutes before the exposure. The irradiance of the device was measured before the study using an Ocean Optics S2000 spectroradiometer by the Nuclear Safety Authority of Finland. The time to irradiate the test area was calculated on the basis of these measurements so that the 4 SED area was irradiated for 6 min 28 sec. One SED is defined as an erythemal effective radiant exposure of 10 mJ/cm^2, and is equivalent to a non-weighted physical UV dose of 172 mJ/cm^2 emitted from our narrowband UVB lamps. The 4 SED thus equals a non-weighted physical dose of 688 mJ/cm^2. Most of the radiation of the narrowband UVB lamp's radiation is in the 311 nm emission peak. The spectrum of the narrowband UVB lamps is presented in Figure 1. Uncertainty (2σ) of the measurement of the OceanOptics S2000 was estimated to be approximately 14% (32). The measurements are traceable to the National Institute of Standards and Technology (Gaithersburg, Maryland, USA).

Sample collection

While the narrowband UVB irradiation was administered in the morning between 7 a.m. and 9 a.m. on day one, the 3 mm skin punch biopsies, consisting of epidermis/dermis and subcutaneous adipose tissue, were sampled 24 hours after the exposure on day two. A non-irradiated skin control biopsy was obtained at least 10 cm away from the irradiated skin square, and had been protected against UVB with impermeable fabric. Lidocaine 1% without epinephrine was used for local anesthesia, and the punch biopsies were obtained thereafter. The subcutaneous adipose tissue and epidermis/dermis were cut separately from each other with a scalpel and incubated separately. All samples were incubated in RNAlater (Thermo Fisher

Scientific, Massachusetts, USA) for 24 hours at +4°C, and thereafter deep-frozen and stored at from −80°C to −20°C until mRNA extraction.

Total-RNA extraction, cDNA synthesis and RT-PCR

Both epidermis/dermis and subcutaneous adipose tissue samples were mechanically homogenized by Ultra-turrax homogenizer (IKA-Analysentechnik) in RLT buffer (Qiagen, Hilden, Germany). To sediment the small tissue pieces and to layer possible fat, the homogenates were centrifuged twice at 12 000 × g for 10 min at +4°C. Total RNA from epidermal/dermal skin biopsies was isolated using AllPrep kit (Qiagen, Venlo, The Netherlands). Four hundred micrograms of RNA was reverse transcribed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, U.S.A.) to cDNA as previously described (33). And from subcutaneous adipose tissue, 80 µg of RNA was reverse transcribed with VILO kit (Thermo Fisher Scientific/Invitrogen, Carlsbad, CA, U.S.A.) according to manufacturer's instructions. The expression of the cytokines and genes involved in the circadian rhythm were studied by TaqMan 7500 Fast System (Applied Biosystems) with PerfeCTa qPCR FastMix (Quanta Biosciences, Gaithersburg, MD, U.S.A.). PCR primers and probes for cytokines and chemokines were obtained as predeveloped assay reagents from Applied Biosystems. Ribosomal 18S gene was used as housekeeping gene in the TaqMan analyses.

Statistics

Gene expressions of selected circadian genes and cytokines were first compared using ordinary one-way ANOVA at different time points. Statistical analysis of target gene expression was performed with the nonparametric Mann–Whitney test before and after UVB treatment. A single apparent outlier from the null samples was excluded from the analysis. The

association between the expression of *CRY1* and *CRY2* in the skin at different time points was examined using the Pearson's correlation test. All statistical analyses were performed with GraphPad Prism 7 Software (GraphPad Software Inc., San Diego, California).

RESULTS

Altogether 12 healthy subjects participated in the study, 2 men and 10 women, aged 41 (26 to 64) years on average and with BMI 23.5 (SD ±3.4) on average. Four presented with anamnestic skin phototype II and eight with phototype III (31). We found the epidermal/dermal skin mRNA of *CRY2* gene to be at a lower level of expression after UVB irradiation than skin without irradiation. We also found that in the null epidermal/dermal skin sample there was 6.5 times more and in the subcutaneous adipose tissue as much as 20 times more *CRY2* than *CRY1* mRNA.

Clock gene expressions after UVB

After the UVB exposure expression of *CRY2* mRNA in epidermal/dermal skin was significantly decreased (p=0.0492), and a similar tendency was seen in *CIART* mRNA (Figure 2a). UVB irradiation of the skin had no statistically significant impact on the expression of epidermal/dermal skin *CRY1* mRNA, but changes in gene expression were seen in the subcutaneous adipose tissue; UVR significantly enhanced the expression of *CRY1* (p=0.0263) (Figure 2b). The expression of *CIART* also enhanced (p=0.0411) and a small increase was seen in *CRY2*. We found a positive linear correlation between *CRY2* and *CRY1* mRNA amounts in epidermal/dermal skin, especially after irradiation (r=0.84) (Figure 3a) and the *CRY1/CRY2* ratio was significantly different after irradiation (p=0.0011). In subcutaneous adipose tissue the correlation was weak (Figure 3b).

UVB modifies cytokine expressions in skin but not in adipose tissue

UVB exposure significantly enhanced the expression of interleukin-1β *(IL-1β)* mRNA in the epidermal/dermal skin (p=0.0011), and a similar tendency was also observed in the expression of *TNF* (Figure 4a). UVB exposure did not induce clear changes in *IL-6* after 24 hours. No significant changes in the subcutaneous adipose tissue were seen in the cytokine expressions studied (Figure 4b). A small increase was also seen after UVB exposure in *POMC* and melanocortin 1 receptor (*MC1R)*, both in the epidermal/dermal skin and subcutaneous adipose tissue. melanocortin 2 receptor (*MC2R*) level showed no significant change (Figure 4a-b).

DISCUSSION

In addition to epidermal/dermal skin UVB radiation seems also to affect mRNA expression in the subcutaneous adipose tissue. We found *CRY2* expression to be down-regulated in epidermal/dermal skin following UVB radiation, and in subcutaneous adipose tissue *CRY1* and *CIART* expression appeared to be up-regulated. The differences in trend in the two tissues with CRY2 and CIART genes may be due to the fact that no NB-UVB penetrates into the subcutaneous adipose tissue.

In humans and other mammals visible light is capable of interacting with the master circadian clock through retinal rods, cones, and ganglion cells that project information from the eyes to the SCN (34-38). Both CRY1 and CRY2 have been detected in the human retina by immunohistochemistry (39) and can function as blue-light photoreceptors, but may even be more sensitive to shorter (from 300 nm to 450 nm) UV- wavelengths (40,41). While the SCN is a pacemaker and the main link between the circadian clock and light exposure, in plants and animals peripheral clocks play a role in perceiving changes in environmental radiation (42), which, based on CRY1 and CRY2 signaling mechanisms, may also be possible in humans (43). In the human retina *CRY2* mRNA is 11 times more abundant than *CRY1* and expected to capture the photons necessary to initiate a light-sensitive response (39). We found that in untreated

control skin there was 6.5 times and in the subcutaneous adipose tissue as much as 20 times more *CRY2* than *CRY1* mRNA, which corroborates the putative role of CRY2 protein interactions with UVB. Although UVB induced mRNA expressions of the clock genes have not been studied earlier *in vivo*, in cultured human keratinocytes UVB downregulated *ARNTL*, *CLOCK* and *PER1* gene expressions. (44).

The observed change in clock genes due to UVB may affect the repair of UVR- induced damage. In mice inflammatory cytokine induction, sunburn apoptosis, and erythema are controlled by circadian rhythm (23). Huber et al. suggested that CRY2 can limit tumor formation by promoting the turnover of c-MYC, a critical regulator of cell proliferation (45). This observation is in line with our earlier finding that human skin with a neglible amount of CRY2 showed more erythema (25). While we now found *CRY2* expression to be down-regulated in epidermal/dermal skin following UVB radiation, is possible that UVB exposure undermines the protective mechanisms in epidermal/dermal skin.

Since after UV exposure proinflammatory cytokines IL-1, IL-6, and TNF are expressed in various skin cell types (9), we expected to detect a significant increase in their mRNA levels. Based on cytokine mRNA levels a slight inflammation occurred in the epidermal/dermal skin but not in the subcutaneous adipose tissue. As UVB induces POMC, we expected to see a significant increase in the expression of *POMC* mRNA when comparing the irradiated and non-irradiated skin specimens. The UVB dose used in our study was large enough to induce visible erythema at 24h, whereas the increase in pigmentation takes more time (46). It is thus possible that later timing of biopsy, e.g. some days later, would have revealed a more pronounced increase in *POMC* mRNA. However, we already detected a small increase in *POMC* mRNA expression both in the epidermal/dermal skin and subcutaneous adipose tissue at 24h after exposure, which may indicate that later sampling might have worked better for

detecting *POMC* mRNA. As we found no proof of the involvement of melanocortin receptors in signaling between UVB and cryptocromes in adipose tissue at 24h, these could be expressed later at 3 to 6 days, when pigmentation reaches its maximum (46).

As we detected an increase in the *CRY1/CRY2* ratio after irradiation, it is possible, in light of earlier findings, that UVB extends the circadian period (17). In addition melatonin produced in epidermal/dermal skin may play a role in the UVB damage responses and mRNA synthesis of clock proteins, but this needs to be clarified in future clinical studies (47). Due to the important role that circadian functions play in the human body, it is essential to understand the mechanisms behind the circadian clock of the metabolic active tissue, the subcutaneous adipose tissue, as part of the skin. As clock genes in adipose tissue have been linked to the metabolic syndrome (48) our data opens up a new perspective when considering the implications of UVR exposure for human health. Unfortunately a limitation of our study is the relatively small number of participants and the fact that we only measured mRNA but not proteins, which is due to invasive nature. Larger cohorts are needed to confirm these links from UVB to adipose tissue disturbances.

CONCLUSION

We investigated the effect of UVB radiation on the expression of key clock genes in epidermal/dermal skin and subcutaneous adipose tissue, showing for the first time that UVB radiation affects expression of circadian genes in the subcutaneous adipose tissue. Variation in the expression of *CIART* gene suggests that it is a possible oscillator of skin functions. More studies are warranted to understand further actions and mechanisms of clock genes in detail. Our findings suggest that UVB may influence peripheral clock genes in the skin to modulate, for example, cellular responses against to UVR-induced damage.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

AUTHOR’S CONTRIBUTIONS

The first author V.N. and M.G. took part in the planning of the study protocol, implemented all UVB irradiations and samplings, authors P.K., M.M. and H.A. performed the mRNA analyses, statistical tests, and produced the figures, L.Y was responsible for UVR dosimetry, all authors took part in the evaluation of results and writing. T.P. and E.S. jointly steered the whole study.

REFERENCES

1. D'Orazio J, Jarrett S, Amaro-Ortiz A, Scott T. UV radiation and the skin. Int J Mol Sci 2013;14:12222–48.
2. Freeman SE, Hacham H, Gange RW, Maytum DJ, Sutherland JC, Sutherland BM. Wavelength dependence of pyrimidine dimer formation in DNA of human skin irradiated in situ with ultraviolet light. Proc Natl Acad Sci USA 1989;86:5605–9.
3. Slominski AT, Zmijewski MA, Skobowiat C, Zbytek B, Slominski RM, Steketee JD. Sensing the environment: Regulation of local and global homeostasis by the skin neuroendocrine system. Adv Anat, Embryol Cell Biol 212: 1-115, 2012.
4. Slominski AT, Zmijewski MA, Plonka PM, Szaflarski JP, Paus R.How UV Light Touches the Brain and Endocrine System Through Skin, and Why. Endocrinology 159(5), 1992-2007, 2018.
5. Slominski AT, Zmijewski MA, Zbytek B, Tobin DJ, Theoharides TC, Rivier J. Key Role of CRF in the Skin Stress Response System. Endocrine Rev 34:827-84, 2013.
6. Slominski A, Wortsman J, Luger T, Paus R, Solomon S. Corticotropin Releasing Hormone and Proopiomelanocortin Involvement in the Cutaneous Response to Stress. Physiol Rev 80, 979-1020, 2000.
7. Slominski A, Tobin DJ, Shibahara S, Wortsman J. Melanin Pigmentation in Mammalian Skin and Its Hormonal Regulation. Physiol Rev 84, 1155-1228, 2004.
8. Slominski A, Tobin DJ, Paus R. Does p53 regulate skin pigmentation by controlling proopiomelanocortin gene transcription? Pigment Cell Res 20, 307-308, 2007.
9. Slominski A, Wortsman J. Neuroendocrinology of the skin. Endocr Rev 2000;21:457–87.

10. Bruls WA, Slaper, H, van der Leun JC, Berrens L. Transmission of human epidermis and stratum corneum as a function of thikness in the ultraviolet and visible wavelengths. Photochem Photobiol. 1984;40: 485-494.

11. Meinhardt M, Krebs R, Anders A, Heinrich U, Tronnier H. Absorption spectra oh human skin in vivo in the ultraviolet wavelength ranga measured by optoacoustics. Photochem and Photobiol. 2009;85;70-77.

12. Dakup P, Gaddameedhi S. Impact of the circadian clock on UV-Induced DNA damage response and photocarcinogenesis. Photochem Photobiol 2017;93:296-303.

13. Matsui MS, Pelle E, Dong K, Pernodet N. Biological rhythms in the skin. Int J Mol Sci 2016;24;17

14. Panda S, Hogenesch JB, Kay SA. Circadian rhythms from flies to human. Nature 2002;417:329–35.

15. King DP, Takahashi JS. Molecular genetics of circadian rythms in mammals. Annu Rev Neurosci. 2000;23:713–42.

16. Goriki A, Hatanaka F, Myung J, Kim JK, Yoritaka T, Tanoue S, et al. A novel protein, CHRONO, functions as a core component of the mammalian circadian clock. PLOS Biol 2014;12:1–15.

17. Papp SJ, Huber A-L, Jordan SD, Kriebs A, Nguyen M, Moresco JJ, et al. DNA damage shifts circadian clock time via Hausp-dependent Cry1 stabilization. eLife 2015;4: e04883.

18. Reppert SM, Weaver DR. Coordination of circadian timing in mammals. Nature 2002;418:935–41.

19. Lowrey PL, Takahashi JS. Genetics of Circadian Rhythms in Mammalian Model Organisms. Adv Genet. 2011;75:173–230.

20. Hatanaka F, Takumi T. CHRONO integrates behavioral stress and epigenetic control of metabolism. Ann Med 2017;49:352–6.

21. Annayev Y, Adar S, Chiou Y, Lieb JD, Sancar A, Ye R. Gene model 129 (Gm129) encodes a novel transcriptional repressor that modulates circadian gene expression. J Biol Chem 2014;289:5013–24.

22. Mure LS, Le HD, Benegiamo G, et al. Diurnal transcriptome atlas of a primate across major neural and peripheral tissues. Science. 2018;359:6381

23. Gaddameedhi S, Selby CP, Kemp MG, Ye R, Sancar A. The circadian clock controls sunburn apoptosis and erythema in mouse skin. J Invest Dermatol 2015;135:1119–27.

24. Geyfman M, Kumar V, Liu Q, et al. 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 2012;109:11758–63.

25. Nikkola V, Grönroos M, Huotari-Orava R, Kautiainen H, Ylianttila L, Karppinen T, et al. Circadian time effects on NB-UVB-induced erythema in human skin in vivo. J Invest Dermatol 2018;138:464-7.

26. Zanello SB, Jackson DM, Holick MF. Expression of the circadian clock genes clock and period1 in human skin. J Invest Dermatol 2000;115:757–60.

27. Anafi RC, Lee Y, Sato TK, Venkataraman A, et al. Machine learning helps identify CHRONO as a circadian clock component. PLoS Biol 2014;12.

28. Otway DT, Mäntele S, Bretschneider S, Wright J, Trayhurn P, Skene DJ, et al. Rhythmic diurnal gene expression in human adipose tissue from individuals who are lean, overweight, and type 2 diabetic. Diabetes 2011;60:1577–81.

29. Vieira E, Ruano Eg, Figueroa AL, Aranda G, Momblan D, Carmona F, et al. Altered clock gene expression in obese visceral adipose tissue is associated with metabolic

syndrome. PLoS One 2014;9:e111678.

30. Kim EJ, Kim YK, Kim JE, Kim S, Kim M-K, Park C, et al. UV modulation of subcutaneous fat metabolism. J Invest Dermatol 2011;131:1720–6.

31. Fitzpatrick TB. The validity and practicality of sun-reactive skin types I through VI. Arch Dermatol 1988:124:869–871.

32. Ylianttila L, Visuri R, Huurto L, Jokela K. Evaluation of a single-monochromator diode array spectroradiometer for sunbed UV-radiation measurements. Photochem Photobiol 2005;81:333-341.

33. Wang G, Savinko T, Wolff H, Dieu-Nosjean MC, Kemeny L, Homey B, et al. Repeated epicutaneous exposures to ovalbumin progressively induce atopic dermatitis-like skin lesions in mice. Clin Exp Allergy 2007;37:151–61.

34. Berson DM. Phototransduction in ganglion-cell photoreceptors. Pflugers Arch. 2007:849–55.

35. Guler AD, Altimus CM, Ecker JL, Hattar S. Multiple photoreceptors contribute to nonimage-forming visual functions predominantly through melanopsin-containing retinal ganglion. Cold Spring Harb Symp Quant Biol 2007;72:509–15.

36. Hannibal J, Hindersson P, Knudsen SM, Georg B, Fahrenkrug J. The photopigment melanopsin is exclusively present in pituitary adenylate cyclase-activating polypeptide-containing retinal ganglion cells of the retinohypothalamic tract. J Neurosci 2002;22:1-7.

37. Hattar S, Liao H-W, Takao M, Berson DM, Yau K-W. Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 2001;295:1065–70.

38. Hannibal J, Hindersson P, Østergaard J, Georg B, Heegaard S, Larsen PJ, et al.

Melanopsin is expressed in PACAP-containing retinal ganglion cells of the human retinohypothalamic tract. Investig Ophthalmol Vis Sci 2004;45:4202–9.

39. Thompson CL, Rickman CB, Shaw SJ, Ebright JN, Kelly U, Sancar A, et al. Expression of the blue-light receptor cryptochrome in the human retina. Invest Ophthalmol Vis Sci. 2003 44:4515-21.

40. Foley LE, Gegear RJ, Reppert SM. Human cryptochrome exhibits light-dependent magnetosensitivity. Nat Commun. 2011: 2:356

41. Hsu DS, Zhao X, Zhao S, Kazantsev A, Wang R-P, Todo T. Putative human blue-light photoreceptors hCRY1 and hCRY2 are flavoproteins. Biochemistry 1996;35:13871–7.

42. Chaves I, Pokorny R, Byrdin M, Hoang N, Ritz T, Brettel K, et al. The cryptochromes: blue light photoreceptors in plants and animals. Annu Rev Plant Biol. 2011;62:335–64.

43. Vieira J, Jones AR, Danon A, Sakuma M, Hoang N, Robles D, et al. Human cryptochrome-1 confers light independent biological activity in transgenic drosophila correlated with flavin radical stability. PLoS One 2012;7: e31867.

44. Kawara S, Mydlarski R, Mamelak AJ, Freed I, Wang B, Watanabe H, et al. Low-dose ultraviolet B rays alter the mRNA expression of the circadian clock genes in cultured human keratinocytes. J Invest Dermatol 2002;119:1220–3.

45. Huber AL, Papp SJ, Chan AB, Henriksson E, Jordan SD, Kriebs A, et al. CRY2 and FBXL3 cooperatively degrade c-MYC. Mol Cell 2016.

46. Suh KS, Roh H J, Choi SY, Jeon YS., Doh KS, Bae JH, et al. A long-term evaluation of erythema and pigmentation induced by ultraviolet radiations of different wavelengths. Ski Res Technol 2007;13:360–8.

47. Slominski AT, Hardeland R, Zmijewski MA, Slominski RM, Reiter RJ, Paus R. Melatonin: A Cutaneous Perspective on its Production, Metabolism, and Functions J

Invest Dermatol 138:490-499, 2018.

48. Gómez-Abellán P, Hernández-Morante JJ, Luján JA, Madrid JA, Garaulet M. Clock genes are implicated in the human metabolic syndrome. Int J Obes 2008;32:121–8.

LEGENDS FOR ILLUSTRATION

Figure 1. **The spectrum of the narrowband UVB lamp TL01.** The spectrum has an emission maximum at 311 nm. 63% of the lamps UV and visible (280 -780 nm) radiation is in the narrow 305 -315 nm band. The UVB (280 – 315 nm) range contains 64%, UVA (315 – 400 nm) 16% and visible light (400 -780 nm) 19% of the lamps UV and visible light radiation.

Figure 2. **Circadian gene expression in a) epidermal/derman skin and in b) subcutaneous adipose tissue** 24h after four SED UVB exposure. The total number of samples in group is 11-12. Nonparametric Mann–Whitney test was used to represent the column of group means, and standard errors of the means (SEM). Ctrl, untreated control skin; UV, UV-treated skin; RU, relative units. *p<0.05

Figure 3. **Correlation between *CRY1* (y-axis) and *CRY2* (x-axis) in UV-exposed and control tissues in a) epidermal/dermal skin and in b) subcutaneous adipose tissue** 24h after four SED UVR exposure. The total number of samples in a group is 11. Linear regression was used to model the data per CRY2, and Pearson's correlation was used to compute the correlation coefficients (r). Ctrl, untreated control skin; UV, UV-treated skin. **p<0.005

Figure 4**. Cytokine, *POMC* and melanocortin receptor gene expression in a) skin and in b) subcutaneous adipose tissue** 24h after four SED UVR exposure. The total number of samples in group is 11-12. Nonparametric Mann–Whitney test was used to represent the group means and standard errors of the means (SEM). Ctrl, untreated control skin; UV, UV-treated skin. *p<0.05, **p<0.005

PUBLICATION
III

Epidermal melatonin levels are higher in the evening than morning but do not account for erythema sensitivity

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