SCIENTIFIC COMMUNIQUÉ IV

CLINUVEL

May 2019

The community of photobiologists and fundamental researchers in melanocyte biology have been preoccupied with a number of key questions posed by the relationship between UV exposure and cell biology and biochemical pathways. One of the questions remaining is that of the exact role of the melanocortin-1 receptor (MC1R) within the skin (dermis and epidermis) and risk of developing skin cancer in one's life.

Scientific experts in the field hope that, in the future, we will be able to forewarn those individuals who are at highest risk of photodamage and contracting skin malignancies through the identification of a number of molecular and genetic criteria. Ultimately, we all strive to pre-select those individuals who are most likely to be prone to photodamage and to develop neoplasms of the skin at a higher rate than the general population. We are not yet at the stage where individuals are genetically screened for risk factors for different types of skin cancers, however the discipline of photomedicine is making steady progress. In this SCIENTIFIC COMMUNIQUÉ further understanding is provided on contemporary views on the role of the MC1R, cellular signalling, and the role of UVR on DNA repair.

In SCIENTIFIC COMMUNIQUÉ I we highlighted MC1R signalling as being pivotal to the physiologic melanogenic response in man. In SCIENTIFIC COMMUNIQUÉ II some of the receptor variants found in Caucasian populations were discussed. In SCIENTIFIC COMMUNIQUÉ III the mechanism of photoprotection, repair and specific types of photodamage were reviewed.

In COMMUNIQUÉ IV we dig deeper into the qualitative and quantitative aspects of this key receptor, since most recently – among other factors – the function of the MC1R has been described as correlating with the risk of non-melanoma and melanoma skin cancers.

MC1R ALLELIC VARIANTS AND RISK

As opposed to historical belief, the polymorphism of the MC1R – encoding from chromosome 16q24.3 - indicates rather a loss of strength of the protein rather than a total loss of its function. Specialised research groups at Dana Farber, Nice University, Oxford Ludwig Cancer Institute, and the Universities of Cincinnati and Queensland have devoted their research to the further understanding of the signalling sequence and impact, with the aim of one day being able to definitively unravel the cellular mechanisms leading to skin cancers. At CLINUVEL we furthered our clinical research by focussing on the MC1R pathway and the main ligand affecting downstream signalling in a wide variety of patient groups.

The research group in Queensland, led by the excellent researchers Dr Sturm and Prof Green, have long focussed on the precise role and risk posed by the compromised function of the MC1R in red haired individuals. Over the years it has been found that MC1R variants behave as recessive mutations in red haired phenotype, i.e. fair skinned individuals. In a study evaluating 2,331 family members and 1,779 individuals the frequency of receptor variants was listed as in the **table 1** below:

Table 1 Most frequent MC1R variants in Caucasians

No.	ALLELIC VARIANT	FREQUENCY (%)
1	V60L	12.2
2	R151C	11
3	V92M	9.7
4	R160W	7.0
5	R163Q	4.7
6	D294H	2.7
7	D84E	1.2
8	1155T	0.9
9	R142H	0.4
	TOTAL	**49.8**

With the relative high frequency of some variants (more than 85 have been identified), the questions for decades have revolved around the clinical implications. In other words, *how do the MC1R variants affect us and what does it mean in terms of UV exposure*? And therefore, *do we all have to be screened and genotyped*?

In a larger meta-analysis conducted by Raimondi and Fargnoli, published in the *International Journal of Cancer* in 2008, it was found that seven of the nine variants listed in table 1 had been associated with red hair phenotype and melanoma development, expressed in odds ratio (OR). In other words, the MC1R status not only determines the skin colour and hair colour one generates, but most likely plays a role in the development of some (but not all) melanomas.

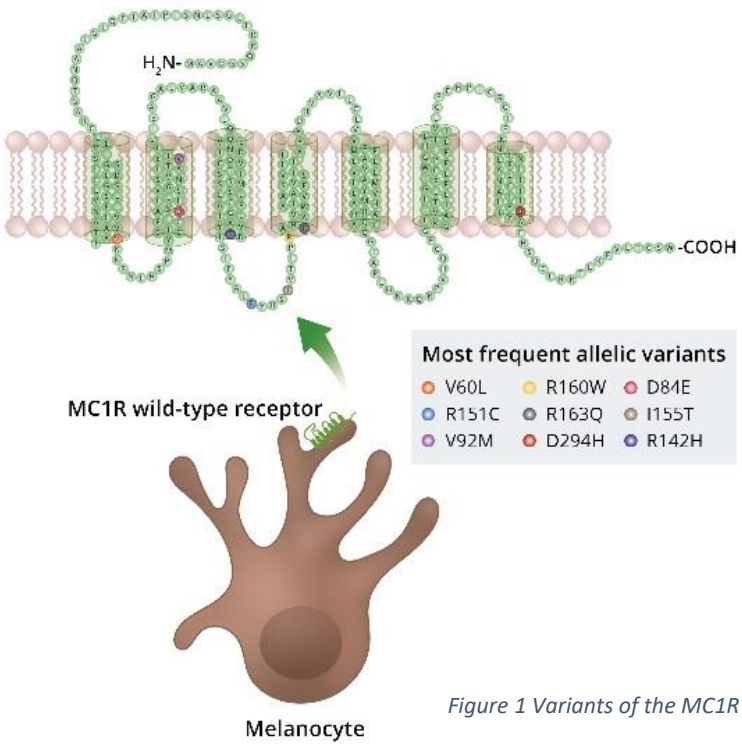

Figure 1 Variants of the MC1R

While remaining with the same scientific question, in 2018 the same Milanese group reviewed 3,830 cases of melanoma and 2,619 controls. Raimondi and many co-workers concluded from this large dataset that the presence of any MC1R variant was associated with melanoma risk with significant higher OR. By using a risk predictive model, since prospective studies in melanoma are challenging to conduct, it was found that MC1R variants played a stronger role in individuals without the red hair phenotype.

In order to analyse the association of skin complexion, phenotype and melanoma, multivariate logistical regression analyses are commonly used for predictive modelling of MC1R status and risk of melanoma. Thereby, adjustments are to be made for confounding factors such as gender, sun exposure, latitude, and/or demographic data, since all individuals live under different circumstances. Useful statistical advances have been made with so-called hierarchical modelling using first- and second-

level parameters in the analyses of genetic association studies which take into account the allelic functions of study participants.

In contrast to a polymorphic receptor, the wild-type MC1R phenotype gives rise to a light coat-colour phenotype expression and ability to provide fully functional melanocyte signalling. The benefits of functional signalling have been discussed in SCIENTIFIC COMMUNIQUÉS I and II, and indirectly in III.

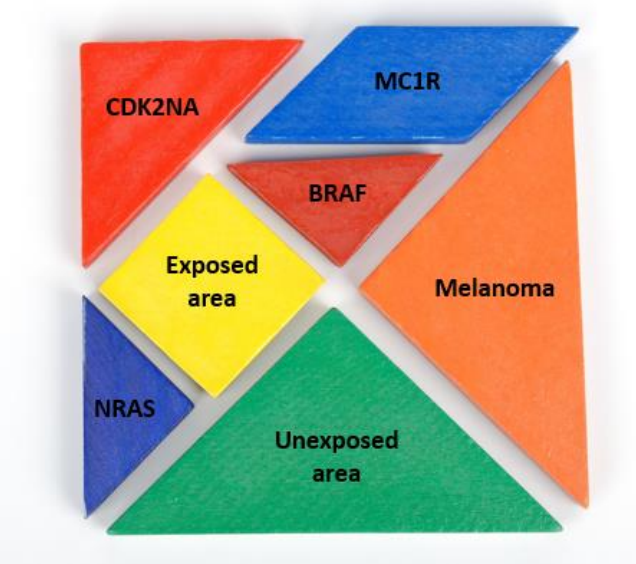

Figure 3 Some of the pieces of the MC1R puzzle

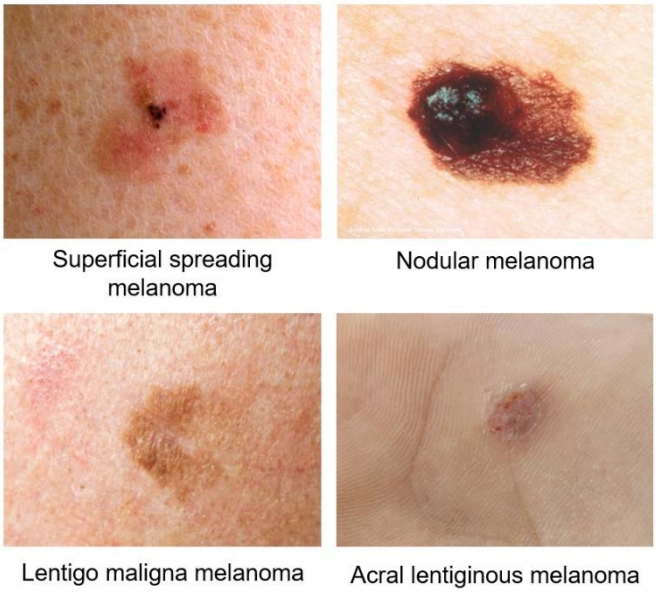

Figure 2 Melanoma is an "umbrella term" for multiple clinical disease entities

PIECES OF THE PUZZLE

Some pieces of the puzzle are still missing, particularly what could be considered the bridge to the MC1R end piece of the sequential processes identifying the correlation with skin cancer genesis. The combination of genetic and epigenetic factors ultimately plays a role in the emergence of skin neoplasms (non-melanoma and melanoma) in individuals at risk. Overall, adequate MC1R-cAMP-CREB-MITF signalling is directly linked to optimum melanogenesis, enhanced cytoprotection to UV damage, and augmented DNA repair response.

US research laboratories and our own team have underscored the existence of rescue systems in human cells, physiology. While questioned for previous decades, it is now widely accepted that the MC1R-cAMP-tyrosinase-CREB-MITF pathway is indeed communicating with adjacent cellular pathways under the hypotheses that “rescue loops” have been identified to compensate for defects in the MC1R pathway. Remarkably, intracellular protein communication consists of a highly sophisticated network, ensuring that a rheostat system is established whereby functions are alternated for up- and down-regulation as the cellular cytoplasm needs to adjust to its environment.

The ultimate objective is to unravel the association between the dose (quantum) of light exposure and UV irradiation, and the temporal relationship with the genesis of skin cancers (non-melanoma and melanoma). Much has been published during the last decade about the initiation and promotion of squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) of the skin, whereby the cellular pathways have been mapped out quite comprehensively. Genetic and epigenetic factors are clearly determinants, and in both cancers the direct impact of the photo carcinogen is well proven.

In melanoma, the scientific status quo is slightly different, and a host of factors play a role in the genesis. In the first place, melanoma is an "umbrella term" for multiple clinical disease entities, such nodular, superficial spreading, melanoma of the acral surfaces, melanoma of the unexposed skin and exposed areas. The biological behaviour of these variants of melanoma differs markedly. Pathologists have accepted that the disease entities require differentiation and present as several variants under one denominator. Hence, the classification and subsequent therapies differ widely.

In the quest to elucidate the role of MC1R in melanoma, various studies assist in our advanced approach. As a surface endocytic receptor, MC1R is mostly considered for diagnostic purposes since most melanoma variants demonstrate an upregulated MC1R status. Recently, a number of therapeutic approaches have been made, such as endo-radiotherapy using Auger electron and α- and β-particle emitters. The results are still being evaluated and remain uncertain.

The research groups in Kentucky have made great strides towards understanding the role of the cKit tyrosine kinase and endothelin B receptors expressed on the melanocyte (see SCIENTIFIC COMMUNIQUÉ II). Whereas CLINUVEL has extensively described the protective role of eumelanin in keratinocyte derived skin cancers (SCC and BCC), the focus on melanoma has been justified by the scientific data generated from immortalised melanoma cell lines illustrating a relationship between MC1R signalling and protection to the human genome. The Kentucky group realised that the annual incidence of melanoma in more than 12,000 US citizens, sunseekers and tanning bed users (more than 25% of US citizens use indoor tanning, amounting to 30 million customers) would warrant further research in MC1R and melanoma.

The first relationship between UV-sun exposure and melanoma is derived as characteristic *signature mutations*, found in sun exposed variants of melanoma. SCIENTIFIC COMMUNIQUÉ II described the typical photolesions found following UV exposure, consisting of 6-4 photoproducts and cyclo-pyrimidine dimers formed in the double helix of the DNA. One distinguishes "light photolesions" formed upon UV exposure and "dark photolesions" seen hours after UV exposure has ceased. As stated in the past, the irradiation of UV follows a stochastic model illustrating that a threshold of UV is tolerated, above which keratinocytes start to die off (the process of apoptosis).

Second, it is found that Caucasian individuals with partial-loss-of-function of the MC1R are more likely to bind antagonistic molecules to the MC1R, such as agouti signalling protein (ASIP) and beta-defensin 3 (βD3). The biochemical consequence is an inferior melanocytic output and lower eumelanin to pheomelanin ratio. This in turn leads to:

1. propensity to sunburn;
2. increased photodamage;
3. less photoprotective melanogenesis; and
4. lessened ability to repair DNA photodamage.

The obvious solution has been to attempt to optimise MC1R signalling through pharmaco-chemical stimulation of the receptor, one of the reasons CLINUVEL's continuous focus on the activity of melanocortins.

Recent studies have focused on inter-pathway communication, unveiling that MC1R signalling induces the transcription of the nuclear factor NR4A in response to UV radiation. When the nucleus of the cell is stressed, intracellular NR4A receptors are upregulated as a nuclear protective mechanism. The possible clinical relevance of this finding is the existence of further pathways initiating protection against UV exposure, beyond the previously presumed limited ones.

Various US research groups have postulated in the past as to how melanocortins could exert their effects on melanoma. These scientific hypotheses stem from a number of studies, such as those experiments described by Froidevaux and Eberle in 2002 (the year V600E BRAF mutations were published in *Nature*). In transplanting two human cell lines – D10 and B16F1 – in mice, they observed down-regulation of the MC1R following the administration of alpha-melanocyte stimulating hormone (α-MSH). While CLINUVEL's earlier work had shown similar results, in the studies conducted by Eberle et al a single injection of 50 to 500 micrograms of alpha-MSH induced a rapid but moderate dose-dependent MC1R down-regulation which could be totally reverted within 16-24 hours. By continuous administration of alpha-MSH via osmotic minipumps, the Swiss researchers showed that MC1R down-regulation was considerably enhanced, thereby confirming the thought that prolonged receptor interaction is necessary to induce a maximum effect.

The importance of the animal studies is often debated in literature (and certainly among clinicians), since a rodent's biology behaves differently to a man's. Nevertheless, as time goes by more work is being published and results point to the protective effect of an optimum alpha-MSH-MC1R-cAMP-PKA-MITF axis.

The role of the MC1R is much more complex than a linear up- and down-regulation. The functionality of the receptor very much depends on the physiologic and pathologic environment of the host cells. In SCIENTIFIC COMMUNIQUÉ II we discussed how the MC1R receptor inhibits activation of p38 MAP kinase, and thereby subsequently enhances syndecan-2 expression, which is of importance for stability and migration of melanoma cells.

SUNBURNS AND RISK OF CUTANEOUS MELANOMA

In a 2008 meta-analysis Dennis and Coughlin had elucidated the odds ratio for melanoma development having retrieved data from 270 articles and pooling 51 studies. The authors analysed that, for those study subjects who incurred a sunburn in their lives, an increased risk of melanoma was found with increasing number of sunburns for all time-periods (childhood, adolescence, adulthood and lifetime). In linear modelling, the magnitude of risk for five sunburns per decade was highest for adult and lifetime sunburns.

In 2014, Quereshi published findings in the *Journal of the American Association for Cancer Research* from a study of 108,916 Caucasian women (registered nurses). This showed that those who had at least five blistering sunburns at the age of 15 to 20 years old had a 68 percent increased risk for BCC and SCC of the skin, and an 80 percent increased risk for melanoma. Of all women participating in the study, 6,955 were diagnosed with BCC, 880 were diagnosed with dermal SCC, and 779 were diagnosed with melanoma. He found that there was a strong relationship between cumulative UV exposure and risk for BCC and SCC of the skin, while no association was found for melanoma. Importantly, in those who had had at least five severe sunburns between ages 15 and 20 an increased probability for developing any of the three types of skin cancers was found, but the greatest risk was assigned to developing melanoma.

In 2016, Wu and Quershi analysed data from 87,166 women and 32,959 men to evaluate overall skin cancer risk associated with history of severe sunburns at different body sites (face/arms, trunk, and lower limbs). In adjusting for other risk factors, overall baseline history of severe sunburn was more apparently associated with risk of melanoma than with risk of SCC and BCC in

men: the multivariable-adjusted hazard ratios were 2.41 for melanoma, 1.48 for SCC, and 1.18 for BCC.

Many other studies have focussed on the association between lifetime sunburn frequency and the three most prevalent skin cancers, BCC, SCC and melanoma. Although melanoma requires further differentiation, the increased risk of incurring melanoma following one or several severe (blistering) sunburns becomes more and more part of mainstream advice by dermatologists and general practitioners. Not surprisingly, the work of CLINUVEL to provide systemic photoprotection is concentrated~~ing~~ towards those individuals at highest risk (constitutional, familial) of contracting melanoma in their lifetime. The prevention of the disease in individuals at risk is an area of intense focus and critique by CLIN~~I~~UVEL's teams, with the objective one day to have the ultimate preventative agent developed. The use of sunscreens and prudent sun behaviour should be practice for all Caucasian children and adults, however for those at increased risk perhaps more can be done and further products be developed. This domain is at the core of CLINUVEL's interest.

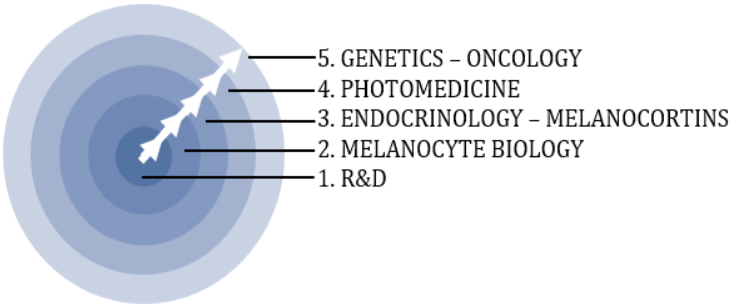

Figure 4 Concentric expansion of CLINUVEL's attention

APPENDIX: SCIENTIFIC COMMUNIQUÉ GLOSSARY

ACTH	adrenocorticotropin
α-MSH	α-melanocyte-stimulating hormone
Apoptosis	programmed cell death in order to regulate balance within the skin
AGRP	agouti-related protein
ARRB1	arrestin-beta-1
ARRB2	arrestin-beta-2
ASIP	agouti signal protein
Bak	Bcl2- homogenous antagonist killer, pro-apoptotic regulator
Bax	Bcl-2 Associated X
Bcl-2	B-cell lymphoma 2, regulator protein involved in apoptosis
BD3	β-defensin 3
Bid	BH3-interacting domain, a pro-apoptotic protein
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
BRN2	a transcription factor, belonging to homeodomain POU3F2, N-Oct-3
Caspase	cysteine-aspartic proteases, protease enzymes involved in apoptosis
CDKN2A	cyclin-dependent kinase inhibitor 2A
CPD	cyclobutane pyrimidine dimer, occurring as fast as 5-90 minutes following first UV and sun exposure
Chromophores	a chemical group of atoms and electrons absorbing light of specific wavelength(s) and providing colour to a molecule
CRE	cAMP-responsive element
Dermis	mid layer of the skin, between the epidermis and hypodermis
Dewar	valence isomers, interrelated isomers (a heterocyclic aromatic organic compound, consisting of a pyrimidine ring fused to an imidazole ring (through pericyclic reactions)
Dimer	a molecular structure or complex comprising of two identical molecules linked together, in this context the pyrimidine dimers from thymine or cytosine
DNA	deoxyribonucleic acid, containing the genetic code
DPD	delayed pigmentary darkening, occurring after days of UV exposure
Endothelin-1	cell receptor on the melanocyte

EPP	erythropoietic protoporphyria: a rare metabolic genetic disorder in man which causes accumulation and storage of phototoxic protoporphyrin IX in the skin and liver and bile ducts
ERK	extra-cellular signal-regulated kinases
Fas	apoptosis stimulating fragment, Apo-1 or CD95
FasL	fas ligand
FasR	apoptosis stimulating fragment receptor
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 propensity
FOXD3	forkhead transcription factor D3
GG-NER	global genomic NER
GPCR	G-protein coupled receptors
GR	global repair, mechanism to repair UV damaged DNA
GRK	GPCR kinase
I-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
IPD	immediate pigmentary darkening
Keratinocyte	keratin producing cells of the epidermis
MAPK	mitogen activated protein kinase
MC1R	melanocortin-1 receptor, a 317 amino acid protein and a seven-pass transmembrane G protein coupled receptor Melanin
MED	minimal erythemal dose
MD	melanin density
Melanocompetent	individuals who can respond to UV exposure with a protective eumelanin response
Melanocompromised	individuals unable to generate sufficient eumelanin and burn as a result of UV exposure. These individuals are at a much higher risk of photodamage and skin cancers.
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 generated within 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.
MGRN1	mahogunin RING finger-1 (ubiquitin E3 ligase with RING-domain)
MITF	microphthalmia-associated transcription factor: protein responsible for – among other activities – melanocyte development, differentiation, and survival
NEDD9	neural precursor cell expressed developmentally down-regulated 9
NER	nucleotide excision repair, mechanism to repair DNA damage
NF-κB	nuclear factor kappa; light chain-enhancer of activated B cells
NIS	Na-I symporter
NR4A	orphan nuclear factors, transcriptional regulators of gene expression in metabolic and vascular anomalies
p38	delta protein kinase which, under normal conditions this protein kinase 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.
PAT	palmitoyltransferase
PAX3	paired box gene 3
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
Photolyase	DNA repair enzyme, belonging to the enzymatic class of flavoproteins
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
6-4 Photoproduct	molecular lesion within DNA following a photochemical reaction
Photothermolysis	thermal damage following a photochemical reaction
PKA	protein kinase A
POMC	proopiomelanocortin
Purine	a heterocyclic aromatic organic compound, consisting of a pyrimidine ring fused to an imidazole ring, such as the case in adenine, guanine
Pyrimidine	a heterocyclic aromatic organic compound similar to benzene and pyridine, containing two nitrogen atoms at positions 1 and 3 of the six-member ring
RHC	Red Hair Colour (phenotype)
RING	real interesting new gene
RNA	ribonucleic acid
Sdc2	syndecane-2
SLUG	SNAI2 transcription factor
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
TCF	transcription factor
TC-NER	transcription-coupled NER
TI	thermodynamic integration
TpT3	Dewar valence isomers
TRAIL	Tumour Necrosis Factor-related apoptosis-induced ligand
USF-1	transcription factor
UV/UVR	ultraviolet radiation, electromagnetic radiation from 10-400nm wavelength, further divided into UVA (320-400nm), UVB (280-320nm) and UVC (100-280nm).
V600E BRAF	mutations found in melanoma whereby the amino acid substitution occurs at position 600 in BRAF, from a valine (V) to a glutamic acid (E).