

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

The role of UV-induced DNA damage response and zinc
homeostasis in dermatological disorders

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ABBREVIATIONS

8 oxo-dG: 7,8-dihydro-8-oxoguanine lesions
AJCC: American Joint Committee on Cancer
Amplex red: 10-acetyl-3,7-dihydroxyphenoxazine
ATR: ataxia telangiectasia and Rad3-related
BCC: basal cell carcinoma
BER: base excision repair
CDK: cycline-dependent kinases
CMM: cutaneous malignant melanoma
CPD: cyclobutane pyrimidine dimer
CTLA-4: cytotoxic T-lymphocyte-associated antigen-4
CYP1B1: cytochrome P450 1B1
DDR: DNA damage response
DSBR: double strand break repair
FBS: fetal bovine serum
GGR: global genome repair
GIF: growth inhibiting factor
GSH: Glutathione
H₂O₂: hydrogen peroxide
HE: dihydroethidium
HIPK2: homeodomain interacting protein kinase 2
HMOX1: heme oxygenase-1
HR: homologous recombination
HRP: horseradish peroxidase
MED: minimal erythema dose
melan-A: melanoma specific antigen
MM: malignant melanoma
MMR: DNA mismatch repair
MRE: metal regulatory element
MT I/II: isoforms I and II of metallothionein
MT: metallothioneins

MTF-1: zinc-sensing transcription factor

MT_n I/II: isoforms I and II of metallothionein expressed in cells of nevi

MT_p I/II: isoforms I and II of metallothionein expressed in cells localized to the peritumoral area

MT_t I/II: isoforms I and II of metallothionein expressed in tumor cells

NADPH: nicotinamide adenine dinucleotide phosphate-oxidase

NER: nucleotide excision repair

NFκB: nuclear factor-kappa B

NHEJ: non-homologous end joining

NM: nodular melanoma

O₂[•]: hydroxyl radical, singlet oxygen

OD: optical density

PBS: phosphate-buffered saline

PBS-T: Tween-20 in PBS

PD1: programmed death protein 1

PI: propidium iodide

POLB: polymerase (DNA directed), beta

PP: pyrimidine 6-4 pyrimidone photoproduct

PTGS2: prostaglandin-endoperoxide synthase 2

RAD51: RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)

ROS: reactive oxygen species

RPA: replication protein A

SALT: Skin Associated Lymphoid Tissue

SCC: squamous cell carcinoma

SOD: superoxide dismutase

SSA: single-strand annealing

SSM: superficial spreading melanoma

TCR: transcription-coupled repair

TLDA: taqMan low-density array

TMA: tissue microarray analysis

TNF: tumor necrosis factor

TNM: tumor-lymph node-metastasis

TTBS: Tween-20 in Tris-buffered saline

UV: ultraviolet

UVR: ultraviolet radiation

ZIP/SLC39: zinc importer

Zn (II): zinc ion

ZnCl₂: zinc chloride

ZnT/SLC30: zinc exporter

INTRODUCTION

The ultraviolet (UV) B-spectrum of sunlight is the major environmental factor responsible for skin cancer formation in susceptible persons ^{1,2}. Photochemical reactions in the DNA generate mutations leading to tumorigenesis, when elimination of damaged cells is incomplete due to ageing, UV-related local immunosuppression, immunosuppressive drugs, chronic inflammation, and hereditary factors ^{3,4}. Modulation of defense mechanisms including DNA-repair, senescence, cell death pathways and inflammation can be realized in several routes e.g. by cytokines, growth factors, plant derived compounds ⁵⁻⁷. Incidence of human cutaneous malignant melanoma (CMM) is rapidly increasing and because of therapy ineffectiveness the prognosis of metastatic cases is very poor. The staging system currently used for cutaneous CMM defines the Breslow thickness, mitotic rate and ulceration of primary tumors and positivity of sentinel lymph nodes to be independent prognostic factors for metastasis development ⁸. However, although these parameters predicts the survival rate of the patients, are not suitable to identify individual risk or therapy ⁹. Many patients, not only with low tumor thickness or sentinel lymph node negativity, can be cured by surgery alone. For a subset of patients, effective immune response can be induced by IFN- α or IL-2 ¹⁰. In addition, we have already enough pharmaceutical knowledge to produce specific molecular inhibitors for cure.

The essential metal zinc is integral part of many structural proteins, enzymes and transcription factors ^{11,12}. In human skin, impact of zinc has been reported on epidermal differentiation, barrier function, inflammatory and antimicrobial regulation, wound healing ¹³. Zinc is bound to macromolecules or stored in cell compartments, however, there is a small and tightly regulated pool of labile zinc, which is assumed to be involved in cell signalling and can act as a redox sensor ^{14,15}. The intracellular concentration of zinc is tightly controlled by zinc importers (ZIP/SLC39), exporters (ZNT/SLC30), buffering proteins (metallothioneins (MT)), and a zinc-sensing transcription factor (MTF-1) ¹⁶. Interestingly, a number of studies have found that increased expression of zinc-transporters occurs in human malignancies suggesting that alteration in the intracellular zinc homeostasis can contribute to cancer development ¹⁷. In addition, high expression of MT has been shown to be associated with worse prognosis of cancers and some workgroups demonstrated a correlation among tumoral MT protein expression and skin cancers ¹⁸⁻²⁰. Nevertheless it has not become widely used prognostic marker yet. One reason for this can be the lack of verification of the results by

different labs, on the other hand, that although change in MT expression is a well known feature of tumor progression in several types of human malignancies ²¹, there is a lack of functional studies on MT, which could explain exactly its role in the process of carcinogenesis ²². Moreover, elevated serum zinc levels have been reported in melanoma patients ²³. Recently, the role of labile zinc pool has been identified in the regulation of immune cell function and in neurotransmission ^{12,24}. There is growing evidence that zinc acts as a second messenger and changes in intracellular free zinc level in response to extracellular stimuli through changes in expression of zinc-transporters and MT contributes to the regulation of intracellular signalling pathways ¹⁵. In addition zinc has been proved to prevent against DNA damage caused by UV exposure in some cases, however its molecular background is not fully understood ²⁵⁻²⁷.

BACKGROUND

THE STRUCTURE AND FUNCTION OF HUMAN SKIN

The skin is the most external part of our body, it forms 15-20% of the total body weight and its surface is $1,6-2,3 \text{ m}^2$ ^{28,29}. It includes three main structures: the epidermis, the dermis and subcutis. The epidermis is the external layer of skin and is mostly composed of keratinocytes going through a vertical differentiation process, forming a multi-layered stratified squamous epithelium. The final steps of keratinocyte differentiation lead to the formation of the stratum corneum, the most external barrier against environmental aggressions. The epidermis can be divided into five layers (**Figure 1.**): stratum basale which consists of pluripotent, cylindrical cells, located in one layer; stratum spinosum which consists of polygonal, irregular shaped cells in multi-layer which are flattened towards the upper layers; stratum granulosum which consists of 3-5 layers of flattened cells with keratohyalin-granules; stratum lucidum which is one layer of flattened cells without nuclei and organelles, except palms and soles, where it is multi-layered and stratum corneum which a multilayer of totally keratinized, flattened cells without any nuclei.

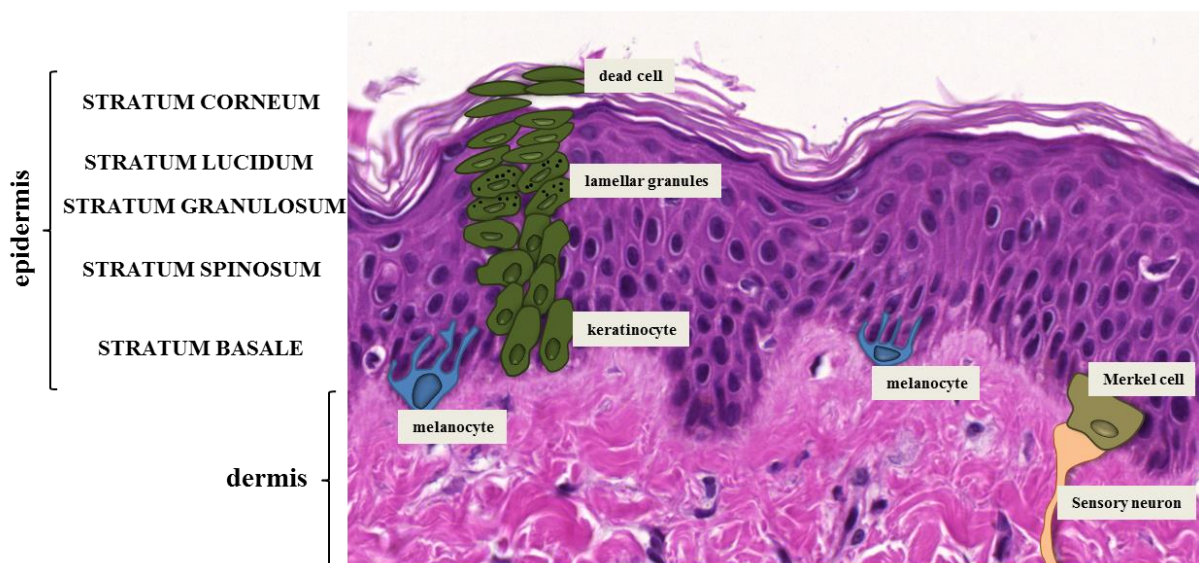


Figure 1. The structure of epidermis

The epidermis also includes melanocytes, Langerhans cells and Merkel cells. Melanocytes are dispersed located between the cells of stratum basale, show dendritic cell morphology and responsible for the production of melanin pigments. Langerhans cells, characterized by Birbeck-granules, are located in stratum basale and spinosum. These cells are members of the monocyte-macrophage system as antigen presenting cells. Merkel cells are located in the stratum basale and interacting with nerve endings.

The dermis is the area of supportive connective tissue between the epidermis and the underlying subcutis. It is a fibrous and elastic tissue that gives the skin its flexibility and strength. It is made up of two layers: stratum papillare which contains skin appendages, blood and lymph vessels and nerve endings. It also includes fibroblasts, which produce extracellular matrix proteins like collagens, elastin and structural proteoglycans, and immune cells such as lymphocytes, mast cells and macrophages; stratum reticulare, a dense fibrous connective tissue (**Figure 2.**).

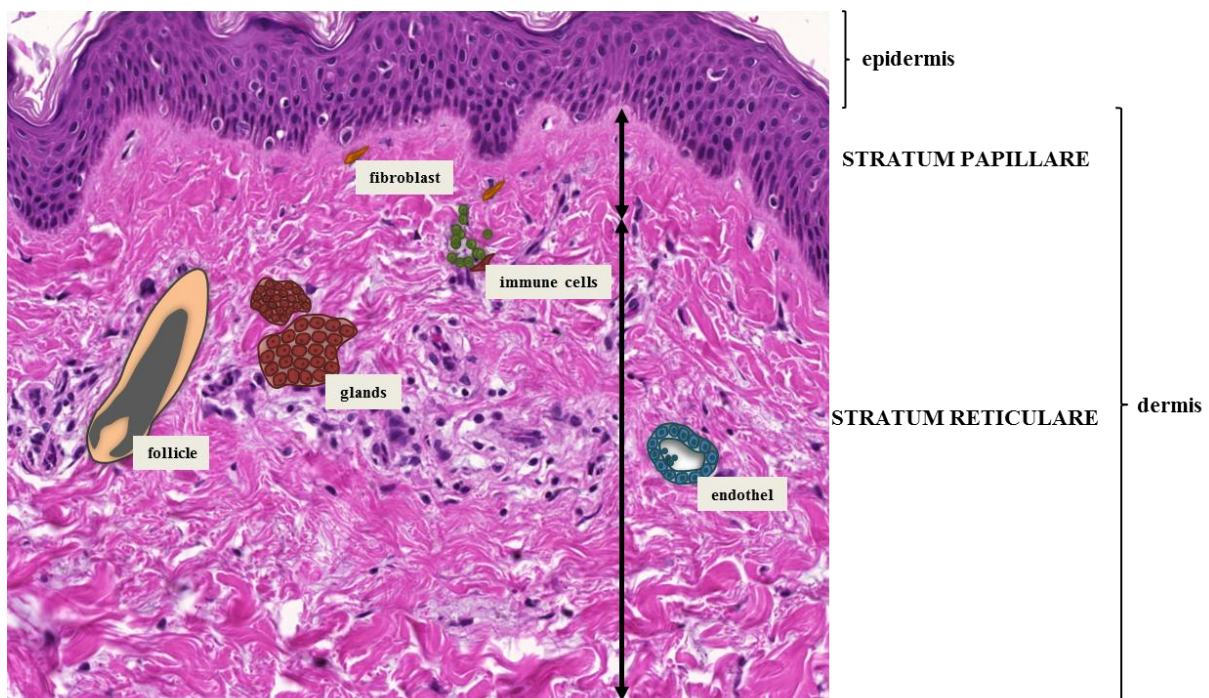


Figure 2. The structure of dermis

The subcutis is the layer of loose connective tissue and fat beneath the dermis. It also includes blood vessels and nerves.

The skin performs multiple functions, such as thermoregulation, barrier, sensory organ, secretion, excretion, thermal insulation, nutrient storage and immunological regulation through Skin Associated Lymphoid Tissue (SIS) ^{30,31}. In the epidermis, there are keratinocytes, which express receptors capable to recognize pathogens, and intraepidermal dendritic cells, called Langerhans-cells, which are involved in antigen presentation to T-cells. In the dermis, there are dendritic cells, mast cells, lymphocytes, macrophages, and fibroblasts, which are also involved in the immune response.

SOLAR UV LIGHT IRRADIATION AND ITS CELL BIOLOGICAL CONSEQUENCES

Solar UV exposure is one of the most important environmental factors affecting skin physiology ¹. UV radiation, expanding the 200 to 400 nm wavelengths of the electromagnetic spectrum, is a high-energy component of solar radiation (**Figure 3.**). UVR is divided into three ranges based on wavelength: UVA (400–320 nm), UVB (320–290 nm), and UVC (290–200 nm). Over 95% of UVA and 1–10% of UVB radiation reaches the earth's surface, whereas almost 100% of solar UVC is absorbed by the atmosphere and the ozone layer.

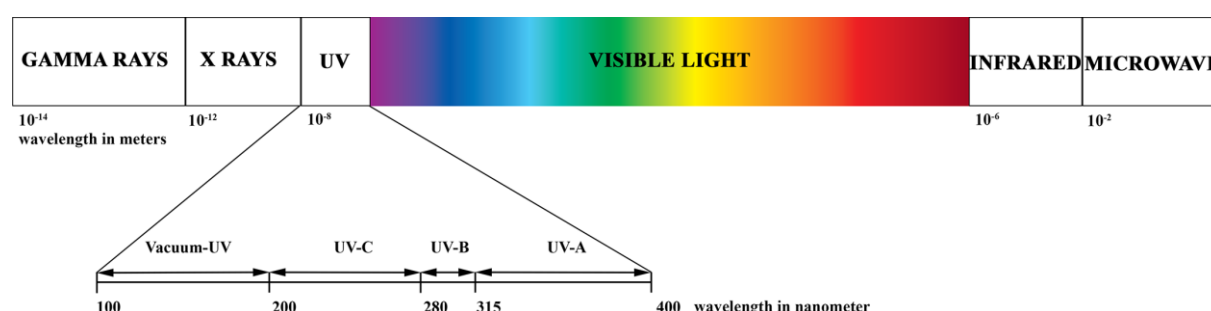


Figure 3. Electromagnetic spectrum

Exposure of human skin to solar UV rays can lead to short and long term consequences including synthesis of vitamin D, erythema (or sunburn reaction), photo-immunosuppression, tanning, photo-aging, and skin cancers. A history of sunburn in childhood and continued unprotected exposure to ultraviolet radiation (UVR) through

adolescence and adulthood contribute to skin cancer risk. UVR directly targets macromolecules in the skin such as proteins, lipids, and nucleic acids. UVB portion of the sunlight has strong effectiveness on photo-carcinogenesis^{32,33}. Wavelength in the UVB range is mainly absorbed by epidermis. Its toxic effects are mainly mediated by the direct absorption of photons by DNA. It causes DNA damage by the formation of dimeric photoproducts between adjacent pyrimidine bases on the same strand. Two forms of pyrimidine dimers are cyclobutane pyrimidine dimers (CPD), which are formed by the covalent binding of carbon atoms at C5 and C6 positions (thymine and/or cytosine) and pyrimidine (6-4) pyrimidone photoproducts (PP), which are resulted from the covalent binding between C6 and C4 positions (**Figure 4.**). Incorrect repair of these damages leads to mutations in epidermal cells that may induce carcinogenesis³⁴. In addition, UVB can damage proteins or cell membranes, and generate oxidative stress according to the workgroup of Kulms³⁵.

UV irradiation can induce production of hydrogen peroxide (H_2O_2), hydroxyl radical, singlet oxygen ($O_2^{\cdot-}$) and peroxy radicals³⁶. Reactive oxygen species (ROS) are generated through photoactivation of endogenous photosensitizers (eg. porphyrins, riboflavin, quinones). It induces then various damages including DNA strand breaks and oxidative modifications of nucleic bases predominantly at guanine bases. This process leads to generation of 7,8-dihydro-8-oxoguanine lesions (8 oxo-dG) (**Figure 4.**), which have been shown to induce specific DNA mutations if not repaired.

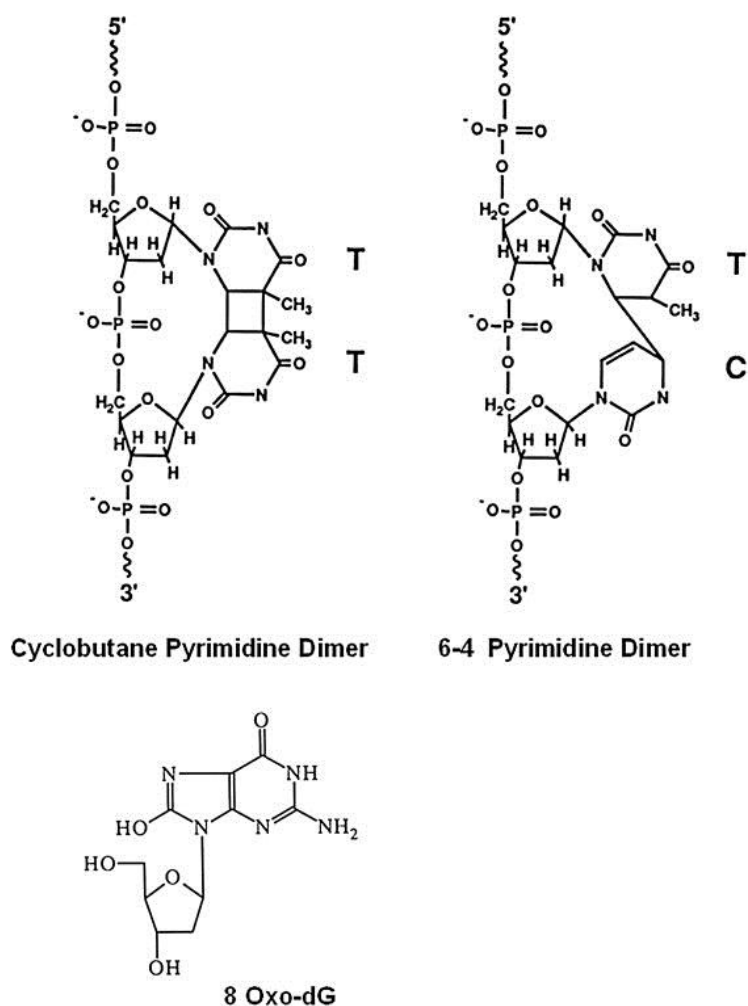


Figure 4. Chemical structures of pyrimidine dimers and 8 oxo-dG. (Forrás: ¹)

UV also activates nitric oxide synthase, and induces lipid peroxidation, thereby increasing production of prostaglandins and induces other inflammatory mediators. Cells have defense mechanisms against oxidative stress. Anti-oxidant molecules detoxify ROS to prevent free radical changes to DNA and other macromolecules^{37,38} (**Figure 5.**). Glutathione (GSH) is a reducing agent to neutralize reactivity of free radicals by donating electrons to otherwise reactive molecules. GSH functions in cooperation with nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxido-reductase system. Catalase is an antioxidant enzyme that detoxifies hydrogen peroxide, whereas superoxide dismutases (SOD) inactivate superoxide anions. Heme oxygenase-1 (HMOX1) is involved in heme metabolism, cleave heme into three products, such as biliverdin, CO and ferrous iron. Biliverdin and its reduced form bilirubin are recognized as antioxidants scavenging ROS. The activation of HMOX1 is a sensitive marker for oxidative stress³⁹.

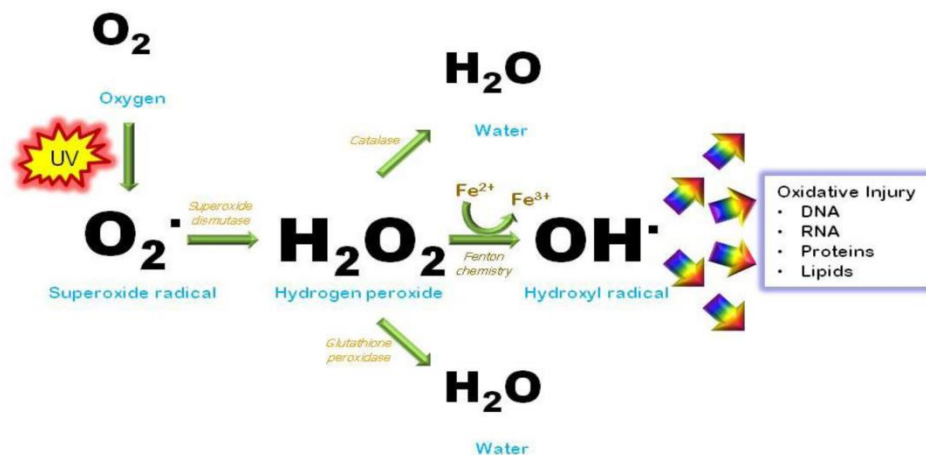


Figure 5. Detoxification of free radicals induced by UV (Forrás: ³⁸)

UV-induced photo-lesions cause cell cycle arrest, DNA repair or in case of irreparable photo-damage cell enter cell death:

1. Cell Cycle arrest: in response to internal and external stress such as genomic DNA damage and suboptimal growth conditions, proliferating cells trigger mechanisms called cell cycle checkpoints that assess and respond to the stress ⁴⁰.
2. To combat the diversity of DNA lesions, cells have evolved a complex DNA damage response (DDR) that can engage many different DNA repair pathways, including nucleotide excision repair (NER) in which process CPDs and (6-4) PPs are repaired ⁴¹. It can be divided into two pathways: global genome repair (GGR), which repairs lesions throughout the genome, and transcription-coupled repair (TCR), which repairs lesions in the transcribed strand of active genes. GGR is initiated by XPC-RAD23B factor, in some cases with the help of UV-damaged DNA-binding protein ⁴². TCR is initiated by RNA polymerase stalled at a lesion with the help of CSA, CSB, and XAB2. Both pathways require the core NER factors to complete the excision process. It recognizes adducts formed by environmental mutagens and cancer chemotherapeutic drugs as well. Other repair mechanisms are base excision repair (BER) which likely cope with damages created upon reactions with ROS ⁴³, DNA mismatch repair (MMR) which promote genomic stability ⁴⁴ and double strand break repair (DSBR) ⁴⁵.

3. Checkpoint-arrested cells resume cell-cycle progression once damage has been repaired, whereas cells with irreparable DNA lesions undergo permanent cell-cycle arrest or cell death:

- a. Apoptosis: there is intrinsic pathway of apoptosis, in which ROS generated upon UV irradiation have been recognized to serve as initiators, which causes mitochondrial damage. UV triggers cell death receptors (CD95/Fas, TRAIL, and TNFR1) by autocrine release of death ligands or in a ligand-independent manner. Therefore, UV additionally induces the extrinsic apoptotic pathway ⁴⁶. The cell shrinks and condenses without damaging the neighbour cells. The cytoskeleton collapses, the nuclear envelope disassembles, and the nuclear DNA breaks up into fragments. The cell surface is altered, displaying properties, such as Annexin V expression that cause the dying cell to be rapidly phagocytized without any leakage of its contents. Cell death can occur through other mechanisms that may not involve an active participation of the dying cell ⁴⁷. Early apoptotic cells are characterized by the retain membrane integrity, in contrast late apoptotic cells are characterized by compromised membranes and act like necrotic cells, which also lack intact membranes, possibly because of the leakage of pro-inflammatory intracellular contents ⁴⁸.
- b. Necrosis: It is a non-apoptotic cell death. Apoptosis is a silent form of cell death, the intact cell disappears through phagocytic uptake, it can be considered to be immunosuppressive. In contrast, necrosis is usually related to an earlier breakdown of plasma-membrane integrity, which may permit the release of pro-inflammatory cellular components, it can be considered to be immunostimulatory. Therefore balance between the two processes plays a substantial role in the decision of whether the uptake of dead cells causes a productive immune response or not.

Other consequences which are induced by UV irradiation is autophagy, which is a catabolic process of “self-eating” aimed to remove long-lived or damaged proteins and organelles, and for recycling of cytoplasmic contents ⁴⁹ and senescence which represents a physiological anti-tumor response. It is an irreversible cell cycle arrest.

These mechanisms can be modulated through several routes e.g. by cytokines such as IL-12, IL-23, growth factors or plant derived compounds such as curcumin or green tea extracts⁵⁻⁷.

CLINICAL ASPECTS OF UV-INDUCED SKIN DAMAGE, MELANOMA AND NON-MELANOMA SKIN CANCERS

Sunburn (erythema), immunosuppression and suntan (pigmentation) are the immediate responses of normal human skin exposed to UVR (short term effects)⁵⁰.

Skin cancers are the most common malignancies of humans, 1 in 5 American will develop skin cancer in their life³⁸. Skin cancer formation is the most important long term effect of UV exposure. There are epidemiologic and molecular evidences connected all forms of skin cancer to UV, and UV is causative factor for approximately 65% of melanoma and 90% of non-melanoma skin cancers³⁸. Both, UVB represent carcinogens for non-melanoma, keratinocyte-derived skin cancer, including squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) and are emerging as causative factors in malignant melanoma (MM) formation.

BCC is the most common cancer type (80% of skin cancers), which occurs frequently on head, neck and other sun-exposed area of body and rarely metastasize (semi-malignant)²⁸. SCC (200,000/year) is more aggressive and can metastasize.

Cutaneous Malignant Melanoma (CMM) occurs less commonly (70,000/year), but is the most dangerous form of skin cancer, with high risk of metastasis. Melanoma arises from single melanocytes in 50% of melanomas, from lentigo maligna in 10-15% of melanomas or from naevus cell nevi and blue nevi. There are different types of benign naevus cell nevi, such as junctional nevi, compound nevi, intradermal nevi and congenital nevi and acquired nevi, respectively. Malignant melanomas are developed with high risk from dysplastic nevi, which are characterized with appearance at a young age.

There are types of factors involved in skin cancer development such as genetic factors, when UV-specific or UV-signature mutations are induced through error-prone translesion DNA synthesis⁵¹. The sites of U-containing CPDs produced by deamination can induce accidentally C → T and CC → TT mutations. p53 is a key tumor-suppressor gene which is

mutated frequently in human skin cancer ¹. Besides induction of DNA damage and p53 mutations immune modulation via inflammatory and immune suppressive pathways are also considered crucial to the process of skin cancer development and independent from p53 signaling pathway ⁵².

CMMs are described with four types: Superficial spreading melanoma (SSM), Nodular melanoma (NM), Acral lentiginous melanoma and Lentigo maligna melanoma. SSM is commonly found on the trunk in men and on the legs and back in women ²⁸. NM is the most aggressive type of melanoma and it is more common in males. To characterize the widespread of the tumor and to find out the risk for metastasis an evidence-based staging system is used.

It is determined by the tumor-lymph node-metastasis (TNM) classification for tumor staging according to 2009 American Joint Committee on Cancer (AJCC) guidelines ⁸ (**Table 1.**). It was made on the basis of a multivariate analysis of 30,946 patients with CMM. CMM is distinguished to 4 stages regarding the severity of the disease. Staging for localized melanoma (stages I and II) includes primary tumor thickness, primary tumor ulceration, primary tumor mitotic rate. Previously Clark level was also a staging criterion, however it was replaced with mitotic rate, since it is not an independent prognostic factor when mitotic rate is included in the analysis. Clark level describes the level of anatomical invasion of the melanoma in the skin and distinguished to five anatomical levels ²⁸. These levels are: CMM limited to the epidermis (level 1), invasion to papillary dermis (level 2), invasion to junction of papillary and reticular dermis (level 3), invasion to reticular dermis (level 4) and invasion to subcutis (level 5) ⁵³. Staging for regional metastatic melanoma (stage III) includes immunohistochemical detection of micrometastases.

Primary melanoma can be treated with surgical resection. However approximately 20 % of all patients with melanoma will develop metastases and in general have a poor prognosis. The AJCC clinicopathological staging system for the prognosis of CMM assigns patients to risk categories, but it does not predict individual patient outcomes. It is highlighted by there are thin (<1 mm) melanomas which eventually metastasize and a significant proportion of melanoma deaths (approximately 15 %) result from metastases of thin primary melanomas according to National Cancer Institute Surveillance Epidemiology and End Results.

Classification	Thickness (mm)	Ulceration Status/Mitoses
T		
Tis	NA	NA
T1	≤ 1.00	a: Without ulceration and mitosis $< 1/\text{mm}^2$
		b: With ulceration or mitoses $\geq 1/\text{mm}^2$
T2	1.01-2.00	a: Without ulceration
		b: With ulceration
T3	2.01-4.00	a: Without ulceration
		b: With ulceration
T4	> 4.00	a: Without ulceration
		b: With ulceration
N	No. of Metastatic Nodes	Nodal Metastatic Burden
N0	0	NA
N1	1	a: Micrometastasis*
		b: Macrometastasis†
N2	2-3	a: Micrometastasis*
		b: Macrometastasis†
		c: In transit metastases/satellites without metastatic nodes
N3	4+ metastatic nodes, or matted nodes, or in transit metastases/satellites with metastatic nodes	
M	Site	Serum LDH
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

Table 1. TNM Staging Categories for CMM

Abbreviations: NA, not applicable; LDH, lactate dehydrogenase.

↵* Micrometastases are diagnosed after sentinel lymph node biopsy.

↵† Macrometastases are defined as clinically detectable nodal metastases confirmed pathologically.

PROMISING BIOMARKERS FOR THE PROGNOSIS OF CMM

1. Immunohistochemically detectable tissue-based prognostic protein biomarkers: Several potential protein biomarkers have been identified, which show statistically significant association with melanoma-specific mortality and disease-free patient survival. Promising protein biomarkers with independent prognostic significance from clinicopathological parameters are AP-2, ATF-2, NCOA3, PRKCA, Bcl-2, Survivin, CEACAM-1, CXCR4, CD44, MCAM, L1-CAM, MMP2, OPN, Tenascin-C, tPA, HMB45, iNOS, p16/INK4A, p27, CyclinA, MAP-2, Metallothionein (MT), p53, Ki67, bFGF, β -Catenin, Bcl-6, Dysadherin, HNK-1, HIF2 α , GADD153, Melastatin, MITF, p-Akt, RGS1, RUNX3, RBM3, PUMA and PHH3⁵⁴. However none of these is introduced in routine clinical practice, because these studies need validation.
2. Genetic biomarkers: There are few studies on uveal melanomas using comparative genomic hybridization which predict melanoma outcomes at gene level⁵⁵.
3. Epigenetic, mRNA biomarkers: there are also evidences for the importance of epigenetic and mRNA alterations in the determination of the progression of CMMs⁵⁶.

CMM FROM THE ASPECT OF TUMOR IMMUNOLOGY

Besides heterogeneity, CMM can also be characterized by its immunogenicity. There are number of clinical studies related to the activity of the immune system in melanoma, and these observations also provide strong evidences that the immune system can naturally react to and destroy or control melanoma⁵⁷. Observations, which show communication between melanoma and tumor microenvironment, are that tumor-associated antigens are expressed by melanoma cells, there is spontaneous tumor regression in patients with primary melanoma or that melanoma regression is related to autoimmune skin depigmentation. In addition there are clinical evidences for the effectiveness of melanoma immunotherapies. Indeed, high-dose IL-2 therapy or adding of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) induced durable responses in patients treated with CMM. However these therapies either have serious side effects or these are effective in only a minority of patients.

The tumor develops and progresses through remodeling of the microenvironment. The interactions between melanoma cells and the microenvironment, including epidermal keratinocytes and stromal fibroblasts, endothelial cells, immune cells, soluble molecules and the extra cellular matrix, depend on cell-cell, cell-matrix contact and on secretion of cytokines or growth factors ^{58,59}. This crosstalk between the melanoma cells and the surrounding cells promote the metastatic process by inducing angiogenesis, invasion, migration, and colonization in a secondary organ (**Figure 6**).

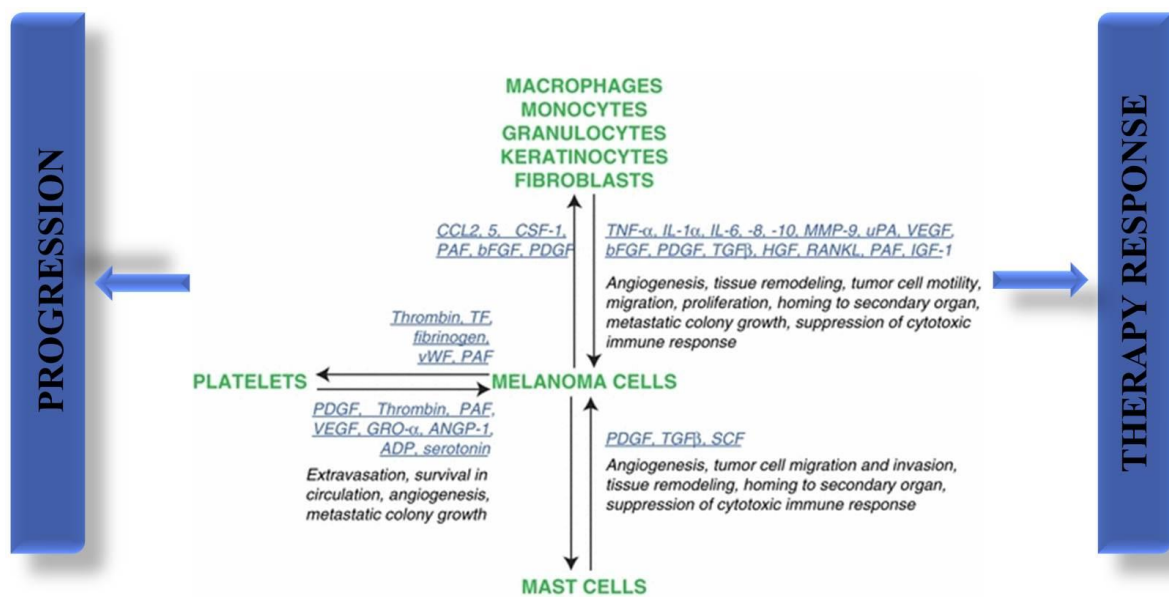


Figure 6: Crosstalk between melanoma cells and tumor microenvironment (⁶⁰)

ROLE OF ZINC HOMEOSTASIS IN SKIN

Zinc is an essential microelement, it has critical role in normal health and development ⁶¹. Zinc content of the body is associated with skin by approximately 9%, primarily with the epidermis (50–70 $\mu\text{g g}^{-1}$ dry weight) ^{62,63}, and 50% of the available zinc is localised to the cytoplasm, while 30–40% is localised in the nucleus, and the remainder is associated with the plasma membrane ⁶⁴.

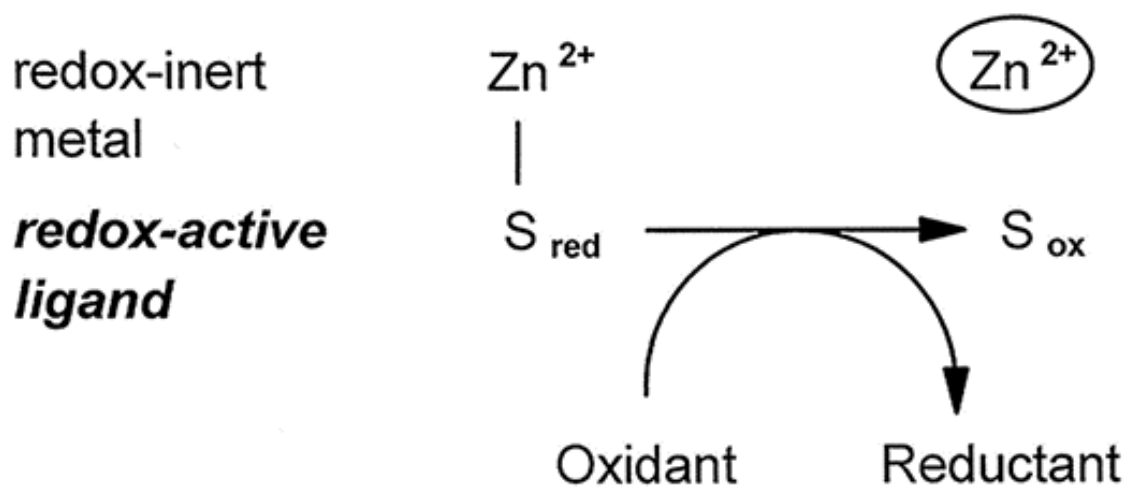
Its importance in skin is shown by the severe skin symptoms of hereditary or acquired zinc deficiency, including erythematous rashes, scaly plaques, ulcers, and the genetic inability to absorb zinc from the intestine results in acrodermatitis enteropathica, which manifests itself as severe dermatitis at orifices and acra^{65,66}. By the ability of systemic or topical zinc preparations zinc improves hair loss, acne and several inflammatory skin conditions⁶¹.

The cellular homeostasis of zinc is mediated by ZIPs (Zrt-, Irt-like proteins), which family contains 14 proteins that transport zinc to the cytosol, and ZnTs, containing 10 proteins which transport zinc out of the cytosol⁶⁷. The intracellular distribution of zinc in the endoplasmic reticulum, Golgi and mitochondria is also regulated.

Zinc coordination motifs have been identified which motifs predict zinc-binding sites on proteins. The prevalence of genes encoding zinc proteins is estimated to be over 3% of the 32,000 identified genes⁶¹. According to Schwartz et al., over 300 zinc-dependent enzymes have been defined and characterised which are related to regulation of gene expression, proliferation, redox homeostasis and immune process.

Zinc occurs in its stable ionic state of +2 in cells, it cannot participate in electron transfer chemistry, it is redox inert. Nevertheless, it has important role in reduction-oxidation processes. There is a dynamic of zinc binding to and release from proteins (**Figure 7.**). In this process MT has crucial role, this characteristic of zinc is maybe connected to its role in protein functions. A close relation between redox homeostasis and the regulation of different protein functions by zinc can provide an important, fine-tuned control mechanism in the cell, but this process has been evidenced biochemically and has not been well studied in biological systems. Zinc also function as a cofactor of superoxide dismutase, and zinc deficiency increases oxidative stress⁶⁸.

A



B

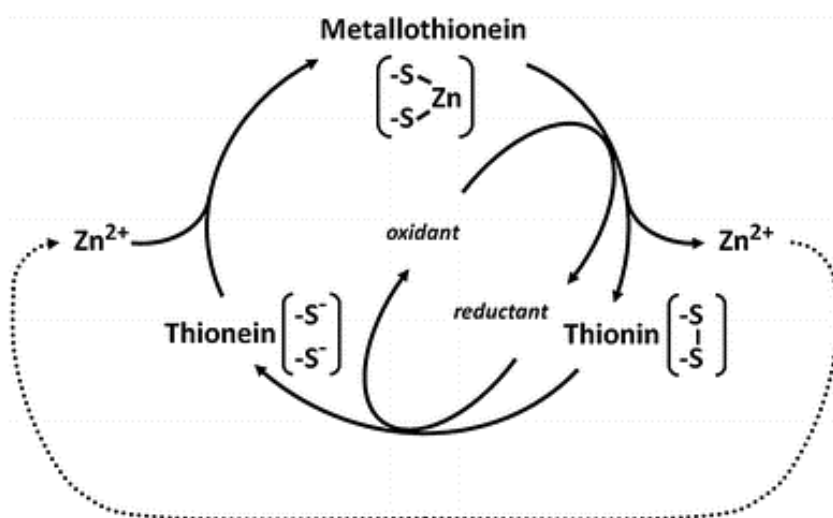


Figure 7. Schematic presentation of zinc/sulphur bond as a redox cycle (A) and MT/thionein sequestration (B) ^{69,70}

METALLOTHIONEIN

MTs are characterized by low molecular weight of 6-7 kDa, high cysteine content and the ability to complex with metals. MT acts as a zinc muffler over several orders of magnitude. MT/thionein couple sequester or release zinc depending on the local redox state, thereby influencing the function of numerous proteins, transcription factors and enzymes⁶⁹. There are at least 10 functional isoforms of MT in human body (**Table 2.**)²². The major isoforms are MT I/II, which are presented in all cells. MT III and MT IV are minor isoforms and found only in specialized cells. The MT III was first isolated as a growth inhibiting factor (GIF) from brain neurons, and the MT IV is found in stratified epithelium. Transcription of MT I/II can be induced by inflammatory cytokines (IL-6, TNF- α , interferons), lipopolysaccharids, glucocorticoids, free radicals, antioxidants or heavy metals.

Based on		Classification
Metals Present		Major –MTI/II
		Minor – MTIII and MTIV
Proteins are encoded by a family of genes which are located on chromosome 16q13	functional (8)	MT1A, MT1B, MT1E, MT1F, MT1G, MT1H, MT1X, MT2A
	non-functional (7)	MT1C, MT1D, MT1I, MT1J, MT1K, MT1L, MT2B
Expressed in tissues	Liver, kidney, skin	MTI/II
	Brain and male reproductive organs	MTIII
	Specific to stratified squamous epithelia	MTIV

Table 2. Classification of MT isoforms^{22,71}

ROLE OF ZINC IN UV-INDUCED DNA DAMAGE RESPONSE AND CARCINOGENESIS

The action of zinc in UVB induced DNA damage response is largely unknown. It has been demonstrated that rapid intracellular elevation of zinc levels occurs in keratinocytes after UVB irradiation^{72,73}, and the level of MT is higher in the epidermis after acute UV exposure, while the skin of MT knockout mice is more susceptible to UVB⁷⁴. In addition apoptosis is

observed in skin lesions of patients with acquired zinc deficiency ⁷⁵ suggesting important regulatory role of zinc in cell death pathways. Previously, it was found that solar (i.e., mixed) UV irradiation of keratinocytes exposed to zinc chloride (ZnCl₂/Zn (II)) enhances cell survival and is able to reduce the immediate DNA damage ^{25,26} and DNA fragmentation after exposure ²⁷.

In the case of cancer, higher zinc intake has been suggested to be related to lower risk of cancer development ⁷⁶, however, it may have opposite effect at extremely high doses or long term supplementation ⁷⁷. This maybe is not due to direct carcinogenicity of zinc, but the effect of zinc on immunosuppression and inflammation can be involved in cancer development. Regarding skin an imbalance in the MT protein level can be detected in different skin cancers ^{19,78}. Expression of MT I/II isoforms in some human tumors have been found to be downregulated, which supports the protective role of MT against malignant transformation ⁷⁹. Epigenetic downregulation of MT1E and MT1G has been reported recently in CMM as well ^{80,81}. However, experimental evidence for tumor suppressor role of some MT isoforms is still lacking. Nevertheless, several types of cancer overexpress MT and the overexpression seemed to correlate with tumor progression and poor survival of patients ⁷⁹. Increased MT I/II protein expression has been observed in keratinocyte- and melanocyte-derived tumors and it correlated with tumor invasiveness. Using whole tumor sections, MT I/II overexpression of primary CMM cells has been shown to be correlated with tumor progression, tumor thickness, invasiveness and reduced survival ⁸²⁻⁸⁴. Further, the overexpression of MT I/II in primary CMM cells appeared to have a higher prognostic value than that of sentinel lymph node positivity ¹⁸. Therefore the protein expression of MT I/II is a promising prognostic immunohistochemical factor in prognosis of CMM.

In summary, UV irradiation is the main carcinogenic factor for skin cancer development. Defense mechanisms against irradiation induced tissue damage and malignant transformation are regulated by several routes. The identification of these regulatory pathways is important for understanding photocarcinogenesis and to develop novel chemopreventive and therapeutic agents, respectively.

CMM is not the most frequent skin malignancy, however, the most aggressive one with poor clinical outcome in metastatic cases. Current therapeutical agents induce only a short term tumor remission or are effective only in a small minority of patients. Importantly, the crosstalk between tumor cells and tumor microenvironment often leads to a rapid tumor escape, which must be taken into consideration thinking about prognosis and effective

therapy. Therefore, it is beneficial to examine the behaviour of primary CMM in conjunction with the characteristics of tumor microenvironment, by which the high risk patients and patient subset for immunotherapy can be identified ⁸⁵. Furthermore, there is a need for predictive biomarkers that are independent from the routinely used clinicopathological prognostic parameters. In addition, understanding the molecular background regarding the function of these marker proteins in CMM progression may justify them as new therapeutic targets for personalized treatment.

MT is a potential immunohistochemical biomarker, since significantly increased expression levels of MT has been shown in melanoma tissue samples by two research groups. However, it is not routinely used as a prognostic marker, because the reported data have not been confirmed yet.

In addition, functional studies on MT could explore its role in the process of carcinogenesis, thereby justifying it as biomarker and, potentially, therapeutic target. On one hand, a multiparametric IHC examination (including those parameters that characterize the tumor microenvironment) of melanoma tissue samples of patients with known clinical outcome can provide an insight into the pathogenetic significance of MT in CMM development. On the other hand, investigation of MT as an important component of cellular zinc homeostasis in the context of the role of zinc in regulation of UV-induced DNA damage response can provide an insight into molecular aspects of MT involvement in skin carcinogenesis.

OBJECTIVES

1. Research data suggest that the up-regulation of MT expression in CMM is a significant and independent factor for reduced patient survival. However, MT has not yet become widely used as a prognostic marker because of the lack of immunohistochemical validation by different workgroups, therefore we aimed to compare the MT I/II protein expression in primary CMM samples without metastases to samples with haematogenous metastases in a retrospective manner using tissue microarray analysis (TMA) of archived paraffin tissue blocks.
2. We hypothesized that there is a relationship between the tumoral MT I/II protein expression and cells surrounding/infiltrating the tumor, thus we aimed to characterize our samples immunologically with such immunohistochemical markers that showed association with CMM prognosis previously and compare these staining patterns to alterations in tumoral MT I/II protein expression.
3. Considering the findings about the connection between MT I/II expression of tumor cells and tumor progression, we were interested in the further role of MT and zinc homeostasis in skin physiology and pathophysiology. The main environmental harmful factor to skin is UVB. We assumed that zinc homeostasis can influence the UVB-induced DNA damage response; therefore we aimed to study the effect of non-toxic Zn (II) exposure on human keratinocyte functions in conventional culture and under stressed conditions using UVB irradiation.

These findings may provide a better understanding of pathogenesis of skin diseases, and may open new therapeutical possibilities.

MATERIALS AND METHODS

Patient selection for tissue microarray

Tissue blocks were chosen from the archive of the Department of Dermatology of the University of Debrecen. Patients were selected retrospectively after considering their clinical outcome and in accordance with ethical rights. Tissue samples were selected by Éva Remenyik, Gabriella Emri and Kristóf Egervári. Formalin-fixed paraffin-embedded tissue blocks of primary CMM without metastases (n= 23, 1998-2003) and with hematogenous metastases (n= 23, 1998-2006) were selected for tissue microarrays (TMAs). Blocks of nevi (n= 6) were also included. TMA was performed as described previously^{86,87}. Briefly, 4-μm sections were prepared from the tissue blocks and stained with hematoxylin-eosin for validation. Thereafter, the areas of interest were selected by a pathologist (Kristóf Egervári) and a dermatooncologist (Gabriella Emri), and multiblocks (6x10; d= 1 mm tissue cylinders/cores) were built by means of TMA MasterTM (TMA Master, USA). The serial sections that were used for immunohistochemical study were previously checked by hematoxylin-eosin staining.

Cell culture, UVB-irradiation apparatus, and ZnCl₂ treatment

HaCaT human keratinocytes were obtained from ATCC and maintained in high-glucose DMEM (PAA, Traun, Austria) supplemented with 10% fetal bovine serum ((FBS) Lonza, Verviers, Belgium), 2 mM L-glutamine (PAA, Traun, Austria), 100 U/mL penicillin G, 0.1 mg/mL streptomycin sulphate, and 0.25 μg/mL amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) in an atmosphere of 5% CO₂ at 37°C. In the UV irradiation experiments, the culture medium was replaced with sufficient phosphate-buffered saline (PBS) (DPBS, Lonza, Verviers, Belgium) to avoid dehydration. The cells were then exposed to UVB (20 mJ/cm²) using a TL20W/12 RS broadband, filtered UVB lamp (Philips, Germany), which had an

emission spectrum of 280–370 nm, with a peak at 312 nm. The UV intensity was controlled with a UV light meter (UVP Inc., San Gabriel, USA). The original medium was replaced after treatment. A 0.1-M stock solution of ZnCl₂ (Sigma-Aldrich, St. Louis, MO, USA) was prepared in deionised water and sterilised with a 0.2-µm sterile filter. A working concentration of 100 µM (unless stated otherwise) was selected according to previous reports²⁷.

Immunohistochemistry

Immunohistochemical staining was performed on serial sections of tissue multiblocks. Endogenous peroxidase was blocked in 1 % H₂O₂-methanol for 20 min at room temperature. Antigen retrieval was achieved by pressure-cooking the tissue samples in 10 mM citrate buffer (pH= 6). After the slides cooled (> 10 min), they were incubated with primary antibody for 1 hour at room temperature (MT I/II – Dako, clone E9, 1:50; melan-A – Dako, clone A103, 1:50; DC-SIGN – R&D Systems, clone 120507, 1 µg/ml; CD68 – Dako, clone KP1, 1:100; CD163 – Novocastra, clone 10D6, 1:100; CD1a – Beckman Coulter, clone O10, 1:3). For visualisation of the reaction, the Envision/HRP detection system (Dako) and the VIP peroxidase substrate kit (Vectorlabs) were used. The slides were counterstained with methyl green. The stained tissue samples were digitalised using Pannoramic Viewer 1.15 (3DHISTECH Ltd.) and evaluated in blinded manner by a dermatooncologist (Gabiella Emri) and a pathologist (Imre Veres). MT I/II expression was evaluated semiquantitatively according to the intensity and extent of the immunoreactivity: score 0 (negative or weak diffuse staining, or strong positivity in < 10 % of the tumor cells) and score 1 (strong positivity in > 10 % of the tumor cells). We used the same criteria for assessing the MT I/II labelling of nevus cells as well. CD1a⁺, DC-SIGN⁺, CD68⁺, and CD163⁺ cells infiltrating or surrounding the tumor and MT I/II expressing cells within the tumor environment were evaluated according to their absence or presence.

Apoptosis assay

Cells were pre-treated with ZnCl_2 for 24 h and then exposed to UVB. Afterward, apoptosis was measured 24 h after UVB exposure. Harvested cells (5×10^5) were washed in ice-cold PBS and stained with Annexin V and propidium iodide (PI) using the Vybrant apoptosis assay Kit (Invitrogen, Eugene, Oregon, USA) according to the manufacturer's protocol. The stained cells were analysed by flow cytometry (CyFlow® Space, Partec, Canterbury, United Kingdom) (Georgina Nagy) using UV/488 nm dual-excitation, measuring the fluorescence emission at 530 and 575 nm. Annexin V⁻PI⁻ cells were identified as viable cells, Annexin V⁺PI⁻ cells were identified as early apoptotic cells, and the sum of the Annexin V⁺PI⁺ and Annexin V⁻PI⁺ cells was identified as late apoptotic plus necrotic cells.

Cell proliferation

Cells were treated with 50 or 100 μM ZnCl_2 for 72 h, and cell proliferation was measured using the EZ4U assay (Biomedica, Vienna, Austria) according to the manufacturer's instructions. The optical density (OD) was determined at 450 nm using an Anthos 2020 microplate reader (Biochrom Ltd., Cambridge, UK).

CPD-ELISA

Cells were treated with ZnCl_2 for 24 h and/or UVB irradiated, and genomic DNA was purified 0, 1, 3, 6, and 24 h after UVB exposure using a QIAamp Blood Kit (Qiagen, Hilden, Germany). Ninety-six-well plates were coated with 0.003% protamine sulphate (Sigma-Aldrich, St. Louis, MO, USA) for 2 days at 37°C. To measure CPD lesions, genomic DNA was diluted to 0.2 $\mu\text{g/mL}$ in PBS, denatured at 100°C for 10 min, and then chilled on ice for 15 min. The denatured DNA solution was transferred to plates pre-coated with protamine sulphate and dried overnight at 37°C. For blocking, the plates were washed four times with

PBS-T (0.05% Tween-20 in PBS) and then incubated in 20% FBS for 30 min at 37°C. The wells were then washed four times with PBS-T and incubated with the primary CPD antibody (#TDM-2, Cosmo Bio Co. Ltd., Tokyo, Japan) at a 1:1000 dilution in PBS for 30 min at 37°C. The wells were then washed and incubated with the horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (BioRad, Hercules, CA, USA) diluted 1:3000 in PBS for 30 min at 37°C. After incubation, the cells were washed four times with PBS-T and then once with citrate-phosphate buffer (pH 5.0). Following buffer removal, the wells were incubated with a substrate solution (o-phenylene diamine (Sigma-Aldrich, St Louis, MO, USA), H₂O₂, and citrate-phosphate buffer (pH 5.0)) for 10 min at 37°C, at which time the enzyme reaction was stopped with 2 M H₂SO₄. The absorbance was measured using an Anthos 2020 microplate reader (Biochrom Ltd., Cambridge, UK) at 492 nm, and the OD values of the untreated cells were subtracted from those of the treated cells.

TaqMan Low-Density Array

Cells were treated with ZnCl₂ for 4 or 24 h, and relative gene expression was measured. In a parallel experiment, cells were pre-treated with ZnCl₂ for 24 h and then UVB irradiated. Six hours after exposure, total RNA was isolated using Trizol Reagent (MRC Inc., Cincinnati, Ohio, USA), and total RNA was quantified using a Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, USA). cDNA was then synthesised using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. We designed Custom TaqMan Array (TLDA) Microfluidic Cards (384-well) by first selecting 91 genes based on their roles connected to zinc and the UVB response. The selected genes were involved in cell cycle progression, inflammation, apoptosis, DNA repair, and antioxidant defence (**Table 3.**).

Gene Symbol	Assay ID	Gene Name
ACTB ^l	Hs99999903_m1	actin, beta
AKT1 ^b	Hs00178289_m1	v-akt murine thymoma viral oncogene homolog 1

APC ^b	Hs01568269_m1	Adenomatous Polyposis Coli
APEX ^d	Hs00172396_m1	APEX nuclease (multifunctional DNA repair enzyme) 1
ATF3 ^{a, b}	Hs00231069_m1	activating transcription factor 3
ATM ^b	Hs01112307_m1	ataxia telangiectasia mutated
ATR ^b	Hs00354807_m1	ataxia telangiectasia and Rad3 related
BAD ^b	Hs00188930_m1	BCL2-associated agonist of cell death
BCL2 ^b	Hs00608023_m1	B-cell CLL/lymphoma 2
BCL2L1 ^b	Hs00236329_m1	BCL2-like 1
C11orf31 ^{h, i}	Hs00415057_m1	chromosome 11 open reading frame 31
CCNE1 ^b	Hs01026536_m1	cyclin E1
CCNF ^b	Hs00171049_m1	cyclin F
CDKN1A ^b	Hs00355782_m1	cycline-dependent kinase inhibitor 1A (p21, Cip1)
CEBPA ^{b, h}	Hs00269972_s1	CCAAT/enhancer binding protein (C/EBP), alpha
CEBPB ^b	Hs00270923_s1	CCAAT/enhancer binding protein (C/EBP), beta
CHEK1 ^b	Hs00967506_m1	CHK1 checkpoint homolog (S. pombe)
CHEK2 ^b	Hs00200485_m1	CHK2 checkpoint homolog (S. pombe)
CYP1A1 ^k	Hs00153120_m1	cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1B1 ^k	Hs00164383_m1	cytochrome P450, family 1, subfamily B, polypeptide 1
DDB1 ^c	Hs00172410_m1	damage-specific DNA binding protein 1, 127kDa
DDB2 ^c	Hs03044953_m1	damage-specific DNA binding protein 2, 48kDa
DDIT3 ^b	Hs01090850_m1	DNA-damage-inducible transcript 3

ERCC1 ^c	Hs01012158_m1	excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)
ERCC2 ^c	Hs00361161_m1	excision repair cross-complementing rodent repair deficiency, complementation group 2
ERCC3 ^c	Hs01554450_m1	excision repair cross-complementing rodent repair deficiency, complementation group 3 (xeroderma pigmentosum group B complementing)
ERCC4 ^c	Hs00193342_m1	excision repair cross-complementing rodent repair deficiency, complementation group 4
ERCC5 ^c	Hs00164482_m1	excision repair cross-complementing rodent repair deficiency, complementation group 5
FOS ^b	Hs00170630_m1	FBJ murine osteosarcoma viral oncogene homolog
GADD45B ^b	Hs00169587_m1	growth arrest and DNA-damage-inducible, beta
GAPDH ^l	Hs99999905_m1	glyceraldehyde-3-phosphate dehydrogenase
GLI1^j	Hs01110766_m1	GLI family zinc finger 1
HBEGF ^{a, b}	Hs00181813_m1	heparin-binding EGF-like growth factor
HIPK2 ^b	Hs00179759_m1	homeodomain interacting protein kinase 2
HMOX1 ⁱ	Hs01110250_m1	heme oxygenase (decycling) 1
HSPA6^k	Hs00275682_s1	heat shock 70kDa protein 6 (HSP70B')
HSPD1 ^k	Hs01941522_u1	heat shock 60kDa protein 1 (chaperonin)
IGF1R ^b	Hs00609566_m1	insulin-like growth factor 1 receptor
IL10^a	Hs00961622_m1	interleukin 10
IL1B ^a	Hs01555410_m1	interleukin 1, beta
IL1RN ^a	Hs00893626_m1	interleukin 1 receptor antagonist
IL8 ^a	Hs00174103_m1	interleukin 8
ITCH ^b	Hs00395201_m1	itchy E3 ubiquitin protein ligase homolog (mouse)
JUN ^b	Hs00277190_s1	jun proto-oncogene
KRT10^j	Hs00166289_m1	keratin 10
LCN2 ^{h,i}	Hs01008571_m1	lipocalin 2

MDM2 ^b	Hs00234753_m1	Mdm2 p53 binding protein homolog (mouse)
MGMT ^k	Hs01037698_m1	O-6-methylguanine-DNA methyltransferase
MMP9^a	Hs00234579_m1	matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
MSH2 ^e	Hs00953523_m1	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)
MSH6 ^e	Hs00264721_m1	mutS homolog 6 (E. coli)
MT1A^{g,h}	Hs00831826_s1	metallothionein 1A
MT1B^{g,h}	Hs00538861_m1	metallothionein 1B
MT1E ^{g,h}	Hs01938284_g1	metallothionein 1E
MT1F ^{g,h}	Hs00744661_sH	metallothionein 1F
MT1G^{g,h}	Hs02578922_Gh	metallothionein 1G
MT1H^{g,h}	Hs00823168_g1	metallothionein 1H
MT1M^{g,h}	Hs00828387_g1	metallothionein 1M
MT1X ^{g,h}	Hs00745167_sH	metallothionein 1X
MT2A ^{g,h}	Hs01591333_g1	metallothionein 2A
MTF1 ^g	Hs00232306_m1	metal-regulatory transcription factor 1
NOTCH1 ^{h,j}	Hs01062014_m1	notch 1
NTHL1 ^d	Hs00959764_m1	nth endonuclease III-like 1 (E. coli)
OGG1 ^d	Hs00213454_m1	8-oxoguanine DNA glycosylase
PARP1 ^d	Hs00242302_m1	poly (ADP-ribose) polymerase 1
PCNA ^{c, d, e}	Hs00427214_g1	proliferating cell nuclear antigen
PGK1 ^l	Hs00943178_g1	phosphoglycerate kinase 1
POLB ^d	Hs01099715_m1	polymerase (DNA directed), beta
PTGS2 ^a	Hs00153133_m1	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
RAD23B ^c	Hs00234102_m1	RAD23 homolog B (S. cerevisiae)
RAD51 ^f	Hs00153418_m1	RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)
RAD52 ^f	Hs00172536_m1	RAD52 homolog (S. cerevisiae)
RPA1 ^{c, f}	Hs00161419_m1	replication protein A1, 70kDa
S100A7^{a,j}	Hs00161488_m1	S100 calcium binding protein A7
SDHA ^l	Hs00188166_m1	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)

SKIL ^b	Hs01045418_m1	SKI-like oncogene
SLC30A1 ^{g,h}	Hs00253602_m1	solute carrier family 30 (zinc transporter), member 1
SMUG1 ^d	Hs00204820_m1	single-strand-selective monofunctional uracil-DNA glycosylase 1
SNAI2 ^a	Hs00950344_m1	snail homolog 2 (Drosophila)
TNF^a	Hs00174128_m1	tumor necrosis factor
TNFAIP3 ^a	Hs00234713_m1	tumor necrosis factor, alpha-induced protein 3
TP53 ^b	Hs01034249_m1	tumor protein p53
TP73 ^b	Hs01056230_m1	tumor protein p73
TREX1 ^e	Hs03055245_s1	three prime repair exonuclease 1
TRIM26 ^a	Hs00758189_m1	tripartite motif-containing 26
TXN ⁱ	Hs01555212_g1	thioredoxin
UNG ^d	Hs00422172_m1	uracil-DNA glycosylase
VEGFC ^a	Hs00153458_m1	vascular endothelial growth factor C
XPA ^c	Hs00166045_m1	xeroderma pigmentosum, complementation group A
XPC ^c	Hs01104206_m1	xeroderma pigmentosum, complementation group C
XRCC1 ^d	Hs00959834_m1	X-ray repair complementing defective repair in Chinese hamster cells 1
XRCC2 ^f	Hs03044154_m1	X-ray repair complementing defective repair in Chinese hamster cells 2
XRCC3 ^f	Hs00193725_m1	X-ray repair complementing defective repair in Chinese hamster cells 3
XRCC6 ^f	Hs00995282_g1	X-ray repair complementing defective repair in Chinese hamster cells 6
ZNRD1 ^k	Hs00205908_m1	zinc ribbon domain containing 1

Table 3. List of selected genes for TaqMan Low-Density Array analysis

Undetermined genes are highlighted in bold.

^a Inflammation

^b DNA-damage response

^c Nucleotide excision repair

^d Base excision repair

^e Mismatch repair

^f Double strand break repair

^g Zinc homeostasis

^h Genes containing MRE sites

ⁱ Oxidative stress

^j Keratinocyte proliferation/differentiation, cell survival

^k Others: ZNRD1, MGMT, HSPA6 (HSP70B), HSPD1 (HSP60), CYP1A1, CYP1B1

^l Housekeeping genes used for normalisation

Genes connected with zinc homeostasis and some with an identified metal regulatory element (MRE) in the promoter region were also added. The TLDA was performed using an Applied Biosystems 7900HT Real-Time PCR System according to the manufacturer's protocol. cDNA (100 ng) in 100 μ L of 1X Taqman Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) was loaded into each port of the TLDA plates, and each sample was examined in pairs. The PCR program used for amplification was as follows: 94°C for 1 min; 40 cycles of 94°C for 12 sec; and 60°C for 45 sec. The data were derived from three independently performed experiments and were analysed using SDS 2.1 software. ACTB, GAPDH, SDHA, and PGK1 were used for normalisation. Absolute and relative gene expression values were calculated using the 2^{-dCt} and 2^{-ddCt} method, respectively^{88,89} (The results were evaluated by Edit Mikó).

Measurement of $O_2^{\bullet-}$ production

After 4 or 24 h of $ZnCl_2$ treatment or after 24 h of $ZnCl_2$ treatment combined with UVB, the production of $O_2^{\bullet-}$ was measured using a dihydroethidium (HE) assay (Sigma-Aldrich, St. Louis, MO, USA) 1, 4, 10, and 24 h after UVB exposure. A working solution of HE at a 2 μ M final concentration was added to the cells and then incubated for 30 min at 37°C. The cells were then harvested, and the fluorescence intensity was measured by flow cytometry with a FACSCalibur (BD Biosciences, San Jose, USA) to determine the fluorescence intensity at 450 nm, with 620 nm as a reference. The production of $O_2^{\bullet-}$ was expressed as the mean of HE-fluorescence intensity⁹⁰.

Detection of hydrogen peroxide

After 4, 10 or 24 h of ZnCl₂ treatment, hydrogen peroxide production was determined using an 10-acetyl-3,7-dihydroxyphenoxazine (Amplex red) assay. In this assay, a working solution of Amplex Red (Invitrogen, Eugene, Oregon, USA) at a 50-μM final concentration and horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) at a 0.1 U/ml final concentration was added to the cells and then incubated for 20 min at room temperature. Fluorescence was subsequently read, with excitation at 530 nm and emission at 590 nm, using a Fluoroskan Ascent FL plate reader (Thermo Scientific, Vantaa, Finland). The MFI values of untreated cells were subtracted from those of the treated cells.

Western blotting

Cells were treated with ZnCl₂ for 4, 24, 48 or 72 h and lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). The protein concentration was then estimated using a BCA protein assay kit (Pierce Biotechnology, Rockford, USA). Proteins (20 μg) were separated on 10 or 12% SDS-polyacrylamide gels and then transferred onto nitrocellulose membranes by electroblotting. The membranes were then blocked with 5% low-fat milk in TTBS (0.1% Tween-20 in Tris-buffered saline) or in PBST (0.05% Tween-20 in PBS) and incubated overnight with primary antibody against heme oxygenase-1 (HMOX1) (#ab13248, Abcam Inc., Cambridge, USA) at 1:250 dilution or MTI/II (#ab12228, Abcam Inc., Cambridge, USA) at 1:1000 dilution in 5% milk at 4°C. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing, the signals were visualised using ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Freiburg, Germany). β-actin (Cell Signaling Technology Inc., Danvers, MA, USA) was used for normalisation.

Immunofluorescence and confocal microscopy

The HaCaT cells were plated on glass coverslips, treated with ZnCl₂ for 24 h or/and then UVB irradiated. 3 h after UVB, cells were fixed (20 min) with 4% paraformaldehyde. They were permeabilised with 1% TritonX-100 (10 min), blocked with 20% FBS, and then incubated overnight with primary antibodies against all MT I/II (#ab12228, Abcam Inc., Cambridge, USA) at a 1:50-dilution in PBS at 4°C. After washing with PBS, the preparations were incubated with a goat anti-mouse IgG Alexa Fluor 555 antibody for 2 h in PBS. Coverslips were mounted using an anti-fade reagent (Vector Laboratories Inc., Burlingame, CA, USA) and analysed using an Olympus FV1000S confocal microscope (Olympus Co. Tokyo, Japan) using the 60x oil-immersion objective (NA: 1.3) (Tamás Juhász). For excitation, a laser of 543 nm was used. The average pixel time was 4 µs. The Z image series of 1 µm optical thickness were recorded in sequential scan mode. Confocal settings were identical for all scans and remained constant during imaging.

Statistical Analysis

The data were statistically analysed using t-test, the Mann-Whitney U-test, Pearson χ^2 -test, Fisher's exact test and Spearman's Rho correlation (GraphPad Prism version 5.03, GraphPAD Software Inc., San Diego, CA, USA; SPSS for Windows, version 19.0, SPSS Inc., Chicago, IL, USA). Correlations were defined as significant if $p < 0.05$ (Katalin Hódosi, Eszter Anna Janka).

RESULTS

MT AS A POTENTIAL IMMUNOHISTOCHEMICAL BIOMARKER FOR CMM PROGNOSIS

Clinicopathological data

We studied 46 primary CMMs after dividing them into 2 groups, one nonmetastatic and the other exhibiting hematogenous metastases. The patients without metastases were followed up for 8-13 years (average 10 years). Comparing the clinicopathological parameters of the tumors with different metastatic potential (**Table 4.**), we found that the average Breslow tumor thickness was significantly higher in the metastatic group ($p= 0.001$) and that there were more metastatic cases among male patients (2.14:1) and fewer among females (1:2) ($p= 0.020$). In the metastatic group, more tumors were localised to the trunk and to the head and neck region than in the nonmetastatic group (78.3 % vs. 56.5 %), and more CMMs were of the nodular subtype (43.5 % vs. 34.8 %). However, these differences were not significant. As expected, the proportion of exulcerated CMMs was higher among the metastatic tumors (56.5 % vs. 13 %). There was no significant difference between the groups in patient age ($p= 0.577$) or in the Clark invasion level ($p= 0.584$).

Parameter	Metastasis			P
	N=46	Nonmetastatic (N= 23)	Metastatic (N= 23)	
Age (year)^a	56.2 ± 14.83	54.96 ± 15.95	57.43 ± 13.86	0.577 ^b
Gender				0.020^c
male	22 (47.8 %)	7 (30.4 %)	15 (65.2 %)	
female	24 (52.2 %)	16 (69.6 %)	8 (34.8 %)	
Localisation				0.303 ^c
trunk	28 (60.9 %)	12 (52.2 %)	16 (69.6 %)	
arm	7 (15.2 %)	5 (21.7 %)	2 (8.7 %)	
leg	8 (17.4 %)	5 (21.7 %)	3 (13 %)	
head-neck	3 (6.5 %)	1 (4.4 %)	2 (8.7 %)	
Type				0.550 ^c
ssm	28 (60.9 %)	15 (65.2 %)	13 (56.5 %)	
exulcerated	6 (21.4 %)	1 (6.7 %)	5 (38.5 %)	
nm	18 (39.1 %)	8 (34.8 %)	10 (43.5 %)	
exulcerated	10 (55.6 %)	2 (25 %)	8 (80 %)	
Breslow tumor thickness (mm)^a	2.84 ± 2.09	1.81 ± 1.22	3.86 ± 2.30	0.001^b
< 1.00 mm	13 (28.3 %)	9 (39.1 %)	4 (17.4 %)	
1.01 - 4.00 mm	25 (54.3 %)	13 (56.5 %)	12 (52.2 %)	
> 4.01 mm	8 (17.4 %)	1 (4.4 %)	7 (30.4 %)	
Clark level				0.584 ^c
II	4 (8.7 %)	3 (13 %)	1 (4.4 %)	
III	20 (43.5 %)	10 (43.5 %)	10 (43.5 %)	
IV	19 (41.3 %)	10 (43.5 %)	9 (39.1 %)	
V	3 (6.5 %)		3 (13 %)	

Table 4. Clinicopathological parameters of 46 primary CMMs subdivided into nonmetastatic and metastatic groups

^amean ± SD

^bt-test

^cMann-Whitney U-test

Values with P < 0.05 are highlighted in bold

ssm, superficial spreading melanoma

nm, nodular melanoma

Tumoral MT I/II (MT_t I/II) expression in primary CMM

The groups of nonmetastatic and metastatic primary CMM were compared with respect to the intensity, extent and distribution of the MT_t I/II staining (**Table 5.**).

		Metastasis		P
		Nonmetastatic	Metastatic	
		N= 46	(N= 23)	(N= 23)
MT I/II expression				
Tumoral				0.018 ^a
score 0	24 (52.2 %)	16 (69.6 %)	8 (34.8 %)	
score 1	22 (47.8 %)	7 (30.4 %)	15 (65.2 %)	
Peritumoral				0.555 ^a
absent	24 (52.2 %)	11 (47.8 %)	13 (56.5 %)	
present	22 (47.8 %)	12 (52.2 %)	10 (43.5 %)	

Table 5. Associations between metastasis formation and tumoral (MT_t I/II) and peritumoral (MT_p I/II) MT I/II expression in primary CMMs

MT I/II expression was evaluated semiquantitatively by considering the intensity and extent of the immunoreactivity: score 0 (= negative staining, weak and diffuse staining or < 10 % of the cells staining positively) and score 1 (> 10 % of the cells were strongly positive).

^a χ^2 probe

Values with P < 0.05 are highlighted in bold

MT I/II, isoforms I and II of metallothionein

The basal keratinocytes, the proliferating epithelium of the follicular bulb and the ductal epithelium of the eccrine and apocrine glands served as positive controls for MT I/II immunohistochemical staining. The cytoplasm and the nucleus of the tumor cells were positively labelled (**Figure 8.a**).

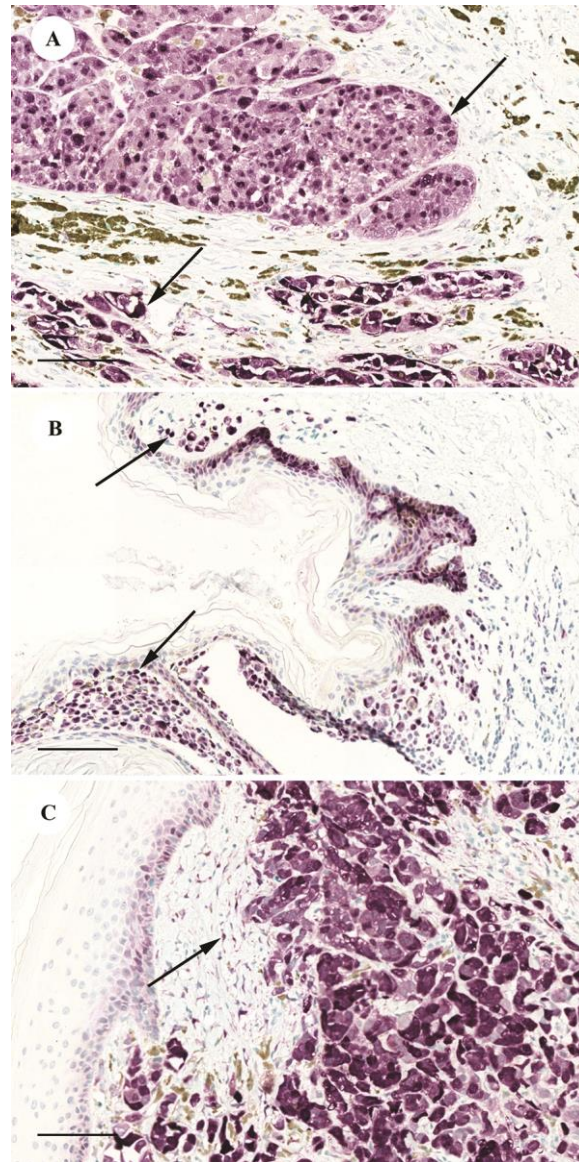


Figure 8. MT I/II immunohistochemistry of pigment cell nevi and primary CMMs

Expression of MT I/II proteins **A** within primary CMM cells (MT_t I+II), **B** in nevus cells (MT_n I/II), and **C** in the peritumoral area of primary CMM (MT_p I/II) detected by immunohistochemistry. Arrows indicate some representative MT I+II-positive cells. The keratinocyte of basal layer served as a positive control. The immunohistochemical detection was based on an immunoperoxidase reaction using VIP chromogenic substrate as described in methods. Original magnification is x 20. The scale bars indicate 100 μm.

To determine the suitability of TMA for the evaluation of MT_t I/II expression in CMM, tissue samples were cut from the epidermal, dermal and central regions of each tumor (at least one section from each area). Although most metastatic CMMs exhibited diffuse staining of all tumor parts, we found that the dermal/central compartments of the melanomas were MT_t I/II-labelled in each positive case. In our preliminary short pilot study, we detected the same

immunoreactivity pattern in whole sections. In several primary tumor samples, single tumor cells at a distance from the main tumor mass could be observed with intensive MT_t I/II staining. Considering the intensity and extent of MT_t I/II labelling, the difference between nonmetastatic and metastatic primary tumors was significant when we defined tumors with less than 10 % of tumor cells positively stained or with diffuse pale staining (score 0) to be negative and tumors with more than 10 % of tumor cells labelled (score 1) to be positive ($p=0.018$).

The Breslow tumor thickness correlated with the risk of metastasis in our CMM cases, but it did not correlate with the MT_t I/II expression (**Figure 9**).

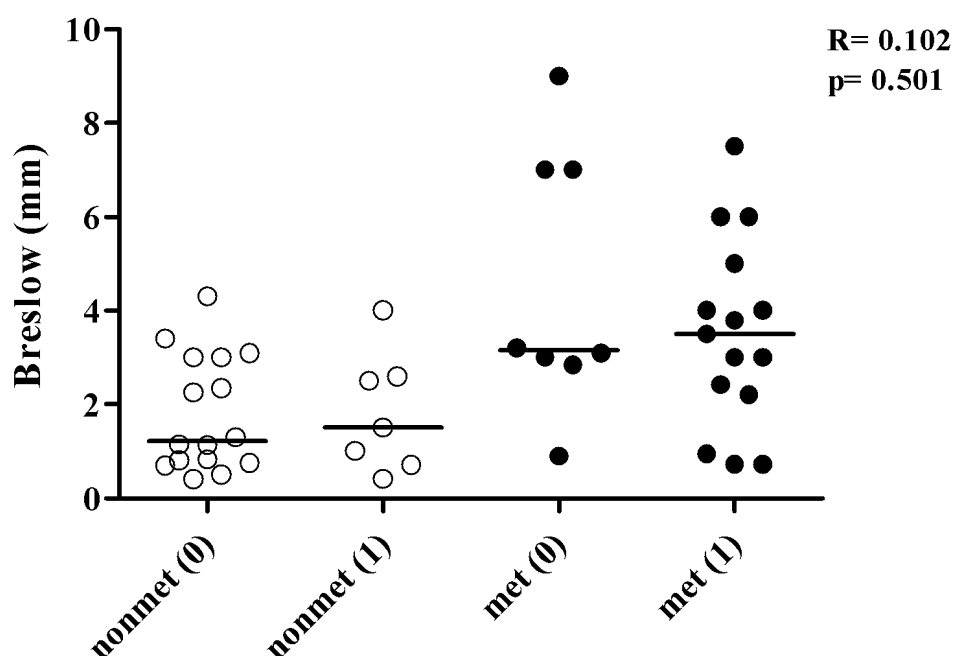


Figure 9. Association between Breslow tumor thickness and MT_t I/II expression of primary CMMs with different metastatic potentials

MT_t I/II expression was scored as 0 in cases with less than 10 % positive tumor cells or diffuse but weak immunostaining and scored as 1 in case with more than 10 % positive tumor cells. Metastatic (met) and nonmetastatic (nonmet) CMMs are indicated separately.

The median tumor thicknesses of the MT_t I/II-positive ($n=22$) and -negative ($n=24$) CMMs were 2.8 mm and 2.6 mm, respectively. The median tumor thicknesses of metastatic MT_t I/II-positive and -negative CMMs (3.5 mm vs. 3.15 mm), as well as the thicknesses of nonmetastatic MT_t I/II-positive and -negative primary tumors (1.5 mm vs. 1.23 mm), were

also similar. Interestingly, the MT_t I/II score correlated with age ($R= 0.349$, $p= 0.017$). There was no correlation between gender and the MT_t I/II score (data not shown).

MT I/II expression in nevi (MT_n I/II)

Benign pigment cell nevi ($n= 6$; male:female= 1:5; mean age= 48 (35-63 years); 4 compound type, 1 junctional nevus and 1 dermal-type nevus) were also investigated for MT_n I+II expression. All the nevi showed MT_n I+II expression (score 1), but the labelling pattern was different from that observed in primary CMM. Namely, nevus cells were stained immediately below the epidermis, whereas MT_t I+II-positive melanoma cell nests were localised to the central/dermal part of the tumor. In addition, we observed pronounced nuclear MT_n I+II staining in the nevi, but the CMM cells showed intensive cytoplasmic and nuclear immunoreactivity (**Figure 8.b**).

Peritumoral MT I/II (MT_p I/II) expression

MT I+II expression of the basal layer of the epidermis is well described and served as a positive internal control in our immunohistochemical study⁹¹. In normal human skin samples, we observed MT I/II expression in dermal cells, which were characterised by dendritic and spindle cell morphologies and by their association with perivascular lymphocytes. MT_p I/II⁺ cells were also observed surrounding the tumor cell nests (**Figure 8.c**); however, their presence did not correlate with the metastatic potential of the primary CMMs ($p= 0.555$).

Tumor-infiltrating and peritumoral CD68, CD163, DC-SIGN and CD1a expression

We determined the presence of CD68⁺, CD163⁺, DC-SIGN⁺ and CD1a⁺ macrophages/dendritic cells separately within the tumor and around the tumor cell nests (**Table 6.**).

		Metastasis			
		N= 46	Nonmetastatic (N= 23)	Metastatic (N= 23)	P
CD68-expression					
intratumoral					0.001^a
	absent	25 (54.3 %)	18 (78.3 %)	7 (30.4 %)	
	present	21 (45.7 %)	5 (21.7 %)	16 (69.6 %)	
peritumoral					0.300 ^a
	absent	11 (23.9 %)	4 (17.4 %)	7 (30.4 %)	
	present	35 (76.1 %)	19 (82.6 %)	16 (69.6 %)	
CD163-expression					
intratumoral					< 0.001^a
	absent	25 (54.3 %)	20 (87 %)	5 (21.7 %)	
	present	21 (45.7 %)	3 (13 %)	18 (78.3 %)	
peritumoral					0.233 ^a
	absent	3 (6.5 %)	0 (0 %)	3 (13 %)	
	present	43 (93.5 %)	23 (100 %)	20 (87 %)	
CD1a-expression					
intratumoral					1.000 ^a
	absent	39 (84.8 %)	20 (87 %)	19 (82.6 %)	
	present	7 (15.2 %)	3 (13 %)	4 (17.4 %)	
peritumoral					0.003^a
	absent	22 (47.8 %)	6 (26.1 %)	16 (69.6 %)	
	present	24 (52.2 %)	17 (73.9 %)	7 (30.4 %)	
DC-SIGN-expression					
intratumoral					0.665 ^a
	absent	40 (87 %)	21 (91.3 %)	19 (82.6 %)	
	present	6 (13 %)	2 (8.7 %)	4 (17.4 %)	
peritumoral					0.179 ^a
	absent	12 (26.1 %)	4 (17.4 %)	8 (34.8 %)	
	present	34 (73.9 %)	19 (82.6 %)	15 (65.2 %)	

Table 6. Association of tumor-infiltrating and peritumoral CD68, CD163, CD1a and DC-SIGN expression with metastatic and nonmetastatic primary CMMs

^a χ^2 probe

Values with P < 0.05 are highlighted in bold

We observed significant differences between the groups of metastatic and nonmetastatic CMM with respect to the presence of tumor-infiltrating CD68⁺ and CD163⁺ macrophages and peritumoral CD1a⁺ DCs (**Figure 10.**).

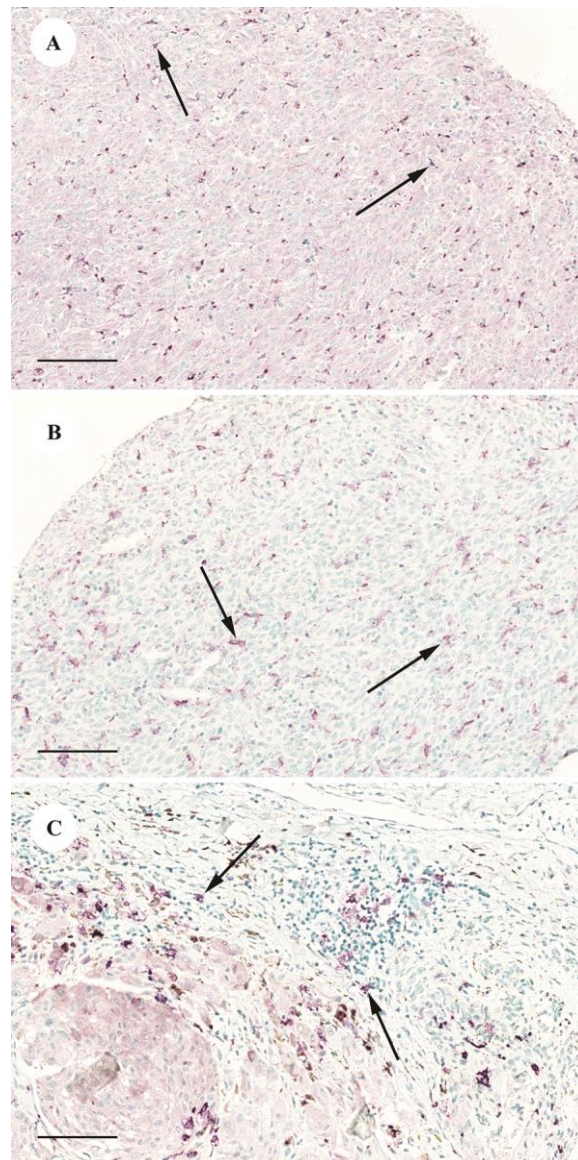


Figure 10. CD68, CD163 and CD1a immunohistochemistry of primary CMM

Immunohistochemical expression of immune markers was evaluated separately within and around the tumor: **A** CD68⁺ cells infiltrating the primary CMM, **B** CD163⁺ cells infiltrating the primary CMM, **C** CD1a⁺ cells surrounding the primary CMM. Arrows indicate some representative positive cells. The detection was based on an immunoperoxidase reaction. Original magnification is x 20. The scale bars indicate 100 μm.

Significantly more metastatic CMM cases were associated with the presence of CD68⁺ (p= 0.001) and CD163⁺ (p< 0.001) cells infiltrating the tumor, but significantly fewer metastatic primaries were associated with the presence of CD1a⁺ cells (p= 0.003) surrounding the tumor. Epidermal CD1a⁺ Langerhans cells were observed in each tissue sample within the epidermis without any obvious difference between the groups. We were then interested whether there is any correlation between the presence of immune cells and CMM MT_t I/II positivity. Some overlap regarding the localisation/distribution of DC-SIGN⁺ cells and MT_p I/II-labelled peritumoral cells could be seen in serial sections, but this observation was not significant. However, we determined a significant linear correlation between tumor-infiltrating CD68⁺ macrophages and the MT_t I/II expression of the melanoma cells (p= 0.003).

BIOLOGICAL ROLE OF ZINC IN HUMAN SKIN HACAT KERATINOCYTE CELL MODEL

Zn (II) increased cell proliferation

To examine the effect of Zn (II) exposure on cell proliferation, HaCaT cells were treated with Zn (II) at concentrations of 50 and 100 μM in growth medium containing 10% FBS, and the effects were measured using an EZ4U assay. We found that 72-h treatment with 100 μM Zn (II) resulted in significantly increased cell proliferation (1.3-fold; $p=0.045$) (**Figure 11.**).

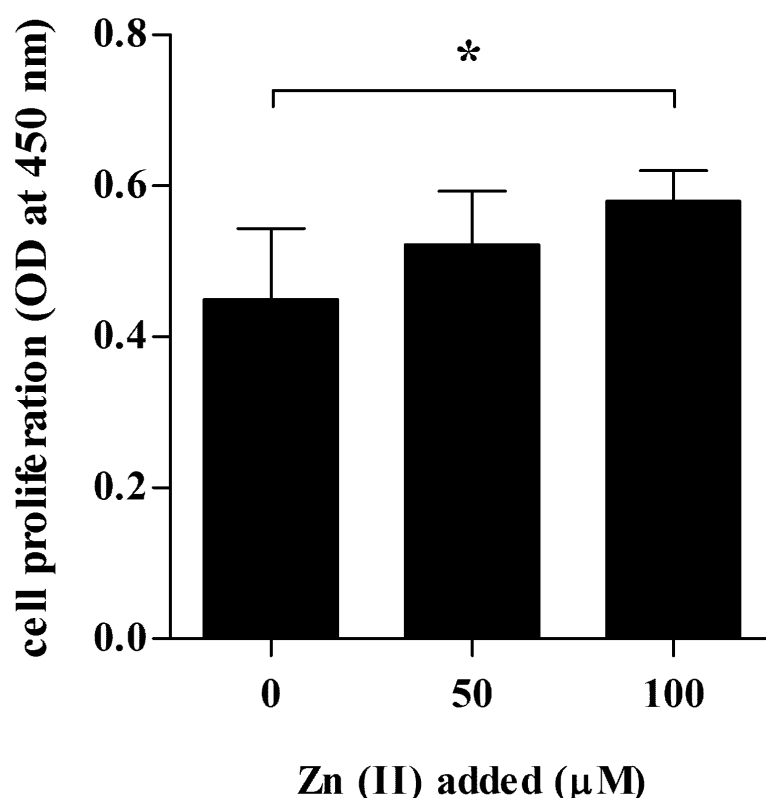


Figure 11. Effect of Zn (II) exposure on cell proliferation in HaCaT keratinocytes.

HaCaT cells were treated with 50 or 100 μM ZnCl_2 for 72 h, and cell proliferation was measured by an EZ4U assay. The data were derived from three independent experiments and are presented as the mean $\pm$ SEM. An asterisk indicates a significant difference between ZnCl_2 -treated and control cells at $p<0.05$, with a 2-tailed Student's t test.

Zn (II) exposure impacted the expression of genes involved in zinc homeostasis, antioxidant defence, cell viability and inflammation in HaCaT cells

Then, we identified genes that showed altered expression in response to 100 μ M Zn (II)-containing medium using TLDA analysis. We detected six significantly up-regulated genes with relative expression levels of at least 1.5-fold in Zn (II)-treated cells compared to the untreated controls (**Table 7.**). Isoforms of MT showed different transcriptional activity at base level. Gene expression of MT2A was the highest, and it was followed by MT1X, MT1E and MT1F (**Figure 12.**).

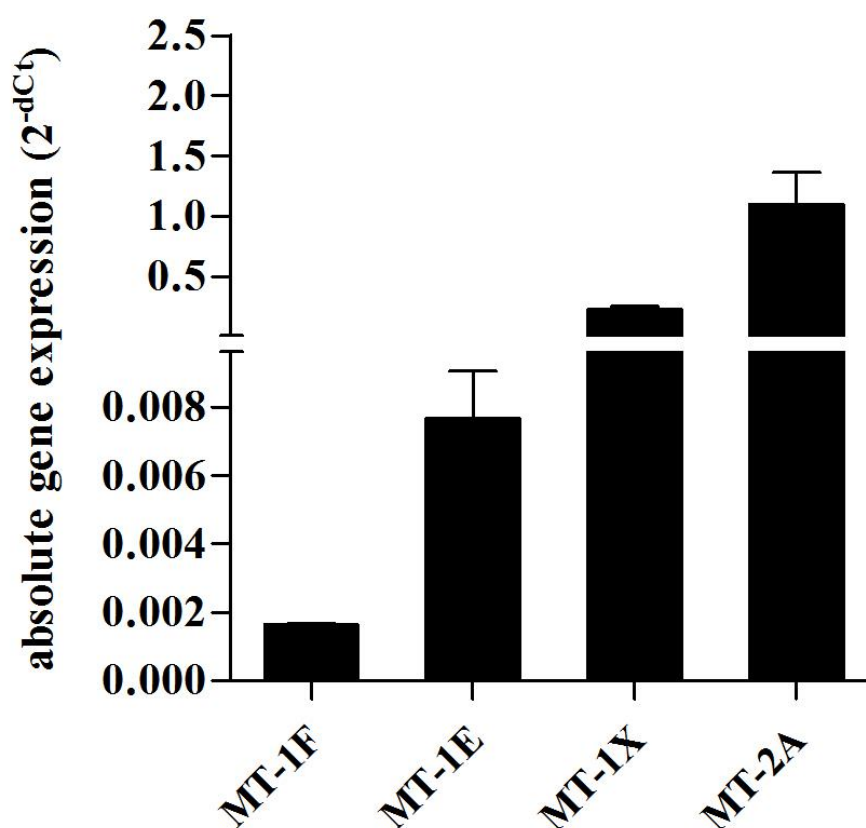


Figure 12. Basal gene expression level of MT isoforms

Absolute gene expression of MT isoforms were measured in untreated HaCaT cells.

Gene Symbol	fold change ($2^{-\Delta\Delta Ct}$)	
	4 h after zinc treatment	24 h after zinc treatment
MT1F	40.86	12.92
MT1X	17.34	3.24
HMOX1	15.33	1.56
MT1E	7.30	2.12
MT2A	6.44	3.03
SLC30A1	6.41	2.48
NOTCH1	1.47	1.06
PTGS2	0.68	1.01
IL8	0.63	0.92
CYP1B1	0.63	1.32

Table 7. Differentially expressed genes upon Zn (II) treatment

The relative gene expression of the listed genes was measured in HaCaT cells treated with 100 μ M Zn Cl₂ relative to untreated cells by TLDA. Values greater than 1.5 but less than 0.75 are highlighted in bold and indicate significant t-test results at $p < 0.05$, using a 2-tailed Student's t test.

The transcriptional change of the isoforms to ZnCl₂ exposure varied from the sequence of the basic expression levels. We detected induction of MT gene expression at 4 h of Zn (II) treatment and the induction of MT1F, MT1X and MT1E was greater than the transcriptional change of MT2A. MT1F gene showed the highest overexpression at both time points (40.86-fold at 4 h; 12.92-fold at 24 h), and MT1X and HMOX1 were expressed more than 10-fold higher in treated cells after 4 h of Zn (II) exposure. The expression levels of MT1E, MT2A and SLC30A1 also showed at least a 2-fold significant difference between treated and untreated cells at both treatment times. The notch homolog 1, translocation-associated (*Drosophila*) (NOTCH1) gene was slightly up-regulated (1.47-fold), and we detected slight but significant suppression of IL8, prostaglandin-endoperoxide synthase 2 (PTGS2), and cytochrome P450 1B1 (CYP1B1) mRNA expression (0.63-fold, 0.68-fold, and 0.63-fold, respectively) after 4 h of Zn (II) treatment. Specific commercial antibodies against MT protein isoforms are not currently available, and the composite of MTI/II can only be investigated at the protein level. To validate our mRNA data we investigated the protein

expression level of MTI/II by western blot, and we observed MTI/II induction compared to control with a maximum at 48 h after Zn (II) treatment (**Figure 13a.**).

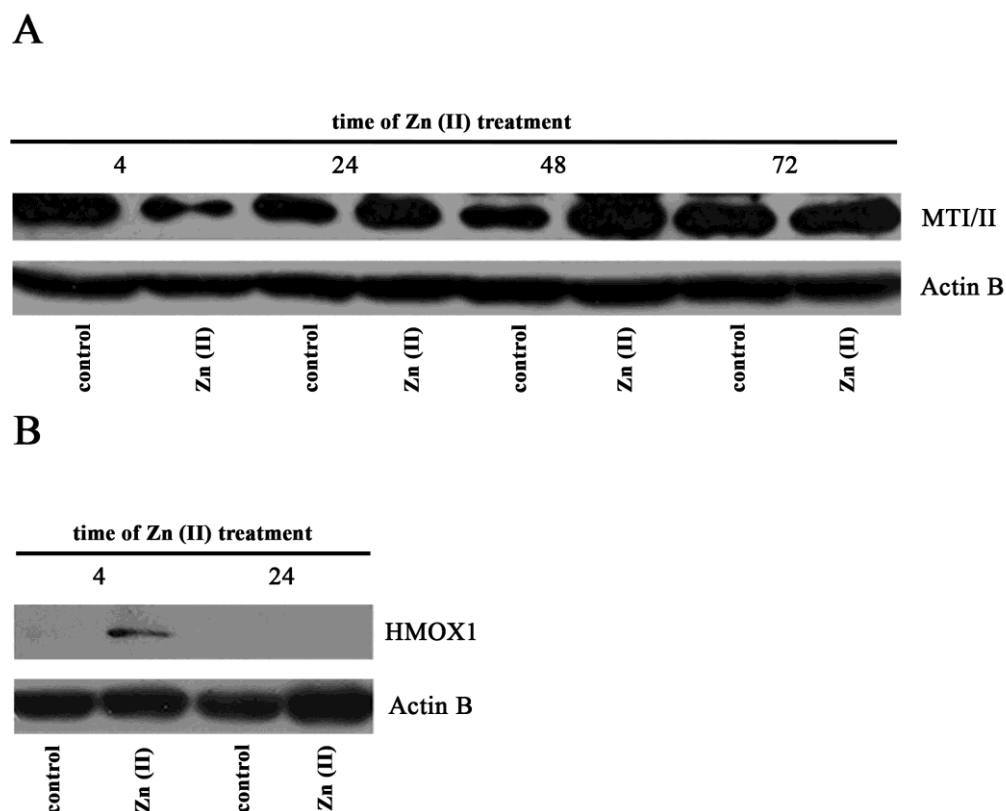


Figure 13. Effect of Zn (II) exposure on the protein expression of MTI/II and HMOX1

HaCaT cells were treated with 100 μ M ZnCl₂ for 4, 24, 48 or 72 h, and western blotting **A** for HMOX1 and **B** for MTI/II were performed. Actin B protein expression was used as a reference; the representative data of three independent experiments are shown.

Zn (II) exposure impacted the expression of HMOX1 and induced significant $O_2^{\bullet-}$ production

We also measured the protein expression of HMOX1 after 4 and 24 h of Zn (II) exposure (**Figure 13b.**). Western blot analysis demonstrated that 4-h Zn (II) treatment induced the production of a 34-kDa HMOX1 protein.

On the other hand, the induction of the antioxidant defence protein HMOX1 upon Zn (II) exposure prompted us to examine whether Zn (II) treatment can induce reactive oxygen species. We measured the generation of $O_2^{\bullet-}$ in response to Zn (II) exposure in cells using a flow cytometry-based HE assay. Our data showed significant production of $O_2^{\bullet-}$ after 4 h of Zn (II) treatment compared to the control ($p=0.006$) (**Figure 14a.**).

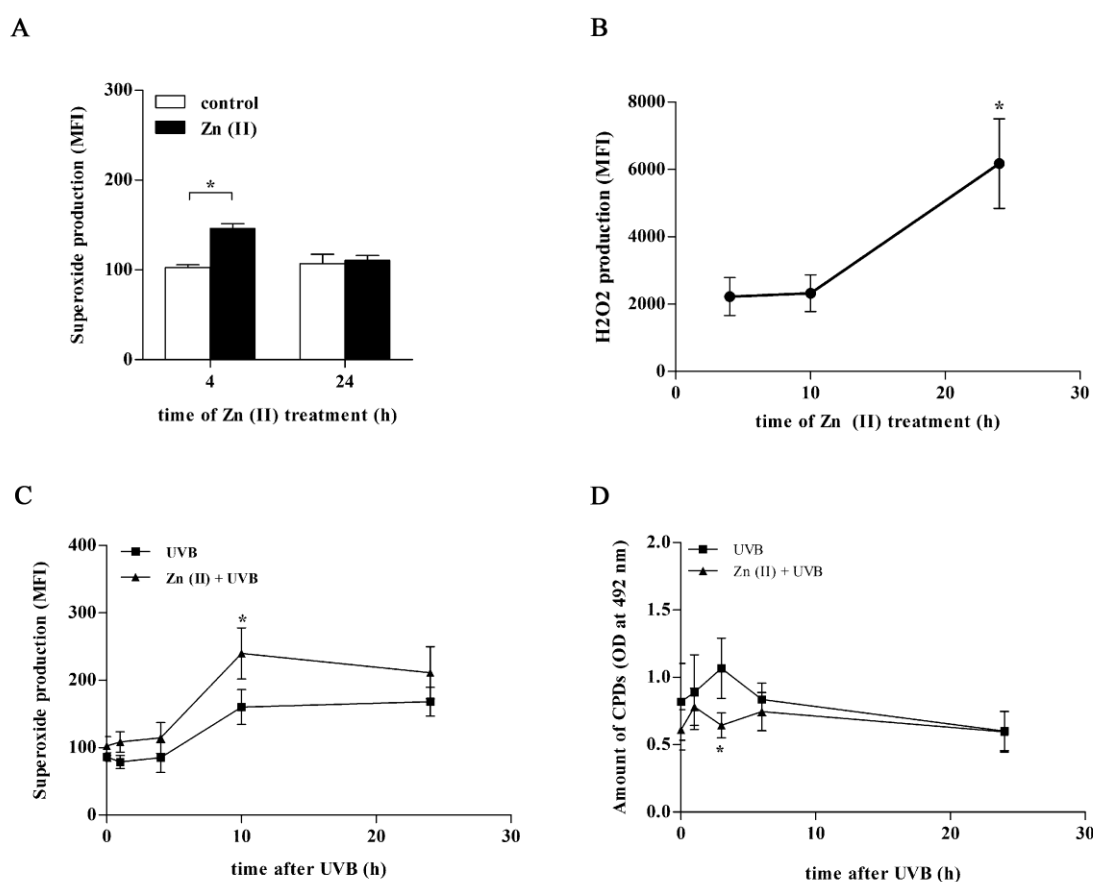


Figure 14. Effect of Zn (II) exposure on the production of reactive oxygen species and on UVB-induced DNA-damage response

HaCaT cells were treated with 100 μ M ZnCl₂ for 4 or 24 h and **A** the production of $O_2^{\bullet-}$ was analysed using a flow cytometry-based dihydroethidium assay. **B** Cells were exposed to 100 μ M ZnCl₂ for 4, 10 or 24 h, and the production of H₂O₂ was measured using a fluorimetric-based Amplex Red assay. **C** HaCaT cells were treated with 100 μ M ZnCl₂ for 24 h followed by 20 mJ/cm² UVB irradiation, and the amount of CPDs was determined at 0, 1, 3, 6, and 24 h after UVB irradiation using a CPD-specific ELISA. **D** The production of $O_2^{\bullet-}$ was measured by a flow cytometry-based dihydroethidium assay 1, 4, 10, and 24 h after UVB treatment.

The data were derived from three independent experiments and are presented as the mean $\pm$ SEM. An asterisk indicates a significant difference between the ZnCl₂ and UVB combination treatment and cells only exposed to UVB at $p<0.05$, using a 2-tailed Student's t test.

To further confirm our data, we investigated $O_2^{\bullet-}$ dismutation by measuring the production of H_2O_2 , and we found extracellular diffusion of H_2O_2 to the growth medium 24 h after Zn (II) treatment ($p=0.019$) (**Figure 14b.**).

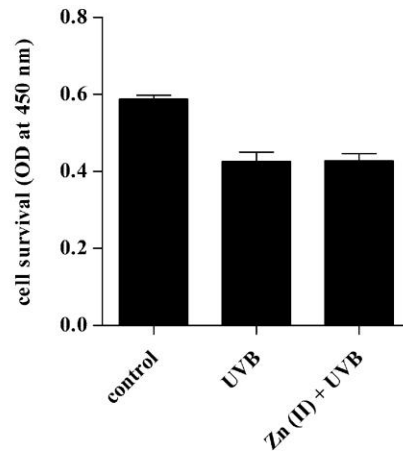
Zn (II) pre-exposure decreased the amount of CPDs but enhanced UVB-induced $O_2^{\bullet-}$ generation

To shed light on the mechanism of the zinc-mediated component of the UVB response, we characterised UVB-induced $O_2^{\bullet-}$ generation and CPD formation after 24-h Zn (II) pre-treatment. We examined the level of $O_2^{\bullet-}$ in Zn (II) pre-treated, UVB-irradiated cells and in cells only exposed to UVB (**Figure 14c.**). We observed an increase in $O_2^{\bullet-}$ generation, with a significant plateau 10 h post-UVB irradiation compared to the control ($p<0.001$), and we detected a time-dependent decrease in the amount of CPDs after UVB irradiation. Zn (II) pre-treatment changed both processes that were induced by UVB (**Figure 14d.**). Our results showed that significantly less CPDs (by 19.64%) were detected 3 h after UVB irradiation in cells pre-treated with Zn (II) for 24 h compared to cells exposed only to UVB ($p=0.0082$). However, at 10 h post-UVB exposure, significant enhancement of superoxide generation was observed when comparing Zn (II) pre-treated, UVB-irradiated cells to cells only treated with UVB irradiation ($p<0.001$).

Zn (II) pre-exposure altered the type of cell death after UVB irradiation, without influencing the overall survival

To investigate the effect of reduced levels of CPDs but elevated $O_2^{\bullet-}$ levels after Zn (II) pre-treatment, flow cytometric apoptosis and EZ4U cell viability assays were used. We found that the cell survival did not change between Zn (II)-treated and control cells 24 h after UVB irradiation (**Figure 15a.**).

A



B

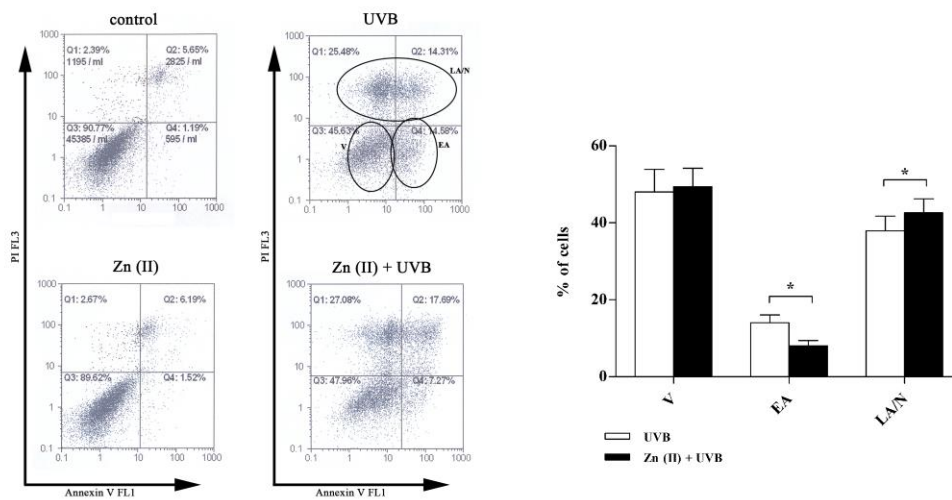


Figure 15. Effect of Zn (II) exposure on the UVB-induced DNA-damage response

HaCaT cells were treated with 100 μ M ZnCl₂ for 24 h followed by 20 mJ/cm² UVB irradiation, and **A** cell survival was measured using an EZ4U assay 24 h after UVB. **B** The rate of apoptosis was investigated by flow cytometry using Annexin V and propidium iodide staining 24 h after UVB irradiation. V=viability; EA=early apoptosis; LA/N=late apoptosis plus necrosis; representative data of three independent experiments. The data were derived from three independent experiments and are presented as the mean $\pm$ SEM, p values were calculated by 2-tailed Student's t test.

Regarding control cells, the proportion of viable cells was 88.69%, and in UVB irradiated cells this ratio decreased to 48.2%. The proportion of viable cells during Zn (II) exposure was 91.71%, and similar decrease was observed in Zn (II) pre-treated, UVB irradiated cells (to 50.1%) (**Figure 15b.**). On the other hand, Zn (II) pre-treatment significantly decreased the proportion of early apoptotic cells (Annexin V⁺PI⁻; by 5.94% or 0.57-fold, p=0.0286) and significantly increased the population of late apoptotic plus necrotic cells (Annexin V⁺PI⁺ and Annexin V⁻PI⁺; by 4.61 % or 1.12-fold, p=0.0026) (**Figure 15b.**).

Zn (II) caused a transcriptional shift in the UVB-modulated expression of genes

To determine the molecular mechanism behind the cellular response to the combined effects of Zn (II) and UVB exposure, we performed a TaqMan Low-Density Array analysis to characterise the gene expression changes in HaCaT cells pre-treated with Zn (II). We identified 39 differentially expressed genes in cells only submitted to UVB irradiation 6 h after exposure compared to non-irradiated control cells (**Table 8.**).

fold change (2^{-ddCt})		UVB					
		≤ 0.5		0.55 – 0.75	0.76 - 1.5 /not significantly different from control*	1.5 - 2	≥ 2
zinc + UVB	≤ 0.5	APC ATM ATR BCL2 BCL2L1 CCNF CHEK2 ERCC3 ERCC4 IGF1R ITCH MDM2 MSH2	MSH6 MT1E MTF1 NOTCH1 RAD52 SKIL TP73 TRIM26 VEGFC XPC XRCC2 XRCC3				
	0.55 – 0.75	ERCC5	CHEK1 ERCC2 SMUG1 TREX1				
	0.76 - 1.5 /not significantly different from control*	HIPK2	DDB2 NTHL1 POLB RAD51				
	1.5 - 2				CEBPA CYP1A1 DDIT3		
	≥ 2			MT1F MT1X MT2A SLC30A1		CCNE1 CDKN1A FOS GADD45B HBEGF IL1B IL8 JUN PTGS2 SNAI2 TNF ATF3	

Table 8. Effect of Zn (II) pre-treatment on transcriptional changes induced by UVB

irradiation

Relative gene expression was measured 6 h after UVB irradiation in HaCaT cells exposed to 20 mJ/cm² UVB or pre-treated with 100 μ M ZnCl₂ for 24 h and then UVB irradiated relative to untreated cells, using TLDA.

Twelve genes were expressed more than 2-fold higher in UVB-irradiated cells than in control cells, and TNF, PTGS2, and IL-8 showed the highest overexpression (>10-fold). We

identified 27 genes that were down-regulated in UVB-irradiated cells compared to the control cells; MT1E was the most repressed (0.12-fold). Furthermore, MTF1 levels were significantly decreased compared to the control (0.32-fold). We observed a modest modulation in gene expression when comparing Zn (II) pre-treated, UVB-irradiated cells to those that were only UVB-irradiated. Zn (II) pre-exposure mainly reduced UVB-induced homeodomain interacting protein kinase 2 (HIPK2) mRNA suppression and caused little modification of polymerase (DNA directed), beta (POLB) and RAD51 homolog (RecA homolog, E. coli) (*S. cerevisiae*) (RAD51) mRNA levels, whose relative gene expression levels were repressed following UVB irradiation (<0.75-fold) but remained unchanged (0.76–1.5-fold change) when pre-treated with Zn (II).

Cellular distribution of MTs in Zn (II) pre-treated cells and after UVB irradiation

We investigated the cellular distribution of MTI/II by confocal microscopy (**Figure 16.**). MTs were present mainly in the nucleus of the untreated cells and we found that Zn (II) induced immunostaining of MTs in the cytosol. UVB treatment changed the picture of Zn (II) pre-exposure, partial translocation of MTs to the nucleus was observed 3 h after UVB irradiation.

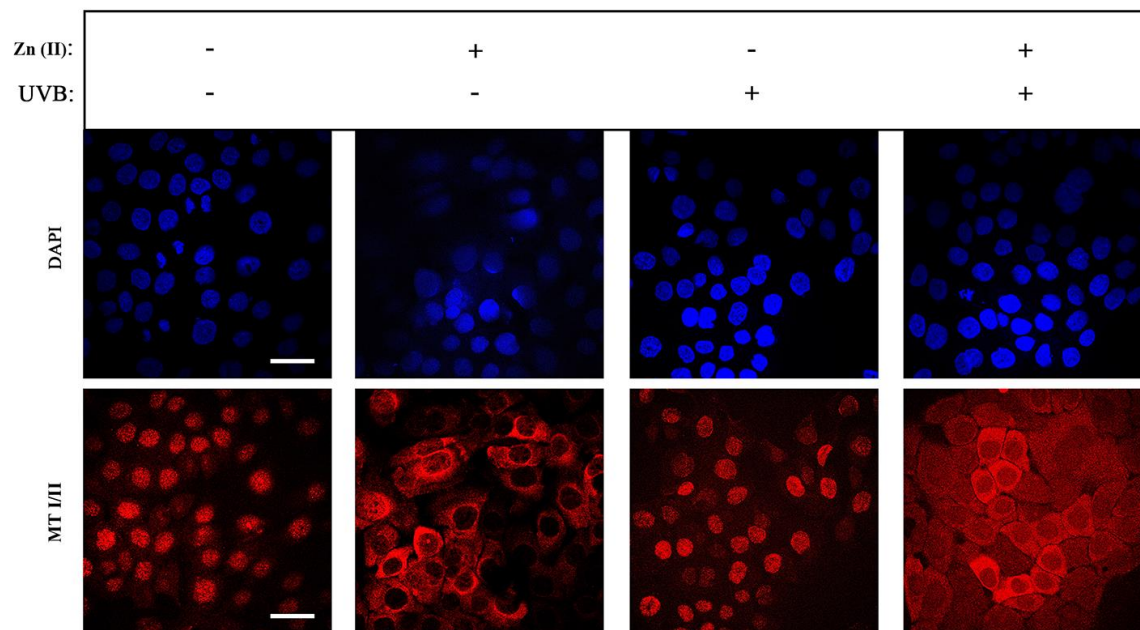


Figure 16. Subcellular localisation of metallothioneins after Zn (II) treatment and UVB irradiation.

HaCaT cells were treated with 100 μM ZnCl_2 for 24 h and/or irradiated with 20 mJ/cm^2 UVB. Localisation of MTs was detected by immunofluorescence staining 3 h after UVB and analysed by confocal microscopy. Original magnification was 60x. Scale bar, 10 μm . Representative data of three independent experiments

DISCUSSION

Zinc is related to a number of physiological functions, and zinc deficiency has enormous consequences as described in skin diseases^{65,66}. The use of zinc-containing topical therapeutics is widespread due to its clear benefit on cutaneous regeneration and on various inflammatory skin conditions⁶¹. However, the events involved in the regulation of zinc homeostasis are complex and incompletely understood¹⁵. Studies investigating the effect of zinc supplementation show that higher zinc intake is related to lower risk of cancer development; however at extremely high doses or long term supplementation it may have opposite effect and drive cancer progression, and serum zinc level is increased in patients with metastatic melanoma. MT has a crucial role in the maintenance of the balance of intracellular labile zinc level in cells. It has a sequestering function, and serves zinc for cell biological processes. It has been demonstrated that it can protect normal cells from damage by protecting against oxidative stress, enhancing tissue repair and modulating the immune response. However, MT overexpression can also contribute to a more aggressive phenotype and therapy resistance in cancer cells, resulting in a worse prognosis^{20,92,93}. The MT proteins are produced in tissues in different isoforms. In the skin the keratinocyte basal layer constitutively expresses MT I, while the differentiating spinous layer produces MT IV⁹². MT is overexpressed in hyperplastic epidermis⁹⁴. Epidermal melanocytes, dermal fibroblasts and endothelial cells are also able to produce MT^{91,92}. Increased MT I/II protein expression is observed in keratinocyte- and melanocyte-derived tumors and it correlates with tumor invasiveness. The role of MT in metastasis formation remains to be confirmed, and experimental evidence for its oncogenic role is still lacking. On the other hand, UVB, the main harmful environmental factor in skin, and crucial risk factor of skin photocarcinogenesis, induces intracellular zinc release in keratinocytes, suggesting a functional role of zinc in the UV stress response^{72,73}. Investigating the role of zinc homeostasis under physiological circumstances and under stress conditions can give an insight into the role of zinc in the course of skin diseases, and can add new therapeutic possibilities.

METALLOTHIONEIN IS A POTENTIAL IMMUNOHISTOCHEMICAL BIOMARKER FOR CMM PROGNOSIS

In the present study, a statistically significant difference was demonstrated between nonmetastatic and metastatic CMM regarding their MT_t I/II expression, where positive immunostaining was defined as intensive MT_t I/II labelling of more than 10 % of the tumor cells. Both the cytoplasm and the nucleus of the cancer cells showed intensive staining. The whole tumor or at least the dermal part of the tumor was positively labeled; therefore, the TMA appears to be appropriate way to investigate MT_t I/II expression in CMM. It is important, because the demand on tissue sample for genetic testing determining the therapy is increasing, but also for more precise estimation of prognosis, and both procedure requires eligible amount of tumor tissue^{95,96}.

We found that there was no significant correlation between the Breslow tumor thickness and the MT_t I/II score ($R = 0.102$, $p = 0.501$), suggesting that the two parameters are independent prognostic factors. In further analyses, we found that the proportion of metastatic CMM with a tumor thickness < 3 mm (29.2 %) was increased in melanomas overexpressing MT_t I/II (45.5 %) but decreased in MT_t I/II negative melanomas (15.4 %). The proportion of metastatic tumors was much higher in CMMs with a thickness ≥ 3.0 mm (72.7 %) whereas the proportion of metastatic tumors was decreased in MT_t I/II-negative CMM (54.5 %) but increased in MT_t I/II-positive primaries (90.9 %). These results confirm the predictive value of MT I/II expression in CMM.

In addition, although both melanomas and nevi exhibited positive MT I/II immunostaining, there were discrepancies in the staining pattern. In the nevi, we observed nuclear MT I/II staining immediately below the epidermis. In contrast, intensive cytoplasmic and nuclear staining was observed in melanomas, particularly in the central and dermal region of the tissue. *In vitro* studies has shown by other workgroups, that MT proteins are localised to the cytoplasm in quiescent cells, but in the S and G₂ phases of dividing cells, MT proteins are localised to the nucleus⁷⁹. The translocation of MT to the nucleus is induced by UV-irradiation, suggesting that the zinc transporter and antioxidant functions of MT are directly required for genomic DNA repair^{91,92}. Therefore, MT I/II expression in nevi is believed to be protective, whereas in melanomas, it is associated with abnormal cell growth. Furthermore, it is possible that circulating cytokines can contribute to increased MT production in CMM

because the increased expression and nuclear translocation of MT can be observed in the basal keratinocyte layer in non-exposed skin areas when other areas are subjected to UV exposure. This phenomenon was connected to increased IL-6 blood levels produced by neutrophils upon UV irradiation ⁹¹.

We detected MT I/II⁺ cells with dendritic and spindle morphologies surrounding the dermal tumor cell nests. These cells were also observed in normal human skin samples associated with perivascular lymphocytes. The stromal localisation of MT expression has been reported in head and neck squamous cell cancers and is related to the host defence ⁷⁹. This is the first report of this phenomenon in melanoma. It is likely that both immune cells and fibroblasts were responsible for MT I/II expression in the tumor microenvironment ^{92,97}. One interesting observation is that melanophages were always negative for MT in our CMM samples. However, we have found that the presence of peritumoral MT I/II⁺ cells did not correlate with a lower or higher metastatic potential of our primary CMM samples, suggesting that these cells may represent the host response to the tumor that is not associated with an effective host defence or tumor promotion. In diffuse large B-cell lymphoma MT I/II⁺ tumor infiltrating macrophages has been observed, but only the MT I/II expression of malignant cells had impact on the clinical outcome ^{21,98}.

For the immunological characterization of our CMM samples we used dendritic cell markers such as, CD1a and DC-SIGN, macrophage markers, such as CD68 and CD163 and T-cell markers, such as CD45RO and CD8 according to ^{99,100}. We have demonstrated a linear correlation between the metastasis risk of primary CMM and the presence of tumor-infiltrating CD68⁺ and CD163⁺ macrophages as well as a correlation between the presence of peritumoral CD1a⁺ cells and a significantly lower risk for CMM metastasis. The tumor microenvironment may play an important role in melanoma progression, and it has been previously demonstrated that the presence of infiltrating macrophages within melanomas is associated with poor prognosis; these fail to induce cytotoxic T-cell response against cancer cells, and are related to tissue remodeling and neoangiogenesis ¹⁰¹. Migration of monocytes and macrophages is thought to be induced by hypoxia due to high rate proliferation of cancer ¹⁰². Recently it was also shown that tumor-infiltrating lymphocytes are able to activate these macrophages at the site of tumor and CMM metastases are even smaller in T-cell deficient mice ¹⁰³. The presence of CD1a⁺ DC infiltrating the tumor cell nests and surrounding the tumor have been reported to be associated with decreased tumor thickness and the radial growth phase of melanomas, respectively ⁹⁹. The peritumoral presence of CD1a⁺ DC in melanomas was also demonstrated in the study by ¹⁰⁴. CD1a⁺ DC are distinct from epidermal

Langerhans cells and were identified as a subset of dermal antigen-presenting cells expressing the maturation marker CD83 on their surface, thereby supporting their role in the initiation of an effective cutaneous immune response¹⁰⁵. Regarding the T cells, we found that the presence of CD8 positive cytotoxic T-cells was inversely correlated with the progression of CMM (unpublished data). The immunostaining for all of these markers were dependent on the tumor thickness.

We found that the MT_{I/II} expression of the melanoma cells was correlated with the presence of CD68⁺ macrophages infiltrating the tumor, which has not been previously reported. Macrophage infiltration of primary tumors is regulated by cytokines, growth factors and enzymes released from the tumor¹⁰⁶. Tumor derived factors can also control the function of the tumor-associated macrophages. Activation of M1 macrophages causes production of pro-inflammatory cytokines and induction of type 1 T-cell response capable of killing tumor cells (arrested tumor growth and invasion). However, during tumor progression M2-type macrophages are induced, which are thought to be the main tumor-associated macrophages. M2-type macrophages suppress the inflammatory response, impair the antigen presentation and type 1 immune responses, induce tissue remodeling, neoangiogenesis (promoted tumor growth and invasion^{107,108}). Most adult mammalian tissues contain low basal levels of MT I/II²⁰ and MT knock out mice develop normally without any phenotypical changes¹⁰⁹. However, it is well known that inflammatory stimuli can upregulate the expression of MT I/II⁷⁹, and in various types of CNS pathology MT knock out animals show enhanced inflammatory response, oxidative stress and tissue damage¹¹⁰. It has been demonstrated that in animal model of brain injury, during stress conditions, MT I/II is produced mainly by reactive astrocytes and the elevation of MT expression level in tissue may act on the inflammatory microenvironment suppressing IL-6 and TNF- α production of macrophages, decreasing apoptotic cell death in neurons and oligodendrocytes, and enhancing tissue repair¹¹¹⁻¹¹⁴. In addition, during endotoxin tolerance M2-like polarization has been found, which was connected with upregulated MT gene expression¹¹⁵. Interestingly, it has also been shown that extracellular MT released from cells might interact with the plasma membrane of immune cells, including lymphocytes and macrophages, resulting in a change in the immune functioning, e.g., although an increase in the number of immature T-cells can be observed, extracellular MT decreases the differentiation of immature T-cells to the effector cytotoxic T-lymphocyte stage thereby contributing to the suppression of cell-mediated immunity¹¹⁶. Furthermore, under inflammatory conditions, extracellular MT seems to operate as a chemotactic factor that governs the trafficking of inflammatory cells¹¹⁷. These findings

suggest that MT might play a role in modulation of immune microenvironment and support that MT over-expression in melanoma might be associated with the macrophage infiltration and tumor promoting microenvironment (**Figure 17.**).

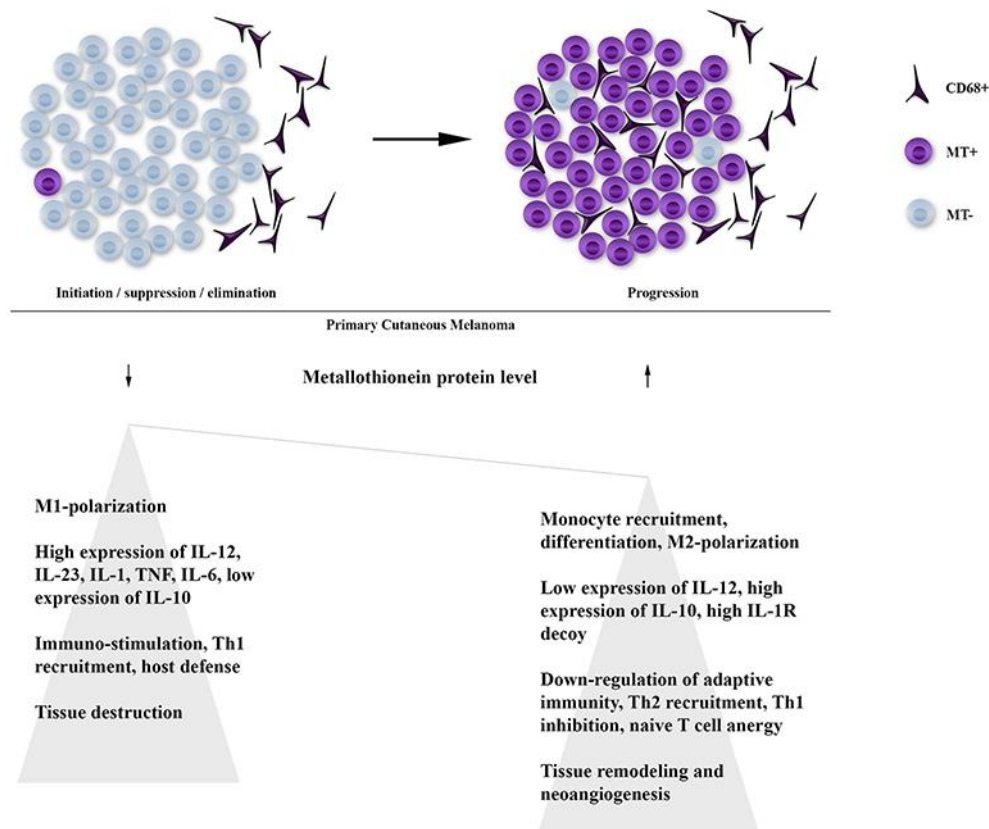


Figure 17. Proposed model of melanoma progression considering the tumoral MT I+II expression and tumor-associated macrophages

Tumoral MT I+II overexpression is associated with higher risk of vascular invasion of CMM and seems to correlate with the presence of prognostic tumor infiltrating CD68+ macrophages. Considering our results and the function of MT^{112,115,118} with the characteristics of the melanoma tumor microenvironment^{107,108} it is assumed that MT has immuno-modulating role in CMM progression. It is proposed that MT can contribute to the switch from M1 macrophage activation towards M2-like phenotype which is then able to promote metastasis by suppressing the anti-tumor immune response, enhancing the tissue remodeling and neoangiogenesis.

Our findings have been cited in a recently published review article as a confirmation of the prognostic value of MT I/II expression level in CMM¹¹⁹. Moreover, another recent study showed that although there is a low MT III expression in normal skin, significant increase in

its level can be detected in SCC ¹²⁰. All these results underline the importance of further investigations on the role of MT proteins in skin cancer development and progression.

METALLOTHIONEIN EXPRESSION IN HACAT KERATINOCYTES

MT is a small protein with 6-7 kDa molecular weight and high cysteine content. It has important role in intracellular zinc sequestration and through this, it is involved in the zinc dependent cell biological processes. Extracellular zinc is thought to enter the cell through plasma membrane zinc importers and then is transported via a muffler with high affinity for zinc (e.g., MT) to intracellular storage sites such as the endoplasmic reticulum ¹²¹. Passive diffusion of zinc across cell membranes is not possible ¹²². Bozym et al. investigated the effect of cell culture medium on the intracellular Zn (II) concentration and found that both the type of cell and the medium influence the intracellular free Zn (II) level during exposure to a certain concentration of Zn (II) and that the toxicity of supplemented Zn (II) must be carefully investigated ¹²³. Previous experiments have shown that the exposure of HaCaT keratinocytes to 100 μ M ZnCl₂ was non-toxic to the skin cells ¹²⁴, and we have not found this concentration to be toxic in our experimental conditions. Based on the calculations of Bozym et al., 100 μ M Zn (II) treatment leads to an approximate 100-nM increase of intracellular Zn (II) ¹²³.

To get a deeper insight into cell biological functions of zinc in human HaCaT keratinocytes, we studied the transcriptional response in cells exposed to nontoxic concentration of ZnCl₂. We also investigated the mRNA expression of MT isoforms. More than 10 functional MT isoforms are present in humans and are classified into four groups; we analysed the mRNA expression of nine of the MT isoforms from the MT I/II groups in keratinocytes in our experiments ¹²⁵. At a baseline level we detected expression of 4 MT-isoforms in HaCaT cells (MT2A>MT1X>MT1E>MT1F). There are only a few studies concerning MT expression in keratinocytes, Lim et al. also have found MT2A to be the most abundant MT-isoform ¹²⁵. MT expression seems to be oscillating during cell cycle and nuclear translocation of MT can be observed during early S-phase ¹¹. The fact that isoforms of MT1 are encoded by different genes ¹¹ and results of the few studies demonstrating prognostically relevant expressional change of distinct MT1 isoforms in cancers ^{20,80} together with our results suggest that the isoforms have specific regulatory functions and it is still little known so far. Zn (II) treatment significantly increased the mRNA expression of all four MT isoforms and SLC30A1. Transcriptional change of MT1F, 1X and 1E was superior to the change of MT2A showing that expression of these are more sensitive to change of intracellular zinc concentration. Specific antibodies against the MT protein isoforms are not available;

therefore, a composite of MTI/II can only be investigated at the protein level. Western blot analysis demonstrated that Zn (II) treatment induced the production of MTI/II protein in a time-dependent manner. In addition, we investigated the cellular localisation of MTs and found that MTs were present mostly in the nuclei of control cells, suggesting a function in defence of genome and/or cell growth ¹¹. It is consistent with our immunohistochemical results demonstrating nuclear MT immunolabelling in nevi. However after 24 h of treatment with Zn (II), we detected high amounts of MTs in the cytoplasm of the cells, and little or no fluorescence staining was detected in the nucleus. The variability of the localisation of MTs upon zinc exposure may reflect the expression of distinct MT isoforms. Unfortunately, specific antibodies against MT isoforms are not available; a composite of MTI/II can only be investigated at a protein level. Notably, in skin biopsy samples from healthy donors the proliferative layer of the epidermis shows high MT expression, which is present in the nucleus as well in the cytosol ⁹¹. Therefore, it must be considered that in in vitro cell cultures the zinc concentration might be lower than in in vivo circumstances, and our investigations are not far from that is feasible physiologically. Interestingly, in aged skin characterized by decreased epidermal renewal the expression of MT is lower ¹²⁶.

THE EFFECTS OF ZINC ON THE FUNCTIONS OF HUMAN HACAT KERATINOCYTES

In the present study we found a significant proliferation advantage upon 100 μ M ZnCl₂ exposure, in accordance with a previous study ¹²⁴. We also tested the effect of 50 μ M ZnCl₂ treatment, but the change in proliferation was not significant. Although the effects of zinc on intracellular signalling events are not completely known, a positive impact of Zn (II) on cell proliferation might be attributed, at least in part, to the inhibition of phosphatases, leading to augmented protein tyrosine phosphorylation ¹²⁷. The proliferation advantage by Zn (II) treatment can also be a consequence of increased calcium uptake ¹²⁸ or up-regulated gene expression (e.g., NOTCH1) ^{129,130}. In addition, as mentioned above, in aged skin, which is characterised by decreased epidermal renewal, the expression of MT is lower ¹²⁶, while the proliferative layer of the epidermis shows high MT expression. Moreover, our finding that

MT is overexpressed in CMM with poor prognosis suggests a role of MT in hyperproliferative capacity of cells.

Consequently from our results zinc homeostasis can be involved in immune regulatory process of keratinocytes. We found significant decrease of COX2, IL8 gene expression compared to control. In human skin, COX-2 expression has been shown to be elevated in human actinic keratoses, SCCs and BCCs and in mouse models, COX-2 is overexpressed in hyperplastic skin, benign papillomas and in SCCs exposed chronic UV light. COX-2 can lead to the production of ROS and contribute to a pro-oxidant state in combination with the release of ROS from activated inflammatory cells. In addition, melanin, which is produced by melanocytes and distributed to the surrounding keratinocytes to protect them against the hazardous UV effects, can also act as a photosensitizer, and therefore related to ROS induction¹³¹.

The mechanism of action of zinc impact on cell signalling is not well understood¹²¹; herein, we found that zinc might affect ROS-sensitive signalling pathways. This is the first report demonstrating high expression of HMOX1 in cultured human keratinocytes upon nontoxic Zn (II) exposure. The HMOX1 gene encodes an important member of the phase-II enzymes, which have cytoprotective roles in ROS scavenging. Furthermore, HMOX1 was shown to prevent cell death and to have a role in the regulation of inflammation¹³²⁻¹³⁴. Nontoxic generation of $O_2^{\bullet-}$ with subsequent H_2O_2 release was detected during nontoxic Zn (II) exposure and could explain the HMOX1 expression. Although Zn (II) itself is reported to be an inducer of oxidative stress by promoting mitochondrial and extra-mitochondrial production of ROS⁷², the generation of ROS at a nontoxic level upon Zn (II) exposure/release has not been described. Nevertheless, $O_2^{\bullet-}$ -producing NAD(P)H oxidases are activated in response to growth factors and oncogenes, and enhanced cell proliferation was observed upon ROS-inducing, low-dose photodynamic treatment¹³⁵⁻¹³⁹. Therefore, simultaneous increases in cell proliferation and ROS levels are not conflicting. In addition, NADPH oxidase mediated the induction of nuclear factor-kappa B (NFκB) and HMOX1 in human colon cancer cells¹⁴⁰ without inducing cell death. Furthermore, the immune response can be redox-regulated¹⁴¹, and we have observed down-regulation of some pro-inflammatory mediators, such as IL8 and PTGS2, which are partly regulated by redox-sensitive transcription factors (e.g., AP-1)¹⁴².

BIOLOGICAL ROLE OF ZINC IN UVB INDUCED STRESS

RESPONSE IN HUMAN SKIN HACAT KERATINOCYTE

CELL MODEL

UVB exposure is the primary risk factor in skin-tumor development¹⁴³. A role of zinc in the mechanism of UVB-induced cell death has already been proposed. Stork et al.⁷² described that the elevation of intracellular zinc levels after UVB irradiation is proportional to the fraction of dying or dead cells, and they concluded that UVB-induced zinc release may be an important step in UVB-induced cell death pathways. The consequences of UVB-induced direct (primarily CPDs) and indirect (free radical generation) DNA damage are cell cycle arrest, DNA repair, and the induction of cell death pathways causing sunburn, inflammation, immunosuppression, and melanogenesis in the skin¹. We measured both the CPD level and superoxide generation. Due to DNA repair processes, the amount of CPDs was decreased with time, and cells with unrepaired DNA damage were eliminated by apoptosis. Similar to the results of Saito et al., pre-treatment of cells with Zn (II) for 24 h was not sufficient to improve cell survival, although the level of induced CPD was lower in these cells 3 h after UVB irradiation¹⁴⁴. We assume that MT could exert some effect on CPD formation because partial translocation of MTs to the nucleus was observed 3 h after UVB irradiation. Nevertheless, when further analysing cell death at 24 h post-UVB irradiation, when UVB-induced apoptosis is maximal¹⁴⁵, we found that the fraction of early apoptotic cells decreased, while the fraction of late apoptotic plus necrotic cells increased in Zn (II) pre-treated cells. In the context of our results, the elevation of intracellular zinc levels after UVB irradiation that was described by Stork et al. was proportional to the fraction of dying or dead cells⁷². When investigating the course of ROS production, the increase in superoxide production upon UVB treatment occurred at a maximum of 10 h, but unexpectedly, this was augmented by Zn (II) pre-exposure. It has recently been described that a vicious circle of ROS-induced zinc release and zinc-driven mitochondrial ROS production is an important neuronal cell death mechanism¹⁴⁶. On the other hand, it has been reported that the induction of a type of cell death other than apoptosis in cancer cells might increase the immunogenic potential of cell death^{147,148}. Whether a change in the death process upon Zn (II) pre-exposure can affect the immune response to UV-damaged cells, which would impact the development of various pathologies, e.g., autoimmunity or cancers, must be further studied.

The mRNA levels of MT1E and MTF1 were strongly suppressed by UVB at 6 h after irradiation. Epigenetic down-regulation of MT1E has been demonstrated in malignant melanoma, suggesting that MT1E exhibits tumor-suppressor function⁸⁰. Only a small fraction of genes showed altered expression upon zinc pre-treatment at 6 h post-UVB irradiation. Interestingly, expression of HIPK2, which is involved in regulatory phosphorylation of key molecules such as p53¹⁴⁹, showed the biggest change in gene expression after combined zinc and UVB exposure. Furthermore, the generated ROS can modulate the selective transactivation of p53 target genes¹⁵⁰.

To summarize the above, CMM is a skin malignancy with high risk of metastasis. There are novel treatment modalities for metastatic cases such as targeted therapies that are effective, but only for short periods, and immune checkpoint inhibitors that are effective, but only in a small minority of patients. There is a need for such prognostic and predictive factors that are independent of routinely used prognostic clinicopathological parameters and allow personalized risk definition and treatment. Herein, we found that MTI/II expression in primary CMM tissue samples correlated with the risk of tumor progression, and that the level of MTI/II expression was independent of other prognostic clinicopathological parameters. Furthermore, the presence of tumor-infiltrating macrophages, which is connected to regulatory T-cell response, T-cell anergy, angiogenesis and tumor progression showed correlation with MTI/II expression. In addition, we proved that a cost- and tissue-saving technology, the tissue microarray is suitable for examination of MTI/II expression in CMM and the proposed location of the sampling is the dermal tumor area. Besides metal detoxification, MT can release/bind zinc with high affinity influencing the function of transcription factors and enzymes. In *in vitro* experiments we found that intracellular Zn(II) levels can influence the proliferation rate and gene expression in skin keratinocytes. Importantly, we observed a link between changes in the levels of zinc and ROS suggesting a possible link between zinc and redox signalling. Moreover, under stressed conditions using UVB irradiation, which is the main environmental harmful factor to skin, we showed that zinc might be involved in a feedforward regulatory loop triggering accumulation of ROS in response to UVB-induced cell damage and switching between cell death pathways. Our findings suggest that Zn(II) is essential in fine-tuned orchestration of basic cell functions such as proliferation, gene expression and stress response. It might explain why we can detect changes in the expression of zinc homeostatic proteins in cancer, and calls for further investigations in this field.

SUMMARY

The purpose of our study was to increase our knowledge on pathogenesis of skin disorders regarding the role of zinc homeostasis and UVB-induced DNA damage response.

Comparison of the MT I/II protein expression of primary CMM tissue samples without metastases to samples with haematogenous metastases:

We found statistically significant difference between nonmetastatic and metastatic CMM regarding their MT I/II expression. The MT I/II expression did not correlate with the Breslow tumor thickness i.e. it seems to be an independent prognostic factor. In addition, this was the first study to investigate correlation between MT I/II expression and composition of tumor immune microenvironment in CMM. We found that MT I/II expression of the melanoma cells was correlated with the presence of CD68⁺ macrophages infiltrating the tumor.

Investigation of the effect of non-toxic Zn (II) exposure on human epidermal keratinocytes under physiological circumstances and stress conditions:

In our study non-toxic elevation in the concentration of extracellular zinc facilitated cell proliferation. This is the first report that this concentration of zinc increased HMOX1 expression and caused non-toxic generation of superoxide in skin keratinocytes. Furthermore, regarding the effects on the UVB-induced toxicity, we could show that although the level of cyclobutane pyrimidine dimers in the keratinocytes pre-treated with zinc was reduced at early time point after UVB irradiation, significantly enhanced superoxide generation was observed later after UVB exposure in the zinc pre-exposed cells. The overall survival was unaffected; however, there was a decrease in the percentage of early apoptotic cells and an increase in the percentage of late apoptotic plus necrotic cells.

In conclusion, by these observations we can assume that MT, and through this, zinc dyshomeostasis might contribute to melanoma progression. Regarding non-toxic zinc exposure, the observed changes in ROS might be attributed to the molecular effects of Zn (II) interactions with cysteinyl thiols, which alters protein functionality and thereby their reactivity and participation in redox reactions. It can affect signal transduction pathways, which further may influence the behaviour of the cell upon stress conditions. Our observations affect our thinking about the pathogenesis of skin disorders and pay attention to further examine the role of the zinc homeostasis in skin.

MAGYAR NYELVŰ ÖSSZEFOGLALÓ

A doktori disszertáció célja az volt, hogy a cink homeosztázis és UVB okozta DNS károsodásra adott válasz szempontjából adjon betekintést a bőrbetegségek patogenezisébe.

MT I/II fehérje kifejeződésének összehasonlítása az áttétet nem adó és hematogén áttétet adó primer kután malignus melanoma (KMM) szöveti mintákban:

Statisztikailag szignifikáns különbséget találtunk a nem áttétes és metasztatikus CMM között a tumorális MT I/II expressziót tekintve, ami a Breslow tumor vastagságtól független prognosztikai faktornak bizonyult. Ezen kívül, elsőként vizsgáltuk az összefüggést a tumor MT I/II expressziója és a tumor immun mikrokörnyezet összetétele között. Azt találtuk, hogy a MT I/II expressziót mutató melanoma sejtek jelenléte korrelált a daganatot infiltráló CD68+ makrofágok jelenlétével.

Nem-toxikus Zn (II) expozíció hatásának vizsgálata humán epidermális keratinocitákban fiziológias és stressz körülmények között:

Vizsgálatainkban a sejtproliferációt fokozta a nem-toxikus cink expozíció. Elsőként írtuk le, hogy ezzel egyidőben toxicitást nem okozó mértékben, de megemelkedik a szuperoxidszint a bőr keratinocitákban és, valószínűleg ezzel összefüggésben, fokozódik a HMOX1 expresszió. Továbbá kimutattuk, hogy bár a cinkkel előkezelt keratinocitákban az UVB besugárzás utáni korai időpontban kevesebb az UVB-indukálta ciklobután pirimidin dimerek mennyisége a kontrollhoz képest, jelentősen nagyobb mértékű a szuperoxid termelődés az UVB expozíció utáni későbbi időpontban. Nem találtunk eltérést az UVB-irradiációt követően 24 órával mért teljes sejttúlélés tekintetében a cinkkel előkezelt és a kontroll sejtek között, azonban a korai apoptotikus sejtek aránya csökkent, míg a késői apoptotikus/nekrotikus sejtek aránya növekedett a cink előkezelés hatására.

Összefoglalva, eredményeink arra utalnak, hogy a megváltozott MT expresszió, és ezen keresztül a cink diszhomeosztázis hozzájárulhat a melanoma progressziójához. A nem-toxikus cink expozíció során egy valószínű Zn (II) és cistein-tiol molekuláris interakció következtében megváltozhat fehérjék funkcionalitása, ezáltal reakciókészsége és redox reakciókban való részvétele, ami ROS képződéshez vezet. Ez hatással lehet további szignál transzdukciós útvonalakra, és megváltoztathatja a sejtek stresszre kiváltott viselkedését. Megfigyeléseink befolyásolják a bőrbetegségek patogenezisééről alkotott felfogásunkat, s felhívják a figyelmet a cink homeosztázis szerepének további vizsgálatára a bőrben.

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PUBLICATIONS



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Ph.D. List of Publications

Candidate: Eszter Emri

Neptun ID: SBIFQ6

Doctoral School: Doctoral School of Health Sciences

List of publications related to the dissertation

1. **Emri, E.**, Mikó, E., Bai, P., Boros, G., Nagy, G., Rózsa, D., Juhász, T., Hegedűs, C., Horkay, I., Remenyik, É., Emri, G.: Effects of non-toxic zinc exposure on human epidermal keratinocytes.
Metallomics. 7 (3), 499-507, 2015.
DOI: <http://dx.doi.org/10.1039/C4MT00287C>
IF:3.585 (2014)
2. **Emri, E.**, Egervári, K., Várvolgyi, T., Rózsa, D., Mikó, E., Dezső, B., Veres, I., Méhes, G., Emri, G., Remenyik, É.: Correlation among metallothionein expression, intratumoural macrophage infiltration and the risk of metastasis in human cutaneous malignant melanoma.
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List of other publications

3. Boros, G., Mikó, E., Muramatsu, H., Weissman, D., **Emri, E.**, van der Horst, G.T.J., Szegedi, A., Horkay, I., Emri, G., Karikó, K., Remenyik, É.: Identification of Cyclobutane Pyrimidine Dimer-Responsive Genes Using UVB-Irradiated Human Keratinocytes Transfected with In Vitro-Synthesized Photolyase mRNA.
PLoS One. 10 (6), e0131141-, 2015.
DOI: <http://dx.doi.org/10.1371/journal.pone.0131141>
IF:3.234 (2014)
4. Boros, G., Mikó, E., Muramatsu, H., Weissman, D., **Emri, E.**, Rózsa, D., Nagy, G., Juhász, A., Juhász, I., van der Horst, G., Horkay, I., Remenyik, É., Karikó, K., Emri, G.: Transfection of pseudouridine-modified mRNA encoding CPD-photolyase leads to repair of DNA damage in human keratinocytes: A new approach with future therapeutic potential.
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DOI: <http://dx.doi.org/10.1016/j.jphotobiol.2013.09.010>
IF:2.803

Total IF of journals (all publications): 12,727

Total IF of journals (publications related to the dissertation): 6,69

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

31 August, 2015



International presentations related to the dissertation:

E Emri: Correlation among metallothionein expression, intratumoral macrophage infiltration and the risk of metastasis in human cutaneous malignant melanoma. 44th ESDR Annual ESDR Meeting, Copenhagen, Denmark, 10-13 September 2014 *oral presentation*

E Emri, E A Janka, G Boros, L Beke, Cs Hegedus, B Nagy, G Mehes, E Remenyik, G Emri: Correlation among anti-tumor T cell response and the redox homeostasis in human cutaneous malignant melanoma. 44th ESDR Annual ESDR Meeting, Copenhagen, Denmark, 2014; J Invest Dermatol 134: S90-S97 *poster presentation*

DOI:10.1038/jid.2014.350

E Emri, E Miko, E Remenyik, G Emri: Zinc chloride exposure modulates cytokine expression and induces heme oxygenase-1 in human keratinocytes. International Investigative Dermatology Edinburgh, Scotland 2013; J Invest Dermatol 133: S191-S208 *poster presentation* **DOI:10.1038/jid.2013.103**

E Emri, G Emri, K Egervari, I Veres, G Mehes, E Remenyik: Prognostic role of CD68, CD163 and CD1a expression in human primary cutaneous malignant melanoma. 75th Anniversary of Albert Szent-györgyi's Nobel Prize Award, 22-25 March, 2012. *oral presentation*

E Emri, E Miko, G Nagy, G Boros, D Rozsa, G Mocsai, G Emri, E Remenyik: Modulating effect of zinc on cutaneous UVB-response. 42th ESDR Annual ESDR Meeting, Venice, Italy, 2012; J Invest Dermatol 132: S118-S123 *poster presentation*

DOI:10.1038/jid.2012.306

E Emri, G Emri, K Egervari, L Beke, T Varvolgyi, I Veres, G Mehes, I Horkay, E Remenyik: Immunohistochemical characterization of metallothionein expression in primary cutaneous

malignant melanoma. 41th ESDR Annual ESDR Meeting, Barcelona, Spain, 2011; J Invest Dermatol 131: S1-S115 *poster presentation*

DOI:10.1038/jid.2011.214

G Emri, T Varvolgyi, A Begany, Z Hargitay, L Toth, **E Emri**, E Remenyik: Immunohistochemical markers as prognostic factors in primary cutaneous malignant melanoma. 39th Annual ESDR Meeting, Budapest, Hungary, 2009; J Invest Dermatol 129: S1-S103 *poster presentation*

DOI:10.1038/jid.2009.232

National oral presentations related to the dissertation:

Emri E, Janka E A, Boros G, Beke L, Hegedűs Cs, Méhes G, Remenyik É, Emri G: A tumor-ellenes T-sejt válasz és a redox homeosztázis közötti összefüggés után melanoma malignumban. Magyar Dermatológiai társulat 87. nagygyűlése, Budapest, Hungary; 27 November 2014. *oral presentation*

Emri E, Miko E, Boros G, Nagy G, Bai P, Mócsai G, Rózsa D, Hegedűs Cs, Remenyik É, Emri G: A cink homeosztázis befolyásolja a citokin-expressziót és hem-oxigenázt indukál humán keratinocitákban. Magyar Dermatológiai társulat 86. nagygyűlése, Budapest, Hungary; 12-14 December 2013. *oral presentation*

Emri E, Miko E, Nagy G, Boros G, Rózsa D, Mócsai G, Remenyik É, Emri G: A cink UVB sugárzás utáni sejtválaszra gyakorolt hatása keratinocitákban. Magyar Dermatológiai társulat 85. nagygyűlése, Budapest, Hungary; 6-8 December 2012. *oral presentation*

Emri E, Emri G, Egervári K, Beke L, Rózsa D, Miko E, Boros G, Nagy G, Várvolgyi T, Veress I, Méhes G, Remenyik É: A metallothionein-expresszió melanomában: tumorbiológiával és immunválasszal összefüggést mutató független biomarker. Magyar Dermatológiai társulat 84. nagygyűlése, Budapest, Hungary; 8-10 December 2011. *oral presentation*

Other presentations:

G Boros, E Miko, H Muramatsu, D Weissman, **E Emri**, G van der Horst, I Horkay, K Karikó, G Emri and E Remenyik: Effect of cyclobutane pyrimidine dimer on gene expression is mediated by activation of c-Jun kinase in UVB irradiated human keratinocytes. 44th ESDR Annual ESDR Meeting, Copenhagen, Denmark, 2014; J Invest Dermatol 134: S83-S89 *poster presentation*

DOI:10.1038/jid.2014.349

Cs Hegedűs, G Boros, **E Emri**, E Mikó, K Karikó, G Emri, P Bai, É Remenyik: The implication of mitochondria in the UVB-driven pathways, 44th Annual ESDR Meeting Copenhagen, Denmark, 2014; J Invest Dermatol 134: S83-S89 *poster presentation*

DOI:10.1038/jid.2014.349

G Boros, E Miko, **E Emri**, G van der Horst, H Muramatsu, D Weissman, I Horkay, G Emri, K Kariko, E Remenyik. 43rd Annual ESDR Meeting. Edinburgh, Scotland, 2013; J of Invest Dermatol 133: S209–S221 *poster presentation*

DOI:10.1038/jid.2013.104

G Boros, E Miko, **E Emri**, G van der Horst, H Muramatsu, D Weissman, I Horkay, G Emri, K Kariko, E Remenyik: CPD-dependent gene expression changes in photolyase mRNA-transfected human keratinocytes irradiated by UVB. 42th ESDR Annual ESDR Meeting, Venice, Italy, 2012; J Invest Dermatol 132: S118-S123 *poster presentation*

DOI:10.1038/jid.2012.306

G Boros, D Rozsa, **E Emri**, G Nagy, E Miko, A Juhasz, I Juhasz, G van der Horst, H Muramatsu, D Weissman, K Kariko, G Emri, E Remenyik, I Horkay: Enhanced repair of UVB-induced DNA lesions in photolyase mRNA-transfected keratinocytes. 41th ESDR Annual ESDR Meeting, Barcelona, Spain, 2011; J Invest Dermatol 131: S1-S115 *poster presentation*

DOI:10.1038/jid.2011.214

KEYWORDS

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metallothionein

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KULCSSZAVAK

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APPENDIX

Original publications related to the dissertation.