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Review

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Oxidative stress and antioxidants in the pathophysiology of malignant melanoma

Abstract: The high number of somatic mutations in the
melanoma genome associated with cumulative ultra vio-
let (UV) exposure has rendered it one of the most diffi-
cult of cancers to treat. With new treatment approaches
based on targeted and immune therapies, drug resist-
ance has appeared as a consistent problem. Redox biol-
ogy, including reactive oxygen and nitrogen species (ROS
and RNS), plays a central role in all aspects of melanoma
pathophysiology, from initiation to progression and to
metastatic cells. The involvement of melanin production
and UV radiation in ROS/RNS generation has rendered the
melanocytic lineage a unique system for studying redox
biology. Overall, an elevated oxidative status has been
associated with melanoma, thus much effort has been
expended to prevent or treat melanoma using antioxi-
dants which are expected to counteract oxidative stress.
The consequence of this redox-rebalance seems to be two-
fold: on the one hand, cells may behave less aggressively
or even undergo apoptosis; on the other hand, cells may
survive better after being disseminated into the circulating
system or after drug treatment, thus resulting in metasta-
sis promotion or further drug resistance. In this review we
summarize the current understanding of redox signaling
in melanoma at cellular and systemic levels and discuss
the experimental and potential clinic use of antioxidants
and new epigenetic redox modifiers.

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Introduction

Human malignant melanoma is an extraordinarily aggres-
sive cancer. The biological basis for the general pharma-
cological resistance of human melanoma, as well as its
ability to adapt to changing microenvironments and to
evade the host response and immunotherapy, remain
poorly understood. Nevertheless, reactive oxygen species
(ROS) and oxidative stress appear central in melanoma
pathophysiology (Liu-Smith et al., 2014).

A unique biochemical feature of the melanocyte is the
generation of melanin, which leads to the generation of
hydrogen peroxide and the consumption of reduced glu-
tathione (GSH). Successful attempts to reverse or inhibit
this process have been initiated (Jenkins and Grossman,
2013) but to date have not been clinically tested.

Malignant melanoma is one of the most aggressive
cancers with a high frequency of metastases at advances
states (Shain and Bastian, 2016). Malignant transforma-
tion of melanocytes is predominantly a disease of the skin
but may also occur at other sites, including the mucous
membranes and the eyes (Bandarchi et al., 2010). Mela-
noma is most likely due to a multistep process of genetic
mutations that alter the cell cycle and render the melano-
cytes more susceptible to carcinogens [mainly ultra violet
(UV radiation)] (Bandarchi et al., 2010). It is also well

known that UV radiation causes ROS-mediated oxidative stress (Sander et al., 2004).

ROS are increased in many different murine and human tumors (Szatrowski and Nathan, 1991; Meierjohann, 2014). The seminal paper by Szatrowski and Nathan concludes that production of large amounts of hydrogen peroxidase (H_2O_2) by human tumor cells was inhibited by diphenyleneiodonium, a flavoprotein binder and diethyldithiocarbamate, a divalent cation chelator, but not by cyanide or azide, inhibitors of electron transport, or by agents that inhibit xanthine oxidase, polyamine oxidase, or cytochrome P450. Thus, suggesting that ROS source is a flavin containing enzyme and not a metabolic process.

Moreover, both intracellular as well as extracellular ROS (e.g. as those produced by phagocytes or the NADPH oxidase-derived extracellular superoxide anions) must be taken into account. Consequently, generation of large amounts of ROS, if it occurs *in vivo*, might contribute to the ability of aggressive cancer cells to mutate, self-renew, inhibit antiproteases, injure local tissues, and therefore promote tumor heterogeneity, invasion, and metastasis. As a result, aggressive cancers maintain high basal levels of ROS compared to normal cells (Liou and Storz, 2010). Paradoxically while high levels of ROS can cause oxidative stress and induce cell death, low levels of superoxide and H_2O_2 can promote G1→S cell cycle transition in different cell systems (Burdon, 1995). Indeed, oxidative stress or redox status shifts may cause cell transition from quiescent to proliferative status, growth arrested or cell death activation according to the duration and extent of the redox imbalance (Ekshyyan and Aw, 2005). There is strong evidence of increased ROS levels in melanoma cells (Liu-Smith et al., 2014). In this regard, as introduced already, extracellular ROS generated by melanoma cells also have pathophysiological implications. For example, it has been shown (Bittinger et al., 1998) that superoxide anions produced by melanoma cells were scavenged by added superoxide dismutase (SOD). As the enzyme cannot penetrate intact cells, this is an indication for extracellular superoxide anion generation (in line with an analogous situation in many other tumor systems).

Therefore, it could be concluded that the use of antioxidants could be a good therapeutic option against melanoma. Besides, therapeutic strategies that promote oxidative stress and eventually tumor cells death have been explored based on the availability of chemotherapy agents that directly induce oxidative stress or inhibit ROS-scavenging systems (Hajibabaei, 2016). These options are reason for an intense debate. This review will analyze the role of ROS (and related species) and antioxidants in regulating melanoma progression, as well as both the beneficial and detrimental effects of using pro-oxidants or antioxidants in melanoma therapy.

Furthermore, we will discuss the role of ROS in melanocyte biology, the role of melanin synthesis in the biology of melanomagenesis, the contribution of disordered melanin synthesis and melanosomal organelle dysfunction as major contributors to the misregulated ROS status in melanoma cells and its contribution to this state and therapeutic resistance.

Oxidative stress and the intracellular redox state in melanoma initiation and progression

An imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to biological damage, is termed 'oxidative stress' (Sies and Cadenas, 1985; Sies, 1997). Oxidative stress in cells is linked to the generation of ROS, formed as natural byproducts of the normal metabolism of O_2 but having key roles in cell signaling and homeostasis (under both physiological and pathophysiological conditions) (Ray et al., 2012) (Figure 1). The sequential reduction of O_2 through the addition of electrons leads to the formation of a number of ROS including: superoxide ($O_2{}^{·-}$), H_2O_2, hydroxyl radical (OH·), or hydroxide ion (OH⁻) (Sies and de Groot, 1992). A rise in intracellular ROS levels has two potentially important effects: damage to various cell components and triggering of the activation of specific signalling pathways. Both of these effects can influence numerous cellular processes linked to carcinogenesis and cancer progression (Milkovic et al., 2014; Gill et al., 2016). Consequently, cells contain antioxidant metabolites (i.e. ascorbic acid, glutathione, lipoic acid, uric acid, carotenes, vitamin E, coenzyme Q) and enzymes (i.e. superoxide dismutases, catalase, peroxiredoxins, sulfiredoxins, and thioredoxin and glutathione systems) that work to prevent oxidative damage and cell death (Davies, 2000; Sies et al., 2017; Hegedűs et al., 2018).

Nevertheless, given the enormous variety and range of prooxidant and antioxidant enzymes and compounds, attempts have been made to classify subforms of oxidative stress and to conceptually introduce intensity scales ranging from physiological conditions (eustress) to excessive and toxic oxidative burden (oxidative distress) (Sies et al., 2017). In addition to ROS, other reactive species have notable impacts on redox biology and, consequently, on oxidative stress. These include the reactive nitrogen species (RNS) [starting with the reaction of nitric oxide
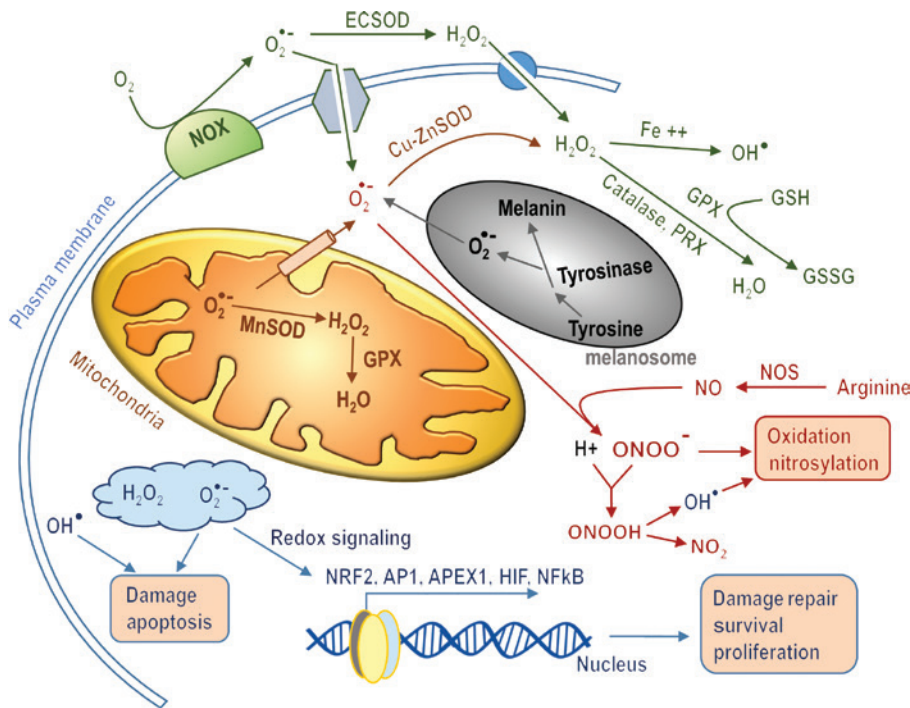
Figure 1: Simplified outline of generic generation and management of ROS in melanocytes and melanoma: The major known source of ROS in melanocytes and melanoma cells includes the mitochondria, melanosomes, the NOX enzymes and the NOS enzymes.
Superoxide is converted to hydrogen peroxide via ECSOD (extracellular), Cu-ZnSOD (cytoplasmic) and MnSOD (mitochondrial), dependent on its subcellular locations. Note that although NOX is shown on plasma membrane, different isoforms are reported to be located in other subcellular compartments. Superoxide passes cellular membrane via specialized membrane pore systems while hydrogen peroxide can diffuse freely or pass through membranes via aquaporins (Bienert et al., 2006, 2007). Hydrogen peroxide is metabolized by catalase, GPX (glutathion peroxidases) and PRX (peroxiredoxins). In the presence of ferrous irons, hydrogen peroxide can generate hydroxyl radical via the Fenton-reaction. In the presence of NO, superoxide forms peroxynitrite which oxidizes or nitrosylates proteins. Peroxynitrite can be protonated and the resulting peroxynitrous acid can be decomposed into hydroxyl radical again which also oxidized proteins. Excessive pools of ROS damage cells and induce apoptosis. However, hydrogen peroxide and superoxide both can serve as signaling molecules to trigger redox signaling cascade involving NRF2, AP1, APEX1, HIF, and NFκB transcriptional factors for damage repair, survival, and proliferation.

(˙NO) with $O_2^{\cdot-}$ to form peroxynitrite (ONOO⁻), Figure 1] (Kruk and Aboul-Enein, 2017), reactive sulfur species (RSS) (i.e. persulfides, polysulfides and thiosulfate, which can oxidize and inhibit thiol-proteins and enzymes) (Nishida et al., 2016), reactive carbonyl species (RCS) (i.e. α,β-unsaturated carbonyl compounds and dialdehydes, which are highly reactive and damage proteins, lipids, and nucleic acids) (Ellis, 2007), reactive selenium species (RSeS) (i.e. selenium nanoparticles and simple selenium salts, seleninic acids, selenoureas and selenoesters, but also multifunctional selenium-based redox catalysts) (for instance, $O_2^{\cdot-}$ and H_2O_2 are byproducts arising from the reaction of selenite and selenocystine with GSH) (Brozmanová et al., 2010), and also chlorine and bromine species (for instance, the UV/chlorine process generates primary radicals of HO˙ and Cl˙ and secondary chlorine-containing radicals such as $Cl_2^{\cdot-}$, ClOH⁻ and ClO˙; whereas it has been shown that, e.g. bromination causes protein oxidative damage) (Halliwell, 2006). In addition, other potential pro-oxidants need to be taken also into account, i.e. transition metals, such as Mg^{2+}, Cu^{2+} or Fe^{2+} (Valko et al., 2016); pro-oxidant vitamins (e.g. vitamin C has anti-oxidant activity when it reduces oxidizing substances such as H_2O_2, however, at high doses it can also reduce metal ions leading to the generation of free radicals through the fenton reaction) (Uetaki et al., 2015); some anticancer drugs (i.e. adriamycin and other anthracyclines, bleomycin, and cisplatin which bind to DNA and generate ROS) (Kovacic and Wakelin, 2001); or quinones (highly redox active molecules which can redox cycle with their semiquinone radicals, leading to formation of ROS) (Benz et al., 2006). The chemical reactivity of the many different reactive species may vary by up to 11 orders of magnitude when assayed against a given target [with comparable rate constants of $7 \times 10^9\, l \times mol^{-1} \times s^{-1}$ for hydroxyl radicals (HO˙) versus $2 \times 10^{-2}\, l \times mol^{-1} \times s^{-1}$ for H_2O_2] (Sies et al., 2017).

Redox state is often used to describe the balance of GSSG/2GSH, NAD^+/NADH and $NADP^+$/NADPH in cells or organs, and is reflected in the balance of several sets of metabolites (e.g. lactate and pyruvate, β-hydroxybutyrate, and acetoacetate), whose interconversion is dependent on these ratio (Krebs and Veech, 1969; Bücher et al., 1972; Schönfeld et al., 1983; Sies et al., 1983; Jones and Sies, 2015). Furthermore, redox proteins and their genes must be co-located for redox regulation according to the 'co-location for redox regulation' hypothesis, which means that the location of genetic information in cytoplasmic organelles permits regulation of its expression by the reduction-oxidation state of its gene products (Allen, 1993).

Cutaneous melanoma arises from epidermal melanocytes in skin, which is a relatively hypoxic tissue. ROS are generated as a result of increased metabolism of transformed cells, immune reaction against the developing tumor, UV radiation, melanin production, and an altered antioxidant system (Wittgen and van Kempen, 2007). ROS in melanocytes can be derived from mitochondria, melanosomes, NADPH oxidase (NOX) family enzymes, different arachidonic acid oxygenase activities, and nitric oxide synthase (NOS) activity/uncoupling (Denat et al., 2014) (Figure 1). Epidermal melanocytes are particularly vulnerable to oxidative stress owing to the pro-oxidant state generated during melanin synthesis, and to the intrinsic antioxidant defenses that are compromised in pathologic conditions.

The presence of melanin in the skin appears to be a double-edged sword: it protects melanocytes as well as neighboring keratinocytes in the skin through its capacity to absorb UV radiation, but its synthesis in melanocytes results in higher levels of intracellular ROS that may increase melanoma susceptibility (Jenkins and Grossman, 2013). Interestingly it has been reported that H_2O_2 is an inducer of elevated tyrosinase levels (an oxidase that is the rate-limiting enzyme for controlling the production of melanin) in melanoma cells (Karg et al., 1993).

Pheomelanin is a lighter pigment form of melanin that produces ROS upon exposure to UV radiation. Black eumelanin can negate this effect if present in sufficient quantity. Thus, higher levels of pheomelanin versus eumelanin (presenting as lighter colored skin) can contribute increased ROS burden and increased risk of carcinogenesis (Shain and Bastian, 2016). Further, MC1R signaling, which is responsible for eumelanin production, also promotes DNA repair and clearance of ROS. Thus, reduced MC1R signaling not only promotes ROS formation upon UV exposure, it also weakens the cells ability to deal with the ROS burden efficiently (Shain and Bastian, 2016). In addition, UV-B exposure of melanin in the presence of metal ions can result in a pro-oxidant activity for melanin

(McNulty et al., 2004; Gidanian et al., 2008). Evidence for the mechanism of melanin 'switching' from its natural anti-oxidant state to the dysfunctional pro-oxidant form was demonstrated by showing UV-B exposure causes morphological changes and bleaching of the melanosome (where melanin is formed). Both of these effects were dramatically increased with co-treatment of the metal ions Cu^{2+} or Cd^{2+} (Gidanian et al., 2008).

Current evidence suggests that NOX1, NOX4 and NOX5 are expressed in melanocytic lineage. While there is no difference in NOX1 expression levels in primary and metastatic melanoma tissues, NOX4 contributes to transformation phenotype of melanoma cells by regulating G2-M cell cycle progression; thus, suggesting specific signals and effects for NOX family enzymes in melanoma (Yamaura et al., 2009). In this sense it has been found that ROS generated by the NADPH oxidase, most likely NOX4, transmits cell survival signals on melanoma cells through the FAK pathway (focal adhesion kinase, a protein that in humans is encoded by the *PTK2* gene), maintaining adhesion contacts and cell viability (Ribeiro-Pereira et al., 2014). Possible differences in signals and effects involving other families of oxidative stress related-enzymes should also be considered and studied.

Inhibitors of both the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism convert mouse melanoma to a non-invasive state by reducing the production of MMP-2, an enzyme required for the degradation of basement membranes (Reich and Martin, 1996). Specific metabolites of each pathway, i.e. PGF2 and 5-HPETE, are able to transcend the block and restore collagenase production, invasiveness *in vitro* and metastatic activity *in vivo* (Reich and Martin, 1996).

Moreover, leukotriene B4 (LTB4), the main product of the 5-lipoxygenase pathway, was able to induce growth of subtumorigenic inocula of melanoma cells, and a LTB4 receptor antagonist inhibited acute inflammation-associated tumor growth (Bachi et al., 2009). Therefore, specific arachidonic acid-related pathways may be required for the invasive and metastatic activity of malignant melanoma cells.

In contrast with normal tissues, an increase in superoxide and peroxynitrite generation associates to higher NOS activities of tumor cells (including melanoma). Based on high-performance liquid chromatography analysis, the underlying mechanism for this uncoupling is a reduced tetrahydrobiopterin:dihydrobiopterin ratio (BH4:BH2) resulting in important tumor growth promoting and anti-apoptotic signaling properties (Rabender et al., 2015). In addition, eNOS uncoupling seems to play a pivotal role in melanocyte malignant transformation induced by sustained anchorage impediment, because no malignant transformation was

observed when L-NAME-treated melanocytes were subjected to sequential cycles of de-adhesion (Melo et al., 2011).

On the other hand, maintenance of cellular redox balance also depends on enzymes that directly remove ROS by providing essential reducing equivalents (Kehrer and Lund, 1994). Melanoma cells indeed overproduce superoxide anions (Bittinger et al., 1998). Interleukin (IL)-1, which inhibits the growth of human melanoma A375 cells, was found to induce selectively and predominantly mitochondrial SOD2 activity in the melanoma cells and also in normal skin fibroblasts and peripheral blood mononuclear cells (Masuda et al., 1988). Later it was reported that SOD2 can be induced by interferon (IFN)-γ in multiple cell types, and synergistically in combination with tumor necrosis factor (TNF)-α or IL-1. In agreement with this it has been observed that overexpression of SOD2 promotes the survival of melanoma cells exposed to IL-1, TNF-α, selected anticancer drugs, and ionizing radiation (Hirose et al., 1993); and that increased SOD2 expression suppresses the malignant phenotype of human melanoma cells (Church et al., 1993), all facts linking ROS control and intercellular cytokine-dependent signaling in melanoma progression. Moreover, overexpression of SOD2 may also promote the survival of melanoma cells exposed to chemotherapy (Suresh et al., 2003).

GSH levels in mitochondria are maintained by glutathione reductase, and NADPH is an essential reducing equivalent for enzyme-linked GSH recycling (Kurosawa et al., 1990). This is important because mitochondria do not synthesize GSH but take it up from the cytosol through a multicomponent transport system (Meister, 1991; Ortega et al., 2003). Mitochondrial GSH depletion or oxidation may facilitate opening of the mitochondrial permeability transition pore, and the release of proapoptotic molecules, such as cytochrome c, to the cytosol (Halestrap et al., 1997). Mitochondrial GSH depletion was found to increase sensitivity of Bcl-2-overexpressing B16 melanoma-F10 cells (high metastatic potential) to TNF-α-induced oxidative stress and death (Benlloch et al., 2006). Interestingly it has been demonstrated that vemurafenib can selectively cause ROS production and depolarization of mitochondrial membranes, potentially initiating apoptosis and growth inhibition of vemurafenib-sensitive BRAF (V600E) melanoma cells (Yu et al., 2014). The BRAF (V600E) mutation is the most commonly observed in patients, confers constitutive kinase activity, accounts for >90% of BRAF mutations in melanoma, and is detected very early in melanoma development (Mourah et al., 2015). Interestingly, BRAF regulates oxidative metabolism via PGC1alpha and MITF (Haq et al., 2013), and PGC1alpha expression in particular has been linked to increased mitochondrial capacity and resistance to oxidative stress in melanoma (Vazquez et al., 2013).

Moreover, the NADPH-dependent thioredoxin reductase (TR)/thioredoxin (TRX) system catalyzes disulfide bond reduction in the cytoplasm and mitochondrion, therefore contributing to preserve cellular thiol homeostasis (Lu and Holmgren, 2014).

Mitochondrial NAD$^+$-dependent isocitrate dehydrogenase (IDH2) catalyzes oxidative decarboxylation of isocitrate into α-ketoglutarate and the reduction of NADP$^+$ to NADPH. Interestingly, IDH2 knockdown inhibits tumorigenicity of highly metastatic B16 melanoma F10 cells (Kim et al., 2014).

Peroxiredoxins (PRDXs) are a ubiquitously expressed family of small (22–27 kDa) non-seleno peroxidases that catalyze the peroxide reduction of H_2O_2, organic hydroperoxides and peroxynitrite (Nicolussi et al., 2017). It has been shown that, e.g. PRDX-2 represses melanoma metastasis by increasing E-cadherin/β-catenin complexes in adherens junctions (Lee et al., 2013). Moreover, PRDX-6 (overexpressed in most melanoma cells) can trigger melanoma cell growth by increasing arachidonic acid-dependent lipid signaling (Schmitt et al., 2015).

Recent findings in human A375 melanoma cells with different phenotypes show that just catalase overexpression could favor reversion of malignant processes, but also the progression to a worse outcome (Bracalente et al., 2016). This work presents a melanoma cell model with low levels of H_2O_2 induced by catalase overexpression to study differentiation/dedifferentiation processes. Three clones (A7, C10, and G10) of human A375 amelanotic melanoma cells with very distinct phenotypes were obtained. These clones faced H_2O_2 scavenging by two main strategies. One developed by clone G10 where ROS increased. This resulted in G10 migration and metastasis. The other strategy was observed in clones A7 and C10, where ROS levels were maintained reversing malignant features. Particularly, C10 was not tumorigenic, while A7 reversed the amelanotic phenotype by increasing melanin content and melanocytic differentiation markers. Thus, these authors conclude that it is necessary to reconsider the use of antioxidants as a strategy against cancer. In fact, antioxidants have been suggested as potential promoters of melanoma metastases in mice (Le Gal et al., 2015).

Melanosomes and melanins

Melanosome generation of ROS, in tandem with those generated by cancer metabolism (Figure 1), cause DNA damage and activate cellular signal transduction pathways that prevent cell death. ROS activation of proto-oncogene

pathways in melanoma contributes to their resistance to chemotherapy (Fruehauf and Trapp, 2008). Different chemical studies show that under oxidative conditions, synthetic melanins demonstrate increased metal affinity and a susceptibility to redox cycling with oxygen to form ROS (Farmer et al., 2003). Increased phaeomelanogenesis in dysplastic naevi cells (a known risk factor for malignant melanoma) is connected with oxidative imbalance, which is reflected by increased intracellular concentrations of ROS and raised calcium and iron concentrations (Pavel et al., 2004). Moreover, it has been hypothesized that the disruption of melanosomal melanin might be an early event in the etiology and progression of melanoma, leading to increased oxidative stress and mutation (Gidanian et al., 2008).

Melanosomes are complex lysosomal-associated organelles best known as the subcellular location within which the pigment melanin is synthesized in the neural crest derivative known as the melanocyte, which finds its way to the skin to function primarily as a protective mechanism against sunlight exposure, as UV radiation is both an effective stimulator of oxidase-stimulating enzymes, and also causes direct mutational damage to DNA. The 50-year history of this field has been summarized in a magnum opus published by two major long-time leaders in the field (Borovansky and Riley, 2011).

The process by which melanosomes are formed is relatively well understood, as are the complex biochemical pathways by which melanin is synthesized within this solid-state matrix. In contrast, our understanding of melanosomal degradation remains limited, although it is better understood than it was just 15 years ago, when the very phenomenon of melanosome degradation was questioned (Borovanský and Elleder, 2003). Much remains to be learned; accumulating biological data in cellular and translational models indicates that melanosomal misregulation or damage occurs during melanocyte carcinogenesis, melanomagenesis, invasive behavior, and metastatic disease spread. Hence, the implications for clinical staging and therapeutic drug resistance should be substantial. These biological and cellular phenomena have been studied extensively in cellular models, including our own work (Gidanian et al., 2008).

Clinical evidence of damage to melanosomes becomes evident early during the carcinogenic process and is seen in dysplastic nevi and during early melanomagenesis, with fragmented melanin and disrupted membranes being prominent, a process identified long ago (Curran and McCann, 1976; Rhodes et al., 1988). Increasing evidence suggests that clinical progression is associated with melanosomal damage that leads to oxidative stress at several levels and further mutational damage of already highly mutated cells. It is likely that these events lead to increased oxidative stress and mutation, although direct evidence is limited.

These observations and others have led to the consideration of new potential therapies for both early, late, and therapy-resistant melanoma. Three such approaches are briefly discussed here:

– Cisplatium, which exhibited some clinical activity in early studies during the 1980s as a therapeutic agent, has shown that its activity is influenced by melanosome dynamics (Chen et al., 2009; Xie et al., 2009) that may be amenable to intervention.
– Nicotinamide derivatives (Chen et al., 2009): In a double-blind trial, niacinamide markedly decreased pigmentation, inflammatory infiltrate, and solar elastasis in patients with melasma (an unusual systemic and proliferative pigmentary disorder limited to the skin). Trials with certain subsets of early melanoma might be reasonable, as the alteration in melanin biochemistry and melanosomal dynamics is similar.
– Immunological modulation: Based on preclinical studies of melanosomal-related proteins and chemotherapy (Hertzman Johansson et al., 2013; Hu-Lieskovan et al., 2015) in human melanosome models and translational studies of BRAF and MEK inhibitors in a mouse model of BRAF (V600E), melanoma inhibitors plus immunotherapy demonstrated increased melanosomal antigen and MHC expression and global immune related gene up regulations (Hu-Lieskovan et al., 2015). Clinical trials are being planned.

Many of the properties ascribed to melanosomes are those deriving from the characteristics of melanin, such as their optimal absorbance, redox properties, and propensity for building cationic materials, including drugs and metals (Meyskens and Yang, 2011). However, their existence as specialized organelles is based on the requirement for cells to contain the hazardous process of melanogenesis. As recognized by melanosome and melanin fans, the presence of melanosomal components and melanin in many organ sites (eye, ear, heart) and its association with amyloid in the brain, raises the ante and opens potential new interventions for their exploration in a range of serious neurologic diseases (Liu-Smith et al., 2015).

Melanoma microenvironments

Tumors are cell masses composed of many different types of cells and extracellular matrix (ECM), including cancer cells, fibroblasts, various types of immune cells

and vascular endothelial cells (Balkwill et al., 2012). This conglomeration of cells and the ECM form a microenvironment for tumor growth, migration/invasion, and respond to treatment. In this section we will briefly summarize current understanding of redox impact on melanoma ECM and on response to treatment, including targeted therapy and immune therapy.

Redox impact on melanoma ECM

The ECM is composed of integrins, collagens, elastins, fibronectin, laminin and other matrix proteins which can often serve as platforms or inter- and intracellular signals for a variety of pathways. Modification of ECMs by enzymes such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) also plays important physiological and pathological roles. Early evidence of redox modification of ECM came from over-expression of SOD2 in melanoma cell lines, which resulted in the inability for the cells to form colonies or tumors *in vitro* and *in vivo* (Church et al., 1993). Over-expression of NOX1, on the other hand, enhanced cell invasion ability associated with the epithelial-mesenchymal transition (EMT), likely via MMP-2 (Liu et al., 2012). Knockdown of NOX1 reduced cell invasion (Liu et al., 2012). Knockdown of NOX4 or inhibiting NOX activities by DPI (diphenyl iodonium, a pan-NOX inhibitor) in the MV3 melanoma cell line led to changes of focal adhesion sites and activities of FAK, a key kinase in regulating cell migration (Ribeiro-Pereira et al., 2014). The mitochondrial ROS was also shown to stabilize HIF1α and thus promote cell invasion and tumor vasculogenic mimicry (Comito et al., 2011). These results collectively support the role of ROS in EMT modification and cell migration/invasion. It should be mentioned that the hypoxia condition in cancer cells was also able to cause chromosome abnormalities in the vicinity endothelial cells (Kondoh et al., 2013), thus the cancer cells and the tumor microenvironment mutually impact each other and form a co-existing feedforward survival niche.

ECM is thus a potential therapeutic target for melanoma. Silencing MMP-13 by siRNA decreased melanoma cell proliferation and enhanced a differentiation phenotype (pigmentation) (Meierjohann et al., 2010). Similarly, siRNA against MMP-2 also showed excellent tumor growth inhibitory effect in a spinal cord melanoma metastasis mouse model (Tsung et al., 2008). MMP-2 is also an independent prognosis biomarker for a poorer survival of melanoma patients (Väisänen et al., 2008). The male patient with over-expression of MMP-2 showed even worse outcome as compared to female patients (Väisänen et al., 2008). Our previous study showed MMP-2 was regulated by NOX1 which was localized on the X chromosome (Liu et al., 2012). Whether the NOX1-regulated MMP-2 expression plays a role in the observed sex difference in melanoma development and survival warrants further investigation (Li et al., 2013; Liu-Smith and Ziogas, 2017; Liu-Smith et al., 2017; Yuan et al., 2018).

Nitric oxide, inflammatory tumor microenvironment (TME), and immune therapy

Melanomas carry the largest average number of mutations among all tumors (Alexandrov et al., 2013), which has rendered melanoma a successful target for immune therapy in general. Recently, therapeutic antibodies against CTLA-4 (Ipilimumab) and PD-1 (Pembrolizumab and Nivolumab) have been approved by FDA for first line treatment in melanoma (Luke et al., 2017). Both CTLA-4 and PD-1 are immune checkpoint proteins expressed in T cells, with the function of inhibiting T cells from recognizing the cancer cells. Once these checkpoint proteins are neutralized by the antibodies, the cytotoxic T cells will be able to recognize the tumor cells and eliminate them. The interactions between T cells and tumor cells are mediated by ligands and receptors, and modulated by many factors from the tumor microenvironment including NO, which is often locally produced and consumed due to their short living time and travel distance (Kelm, 1999).

NO in TME can be contributed by tumor cells or tumor-associated macrophages (Salimian Rizi et al., 2017). As stated already, melanoma cells expressed both iNOS and nNOS. The elevated NO levels in TME help melanoma tumors to maintain a chronic inflammatory status, which in turn maintain the TME in an immune suppressive microenvironment (Tanese et al., 2012), as increased NO levels can lead to decreased leukocytes proliferation and decreased infiltration (Choudhari et al., 2013). In addition, NO can also inhibit leukocytes adhesion to target cells (Kubes et al., 1991). In a preliminary mouse study combination treatment with a NOS inhibitor and an anti-PD1 antibody showed better response rates than single agents (Davila-Gonzalez et al., 2017). This strategy is expected to be effective in melanoma, but no report has been published yet up to date. However, it was reported that a BRAF inhibitor triggered NO production in melanoma cells for promoting drug resistance (Yu et al., 2014). Therefore, NO inhibitors may be also used in combination with BRAF inhibitors in melanoma therapy.

Systemic mechanisms

It is well known that (autocrine, paracrine, or endocrine) cytokines/chemochines and growth factors have biologic effects that could stimulate tumor growth, invasion and angiogenesis (Jin et al., 2017). Though normal epidermal melanocytes require a number of exogenous growth factors, nevi require fewer growth factors, and most metastatic melanomas are frequently capable of growing without an exogenous supply of growth factors. This is apparently caused by endogenous production of essential growth factors (Richmond, 1991). Over 80% of the human melanoma cell lines express TGF-β, IL-8, IL-6, VEGF, PDGF-AA, OPN, MGSA-GROalpha-gamma, and monocyte chemotactic protein-1. Significantly higher TGF-β, IGF-1 and IL-15 were determined in primary lesions compared to distant metastases (Payne and Cornelius, 2002; Elias et al., 2010). Nevertheless, a possible role of specific neuroendocrine system-related molecules in regulating systemically melanoma progression remains an open question. For instance, coupled with genetic and environmental factors, stress appears to play a role in melanoma formation and progression (Sinnya and De'Ambrosis, 2013). In fact, the corresponding author of this review can state that different oncologists attending melanoma patients refer that over half their patients reported extreme or very high stress in the previous 12 months before a melanoma diagnosis.

Interaction of metastatic melanoma cells with the vascular endothelium activates local release of proinflammatory cytokines, which act as signals promoting cancer cell adhesion, extravasation, and proliferation. Recent work shows that a high percentage of metastatic cells with high GSH levels survive the combined nitrosative and oxidative stresses elicited by the vascular endothelium and possibly by macrophages and granulocytes (Carretero et al., 1999, 2001; Ortega et al., 2003; Estrela et al., 2006).

Hepatic GSH release increases in metastatic B16 melanoma-bearing mice (as compared with non-tumor-bearing controls), and this increased release appears to be channeled through an Oatp1/MRP1/MRP2-independent system (Obrador et al., 2002). We found that GSH efflux increased in hepatocytes isolated from mice bearing liver or lung B16-F10 melanoma metastases (Obrador et al., 2011). Fractionation of serum-free conditioned medium from cultured B16-F10 cells and monoclonal antibody-induced neutralization techniques facilitated identification of IL-6 as a tumor-derived molecule promoting GSH efflux in hepatocytes (Obrador et al., 2011). IL-6 activates GSH release through a methionine-sensitive/Oatp1-and MRP1-independent channel located on the sinusoidal site of hepatocytes (Obrador et al., 2011). This work identified

IL-6 (mainly of tumor origin) as a melanoma-derived systemic signal inducing GSH release from hepatocytes and its interorgan transport to growing metastases. The strong association between inflammation and cancer is reflected by the high IL-6 levels in the tumor microenvironment, where it promotes tumorigenesis by regulating all hallmarks of cancer and multiple signaling pathways, including apoptosis, survival, proliferation, angiogenesis, invasiveness and metastasis, and, most importantly, the metabolism (Kumari et al., 2016). IL-6 alone can enhance melanoma metastatic potential (in part) by regulating the expression of Twist and N-cadherin (Na et al., 2013). Simultaneous blocking of IL-6 and IL-8 is sufficient to fully inhibit cancer-associated fibroblasts-induced human melanoma cell invasiveness (Jobe et al., 2016). Furthermore, IL-6 may induce in tumor cells increased expression of several survival proteins (Bcl-2, Bcl-xL, Mcl-1, survivin and XIAP) to escape cell death induced by stress and cytotoxic drugs (Moreno-Smith et al., 2010).

In Table 1 it is shown that melanoma patients bearing skin metastases (with different genetic backgrounds) show higher GSH levels in tumor tissues than in normal skin, lower blood GSH levels, and higher IL-6 levels in the serum (Estrela et al., unpublished results). These results are coherent with the concept of an IL-6/GSH interorgan cycle proposed as a melanoma-promoting mechanism (Obrador et al., 2011).

The hypothalamus-pituitary-adrenal (HPA) axis, a key coordinator of the stress response, can be stimulated by cytokines (e.g. IL-1, IL-6 or TNFα) during the course of immune, inflammatory and neoplastic processes (Besedovsky et al., 1991). IL-6, in particular, is a corticotropin releasing hormone (CRH)-independent stimulator of the pituitary-adrenal axis (Bethin et al., 2000). Besides noradrenaline (NORA), at stress-related concentrations, has been shown to up-regulate VEGF, IL-1 and IL-6 expression in different human melanoma cell lines (Yang et al., 2009).

Plasma levels of stress-related hormones [adrenocorticotropic hormone (ACTH), corticosterone, and NORA] increased in a circadian rythm, as compared to non-tumor controls, in mice bearing B16-F10 melanoma metastases (Valles et al., 2013). Corticosterone and NORA, at pathophysiological levels increased expression and secretion of IL-6 in B16-F10 cells *in vitro*. Corticosterone- and NORA-induced transcriptional up-regulation of the IL-6 gene promoted DNA binding activity of NF-kB, cAMP response element-binding protein, AP-1 and nuclear factor for IL-6. *In vivo* inoculation of B16-F10 cells transfected with anti-IL-6-siRNA or treatment with a glucocorticoid receptor blocker (RU-486) or with a β-adrenoceptor blocker (propranolol)

Table 1: GSH levels in skin melanoma metastases and in circulating blood.

	Normal skin (nmol/mg prot.)	Cutaneous metastases (nmol/mg prot.)	GSH Blood (µmol/g Hb)	IL-6 (pg/ml serum)	Genetic background BRAF (V600E)	NRAS	TP53
Healthy volunteers (n = 10)	34 ± 7		7.7 ± 1.0	12 ± 4			
Melanoma patients (n = 11)		67 ± 12[a]	5.0 ± 0.7[a]	106 ± 15[a]	Mutant	WT	WT
Melanoma patients (n = 9)		59 ± 9[a]	5.3 ± 1.1[a]	84 ± 13[a]	WT	WT	V2741

Tumor and blood samples were obtained from patients bearing skin melanoma metastases (all clinically classified as Stages III or IV based on the internationally accepted TNM staging system). GSH levels in metastases and blood were determined, following procedures described previously (New and Chan, 2008), by liquid chromatography-mass spectrometry using a Quattro microTM triplequadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Shimadzu LC-10ADVP pump and SCL-10AVP controller system with an SIL-10ADVP autoinjector (Shimadzu Corp., Kyoto, Japan). Tissue/blood sample collection and processing were performed according to a published methodology (Asensi et al., 1994), where rapid N-ethylmaleimide derivatization was used to prevent GSH auto-oxidation. IL-6 in the serum was determined using commercially available human cytokine ELISA kits from Innovative Research (Novi, MI, USA). BRAF and NRAS mutational status was determined by direct sequencing of polymerase chain reaction (PCR)-amplified genomic fragments of exons 15 and 3, respectively; and p53 mutational status was determined by direct sequencing of exons 2–10 by real-time (RT)-PCR (Benlloch et al., 2016). [a]$p < 0.01$ as compared to control values (Student's t test).

increased hepatic GSH, whereas they decreased plasma IL-6 levels and metastatic growth (Valles et al., 2013). Corticosterone, but not NORA, also induced apoptotic cell death in metastatic cells with low GSH content (Valles et al., 2013). Thus, suggesting an interorgan system where stress-related hormones, IL-6 and GSH coordinately regulate metastatic melanoma growth. Furthermore, GR knockdown decreased the expression and activity of γ-GCL in metastatic cells in an *in vivo* experiment, independently of the tumor location (liver, lung, or subcutaneous) (Obrador et al., 2014). The decrease in γ-GCL activity associated with lower intracellular GSH levels. Nrf2- and p53-dependent down-regulation of γ-GCL associated with a decrease in the activities of SOD1 and SOD2, CAT, GPX and GSR, but not of the $O_2^{.-}$ generating NADPH oxidase (Obrador et al., 2014). The decrease in antioxidant protection caused a drastic decrease in the survival of metastatic cells during their interaction with endothelial cells, both *in vitro* and *in vivo*.

As an example, Figure 2 schematically summarizes the molecular events that occur during B16-F10 melanoma cell attachment to the hepatic sinusoidal endothelium and subsequent tissue invasion. This figure also displays potential metabolic and neuroendocrine mechanisms that may favor metastatic growth.

Whether specific tissue/organ-derived factors (still undefined) contribute to GR expression by melanoma cells is another open question. Moreover, interestingly p53 can physically interact with the GR to form a complex that results in cytoplasmic sequestration of both p53 and GR (Yu et al., 1997). Consequently, it may be concluded that an increase in p53 could represent a therapeutic advantage. However, paradoxically, it has been reported that upon p53 activation and subsequent glutaminase 2 activation,

glutamate and α-ketoglutarate levels, mitochondrial respiration rates and GSH levels increase (Hu et al., 2010). In fact, HeLaR xenografts with glutaminase 2 silenced were more sensitive to radiation. At the molecular level, knock-down of glutaminase 2 increased the intracellular ROS levels of HeLaR exposed to radiation by decreasing the productions of antioxidant GSH, NADH, and NADPH (Xiang et al., 2013). Nevertheless, the mentioned paradox may involve additional paradoxes.

Studies in different tumors, including melanoma, have demonstrated that L-Gln utilization correlates with: (a) increased mitochondrial glutaminase 2 activity that is dependent on Rho GTPases and NF-kB activity; and (b) oncogenic levels of Myc which induce a transcriptional program that promotes glutaminolysis and trigger cellular addiction to L-Gln as a bioenergetic substrate (Scalise et al., 2017). Tumors with high rates of glutamine uptake and metabolism can behave as glutamine traps, depleting host glutamine stores, producing glutamate rapidly, and resulting in cachexia (Klimberg and McClellan, 1996). In fact, glutamine-fueled mitochondrial metabolism is decoupled from glycolysis in melanoma (Filipp et al., 2012), thus expressing an alternate wiring required by melanoma cells to remain metabolically versatile. However, a high rate of L-Gln oxidation may render the mitochondria more susceptible to ROS-mediated cytotoxicity by TNFα (Obrador et al., 2001). In studies in Ehrlich ascites and metastatic B16-F10 melanoma tumor cells, we found that L-Glu derived from L-Gln competitively inhibited GSH transport into mitochondria (Benlloch et al., 2006). Thereby depleting selectively tumor mitochondria GSH under *in vivo* conditions and rendering tumor cells more susceptible to oxidative stress-induced mediators (Benlloch et al., 2006).
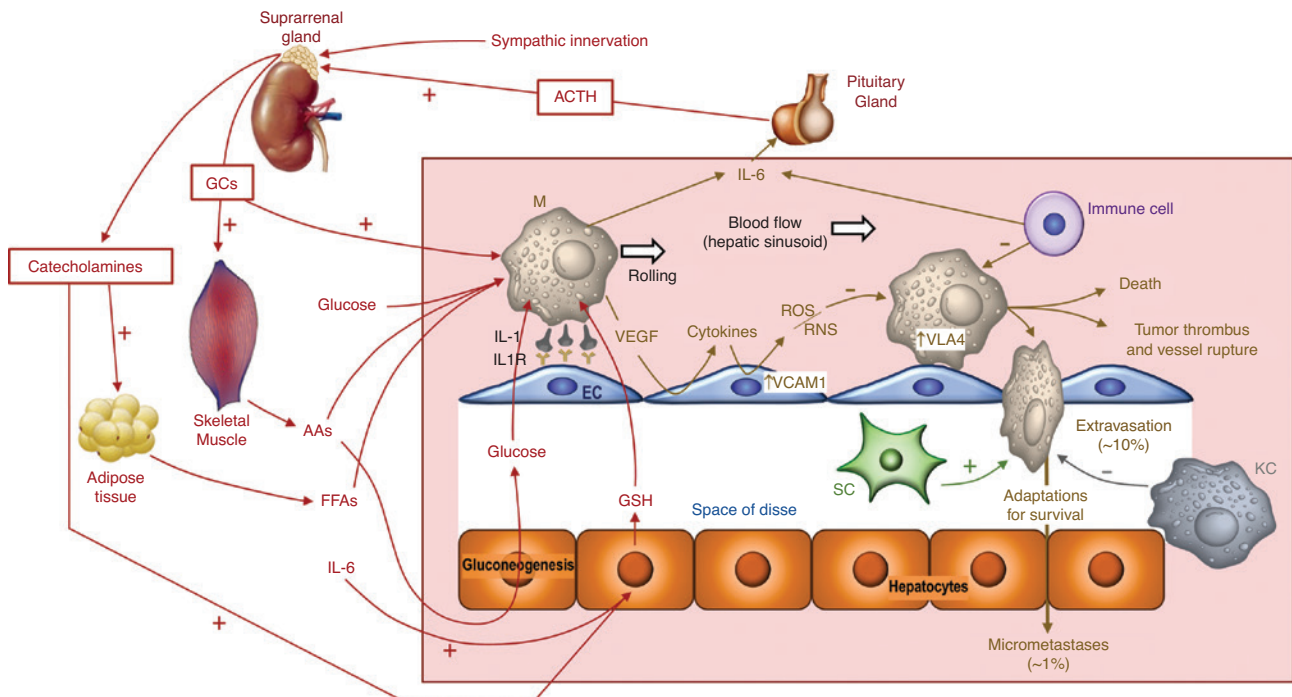
Figure 2: Organ and interorgan molecular signals promoting melanoma metastatic invasion and proliferation.
Highly aggressive B16-F10 melanoma cells (used as an experimental model to generate liver metastases), after reaching the hepatic sinusoids, establish a first molecular bridge (IL1-IL1R, docking) with the endothelial cells. Subsequent rolling associates with the release of metastatic growth factors, which induce endothelial cytokine release and generation of ROS and RNS. These reactive species, in cooperation with the immune cells, cause tumor cytoxicity. Surviving melanoma cells establish a second molecular bridge with the vascular endothelium (VLA4-VCAM1, locking) before migration through vessel fenestrae and extravasation. Alternatively, metastatic cells may also grow intravascularly promoting tumor thrombus formation and vessel rupture. Invading cancer cells will form micrometastases within the lobular hepatic architecture via a mechanism regulated by cross-talk with the stroma and by multiple microenvironment-related, and also systemic, molecular signals. Activation of angiogenesis will facilitate metastatic growth and spread. Cathecholamines released by the suprarenal glands upregulate IL-6 expression and secretion by metastatic cells, which in turns increases GSH release from the hepatocytes into the blood. Tumor γ-glutamyl transpeptidase activity degrades plasma (or extracellular) GSH, providing extra Cys for metastatic cell GSH synthesis. IL-6 (mainly of tumor origin) and stress-related dysregulation of central pacemakers potentiate the release of pituitary ACTH. Glucocorticoids (GCs), at pathophysiological levels, upregulate Nrf2-related defense systems in metastatic cells. Adrenal gland-released GCs and catecholamines also favor tumor feeding while repress our physiological immune defense.

Furthermore, knockdown of P53 in melanoma cells resulted in decreased proliferation (Avery-Kiejda et al., 2011). Thus, indicating that p53 target genes involved in apoptosis and cell cycle regulation are aberrantly expressed in melanoma and that this aberrant functional activity of P53 may contribute to the proliferation of melanoma.

It is also feasible that metastatic melanoma cells activate additional defense mechanisms in order to prolong their survival. For instance, it has been shown that, e.g. treatment with dexamethasone plus the GR antagonist, RU486, blocked apoptosis and (pro-apoptotic) BIM expression in glucocorticoid-resistant acute lymphoblastic leukemia cells (Zhao et al., 2011). And it is well known that ROS can control expression of Bcl-2 proteins by regulating their phosphorylation and ubiquitination (Li et al., 2004). Interestingly, Bcl-2 specifically inhibits GSH efflux

from melanoma cells thereby facilitating its intracellular accumulation (Ortega et al., 2003). All facts coherent with observations indicating that Bcl-2-overexpressing melanomas appear more aggressive (Trisciuoglio et al., 2005; Mena et al., 2012); and that in melanoma, the mitochondrial apoptosis pathways and Bcl-2 proteins appear of particular importance for apoptosis resistance (Eberle and Hossini, 2008).

Evidence of oxidative stress in melanoma tumors and patients

There have been numerous publications demonstrating that oxidative stress is elevated in melanoma cells *in vitro*

(Yamanishi et al., 1991; Meyskens et al., 1997, 2001; Sander et al., 2004; Liu et al., 2012; Denat et al., 2014). Thus it has been proposed that melanoma is a reactive oxygen-driven tumor (Yamanishi et al., 1991; Meyskens et al., 1997, 2001; Fried and Arbiser, 2008; Denat et al., 2014), as the transformation occurs at cellular level. The status of cultured cancer cells often do not reflect what happens in patients (Meyskens et al., 1989). Whether oxidative stress is systematically higher in melanoma patients, and if so, whether the oxidative stress is associated with melanoma stages is less studied. Also, because inflammation is usually associated with oxidative stress (Meyskens et al., 1999), the status of chronical inflammation in melanoma tumors and patients is also summarized in this section.

Diagnosis of melanoma is still based on traditional biomarkers including S100, MiTF, tyrosinase, Melan-A, Pmel17, SM5-1, and CSPG4 (Weinstein et al., 2014); none of them is directly a biomarker for oxidative stress or inflammation. This is perhaps due to the requirements of specificity in diagnosis, as the inflammation and oxidative stress are not specific for melanoma. In melanoma tumor tissues, however, oxidative stress and inflammation markers are frequently found to be much higher than the surrounding control tissues, or even non-melanoma skin cancer. In a small study with 10 superficial spreading melanoma samples, catalase, SOD1, and SOD2 levels were significantly higher in melanoma tissue than in age-matched control tissue, nevi, or tumor tissues from squamous cell carcinoma or basal cell carcinoma (Sander et al., 2003). Interestingly, the malondialdehyde (MDA) levels (a lipid peroxidation marker) were also significantly higher in melanoma tissues than the control tissues in the same study (Sander et al., 2003).

Several studies also confirmed that the systemic level of MDA was higher in melanoma patients or mice models than that in healthy control subjects. Mice groups of different ages were examined for their MDA levels in different organs (Woźniak et al., 2004). Some mice were intraperitoneally inoculated with mouse melanoma cells, while the control group was age-matched healthy mice with no melanoma. MDA levels were higher in brain, liver, lungs, and erythrocytes of the melanoma-inoculated mice. Additionally, the difference was enhanced in younger mice as compared to that in the older ones (Woźniak et al., 2004). As oxidative stress is also a hallmark for aging, thus it is possible that aging masked some differences between the melanoma and healthy groups. In patients, the serum MDA levels are elevated in all stages of melanoma, and patients with Stage IV tumors showed the highest levels as compared to other stages (Bisevac et al., 2018). Serum superoxide levels were significantly elevated in melanoma

patients compared with control healthy subjects, and the levels increase with stages of melanoma (Bisevac et al., 2018). However, total serum SOD activity was only elevated significantly in Stage III melanoma, while SOD2 activity was only elevated in Stage IV melanoma. Catalase activity was elevated in Stage I, II, and III melanoma but not in Stage IV melanoma patients as compared to the healthy control group (Bisevac et al., 2018). A recent study further validated these observations (Santos Bernardes et al., 2016). Erythrocyte MDA levels were elevated in melanoma patients with either primary or metastasis tumors, but increased total radical-trapping antioxidant parameter (TRAP), thiol, and AOPP (advanced oxidation protein products) levels were found only in metastatic patients (Santos Bernardes et al., 2016). Due to the systemic effect, the normal melanocytes from some melanoma patients also showed lower catalase activity and concomitant higher total SOD activity, strongly suggest elevated ROS levels in the normal melanocytes from melanoma patients (Grammatico et al., 1998). These results fully demonstrated a very dynamic redox imbalance and rebalance with melanoma progression.

Melanomas of early stages are often removed by surgical procedures. It was evident that even after tumor removal, the MDA levels remained higher in patients who had thick tumors (Breslow thickness ≥2 mm) than the healthy controls or patients who had thin tumors (Bernardes et al., 2015). Specifically, 6 months to 5 years after surgical removal of tumors, with no sign of recurrence, patients who initially carried thick tumors showed significant higher MDA levels and higher inflammation biomarker C-reactive protein. Breslow thickness is an important prognosis index. The authors speculated that the higher level of oxidative stress was due to the sustained inflammatory status (Bernardes et al., 2015). Yet it was also possible that patients with thick tumors had more tumor cells disseminated into the circulating system resulting in higher oxidative stress status.

In a recent study 23 single nucleotide polymorphisms (SNPs) in eight redox-related genes (*NOX1*, *NOX4*, *CYBA*, *RAC1*, *SOD1*, *SOD2*, *SOD3*, and catalase) were genotyped in a case-control study of melanoma patients and healthy controls (Yuan et al., 2018). The minor allele from rs10951982 in RAC1 showed a significant association with melanoma with elevated risk [odds ratio (OR) 6.15, 95% confidence interval (CI): 2.98 to 13.41; $p < 0.001$], even after multivariate adjustment against age, sex, UV exposure, and family history (Yuan et al., 2018). While RAC1 itself is a melanoma oncogene (Krauthammer et al., 2012), it is also an activator for NOX1 and is potentially associated with elevated cellular ROS levels (Cheng et al., 2006). For NOS enzymes, four

SNPs from iNOS and nNOS were genotyped in 603 cases and matched controls, only nNOS–84G→A was significantly associated with melanoma risk (OR = 1.49, 95% CI 1.05–2.13) (Li et al., 2007).

The role of NO in melanoma is not well defined. Recently several epidemiological and meta-studies revealed that men taking pills for erectile dysfunction (PDE5 inhibitors including sildenafil, tadalafil, and vardenafil) showed small but significantly increased risk for melanoma (Li et al., 2014; Loeb et al., 2015, 2017; Matthews et al., 2016). Interestingly it has been shown that a PDE5 inhibitor (like sildenafil) may restore nNOS effects (Witting et al., 2014). The expression of nNOS enzyme in normal melanocytes remains controversial in different studies (Ahmed and Van Den Oord, 1999; Yang et al., 2013; Vaccaro et al., 2017), but it seems to be expressed in melanoma cells (Yang et al., 2013). Expression of iNOS was not found in normal melanocytes from a biopsy of healthy skin but was detected in a biopsy from vitiligo lesions (Vaccaro et al., 2017). Only a subset of melanoma cell lines is known to express iNOS (Ekmekcioglu et al., 2000; Fecker et al., 2002). Overall iNOS seems to be responsible for inflammation-induced intracellular NO levels (Grimm et al., 2013), and thus may regulate the inflammatory tumor microenvironment (Tanese et al., 2012). The role of nNOS seems to be associated with proliferation as nNOS-specific inhibitors showed an inhibitory effect against melanoma cell growth (Huang et al., 2014). It is difficult to directly measure NO levels in tumors, therefore the nitrotyrosine levels were often used as a surrogate biomarker. High nitrotyrosine levels (as well as iNOS levels) in tumors were significantly associated with poor survival of melanoma patients (Ekmekcioglu et al., 2000).

Antioxidants and prooxidants: good or harmful?

Classical theories of ROS in cancer simplistically agreed that ROS promoted cancer initiation and progression while antioxidants were anticancer agents. The role of ROS is cancer (and melanoma in particular) has been studied extensively over the last few decades and one thing is clear, there is no simplistic answer to the question. Abundant evidence supports the role of ROS as both a tumor-promoting and tumor suppressing agent (Liu-Smith et al., 2014; Assi, 2017; Chio and Tuveson, 2017). This apparent oxygen-related paradox is currently explained as adaptive homeostasis; the balance that exists intracellularly when reactive species that can potentially cause cellular damage also activates biological pathways that protect the cell from their damage. Of course, this balance depends on concentration as too high a concentration of the reactive species can be toxic (Davies et al., 2017).

When administration of vitamin C was shown to prolong survival in terminal cancer patients (Cameron and Pauling, 1976, 1978) a new field of cancer therapy materialized. The characterization of oncogenes as drivers of uncontrolled signaling leading to elevated ROS levels and tumorigenesis (Szatrowski and Nathan, 1991; Sabharwal and Schumacker, 2014) also supported the idea that some antioxidants may not be only anti-cancer agents, they may also be able to prevent carcinogenesis. In fact, ROS had been shown to induce cancer cell proliferation, evasion of cell death pathways, angiogenesis, and metastasis in a wide array of human cell lines and mouse models (Sabharwal and Schumacker, 2014; Chio and Tuveson, 2017).

Melanoma is no exception, it is evident that ROS play crucial roles in almost every aspect of melanoma development, from cell proliferation, DNA damage, to invasion and drug resistance. Thus, melanoma can be argued to be the prototypical cancer to treat melanoma with antioxidants to counter-act the increased ROS levels, or with pro-oxidants that could elevate oxidative stress to a level that is beyond the tolerance range of cells (Fruehauf and Trapp, 2008). Yet, use of antioxidants to treat melanoma have proved a double edge sword and the best example of this is research surrounding the use of the antioxidant N-acetyl cysteine (NAC) for the prevention/treatment of melanoma.

In humans (19 volunteers) a nevus was removed before ingestion of a single dose of the antioxidant NAC, whereas other similar nevus was removed 3 h after ingestion of NAC. The *ex vivo* nevi were then exposed to UV radiation. UV-induced GSH depletion was attenuated in nine of the patients (in all cases in nevi removed after ingestion of NAC), suggesting that NAC could be used for melanoma prevention (Goodson et al., 2009). In stark contrast, a subsequent study in mice showed that NAC increased lymph node metastasis of melanoma without impacting cell proliferation (Le Gal et al., 2015). A soluble vitamin E analog Trolox showed similar effect in cells. Both NAC and Trolox stimulated cell migration through RHOA GTPase (Le Gal et al., 2015), in which a redox sensitive cysteine may play an important role (Aghajanian et al., 2009). Additionally, NAC-treated mice exhibited higher intratumoral GSH levels (Le Gal et al., 2015). Although the first evidence showing that NAC is effective in activating GSH synthesis in cancer cells was published by our group, using Ehrlich ascites cells (Estrela et al., 1992), it was also our group who were the first to show that a close relationship between

GSH levels in melanoma cells and their metastatic growth: intrasplenic injection of B16-F10 melanoma cells with high GSH content showed higher metastatic activity in the liver than cells with low GSH content (Carretero et al., 1999). Furthermore, a clinical trial in 1994 showed that daily high doses of the antioxidant beta-carotene resulted in an 18% increased risk of lung cancer in male smokers [alpha tocopherol, B.C.C.P.S.G. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers (Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, 1994, p. 330)]. Another human trial similarly observed that high doses of beta carotene and retinol increased lung cancer risk by 28% in smokers and workers exposed to asbestos (Omenn et al., 1996).

Other human studies have failed to show a benefit for antioxidants and melanoma. A meta study of nine prospective observational studies and randomized controlled trials (RCTs) concluded that 'neither antioxidant nutrients, individually or combined, nor fruit and vegetable intake showed any strong and significant associations with melanoma' (Miura and Green, 2015). Dr. D'Andrea went one step further and suggested that antioxidants use should be avoided during chemotherapy (D'Andrea, 2005). The evidence from previous clinical trials and the β-Carotene and Retinol Efficacy Trial (CARET) (Omenn et al., 1996) support the idea that these antioxidants did not prevent tumorigenesis. As discussed already, these studies showed in increased risk of lung cancer associated with antioxidant interventions. Molecular and cellular studies revealed the master redox regulator Nrf2, may even promote tumorigenesis through detoxifying ROS (DeNicola et al., 2011). The Selenium and Vitamin E Cancer Trial (SELECT) also suggested similar results (Vinceti et al., 2014). However, the selenium effect (and possibly the effect of many other antioxidants) may be dependent on the dosage. A moderate dose may stimulate tumor growth while only a high dose would show an inhibitory effect (Cassidy et al., 2013). To the best of our knowledge, none of the antioxidants was developed into a clinical drug for melanoma, either for prevention or for treatment.

Targeting proliferation and the prevention of metastasis of melanomas though ROS remains a viable strategy. The antioxidant lupeol has been shown to reduce melanoma tumor volume in mouse xenopgraphs via prevention of proliferation and induction of apoptosis (Saleem et al., 2008). A follow-up study demonstrated that lupeol reduced melanoma tumor growth through decrease in Wnt signaling including a decrease in proliferation and invasion markers (Tarapore et al., 2010). Both of these papers propose lupeol as a promising adjunct therapy in melanoma. Similarly, the antioxidant fisetin has also been shown to inhibit melanoma tumor growth (in mice xenographs) through disruption of Wnt signialling (Syed et al., 2011). Fisetin also shows promise as a combination therapy with sorafenib (Pal et al., 2015), this combination was reported to be more effective than either treatment alone reducing the growth for BRAF-mutant tumors in mouse models (Pal et al., 2015). A key follow-up study from the same group demonstrated that the combination of fisetin with sorafenib inhibited the epithelial to mesenchymal transistion in BRAF-mutant mouse models indicating this combination may be effective against metatsasis (Pal et al., 2014). Yet another antioxidant shown to work as an effective combination in a mouse model of melanoma is EGCG in combination with IFN, demonstrating reduced tumor growth (Nihal et al., 2009).

In contrast to these studies, another group discovered that oxidative stress experienced by disseminated melanoma cells in the circulating system could effectively inhibit the metastasis process (Piskounova et al., 2015), indicating that antioxidant treatment would be detrimental. Furthermore, vitamin C at low (micromolar range) and high (millimolar range) showed proliferation-stimulating and cytotoxicity effect, respectively (Yang et al., 2017). Time and again, antioxidant use and related confusions are heavily dependent on dose and dose-dependent redox balance and rebalance process.

The NOX enzymes and NOS enzymes may be ideal targets for melanoma treatment. NOX1 expression was elevated in all melanoma cells and stages (Liu et al., 2012; Liu-Smith et al., 2014); and NOX4 was associated with a subset of metastatic melanoma (Govindarajan et al., 2007; Liu et al., 2012). A number of NOX and NOS inhibitors were tested in Meyskens' and Liu-Smith's laboratory and other laboratories. The preliminary results revealed that nNOS-specific inhibitors, compound 13 and 14, showed promising efficacy against melanoma cells *in vitro* (Huang et al., 2014). An iNOS-specific inhibitor, N6-(1-iminoethyl)-L-lysine dihydrochloride(L-nil), also inhibited tumor growth in a pre-clinical mouse model (Sikora et al., 2010). Due to the crucial role of nitric oxide in inflammatory and immune response, it was proposed that NOS inhibitors could be excellent adjuvant agents for immune therapy (Ekmekcioglu et al., 2017). However, the actual clinical use of these approaches still requires further *in vitro* and *in vivo* investigations.

In contrast to selective NOS inhibitors, it has been a challenge to develop selective NOX inhibitors (Cifuentes-Pagano et al., 2014). A number of pan-NOX inhibitors have been identified and tested in anti-cancer experiments,

but few were tested in melanoma. We tested NOX1 inhibitor VAS2870 and found a moderate cell growth inhibitory effect against melanoma (unpublished data, Meyskens' and Liu-Smith's laboratory). Honokiol, a lignan isolated from the bark, seed, and leaves of trees belonging to the genus Magnolia, showed an inhibitory effect against NOX1 and reduced cellular ROS levels, and thus reduced migratory potential of melanoma cells in an *in vitro* model (Prasad et al., 2016). Overall, much effort is required to take the current discoveries into clinic use.

Oxidative phosphorylation and Nrf2-mediated antioxidant response have been implicated in tumor adaptation and resistance to current approved therapies for melanoma. Specifically, since its introduction (Collisson et al., 2003), the targeted combination therapy of BRAF+MEK inhibitors to treat melanoma has seen rapid acquired resistance resulting in tumor progression within a few months of the therapy. A recent mouse model was developed to recapitulate *in vivo* acquisition of resistance to BRAF and MEK inhibitors (Khamari et al., 2018). Using this model, the BRAF inhibitor-acquired resistance was shown to be due to mitochondrial metabolism adaptation to increased glucose-derived glutamate synthesis resulting in higher GSH levels (Khamari et al., 2018). Concomitantly, these BRAF-inhibitor resistant melanomas exhibit increased Nrf2-mediated antioxidant response signaling. Taken together, the resistant melanoma has a heightened ability to combat oxidative stress, achieve redox balance and survive. Conversely, melanomas can also acquire new vulnerabilities by upregulating antioxidant response mechanisms. A recent *in vitro* study demonstrated that addition of the histone deacetylase inhibitor vorinostat (designed to suppress the cysteine/glutamate transporter SLC7A11) to BRAF+MEK resistant melanoma can lead to a lethal increase in ROS levels (Wang et al., 2018).

Drug metabolism enzymes have a well-established role in chemotherapeutic resistance. The UDP-glucuronosyltranserases (UGTs) metabolize a wide range of xenobiotic and endogenous compounds. Three UGT family members are normally expressed in melanocytes (UGT2B7, UGT2B10, and UGT2B15) (Dellinger et al., 2012). The expression of all three UGTs has been shown to be lost during melanomagenesis. However, melanoma cells retain the ability to re-express the same three UGT family members in response to exposure to xenobiotics (Dellinger et al., 2012). In turn, the UGTs can metabolize the anti-cancer agent before it can kill the melanoma. However, the UGTs also metabolize endogenous compounds such as the products of the lipoxygenases and expression of 12-lipoxygenase has been shown to increase during melanoma progression (Winer et al., 2002). Furthermore, 12-lipoxygenase signaling has been shown to

activate MEK signaling, another known resistance mechanism melanoma employs to evade apoptosis (Kang et al., 2013). Taken together, all of the apparent dichotomies mentioned underscore the need to understand adaptive homeostasis when developing anti-cancer strategies.

One class of antioxidant compounds that have been studied extensively as anticancer agents are the polyphenols. An abundance of evidence has demonstrated polyphenols have anticancer activity *in vitro* and in animal models (Estrela et al., 2017). Adverse effects of polyphenols have also been reported. High concentrations of some polyphenols may have carcinogenic and/or genotoxic effects. Specifically, caffeic acid demonstrated pro-carcinogenic effects in a multi-organ mouse model (Hirose et al., 1998), green tea catechins increased colon tumor development in rats (Hirose et al., 2001), and quercetin increased severity of kidney tumors in hamsters (Zhu and Liehr, 1994). While these antioxidants are readily available for human consumption as dietary supplements, one human study did show that an IV bolus of high dose quercetin (1400 mg/m^2) once a week for 3 weeks, was associated with renal toxicity in two of ten patients (Gugler et al., 1975). Importantly, kidney function did improve within 1 week, and this effect was not observed to be cumulative over subsequent doses in a phase I trial in advanced cancer patients (Gugler et al., 1975).

Polyphenols may also trigger prooxidative effects, based on their ability to donate electrons. For instance, salicylic acid and anthocyanidins inactivate tumor cell protective catalase and thus reactive apoptosis-inducing intercellular ROS signaling of tumor cells and the mitochondrial pathway of apoptosis (Scheit and Bauer, 2015).

The antioxidant pterostilbene has also been shown to be effective as a single anticancer agent against various cancers. Pterostilbene was confirmed *in vivo* to block tumor growth through inhibition of NF-κB signaling in a mouse model of skin cancer (Tsai et al., 2012). Pterostilbene was also shown to be effective in the treatment of melanoma (Benlloch et al., 2016). Counterintuitively, pterostilbene is not effective (cytotoxic) *in vitro*, only *in vivo*. This study elucidated that pterostilbene can be effective against melanoma through inhibition of ACTH production in the brain of a mouse, which weakens the Nrf2-dependent defenses of melanoma and pancreatic cancers. This both inhibits tumor growth and sensitizes the tumor to oxidative stress (Benlloch et al., 2016). Importantly, this only works if the tumor model is under pituitary hormone control and was effective in significantly reducing tumor burden against three different melanoma xenograph mouse models (Benlloch et al., 2016). Other reports have shown pterostilbene to activate the Nrf2 pathway (Chiou et al., 2011; Saw et al., 2014; Li et al., 2017). This apparent paradox may be

explained by the concentration of pterostilbene shown to be effective against melanoma; a low, physiologically relevant dose of pterostilbene was shown to be effective (Benlloch et al., 2016). This represents a potential paradigm shift in how antioxidant-based cancer treatments are developed. Moreover, it is also essential to keep in mind that combination of two or more polyphenols (at non-toxic and pharmacologically acceptable doses in humans) may show synergic/additive effects (Estrela et al., 2017).

New anti-melanoma therapeutic approaches based on redox-related science

In the last 5–10 years, there has been a remarkable surge in attempts to use the tremendous increase in basic knowledge about the redox process in cells to develop new therapeutic approaches. The areas of interest emphasizing some of these activities in melanoma can be grouped into two major areas of investigation and have recently been reviewed in detail: energy metabolism and mitochondrial interactions (Marchetti et al., 2014; Theodosakis et al., 2014; Hosseini et al., 2017) and nano-technology (Mishra et al., 2018).

The major interest related to energy metabolism has involved the modulation of downstream mitochondria dependent pathways rather than direct effects on the mitochondria *per se* (Marchetti et al., 2014). A large number of basic therapeutically-oriented studies across a range of strategies has recently emerged involving redox modulation via affecting a broad range of targets (Marchetti et al., 2014). Some different examples include:

- Targeting mitochondrial oxidative metabolism in melanoma causes metabolic compensation through glucose and glutamine utilization (Lim et al., 2014). The results of this detailed study hold promise but clearly demonstrate that successful treatment of melanoma will require combinational therapy that involves multiple metabolic components, not a trivial task.
- The use of components of candidate anti-oxidant natural products has become popular. Three excellent examples that have the eventual goal of synthesizing a new drug include:
- A study of cantharidin, a component of natural mylabris which triggers apoptosis through mitochondrial-dependent pathways in human melanoma cells (Hsiao et al., 2014).
- A second intriguing study involves the characterization of the natural phytochemical Zerumbone in

BRAF-mutated melanomas (Wang et al., 2016). This compound suppressed these cells both *in vitro* as well as in xenografts in mice.
- Using an herbal antipyrelic formula UVA-A mediated melanogenesis was inhibited through the activation of Nrf2 regulated antioxidant defenses (Onkoksoong et al., 2018).
- A recent broad-based mechanistic study of inhibitors of cytosolic thioredoxin reductase 1 in many tumor types produced impaired growth of syngeneic mouse tumors and viability of human tumors xenografts with little mitochondrial toxicity (Stafford et al., 2018). However, specific studies involving melanoma will need to be explored.

The increased understanding of the delineation of changes in mitochondria-driven energetics during melanoma formation offers an expanded repertoire for new targets to be developed for clinical intervention, although to date translation has been slow in coming without substantial translational success.

A wide spectrum of biological metabolic interactions, modulated through a diversity of nanotechnology approaches, have also been shown to reverse drug resistance mechanisms in melanoma. A very nice summary review of this complex topic recently appeared and should be required reading for anyone interested in delving into this very complex field (Mishra et al., 2018).

Using nano-tech technologies, several hundred publications using both mouse and human and melanoma models have appeared. Several different types of studies are briefly reviewed here.

- In an early study, cuprous oxide nanoparticles inhibited the growth and metastasis of mouse melanoma (B16-F10) by targeting mitochondria (Wang et al., 2013). Translation of these results to human melanoma represents an exciting opportunity but has yet to be followed up on.
- Interactions of redox parameters and mitochondrial interactions on autophagy and mitophagy and its role in melanoma progression (Soengas, 2012; Maes and Agostinis, 2014) has provided an intriguing approach that has yet to be capitalized on. Nevertheless, the use of functional nano-particles for tumor-targeting co-delivery has become increasingly popular (Xia et al., 2018a,b).
- In a recent study nanocarrier-mediated chemo-immunotherapy arrested progression and induced tumor dormancy in desmoplastic melanoma (Liu et al., 2018). A fascinating observation – but how to follow-up?

Many different nano-tech approaches have been pursued in an attempt to reverse BRAF resistance.

– In a very sophisticated structural characterization study, the 'DFG-flip', the three-dimensional conformational transitions of BRAF kinase was determined (Shao et al., 2017). Study of this phenomenon in cells, if promising, could lead to a new array of therapeutic compounds, if vigorously pursued.

– Suppression of BRAF (V600E) using different types of compounds that induce mitochondria reprograming has already shown considerable progress. In a unique recent approach triphenylo-phosphonium-conjugated nitroxide or ubiquinine was used to produce mitochondrial-membrane depolarization and subsequent oxidative stress (Hong et al., 2017). The results showed that PLX4032 resistant melanoma cells become sensitive *in vitro* and as well in murine SK-MEL28 xenografts without serious effects.

The conclusions from these studies and many other recent results is that mitochondrial targeting agents effectively suppress pathways in BRAF-mutated melanoma that are distinct from those of BRAF inhibitors (Gu et al., 2018; Marchetti et al., 2018). Thus, offering rich opportunities for the development of new preventative and therapeutic approaches, and perhaps new pharmaceutical compounds. The richness and complexity of redox, mitochondrial energetics, and cellular nanotechnology have come together to provide novel and exciting opportunities for the development of new drugs that should impact the prevention and progression of human melanoma.

However, we are still early in the game – much work lies ahead.

Conclusions

Although recent advances have led to genuine meaningful and long-lasting clinical responses in patients using immune modulation (Rozeman et al., 2018), the initial enthusiasm for molecular and biochemical approaches to the treatment of metastatic melanoma has waned due to the almost inevitable emergence of drug resistance – and means to reverse this process effectively have been wanting (Szczepaniak Sloane et al., 2017).

The majority of melanomas are caused by UV exposure; thus, particular attention has been paid to the impact of oxidative stress on these tumors. It is now evident that oxidative stress is involved (directly or indirectly) in all the stages of carcinogenesis and malignant melanocyte transformation. Melanomas undergo functional and metabolic changes during metastasis that increases their capacity to withstand oxidative stress, which implies that melanoma balances intracellular ROS levels to favor their own survival. This adaptive process seems to lead to the generation of more resistant cell subclones. Paradoxically chronic antioxidant treatment (as those that-depending on the antioxidant and its doses-may induce higher melanoma GSH levels) could favor circulating melanoma cell survival and distant metastases. Figure 3, in the form of a timeline, briefly summarizies which may represent, in our opinion, the more relevant publications in the field.
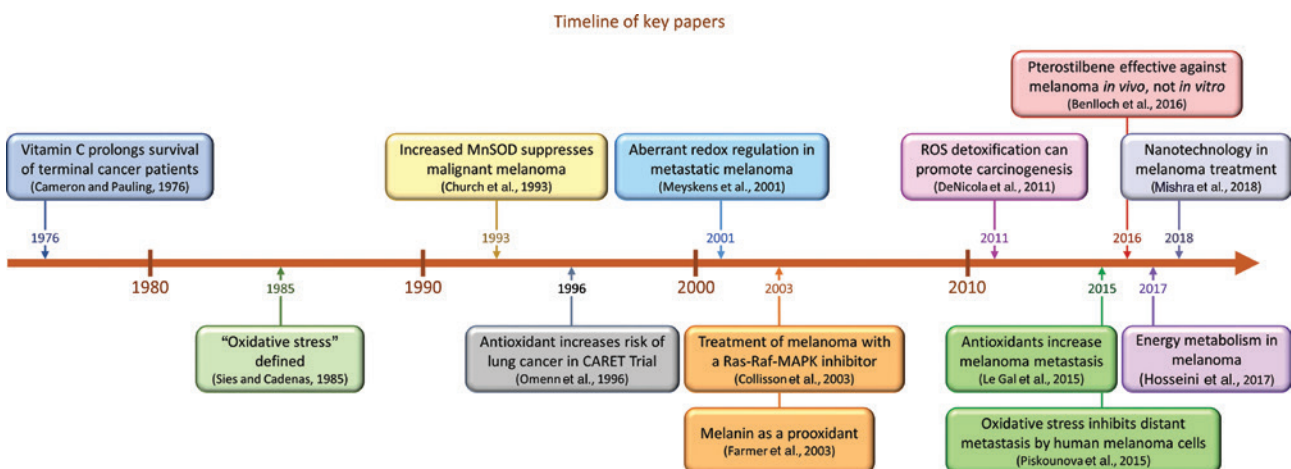

Figure 3: Timeline of key achievements in the field of melanoma, oxidative stress and antioxidants.
The focus of this figure is to provide a list of significant discoveries that, in the opinion of the authors of this review, offer a general overview of what may represent key milestones in the field.

Melanoma cells that survive the circulatory system and reach different organs/tissues interact with the vascular endothelium to begin secondary colonization. The interaction of melanoma and endothelial cells in capillary beds, a critical step in the initiation of metastasis, involves mechanical contact and transient adhesion. This interaction initiates a cascade of activation pathways involving cytokines, growth factors, bioactive lipids, ROS, and RNS produced by melanoma cells and the endothelium. Immune cells are also present in the metastatic microenvironment. Both innate and adaptive immunity participates in antitumor effects, including the activity of natural killer cells, natural killer T cells, macrophages, neutrophils, eosinophils, complement proteins, various cytokines, specific antibodies, and specific T cytotoxic cells. Upon activation, macrophages and neutrophils are able to release tumoricidal ROS/RNS and kill cancer cells, and also angiogenic and immunosuppressive substances. In this complex scenario, the antioxidant defenses of the metastatic melanoma cells appear to be important for their survival and invasive activity.

We note that the brain monitors immune status and sense peripheral metastases-related inflammation through neural and humoral mechanisms. Therefore, it is plausible that metastatic melanoma cells also use physiological neuroendocrine mechanisms to promote survival and growth of highly aggressive (high GSH content) cell subsets. Although the mechanism of traverse remains to be defined.

There are controversial (even opposite) results involving the use of potential antioxidant molecules in cancer therapy. Nevertheless, both anti- and procancer effects appear to depend on the chemical structure and on the dose and *in vivo* achievabable concentration. The development of successful treatments targeting melanoma clearly depend on a more complete understanding of the role of oxidative stress throughout the melanoma initiation, development and metastatic spread.

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