Effects of antioxidant supplementation on cancer progression

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Gothenburg 2018

Cover illustration: “Not all heroes wear capes” by Kristell Le Gal. Picture of a NAC-treated BPT mouse in the style of “The starry night” by Van Gogh. Image generated with Deep Dream Generator.

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ISBN 978-91-7833-033-1 (PRINT)
ISBN 978-91-7833-034-8 (PDF)

Printed in Gothenburg, Sweden 2018
Printed by BrandFactory

True science teaches us, above all, to doubt and to be ignorant – **Miguel de Unamuno**

ABSTRACT

Popular wisdom holds that antioxidants protect against cancer because they neutralize reactive oxygen species (ROS) and other free radicals which can otherwise cause cancer by damaging DNA. This has been the rationale behind many clinical trials with antioxidants, which in most cases failed to show a beneficial effect and in others even increased cancer incidence. Our group believes that these inconsistencies can be explained by the idea that antioxidants have opposite effects on tumor initiation and progression, and that tumor cells benefit from low ROS levels which is facilitated by antioxidant supplementation. In this thesis we describe the effects of two widespread antioxidants, N-acetylcysteine and vitamin E, on malignant melanoma progression, a cancer known to be sensitive to redox alterations, using a transgenic mouse model and a panel of human cell lines. Because strong evidence links mitochondria-associated ROS to tumor progression, we also define the impact of targeting mitochondrial ROS on malignant melanoma and lung cancer progression. The results show that dietary antioxidant supplementation increases metastasis in malignant melanoma, and that this is dependent on new glutathione synthesis and activated RHOA. The data also indicates that mitochondria-targeted antioxidants do not inhibit cancer progression. These results suggest that cancer patients and people with high risk of developing cancer should avoid the use of antioxidant supplements.

Keywords: antioxidants, ROS, cancer, metastasis

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SAMMANFATTNING PÅ SVENSKA

Det är allmänt vedertaget att antioxidanter skyddar mot cancer eftersom de neutraliserar reaktiva syreföreningar (ROS) och andra fria radikaler som annars kan orsaka cancer genom att skada DNA. Detta har varit grunden till många kliniska prövningar med antioxidanter, vilka i de flesta fall misslyckades med att visa en fördelaktig effekt och där vissa även ökade cancerincidensen. Vår grupp anser att dessa inkonsekvenser kan förklaras av att antioxidanter har motsatta effekter på tumörinitiering och progression, och att tumörcellerna drar nytta av låga ROS-nivåer, vilket underlättas av antioxidanttillskott. I denna avhandling beskrivs effekterna av två väl använda antioxidanter, acetylcystein och E-vitamin, på malignt melanomprogression, en cancer som är känd för att vara känslig för redoxförändringar, genom att använda en transgen musmodell och en panel av humana cellinjer. Eftersom starka bevis kopplar mitokondrie-associerade ROS till tumörprogression definierar vi också effekten av att rikta antioxidanter specifikt mot mitokondriella ROS på malignt melanom och lungcancerprogression. Resultaten visar att kosttillskott av antioxidanter ökar metastasering i malignt melanom och att detta är beroende av ny glutationsyntes och aktiverad RHOA. Uppgifterna indikerar också att mitokondrie-riktade antioxidanter inte hämmar cancerprogression. Dessa resultat tyder på att cancerpatienter och personer med hög risk att utveckla cancer bör undvika användning av kosttillskott som innehåller antioxidanter.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Le Gal, K. *et al*. Antioxidants can increase melanoma metastasis in mice.
Science Translational Medicine 2015; volume 7, issue 308.

II. Le Gal, K. *et al*. Mitochondria-targeted antioxidants do not influence malignant melanoma and lung cancer progression in mice.
Manuscript.

CONTENT

ABBREVIATIONS

BSO	Buthionine sulfoximine
COPD	Chronic obstructive pulmonary disease
DHE	Dihydroethidium
DHR	Dihydrorodamine
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ETC	Electron transport chain
GFP	Green fluorescent protein
roGFP	Redox-sensitive green fluorescent protein
GRX	Glutaredoxin
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H2DCF	2´,7´-dihydrodichlorofluorescein

4-HT	4-hydroxytamoxifen
MAPK	Mitogen-activated protein kinase
NAC	N-acetylcysteine
NOX	NAD(P)H oxidase
PDGF	Platelet-derived growth factor
PRX	Peroxiredoxin
PTP	Protein tyrosine phosphatase
ROCK	Rho-associated protein kinase
ROS	Reactive oxygen species
SOD	Superoxide dismutase
dTPP	Decyltriphenylphosphonium
TRX	Thioredoxin
UV	Ultra-violet

INTRODUCTION

If I had a world of my own, everything would be nonsense. Nothing would be what it is, because everything would be what it isn't. And contrary wise, what is, it wouldn't be. And what it wouldn't be, it would. You see? – **Lewis Carroll**

ANTIOXIDANTS AND ROS: REACHING FOR THE GOLDEN MEAN

The concept of balance as a centerpiece of harmony and wellbeing is common to most societies and cultures. And thus we read of virtue and *aurea mediocritas* or Golden Mean from classic Greek philosophers like Aristotle, the Middle Path from Buddha, moderation in all monotheistic religions, and we even encounter the notion of "lagom" in the everyday Swedish life.

This idea is but a reflection of life itself where organisms adapt to their environment and find stability to exist, and where cells regulate their internal state in search for an equilibrium or homeostasis. This homeostasis however, is not static and it is subjected to necessary fluctuations; hence, there is a need for systems with the ability to detect these alterations in the equilibrium and counteract the extremes.

An example of environmental adaptation would be the evolutionary selective pressure occurred during the "Great Oxidation" around 2.4 billion years ago, which favored life adjusted to the presence of oxygen [1, 2]. Although a toxic agent, oxygen increased the production of energy in aerobic organisms by becoming the final electron acceptor in the electron transport chain (ETC), and consequently some oxygen biproducts were generated during the process [3]. These agents are known as reactive oxygen species (ROS) because they have the capability to interact with other molecules and alter their oxidative status and their function [4]. Upon excessive activity of these ROS, a situation known as oxidative stress, and in order to reach back to homeostasis, cells have developed antioxidant defenses that neutralize ROS by giving back the electrons taken [5, 6]. These antioxidants can be produced endogenously, but they can also be supplied in the diet [7].

ROS were initially regarded as purely damaging agents, the toll we paid for using oxygen to produce more energy. But thanks to advances in the redox field, in charge of studying reduction-oxidation reactions, we now know that they also regulate a wide variety of cell signaling events that are essential to the normal function of cells and organisms [8]. Therefore, understanding their role in health and disease is of great interest in medicine.

ROS CAN CAUSE CANCER

As previously mentioned ROS can modify proteins and DNA and therefore regulate signaling pathways. For example, they can inhibit or activate them by reversible oxidation of cysteine residues in proteins. The advantage of this type of regulation is that ROS have a short half-life and are able to easily diffuse across membranes, making available both intra- and intercellular control [9]. Examples of ROS mediated signaling are the response to growth factors, such as EGF or PDGF, which upon binding to their receptors increase ROS production through NAD(P)H oxidases (NOXes) located in the cellular membrane or the response to steroid hormones, which can change intracellular levels of calcium and dephosphorylate cytochrome c oxidase, thereby increasing the mitochondrial membrane potential and consequently, the production of superoxide ($O_2^{\cdot}$) [10-12]. Conversely, antioxidants can inhibit growth factor signaling. One way in which ROS regulate these signaling cascades is the inhibition of neighboring phosphatases. Hydrogen peroxide (H_2O_2) is a well-known inhibitor of protein tyrosine phosphatases (PTPs), such as RPTP-α, PTP-1B, SHP-2 and MKPs. ROS can also inhibit antioxidant proteins that are normally bound to kinases, like thioredoxins (Trx) or peroxiredoxins (Prx)[13].

Increased ROS production has been observed in a variety of cancers [14, 15]. This exacerbated production can come from NOXes in the cellular

membrane, the ETC in the mitochondria or from xanthine oxidase [6]. A decrease in endogenous antioxidant activity can also increase ROS content in the cell. The exact mechanisms that trigger the uncontrolled production of ROS remain largely unknown. Nevertheless, altered ROS levels can cause mitochondrial and genomic DNA damage. It also affects the regulation of transcription factors that are involved in apoptotic signaling by regulating their DNA binding activity. For instance, the tumor suppressor p53 requires its reactive cysteines to be reduced in order to bind to DNA [16].

Since ROS can modify proteins and DNA, they can cause the formation of protein and DNA adducts, that in turn favor the propagation of mutations in the highly proliferative cancer environment [17, 18]. The formation of these adducts can affect gene expression by interfering with methyltransferases and producing hypomethylation of promoters, such as those of oncogenes [19, 20]. Combined with the mutational silencing of tumor suppressor genes, there is no question that ROS can contribute to carcinogenesis [21].

ROS LOCALIZATION AFFECTS THEIR ROLE

Mitochondria largely contribute to the production of ROS in the cells; in fact, they are the major source due to the production of $O_2^{\cdot}$ in the ETC from complexes I, II and III [3]. The $O_2^{\cdot}$ produced is taken care of by the antioxidant enzymes superoxide dismutases (SODs) and rapidly turned into H_2O_2 [22]. This mitochondrial-associated H_2O_2 can diffuse from the mitochondria into the cytosol and the extracellular environment and trigger signaling pathways [9]. In addition to cell signaling, mitochondrial ROS contribute to carcinogenesis by mutating mitochondrial DNA (mtDNA). This mtDNA is susceptible to mutations because of its proximity to the source of ROS, lower level of histones and limited proofreading [23-26]. Mutations in the ETC have been reported in many forms of cancer [27, 28].

Hence, cancer cells could use mitochondrial ROS production to their advantage [29]. Along those lines, several mitochondria-targeting antioxidant compounds have been developed and some promising results have been reported [30-32]. However, their impact on endogenous mouse models of cancer with an intact immune system has yet to be evaluated.

ANTIOXIDANT SUPPLEMENTS AFFECT CELL SIGNALING BY TARGETING ROS

As presented so far, the dual character of ROS, cell signaling molecules vs damaging agents, requires some fine tuning to keep cellular balance. It has also been shown that high oxidative stress levels correlate with malignant progression. Thus it was thought that antioxidant supplementation would counteract the damaging effects of ROS and promote a healthy cellular state. In addition, several epidemiological studies show an inverse correlation between cancer and antioxidant-rich diets [33].

To that end, numerous clinical trials have been conducted to test whether antioxidant supplementation could be used to fight and prevent cancer. But the results are somewhat mixed and it would seem that general conclusions cannot be drawn. The effects varied depending on the population, the type of cancer and the type of antioxidant used. For instance, the Linxian Nutrition Intervention Trial showed a decrease in gastric cancer incidence for participants who were supplemented with beta-carotene, vitamin E and selenium, but not with retinol and zinc, riboflavin and niacin, or vitamin C and molybdenum [34, 35]. However, the protective effect of beta-carotene, vitamin E and selenium was lost after 10 years post-intervention and increased risk for esophageal cancer was observed in participants who were 55 years old or above at the time of inclusion [36]. In an independent study where Finnish male smokers were given alpha-tocopherol and beta-carotene

(ATBC trial), higher incidence of lung cancer was observed in the beta-carotene treated group [37, 38]. The results were additionally confirmed in another large trial involving men and women at risk of developing lung cancer who were given beta-carotene and retinol (CARET trial); the trial had to be prematurely stopped due to significantly higher incidence and death rate in the antioxidant-supplemented group [39]. In a third study where apparently healthy women were given beta-carotene to assess its usefulness in preventing cancer and cardiovascular diseases, no harm nor benefit was observed [40]. In another large trial where the effects of selenium and vitamin E on prostate cancer prevention were assessed (SELECT trial) no significant differences were seen at first between treatment groups. However a statistically significant increase in tumor incidence was later observed in the vitamin E treated group [41, 42].

These inconsistencies are perhaps a result of a vague scientific question: "are antioxidants beneficial in fighting cancer?" which we think should be split into two different ones:

1. Can antioxidants prevent tumor initiation?
2. Do antioxidants hinder tumor progression?

The answer to these questions is not an easy one. Tumor cells do have elevated levels of ROS in comparison to normal cells, but they are also vulnerable to further increases, and therefore are dependent on the use of antioxidant defenses. In addition, decreases in reduced glutathione (GSH) and increases in ROS have been shown to delay cell cycle progression through G1 & S phases and led to G2 cycle arrest [43]. Nonetheless, the metabolic plasticity of cancer cells allows them to adjust pathways to ensure the supply of antioxidant molecules and regulate multiple antioxidant enzymes [44, 45].

CHOOSING CANCER MODELS TO DEFINE EFFECTS OF ANTIOXIDANTS ON CANCER

Melanoma is the deadliest form of skin cancer and its prevalence has increased over the past decades [46, 47]. It can develop anywhere in the body and most commonly does in the skin (cutaneous melanoma). However, it is the metastases that arise from the primary skin tumor which determine patient prognosis and survival [48-50].

Our current knowledge and understanding of the genetic changes present in melanoma is vast, but the molecular mechanisms that trigger and regulate the progression of the disease remain largely unknown [51]. Some oncogenic mutations have been well described; For instance, the *BRAF* p.V600E mutation that leads to the activation of the mitogen-activated protein kinase (MAPK) pathway is present in roughly 50% of all cutaneous melanomas. Another classical melanoma oncogene is *NRAS*, which is found mutated in 15-20% of melanomas; In addition to activating the MAPK pathway, oncogenic *NRAS* also triggers the phosphatidyl-inositol 3-kinase (PI3K) pathway [52, 53]. However, expression of mutant *BRAF* alone does not progress into melanoma unless accompanied by other events [54], such as loss or alteration of tumor suppressors like *PTEN* or *CDKN2A* [55].

The primary identified mutagen in malignant melanoma is UV light exposure, but it does not account for the driving mutations that regulate known oncogenes in melanoma at the molecular level, leaving room for other processes such as oxidative stress to have an important role in the development of the disease [56, 57]. In addition, the skin can be exposed to antioxidant supplementation from different sources, such as topical and dietary [58].

Lung cancer has also caught the attention of the antioxidant field. Being the deadliest and most common form of cancer, it is not strange that one of the

largest clinical trials on antioxidant supplementation ever conducted assessed their efficacy in preventing it. Although its incidence among men has declined over the years, it is still the leading cause of cancer death among this gender [59].

The use of tobacco is the main risk factor associated with the disease [59], and longtime smokers are at high risk of developing chronic obstructive pulmonary disease (COPD) [60, 61]. To those affected, N-acetylcysteine (NAC) is often prescribed as a mucolytic to facilitate respiration.

In order to evaluate the impact of antioxidant supplementation and redox modulation on these forms of cancer, we need to make use of specific research tools.

RESEARCH METHODS

Climate is what we expect, weather is what we get. – **Mark Twain**

THE MOUSE AS A RESEARCH TOOL

Since the times of Ancient Greece, scientists have used animal experimentation to study and understand the complexity of life and biological processes. As early as in the 4th century BC, Aristotle observed differences in the anatomical content and placement of organs across species through dissections, and Erasistratus was the first to document experiments on living organisms. Science and medicine have been able to develop to their current state thanks to the use of animal models. These organisms have offered the possibility of researching questions that were relevant to another species without direct intervention, and they have contributed to the validation of the scientific method in multiple disciplines [62]. However, the model chosen to answer to a specific physiological or pathological question should be carefully considered and should be relevant to the research problem at hand [63].

For this theis, I used one particular and well-known model organism to understand and monitor key events in cancer progression: *Mus musculus*, commonly known as the house mouse.

Humans and mice have shared habitats since about 12,000 years ago, by the time of the Neolithic Revolution. It is not surprising then that these animals were picked as research models in the early stages of science. They are small, easy to breed, strains can be highly standardized through inbreeding, and their genetic mutations often represent human disease.

MICE ARE VALUABLE IN CANCER RESEARCH

Given that around 99% of the mouse genes have a human homologue, we can model a large variety of human pathologies by altering the mouse genome [64]. Additionally, although a rare event in wildlife, every mouse tissue is

potentially subjected to the development of neoplastic events, just like their human counterparts. In order for that to happen, two types of genes can be manipulated: tumor suppressor genes (loss of function) and oncogenes (gain of function) [65].

THE CRE-LOXP SYSTEM ALLOWS FOR GENOME EDITING

One of the most common methods used to modify genes is the Cre-loxP technique, which relies on the use of the bacteriophage P1 cyclic recombinase (*Cre*) which recognizes DNA sequences called locus of crossing over (*loxP*). The *loxP* sites consist of 34 base pair (bp) long DNA fragments formed by two 13 bp inverted repeats separated by an 8 bp spacer region. The enzyme *Cre* cleaves sequences of DNA flanked by two *loxP* sites with the same orientation, and the resulting cleaved sequence is excised in a circular loop of DNA. The expression of *Cre* can be regulated temporally and/or spatially by exogenous *Cre* expressing vectors (plasmid or viral particles) or by inserting *Cre* behind tissue-specific promoters.

A MOUSE MODEL TO STUDY METASTASIS

With the aim of studying the effects of antioxidant supplementation on metastasis, we used the *Braf* $^{CA/+}$ *Pten* $^{f/f}$ *Tyrosinase-Cre* (BPT) mouse model of malignant melanoma. This model is used in both paper I and II.

The BPT mice conditionally express oncogenic mutant *Braf*^{V600E} and loose expression of *Pten*. The conditional *Braf* transgene expresses normal BRAF until activated by *Cre*, upon which wiltype exons 15-18 and a STOP cassette flanked by *loxP* sites are excised and replaced by a mutant exon 15 followed by wildtype exons 16-18. Additionally, both alleles of the tumor suppressor *Pten* have their exon 5 flanked by *loxP* sites, which leads to the expression of a non-functional PTEN protein when cleaved by *Cre* [66]. In this model the expression of *Cre* is spatially limited to melanocytes and some cells of the central nervous system, as it falls under the control of the *Tyrosinase* promoter, which regulates the expression of the skin pigment melanin [67, 68].

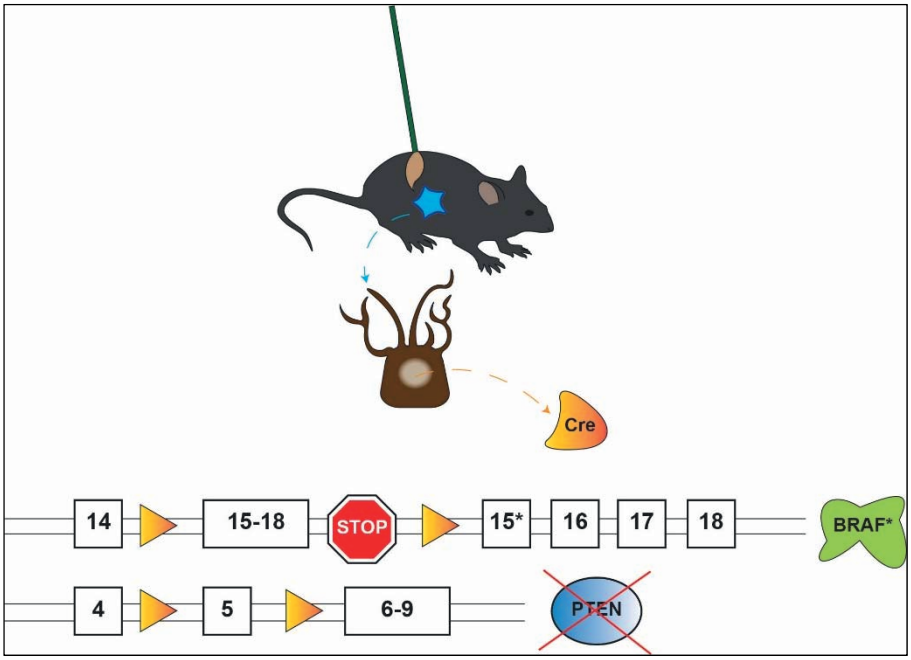

Figure 1. Genetic strategy to generate mice with malignant melanoma. After painting the skin of 2 days-old pups with 4-HT, the mice express mutant BRAF and inactive PTEN in melanocytes, which will lead to the formation of skin tumors and eventually metastases.

By painting the right flank of the animals at postnatal day 2 with 4-hydroxytamoxifen (4-HT), *Cre* is induced in melanocytes and thus mutant protein BRAF is expressed and PTEN is lost; all of which leads to the formation of skin tumors that eventually metastasize to regional lymph nodes and in some cases lungs. Despite recapitulating most of the events leading to the development of the disease in humans, this model is limited by the fact that the mice often come to a humane endpoint due to the size of the primary tumor and not due to the metastatic burden, which is the leading cause of death in humans.

THE KRAS^{LSL} MODEL RECAPITULATES EVENTS IN HUMAN LUNG CANCER

To analyze the effects of mitochondria-targeted antioxidants on tumor proliferation, we used a mouse model of lung cancer in paper II.

In this model, the expression of the oncogenic *Kras* allele, *Kras$^{LSL\text{-}G12D}$*, is controlled by exogenous *Cre* expressing virus which can be delivered by intratracheal instillation directly to the lungs or inhaled through the nose; in this study we used nasal inhalation of adenovirus. The mice carry a *Kras* allele with a *LoxP* flanked STOP cassette (LSL) followed by an activating *Kras*^{G12D} mutation, which results in a null mutation. Without *Cre* expression the mice only produce one copy of wildtype K-RAS and are unaffected; with *Cre* expression, the STOP cassette is cleaved and mice express one copy of K-RAS^{G12D}, which is enough to induce disease.

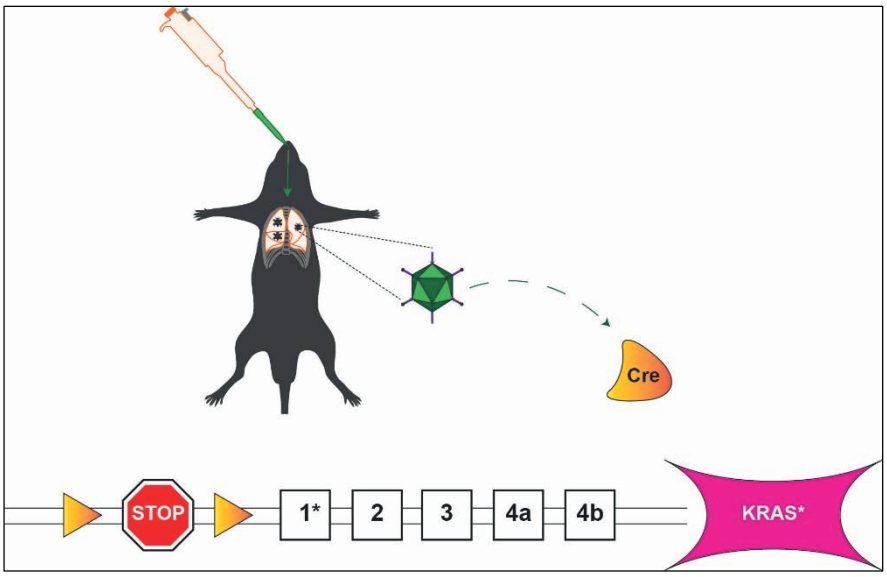

Figure 2. Genetic strategy to generate mice with KRAS-induced lung cancer. After inhaling adenovirus into the lungs, mice will express one copy of wildtype Kras plus one copy of mutant Kras, which is enough to induce the development of tumors in the lungs.

The consequent activation of the oncogene in the lung epithelium leads to increased proliferation and progression to atypical adenomatous hyperplasia, adenomas and, finally, adenocarcinomas [69, 70].

ETHICAL CONSIDERATIONS ARE NEEDED WHEN WORKING WITH ANIMAL MODELS

Even though animal experimentation has led to enormous advances in the field of scientific medicine, it has been accompanied since its origins by a growing criticism of the use of animals in science. These concerns were aggravated after the publication of Darwin´s theory of evolution, which made many question the line that separated animals and humans and prompted the creation of societies against animal cruelty [71].

Nevertheless, in comparison to other industries where animals are exploited for human benefit, such as farming, the use of animals in experimental research is tightly regulated and controlled at several levels.

All animal experiments performed during the development of this thesis were evaluated and approved by the Research Animal Ethics Committee in Gothenburg, and all researchers involved strived to follow the 3Rs principle.

ANALYZING ROS IN CELLS: WHEN AND WHERE?

Contrary to popular belief, redox couples are not found in thermodynamic equilibrium in cells; they vary in their subcellular localization and differ in their kinetics [72]. Hence, it is necessary to use tools that allow us to gain a better understanding of the context in which redox reactions occur. However, whole-cell extract based assays can be useful to obtain an overall look and determine whether certain conditions are pro-oxidative or reducing at a general level, for example, by measuring glutathione; and even though they are usually specific, reproducible and sensitive, they do not give any information about specific compartments.

FLUORESCENT PROBES FACILITATE MONITORING OF ROS IN CELL CULTURES

A variety of redox-active fluorescent probes that are triggered by different oxidative species are commercially available. They enable monitoring of redox processes in the cell through microscopy techniques, and can be combined with compartment-specific dyes to increase spatial-specificity of the reactions studied. They are easy to use in culture and some of them can be used to stain tissues too. The use of general probes, such as 2′,7′-dihydrodichlorofluorescein (H_2DCF), dihydroethidium (DHE), cellROX,

dihydrorodamine (DHR) or mitochondria-targeting ones, like mitoSOX or mitoPY1, are widely spread in the literature. Though useful, a major caveat is their partial non-specific behavior, meaning that they can be triggered by several oxidative reactions, and their activation is irreversible, making the analysis of redox kinetics impossible.

GENETICALLY ENCODED BIOSENSORS INCREASE SPATIO-TEMPORAL RESOLUTION

In order to define redox processes in their natural context, genetically encoded redox probes based on green fluorescent protein (GFP) were developed. In this thesis redox-sensitive GFP (roGFP) biosensors were used, but there are other biosensors available, such as redox-sensitive yellow FP (rxYFP) and HyPer. Some of the major advantages of roGFP is its ratiometric fluorogenic behavior, and the possibility of engineering redox relays between redox enzymes and roGFPs to increase its specificity and sensitivity, and equal response of the fluorescent protein in different tissues.

In papers I and II we used biosensors based on enhanced GFP (EGFP) developed by Tobias Dick´s lab [73]. Briefly, two reactive cysteines were engineered in positions S147 and Q204, located on β-strands 7 and 10 of EGP. Excitation maxima from GFP are preserved (400 nm for A-band and 475-490 nm for B-band), but oxidation results in an increase in excitability in the A-band and a decrease in the B-band and a reverse behavior during reducing conditions. Analyzing the ratio of fluorescence intensity between the 405 and 488 excitation maxima, one can conveniently visualize oxidative processes (increased ratio) or reducing reactions (decreased ratio). By fusing roGFP with human glutaredoxin-1 (Grx1) real-time equilibration between the sensor protein (Grx1-roGFP) and the glutathione redox

couple (GSH/GSSG) is facilitated, [74], and fusion to the yeast peroxidase Orp1 mediates oxidation of roGFP by H_2O_2 [75]. Versions of the probes that target specifically to the mitochondrial matrix are also available.

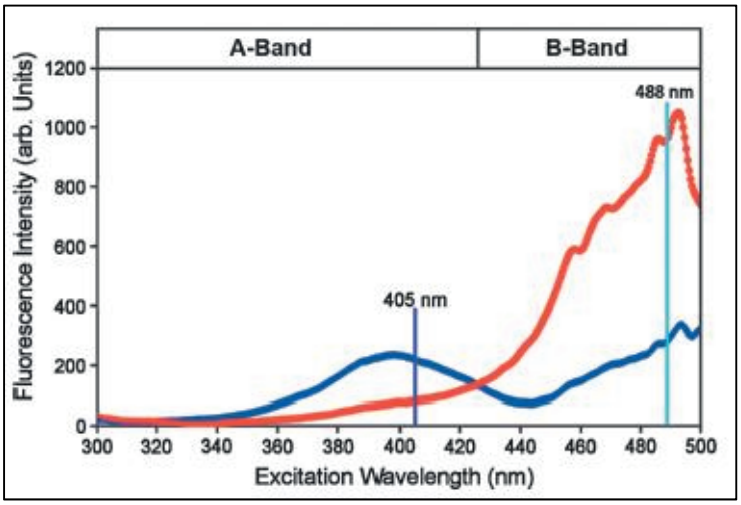

Figure 3. roGFP2 excitation is dependent upon redox changes (Meyer and Dick, 2010). When roGFP is oxidized, the fluorescence intensity increases when excited at 405 nm (blue line). When it is reduced, its maximum fluorescence peak appears when excited at 488 nm (red line). Increases in the 405/488 fluorescence ratio, indicate an oxidative condition or process.

RATIONALE, RESULTS & DISCUSSION

Trust those who seek the truth, doubt those who found it; doubt everything; but don't doubt yourself. – **André Gide**

RATIONALE

The overall aim of this thesis was to evaluate the effect of antioxidant supplementation in the progression of cancer, with special focus on malignant melanoma.

The specific aims of the two papers included in the thesis were:

I. **Antioxidants can increase melanoma metastasis in mice**
   The rationale behind this first paper was to assess the impact of NAC and vitamin E as dietary antioxidants on the progression of a malignant melanoma mouse model, in order to validate the hypothesis that tumors, with high endogenous ROS levels, benefit from additional antioxidant supplementation.

II. **Mitochondria-targeted antioxidants do not influence malignant melanoma and lung cancer progression in mice**
   The aim of this second study was to determine whether targeting mitochondria, the main source of cellular ROS, with antioxidant compounds would hinder cancer progression in mouse models of lung cancer and malignant melanoma.

PAPER I: ANTIOXIDANTS CAN INCREASE MELANOMA METASTASIS IN MICE

Following up on a study published by Sayin and colleagues in 2014 [76], we decided to investigate whether the accelerated proliferation observed upon antioxidant treatment was exclusive to lung cancer or if it could be extrapolated to other forms of cancer.

THE GENERAL ANTIOXIDANTS NAC AND VITAMIN E ACCELERATE METASTASIS

In this study we show that, dietary supplementation of NAC in the drinking water doubled the number of lymph metastases in BPT mice [77]. In addition, these metastases showed increased S100B and Nestin staining, both markers of malignancy [78, 79].

Concordant to our *in vivo* observations, NAC and Trolox, an analogue of vitamin E, increased migrating and invasive properties in a panel of human melanoma cell lines.

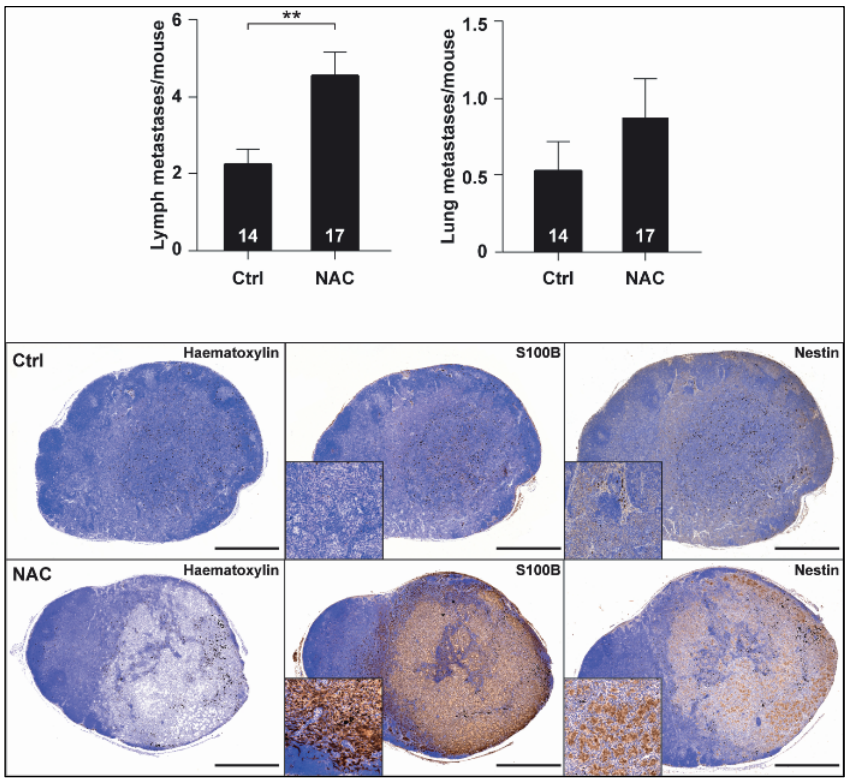

Figure 4. NAC administration increases metastasis in mice with malignant melanoma. NAC was administered in the drinking water to newly weaned BPT mice. Upper panel shows number of lymph metastases (left) and surface lung metastases (right). Lower panel shows immunochemical detection of S100B and Nestin in lymph metastases.

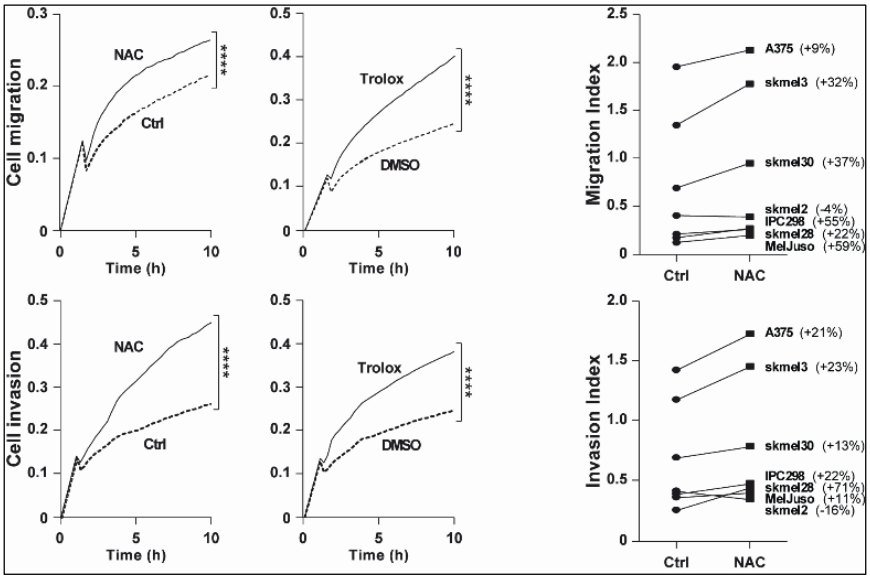

Figure 5. NAC and Trolox increase migrating and invasive properties of human melanoma cells.Real-time analysis of migration (upper panel) and invasion (lower panel) of cell line sk-mel-28. Right panels show migration and invasion indices at the 10-hour time point from real-time analyses of these parameters in seven melanoma cell lines incubated with control medium or medium supplemented with NAC.

Follow-up studies revealed that: dietary vitamin E markedly increased the number of lymph metastases but not primary tumors in mice, which was in agreement with our previous *in vitro* observations.

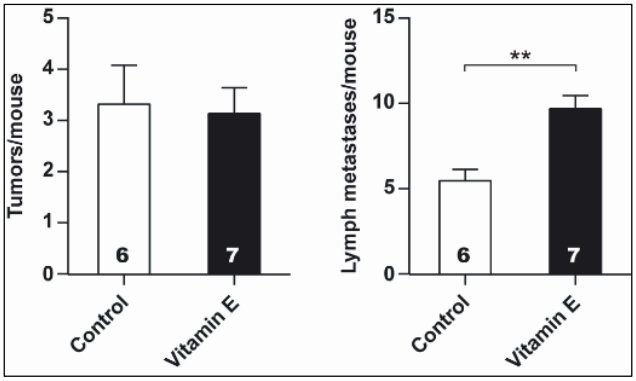

Figure 6. Vitamin E increases metastasis in mice with malignant melanoma. Vitamin E was supplemented in the chow diet to newly weaned BPT mice. Left panel shows the number of skin tumors and right panel shows the number of lymph metastases.

THE INCREASED MIGRATION DEPENDED ON GSH SYNTHESIS

Consequent with NAC supplementation, the levels of GSH were increased, but only significantly in the lymph metastases. These results were supported by *in vitro* analyses of GSH/GSSG content of antioxidant treated cell lines, and unexpectedly they were also elevated by Trolox.

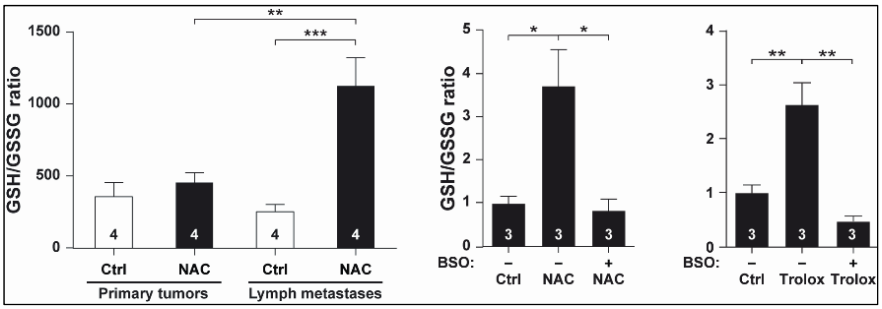

Figure 7. Levels of reduced glutathione are increased by antioxidant treatment. Left panel shows markedly increased GSH/GSSG ratios in lymph metastases of NAC treated mice. Center and right panels show increased GSH/GSSG ratios in human melanoma cells treated with NAC or Trolox.

We then proceeded to investigate whether the increased migration was dependent on GSH by inhibiting *de novo* synthesis of glutathione with buthionine sulfoximine (BSO). Indeed, upon BSO treatment migration was brought back to baseline, indicating that the increased antioxidant-triggered migration depended on GSH.

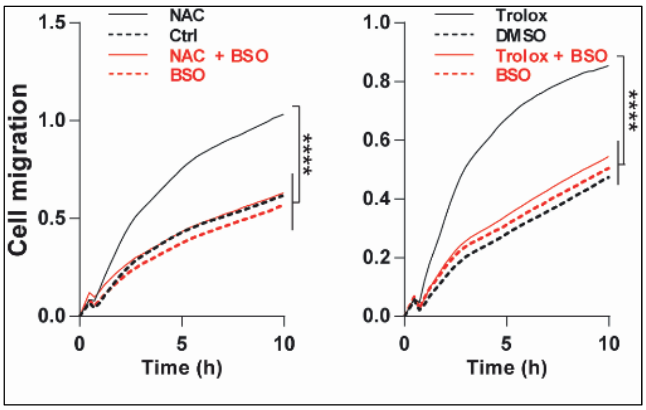

Figure 8. NAC- and Trolox-triggered migration depends on GSH. Real-time analyses of sk-mel-28 migration in response to NAC, BSO, and NAC +BSO (left panel) and Trolox, BSO, and Trolox + BSO (right panel).

Next, we looked into the RHO family proteins RHOA and RAC1, which mediate cytoskeletal changes during migration and invasion and have been shown to be redox regulated [80]. RAC1 oxidation increases its activity and promotes lamellipodia formation [81]. In opposition, oxidative stress leads to the formation of an intramolecular disulfide bridge in RHOA which prevents guanine nucleotide exchange, therefore inactivating the protein. Disulfide formation can be reversed by the addition of reductants [82]. Indeed, RHOA activity was elevated in antioxidant treated cells and further downstream signaling inhibition of Rho-associated protein kinase (ROCK) reverted the antioxidant-dependent increased migration.

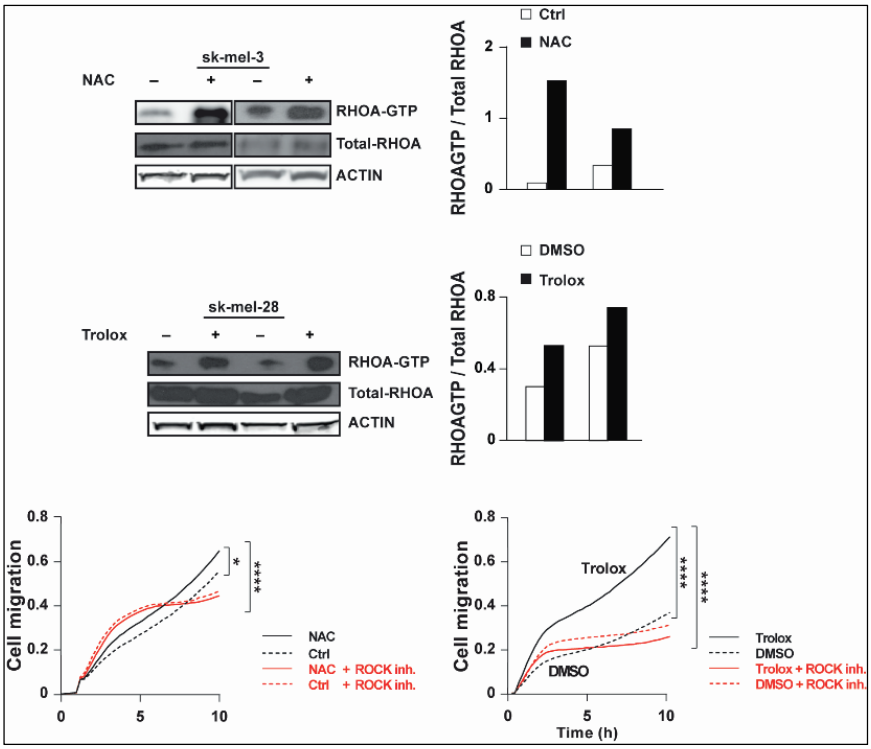

Figure 9. Antioxidant increased migration correlates with elevated levels of active RHOA. Human melanoma cells treated with antioxidants show higher levels of GTP-bound RHOA (upper and middle panels). The increased migration is reverted when cells are subjected to treatment with a ROCK inhibitor (lower panels).

CANCER PATIENTS AND SURVIVORS SHOULD AVOID ANTIOXIDANT SUPPLEMENTS

In this study we use two different antioxidants with distinct chemical structures and properties: NAC which is hydrophilic and can act as a precursor of cysteine and glutathione synthesis, and Trolox, which is a soluble analogue of the lipophilic peroxyl radical scavenger vitamin E. We additionally treated mice with chow diet containing supplementary vitamin E (in the form of Dl-α-tocopheryl acetate), although this was not included at the time in the publication. In all cases the net result was the same: mice had significantly

more metastases at endpoint, and human malignant melanoma cells migrated and invaded more.

In order to ensure that the doses administered *in* vivo were in accordance to human doses, we used a body surface area conversion [83]. NAC supplementation was in range of what it is prescribed to COPD patients and vitamin E doses were adjusted to 20 times the recommended daily intake, which can be found in vitamin supplements.

Shortly after the release of this article, other publications showed that oxidative stress limits metastasis of human malignant melanoma cells injected into immunocompromised mice [84], and it also impairs tumor invasion *in vivo* by suppressing Rho-ROCK activity through mechanisms involving p53 [85], all of which further supported our findings. Additionally, another group reported that several antidiabetic drugs with antioxidant properties accelerated metastasis in mouse models of cancer [86].

Although it remains to be seen whether these results can be directly translated into the context of human health care, all of the studies above mentioned together with the lack of evidence showing beneficial effects of antioxidant supplementation in the vast majority of cancer clinical trials suggest that cancer patients and people at risk of developing cancer should avoid the use of antioxidant supplementation [29].

PAPER II: MITOCHONDRIA-TARGETED ANTIOXIDANTS DO NOT INFLUENCE MALIGNANT MELANOMA AND LUNG CANCER PROGRESSION IN MICE

Following our observations that dietary antioxidant supplementation accelerated proliferation and metastasis in lung cancer and malignant melanoma respectively, we decided to target ROS at its main production site. Previous studies hypothesize that mitochondria-associated and not cytosolic ROS are responsible for the pro-tumorigenic signaling [87-89]. This raises the possibility of using mitochondria-targeted antioxidants to inhibit tumor growth.

In order to target mitochondrial ROS we used two different antioxidant compounds conjugated to a lipophilic cation, which ensures uptake through the phospholipid bilayer and mitochondrial accumulation by plasma membrane potential.

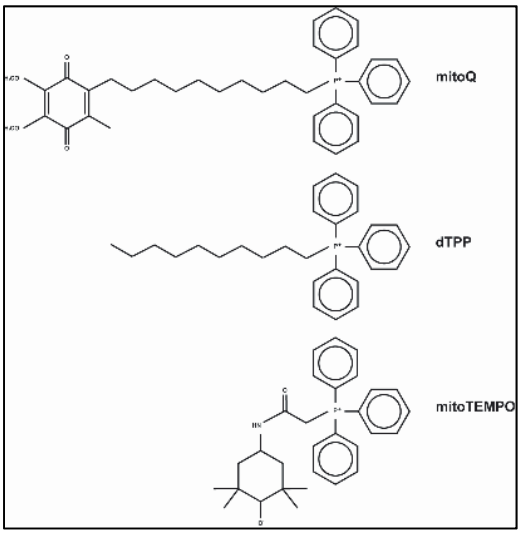

Figure 10. Chemical structure of the mitochondria-targeted antioxidants used in Paper II. The compounds mitoQ and dTPP share the same 10-carbon lipophilic cation moiety, while mitoTEMPO has a shorter chain.

MitoQ is a ubiquinone conjugated to a decyltriphenylphosphonium (dTPP) cation [90-92], that is recycled by the ETC. Its main antioxidant function is preventing mitochondrial lipid peroxidation [93], although it is also suggested that it acts upstream of H_2O_2 production [94]. MitoTEMPO on the other hand, is the combination of the antioxidant piperidine nitroxide with a lipophilic cation. It acts as a SOD mimetic and detoxifies $O_2^{\cdot}$ [95].

MITOCHONDRIA-TARGETED ANTIOXIDANTS DO NOT INHIBIT CANCER PROGRESSION

In this study we added mitoQ and its control compound dTPP to the drinking water of BPT and lung cancer mice. MitoQ treatment did not increase survival nor reduced other parameters of progression such as tumor growth or metastasis. Intra-peritoneal injection of mitoTEMPO in BPT mice however, reduced survival and accelerated the kinetics of primary tumor growth.

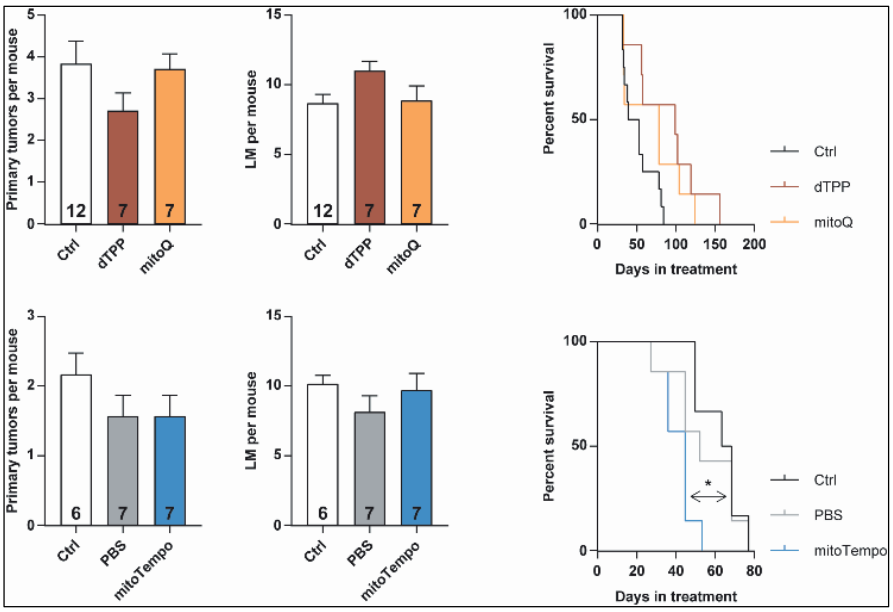

Figure 11. Mitochondrial antioxidants do not slow down malignant melanoma progression in mice. Upper panels show number of skin tumors, lymph metastases and survival of mice treated with mitoQ, dTPP and water controls. Lower panels show number of skin tumors, lymph metastases and survival of mice injected with mitoTEMPO, PBS or controls.

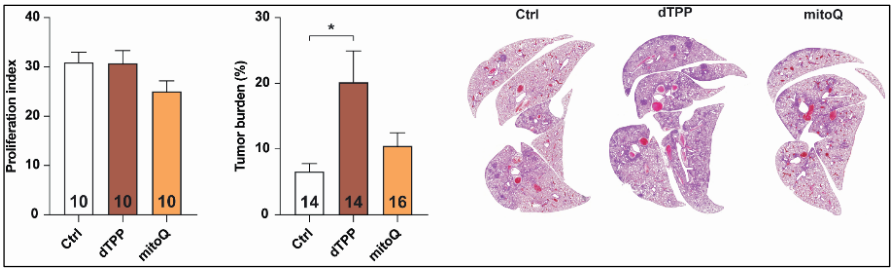

Figure 12. MitoQ does not decrease tumor burden in a mouse model of lung cancer. Left panel shows proliferation index in tumors, central panel shows tumor burden per mouse and right panel shows hematoxylin and eosin staining of mouse lungs.

In vitro results indicate that mitoQ and dTPP disrupt the ETC and affect tumor cell proliferation. As these effects are achieved by both substances, we hypothesize that they are due to non-antioxidant related cytotoxic effects. Indeed, accumulation of lipophilic cations in the mitochondrial matrix surface

of the inner membrane can disrupt membrane permeability and affect enzymatic transporter activity [96-98].

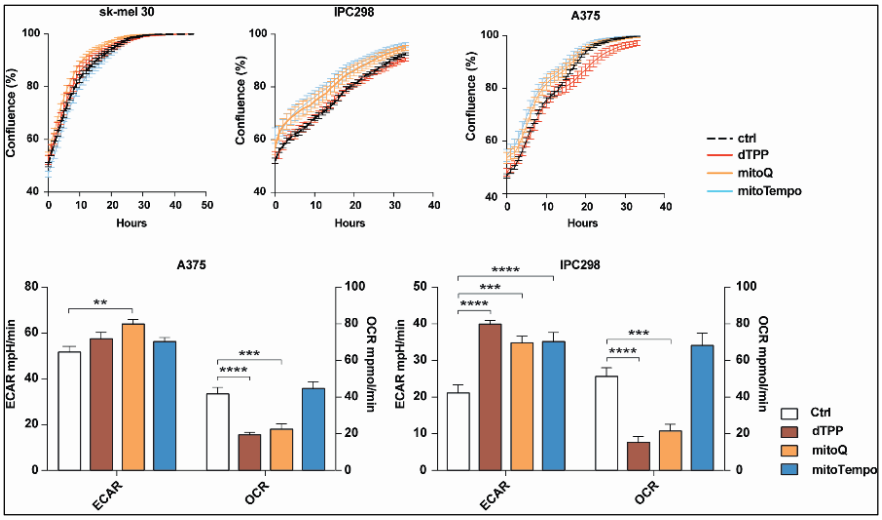

Figure 13. MitoQ effects on proliferation and ETC are non-antioxidant related. Upper panels show proliferation of mitochondria-targeted antioxidant-treated human melanoma cells over a course of 48hours. Lower panels show the effects on the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) of mitochondria targeted antioxidants on human melanoma cells.

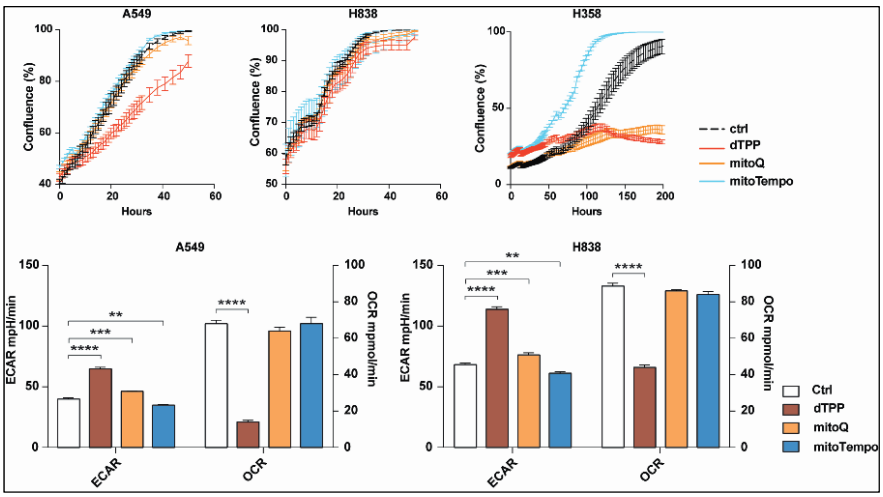

Figure 14. The mitochondria-targeting cation dTPP disrupts proliferation and ETC in human lung cancer cells. Upper panels show proliferation of mitochondria-targeted antioxidant-treated human lung cancer cells. Lower panels show the effects of antioxidant treatment on the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in human lung cancer cells.

In addition, treatment of human melanoma cells with antioxidants show significant hyperpolarization of the mitochondria at the concentrations used during proliferation assays as indicated by JC-1 staining. Furthermore, increases in membrane potential are associated with a lower respiration rate and increased ROS production at complexes I and III of the ETC [99-101], indicating that the accumulation of the compound in the mitochondria might render an effect opposite to the one desired.

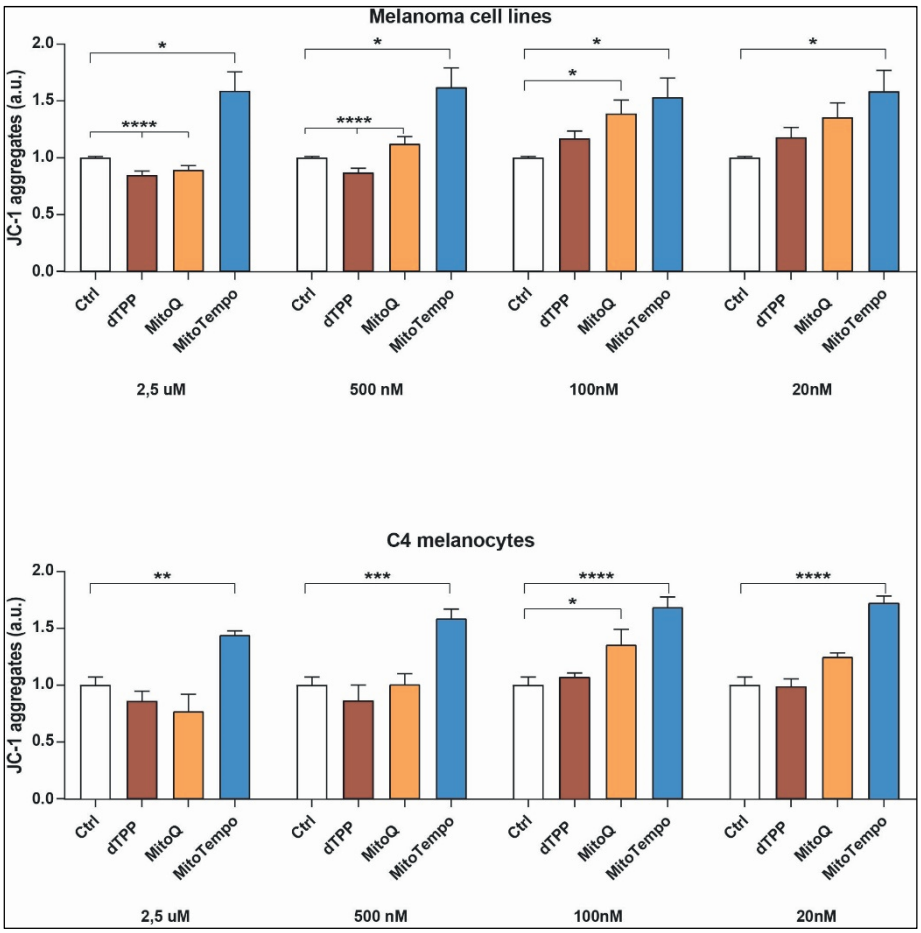

Figure 15. Accumulation of JC-1 aggregates in the mitochondria of antioxidant-treated human melanoma cells and melanocytes. Upper panel shows the mean of 4 different melanoma cell lines with 3 wells per treatment and cell line and 15 fields of view per well. Lower panel shows the mean of human C4 melanocyte with 3 wells per condition and 15 fields of view per well. Increases in aggregates indicate higher mitochondrial membrane polarization.

Interestingly, oxygen consumption was not affected by mitochondria-targeted antioxidants in lung cancer cells, and only mitoQ but not mitoTEMPO affected it in melanoma cells.

To look at the possibility that mitochondria-targeted antioxidants might increase ROS production, we used different genetically encoded biosensors in human melanoma cells. We found that cytosolic oxidation was increased

at basal levels after 48 hours of treatment with mitoTEMPO in both cell lines assayed.

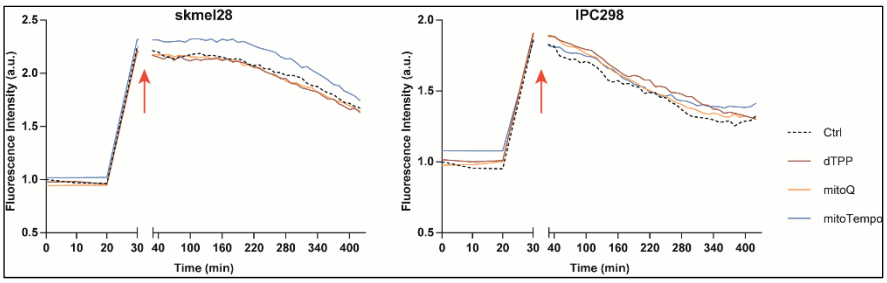

Figure 16. Ratio of cytosolic glutathion oxidation/reduction in human melanoma cells assessed with a Grx1-roGFP2 biosensor. Red arrows indicate addition of diamide to induce further oxidation. Incubation of cells with 100 nM mitoTEMPO for 48 hours induced cytosolic oxidation in both cell lines.

MITO-TEMPO INCREASES CYTOSOLIC OXIDATION

Our results suggest that by acting as a SOD mimetic, mitoTEMPO might detoxify $O_2^{\cdot}$ to H_2O_2 which can then diffuse to the cytosol where it can act as a signaling pathway regulator. In order to look further into the role of mitoTEMPO as a ROS scavenger, we used genetically encoded biosensors to look into differences in H_2O_2 content in the mitochondria of human melanoma cells.

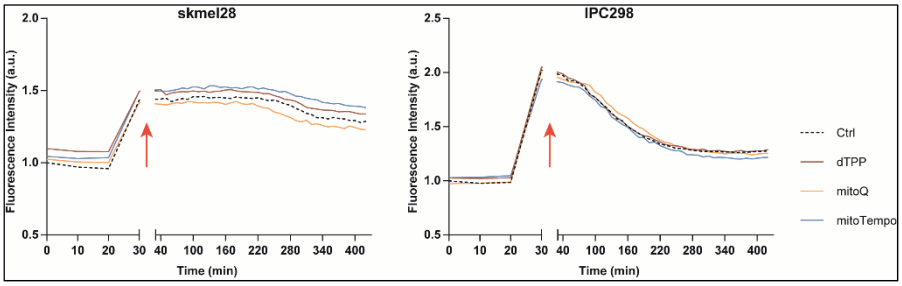

Figure 17. Ratio of mitochondrial oxidation assessed with the H_2O_2- sensitive Orp1-roGFP2 biosensor in human melanoma cells. Red arrows indicate addition of diamide to induce further oxidation. Incubation of cells with 100 nM mitoTEMPO and dTPP for 48 hours induced mitochondrial oxidation in both cell lines.

Additionally, gene expression analysis of primary tumors from mitoTEMPO –treated mice showed increased expression of *Krt1*, *Alb*, *Gpx2*, *Duox1*, *Ucp3*, *Mb* and *Hspa1a* when compared to their control counterparts. Although indirectly, these gene expression changes indicate a response to increased ROS levels; Keratin 1 (*Krt1*) levels have been shown to increase under H_2O_2 stimulation [102], albumin (*Alb*) is a reported oxygen scavenger *in vivo* [103, 104], glutathione peroxidase 2 (*Gpx2*) is a H_2O_2-reducing enzyme that has been linked to increased metastasis [105, 106], and the uncoupling protein 3 (*Ucp3*) can mildly uncouple the ETC to reduce mitochondrial ROS levels [107-110]. Myoglobin (*Mb*) is inactivated at protein level by oxidation, although it has also been reported to propagate oxidation by interacting with hydrogen peroxide, and the increased transcriptional levels observed might

be a compensatory mechanism to such protein inhibition [111, 112]. The heat shock protein family A member 1A (*Hspa1a*) has been reported to participate in the removal of proteins damaged by oxidation [113]. Interestingly, the H_2O_2 producing dual oxidase 1 (*Duox1*) was also overexpressed. This result is in opposition to what Dikalov and colleagues have previously described on how blocking mitochondrial $O_2^{\cdot}$ production with mitoTEMPO downregulated cytosolic $O_2^{\cdot}$ production by NOXes, breaking a forward feed loop [114].

Overall we conclude that mitoTEMPO acts as a mitochondrial antioxidant/cytosolic pro-oxidant in our system. To validate such hypothesis we could isolate mitochondria and look at excreted H_2O_2 upon mitoTEMPO treatment. If our hypothesis was confirmed, we could conditionally overexpress catalase in human melanoma cells *in vitro* or in BPT mice *in vivo* to see whether the phenotypes observed can be reverted.

MITOCHONDRIA-TARGETED ANTIOXIDANTS: THESE ARE NOT THE COMPOUNDS YOU ARE LOOKING FOR

Previous studies have shown that mitochondria-targeted antioxidants could potentially inhibit tumor development. Indeed, combined inhibition of mitochondrial ROS and glycolysis successfully decreased ATP production and induced apoptosis in hepatocellular carcinoma [30]; targeting mitochondrial ROS decreased KRAS-mediated tumorigenicity by increasing ERK 1/2 signaling [32]: it also reversed superoxide-dependent migration upon partial ETC inhibition [31].

Although promising, none of these studies have evaluated the impact of such compounds in transgenic mouse models. Even with similar doses, neither mitoQ nor mitoTEMPO blocked disease progression. In fact, mitoTEMPO

decreased survival which is in concordance with the work of Wang and colleagues, where mitochondria-targeted antioxidants aggravated tumorigenesis by affecting DNA-damage repair in a chemically induced model of hepatocellular carcinoma [86].

In addition, our *in vitro* results show that no direct translation can be drawn to an *in vivo* context, which might explain the conflict with previous studies. Furthermore, the effects observed with mitoQ treatment were recapitulated by the control substance, suggesting that the decrease in proliferation observed is related to cytotoxic effects coupled to the targeting moiety rather than to antioxidant properties of the ubiquinone. It has been proposed that genetic therapy with alternative oxidase, an enzyme present in plants and lower animals, could potentially reduce mitochondrial ROS formation by bypassing the ETC when disrupted and maintaining the electron flow and redox homeostasis in the cell [115, 116]. However, preliminary histological data indicates that the ETC complexes remain unaltered in the mitochondria-targeted antioxidant-treated mice, questioning the usefulness of such treatment in our model (data not shown).

Mitochondrial antioxidants have been successfully used in other areas and models, such as in acute hypoxia, inflammation, cardiovascular diseases, and ischemia reperfusion [117-120], but our results demonstrate that they are unlikely to be useful in cancer therapy.

GENERAL DISCUSSION

&

FUTURE WORK

One never notices what has been done; one can only see what remains to be done. – **Marie Sklodowska-Curie**

THE ANTIOXIDANT/ROS DOGMA NEEDS TO BE RECONSIDERED

ROS are not only damaging products, they are important players in the maintenance of cell signaling and homeostasis.

Antioxidant supplementation has been traditionally seen as a way to protect against oxidative stress-related damage. However, antioxidants protect both healthy and tumor cells. The latter have elevated levels of ROS and rely on antioxidant defenses to protect themselves from further damage. Antioxidants give them the additional help they need.

In paper I we show that general antioxidants supplied in the diet accelerate metastasis in *in vivo* and *in vitro* models of malignant melanoma.

In paper II we show that mitochondria-targeted antioxidants did not inhibit cancer progression *in vivo*. In fact, one of the compounds, mitoTEMPO, reduced survival of mice with malignant melanoma and this was accompanied by increased levels of cytosolic H_2O_2.

ARE HYPOTHETICAL BENEFITS OF ANTIOXIDANTS WORTH THE RISK?

Clinical trials have consistently failed at showing the value of antioxidant supplementation for the prevention and treatment of cancer. In fact, meta-analysis studies of clinical trials show that antioxidant supplementation lacks support for beneficial effects and may increase mortality of certain forms of cancer [121-124].

In addition, there is a widespread use of antioxidant supplements by cancer patients [125-128] partially to alleviate toxic radiotherapy and chemotherapy side-effects, but also prompted by the popular conception that antioxidants help fighting cancer. Interestingly, the published literature suggests growing

concern and debate amongst clinicians on the potential interference of such supplements with therapy that relies on the production of ROS and induction of apoptosis [129-133].

Furthermore, although some experimental studies of chemically- and radiation-induced cancers have displayed potential therapeutic effects of the use of antioxidants [134, 135], there is an increasing body of evidence showing their role in the acceleration of progression [136-138].

Overall, there is no doubt that redox regulation plays an important role in the development and progression of cancer. We therefore think that the study of redox-regulated pathways, proteins and genes might reveal new drug targets and offer new and reliable therapeutic possibilities [139].

FUTURE WORK

One of the main difficulties in the field is the study of redox reactions *in vivo.* Indeed, we have to rely on methods that can give an overall idea of whether certain conditions are pro-oxidative or reductive. As described in the methods section, the use of genetic encoded biosensors has revolutionized the field by giving the possibility to analyze when and where in the cell these redox reactions occur. This tool is now being expanded to *in vivo* models in Tobias Dick´s group, where genetically encoded biosensors have been stably expressed in mouse tissues [140]. This opens many possibilities if combined with our cancer models, since it would be easier to pinpoint where and when during the development of the disease redox alterations occur with and without the use of antioxidants.

For instance, in Paper I we saw that the increased migrating and invasive properties of cancer cells were dependent on new synthesis of GSH [141]. We also saw that the GSH/GSSG ratio was increased in lymph metastases of NAC treated mice according to a whole cell extract assay. It would be

interesting to combine the BPT model with the expression of a glutathione biosensor to observe whether this increase is particular to the cells that survive transit from the primary tumor to the lymph nodes, or whether it is only those cells within the primary tumor that show increased GSH that migrate. It would also allow us to see what happens with the rest of the cells in the neoplastic niche. For instance, it is well known that redox regulation plays an important role in the vascularization of tissue. It has been proposed that low levels of ROS can stimulate angiogenesis and therefore influence tumor progression [142]. Indeed, the accelerated growth kinetics observed in mitoTEMPO-treated mice might be related not so much to tumor cell proliferation in itself as to a better vascularization of the neoplastic tissue, prompted by the excretion of H_2O_2.

Another important point from Paper I was that migration was also dependent on RHOA signaling, and we hypothesize that this increase in signaling is due to either the inhibition by reduction of RAC1 (and hence de-repression of RHOA) or activation of RHOA by reduction. But we cannot rule out other effects of redox regulation of the cytoskeleton. One way of analyzing this would be to study the thiol proteome by mass spectrometry and study potentially GSH-regulated cysteines.

We could combine these results with RNAseq analysis of primary tumors and lymph metastases from NAC and vitamin E treated mice, to get a better landscape of redox regulation by antioxidant supplementation. Although the phenotype exhibited by both treatments is the same, we cannot rule out that the underlying mechanisms are different.

To that end, it would be interesting to perform the same experiments in immunodeficient mice to rule out the possibility that effects on the immune system are responsible for the observed metastasis.

In Paper II we observed a decreased survival by mitoTEMPO and we argue that growth kinetics were affected by the treatment. To challenge this idea, we are now repeating a new study where mice will be sacrificed after five weeks of treatment. We also observed that scavenging of mitochondrial $O_2^{\cdot}$ resulted in increased levels of cytosolic ROS and we hypothesize that this in turn triggers cellular signaling cascades that accelerate growth. To verify the hypothesis we could overexpress a mitochondrial catalase to decrease pro-tumorigenic signaling from mitochondrial H_2O_2 or isolate mitochondria and measure H_2O_2 excretion.

In addition to these studies, we are interested in comparing different methods of antioxidant delivery to the skin in the context of malignant melanoma. Skin lotions often contain different forms of antioxidants, whether with the purpose of stabilizing formulation and avoiding rancidity or with the promise of improving skin texture and condition. We are interested in studying how these will affect malignant melanoma progression. I have administered vitamin E in the diet, and as a lotion to BPT mice, and I am going to test dietary mitochondrial targeting with mitoE.

ACKNOWLEDGEMENT

What is considered the end of the world for a caterpillar is the beginning of Mariposa´s story **– Sayin, 2018.**

All good stories come to an end, and what a journey this one was! I would like to thank, in no particular order, the following people for keeping me good company along the way:

My supervisor, **Martin Bergö**, for believing in my potential when I was about to give up and allowing me to develop into an independent researcher.

My co-supervisors, **Levent Akyürek** and **Per Lindahl**, for all the fruitful scientific discussions and career advice over the years.

The current and previous members in the Bergö lab, for all the time (and it´s been a lot of hours) shared together. **Martin Dalin,** for introducing me to the BPT model, always with a smile on your face, even though you had to write your own thesis. **Christin**, for making sure I´m feeling fine and I have everything I need to do my work. You´re the best lab-mum ever! **Volkan "Just" Sayin**, you have been a friend, a mentor, and one of my biggest supports during these years. Thank you for the laughs, the hugs, and the Hello Kitty candy! **Clotilde**, thank you for walking the redox rocky road with me, even though we get mixed up sometimes. Your talent and ambition inspire us all, but what I like the most is your sarcasm and availability to get drinks with me! **Ella**, for always making sure everyone is feeling well and included. You´re beautiful both outside and inside. **Mohamed**, for welcoming me to the group before it was even official. I sincerely don´t know what´s bigger, your brain or your heart. **Murali "baba"**, for singing, huffing and puffing around the lab, and bringing joy to our lives! **Jaroslaw**, for the scientific discussions and twisted sense of humor. We definitely need the same centrifuge as in CSI. **Tony**, for all the spontaneous neck massages, your energy, your friendship

and trust. We will for sure fix your "problem". **Oskar "den starke"**, for all your hard work and help with the mice and stubborn bottles. We wouldn't be able to do all this without you! **Julia**, **Josefin**, and **Louise**, for lightening up the mood in the lab! Your enthusiasm and hunger for knowledge is contagious.

The members of SCC for creating such a great research family! **Camilla**, for the words of wisdom (and the heavy metal!). **Mattias**, for the math lessons: double nothing is still nothing. **André**, for all the insightful conversations. It´s gonna be great to have you back. **Patricia**, for all the advice and the WhatsApp chats! I really wish you had stayed around longer. **Jonas**, **Lisa**, **Berglind**, **Som, Joy**, **Elin**, **Gülay**, **Emman**, **Yvonne**, **Tobias**, **Gautam**, **Dorota**, **Agnieszka**, and **Toshima** for the fun times at the sixth floor. **Emma**, **Paloma**, **Tajana**, and **Marta**, for being awesome office mates and cheering up my days. **Anna**, **Karoline**, **Christoffer**, **Sara**, **Emma**, **Stefan**, **Mamen**, **Elena**, **Gustav**, **Pernilla**, **Malin**, **Shawn**, **Valerio**, **Ágota**, **Daniel**, **Sara**, **Andreas**, and **Mila**, for the lunch and corridor talks. **Paul**, for the singing, the documentary suggestions, and for sharing a dark sense of humor with me. You make me feel a little bit more normal (but not too much).

Aditi, **Ahmed**, **Octavia**, **Sally**, **Jonathan** and **Peidi**, for being friends with this lab-addict and all the happy moments shared over the years. Let's create some more soon?

Lydia and **Dzeneta**, for inspiration. You two are amazing in every aspect possible. Never forget that, and never let anyone take that away from you. **Amiko** and **Tomiko**, for being extraordinary friends despite the distance. Thinking about all the moments we had together in the lab always warms my heart.

Soheila, for friendship and opening the doors to your "fun place". Thanks for trusting me despite my proficiency at winning lying games! **Maryam**, for

being kindness personified. I´m blessed to count you among my friends. **Ángela** and **Esther**, for always being there for me no matter where, when or why. Words cannot tell how thankful I am for having you both in my life. **Roberto**, **Llanos**, and **Sabrina**, for always taking the time to meet and listen to me whenever I travel back home. You guys are gold.

My parents, **Michel** and **María Teresa**, for being the best teachers one could ever wish for. I owe you so very much, and I hope I will be able to repay you some day. My grandparents, **Encarnación** and **José**, and my auntie, **Tere**, for all the love and care. My brother, **Mikael**, for being my first lab assistant and for your unconditional support. You know you will always have mine. I love you all more than words can tell.

And last, but absolutely not least: **Emil**. Thank you for shining bright when I'm at my darkest, and making me laugh when I don't even feel like smiling. For your patience with this free and radical "Spanish temper" of mine. For love.

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