Ph.D. Thesis

The cutaneous redox system as a driver of skin pigmentation and skin cancer risk

Elisabeth Roider, M.D.

Supervisor: István Németh, M.D., Ph.D.

Department of Dermatology and Allergology
Doctoral School of Clinical Medicine
University of Szeged

Szeged
2021

Original publications related to the thesis

1. Allouche J[*], Rachmin I[*], Adhikari K, Pardo LM, Lee JH, McConnell A, Kato S, Fan S, Kawakami A, Suita Y, Wakamatsu K, Igras V, Zhang J, Navarro PP, Makhlouta Lugo C, Noonan H,. Christie KA, Itin K, Mujahid N, Lo JA, Won CH, Evans CL, Weng QY, Wang H, Osseiran S, Lovas A, Németh I, Cozzio A, Navarini AA, Hsiao J, Nguyen N, Kemeny LV, Iliopoulos O, Berking C, Ruzicka T, Gonzalez-José R, Bortolini MC, Canizales-Quinteros S, Acuna-Alonso V, Gallo C, Poletti G, Bedoya G, Rothhammer G, Ito S, Schiaffino MV, Chao LH, Kleinstiver BP, Tishkoff S, Zon L, Nijsten T, Ruiz-Linares A, Fisher DE[**], **Roider E**[**#]. NNT mediates redox-dependent pigmentation via a UVB- and MITF-independent mechanism. **Cell.** 2021. Jun 29;S0092-8674(21)00757-1. Online ahead of print. ([*, **] shared, [#] lead author), **Impact Factor (IF) 38.6**
2. **Roider E,** Adhikari K, Kempter F, Kemeny L, Nemeth I. Identification of ACSL4 as a prognostic marker for melanoma survival. *Manuscript in preparation.*

Original publications not related to the thesis

1. Wang P, **Roider E**, Coulter ME, Walsh CA, Kramer CS, Beuning PJ, Giese RW. DNA Adductomics by Mass Tag Prelabeling. **Rapid Commun Mass Spectrom**. 2021 Apr 6:e9095. *Online ahead of print.* **IF 2.2**
2. Lo JA, Kawakubo M, Juneja VR, Su MY, Erlich TH, LaFleur MW, Kemeny LV, Rashid M, Malehmir M, Rabi SA, Raghavan R, Allouche J, Kasumova G, Frederick DT, Pauken KE, Weng QY, Pereira da Silva M, Xu Y, van der Sande AAJ, Silkworth W, **Roider E**, Browne EP, Lieb DJ, Wang B, Garraway LA, Wu CJ, Flaherty KT, Brinckerhoff CE, Mullins DW, Adams DJ, Hacohen N, Hoang MP, Boland GM, Freeman GJ, Sharpe AH, Manstein D, Fisher DE. Epitope spreading toward wild-type melanocyte-lineage antigens rescues suboptimal immune checkpoint blockade responses. **Sci Transl Med.** 2021 Feb 17;13(581):eabd8636. **IF 16.3**
3. Jamiolkowski D, Mühleisen B, Müller S, Navarini A, Tzankov A, **Roider E.** SARS-CoV-2 PCR testing of skin for COVID-19 diagnostics: a case report. **Lancet.** 2020 August; 396(10251):598-599. **IF 60.3**
4. Maul LV, Vasconcelos Berg R, Navarini AA, Rümmelein B, **Roider E**. Komplikationsvermeidung bei Laserbehandlungen. **ästhet dermatol kosmetol.** 2020 August; 12 (4):32-39. **IF 1.6**

5. Wang P, Shah GL, Landau H, Coulter ME, Walsh CA, **Roider E**, Kramer CS, Beuning PJ, Giese RW. Jettison-MS of Nucleic Acid Species. **J Am Soc Mass Spectrom.** 2020 Jul 9. doi:10.1021/jasms.0c00084. **IF 3.2**
6. Vukovic S, Anagnostopoulos A, Zbinden R, Schönenberger L, Guillod C, French LE, Navarini A, **Roider E**. Painful lymphadenopathy after an insect bite-a case report. **Hautarzt.** 2019 Jan;70(1):47-50. **IF 0.7**
7. **Roider E**, Signer C, Fehrenbacher B, Metzler G, Schaller M, Kamarachev J, Kerl K, Balabanov S, Jochum W, Hoetzenecker W, Cozzio A, French LE, Dummer R, Guenova E. Individualized Treatment Approaches for Langerhans Cell Histiocytosis**. Br J Dermatol.** 2018 Jun;178(6):1423-1424. **IF 7.0**
8. Sam Osseiran S, **Roider EM**, Wang H, Suita Y, Murphy M, Fisher DE, Evans CL. Non-Euclidean phasor analysis for quantification of oxidative stress in ex vivo human skin exposed to sun filters using fluorescence lifetime imaging microscopy**. J Biomed Opt.** 2017 Dec; 22(12):1-10. **IF 2.8**
9. Viswanathan VD, Ryan MJ, Dhruv HD, Gill S, Eichhoff OM, Seashore-Ludlow B, Kaffenberger SD, Eaton JK, Shimada K, Aguirre AJ, Viswanathan SR, Chattopadhyay S, Tamayo PS, Yang WS, Rees MG, Chen S, Boskovic ZV, Javaid S, Huang C, Wu X, Tseng YY, **Roider EM**, Gao D, Cleary JM, Wolpin BM, Mesirov JP, Haber DA, Engelman JA, Boehm JS, Kotz JD, Hon C, Chen Y, Hahn WC, Levesque MP, Doench JG, Berens ME, Shamji AF, Clemons PA, Stockwell BR, Schreiber SL. Dependency of a therapy-resistant state of cancer cells on a lipid peroxidase pathway. **Nature.** 2017 Jul 27;547(7664):453-457. **IF 42.8**
10. Mujahid N, Liang Y, Murakami R, Choi HG, Dobry AS, Wang J, Suita Y, Weng QY, Allouche J, Kemeny LV, Hermann AL, **Roider E**, Gray NS, Fisher DE. A UV independent topical small molecule approach for melanin production in human skin. Cell Rep. 2017 Jun 13;19(11):2177-2184. **IF 8.1**
11. Laviolette LA, Mermoud J, Calvo IA, Olson N, Boukhali M, Steinlein O, **Roider E**, Sattler EC, Huang D, The BT, Motamedi M, Haas W, Iliopoulos O. Negative Regulation of EGFR Signaling by the Human Folliculin Tumor Suppressor Protein. Nat Commun. 2017 Jun 28;8:15866. **IF 12.1**
12. Wang H, Osseiran S, Igras V, Nichols AJ, **Roider EM**, Pruessner J, Tsao H, Fisher DE, Evans CL. In vivo coherent Raman imaging of the melanomagenesis-associated pigment pheomelanin. **Sci Rep.** 2016 Nov;6:37986. **IF 3.9**
13. **Roider EM** und Fisher DE. Red hair, light skin, and UV-independent risk for melanoma development in humans. **JAMA Dermatol.** 2016 Jul;152(7):751-3. **IF 7.7**

14. Saulite I, **Roider E**, Darlenksi R, Jalili A, Guenova E. Cutaneous oncology: from research to diagnosis and management. **Biomed Res Int.** 2016 Jun 2; 2016:7819428. **IF 2.6**

15. Tateishi K, Wakimoto H, Iafrate AJ, Tanaka S, Loebel F, Lelic N, Wiederschain D, Bedel O, Deng G, Zhang B, He T, Robert GE, Zhang Y, Yeh JJ, Curry WT, Zhao D, Sundaram S, Nigim F, Koerner MVA, Ho Q, Fisher DE, **Roider EM**, Kemeny LV, Samuels Y, Flaherty KT, Batchelor TT, Chi AS, Cahill DP. Extreme vulnerability of IDH1 mutant cancers to NAD+ depletion. **Cancer Cell.** 2015 Dec; 28(6):773-84. **IF 26.6**

16. **Roider EM**, Fisher DE. The Impact of Pheomelanin On Melanoma Formation. **The Melanoma Letter.** 2015 Oct; 33(2)1-5. **IF 2.8**

17. Horváth ON, Borovaya A, **Roider E**, Klose J, Hartlieb E, Waschke J, Ruzicka T, Sárdy M. Successful Methotrexate Treatment of Oesophageal Pemphigus Vulgaris in an Immunosuppressed Patient with Crohn's Disease. **Acta Derm Venereol.** 2015 Sep; 95(7):868-9. **IF 4.0**

18. **Roider E**, Gauglitz G, Flaig MJ, Ruzicka T, Schauber J. Massive granuloma formation after long-term use of dermal fillers. Hautarzt. 2015 Aug;66(8):620-624. **IF 0.6**

19. **Roider EM**, Fisher DE. The Impact of MITF on Melanoma Development: News from Bench and Bedside. **J Invest Dermatol.** 2014 Jan;134(1):16-17. **IF 7.1**

20. Porto AC, **Roider E**, Ruzicka T. Cowden Syndrome: report of a case and brief review of literature. **An Bras Dermatol.** 2013 Nov-Dec;88:52-55. **IF 1.2**

21. **Roider E**, Ruzicka T, Schauber J. Vitamin D, the cutaneous barrier, antimicrobial peptides and allergies: is there a link? **Allergy Asthma Immunol Res.** 2013 May;5(3):119-128. **IF 5.0**

22. **Roider E,** Schneider J, Flaig MJ, Ruzicka T, Kunte C, Berking C. Hypopigmentation in the sites of regressed melanoma metastases after successful dacarbazine therapy. **Int J Dermatol.** 2012 Sep;51(9):1142-1144. **IF 1.8**

23. **Roider E**, Panthel K, Köhn K, Jellbauer S, Berchtold C, Partilla M, Busch DH, Rüssmann H. Invasion and destruction of a murine fibrosarcoma by Salmonella-induced effector CD8 T cells as a therapeutic intervention against cancer. **Cancer Immunol Immunother.** 2011 Mar;60(3):371-380. **IF 5.4**

24. Berchtold C, Panthel K, Jellbauer S, Köhn B, **Roider E**, Partilla M, Bourquin C, Endres S, Rüssmann H. Superior protective immunity against murine listeriosis by combined vaccination with CpG DNA and recombinant *Salmonella enterica* serovar typhimurium. **Infect Immun.** 2009 Dec;77(12):5501-5508. **IF 3.3**

1 Introduction

Pigmentation of human skin, which confers protection against skin cancer, evolved over one million years ago in the setting of evolutionary loss of body hair (Jablonski & Chaplin, 2017). Human skin color results from the relative amounts of yellow-orange pheomelanin and black-brown eumelanin (Del Bino et al., 2015). Darker pigmented individuals are more protected from, oncogenic UV radiation by the light scattering and antioxidant properties of eumelanin (Jablonski & Chaplin, 2012). Pigment dictates how light is absorbed and disseminated in skin (Pathak et al., 1962). UV can interact photochemically with DNA to form cyclobutane pyrimidine dimers (CPD) and 6,4-photoproducts and causes production of reactive oxygen species (ROS) through multiple mechanisms, increasing the risk of skin cancer (Premi et al., 2015). Whereas eumelanin has antioxidant activity, ROS-mediated oxidation of DNA bases and lipid peroxidation are elevated in mice that produce pheomelanin only (Mitra et al., 2012). Melanocytes produce melanin within subcellular organelles called melanosomes which mature from early, unpigmented (stages I-II) towards late, pigmented states (stages III-IV). Early-stage melanosomes are recognized by proteinaceous fibrils within the melanosomal lumen. In the late stages melanin is gradually deposited on the fibrils (Raposo & Marks, 2007). These mature melanosomes are ultimately transferred to keratinocytes (Park et al., 2009) where they coalesce in a supranuclear location on the sun-facing side. UV radiation triggers tanning through p53-mediated induction of POMC peptides in keratinocytes, leading to MC1R activation on melanocytes and cAMP-mediated induction of the microphthalmia-associated transcription factor (*MITF*), that induces expression of tyrosinase-related protein 1 and 2 (*TYRP1* and *DCT*) (Lo & Fisher, 2014) and tyrosinase, which drive melanosome maturation (Paterson et al., 2015) and increased production of eumelanin (Iozumi et al., 1993). The enzyme nicotinamide nucleotide transhydrogenase (NNT) is located in the inner mitochondrial membrane. It regulates mitochondrial redox levels by coupling hydride transfer between β-nicotinamide adenine dinucleotide NAD(H) and β-nicotinamide adenine dinucleotide 2'-phosphate NADP (+) to proton translocation across the inner mitochondrial membrane (Earle & Fisher, 1980; Rydstrom et al., 1970; Zhang et al., 2017). Even though The Human Protein Atlas ("Human Protein Atlas available from http://www.proteinatlas.org," ; Uhlen et al., 2015) showed expression of NNT in human melanocytes, fibroblasts, keratinocytes, and other epidermal cells, so far, NNT has not been described to be involved in mechanisms of direct regulation of skin pigment. Here, we report a role for NNT in modulating melanosome maturation and pigmentation. A connection between melanoma and oxidative stress has been suggested by Doll et al. (Doll et al., 2017) when identifying increased ACSL4 levels in metastasized melanoma. ACSL4 is a marker for ferroptosis, which is an iron- and oxidative stress-driven mechanism of cell death (Dixon et al., 2012).

2 Objectives

The goal of this work is to understand how oxidative stress may drive pigmentation and finally also melanoma risk. The skin's main function is to protect the body from harmful pathogens and UV radiation. The need to protect skin from these oxidative stress-inducing events suggest a biological connection between oxidative stress, skin pigmentation and skin cancer risk.

3 Methods

A variety of methods has been applied as outlined in the long version of this thesis. All IRB approvals were granted. Mice were bred on a heterozygous MiWhite background (*Mitf* white) (Steingrimsson et al., 2004). C57BL/6J mice (Jackson Laboratory, Stock No: 000664) displaying a 5-exon deletion in the *Nnt* gene resulting in a homozygous loss were compared to *Nnt* wild type C57BL/6NJ mice (Jackson Laboratory, Stock No: 005304). Zebrafish overexpressing human NNT were generated by using a MiniCoopR plasmid and zebrafish lacking NNT were generated by using a SpCas9 guide RNAs (gRNAs). Pigmentation of free-standing melanocytes were identified at high magnification, making sure no overlapping signal was included into the analysis. The intra-melanocytic region was marked and the brightness was measured using the FIJI software. Primary human melanocytes were isolated from normal discarded foreskins as previously published (Allouche et al., 2015). Human melanoma cell lines were obtained the National Cancer Institute (NCI), Frederick Cancer Division of Cancer Treatment and Diagnosis (DCTD) Tumor Cell Line Repository, the Memorial Sloan Kettering Cancer Center and the Wellcome Trust Functional Genomics Cell Bank. siRNA experiments were performed by using Lipofectamine RNAiMAX (Life Technologies, #13778150). Human *NNT* fused by multiple cloning procedures. Chemicals were purchased as follows: 2,3-Butanedione 97% (2,3 BD) (Sigma Aldrich, #B85307), N,N-Dicyclohexylcarbodiimide (DCC) (Sigma Aldrich, #D80002), and Palmitoyl coenzyme A lithium salt (Sigma Aldrich, #P9716) (10 uM, 2 mM). For Western Blotting were primary antibodies used as indicated in the long version of the manuscript. For RT-PCR primers were used as outlined in the manuscript. For melanin quantification equal numbers of cells were were resuspended in 60 µl of 1 N NaOH solution and incubated at 60°C for 2 h or until the melanin was completely dissolved. After cooling down to room temperature, samples were centrifuged at 500 × g for 10 min and the supernatants were loaded onto a 96-well plate and measured by the absorbance at 405 nm. For HPLC analysis were lyophilized cells were ultrasonicated and analyzed according to published protocols (Wakamatsu et al., 2002) (d'Ischia et al., 2013). Skin color measurements have been performed as outlined by (Park et al., 1999). Cyclic adenosine monophosphate (cAMP) was measured directly using an enzyme-linked immunosorbent assay (ELISA) (Enzo Life Sciences, #ADI-901-0) and cell viability was evaluated by the CellTiter-Glo Luminescent Cell Viability Assay (Promega, #G7570). NADPH/$NADP^+$ ratios were determined using the NADP/NADPH-Glo Assay (Promega, #G9082) following the manufacturer's protocol and

luminescence was measured using an EnVision 2104 Multilabel Reader (PerkinElmer). To measure MITF transcriptional activity cell lines were infected with the dual-reporter system (GeneCopoeia, #HPRM39435-LvPM02), which expresses secreted Gaussia luciferase (GLuc) under the TRPM1 promoter and SEAP (secreted alkaline phosphatase) as an internal control for signal normalization. For histology, paraffin sections were prepared and stained with hematoxylin and eosin (H&E) using the ihisto service (https://www.ihisto.io/). For visualization of melanin, paraffin sections were stained using a Fontana-Masson Stain kit (abcam, #ab150669). To detect cellular reactive oxygen species (ROS) the kits ThermoFisher Scientific, #C6827 and ThermoFisher Scientific, #M36008 were used. Transmission electron microscopy was performed as outlined in the long version of the thesis. For human genetic association studies the GRCh37/hg19 human genome build was used. SNPs with minor allele frequency less than 1% were excluded from each cohort. In total were four cohorts used: The Rotterdam Study (Ikram et al., 2017). The RS consists of 4,694 people of predominantly North European ancestry. The CANDELA cohort, a GWAS study of skin color (Adhikari et al., 2019) and the East and South African cohort (Crawford et al., 2017) as well as the UK Biobank cohort (Jiang et al., 2019). The meta-analysis of these cohorts was performed by the Fisher's method (Won et al., 2009) of combining p-values from independent studies was used, in which p-values for one marker across different cohorts were combined to provide an aggregate p-value for the meta-analysis of that marker. Multiple test adjustment has been performed by using the Benjamini-Hochberg procedure. Additional statistical tests have been outlined in the long version fo the thesis.

For the ACSL4-based tumor analysis were the paraffin-embedded archived database of Szeged Department of Dermatology and Allergology was used for the retrospective tissue biomarker study. The patient clusters (primary non-metastatic melanomas, primary metastatic melanomas, solid melanoma metastases, primary melanomas and their metastases-pairs) were selected from the database of routine medical care (Medsol). The histopathological cases were reanalyzed according to the 4th WHO and 8th AJCC guidelines, the standardized histopathological dataset included the subtypes, Breslow thickness, Clark levels, ulceration, regression, mitotic index, peritumoral host reaction and their derivate, the pathological stage (pT). For the clinical stage (cT), archived diagnostical reports were used. Finally, progression-free survival (PFS) and overall survival (OS) variables were counted in months serving for the outputs of the biomarker study. Ethical approval: MEL-BIOCHIP-001 (4321 (142/2018). Histology was performed as outlined in the long version of the thesis. Publicly available data from the TCGA dataset from the cBioPortal (https://www.cbioportal.org/study/clinicalData?id=skcm_tcga) has been downloaded and analyzed via the Cistrome (http://timer.cistrome.org/) or the TCGA Cancer Browser (http://tcgabrowser.ethz.ch:3838/PROD/) (Cancer Genome Atlas, 2015). Survival analysis was conducted using the Cox Proportional Hazards model, which provided the effect size parameter

estimate and p-value. The relationship of ACSL4 level with the survival variables was visually represented using Kaplan-Meier survival curves. Finally, a multivariate Cox PH model was used to analyse the survival variables, where in addition to ACSL4 some covariates of known relevance (such as sex) were jointly included. This allows the effect of ACSL4 to be estimated why controlling for the effects of major convariates.

Levels of significance are indicated by *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; ns, not significant.

4 Results

To show the impact of NNT on pigmentation in vitro experiments were performed. NNT was depleted using a pool of siRNAs (siNNT) in human melanoma cell lines UACC257 and SK-MEL-30, and in primary human melanocytes. In all three cell models knockdown of NNT led to a significant increase in melanin content (Figures 2). NNT has been described to increase GSH in Nnt wild type versus Nnt mutant C57BL/6J mice (Ronchi et al., 2013), as well as in human myocardium (Sheeran et al., 2010). Cysteine or reduced glutathione is a required component for pheomelanin synthesis (Ito & Ifpcs, 2003; Jara et al., 1988), suggesting that NNT may modulate pigmentation via its role in regenerating GSH and thereby affecting the pheomelanin to eumelanin ratio (Figure 2B, Left). To investigate this possibility, high-performance liquid chromatography (HPLC) was utilized and demonstrated significantly increased absolute levels of eumelanin, but not pheomelanin, upon NNT knockdown (Figure 2B, Middle). The eumelanin to pheomelanin ratio also showed a significant increase, (Figure 2B, Right). Tyrosinase silencing was used as a positive control showing efficient and quick depigmentation five days after transfection (Figure 2A). Due to NNT's essential role as an antioxidant enzyme against ROS by controlling the NADPH conversion, we hypothesized that the increase in pigmentation following silencing of NNT is driven by an oxidative stress-dependent mechanism. Adding thiol antioxidant N-acetylcysteine (NAC), or mitochondria-targeted antioxidant MitoTEMPO to siNNT, inhibited the siNNT-mediated increase in pigmentation (Figures 2C), demonstrating the dependence of siNNT-mediated pigmentation on oxidative stress. To understand how cytosolic and mitochondrial oxidative stress levels are connected, isocitrate dehydrogenase 1 (IDH1), a source of cytosolic NADPH (Zhao & McAlister-Henn, 1996) was depleted in UACC257 cells (Figures 2D). Interestingly, while siNNT alone increased pigmentation, siIDH1 alone had no significant effect on pigmentation (Figure 2D). However, the double knockdown of NNT and IDH1 increased the intracellular melanin content further, exceeding the siNNT-induction of pigmentation (Figure 2D). To exclude the possibility that siIDH1 or siIDH1-induced oxidative stress may increase NNT levels, NNT mRNA levels were measured, which showed no changes (data not shown). In order to clarify the role of mitochondrial oxidative stress, we investigated the participation of peroxisome

proliferator-activated receptor gamma coactivator 1-alpha (PGC1a). As shown previously, intramitochondrial concentrations of ROS were significantly increased in PGC1a-depleted melanoma cells, associated with decreased levels of reduced glutathione (GSH), cystathionine, and 5-adenosylhomocysteine (Vazquez et al., 2013). However, no change of pigmentation was detected in PGC1a-depleted human UACC257 melanoma cells (Figures 2E), thus emphasizing the specific role of NNT and especially NNT-induced cytosolic oxidative stress for the pigmentation response. As opposed to the increase in pigmentation observed with silencing of NNT, overexpression of NNT induced a significant decrease in pigmentation (Figure 2F), confirming the relationship between NNT and pigmentation in both directions. Taken together our data suggest that NNT affects pigmentation via a redox-dependent mechanism.

In order to elucidate the mechanism underlying hyperpigmentation after NNT knockdown, we investigated its effects on key melanin biosynthesis factors in UACC257 cells (Figure 3). NNT knockdown revealed a significant increase in the levels of the melanin biosynthesis enzymes, TYRP1, TRP2/DCT and tyrosinase. In contrast to these late stage melanosome markers, did Pmel17, a marker for early melanosome development, not change upon depletion of NNT. Since MITF is the main regulator of these enzymes and the master regulator of melanogenesis, we measured MITF protein levels and its transcriptional activity. Upon silencing of NNT, neither MITF protein levels, nor mRNA levels were significantly changed and no significant change in the mRNA level of TYRP1, TRP2/DCT or tyrosinase was observed (data not shown). This suggests that NNT can impact tyrosinase, TRP2/DCT and TYRP1 protein levels without affecting their mRNA levels. Together, these data suggest the existence of an NNT-dependent pigmentation mechanism, independent of the previously established cAMP-MITF-dependent pigmentation pathway.

Since altering NNT was found to impact the protein levels of tyrosinase and related key melanogenic enzymes (Figure 3) without impacting their mRNA levels, we hypothesized that NNT can affect the stability of certain melanosomal proteins. The impact of NNT-mediated redox changes on tyrosinase protein stability was investigated by knockdown of *NNT* mRNA in the presence or absence of an antioxidant, followed by inhibition of protein synthesis with cycloheximide (CHX) and measurements of the rate of decay of tyrosinase protein. Silencing of *NNT* increased tyrosinase protein stability significantly, and this effect was prevented by antioxidant treatment with either NAC, NADPH or Mito-Tempo (Figures 4A-C). The mechanism of tyrosinase degradation is not fully understood, although it has been shown that tyrosinase is degraded via the ubiquitin-proteasome system (Bellei et al., 2010). Addition of carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132), a cell-permeable, reversible proteasome inhibitor prevented an NNT overexpression-induced decrease in Tyrosinase protein stability in UACC257 cells (Figure 4D), suggesting that NNT induces changes in melanin levels is through proteasome-mediated degradation of Tyrosinase protein.

Due to siNNT-induced increases in melanogenesis enzymes, NNT's role in NADPH and GSH generation and its location in the inner mitochondrial membrane, we hypothesized that NNT function might be connected to the maturation of melanosomes. The effects of modulating NNT expression on the ultrastructure of melanosomes was assessed by electron microscopy in primary human melanocytes. Knockdown of NNT resulted in a striking increase in late-stage/pigmentated melanosomes (stages III and IV) (Figures 5A), while overexpression of NNT resulted in a switch towards early-stage/unpigmented melanosomes (stages I and II) (Figure 5B), establishing a role for NNT in regulating melanosome maturation. In line with the pigmentation data (Figure 2C), cotreatment with either NAC or MitoTEMPO prevented the siNNT-induced phenotype (Figures 5A). The absolute number of melanosomes per cytosolic area was not affected by NNT knockdown or overexpression (data not shown), which is in line with the observation that the pre-melanosome protein Pmel17, a marker for early melanosome development, did not change upon depletion of NNT (Figure 3A).

Currently, only a limited number of topical drugs are capable of altering pigmentation in human skin (Rendon & Gaviria, 2005). No topical skin darkeners are available for clinical use. Systemic administration of peptides such as a-MSH analogs (e.g., Melanotan) has been used to successfully increase skin pigmentation (Ugwu et al., 1997). Three NNT inhibitors (*N,N'*-Dicyclohexylcarbodiimide [DCC], 2,3-Butanedione [2,3BD], Palmitoyl-CoA) have been described previously (Rydstrom, 1972). DCC is commonly used as a peptide-coupling reagent and 2,3BD is used as a flavoring agent (Rigler & Longo, 2010). Both are low molecular weight compounds (DCC: 206.33 g/mol; 2,3BD: 86.09 g/mol) potentially capable of penetrating human epidermis. Palmitoyl-CoA, like 2,3BD, is a natural product, but has a high molecular weight (1005.94 g/mol), making skin penetration challenging. The effects of all three compounds on pigmentation of intermediately pigmented murine Melan-A cells (Figure 6A) were assessed. Both 2,3BD and DCC significantly increased the melanin content in intermediately pigmented murine Melan-A cells (Figure 6A). In vitro toxicity was assessed in primary human melanocytes, dermal fibroblasts and keratinocytes showing no significant toxicity in doses up to 10uM respectively, 100uM for 2,3BD in primary melanocytes (data not shown). To verify the effects of the small molecular weight compounds on NNT function, the GSH/GSSG ratio, an indirect endpoint of NNT enzyme activity, was measured, revealing decreased GSH/GSSG ratios induced by DCC and 2,3 BD in primary melanocytes (Figures 6B and 6C) and by DCC, without significant toxicity (data not shown).

Next, we tested the compounds on human skin explants from different skin types. As suggested above, palmitoyl-CoA did not penetrate the epidermis and had no effect on pigmentation (data not shown). In abdominal skin from individuals of fair skin phototype 1-2, 2,3BD yielded a strong induction of pigmentation at relatively high doses (Figure 6D). Histology with Fontana-

Masson staining showed increased melanin in the 2,3BD treated skin (Figures 6Ei) and no obvious cell damage or inflammation by H&E staining (Figure 6Eii), although the volatility of 2,3BD produces a strong butter-like aroma, potentially limiting its future clinical use. Importantly, keratinocytic supranuclear caps (Figures 6Eiii) were present, suggesting the formation of functional melanosome/melanin transfer to keratinocytes, which allows cells to protect their nuclei from UV radiation. Daily application of 50mM 2,3BD or DCC on skin from intermediately pigmented skin type 3-4 individuals yielded significantly increased pigmentation after 5 days (Figure 6F). Due to the activity of DCC as a coupling agent and its corresponding unclear toxicity risks, only 2,3BD was used in subsequent experiments. UV radiation interacting with DNA can directly produce cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts, whereas ROS-mediated DNA modifications produce alternative nucleotide adducts including 8,5-cyclo-2-deoxyadenosine, 8,5-cyclo-2-deoxyguanosine, and 8-oxo-deoxyguanine (Jaruga & Dizdaroglu, 2008; Wang, 2008).While superficial epidermal cells containing modified proteins, lipids and DNA are continuously shed through corneocyte desquamation, durable basal cells require active DNA repair machinery for their maintenance. Melanomas have been found to contain high frequencies of somatic mutations with characteristic UV-induced signatures of C to T and G to A transitions (Berger et al., 2012). Protecting human skin from these intermediates is a major goal of skin cancer prevention strategies. As shown in previous studies, increased pigmentation can help to protect against CPD formation (D'Orazio et al., 2006; Mujahid et al., 2017). We tested if 2,3BD-induced pigmentation can protect skin from UVB-induced CPD formation. After inducing a visible increase in pigmentation of human skin by application of 50 mM 2,3BD to skin type 2-3 for 5 days (Figure 7A), UVB was applied and CPD formation was detected by immunofluorescence staining and normalized to the total number of cells. It was observed that 2,3BD treatment protected against formation of UVB-induced CPD (Figure 7A). We then measured γ-H2AX, a marker of DNA double-stranded breaks, in order to investigate potential 2,3BD-mediated toxicity as well as whether 2,3BD-mediated skin pigmentation could protect from UVB-induced γ-H2AX induction (Figure 7B). 2,3BD was observed to be non-toxic and the pigmentation it produced could protect human skin from UVB-induced γ-H2AX induction.

C57BL/6J and C57BL/6NJ mice are substrains of the C57BL/6 mouse with known genetic differences. While C57BL/6NJ mice are homozygous for the *Nnt* wild type allele, C57BL/6J mice are homozygous for the *NntC57BL/6J* mutation. This mutant allele is missing a stretch of 17,814 bp between exons 6 and 12, resulting in a lack of mature protein in these mutants (Toye et al., 2005) (Huang et al., 2006). In our experiments, C57BL/6J mice that are homozygous for the *Nnt* mutation showed increased fur pigmentation compared with C57BL6/NJ control (wild type *Nnt*) mice (Figure 8A, Left panel). Quantification of pheomelanin and eumelanin levels in mouse hair by HPLC shows higher eumelanin, but not pheomelanin, in C57BL/6J mice compared with C57BL/6NJ mice (Figure

8A, Right panel). Next, a zebrafish (*Danio rerio*) model that overexpresses NNT selectively in melanocytes was engineered. Similar to humans and mice, zebrafish melanocytes originate from the neural crest, and the pathways leading to melanocyte differentiation and pigment production are conserved. Many human pigmentation genes and disorders have been successfully modeled in the zebrafish, highlighting the striking similarity between zebrafish and human melanocytes. Unlike humans, zebrafish have xanthophore and iridophore pigmentation cells, however in this manuscript we restrict our studies to melanocytes (van Rooijen et al., 2017). Five days after NNT overexpression, a decrease in intramelanocytic pigmentation was observed in NNT-overexpressing zebrafish compared with empty plasmid Zebrafish embryos (Figure 8B). This observation was confirmed by pixel-based brightness quantification analysis. Deletion of *nnt* using CRISPR-Cas9 resulted in darkened melanocytes (Figure 8C). Similar to the genetic deletion of *nnt*, treatment of zebrafish embryos for 24 hours with the chemical NNT-inhibitors (DCC and 2,3BD), resulted in a significant darkening (Figure 8D). Together this suggests a role for NNT in driving *in vivo* skin and hair pigmentation.

Next, we examined the status of NNT in human hyperpigmentation disorders including post inflammatory hyperpigmentation (PIH) and lentigo. Skin biopsies of nine Asian patients were co-stained for NNT and 4',6-diamidino-2-phenylindole (DAPI) immunofluorescence. NNT intensity was normalized to the sample's DAPI intensity and cell count. Both epidermal and upper dermal skin were investigated. In line with the Human Protein Atlas, NNT is expressed in different epidermal cells including keratinocytes, fibroblasts, and melanocytes ("Human Protein Atlas available from http://www.proteinatlas.org," ; Uhlen et al., 2015), were moderate levels of NNT expression (red) detected throughout the epidermis and upper dermis (Figure 9, Left panel). While non-inflammatory skin disorders, such as ABNOM (Acquired, bilateral nevus of Ota-like macules, also known as Hori nevus), displayed NNT expression levels similar to those of healthy skin (data not shown), skin of patients with inflammation-induced disorders displayed decreased NNT expression levels. Disorders where intrinsic inflammation was present, such as post-inflammatory hyperpigmentation, or where extrinsic inflammation was present, such as UV-induced lentigo, NNT expression was significantly lower compared with healthy skin (Figure 9, middle and right panels).

To investigate whether NNT plays a role in normal skin pigmentation variation in humans, we examined associations between pigmentation and genetic variants within the ~1.1 Mb *NNT* gene region. A meta-analysis was performed to combine P-values from Genome-Wide Association Studies (GWAS) conducted in 4 diverse population cohorts with a total of 462,885 individuals: two Western European cohorts (Rotterdam Study (Jacobs et al., 2015), UK Biobank (Hysi et al., 2018; Loh et al., 2018)), a multi-ethnic Latin American cohort (CANDELA (Adhikari et al., 2019)), and a multi-ethnic cohort from Eastern and Southern Africa (Crawford et al., 2017). In these studies skin pigmentation

was measured either quantitatively by reflectometry or by an ordinal system (see Methods). UK Biobank summary statistics were also available for ease of skin tanning (sunburn) and use of sun protection. 332 variants were available in the combined dataset; using a P-value significance threshold of 1.01E-3 (adjusted for multiple testing, see STAR Methods), 11 variants were significantly associated with skin pigmentation in the meta-analysis (Figure 5A). The variants were present in all worldwide populations, with the alternative alleles having the highest frequency in Africans (Supplementary Table 1 and Figure 6A) and associated with darker skin color. The strongest association (P = 4.94E-05) was observed for an intronic variant rs561686035. It was also the strongest associated variant for sun protection use in the UK Biobank cohort (P = 4.15E-04, Figure 5B), the minor allele being associated with increased use. The UK Biobank cohort also showed a significant association with ease of skin tanning (sunburn), the lowest P-value being 1E-3 for the intronic SNP rs62367652, the minor allele being associated with increased tanning (Figure5B, S6B). All the 11 variants that were significant in the meta-analysis of pigmentation are in linkage disequilibrium (LD) ($r^2 > 0.7$), and they span a 11 KB region at the beginning of the NNT gene overlapping its promoter (ENSR00000180214) (Figure 10A), which shows regulatory activity in melanocytes and keratinocytes (according to the Ensembl database). Furthermore, several of these variants are highly significant eQTLs for the NNT gene in both sun exposed and unexposed skin tissues (according to the GTEx database). Subsequently, we sought to understand the direction of effect of the NNT genetic variants on these traits and on the expression of NNT. We calculated the correlation between the GWAS effect sizes of the alternative allele of each genetic variant within the *NNT* region with their effect sizes as eQTLs on the expression of the *NNT* transcript according to GTEx in the two skin tissues (see Methods). The results are consistent with the direction of association between the *NNT* transcript expression and skin color as described earlier: expression levels of the *NNT* transcript in both tissues was negatively correlated with darker skin color (especially in sun unexposed skin tissue, where the effect of external factors such as sunlight is less prominent), and sun protection use (especially in sun exposed skin tissue) as well as sunburn (especially in sun exposed skin tissue). Therefore, several intronic SNPs within the *NNT* genomic region were associated with skin pigmentation, tanning, and sun protection use in 4 diverse cohorts including 462,885 individuals. Using eQTL expression data for *NNT*, we observe that lower expression of the *NNT* transcript in skin tissues correlates with darker skin color, and consequently less sunburn and less sun protection use.

In addition to the above-described effect of oxidative stress on physiological pigmentation, we aimed to understand how oxidative stress may impact melanoma. The role of oxidative stress in melanoma is poorly understood. In general, it is suspected that melanoma is oxidative stress associated and connected to mitochondrial dysfunction (Bisevac et al., 2018). Therefore the role of ACSL4 in human melanoma has been investigated. Staining of 76 human melanoma samples originating from patients

with primary and metastatic melanoma has been performed. In addition to the above-described effect of oxidative stress on physiological pigmentation, we aimed to understand how oxidative stress may impact melanoma. The role of oxidative stress in melanoma is poorly understood. In general, it is suspected that melanoma is oxidative stress associated and connected to mitochondrial dysfunction (Bisevac et al., 2018). Therefore, the role of ACSL4 in human melanoma has been investigated. 454 human melanoma samples from the TCGA dataset (Cancer Genome Atlas, 2015), correlating patient survival and ACSL4 gene expression (Figure 11A). In addition, were 63 human melanoma samples originating from patients with primary and metastatic melanoma from the University of Szeged biobank stained for ACSL4 protein by immunohistochemical methods. Spearman correlation analysis was done, correlating different variables with ACSL4 expression (Figure 11B). Here, due to the small sample size reached only DFS and PFS statistical significance in primary melanoma. Other significantly correlated markers included sex, BRAF status, Clark level, Breslow thickness, number of metastases, tumor subtype (specifically acrolentiginous melanoma). Other non-significantly correlated markers included other tumor subtypes, but interestingly also age, tumor thickness, ulceration and regression. In addition to the above-investigated correlation between various markers and ACSL4 was an obvious loss of this correlation observed in metastatic melanoma. While metastatic melanoma displayed in average significantly higher ACSL4 levels were differentially expressed between primary and metastatic melanoma samples, displaying in average higher levels of ACSL4 in metastatic samples.

5 Conclusion

This report presents evidence for the existence of a novel mechanism of skin pigmentation, which can be used for preventing skin cancer and treating pigmentary disorders. Lightly pigmented individuals may benefit from increasing their skin color in a transient, safe and efficient way, thereby preventing skin cancer formation. This work presents a paradigm shift in skin physiology and pathophysiology, allowing the treatment of pigmentary disorders, which are among the most common reasons for dermatological consultations and for which until today very limited treatment options exist. In addition, lays this work the groundwork for a first understanding of how skin pigmentation and oxidative stress signals are interconnected enabling the development of predictive melanoma markers, finally guiding treatment decisions in patients.

6 Thesis points

- Discovery of the existence an additional (so far second known) human pigmentation mechanism.
- This mechanism is redox-dependent, and independent of the traditional UVB- and MITF-mediated skin pigmentation mechanism.

- Modification of NNT impacts ubiquitin-proteasome mediated tyrosinase degradation and melanosome maturation.
- Topical compounds that inhibit NNT yielded human skin darkening.
- Several human NNT SNPs associate with skin color, tanning, and sun protection use.
- ACSL4 has been described as the first marker for survival in melanoma.

7 Disclosures

With the kind permission of the PhD program committee of the University of Szeged, is the NNT-related part of this thesis in large parts identical to our most recent publication in *Cell* (Allouche et al., 2021). Standard rules of plagiarism do with the kind allowance of the committee not apply to this work.

Regarding the ACSL4-related, data are we currently preparing a publication containing the above-presented data.