SCIENTIFIC COMMUNIQUÉ II

CLINUVEL

May 2018

Following the positive response to SCIENTIFIC COMMUNIQUÉ I, released in April 2018, CLINUVEL will now publish a longer SCIENTIFIC COMMUNIQUÉ series throughout the course of 2018. The goal of the SCIENTIFIC COMMUNIQUÉS is to share the current understanding and progress in proopiomelanocortin (POMC) science and photomedicine with various interested audiences. SCIENTIFIC COMMUNIQUÉ I provided an outline of the various physiological modifications taking place on proteins and the clinical relevance to our technology programs. In SCIENTIFIC COMMUNIQUÉ II we delve into how ligands bind to the various cellular receptors, signalling cascades and output to arrive at therapeutically meaningful applications. In SCIENTIFIC COMMUNIQUÉ III –scheduled for June – we will review the effects of afamelanotide, CLINUVEL's lead drug, on the human genome, based on existing literature. These three pieces will then be used as a basis for further COMMUNIQUÉS later in the year. After the COMMUNIQUÉS we hope the reader will be able to grasp the, often opposing, opinions found in the abundance of literature on relevant topics. A library of scientific terms has been established on CLINUVEL's website allowing the reader to follow in time.

LIGANDS – MOORING AND BERTHING OF THE VESSEL

CLINUVEL's attention has long been on the family of melanocortin receptors, and specifically the melanocortin-1 receptor (MC1R). This receptor is expressed on a number of epidermal and dermal cells but also at various other locations in the human body. In our journey, we simultaneously started to investigate the endothelin-1, cKIT, CSF and MAP kinases. All of these deserve further discussion in a subsequent COMMUNIQUÉ, but it is sufficient here to state that our scientific teams are taking a broader direction when evaluating the applicability of the melanocortin family.

The study of the cellular membrane receptors, their expression, behaviour, and up and down regulation under various conditions, makes pharmacology and the area of pharmacogenomics a dynamic subject.

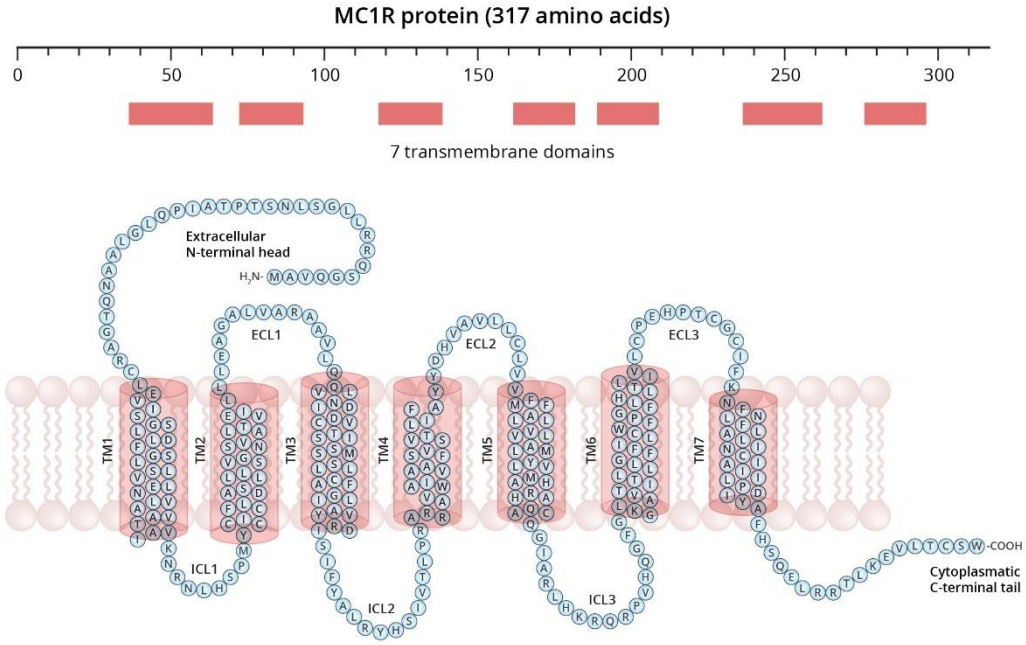

Figure 1: MC1 receptor

The MC1R consists of 317 amino acids and belongs to the family of G-protein coupled receptors (GPCRs), a receptor spanning the cell membrane over seven passes (seven winded pathways), also called transmembrane. Common to all GPCRs is that they pick up extracellular signals to pass them on to the cellular environment. Of high interest here are the signals provided by hormones, peptides which are transmitted through a cascade further into the cell. MC1R was first found in 1994 to encode for chromosome 16q24.3, and further genotyping followed years later. In reference to SCIENTIFIC COMMUNIQUÉ I, proteins undergo a wide variety of processes, and the protein receptor MC1R is subject to palmitoylation at one end of the molecule within the cell. The function of adding cysteine thiols on a transmembrane protein has been widely speculated, yet the consensus is that it regulates cell function and homeostasis and, critically, acts as a protein membrane associative signal. Conversely, the lack of the critical palmitoyltransferase (PAT) – an enzyme active during the process – is associated with various diseases of the nervous system and cancer progression. Humans are known to have 23 different palmitoyltransferases, and many proteins which are palmitoylated are associated with neuronal development.

Stepping back to the subject of the receptor itself, the further study of exploring a *mooring and berthing site* has proven essential to grasp the quality of the cellular signalling origin, in this case the melanocyte and other epi-/dermal cells, but also white blood cells. In the 1990's, a number of research groups started to hone in on the importance of the MC1R quality and its possible consequences on the human response. In 2007, a Queensland research group started reporting its findings that loss-of-function alleles of the MC1R seen in Caucasian individuals made them more sensitive to ultraviolet (UV) radiation, a long-held view but now demonstrated in a series of human experiments. Large scale studies found that the polymorphisms of this receptor were mostly found in red haired individuals, this became later known in the field as the red hair colour (RHC)-blue eyes-freckled phenotype. These individuals are known to burn fast and intensely following sun and UV exposure, and poorly respond through melanogenesis ('tanning response'). The RHC is a group of the population which benefits from local or systemic photoprotection. In subsequent SCIENTIFIC COMMUNIQUÉS we will expand our views on this subset, in which it was

supposed that the degree of MC1R function correlated with the extent of pigmentation in RHC individuals.

In Australia, where we tend to be most interested in the role of the skin's response to longer term UV irradiation, research was well under way to clarify the precise role of the MC1R in photoprotection. Since many individuals in Australia are of Anglo Saxon descent, this research deserved much attention from the Australian public and was well received among melanocyte biologists.

Simultaneously, other research groups started to look into the relevance of the MC1R function and its cellular output. As mentioned in SCIENTIFIC COMMUNIQUÉ I, the quality of the binding agent, but also of the receptor, is key in transmitting and provoking an adequate cellular response. Which ligand selects the relevant receptor is subject of pharmacokinetic experiments. Perhaps a maritime analogy is most appropriate: the quality of the berthing site attracts the best vessels, who in turn select the preferable mooring sites.

The interplay of agonist-receptor is most relevant in the various layers of the skin.

The advanced step in the evolution of MC1R is its correlation with the risk of skin cancers. It was somewhat logical to further the scientific argument that RHC individuals – with poorly functioning MC1R – would be more prone to skin cancers. Here we will elaborate later on in the COMMUNIQUÉ series, since it has become one of our objectives to evaluate whether optimising the MC1R would lead to a decrease in skin cancer risk.

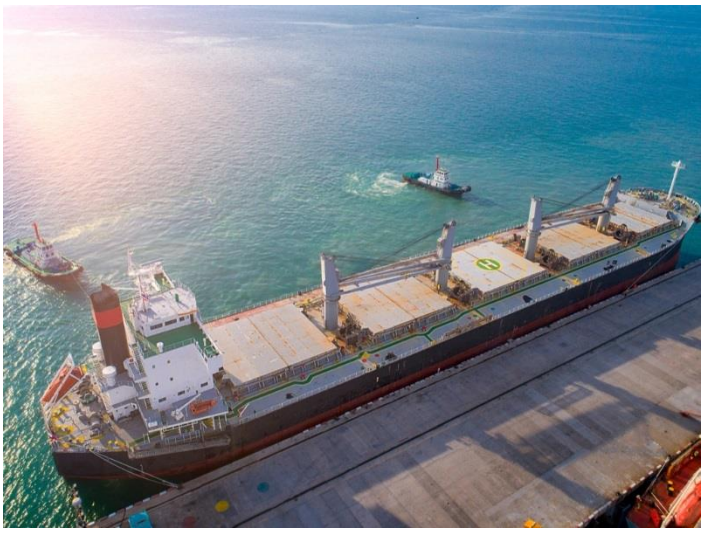

Figure 2: The quality of the MC1R resembles the optimum mooring site

MOORING AND SIGNALLING

When reviewing the ability of drugs to bind to GPCRs, a number of analytical methods serve us to assess the strength of binding as well time to dislodge from the receptor. Classical assays were once the common method of evaluating the interaction between receptor and drug. Now we use methods such as free-energy perturbation (FEP) and thermodynamic integration (TI) which hold the promise of more accurately predicting the free energy of binding. FEP and TI are, however, still subject to debate as to their wider applicability in drug development and clinical interpretation of results.

In coming back to our main focus, the MC1R, CLINUVEL's early knowledge gained on the strength of melanocortin binding and dissociation from the receptor has proven essential in choosing a clinical development program. Specific data and intelligence gained during investigative pharmacology ultimately decide the *fate of a molecule*. Thorough understanding of the differences of the pharmacology in vitro and in humans is essential to arrive at the desired application. In addition, the advances in modern medicine may confirm or negate one's decisions on deploying

new drugs; within CLINUVEL this is part of a scientific awareness which needs to be maintained at all times. An applicable example is how only recently the medical community has acknowledged the differences of MC1R function in various species of animals, and even in man.

Classically, scientists compared the binding strength of various drugs to each of the five melanocortin receptors, MC1R, MC2R, MC3R, MC4R and MC5R. However, individual assays are now able to compare ligands binding to the same receptor to make a faint prediction of how the molecule would behave in man. Outside the scope of this piece, the five melanocortin receptors are found in different parts and organs of the human body, and all regulate various functions such as obesity, thermoregulation, inflammation, immunomodulation, sexual behaviour, and pigmentation. As stated the MC1R is mainly found on the plasma membrane of the melanocyte – pigment producing cell in skin – but also on fibroblasts, cells lining blood vessels and white blood cells. How various molecules behave in interaction with the target receptors depends on a multitude of factors and is the subject of modelling studies attempting to replicate the desired or even undesired physiological effect(s). The distinction in modelling for use under physiological and pathological conditions is vast, and goes beyond this piece, but is relevant to CLINUVEL's work.

MC1R VARIANTS AND IMPACT

Due to the thoughts of Dr T.B. Fitzpatrick, first published in 1975 and later expanded upon, dermatologists, photobiologists and physicists have followed his classification of skin types, consisting of skin types found in a sample of the general population. In this original classification, only skin types V and VI enjoy full melanoprotection. These thoughts are the focus of fierce debate in the relevant fields of dermatology, but are maintained due to lack of a better classification.

Skin Type	1	2	3	4	5-6
Sunburn and Tan Tendency	Always burns Seldom tans	Usually burns Sometimes tans	Sometimes burns Usually tans	Seldom burns Always tans	Never burns Always tans
Skin, Hair and Eye Colour	*White skin, freckles, blond or red hair, blue or green eyes*	*White skin, blond hair, blue or green eyes*	*White skin, usually dark hair and brown eyes*	*Darker skin, usually dark hair and brown eyes*	*Naturally brown to dark skin, brown or black hair, brown eyes*
Tanning and Sunburn History	Develops red sunburn; painful swelling, skin peels	Usually burns, pinkish or red colouring appears; skin can gradually develop light brown tan	Rarely burns; skin shows moderately rapid tanning response	Rarely burns; skin shows very rapid tanning response	Rarely burns; skin shows very rapid tanning response
Melanin (Pigmentation) of the Skin	**Melanocompromised**		**Melanocompetent**		**Melanoprotected**

Figure 3: The Fitzpatrick skin types classification

Although our teams still work with the Fitzpatrick skin types classification it is deemed too crude to accurately describe a patient's (in)tolerance to light and sun. In time, we strive to introduce a new classification in the field of photomedicine which will need to be more precise in reflecting

the constitutive dermal and epidermal properties of patients of different melanin density, and tolerance to light and sun.

Essential work was performed for decades by various Australian, European and American universities on the role of receptor variants and risk of skin cancer. Simultaneously with our research, the understanding of the ability of alpha-melanocyte stimulating hormone to 'override'

Figure 4: Variety in skin complexions, with lower melanin density increasing the propensity to 'burn' in response to UV radiation

partial-loss-of-functions with new ligands has grown. One of the research objectives has been to investigate the signalling functions of MC1R in models, but mostly relevant to man.

When looking at the functionality of the MC1R and risk for incurring skin cancer(s), we tend to define the MC1R gene as one which is polymorphic and gives rise to *allelic variants* of the receptor. During the past two decades we have come to know the most frequent MC1R variants, Arg160Trp, Asp294His, Arg151Cys, Asp294His and Val92Met, but there are many more. These variants cause the receptor to function in a slightly deficient manner and, as highlighted at the start, *partial loss of function* results in greater human susceptibility to both various disease and damage from UV radiation. This is the subject of genetic and epigenetic studies, whereby major scientific leaps have been made the past decades. It is predicted that the individual susceptibility to various skin cancers will become relevant in finding targeted treatments, and this is highly relevant to our fields of research and interest. However, to add to another part of the puzzle, it was Dwyer and colleagues who had demonstrated a decade ago that Caucasian men living in Australia, those with 0–1 melanin density (MD) units, had six or more times risk of skin cancer than those with three or more MD units. Put simply, greater melanin equals greater protection and this in turn depends on good signalling of the melanocyte.

In summary, increasing evidence points to the MC1R, the quantum of melanin output, and constitutive propensity to respond to UV radiation, as main determinants of an individual's skin cancer risk.

OUTPUT

Melanocytic activity – originating from MC1R and cAMP (intracellular messenger) – aims to provoke eumelanisation of the skin (see SCIENTIFIC COMMUNIQUÉ I). While eumelanin is seen as photoprotective, the counterpart pheomelanin is largely viewed as photoreactive, mostly in response to UVA (wavelength 320-400nm). In photophysics we investigate the absorptive, reflective, refractive and other optical properties of various pigments. In medical applications, we collectively have researched the ability to block emitted photons for some time, but also looked at using the absorptive capacity in techniques such as photodynamic therapy in superficial tissue lesions. Eumelanin comes in various forms, granules, particles and 'dust', depending on constitutive skin type. Therefore, the photoabsorptive properties differ between individuals and skin types.

Our work focused on thoroughly understanding UV-induced cellular damage due to radical and reactive oxygen species – mostly abbreviated as ROS – and oxidative injury. The current work and

future technology will most certainly address these highly relevant issues. Cellular and proteonomic work is progressing, and understanding of the function of receptor, cellular environment, genomic mechanisms, and external variables are coming together in CLINUVEL's next generation of pharmaceutical products.

In deepening our understanding, we typically find that MC1R protein expression is typically low, with approximately 700 units expressed per healthy melanocyte, and significantly higher numbers on mutated cells such as melanoma cells.

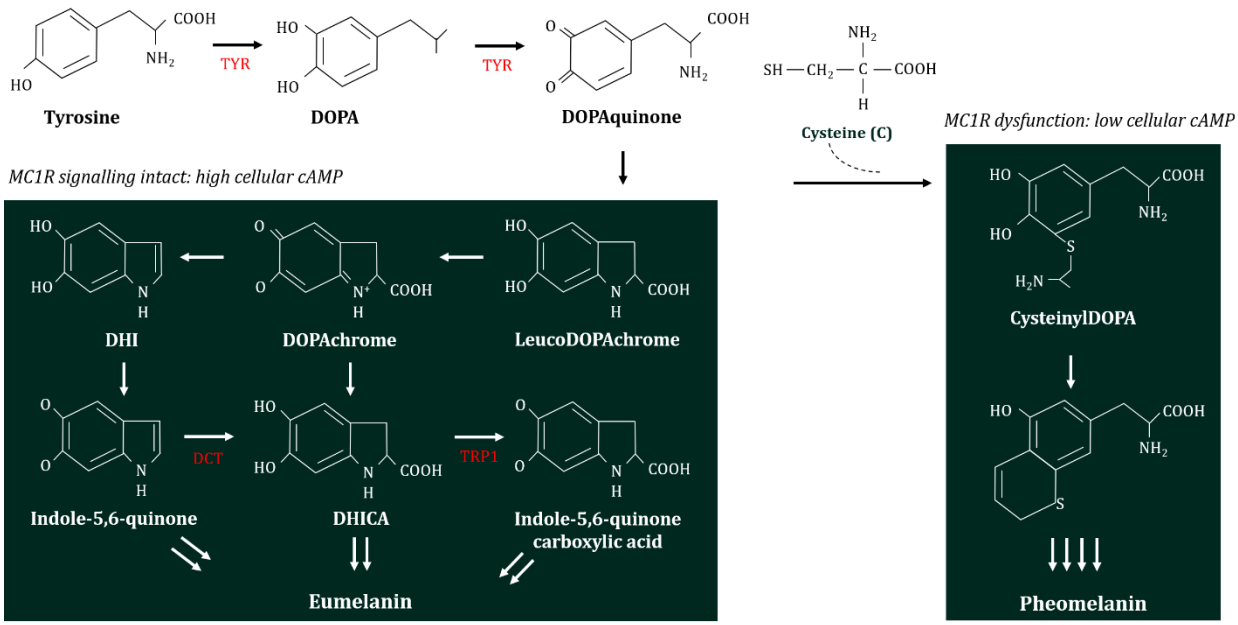

Figure 5: Melanin distinction to produce either photoprotective eumelanin or photoreactive pheomelanin

RELEVANCE TO CLINUVEL'S TECHNOLOGY

Historically our scientific professionals paid attention to the pigmentary response induced by UV exposure. The irradiation of the epi-/dermis brings about a number of instantaneous and delayed changes which are of great relevance.

In contrast, as we have discussed previously, the use of a hormonal analogue elicits various responses, of which one is *the melanogenic response without UV radiation*. Our scientific teams view this application of the analogue as a form of **biomimicry**, a way to emulate the biological response seen to light and UV irradiation under daily conditions.

We understood, early on, the potential and limitations of the melanogenic system and its pharmacological applications. Questions arose on the desired and ideal depth, length, location, and origin of pigmentation. By systematically working along decision trees, we arrived at working hypotheses and further applications of our hormonal therapy targeting MC1R. Decisions are continuously taken as CLINUVEL's teams generate more data to further our knowledge. The colloquial "tip of the iceberg" is applicable here.

In the past, we discussed the strengths and weaknesses of pioneering in technology, but for this purpose it suffices to state that our collective attitude is to incrementally, and more so **concentrically**, expand our applications from the most recent knowledge gained. This approach reduces the risk of venturing into costly R&D projects with all too uncertain outcomes.

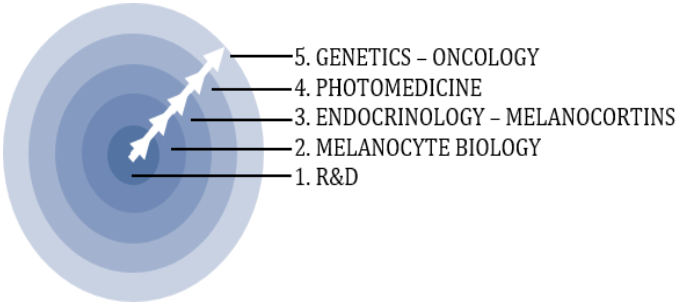

Figure 6: Concentric expansion of CLINUVEL's attention

Originally, the scientific community debated the function of MC1R and its association with the pigmentary output; as discussed in SCIENTIFIC COMMUNIQUÉ I. Whether the melanocytic output is eumelanin (brown) or pheomelanin (red-yellow) is predominantly determined by our constitution and genetic makeup, however – as we now know – activity of the cell through strong receptor binding is important to generate the required cellular output.

In the past decade our scientific understanding has progressed by finding epidemiological and statistical relations between the functioning of MC1R and individual sensitivity to UV radiation and skin cancer. This link between MC1R function and skin cancer has been of immense importance to forewarn individuals at high risk of contracting the disease at a later age.

APPENDIX: SCIENTIFIC COMMUNIQUÉ – SCIENTIFIC REFERENCE TERMS

6-4PP	Pyrimidine (6-4) pyrimidone photoproducts
αMSH/MC1R complex	α-Melanocyte-Stimulating Hormone/Melanocortin 1 Receptor complex
AAF-G	Acetylaminofluorene-guanine
ACF	Asymmetric Crying Facies
ACTH	Adrenocorticotropic Hormone
AICAR	5-aminoimidazole-4-carboxamide ribotide
Akt	V-akt murine thymoma viral oncogene homolog
ALC1	Amplified in liver cancer 1
AMPK	AMP-activated protein kinase
AP-1	Activator protein-1, member of the transcription factor family
ARF	Alternative reading frame
ATF2	Activating transcription factor 2
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
β-catenin	A transcription co-activator involved in embryonic development and cellular homeostasis
BCC	Basal cell carcinoma
BRAFV600E	V600E mutant V-Raf murine sarcoma viral oncogene homolog B
BRN2	A transcription factor, belonging to homeodomain POU3F2, N-Oct-3
cAMP	Cyclic adenosine monophosphate or cyclic AMP
CBP	CREB binding protein
CEBPG	CCAAT/enhancer-binding protein gamma
CPD	Cyclobutane pyrimidine dimers
CREB	cAMP response element binding protein
CS	Cockayne syndrome
CsA	Cyclosporin A
CSA	Cockayne syndrome group A
CSB	Cockayne syndrome group B
CSN	Constitutive photomorphogenesis 9 (COP9) signalosome
Cul4A	Cullin-4A

DDB1	Damage-specific DNA binding protein 1
DDB2	Damage-specific DNA binding protein 2
Def1	RNA polymerase II degradation factor 1
Dermis	Mid layer of the skin, between the epidermis and hypodermis
DNA	Deoxyribonucleic acid, containing the genetic code
DOT1L	DOT1-like protein
DP1	DRTF polypeptide 1
E2F1	E2F transcription factor 1
E2F4	E2F transcription factor 4
Endothelin-1	Cell receptor on the melanocyte
Epac	Exchange protein activated by cyclic AMP
EPP	Erythropoietic protoporphyria: a rare metabolic genetic disorder in man which causes accumulation of phototoxic protoporphyrin IX in the skin and liver
ERCC1	Excision repair cross-complementation group 1
ERCC5	Excision repair cross-complementation group 5
FEP	Free-energy perturbation
FGF	Fibroblast growth factor
Fitzpatrick skin type	First described by Fitzpatrick in 1975, classifies skin in six distinct types based on melanin density and tanning/burning propensity
FOXD3	Forkhead transcription factor D3
Gadd45	Growth arrest and DNA-damage-inducible
GCN5	General control of amino-acid synthesis 5
GG-NER	Global genome nucleotide excision repair
GPCR	G-protein coupled receptors
H2A.Z	H2A histone variant HTZ1
H3K9	Histone H3 lysine 9
HIF-1α	Hypoxia-inducible factor-1 alpha
HIRA	HIR (histone cell cycle regulation defective) homolog A
HMGN1	High mobility group nucleosome binding domain 1
HR23B	RAD23 homolog B
HRE	Hypoxia response element

IFN-β	Interferon β
Keratinocyte	Keratin producing cells of the epidermis
LEF	Lymphoid enhancer binding factor
MAPK	Mitogen activated protein kinase
MC and EC	Total melanin and eumelanin contents
MC1R	Melanocortin-1 receptor, a 317 amino acid protein and a seven-pass transmembrane G-protein coupled receptor melanin
MD	Melanin density
Melanocortin	Peptide belonging to the group of proopiomelanocortin, such as ACTH, α-MSH, β-MSH, γ-MSH
Melanocyte	Pigment producing cell
Melanogenesis	The process by which melanin is produced in the melanocyte and transferred to the keratinocyte
Melanoma	A malignancy originating from the melanocyte and now known to be linked to a variety of biochemical and genetic defects. Melanoma is an umbrella term for a variety of tumours with diverse biological behaviour
MFA2	Mating Factor A
MITF	Microphthalmia-associated transcription factor: protein responsible for – among other activities – melanocyte development, differentiation, and survival
MSH	Melanocyte stimulating hormone
NAP1L1	Nucleosome assembly protein 1-like 1
NAP1L4	Nucleosome assembly protein 1-like 4
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa beta
NOX1	NADPH Oxidase 1
Nrf1	Also called NFE2L1, nuclear factor erythroid 2-related factor 1
p130	130 kDa retinoblastoma-associated protein
p38	Delta protein kinase which, under normal conditions, controls cell differentiation, but under stress it regulates a cellular distress response
p53	Ubiquitous human tumour suppressor protein controlling the cellular response to DNA damage, cycle progression and apoptosis by regulating its targets transcriptionally. p53 plays a critical role in the normal UV

	stress response and activation of pigmentation by transcriptional activation of the POMC gene
PARP-1	Poly (ADP-ribose) polymerase 1
PARylation	Poly (ADP)-ribosylation
PAT	Palmitoyltransferase
PAX3	Paired box gene 3
PCNA	Proliferating-cell nuclear antigen
PGE2	Prostaglandin E2: a lipid signalling intermediate
Photodermatology	A sub-specialty of photobiology including all aspects of photobiology related to the skin ranging from sun exposure and its consequences (both short-term and long-term) to the therapeutic effects derived from exposure to natural or artificial radiation
Photomedicine	Deserves a broad definition spanning all aspects of photobiology, photophysics and photochemistry, investigating the interaction of light and human matter and tissues
Photophysics	Concerned with processes that occur when light and sunlight, filtered through the Earth's atmosphere, interact with matter (atoms and molecules) present, with particular attention to the spectrum of solar radiation striking the organic matter
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein Kinase A
Pol	Polymerase
POMC	Proopiomelanocortin
PPIX	Protoporphyrin IX
PTEN	Phosphatase and tensin homolog
R4B	Rad4 binding domain
RB	Retinoblastoma protein
RHC	Red hair colour (phenotype)
RNA	Ribonucleic acid
RNA pol II	RNA polymerase II
RNF111	RING finger protein 111
ROC1	Regulator of Cullins-1
ROS	Reactive oxygen species
RPA	Replication protein A

RPA3	Replication protein A3
SCC	Squamous cell carcinoma
SHM	Somatic hypermutation
SIRT1	Sirtuin 1
SLUG	SNAI2 transcription factor
Smad4	SMAD family member 4
SNAII	SNAI1 transcription factor
SOX9/10	HMG-box of the sex-determining gene SRY on the Y-chromosome
Squamous Cell Carcinoma	Epidermal tumours (skin cancers) caused by chronic sun damage
SWI/SNF	SWItch/sucrose nonfermentable
TCF	Transcription factor
TC-NER	Transcription coupled nucleotide excision repair
TFIIH	Transcription factor II H
TI	Thermodynamic integration
TLS	Translesion synthesis
TTD	Trichothiodystrophy
UBL	Ubiquitin-like domain
USF-1	Upstream stimulatory factor 1
USP7	Ubiquitin-specific-processing protease 7
UV/UVR	Ultraviolet radiation, electromagnetic radiation from 10-400nm wavelength, further differentiated into UVA (320-400nm), UVB (280-320nm) and UVC (10-280nm)
UVSSA	UV-sensitive syndrome protein
VHS	Vps-27, Hrs and STAM domain
XAB1	XPA-binding protein 1
XP	Xeroderma pigmentosum
XPA	Xeroderma pigmentosum, complementation group A
XPB	Xeroderma pigmentosum, complementation group B
XPC	Xeroderma pigmentosum, complementation group C
XPD	Xeroderma pigmentosum, complementation group D
XPE	Xeroderma pigmentosum, complementation group E

XPF	Xeroderma pigmentosum, complementation group F
XPG	Xeroderma pigmentosum, complementation group G
YY1	Yin Yang 1
ZEB1	Zinc finger E-box binding homeobox 1