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Reactive Oxygen Species-Associated Risk and Gender-Specific Risk Disparities of Cutaneous Melanoma

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Public Health

by

Tze-An Yuan

Dissertation Committee:
Professor Frank L. Meyskens, Jr., Chair
Professor Scott Bartell
Assistant Professor Feng Liu-Smith

2020

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DEDICATION

To

my parents and my husband

in recognition of their full support and unconditional love

Today was no mere dalliance
but the sharing of the cup,
a spiritual union that
blesses you for eternity.

You have been given an abundance of gifts
for which you must be a careful steward.
Use them well to help others
become what they should be.

When the ashes become too thick
and others have lost their way,
bind your belt to theirs
on the path to safer ground.

Frank L. Meyskens, Jr.
“A Bouquet for Vicente”
in *Believing in Today*, p.56 © 2014 Fithian Press

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LIST OF ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
CI	Confidence interval
CI5	Cancer Incidence in Five Continents
ER/ESR	Estrogen receptor
GEM	International Gene, Environment, and Melanoma Study
GENEVA	Gene Environment Association Studies Initiative
HWE	Hardy-Weinberg equilibrium
IARC	International Agency for Research on Cancer
IGF1/R	Insulin-like growth factor 1/receptor
NOX	NADPH oxidase complex
OR	Odds ratio
ROS	Reactive oxygen species
SEER	The US Surveillance, Epidemiology, and End Results Program
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
US	The United States
UV	Ultraviolet radiation
WHO	World Health Organization

ACKNOWLEDGMENTS

I would like to express the deepest appreciation to my committee chair, Professor Frank L. Meyskens, Jr., who believed in me during the admission process and gave me a chance to try things out in the combination of clinical medicine and population health. Over the course of my Ph.D. training, he actively held meetings with me to go over my research aims, progress, career goals, as well as the difficulties and hardships I faced. Without his guidance and persistent help, this dissertation would not have been possible.

I would like to thank my co-adviser, Assistant Professor Feng Liu-Smith, whose passion for advancing science and improving precision medicine has always been an inspiration to me. Her meticulousness in guiding me in every single step in a research study never ceases to amaze me. In particular, the same religion in Tibetan Buddhism we share has made not only the professional discussions but also general conversations between us much easier and understandable.

In addition, a thank you to my committee member Professor Scott Bartell, who provided tremendous help in research design and statistical analysis. He always has this non-stop energy to help students tackle the complexity in statistics and epidemiology. Along the way in my dissertation research, he used his super clear and logical mind to identify analytic issues neglected by me that would seriously affect the significance in my results. I would not have made these publications without his eagle eyes.

I thank the financial support provided by the University of California, Irvine, Program in Public Health, to sustain me over the years in my doctoral education. I thank Terese Winslow LLC, Springer Healthcare, CRC Press, The Company of Biologists Ltd, and U.S. Government: The American Society of Photobiology for permissions to include copyrighted images as part of my dissertation in Chapter One. I thank Multidisciplinary Digital Publishing Institute (MDPI) for permission to include Chapter Two of my dissertation, which was originally published in the International Journal of Molecular Sciences. I also thank Springer International Publishing AG for permission to include Chapter Three of my dissertation, which was originally published in Cancer Causes & Control. I thank MDPI again for permission to include Chapter Four of my dissertation, which was originally published in the International Journal of Environmental Research and Public Health. Lastly, one more thank you to MDPI again for permission to include Chapter Five of my dissertation, which was originally published in the International Journal of Molecular Sciences. The co-authors listed in these publications directed and supervised research which forms the basis for the dissertation. Special thanks to Mr. Vandy Yourk and Mr. Ali Farhat who contributed heavily to the laboratory experiments.

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Genetic and Gender Risk Factors for Cutaneous Melanoma

PUBLICATIONS

Yuan TA, Yourk V, Farhat A, Guo K, Garcia A, Meyskens F, Liu-Smith F. A possible link of genetic variations in ER/IGF1R pathway and risk of melanoma. International Journal of Molecular Sciences. 2020; 21(5): 1776-1794.

Yuan TA, Lu Y, Edwards K, Jakowatz J, Meyskens F, Liu-Smith F. Race-, age-, and anatomic site-specific gender differences in cutaneous melanoma suggest differential mechanisms of early- and late-onset melanoma. International Journal of Environmental Research and Public Health. 2019; 16(6): 908-923.

Yuan TA, Meyskens F, Liu-Smith F. A cancer registry-based analysis on the non-white populations reveals a critical role of the female sex in early-onset melanoma. Cancer Causes & Control. 2018; 29: 405-415.

Yuan TA, Yourk V, Farhat A, Ziogas A, Meyskens F, Anton-Culver H, Liu-Smith F. A case-control study of the genetic variability in reactive oxygen species-metabolizing enzymes in melanoma risk. International Journal of Molecular Sciences. 2018; 19(1): 242-260.

ABSTRACT OF THE DISSERTATION

Reactive Oxygen Species-Associated Risk and

Gender-Specific Risk Disparities

of Cutaneous Melanoma

By

Tze-An Yuan

Doctor of Philosophy in Public Health

University of California, Irvine, 2020

Professor Frank L. Meyskens, Jr., Chair

Cutaneous melanoma disproportionally affects the fair-skinned populations in the US and worldwide. UV radiation has long been recognized as the primary environmental cause of cutaneous melanoma. However, counterfactual evidence has indicated a non-straightforward relationship between UV radiation and melanoma transformation. For instance, melanoma tumors that grow on the body surface can be found in the non-UV-exposed regions such as the trunk, hip, and lower extremities. Melanoma incidence rates tend to be higher in adolescent and young adult females without a direct connection to excessive UV exposure. Nevertheless, few studies have investigated the impact of additional secondary drivers which contribute to the heterogeneity of melanoma tumors.

The overarching goal of the current dissertation was to make a contribution to the research of secondary drivers in addition to UV radiation that play a role in the development of melanoma heterogeneity in diverse ethnic backgrounds. The specific aims include 1) determine the association between reactive oxygen species (a leading secondary driver) and risk of melanoma;

2) examine the age-dependent gender differences in the incidence rates of melanoma in multiple ethnic groups; 3) investigate the relationship between estrogen receptors' signaling network and risk of melanoma.

Utilizing the International Gene, Environment, and Melanoma Study dataset, a case-control design was performed to study the first aim. It turned out that the activator of NADPH oxidase complex 1 enzyme – the RAC 1-GTPase, contributed to an oxidative stress-associated predisposed risk of melanoma in the fair-skinned population. The second aim was explored by using the US Surveillance, Epidemiology, and End Results Program (SEER) dataset. The results demonstrated a shared phenomenon of early disease onset in adolescent and young adult females in multiple ethnic groups regardless of skin color variations that have different sensitivities to the sun. In addition, the darker-skinned populations presented an even younger age of disease diagnosis than the fair-skinned population and the tumors that grow on the non-UV-exposed regions exhibited the fastest increase in incidence rates over the years. These findings suggested that female-sex is a pivotal secondary driver in addition to UV radiation in melanoma transformation. Therefore, the third aim carried out a genetic analysis of estrogen receptors' signaling network in the fair-skinned population, in our preliminary attempts to explain the early melanoma-onset tendency introduced by female-sex. Using the NCBI High Density SNP Association Analysis of Melanoma: Case-Control and Outcomes Investigation dataset, findings showed that estrogen's downstream IGF1 and its receptor IGF1R may play a critical role in the predisposed gender disparity of melanoma in the fair-skinned population.

This current dissertation should lead to an improved melanoma UV-protection message that is currently conveyed to the public. Additionally, a female-sex-oriented message should be incorporated into educational campaigns to help improve melanoma primary prevention strategy.

CHAPTER 1 INTRODUCTION

1.1 Melanoma Pathology

1.1.1 Anatomy

The skin is the largest organ of the human body. It is composed of three major layers – from top to the bottom – the epidermis, the dermis, and the subcutaneous fat tissue[1,2] (Figure 1.1). The epidermis is the outermost layer of the skin. It provides a waterproof barrier, renews the surface cells, and protects the body from external environments[3]. The dermis regulates the body temperature through sweat glands and enables the feelings of touch, heat, and cold[4]. The subcutaneous tissue is made of fat and connective tissue to attach the dermis to the muscles and bones (Figure 1.1).

Melanocytes reside in the epidermis[5] (Figure 1.1). These cells originate from the ectoderm – the outermost layer of an embryo where the neural crest is formed[6]. The neural crest not only develops into the peripheral nervous system but also produces melanocytes. Melanocyte begins with the embryonic melanoblasts (i.e. melanocytic precursor cells) that migrate to specific locations in the human body, proliferates at the specific location, differentiates from melanoblasts into melanocytes, and matures into melanin-producing melanocytes[5]. Melanoblasts not only can differentiate into "classical melanocytes" that reside in the skin[7], but also can mature into "non-classical melanocytes" that can be found in the meninges, eyes, inner ears, fat tissue, and heart[8].

Skin pigmentation involves melanin biosynthesis (or melanogenesis) that takes place in melanosomes, a membrane-bound organelle in mature melanocytes[9]. On average, melanoblasts proliferate into about 1500 mature melanocytes/mm^2 in the skin epidermis and the total number of epidermal melanocytes in an adult is about 2000 million[10]. There are no regional differences

in the number of melanocytes between genders although the cheek and forehead contain two to three times higher the number of melanocytes in the epidermis in both genders. The distribution and frequency of epidermal melanocytes do not vary among human races[10]. The proliferation of melanoblasts into approximately a fixed number of mature melanocytes is strictly regulated by genetic factors and environmental influences[11]. If this process is damaged, defective melanoblast development may occur and melanoma may be induced.

Melanocytic neoplasms range from benign and dysplastic naevi to melanoma *in situ* (localized melanoma or primary melanoma)[12]. Freckles are just a result of melanin overproduction in the melanocytes with no proliferation of melanoblasts[13]. Freckles only grow on the outermost layer of the skin surface (i.e. in the keratinocytes). Benign naevi, or moles, are a result of normal proliferation of melanoblasts[14], they do not necessarily progress to melanomas[15,16]. Genetic mutations[17], either hereditary[18] or sporadic[19], are usually found in those naevi that progress to dysplastic naevi and melanoma *in situ*. Once the localized melanoma cells penetrate the epidermis and reach the dermis, the melanoma has become invasive[20]. When the melanoma cells are discovered in the local lymph nodes nearby the primary site, the melanoma is malignant and ready to spread (i.e. metastasis).

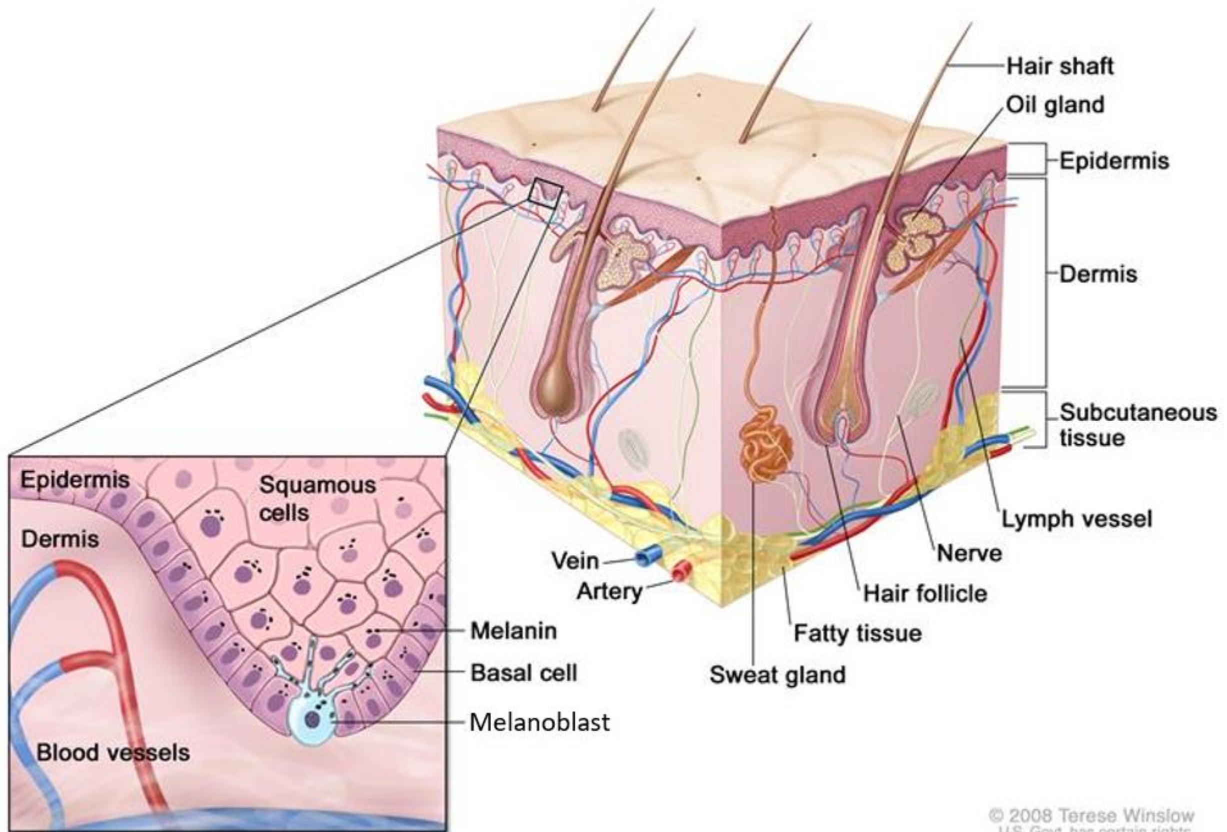


Figure 1.1 The skin dissection. The skin is composed of three major layers – the epidermis, the dermis, and the subcutaneous fat tissue. Mature melanocytes are originated from the melanoblasts that reside at the bottom of the epidermis and align with the basal cells. The outermost layer of the skin is consisted of squamous cells, of which are keratinocytes in squamous shape. Mature melanocytes produce melanin that is packaged in melanosomes to transport to the surface keratinocytes to protect the skin cell DNA damage from UV radiation (Adapted from the National Cancer Institute. © 2008 Terese Winslow LLC, U.S. Govt.)

1.1.2 Histology

Localized or primary melanoma was first categorized into three histologic forms about five decades ago, namely lentigo maligna melanoma, superficial spreading melanoma, and nodular melanoma[21] (Figure 1.2). Lentigo maligna, the precursor of lentigo maligna melanoma, is confined to the epidermis and is, therefore, called melanoma *in situ* (Figure 1.2A)[22]. It first appears as a flat and tan lesion and in time increases in size by centrifugal spread. When lentigo maligna invades into the dermis, it is called lentigo maligna melanoma.

Superficial spreading melanoma is the most common form of melanoma[23] (Figure 1.2B). In it's *in situ* phase, superficial spreading melanoma also stays in the epidermis where it grows horizontally on the skin surface, known as the radial growth phase[24]. It tends to be circular with an irregular outline, and the margins of the lesion are usually protruding and palpable. The lesion is usually a mix of color, from black, brown, to gray, blue, and white[25]. When superficial spreading melanoma invades into the dermis, it is usually seen with the invasion of a nodular melanoma[26] (Figure 1.2C), of which no intraepidermal growth can occur without a dermal invasion. Nodular melanoma is the most invasive form of melanoma due to its vertical growth nature[24]. It does not have a detectable precursor lesion in the epidermis. The lesion usually appears as a lump with uniform color in black or rose-gray; however, it occasionally can be colorless.

More recently, many other histologic forms of localized melanoma were discovered. For instance, acral lentiginous melanoma (Figure 1.2D) which grows specifically on the palms of the hands, soles of the feet, and under the nails[27]. Desmoplastic melanoma[28] and amelanotic melanoma[29] are both very rare forms of melanoma[30]. Desmoplastic melanoma that grows in the

dermis is usually surrounded by fibrous tissue and can be easily confused with a fibrosarcoma[28], whereas amelanotic melanoma has little to no pigment and is thus very difficult to diagnose[31].

Non-cutaneous melanomas are a type of melanoma that do not grow on the skin surface and include mucosal melanoma[32], ocular melanoma[33], and leptomeningeal melanoma[34]. They originate from the melanocytic precursor cells that generate "non-classical melanocytes" in these organs. In this current dissertation, melanoma refers to cutaneous melanoma – melanomas that grow on the skin surface which are originated from the melanoblasts – the melanocytic precursor cells that generate "classical melanocytes" in the skin dermis and epidermis.

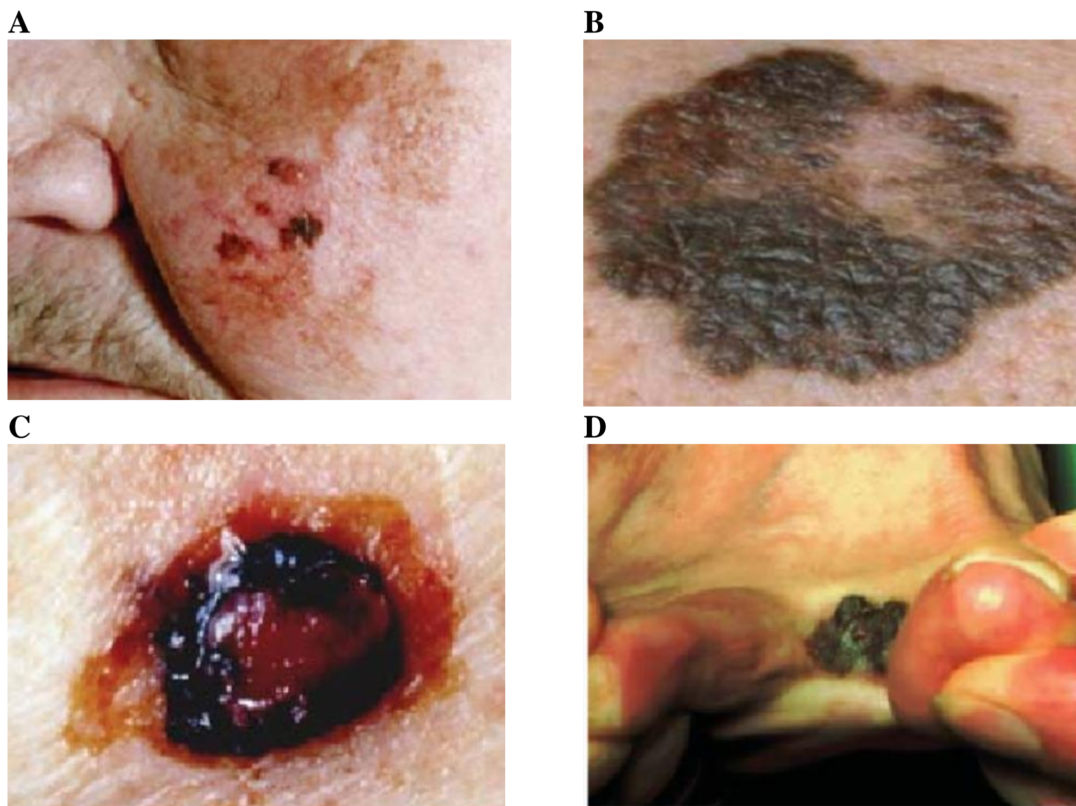


Figure 1.2 The four major localized melanoma histologic forms. **A** Lentigo maligna melanoma. **B** Superficial spreading melanoma. **C** Nodular melanoma. **D** Acral lentiginous melanoma (Adapted from D. Schadendorf et al., *Handbook of Cutaneous Melanoma.* DOI: 10.1007/978-1-908517-98-2_2. © 2013 Springer Healthcare.)

1.1.3 Staging

Clinically, melanoma is examined by the ABCDE screening system[35]. ABCDE stands for "Asymmetry", "Border" irregularity, "Color" variegation, "Diameter" larger than 6 mm, and "Evolution" of lesion growth. Once a lesion is suspected by a dermatologist, subsequent biopsy procedures might be administered.

There are two staging systems to determine the depth of tumor invasion, namely the Clark level and Breslow thickness. Wallace Clark, MD, first developed a staging system to determine tumor penetration in 1969[26,36]. The Clark five-level tumor invasion scale was then the dominant staging system for 40 years[37]. The five levels describe which anatomic compartments of the skin is invaded by melanoma tumors. These levels include Level 1: melanoma is confined to the epidermis; Level 2: invasion into the papillary dermis; Level 3: invasion to the junction of the papillary and reticular dermis; Level 4: invasion into the reticular dermis; and Level 5: melanoma invades into the subcutaneous fat tissue. However, the Clark staging system is no longer recommended as a staging criterion due to its subjectivity and lower predictivity of the outcome[38,39].

The Breslow thickness was invented by Alexander Breslow, MD, in the 1970s[40,41] and has replaced the Clark level after being 40 years as a chief component of melanoma staging[37]. Generally, the Breslow thickness reflects how deep the tumor invades which correlates with worse outcomes. It is now estimated that, on average, if the tumor thickness is less than 1 mm, the five-year survival can reach 92% to 97%[42]. On the other hand, if the tumor thickness is greater than 4 mm, the five-year survival drops to 53% to 70%. Beginning in 2001, the American Joint Committee on Cancer has developed a staging system by TNM scoring, namely the tumor

size (using Breslow thickness), lymph nodes affected, and metastases. Melanoma has then been classified from Stage 0 – melanoma *in situ*, to Stage IV – distant metastasis (Table 1.1).

Table 1.1 Melanoma Staging.

Anatomic Stage/Prognostic Groups							
Clinical Staging				Pathologic Staging			
Stage 0	Tis [1]	N0 [2]	M0 [3]	0	Tis	N0	M0
Stage IA	T1a	N0	M0	IA	T1a	N0	M0
Stage IB	T1b	N0	M0	IA	T1b	N0	M0
	T2a	N0	M0	IB	T2a	N0	M0
Stage IIA	T2b	N0	M0	IIA	T2b	N0	M0
	T3a	N0	M0		T3a	N0	M0
Stage IIB	T3b	N0	M0	IIB	T3b	N0	M0
	T4a	N0	M0		T4a	N0	M0
Stage IIC	T4b	N0	M0	IIC	T4b	N0	M0
Stage III	Any T, Tis	≥N1	M0	IIIA	T1a-b	N1a	M0
					T2a	N2a	M0
				IIIB	T0	N1b-c	M0
					T1a-b	N1b-c	M0
					T1a-b	N2b	M0
					T2a	N1b-c	M0
					T2a	N2b	M0
				IIIC	T0	N2b-c	M0
					T0	N3b-c	M0
					T1a	N2c	M0
					T1a	N3a-c	M0
					T3a	N2c	M0
					T3a	N3a-c	M0
					T3b	Any N≥N1	M0
					T4a	Any N≥N1	M0
					T4b	N1a-N2c	M0
				IIID	T4b	N3a-c	M0
Stage IV	Any T	Any N	M1	IV	Any T, Tis	Any N	M1

[1] Primary tumor (T): TX-primary tumor thickness cannot be assessed; T0-no evidence of primary tumor; Tis-melanoma *in situ*; T1-melanoma 1.0 mm or less; T2-melanoma 1.1-2.0 mm; T3-melanoma 2.1-4.0 mm; T4-melanoma more than 4.0 mm. a-without ulceration; b-with ulceration.
[2] Regional lymph nodes (N): N0-no regional metastases detected; N1-3-regional metastases based upon the number of metastatic nodes and presence or absence of intralymphatic metastases (in transit or satellite metastases). a-micrometastasis; b-macrometastasis; c-in transit met(s)/satellite(s) without metastatic nodes.
[3] Distant metastases (M): M0-no detectable evidence of distant metastases; M1a-distant skin, subcutaneous, or nodal metastases (normal serum LDH); M1b-lung metastases (normal serum LDH); M1c-all other visceral metastases (normal serum LDH) or any distant metastases (elevated serum LDH).
Adapted from the American Joint Committee on Cancer, Melanoma of the Skin Staging, 8th edition (2017).

1.2 Melanoma Risk Factor: Environmental

As previously mentioned in section 1.1, melanoma can be triggered by multiple mechanisms during melanoblast development. The long recognized primary environmental causal factor has been ultraviolet (UV) radiation[43-45]. In 1992, the International Agency for Research on Cancer (IARC) stated that UV radiation is a cause of melanoma[46]. Since then, solar radiation has been listed in cancer risk Group 1 (Carcinogenic to Humans[47]).

1.2.1 Ultraviolet Radiation

On the electromagnetic spectrum, ultraviolet (UV) radiation ranges from 10 nanometers (nm) to 400 nanometers (nm)[48]. Recommended by the ISO standard, UV radiation can be subdivided into several categories based on the wavelength. The most well-known categories are UVA (315-400 nm), UVB (280-315 nm), and UVC (100-280 nm)(Figure 1.3). In the atmosphere, the ozone layer absorbs UVC entirely and UVB partially[49], and therefore, the ambient sunlight is composed of mainly UVA (90% – 95%) and UVB (5% – 10%)[50].

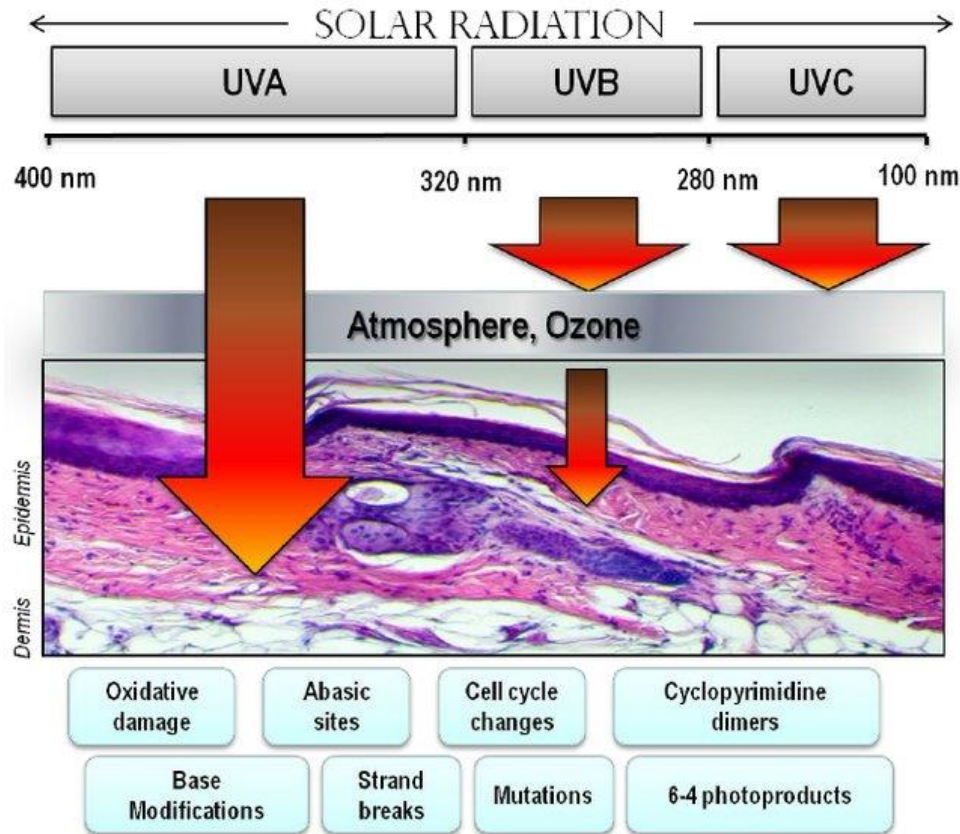


Figure 1.3 The spectrum of UV radiation and the biological effects on the skin. Solar UV radiation can be subdivided into UVA, UVB, and UVC categories. The atmospheric ozone layer absorbs UVC, therefore, the ambient sunlight is predominantly UVA (90% – 95%) and UVB (5% – 10%). UV penetrates the skin in a wavelength-dependent manner. Longer wavelength UVA penetrates deeply into the dermis, while UVB is largely absorbed by the epidermis. UVA is efficient at generating reactive oxygen species that can damage DNA via indirect photosensitizing reactions. UVB is directly absorbed by DNA which causes molecular rearrangements forming the specific photoproducts such as cyclobutane dimers and 6-4 photoproducts (Adapted from John A. D'Orazio et al., *UV radiation and the skin.* DOI: 10.3390/ijms140612222. © 1996-2013 MDPI.)

Upon human exposure, UVA can penetrate the skin and reach the dermis, while UVB only invades superficially into the epidermis[51]. The energy of UV radiation first induces DNA damage in the keratinocytes – the outermost layer of skin cells in the epidermis that protects the basal cells and dermis. A distress call is then sent by the p53 tumor suppressor protein in the keratinocytes to initiate target gene transcription and produce α-melanocyte stimulating hormone (α-MSH)[52] (Figure 1.4). The α-MSH is secreted by keratinocytes and binds to the melanocortin 1 receptor (MC1R) on melanocytes (Figure 1.4). Melanocytes are then activated to initiate pigmentation gene[53] transcription to synthesize melanin. A cascade of amino acids is involved in melanin production[54]. Upon melanocyte activation, the cAMP level in the melanocyte is upregulated, which in turn stimulates the expression of microphthalmia-associated transcription factor (MITF). MITF then activates the enzyme tyrosinase (TYR) and tyrosinase-related protein 1 (TRP1) which converts tyrosine to DOPA and its oxidative intermediate dopaquinone. Dopaquinone combines with cysteine to form the precursor of pheomelanin or channels through the TRP1 pathway which leads to eumelanin, the second type of melanin[55]. Mature melanin is eventually packaged and transported by melanosomes to keratinocytes which protects DNA from further UV radiation damage.

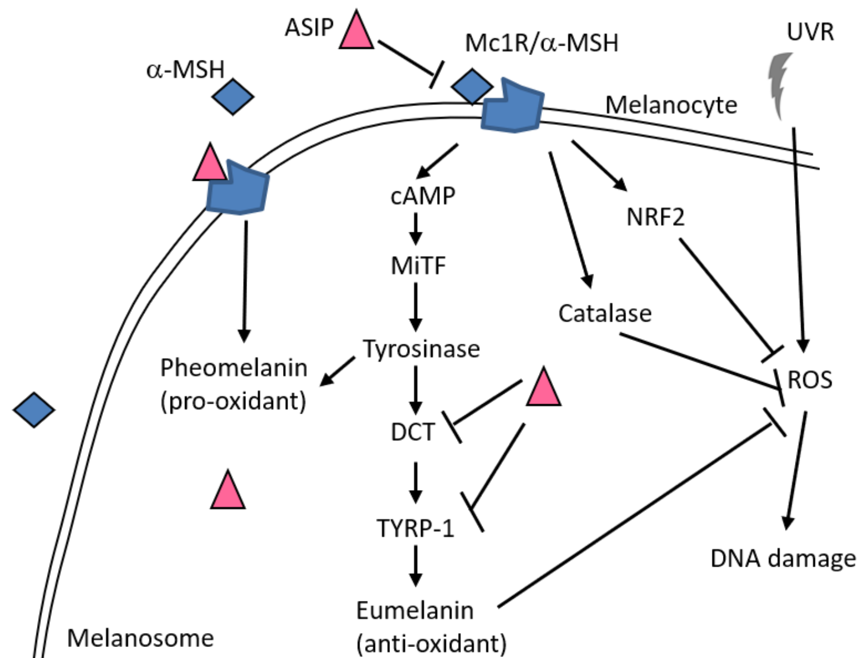
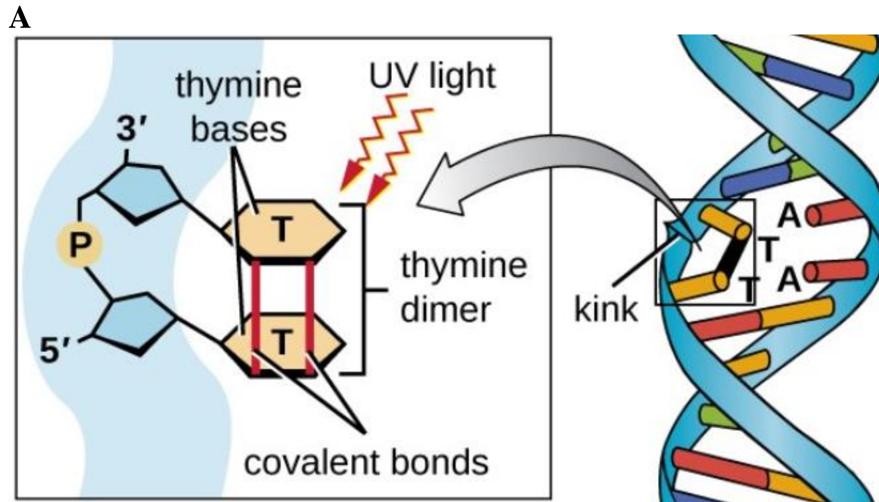


Figure 1.4 Melanin synthesis. The hormone alpha-melanocyte stimulating hormone (α-MSH) interacts with the melanocortin 1 receptor (MC1R) on the outside membrane of melanocytes. cAMP levels increase. A cascade of reactions converts the amino acid tyrosine to the skin pigments. Tyrosinase (coded by the TYR gene) is the primary enzyme involved in the conversion of tyrosine to melanin. The skin pigments, eumelanin and pheomelanin, are synthesized in melanosomes, of which form in the melanocytes. Melanosomes subsequently are transported to keratinocytes where they protect the skin cells from UV radiation (Adapted from Feng Liu-Smith, Chapter 19: Reactive Oxygen Species in Melanoma Etiology in *Reactive Oxygen Species in Biology and Human Health*, page 259-275. ISBN:9781498735452. © 2016 CRC Press, Boca Raton, Florida.)

Melanin pigmentation in the keratinocytes is the first line of defense absorbs 50% to 75% of the UV radiation[56] and darkens (or tans) the skin[57]. This process is still unable to completely prevent UV radiation from reaching the skin cells in the epidermis and dermis[51]. UVA is able to penetrate the epidermis and reach the dermis, but it does not directly damage the DNA[58]. Instead, an indirect photosensitizing response that involves reactive oxygen species (ROS) [59-61] production creates oxidative stress to DNA and causes prolonged damage (summarized in the next subsection 1.2.2). In contrast, UVB can directly damage DNA in the epidermal cells (including the squamous cells, basal cells, and melanoblasts/melanocytes) by inducing three "signature" DNA lesions, i.e. cyclobutane pyrimidine dimers (CPDs), pyrimidine 6-4 pyrimidone photoproducts (6-4PPs), and their Dewar isomers[44,62] (Figure 1.5A). Normally, these "signature" DNA pyrimidine dimers can be repaired by the nucleotide excision repair system[63,64](Figure 1.5B). However, if the DNA damage that UV radiation exerts is too great, such as the formation of sunburns that are readily visible[65] – an acute inflammatory response of the skin after exposed to UV radiation, characterized by erythema, edema, and pain, etc.[66,67] – will eventually lead to cellular apoptosis[68] in order to remove potential cancerous cells. Nevertheless, "signature" DNA pyrimidine dimers may reside in the nucleus for a long-term period[44]. Failure to restore DNA to a normal structure in the long run may result in loss of control of cell proliferation through mechanisms such as inactivation of tumor suppressor genes[68] and activation of oncogenes[17].

A



B

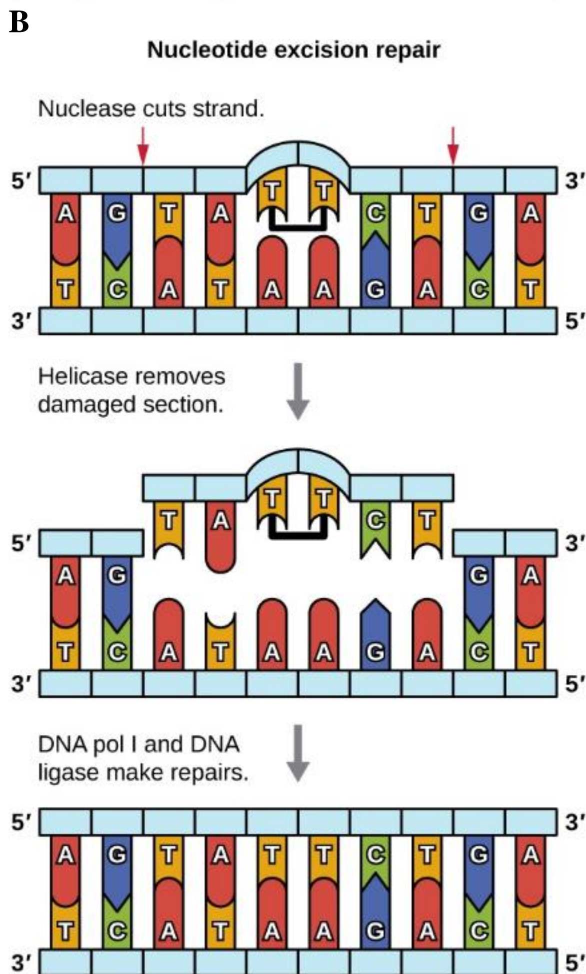


Figure 1.5 UVB-induced DNA damage. **A** An example of UV-induced pyrimidine dimers, of which can stop DNA replication, transcription, and introduce frameshift or point mutations. **B** During nucleotide excision repair process, an enzyme complex recognizes the pyrimidine dimers in the DNA complex, cuts, and removes the damaged DNA strand. The correct nucleotides are replaced by DNA polymerase I (pol I) and the nucleotide strand is sealed by DNA ligase (Adapted from OpenStax Microbiology, *Mutations.* © 1999-2019 Rice University.)

UV radiation was first considered a causal factor for melanoma because the other two commonest types of skin cancer that also originate from the epidermis are caused by cumulative sun exposure, namely basal cell carcinoma and squamous cell carcinoma. Basal cell carcinoma is the most frequent type of skin cancer[51]. It is slow growing, invades locally in the epidermis, and rarely metastasizes. Squamous cell carcinoma is the second most common type of skin cancer. This type of skin malignancy can be fast growing, and it does metastasize when left untreated. Melanoma has the least number of incident cases compared to the previous two skin cancers. Nevertheless, it has the highest mortality among all skin cancers, which is discussed in section 1.8.

In early epidemiology studies, basal cell carcinoma and squamous cell carcinoma were usually lumped together as non-melanoma skin cancer and were clearly found to be associated with cumulative UV exposure[69,70]. However, when these two types of skin cancer were analyzed separately, only squamous cell carcinoma remained a clear positive association with accumulated sun exposure[71,72]. Although basal cell carcinoma does appear to be frequent on the most sun-exposed skin regions[69,73] and the risk goes up with cumulative sun exposure in these areas[71], it rarely grows on the backs of the hands where this region is subject to intense sun exposure and more lesions appeared on the UV-protected trunk region[72,73]. Melanoma has somewhat similar characteristics to basal cell carcinoma because of its origin at the epidermis/dermis border aligned with the basal cells[2].

In fact, beginning in the 1990s, researchers started to realize that the principle causative factor for basal cell carcinoma was not UV radiation alone. Due to the clinical manifestation of various histologic subtypes of basal cell carcinoma, the etiology of nodular and superficial basal cell carcinoma, for example, is believed to be different. For instance, nodular basal cell

carcinoma tends to appear on the head and neck, while superficial basal cell carcinoma is usually found on the trunk[74]. In addition, patients with superficial basal cell carcinoma are usually diagnosed younger[75] with a predominance in females[76] than the nodular subtype, which further suggests different etiologic factors. Interestingly, severe acne is found to increase the risk of developing basal cell carcinoma in sun-protected regions, while the presence of more than five naevi is associated with a decreased risk at the covered sites[77]. Additional non-UV risk factors include exposure to arsenic and therapeutic ionizing radiation, a history of chronic trauma, immunosuppression, and HIV/AIDS seropositivity[78,79]. Although more secondary drivers are remained to be found, one important causal pathway has been indicated in the non-UV etiology of basal cell carcinoma. The hedgehog pathway is essential to normal embryonic development in all living organisms with its relevance to cell proliferation, differentiation, and development into specific tissues. Mutation in the components of this pathway has been found in basal cell carcinoma[80]. Basal cell carcinoma carcinogenesis is, therefore, an interaction between intrinsic genetic factors and environmental influences.

Indeed, melanoma was initially examined by tumor distributions on the body surface to determine UV causation as well. Early epidemiology studies in the 1980s and 1990s had shown that melanoma grew densely on the face, ears, neck, and shoulders where these areas are subject to intense sun exposure[81,82]. Nevertheless, the body site-specific melanoma distribution became less easy to explain by a UV causation when the trunk, hip, and lower limbs – locations where sun exposure is more infrequent than the head areas – appeared to be favorable niches for tumor growth[83]. The comparison between occupations, indoor versus outdoor, had revealed that office work was related to an excessive number of melanomas on the trunk and limbs, while outdoor workers presented higher melanoma tumors in the head regions[84]. Considering that social class

(e.g. educational level) might confound these findings, controlling for social class factors only strengthened the differences of melanoma distribution between indoor and outdoor workers[85]. Interestingly, the age of diagnosis on the trunk was made even earlier than the tumors diagnosed in the head areas and on the extremities[86]. Melanoma was thus suggested by different researchers to either present various subtypes[87] on the body surface or arise through distinct causal pathways per anatomic region[88,89].

Later in the 2000s, researchers started to recognize that the incidence rate of melanoma did not have a straightforward positive correlation to cumulative levels of UV radiation. Using population-based cohort data as early as from 1977[90], researchers found that, first, UV radiation explained a higher percentage of age-specific incidence rates in non-melanoma skin cancers than melanoma in the fair-skinned population. Second, in a regression model of incidence rate against the power function of age and UV, researchers discovered that melanoma showed a more scattered plot than a linear relationship found in non-melanoma skin cancers. Lifetime cumulative UV exposure appeared to be more correlated to non-melanoma skin cancers than melanoma. The same group of researchers later analyzed melanoma incidence rates against UV radiation by anatomic site[91]. They discovered that, only in the fair-skinned men, the UV impact was positively correlated to the incidence rates in the sun-exposed body areas (e.g. head, neck, arms, and shoulder). However, in the fair-skinned women, it was the opposite. The highest incidence rates were seen on the legs and feet regardless of UV radiation. Indeed, these findings have been continuously validated in the literature over the past decade. In the most recent study done by Liu-Smith et al. in 2017[92], the researchers retrieved fair-skinned melanoma incident cases from 31 European cancer registries as well as from the US Surveillance, Epidemiology, and End Results Program (SEER) database to examine if UV radiation differentially affected

melanoma incidence rates between genders. They pointed out that, although there was no correlation found between melanoma incidence rates in females and UV radiation, males did show a significant and positive lifetime correlation to different levels of UV exposure. Liu-Smith et al. concurrently studied the interactions between UV radiation, gender, and age and published another article in 2017[93]. It has further shown that the female-sex was an independent predictor of early-life melanoma incidence rates regardless of UV radiation, while after the age of 50 years, there was a positive correlation between UV radiation and melanoma incidence rates in both genders.

Geographic location might confound the extent of UV radiation imposed on melanoma incidence rates. It has been found that people living closer to the equator developed more numbers of naevi arose from proliferated melanoblasts[94]. These proliferated melanoblasts have a 10% to 20% chance of developing to melanoma[15,16]. A latitude gradient for melanoma incidence rates was then identified in the United States (US) and in the fair-skinned population in the 1960s[95,96]. Similar latitude gradient against an inverse melanoma incidence rate was also found in other continents such as in Australia[97] and in India[98]. But in more recent years, the Western states in the US began to contribute to more incident cases of melanoma than the Southern states (i.e. closer to the equator)[99], especially those tumors occuring in the sun-exposed regions[100], suggesting a non-geographic UV impact on these changes. In Chile, an example of the Southern Hemisphere, the latitude gradient of an inverse incidence rate was only seen in non-melanoma skin cancers but not in melanoma[101]. Evidence from these observations seemed produce an inconsistent association between geographic UV radiation and risk of melanoma.

Some researchers suggested that socioeconomic factors might confound the geographic UV radiation influences on melanoma[102]. For instance, in countries and cities with a narrow

latitudinal belt such as in Canada[103] and in New York City[104], the residential choice made by people due to race[95], educational attainment[105], occupation and household income[104], and healthcare access[106] was found to be highly associated with the level of ambient UV exposure and risk of melanoma. Nevertheless, in Liu-Smith et al.'s 2017 study[93], they substituted the measure of UV radiation with geographic latitudes and verified the same impact of UV radiation on gender-specific melanoma incidence rates stratified by age. Therefore, although the impact of geographic UV radiation on melanoma incidence rates seemed to be controversial in the literature, the patterns of melanoma gender-specific and age-dependent incidence rates were likely not affected by geographic locations.

Because of the conflicting evidence on cumulative UV radiation and the risk of melanoma, researchers had then attempted to explain this relationship by grouping UV radiation into chronic or intermittent exposure in the 1980s[90]. It was presented that, recreational and vacation exposure was associated with an increased risk of melanoma, while occupational exposure showed a protective effect on melanoma[45]. Indeed, long-term UV exposure has been found to increase the thickness of the skin[107-109], and therefore, creates a natural photoprotective barrier to the sun. On the other hand, chronic exposure to UV radiation also creates more solar elastoses in the dermis, or the so-called "sailor's skin", "farmer's skin", or "collagen degeneration" that are never found in the non-exposed skin[110]. These lesions require a longer time to develop into melanomas in the head area and on the distal extremities, and therefore, are usually found in older adults with a cumulative history of a high dose of UV exposure[111]. A history of sunburns is an alarming indicator of the past or ongoing UV-induced "signature" DNA lesions[44,62] that results from a sudden and unusual UV exposure[65] which exemplifies intermittent UV radiation. Most importantly, the $BRAF^{V600E}$ gene mutation (discussed in subsection 1.3.2) is a distinct oncogene

that is predominant in the intermittent UV-induced melanomas, and is seen on the trunk and proximal extremities in younger individuals but not in those with chronically UV-induced melanomas[112].

In summary, a large body of literature and ongoing etiologic research on melanoma and UV radiation indicates that the cause of melanoma cannot be explained by UV radiation alone. After the initial exposure to UV radiation, the neoplastic progression from proliferative melanoblasts to melanomas seems to not demand a further need for UV radiation to progress. In addition, the chronically sun-damaged and intermittently sun-damaged melanomas can still be divided based on host factors (e.g. race, age, gender, and behavioral components), mutation burdens, and the types of oncogenic alterations[12] and will be discussed in sections 1.3 to 1.7. Melanoma carcinogenesis, therefore, highly likely resembles basal cell carcinoma etiology that is an interaction between host intrinsic factors and environmental influences. Most interestingly, currently in the literature it remains elusive why women develop melanoma at an earlier age than men[113], particularly in adolescent and young adult women where between the age of 20 and 24 years there is a highest increase in the incident cases in women discovered by Liu-Smith et al. in 2013[114]. After the age of 50 years, there is a rapid increase in the incidence rates in men[115,116] while old adult women present a relatively stable[117] to slightly declining[118] incidence rates. The influence of female-sex is indicated in this gender disparity of melanoma and is discussed in detail in section 1.6.

1.2.2 Reactive Oxygen Species

Reactive oxygen species (ROS) (Figure 1.6), or free radicals in common language, were first described a century ago[119]. In the 1950s, ROS were considered solely deleterious to the biological system[120], not until the 1970s when ROS were found in the immune system to be

responsible for eliminating bacterial infection[121,122]. Since then, ROS were discovered in various physiological activities normally regulated in the human body, including the vascular system[123], intracellular signaling[124], and insulin metabolism[125]. For instance, ROS are essential to normal growth factor signaling. If ROS are depleted, the growth factor is not able to exert its downstream effect, such as blood vessel formation induced by platelet-derived growth factor[126].

ROS are a collective term for molecular oxygen (O_2)-derived compounds (Figure 1.6A). The human body acquires energy from the consumed nutrients through cellular aerobic respiration that takes place in the mitochondria[127]. During this complex cellular aerobic respiration process, around 99% of the molecular oxygen consumed is converted to water (H_2O), while about 1-2% of the molecular oxygen consumed is converted to superoxide anion ($O_2^{\cdot -}$)[128]. Mitochondria presents its own ROS metabolic enzyme – the manganese superoxide dismutase (MnSOD) – to convert superoxide anion to hydrogen peroxide (H_2O_2). Hydrogen peroxide can either leak to the cytosol or be further cleaved into hydroxyl radical ($HO^{\cdot}$) and hydroxyl anion (OH^-). Finally, hydroxyl radical can be converted to water, but to date there has not been defined a further catabolic pathway for hydroxyl anion[129]. The ROS produced during mitochondrial aerobic respiration (Figure 1.6B) are: superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), and hydroxyl anion (OH^-). Among these ROS species, hydrogen peroxide (H_2O_2) is the most important cellular signaling messenger[130] due to its ability to cross between cellular and organelle membranes[131] and the ability to catalyze cysteine residues in proteins[132,133], resulting in activation of their functions. Of particular note, ROS include many other compounds such as lipid peroxides[134,135], protein peroxides[136,137], and peroxides of the nucleic acids[138]. There is a group of reactive nitrogen species (RNS) that are produced by nitric oxide synthase (NOS)[139] which are also free radicals and can directly interact with molecular oxygen and superoxide

anion[140]. However, to stay focused on the central topic, these compounds are not discussed in the current dissertation.

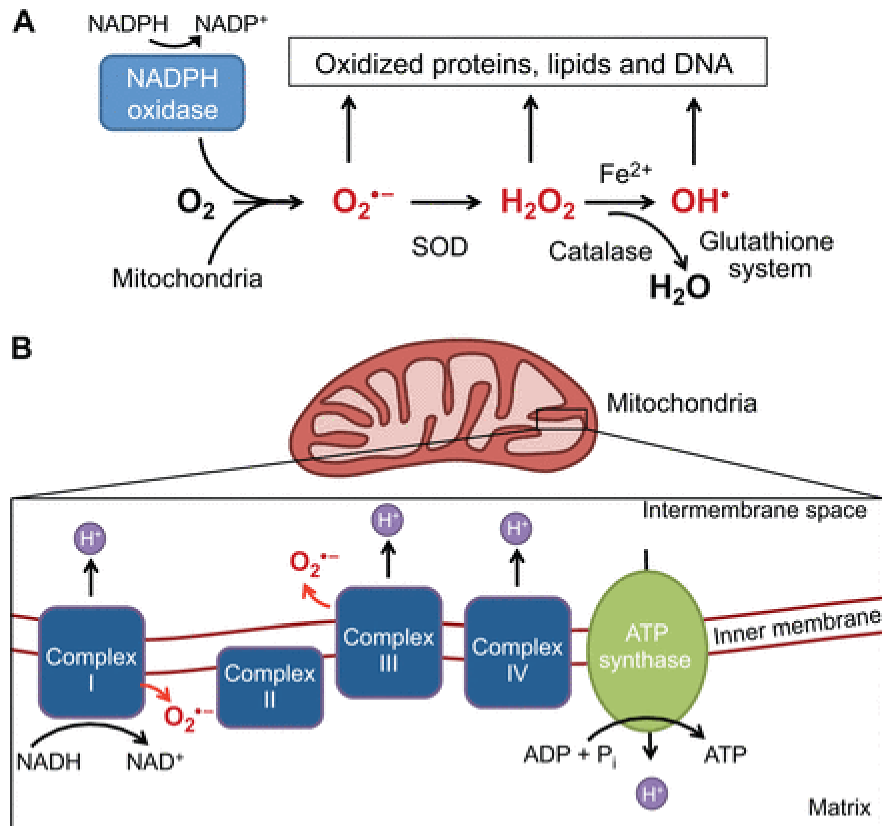


Figure 1.6 Reactive oxygen species production and metabolism. **A** Reactive oxygen species (ROS) include superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$). $O_2^{\cdot -}$ can be produced by mitochondria or by NADPH oxidase. $O_2^{\cdot -}$ is metabolized to H_2O_2 by superoxide dismutase (SOD). H_2O_2 can be further metabolized to water (H_2O) by catalase or cleaved by Fe^{2+} into $HO^{\cdot}$**.** Under conditions of oxidative stress, when ROS production outpaces ROS metabolism, accumulated levels of ROS oxidize and damage various cellular components (e.g. proteins, lipids, and DNA). **B** The human body acquires energy from the consumed nutrients through cellular aerobic respiration that takes place in the mitochondria. During this complex cellular aerobic respiration process, around 99% of the molecular oxygen consumed is converted to water (H_2O), while about 1-2% of the molecular oxygen consumed is converted to superoxide anion ($O_2^{\cdot -}$) (Adapted from Carolina L. Bigarella et al., *Stem cells and the impact of ROS signaling.* DOI: 10.1242/dev.107086. © 2014 The Company of Biologists Ltd.)

Although the majority of ROS are produced as by-products of mitochondrial aerobic respiration (Figure 1.6B), there are some other cellular enzymes which have the potential to produce ROS[141], including xanthine oxidase[142,143], cyclooxygenases[144], cytochrome p450 enzymes[145], lipoxygenases[146], and NADPH oxidase complex (NOX)[147]. These enzymes produce ROS secondary to their chief cellular catabolic functions, except for NADPH oxidase complex (NOX): its primary function is to produce ROS[148] (Figure 1.7). NOX is highly organ-specific[149] with seven different homologs[150-152] and rely on transient stimuli such as UV radiation[153], chemicals[154], and proteins[155] to be activated. NOX has a long history of its discovery. Dr. Otto Warburg first unveiled that when sea urchin's eggs were fertilized, the level of oxygen consumption spiked to be 6 times higher than the unfertilized eggs. He originally proposed that this was a result of mitochondrial aerobic respiration. Although this hypothesis was later proven wrong[156], his discovery still won him a Nobel Prize in 1931[157]. Now we know that the spiked oxygen was used by NOX on the egg's surface to produce hydrogen peroxides[156]. In fact, the NOX homologs either produce superoxide anions or hydrogen peroxides: NOX homologs 1-3 and 5 produce superoxide anions only[155], while NOX 4[158] and duo oxidases (DUOXs) 1-2[159] produce hydrogen peroxides mainly. Upon stimuli activation, NOX homologs 1-4 require dimerization with a $p22^{phox}$ small membrane-bound protein for their stability, while NOX 5 demands no dimerization with a small protein for its stability that is known to date[155]. NOX homologs 1-3 depend on a few more small proteins to reach their full activation state: $p47^{phox}$ (or NOXO 1[147] for NOX 1), $p67^{phox}$ (or NOXA 1[147] for NOX 1), and RAC 1 GTPase (or RAC 2[155] GTPase for NOX 2), while NOX 4 only needs to dimerize with $p22^{phox}$ to be constitutively activated[147] (Figure 1.7). The extracellular superoxide anions produced by NOX are soon converted to hydrogen peroxides to cross the plasma membrane and enter the cells[160].

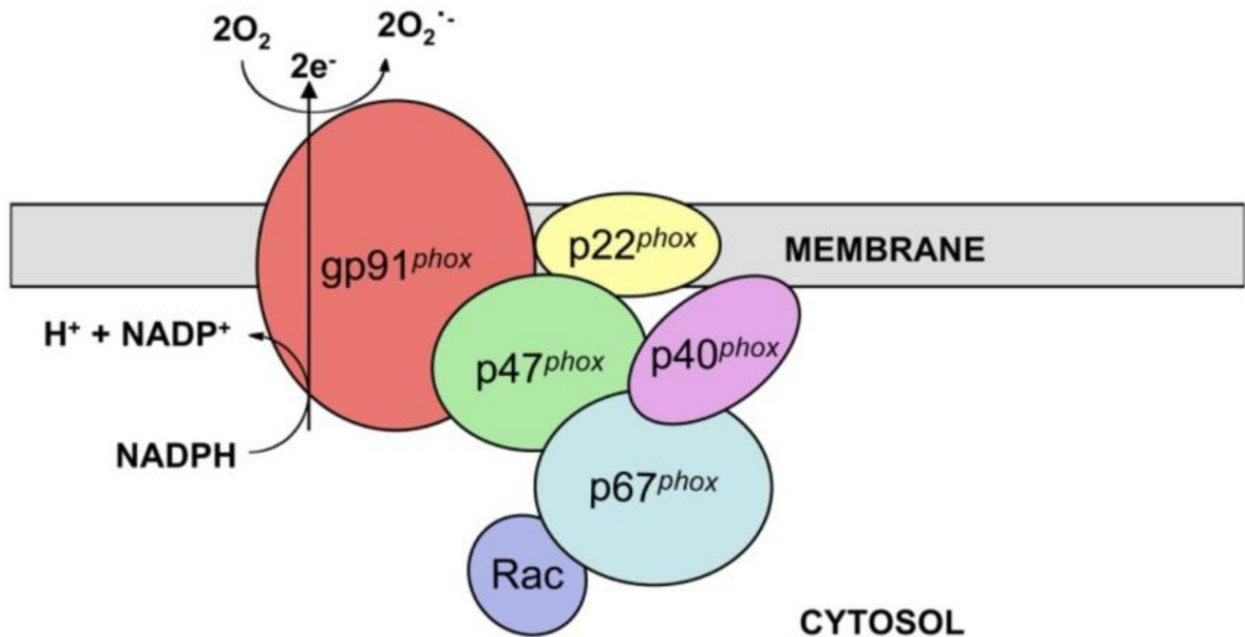


Figure 1.7 NADPH oxidase complex structure. There are seven NADPH oxidase complex (NOX) homologs, namely NOX 1-5 and duo oxidase (DUOX) 1-2. NOX is a multi-subunit enzyme complex. NOX 2, originally identified as a NOX 1's gp91^{phox} homolog, was renamed in 2006. NOX homologs 1-4 require dimerization with a p22^{phox} small membrane-bound protein for their stability, while NOX 5 demands no dimerization with a small protein for its stability that is known to date. NOX homologs 1-3 depend on a few more small proteins to reach their full activation state: p47^{phox}, p67^{phox}, and RAC1-GTPase, while NOX 4 only needs to dimerize with p22^{phox} to be constitutively activated (Adapted from Gail J. Gardiner et al., *A role for NADPH oxidase in antigen presentation*. DOI: 10.3389/fimmu.2013.00295. © 2013 Gardiner, Deffit, McLetchie, Pérez, Walline, and Blum.)

Oxidative stress is induced when there is an excessive increase in cellular ROS level, whether it is transient or chronic, that has resulted in the disturbance or damage of cellular metabolisms and regular functions[161]. There are three phases of cellular responses to oxidative stress[129]. When the ROS level is relatively low, the NF-E2-related transcription factor (NRF2) together with Kelch-like ECH-associated protein 1 (KEAP1) are activated and translocated to the nucleus to interact with antioxidant response elements[162]. This results in the up-regulation of antioxidant genes such as superoxide dismutase (SOD), catalase, peroxidase, glutathione (GSH), GST-transferase, thioredoxin (TRX), and gamma-glutamylcysteine synthetase. SOD works closely with catalase[163] to metabolize superoxide anions to hydrogen peroxides and water. In addition, SOD is highly organelle-specific with three different homologs[164]. For instance, MnSOD is in the mitochondria, while the copper-zinc (Cu-Zn) SOD is in the cytoplasm, and extracellular SOD is not located inside the cell. When the ROS are at intermediate level, in addition to the up-regulation of antioxidant genes, the cell will also initiate genes that are involved in inflammation and cellular reprogramming, such as NF-κB, AP-1, and MAP kinase[165]. Finally, when the ROS level is intensively high, the cell will eventually go through apoptosis.

Failure to restore the cellular ROS level back to homeostasis, or a balanced state, causes harm to cells, one outcome being tumorigenesis. Cancer cells are under chronic oxidative stress[166] – experiencing increased levels of ROS compared to their normal counterparts (Figure 1.8). In order to maintain a low to intermediate level of ROS to avoid apoptosis, the cancer cells present a highly active antioxidant system[167,168] that prevents the high levels of ROS that will induce apoptosis.

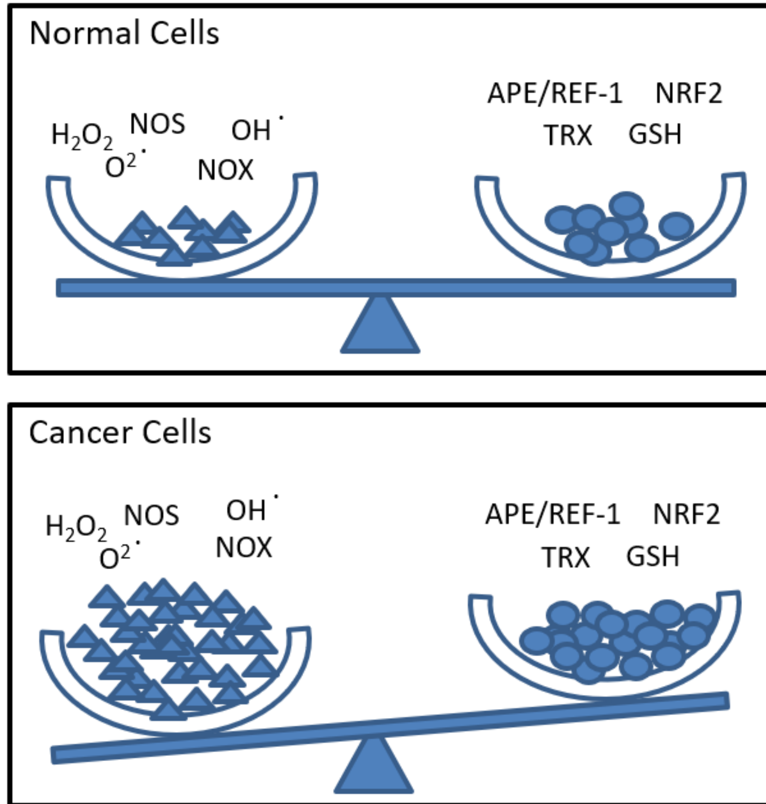


Figure 1.8 Chronic oxidative stress in cancer cells. Cancer cells are under chronic oxidative stress, experiencing increased levels of reactive oxygen species (ROS) compared to normal cells. ROS are produced by NADPH oxidase complex (NOX). ROS include superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$). Nitric oxide synthase (NOS) produces reactive nitrogen species (RNS) that are not discussed in the current dissertation. When the ROS level is increased in normal cells, the NF-E2-related transcription factor (NRF2) is activated and translocated to the nucleus to interact with antioxidant response elements. This results in the up-regulation of antioxidant genes such as superoxide dismutases (SODs), catalase, apurinic (apyrimidinic) endonuclease/redox-factor 1 (APE/REF-1), glutathione (GSH), and thioredoxin (TRX). Failure to restore the cellular ROS level back to homeostasis, or a balanced state, causes harm to cells, one outcome being tumorigenesis (Adapted from Feng Liu-Smith, Chapter 19: Reactive Oxygen Species in Melanoma Etiology in *Reactive Oxygen Species in Biology and Human Health*, page 259-275. ISBN:9781498735452. © 2016 CRC Press, Boca Raton, Florida.)

In addition, the inflammatory responses induced by intermediate levels of ROS[165] also create a more favorable niche for tumor growth. For instance, the innate immune cells including neutrophils and macrophages are able to produce a rapid burst of superoxide anions which can be further converted to hydrogen peroxides that can easily diffuse through the plasma membranes of cancer cells nearby[169]. Historically, ROS were believed to cause tumorigenesis only through damaging cellular DNA such as creating chromosomal aberrations[170]. Later, researchers found that cellular signaling is also dependent on ROS to facilitate the progression of tumorigenesis, such as tumor proliferation, invasion, and metastasis[171]. For instance, tumorigenesis is initiated by oncogenic activation[172] and inhibition of tumor suppressor genes[173] that are subject to oxidative stress. Subsequently, tumor cell division requires cell cycle progression to proliferate[174]. ROS initiate tumor cell expansion through mimicking the effects that are supposed to be exerted by growth factor stimulation[175]. And eventually, tumor cells acquire the ability to migrate, invade, and metastasize via epithelial-mesenchymal transition[176] and angiogenesis[177] that are reliant on ROS-dependent mediators[178,179]. SODs are also playing a double-edged sword role in oxidative stress and tumorigenesis as they are responsible for metabolizing superoxide anions to hydrogen peroxides[164], and elevated levels of hydrogen peroxides are important in tumor progression and metastasis. For instance, MnSOD overexpression is found in advanced lung[180], esophageal[181], gastric[182], prostate, and colon cancers[183]. In contrast, MnSOD is found to be depleted in breast[184], pancreatic[185], and ovarian cancers[186]. MnSOD overexpression has also been found to suppress colorectal and breast cancers[184,187].

Early researchers found that both NOX 2 (originally identified as a NOX 1's gp91^{phox} homolog[188], renamed in 2006[189]) and NOX 4 homologs were expressed in malignant melanoma cells, but only NOX 4 and p22^{phox} were first reported in normal melanocytes in 2002[190]. Later in 2012, NOX 1 was reported by Liu-Smith et al. to be expressed in normal melanocytes and overexpressed in melanoma cell lineages[191]. In this same report, Liu-Smith et al. also pointed out that NOX 4 was, instead, not expressed in normal melanocytes nor primary melanoma cell lineages but was only expressed in malignant melanoma cell lineages. Liu-Smith et al. further examined all 7 NOX homologs and again, confirmed that only NOX 1 was expressed in normal melanocytes but both NOX 1 and NOX 4 were expressed in melanoma cell lineages. No signs of NOX 2 and NOX 5 were found in either normal melanocytes or melanoma cell lineages, even though NOX 5 was identified in only one malignant melanoma cell lineage in 2013 but awaits further verification[192]. These inconsistencies that impede scientific progress are largely due to technical difficulties to precisely pinpoint specific NOX homologs[192,193].

As previously mentioned, NOX 4 only needs to dimerize with p22^{phox} to be constitutively activated, while NOX 1 requires stimuli to activate. To date, studies only established NOX 1's response to UV radiation in the keratinocytes[153,194,195]. Since these articles, it has been widely accepted that NOX 1 in both normal melanocytes and melanoma cells are also responsive to UV radiation. Indeed, the unpublished data incorporated into Liu-Smith et al.'s 2014 report[61] stated that NOX 1 in normal melanocytes is activated when exposed to both UVA and UVB. Interestingly, Cooper et al.[196] compared the UV responses between keratinocytes and melanocytes. They discovered that ROS produced in keratinocytes only spiked 15 minutes after exposure and dropped after 30 minutes back to the baseline level, with no differences when UV doses increased. In contrast, upon UV exposure, melanocytes produced a rapid burst of ROS and

the level maintained up to 120 minutes. This phenomenon was enhanced when UV doses increased. The authors further compared the NADPH oxidase activity between keratinocytes and melanocytes. The result showed that the activity persisted up to 60 minutes in melanocytes compared to 30 minutes in keratinocytes. Although the NADPH oxidase activity was determined using the Lucigenin Illumination assay which does not distinguish among NOX homologs[191] that was working in Cooper et al.'s study, it is believed to be NOX 1.

Although NOX 1 seems to play an earlier role in melanoma transformation via prolonged ROS production and eventually tumorigenesis[171], the increased mRNA/protein level of NOX 1 only differs between normal melanocytes and melanoma cell lineages[191] but does not differentiate between primary melanomas and malignant melanomas[61]. Moreover, RAC 1 GTPase, of which is one of the small proteins that dimerizes with NOX 1 to activate NOX 1 (Figure 1.7), has been identified as the 3rd common hotspot mutation in primary melanomas in 2012 and is expressed among 4% to 7% of the primary melanoma patients[197,198]. Interestingly, this highly activated RAC 1^{P29S} hotspot mutation is a "signature" DNA pyrimidine dimer mutation caused by direct UVB damage[44,62]. This mutation only expresses in sun-exposed melanoma lesions but does not present in benign naevi. This evidence of RAC 1 GTPase hotspot mutation not only validates the initiative role of UV radiation but also NOX 1 in primary melanomas[199]. With this RAC 1^{P29S} hotspot mutation, NOX 1 becomes constitutively active[200], no further stimuli are needed, just like the outcome when NOX 4 dimerizes with p22^{phox}. On the other hand, NOX 4, which does not have a direct link to UV radiation, is overexpressed in its level in malignant melanomas than primary melanomas[61,191,201,202]. How melanoma transformation acquires NOX 4 expression from no-expression in normal melanocytes is currently unknown[203]. One theory is that when melanoma acquires its invasive ability, which is

the turning point between radial growth and vertical growth[24,204], a signaling cue from AKT activates the expression of NOX 4 in the melanoma cells[201]. This, in turn, allows melanoma cells to progress to an aggressive tumor growth phase. Therefore, NOX 4 is one of the driving forces of melanoma spreading due to its production of hydrogen peroxides that can be readily used in tumor progression signaling[130] than superoxide anions produced by NOX 1 that have to be further converted before the tumor cells can use. The hydrogen peroxides produced spontaneously by NOX 4 provide cell cycle progression that is critical in melanoma cell survival, proliferation[202], and the signaling involved in cell viability and migration[205].

Melanoma incidence rates have long been reported disproportionally high in the fair-skinned populations in the US[206] and worldwide[207,208]. This multifactorial process of melanoma transformation involves defective melanoblast development (subsection 1.1.1), a burden of UV-induced DNA pyrimidine mutations (subsection 1.2.1), the tumorigenic source of ROS (subsection 1.2.2) from mitochondria, NOX homologs, peroxisomes (where catalase are located, Figure 1.6A), and melanosomes (where melanin is produced: More details are discussed in subsection 1.4.1). Mutations in mitochondrial DNA are rare in cancers[209]. On the other hand, the pheomelanin is abundant in the fair-skinned populations. During pigmentation, superoxide anions and hydrogen peroxides are produced as by-products of melanin biosynthesis[210]. Although the antioxidative potential within pheomelanin in the fair-skinned populations is weaker than eumelanin in the black-hair populations, the ROS produced by pheomelanin during pigmentation does not reach a significant level[211]. In addition, pigmentation induced by pheomelanin can be done in the dark[211]. Also, the connection between pigmentation and melanoma is unusual[212]. Hence, melanin’s ROS production during pigmentation upon UV stimulation does not fully explain melanomagenesis. The reason why melanoma is predominant

in the fair-skinned populations is likely to be associated with the NOX homologs. Both NOX 1 and NOX 4 are expressed in melanoma cell lineages. In normal melanocytes, NOX 1 which is one of the initiative drivers of melanoma is responsive to UV radiation. Also, NOX 1 is the long-lasting source of ROS in melanocytes[44,196] which facilitates tumorigenesis[171]. These cumulative reasons lead to the central focus of genetic susceptibility of NOX homologs in ROS production in the fair-skinned population in Chapter 2 of the dissertation[213].

1.2.3 Tanning

In 2009, the International Agency for Research on Cancer (IARC) moved UV tanning beds to the highest cancer risk category – Group 1: Carcinogenic to Humans[47]. Prior to this new announcement, tanning beds were in Group 2A, that is Probably Carcinogenic to Humans. The UV radiation source used in tanning lamps is UVA[214]. As UVA is not as harmful as what UVB does to the skin DNA[44,62], the tanning salons used to promote artificial tanning as a means to induce skin pigmentation that works like sunscreen to protect the skin DNA from further UV damage[215,216]. However, the mechanism of UV radiation to skin damage has been revealed by many scientists. UVA is capable of producing a prolonged ROS pool in the melanocytes, which is strongly tumorigenic[217]. In addition, the occasional use of a tanning device also satisfies the classification of intermittent UV exposure which has been found to increase the risk of melanoma[112]. Therefore, artificial tanning device use is an extremely preventable UV radiation source that adds to the personal risk for melanoma in certain populations. For instance, indoor tanning is the most popular among the fair-skinned population in the US[218], followed by Hispanics[219], African Americans[220], and Asian Americans[221], although the numbers from the non-fair-skinned populations are small and non-representative. There is no strong evidence of the popularity of tanning device use in people of color[222,223] mostly because of the social norms of

appearance are perceived differently among the non-fair-skinned populations[224,225]. Of the people who use sunbeds, females are the most frequent users[226], especially among those adolescents[227] and young adults between 20 and 30 years old[214]. In the US in 2014, the Food and Drug Administration (FDA) stated that tanning beds were moderate risk devices and required a warning sign labeled on the device restricting the minors under the age of 18 to use. However, the FDA did not ban the use by minors and by the general adult population[228,229]. In 2010, the Affordable Care Act required a 10% tanning tax charged by tanning salons[230]. Effected since January 1st, 2017, 15 states and the District of Columbia have banned the use of tanning beds by minors under the age of 18[231]. Similar regulations have been published in other countries, such as Australia, Brazil, Canada, the European Union, and New Zealand[232,233]. It is of particular importance that the public is aware of the substantial risk of using tanning devices on the skin, as well as the complementary legislation that helps restrict the access to tanning services[234].

1.3 Melanoma Risk Factor: Genetic

Point mutations are frequently found in melanoma lesions and are fundamentally different than the "signature" DNA pyrimidine dimers resulting from UV radiation[235,236]. In fact, these mutations are sporadic and non-inheritable. However, a group of germline mutations do exist in some families predisposed to melanoma.

1.3.1 Familial Mutations

Individuals with hereditary melanoma usually pass on multiple generations, present multiple primary lesions clinically, and have an early onset of the disease[237]. This tendency had been recognized as early as 1820[238], but its official diagnostic name, the familial atypical multiple mole melanoma (FAMMM), was only formally identified in 1968[239]. Since then, the germline mutation in the cell cycle gene, p16 (now CDKN2A)[240,241], has been recognized as the most common and signature mutation in FAMMM. CDK4, which is a downstream effector of CDKN2A, has also been found to mutate in FAMMM, although this is a relatively rare occurrence[242]. CKD4 mutation disrupts its binding with CDKN2A, which in turn cannot stop the proliferation of tumor cells[243].

Loss-of-function of melanocortin 1 receptor (MC1R) in the fair-skinned population is the signature genetic mutation that predisposes the fair-skinned population to be more susceptible to melanoma[244]. MC1R is crucial in receiving the signal from the UV-damaged keratinocytes to start biosynthesizing melanin in melanocytes (Figure 1.4). The higher the receptor activity, the more black-brown eumelanin is produced, otherwise the blond-red pheomelanin is by default produced[245]. Pheomelanin is a subtype of melanin that is very weak at shielding the skin against UV radiation[55] This is reflected in the fair-skinned population as not able to tan[246]. More details are discussed in subsection 1.4.1.

1.3.2 Sporadic Mutations

Discovered in 2002, the two most common point mutations found in melanoma tumors are BRAF (found in 33% to 66% of primary melanoma patients)[236,247-250] and NRAS (found in 18% to 33%)[251-254]. In 2012, a 3rd common point mutation was found is RAC 1 (found in 4% to 7%)[197,198]. This observation has been previously discussed in detail in subsection 1.2.2.

The most frequently (> 90%[255]) found BRAF point mutation is at the position of codon 600 of exon 15. It is the signature $BRAF^{V600E}$ mutation (GTG to GAG) that resulted from an amino acid change from valine (V) to glutamic acid (E)[236]. Additional mutations at the position of codon 600 are found in fewer patients, such as V600D (GTG to GAT), V600K (GTG to AAG), V600E2 (GTG to GAA), and V600R (GTG to AGG or CGG)[256]. Interestingly, these mutations are mutually exclusive to each other[257]. On the other hand, mutations at exon 11 have also been identified, but only in patients with the relatively benign lentigo maligna melanoma[258]. BRAF mutations at the position of codon 600 are mostly associated with intermittently sun-exposed melanoma lesions[244] with low frequency found in chronically-exposed lesions[259] and non-exposed regions[260]. The comparison between primary and metastatic melanoma revealed that BRAF mutations are more commonly seen in primary melanoma tumors[261]. Both BRAF and NRAS effectors are involved in the cellular MAPK (mitogen-activated protein kinase) pathway[262]. BRAF parts ways with NRAS by its downstream effectors. BRAF goes through the RAS-(B)RAF-MEK-ERK pathway[263]. BRAF mutations resulted in constant activation of MEK that drives cell growth-promoting ERK (extracellular signal-regulated kinase) signaling, which is considered an early-stage genetic alteration in melanoma transformation[263]. Nonetheless, BRAF mutations alone cannot induce melanoma development, a series of other genetic mutations are usually found to co-express with BRAF mutations in melanoma[264].

NRAS point mutation at the position of codon 61 of exon 2 is the most frequently found genetic aberration hotspot in addition to $BRAF^{V600E}$ in melanoma. It generates the Q61R (glutamine to arginine), Q61K (glutamine to lysine), or Q61L (glutamine to leucine) mutations[265]. Similar to BRAF mutations, NRAS mutations are mutually exclusive to each other and to BRAF mutations[257]. Additionally, point mutations at the position of codon 12 of exon 1 were also discovered[266] but no distinct clinical behavior was identified differently between mutations at exon 1 and 2[267]. Unlike BRAF mutations, NRAS mutations rarely present in benign lesions[265]. These mutations are usually seen in patients with invasive nodular melanoma[254], in tumors grown on the extremities, and in chronically sun-damaged lesions in older adults[254,268]. In other words, NRAS mutations are associated with malignant and metastatic melanomas rather than in primary melanomas and are found in both the sun-exposed and non-exposed regions rather than in intermittently-exposed regions that harbor BRAF mutations. NRAS drives both the (N)RAS-RAF-MEK-ERK pathway and the (N)RAS-PI3K-AKT pathway, of which the latter is rarely driven by BRAF[197]. The PI3K and AKT effector proteins are essential in cell expansion and survival[269]. Mutant NRAS locks itself into an active state constantly[270] and thus constitutively activates PI3K-AKT signaling to maintain tumor cell survival and subsequent proliferation.

NRF2 and KEAP1 proteins[162] are crucial sensor systems in response to oxidative stress in melanocytes that activate the transcription of antioxidant genes (subsection 1.2.2). However, mutations in either NRF2[271] or KEAP1[272] gene will increase tumor cell tolerance to oxidative stress and maintain cellular ROS levels that facilitate tumorigenic than apoptotic responses[273]. Although mutations in the NRF2 and KEAP1 systems are not as common as BRAF and NRAS mutations in melanoma, their dysfunctional regulation of cellular ROS levels is crucial in

melanoma transformation. Similar to NRF2 and KEAP1, the less common genetic alterations still include CCND1, cKIT, TP53, and more[274]. The CCND1 gene, which transcribes the cyclin D1 protein, regulates cell proliferation through the binding of CDK4[275]. Amplification of the CCND1 gene has been found to increase the resistance to $BRAF^{V600E}$ inhibitor treatment[276]. However, amplification of the cKIT protooncogene[277], which is mainly considered a stem cell factor[278], has been found in around 7% of all melanomas[279] and the frequency is higher in those chronically UV-damaged lesions[280] as well as in the acral lentiginous melanoma in the Chinese population[281]. The tumor protein 53 (TP53) gene mutations are universal across diverse cancer types[282]. The TP53 gene, which encodes the p53 transcription factor protein, is known for its key function as a tumor suppressor[283]. Therefore, its mutation is non-specifically associated with all types of cancer because of its dysregulation of cell cycle arrest, DNA repair, and cell apoptosis[284]. In melanoma, TP53 mutations are found to accelerate $BRAF^{V600E}$ melanoma formation[285]. Overall, these point mutations are often non-causal aberrations that are sporadically introduced during melanoma evolution[274]. Yet, there are clearly more melanoma predisposing genes remain to be discovered.

1.4 Melanoma Risk Factor: Race

The correlation between skin color and the sensitivity to UV radiation was first observed in non-melanoma skin cancers. By comparing the number of total skin cancer cases, 35% to 45% were seen in the fair-skinned populations[79], while Hispanics represented 4% to 5%. 2% to 4% were observed in Asians, and only 1% to 2% found in African Americans[286]. The photoprotective role provided by skin color is primarily dependent on the amount of melanin (Figure 1.9) in the epidermal melanocytes. Of particular note, the number of melanocytes[10], the skin structure[287], and the rate of DNA repair[288,289] do not vary among racial groups. More striking evidence has been generated by studies of African albinos. Albinism is an inheritable genetic disease characterized by hypopigmentation in the skin[290]. The African albinos present melanocytes without melanin production and their risk of developing non-melanoma skin cancers spiked to be 1000 times higher than their healthy counterparts[291], but melanoma is relatively rare[292], suggesting an even more complex situation of melanoma transformation in people of color. The discoveries from non-melanoma skin cancers led to a conclusion that UV radiation does not impose a significant role in people of color. However, when melanoma does happen in people of color, the clinical diagnosis usually manifests at an advanced stage with worse outcomes[293]. The current hypothesis is that late diagnosis or misdiagnosis in people of color results in a poor prognosis[294]. The epidemiology of melanoma in different racial groups is discussed as follows.

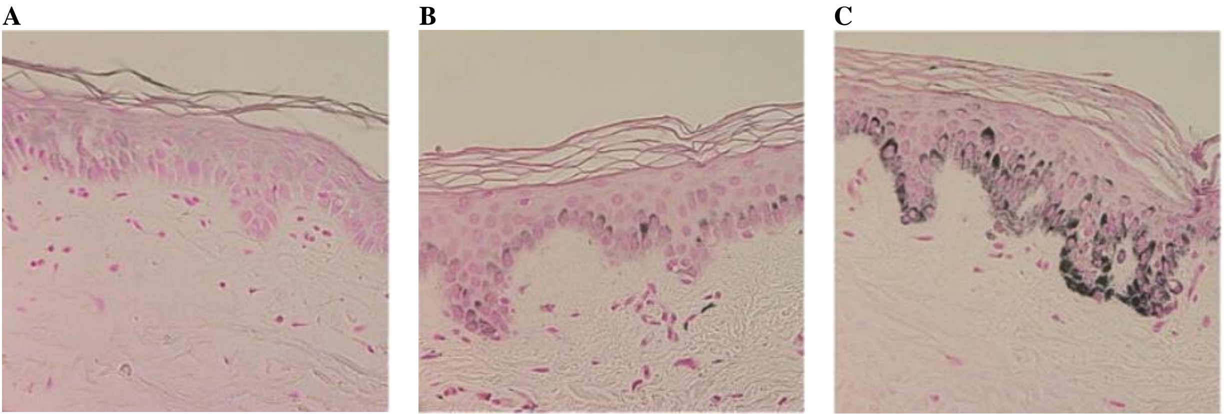


Figure 1.9 Total melanin content in the epidermis. **A** The fair skin. **B** The Asian skin. **C** The black skin. The Fontana-Masson staining of skin dissections shows total melanin content (eumelanin and pheomelanin) at the bottom of the epidermis. Total melanin content is significantly the highest in the black skin as compared to the fair skin and Asian skin. The Asian skin presents the intermediate level of total melanin content among the racial groups. Of particular note, the number of melanocytes is identical in the skin across racial groups[10]. (Adapted from M. Brenner and V.J. Hearing, *The protective role of melanin against UV damage in human skin.* DOI: 10.1111/j. 1751-1097.2007.00226.x. © 2007 U.S. Government. The American Society of Photobiology.)

1.4.1 Caucasian or European Ancestry White

In general, the Caucasian or European ancestry white population refers to light-skinned, blond to red hair, blue to green eyes, and usually is the decedent of European ancestry[295-297]. These individuals characterized by a disproportionally high melanoma incidence rate worldwide[208] and in the US[206]. The racial differences in skin coloration are attributable to melanin amount (Figure 1.9) and melanin subtype in the skin[298]. To be more specific, the melanin amount and subtype in the keratinocytes, where mature melanin is transported from the melanocytes to the keratinocytes and visible as a tan[299]. As mentioned earlier, there are two major subtypes of melanin, the black-brown eumelanin, and the blond-red pheomelanin[55]. The fair-skinned population is susceptible to melanoma because of their higher ratio of pheomelanin present in the skin[298]. The melanosomes that encapsulate the melanin are also smaller in size in the fair-skinned population, but the sizes grow larger in racial groups of darker skin colors[300]. Also, the structure of the two melanin subtypes are different: eumelanin is ellipsoidal while pheomelanin is spherical[301]. In the keratinocytes, the mature eumelanosomes are usually dispersed individually in the cytosol and located closer to the nucleus of keratinocyte, whereas the pheomelanosomes are clustered in groups[298,302]. Although the pheomelanosomes are also located near the nucleus of keratinocyte, the nucleus coverage is less efficient than the eumelanosomes due to the smaller size and the clustered pattern.

Melanin works as an UV absorbent physically darkening the skin which scatters UV radiation[299]. Also, melanin absorbs UV radiation to reduce its penetration to the deeper epidermis and dermis[56]. In terms of the UV absorption ability, pheomelanin in the fair-skinned population is 5 times weaker than eumelanin in the darker-skinned populations[303,304]. In addition, the pheomelanosome is more easily degraded by UV radiation than the eumelanosome[305]. Melanin is

usually rapidly solubilized when exposed to UV radiation[44]. This UV-degradation phenomenon was reported by early researchers[306] and the presence of immature melanin or melanin fragments in the blood is an important biomarker of melanoma progression[307]. Moreover, the debris of melanin is able to diffuse into the nucleus of the melanocyte. Researchers have shown that human melanocytes isolated from fair-skinned neonatal foreskin (with the highest melanocyte density compared to other skin regions[10,308] and replicate more rapidly than adult foreskin melanocytes *in vitro*[309]) are able to produce the "signature" DNA pyrimidine dimers as early as 2 hours after UV radiation in the nucleus which can be sustained in the nucleus up to 12 hours, although variations were great due to genetic differences between donors[44]. It was also impossible to distinguish if the variations were because of a specific melanin subtype due to ethical restrictions about revealing newborn donor identities. Nevertheless, pheomelanin was found to be able to induce pigmentation in the dark. Premi et al. identified that melanin precursors, both immature pheomelanin and eumelanin, were able to generate DNA pyrimidine dimers under certain oxidative states in the dark without exposure to UV radiation[44]. Indeed, melanin biosynthesis creates ROS as by-products[210] and this phenomenon is especially evident in pheomelanin biosynthesis[306]. UV radiation adds to this melanin biosynthesis by inducing more superoxide anion production, especially in the pheomelanosomes[310]. This eventually leads to melanosome degradation. Although the ROS produced during melanin biosynthesis in the melanosomes does not reach a significant level[211], the melanin debris not only can result in DNA pyrimidine dimer mutations[44] but also can release ROS from degraded melanosomes into the cytosol[212]. This phenomenon adds to the existing ROS pool produced by mitochondria, peroxisomes, nitric oxide synthases, and NOX homologs[61]. The elevated ROS level will eventually become tumorigenic. Overall, eumelanin has better photoprotective potential than

pheomelanin. Nevertheless, UV-driven DNA mutations constitute only the first hit[311] of melanoma transformation in the fair-skinned population. There are additional risk factors that add to the complexity of melanoma heterogeneity in the fair-skinned population. For instance, the gender differences in melanoma incidence and mortality rates has been observed and described since 1975. Men seemed to have a higher risk. The most recent statistics available in 2016 from the CDC showed that 82,476 people from all races in the US were diagnosed with melanoma, including 48,762 (59%) men and 33,714 (41%) women; 8,188 people in the US died from melanoma, including 5,425 (65%) men and 2,763 (35%) women[99].

However, contradicting findings were found when stratifying the rates by age. Using the SEER*Explorer interactive tool provided by the US Surveillance, Epidemiology, and End Results Program (SEER)[312], data available from 2012-2016 showed that under the age of 50 years, women from all races exhibited about 1.4 times higher incidence rates as compared to men. The highest female to male incidence rate ratio was further reported by Liu-Smith et al. in 2013 to be between the age of 20 and 24 years in the fair-skinned population[114]. However, this phenomenon switched after the age of 50 years. Based on these numbers, there is clearly a gender-specific and age-dependent risk disparity in melanoma transformation in the fair-skinned population. Possible influencing factors are discussed in sections 1.5 to 1.7.

1.4.2 Hispanic

Before 1992, Hispanics in the US were included as a fair-skinned population. However, after 1992, Hispanics were seen as an independent racial group[313]. In 2015, Hispanics produced 1,740 new melanoma cases in the US, which translated to an incidence rate of 4.4 per 100,000 person-years[206]. Compared to global statistics in 2015, Latinos from South America had incidence rates ranging from 2.8 per 100,000 person-years in the Andean region to 5.5 per

100,000 person-years in the Tropical region[208]. Hispanics contributed to the second most common racial group to develop melanoma of the skin. They used to be called “white Hispanics” secondary to the color of their skin under the skin category of “white” that ranged from skin types I through IV based on MD Thomas Fitzpatrick’s definition[314] (whereas skin type V is brown and VI is black). Indeed, findings from California[315] and Florida[316] showed that melanoma incident cases were increasing annually in the white Hispanics between the 1990s and 2000s. Although the diagnosis in Hispanics was usually made at an advanced stage with thicker melanoma tumor and distant metastasis, the causal relationship to UV radiation was not debated. Instead, the more aggressive melanoma diagnostic status in the Hispanics was attributed to a lower educational attainment, a poorer socioeconomic status, and a decreased access to healthcare[317,318]. Wagner et al. studied the skin responses to UV radiation among different racial groups in 2002[319]. Interestingly, European ancestry Americans showed the lowest pigmentation after UV radiation, while Hispanics and East Asian ancestry Americans presented similar tanning responses after UV exposure. Similar findings had been pointed out by others as well, that the UV radiation responses and the latitude gradient of melanoma incidence rates were only seen in the European ancestry whites but not in other racial groups[320]. Therefore, the role of UV radiation in melanoma transformation in Hispanics is less clear in the current literature and awaits more etiologic studies in this population.

1.4.3 Asian and Pacific Islander

In 2015, Asian Americans and Pacific Islanders overall presented 257 melanoma incident cases, which translated to a 1.3 per 100,000 person-years incidence rate in the US[206]. Compared to world statistics, melanoma incidence rates ranged from 1.4 per 100,000 person-years in East Asia, 1.3 in Southeast Asia, 1.1 in South Asia, to 0.7 per 100,000 person-years in Asia Pacific[208].

Melanoma cases were consistently low in Asians. Interestingly, Central Asia had an incidence rate of 3.7 per 100,000 person-years in 2015, this is due to the fact that people live in Central Asia are not homogeneously typical Asians. Instead, this region had a long history of recurrent immigration and thus created a great population diversity[321]. Nevertheless, the acral lentiginous tumor that grows on the soles of the feet, similar to African Americans, is also the most common subtype of melanoma in Asians[322,323], followed by non-cutaneous melanomas e.g. the mucosal[324] and ocular melanomas[325,326]. Since melanoma is very rare in Asians, the etiology of these melanoma subtypes remains poorly understood[327]. As both the BRAF and NRAS mutations are rarely found in Asian melanoma patients[328-330], this has hindered the development of a proper treatment plan for Asians[331] and led to poorer survival in this population.

1.4.4 American Indian and Alaska Native

American Indians and Alaska Natives in the US, in 2015, had an overall 208 melanoma incident cases and an incidence rate of 5.9 per 100,000 person-years[206]. The closest incidence comparable with these Americans occurs in people from Oceania regions, which represented a 2.6 incidence rate per 100,000 person-years in 2015[208]. Subungual tumors, the subtype of melanoma that grows under the fingernails or toenails, are mostly seen in the American Indians and Alaska Natives[332]. The rarity of melanoma in these populations[333] is still explained by the UV radiation-associated darker skin color theory[334]. However, studies have shown that the acral lentiginous subtype of melanoma did not correlate with UV radiation overexposure[27]. In fact, the nails are a natural barrier to UV radiation[335]. Nevertheless, there is still a significant diagnostic disparity in these populations when compared to the fair-skinned population. Because of a mutual lack of awareness from healthcare professionals and these ethnic groups, melanoma diagnosis is often delayed, resulting in a worse prognosis[336].

1.4.5 African American

The black skin is four times more effective than the fair skin in blocking UVB penetrance to the epidermis[303] (Figure 1.9C). Indeed, in 2015, there were only 394 new melanoma incident cases in African Americans, of which constituted an incidence rate of 1.0 per 100,000 person-years, compared to 75,199 incident cases in the US fair-skinned population with an incidence rate of 25.1 per 100,000 person-years[206]. However, as compared to world statistics, melanoma incidence rates in sub-Saharan Africa areas ranged from 2.1 to 6.4 per 100,000 person-years, 2.8 in Caribbean, and about 1.7 per 100,000 person-years in North and Middle East Africa[208]. The melanomas diagnosed in African Americans are usually the acral lentiginous subtype, of which the tumors grow on the soles of the feet are most often seen[337]. Studies showed that the melanoma tumors grow in African Americans rarely present BRAF mutations[338], of which is an indicator of intermittent sun exposure, further suggests a different melanoma transformation pathway in African Americans rather than UV radiation. Moreover, with feet melanomas, African Americans presented a very poor 5-year survival, as low as to 23%[337]. This is a collective result of a lower income, a barrier to healthcare, a lower educational attainment, and a lower level of melanoma awareness and thus early screening[339]. Substantial gaps still exist in this population regarding the etiology and survival disparity in melanoma.

Grouping people by skin color or region of living is a very coarse way to differentiate race and ethnicity since multiethnic decedents from cross-cultural marriages are not unusual nowadays. Indeed, when analyzing the genetic makeup of people in the same racial category, the minute genetic differences may be contributing to a great diversity of disease susceptibility[340,341]. Nevertheless, whether the patterns of melanoma gender-specific and age-dependent incidence

rates differ between the darker-skinned populations and the fair-skinned population is currently unclear in the literature, which is further discussed in Chapter 3 of the dissertation[342].

1.5 Melanoma Risk Factor: Age

The incidence rate of melanoma was first linked to age because of the cumulative UV radiation theory for all types of skin cancer[69,70]. In other words, the melanoma incidence rate is observed to increase with age to reflect the accumulated years of UV exposure[343]. However, beginning in the 1970s[344], researchers started to notice that, this age-dependent trend of melanoma incidence rate was not showing a strong positive relationship to cumulative UV radiation, particularly when the population-based cohort data were stratified by gender[115]. Indeed, it was later discovered by researchers that only males were showing a stable and positive relationship to accumulated UV exposure, indicated by an increased incidence rate with age and over time[92,93] and surged in older men age 60 years and older[116,345]. In contrast, it was continuously revealed by the scientists that females, especially the adolescents and young adults under the age of 40 to 50 years, presented a spiked incidence rate of melanoma, as compared to their male counterparts at the same age range[113,346]. The most prominent incidence rate difference between females and males was reported in 2013 by Liu-Smith et al. at the age of 20 to 24 years[114]. A few hypotheses have been presented to explain this age-dependent variability in melanoma incidence rate introduced by gender, including the famous intermittent versus chronic sun exposure theory[90] (subsection 1.2.1) based on gender-oriented occupations[347-349], the preferable use of tanning devices among adolescent and young adult women[226,227,350] (subsection 1.2.3), the influence of female sex-hormone[351], pregnancy[352], and the sex-specific genetic effects[353], and a mix behavioral effect of educational attainment, healthcare access, health consciousness, cosmetics and sunscreen use, and attention to the appearance, that leads to an earlier detection of melanoma in women[354,355]. The following sections will discuss the sex-specific effects of female sex-hormone and the behavioral components.

1.6 Melanoma Risk Factor: Gender

"Sex differences in cancer is an old problem," said Dr. Paul Boutros, professor of University of California, Los Angeles, Department of Human Genetics and director of Cancer Data Science at the Jonsson Comprehensive Cancer Centre. "Sex is important in cancer and not only because of lifestyle differences. The source of that bias, however, has remained unclear." Dr. Kenneth Buetow, director of Computational Sciences and Informatics Program, Complex Adaptive Systems Initiative at the Arizona State University, also commented on recent news released by Nature on February 13th, 2019 titled "Biological sex shapes tumour evolution across cancer types." Because of a large number of tumor subtypes, distinct molecular profiles, and various outcomes, sex differences as one of the crucial determinants of the origin of cancer differences may influence the kinds of cancer-causing mutations, resulting in different cancer formation trajectories. Indeed, started in 2014, the US National Institutes of Health (NIH) has been encouraging researchers to consider sex differences in preclinical studies[356]. Some studies have since found a few sex-associated biases in the frequency of gene mutations in certain cancers e.g. brain tumors[357] and metastatic melanoma[358]. In the metastatic melanoma study, the researchers pointed out a greater DNA missense mutation burden in males than in females, and these mutations were not the "signature" UV-induced DNA pyrimidine dimer mutations. These genetic biases may lead researchers to a better understanding of how melanoma develops and evolves between genders. However, the database the researchers utilized, The Cancer Genome Atlas (TGCA), only provides the exome sequence, which only encodes genes that are transcribed to functional proteins. The non-coding regions that usually play a regulatory role[359] remain undetermined. Moreover, the patient biospecimens retrieved from the TCGA database were melanoma tumors and the genetic profile of healthy tissues was not examined. Therefore, the

mutation burden was already the end result of the cumulative tumorigenic progression process. An examination of these samples seemed not able to fully explain the propensity for gender disparity in early-onset melanoma.

Dr. Paul Boutros aimed to fill this gap by looking at not only the protein-coding genes but also the non-coding regions where the DNAs are controlling for genes to turn on or off as well as many other functions that have remained undetermined. In 2019, Dr. Boutros and his research team studied 1,983 patients of 28 cancer types retrieved from the International Cancer Genome Consortium[360]. They first studied 174 known cancer-driving mutations in the coding and non-coding DNA regions across cancers and in each specific cancer type. The results showed sex-biased mutation frequencies between genders across these driver mutations. Next, they screened the entire genome and found 4,285 more sex-dependent differences in the loss or duplication of DNA segments. Some of these differences arose at different cancer stages, suggesting different cancer trajectories between genders. This study provides a foundation for other researchers to generate hypotheses based on sex-dependent genetic differences in cancer etiology.

On the other hand, Dr. Boutros and his research team also studied germline-predisposed pathogenic (i.e. cancer-driving) genetic variants in 2018[361]. Among 10,389 patients of 33 cancer types, they discovered 853 potential cancer-driving genetic variants in 21 genes, including some novel ones that have not been published to date. Nevertheless, germline-predisposed cancer-driving genetic variants that differ between genders has not yet been clarified.

Although gender differences in melanoma have been continuously reported in the literature since 1975, it is still elusive as to why women develop melanoma at an earlier age than men[113], particularly in adolescent and young adult women where between the age of 20 and 24 years there is a highest increase in the incident cases discovered by Liu-Smith et al. in 2013[114]. After

the age of 50 years, there is a rapid increase in the incidence rates in men[115,116] while old adult women present a relatively stable[117] to slightly declined[118] incidence rates. A qualified biological explanation on this phenomenon has not yet been established.

In the early 1970s, Sadoff et al. first proposed that melanoma should be grouped as one of the "estrogen-dependent" tumors[362]. Noticeable patterns that came to this hypothesis included darkened naevi during pregnancy[363-365], more dysplastic naevi found during pregnancy[366], and hyperpigmentation of the face (i.e. chloasma) during pregnancy or due to the use of oral contraceptives[367]. When estrogen receptor alpha (ERα) was first cloned in 1986[368], it was believed that ERα played a critical role in melanoma progression[369,370]. However, the field of melanoma research soon dropped this hypothesis because ERα was unable to be constantly detected in melanomas and the findings were inconsistent[371]. The association between pregnancy and the prognosis of melanoma was also found to be controversial. One aspect of the research suggested an increased risk of dysplastic naevi transformed to malignant melanoma during pregnancy[372] and an increased risk of metastasis[373] because of a blunting state of immune surveillance during pregnancy promoting tumor progression[374]. In women, malignant melanoma is rare before adolescence[375] but increases significantly during reproductive age until the age of 50[376]. But an earlier age of childbearing and multiparity also decrease the risk of developing melanoma in women[377]. Indeed, other researchers suggested that these results reflected that these findings was just an end result of delayed diagnosis and treatment due to pregnancy[378,379]. From the Nurses' Health Study (NHS) between 1976 and 2016, current or recent use of oral contraceptives was found to be associated with an increased risk of melanoma during the reproductive age[380]. The association was enhanced in long-term users and with higher doses[381]. However, there was another group of researchers who did not find an association between use of

oral contraceptives and the risk of melanoma in case-control[382-384] and population-based cohort[385,386] studies. Menopausal women on hormone replacement therapy were also found to be at risk of developing malignant melanoma[387,388] although contradicting findings remained[389,390].

Since in 1996 when the second ER subtype the ERβ was cloned[391], many studies have now shown that melanoma cell lineages[392] and normal melanocytes[393] responded well to 17β-estradiol, the major female-sex estrogen steroid hormone, possibly via ERβ instead of ERα. Since then, estrogen receptors have made a comeback in the field of melanoma research.

The estrogen steroid hormone initiates its cellular responses via the two major ERs, ERα and ERβ. The "classical response" is represented by the diffusion of estrogen hormone into the cell and binding to the nucleus membrane-bound ER to translocate together into the nucleus and initiate the transcription of estrogen-responsive genes[394] that are involved in cell growth, reproduction, development, and differentiation[395]. In reproductive-competent women, this "classical response" takes place in the ovaries where estrogen hormone is synthesized as its predominant form 17β-estradiol and utilized locally in the ovaries or systematically in the target organs such as the uterus and breast[395]. In men, 17β-estradiol is converted by the circulating male-sex hormone androstenedione and acts locally on the target organs such as the breast, brain, and fat tissue[395]. The reproductive functions of estrogen as a sex hormone are detailed in excellent textbooks[396-398] and are thus not discussed in the current dissertation. The "non-classical response" acts more quickly, through the binding of plasma membrane-bound ERs to increase cellular calcium ion (Ca^{2+}) or nitrate oxide (NO) levels, or to activate protein kinases[399], the cellular enzyme that catalyzes the phosphate groups between molecules to initiate or stop protein functions[400]. Interestingly, the binding of estrogen hormone to plasma membrane-bound ERα and ERβ initiates diverted downstream responses which counteract each other[401]. For

instance, ERα induces cell growth and expansion[402], while ERβ controls cell cycle progression to prevent overproduction and eliminate erroneous cells through cell apoptosis[403]. ERα but not ERβ is also found to co-express with a protein kinase, the insulin-like growth factor 1 receptor (IGF-1R), which has been continuously implicated in tumorigenesis[404-406]. ERα is found in 70% of breast cancers[407,408], with a role in progression and metastasis[409], as well as predicting a poorer prognosis in lung[410], gastric[411], prostate[412], and thyroid cancers[413]. On the contrary, ERβ functions as a tumor suppressor in most malignancies. For instance, ERβ is crucial in restoring the sensitivity to chemotherapy in ovarian cancer, as well as the inhibition of tumor cell survival, migration, and invasion[414,415]. In addition, ERβ can induce colorectal cancer cell apoptosis and reduce the formation of colorectal polyps[416].

Indeed, ERβ has been found to be the predominant ER subtype in the normal skin[417], melanocytes, and benign melanocytic neoplasms such as dysplastic naevi and lentigo maligna[418], while ERα is absent in the normal skin[417]. ERβ has been suggested to regulate the normal estrogen-dependent melanocytic physiology. However, ERβ detected in the skin drops remarkably in melanoma tumors with increasing Breslow thickness and in metastatic melanoma[418]. ERβ might thus be essential in monitoring melanocytic neoplasm development, while ERα is critical in promoting progression to melanoma.

In the 1980s, researchers found that there was no difference in the expression level of ER between men and women in the normal skin but a great difference was shown between genders in melanoma tumors with a higher ER concentration detected in female-origin melanomas[419]. At that time, ER had not been discovered to have different subtypes and therefore it was not specified which ER was showing differences between genders in melanoma tumors. Later in 2007, Susan Stevenson and Julie Thornton summarized that ERβ expression in the normal skin is

sex- and age-dependent[420]. In men, the skin levels of ERβ are lower than women. In women, the skin levels of ERβ drops with age and drops more rapidly after menopause. In 2014, a group of researchers analyzed the expression frequencies of ERα, ERβ, and androgen receptor in melanoma tumors of stage- and age-matched pregnant women (n=18), non-pregnant women (n=18), and men (n=18)[421]. Because of the small sample size, ERα was only detected in one pregnant patient and one male patient. But there was significantly more ERβ expressed in pregnant patients than male patients, while no difference was found between pregnant patients and non-pregnant patients. None of these melanoma tumors expressed androgen receptor, the major receptor that interacts with male-sex androgens (e.g. testosterone and dihydrotestosterone), primarily reflecting that the skin locations of androgen receptor were only present in hair follicles and sebaceous glands[417]. Women usually have noticeable changes of skin darkening in the genital and areolar areas during puberty[422] and epidermal pigmentation during the menstrual cycle[423]. Since ERβ expression in the skin is higher in premenopausal women than in men and can be found not only in the healthy skin but also in melanocytic neoplasms, perhaps the fluctuation of estrogen hormone from adolescence throughout the reproductive age might affect the ratio of ERβ to ERα expression, which would make it one of the chief determinants in women developing melanoma at an earlier age than men and the highest increase in melanoma incident cases during 20 to 24 years in young women.

Recently, ERα and ERβ signaling have been implicated in inducing oxidative stress, although results to date have been repeated using experimental animal models. Myocardial oxidative stress induced by ethanol is dependent on estrogen, and this signaling is mediated by ERα and ERβ[424-426]. When estrogen is absent, the coronary resistance to oxidative stress is resumed[427]. Interestingly, these studies were done in female rats. In chemotherapy-treated

animals, female mice were found to be more prone to oxidative stress-induced DNA damage than the male mice[428]. These animal experiments indicated that females are under natural oxidative stress because of estrogen-dependent responses, and oxidative stress weighs heavily in its effects on melanoma progression (subsection 1.2.2).

The patterns of melanoma tumor distributions on the body surface, stratified by gender and age, have been widely studied in the fair-skinned populations[81-83,86,88,429] but rarely been examined in the darker-skinned populations. This observation has led to Chapter 4 of the dissertation[430]. In addition, if this gender-specific and age-dependent distributions of melanoma tumors on the body surface present a universal pattern between the fair-skinned and the non-fair-skinned populations, regardless of the UV radiation impact on different skin colors, then a gender-oriented factor that predisposed the women to develop melanoma earlier than men is expected. An additional biological explanation of melanoma gender disparities which lies in estrogen receptor signaling is included as Chapter 5 of the dissertation.

1.7 Melanoma Risk Factor: Behavioral

The whole-body "ABCDE" screening[35] of cutaneous melanoma done by primary care practitioners and dermatologists usually identifies melanomas accidentally[354]. However, the US Preventive Services Task Force did not find the benefits of a whole-body screening to outweigh the harms in early skin cancer detection[431,432], e.g. overdiagnosis, procedure-related adverse effects, and psychological harms[433], and therefore, lower than 25% of American adults reported ever receiving a whole-body skin examination[434]. The majority of melanomas is still a result of self-detection[354,435-437] of some clinical manifestations[354,438] such as noticeable naevi size change, itching, or bleeding that drove the patients to their healthcare practitioners for further examination. Interestingly, women are usually more likely to notice changes on the appearance of themselves or of their partners[354,435,436]. This results in smaller and thinner melanomas detection with fewer metastases and fewer associated deaths[354]. Furthermore, among those surgically removed melanoma survivors, women are more likely to adopt behavioral changes to accommodate sun protection. For instance, women are found to be more likely than men to limit outdoor activities and to seek shade when being outside[355].

Cosmetics with sun protection factor (SPF) added or sunscreen use does not always decrease the incidence rates of melanoma[439]. Not surprisingly, this requires a constant reminder sent to the public, otherwise the old behavior will replace the sun protection practices[440]. This behavioral retroversion is especially prevalent in men older than 50 years old[441]. In fact, the benefit of applying sunscreen to reduce the incidence rates of melanoma has not reached any type of consensus in the literature[442]. On the other hand, the socioeconomic status that leads to improved educational attainment, household income, and healthcare access is well related to melanoma detection and prognosis[354,355]. Education level increases with melanoma awareness as does self-

examination[443]. The lowest melanoma knowledge and attitude was found in demographic groups with lowest level of education and income[444]. Nevertheless, within one year after melanoma diagnosis, only an estimated 86.7% of patients had received surgical removal, while about 10.6% of patients had received inadequate or no treatment after diagnosis[445]. A lack of proper referral to the sub-specialist is, therefore, clearly impeding the immediate treatment of primary melanoma. Public health insurance[446] and education level are, not surprisingly, the strongest predictors of advanced melanoma, especially in the Hispanics and high-poverty neighborhoods[447]. Targeting underserved populations is another critical issue in lowering melanoma mortality.

Women are found to have a better health consciousness that leads to melanoma early detection because of a better knowledge of melanoma[448]. Knowing that melanoma is a kind of skin cancer is more frequent in adult women[443]. In addition, they are usually more likely to perform sun protection secondary to a higher risk awareness[449], while sunburns are reported more frequently in men[450]. However, melanoma early detection usually refers to a diagnosis made at an earlier stage of the disease but does not necessarily mean at a younger age. And therefore, a better health consciousness is still insufficient to support a causal relation to the disparity of melanoma gender differences in the age-dependent incidence rate.

1.8 Melanoma Treatment and Survival

The 5-year survival of melanoma improved from 60% in the 1960s to 91% in 2015 in the fair-skinned population[451]. However, the 5-year survival remained relatively low in the darker-skinned populations[452], with 70% in Hispanics, 58% in African Americans, 70% in American Indians, and 71% in Asian Americans[453]. Early detection not only makes a difference in survival[454] but also the cost of treatment[455]. The annual cost of melanoma treatment had increased by 288% from 2002 to 2011 and reached $3.35 billion[456]. The delayed diagnosis could arise from attention to the lesions only when clinical manifestations occurred e.g. bleeding and ulceration[457], common beliefs that melanoma does not happen to a specific racial background[458], and the misdiagnosis made by the practitioners due to unnoticeable melanoma subtypes e.g. acral lentiginous melanoma and amelanotic melanoma[459], and lack of time and training[460]. Melanoma has been found to be irrelevant in Asians and African Americans, and therefore, the awareness of the term “melanoma” itself is lacking in these populations[458]. Adding “skin cancer” to “melanoma” is suggested to be a way to promote awareness in these ethnic minorities of melanoma[458]. Moreover, melanoma is diagnosed in non-UV-exposed regions with most lesions found on the soles of the feet[322,323,337,461] in the darker-skinned populations, and therefore, the traditional treatment based on melanoma diagnosed in the fair-skinned population might not apply to this subtype of melanoma in the darker-skinned populations and hence correlates to a poorer survival rate.

While most melanoma tumors can be surgically removed, some can be fatal. Primary melanoma is first diagnosed with a biopsy since this is the only way to determine the Breslow thickness (i.e. staging) of the tumor[462]. After the diagnosis, the melanoma tumor lesion will be removed with the resection of some healthy skin nearby, depending on the thickness of the

tumor[463]. Subsequently, the lymph nodes nearby the primary lesion will be examined by a biopsy to see if there is any sign of tumor migration[464]. Radiotherapy will be applied if a large number of lymph nodes are found to contain melanoma tumor cells[462]. Lastly, if melanoma tumor has spread to other parts of the body revealed by a CT or MRI scan[465], chemotherapy will be applied to treat stage IV metastatic melanoma. Currently, there are over 20 metastatic melanoma treatments approved by the US Food and Drug Administration (FDA)[466]. Dacarbazine, approved in 1974, was the first standardized treatment for stage IV melanoma[467]. However, the median survival reached only 5.6 to 7.8 months for stage IV melanoma patients after dacarbazine treatment[468]. Zelboraf (vemurafenib), approved in 2011, was the first standard treatment for $BRAF^{V600E}$-mutant metastatic melanoma, reaching an overall patient survival at 84% at 6 months[468]. This is due to the advancement in discovering sporadic genetic mutations in melanoma tumors in order to develop targeted chemotherapy[465]. Ipilimumab, also approved in 2011, was a monoclonal antibody used to increase T lymphocytes production. It worked as an immune system booster to manage metastatic melanoma co-treated with targeted chemotherapy[469]. In more recent years, the FDA approved the combination of targeted therapeutics as a better way to improve stage IV patient survival. For example, dabrafenib (Tafinlar) plus trametinib (Mekinist)[470-473] was approved in 2014, and vemurafenib plus cobimetinib (Cotellic)[474,475] was approved in 2015. Both regimens were used for $BRAF^{V600E}$-mutant metastatic melanoma targeting the MAPK tumorigenic signaling pathway[476]. The median survival for the dabrafenib (Tafinlar) plus trametinib (Mekinist) combination had been found to reach 25.1 to 26.1 months, while the median survival for the vemurafenib plus cobimetinib (Cotellic) combination was 22.3 months[477]. The third combination therapy approved by the FDA in 2018 was encorafenib (Braftovi) plus binimetinib (Mektovi). This combination targeted

multiple $BRAF^{V600}$ mutations and the MAPK pathway. In phase 3 trial results, this combination reached a median progression-free survival by 12.9 months[478]. As the trial is still ongoing, the overall median survival has not been reached thus far.

Nevertheless, these various treatments currently available for stage IV metastatic melanoma still provided a very poor life expectancy, with the majority of the treatments demonstrating a 3-year survival lower than 50%[477]. This has reflected to the rapidly-developed resistance to therapy[479] as most patients progressed within one year[480]. Therefore, the most effective way to manage the epidemic of melanoma is still to prevent the disease from happening or an early detection when surgical removal is still feasible, and the best survival rate can be reached. Melanoma prevention programs are proven to show promising effectiveness in secondary prevention in the high-risk populations[481]. For instance, the mass media campaigns started in the 1980s in Australia has led to a higher detection rate of melanoma in early stages[482], and evidence from Germany also demonstrated a higher diagnosis rate for skin cancers after the implementation of the nationwide population-based skin cancer screening program in 2008[483]. However, in the US, there is still limited evidence to support a nationwide skin cancer screening program that leads to a decrease melanoma mortality rate[444,484]. The sustainability of the short-duration intervention programs is the reason why the evidence is difficult to show effectiveness in the long term[485,486]. A possible strategy to further improve the effectiveness of current prevention status is to educate primary care practitioners to be aware of the ABCDE whole-body screening for the high-risk individuals who come annually for a health checkup[487]. As an old idiom goes, "*prevention is better than cure* (by Henry De Bracton, in *De Legibus*, was published in 1240)." This statement surely applies to melanoma and thus encourages continuous research,

surveillance, monitoring, and assessment of more desirable prevention strategies as a cure of late primary melanoma and metastatic melanoma is still a long way away.

CHAPTER 2 NADPH ENZYME COMPLEXES-ASSOCIATED RISK OF CUTANEOUS MELANOMA

2.1 Introduction

UV rays are capable of inducing melanin production in melanocytes and promoting melanin transportation to the outermost layer of the skin—the keratinocytes. These melanins form a cap over the nucleus of both cell types and protect DNA from direct energy destruction[50,488]. On the other hand, UV rays are also able to initiate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) dominated reactive oxygen species (ROS) production and chemiexcitation of melanin fragments that affect DNA stability in melanocytes[44,153,212]. The oncogenic characteristics of UV-induced ROS signaling have not yet been fully elucidated, particularly in the transformation of melanocytes to melanomas.

Recent understanding of melanoma photobiology has implied the etiological role of NOX enzymes, particularly NOX1 and NOX4[61,191,489]. NOX enzymes produce superoxide and/or hydrogen peroxide when coupled with CYBA (p22phox) membrane protein[490]. RAC1, a newly defined melanoma oncogene[197], is shown to enhance NOX1 activity[491]. The downstream ROS metabolizing enzymes, e.g. copper-zinc superoxide dismutase (Cu-ZnSOD, *SOD1*), manganese superoxide dismutase (MnSOD, *SOD2*), and extracellular superoxide dismutase (ECSOD, *SOD3*), convert superoxide to hydrogen peroxide. Catalase then transforms hydrogen peroxide to water molecules (Figure 2.1). The cellular locations of NOX1, RAC1, NOX4, CYBA, and SOD enzymes, and their functions in ROS production and metabolism are illustrated in Figure 2.1. Little is known about the comprehensive role of this entire pathway in melanoma formation. However, the risk associated with these genes has been reported in various health conditions. For example, the V16A variant in *SOD2* (rs4880) showed an impaired mitochondrial

importing function and was associated with prostate cancer risk[492]. The rs7277748 and rs4998557 variants in *SOD1* were found to be associated with amyotrophic lateral sclerosis[493]. Variants rs2536512 and rs699473 in *SOD3* were linked to cerebral infarction[494] and brain tumor[495].

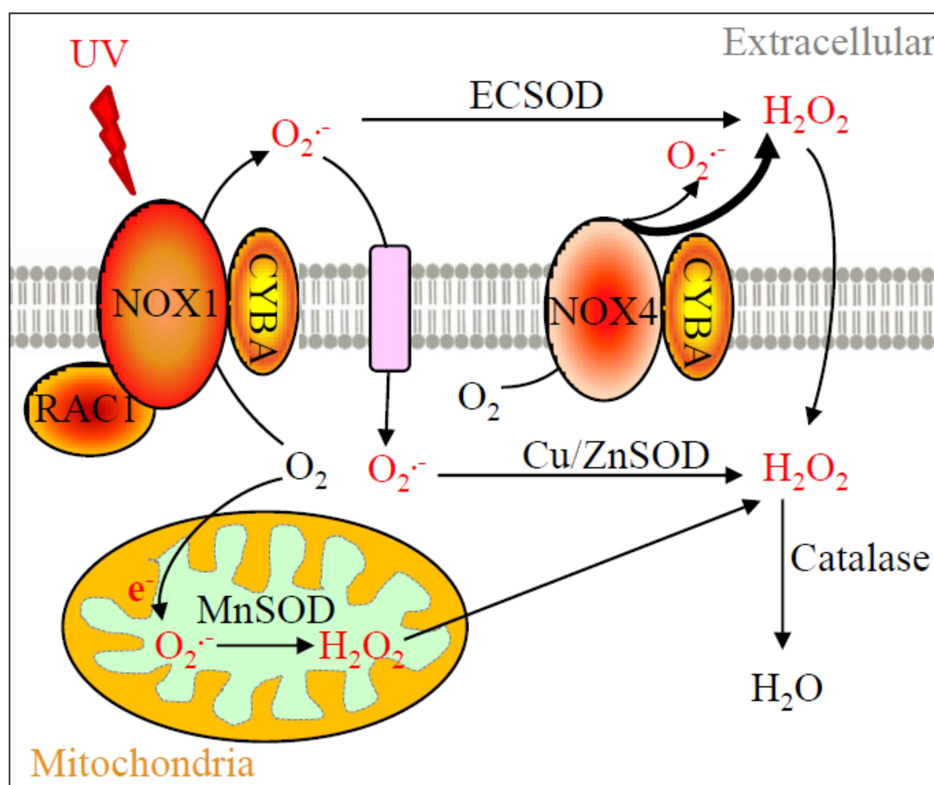


Figure 2.1 Diagram of the relevant reactive oxygen species (ROS) production pathway. NOX1, NOX4, CYBA, RAC1, SOD enzymes, catalase, their subcellular locations, and their functions in ROS production and metabolism are depicted in this diagram. NOX1 enzyme complex utilizes CYBA as one of its subunits and is activated by RAC1-GTPase to produce superoxide. On the other hand, NOX4 only couples with CYBA to generate hydrogen peroxide and superoxide. Of particular note, only plasma membrane NOX4 is shown in this diagram but mitochondrial or nuclear NOX4 has also been reported[496]. NOX1 is activated by UV to enhance its superoxide production, which requires the GTPase activity of RAC1. Superoxide is further metabolized into hydrogen peroxide at various subcellular locations by different SOD isozymes. Hydrogen peroxide is then converted into water molecules by catalase. Other additional redox enzymes (e.g., glutathione peroxidases, which also convert hydrogen peroxide into water) are not the focus in this study and therefore not included.

Although the causal network of melanoma has not yet been fully elucidated[497], UV exposure is the most tangible environmental risk factor that can be readily modified by behavioral precautions[311]. Therefore, the purpose of this study was to explore the relationship between the hypothesized photobiological pathway and risk of melanoma. Specifically, our aim was to use the candidate gene approach to discover the association of variations in the genetic profile of the redox enzymes with melanoma (Figure 2.1). Building upon this rationale, functional genetic variants, namely single nucleotide polymorphisms (SNPs), were identified in this study with a priori chance of being associated with the risk of melanoma based on the following criteria: (1), not a well-known somatic mutation found in tumors with an established causality; (2) presented strong associations with many other health conditions in humans; and (3) with a potential to alter normal protein function based on the nucleotide substitution. For instance, variant rs8031 in *SOD2* was found to be associated with kidney complications in subjects with Type 1 diabetes[498]. Variant rs10951982 in *RAC1* has been implied in the increased risk of hypertension[499]. Even though rs10951982 in *RAC1* has not yet been reported in ROS-related malignancies, somatic mutations of *RAC1* (e.g., *RAC1*^{P29S}) were found in 9.2% of sun-exposed melanoma tumors[198,500].

With this genetic profiling information in hand, we hope to lay a foundation to identify those individuals predisposed to UV exposure and risk of melanoma. This, in turn, will contribute to a better primary prevention strategy, such as earlier-life behavioral precautions. To the best of our knowledge, our work was the first to use a hypothesis-driven and pathway-based approach to study the association between genetic variations in the ROS pathway and risk of melanoma.

2.2 Materials and Methods

2.2.1 Ethics statement

We obtained approval from the Institutional Review Board of the University of California Irvine Office of Research.

2.2.2 Study population

Our study subjects were adopted from a previously designed case-control study (the international Genes, Environment, and Melanoma study, the GEM study), although we made considerable modifications. The original GEM case-control study compared white multiple melanomas patients to primary melanoma patients[501]. In total, 177 patients were recruited between 1998 and 2003 in the southern California area as part of the GEM study, and consent forms were obtained accordingly[501]. In our study, we used both of these patients as our cases and we recruited additional healthy participants as controls. Healthy white volunteers from Orange County were recruited through random-digit-dialing by trained interviewers from 1999 to 2006.

Demographic information regarding age, sex, family history of melanoma, and lifetime sun exposure were recorded via in-person questionnaires and phone interviews, with written consents from the patients and their physicians[501-507]. Random-digit-dialing healthy respondents completed eligibility screening questions over the phone, including being Orange County residents and having no personal history of melanoma or any other types of cancer. Eligible respondents were asked for their verbal informed consents for a 20 min standardized phone interview[501], in which they were asked questions about basic demographics, personal medical history, and family cancer history. In total, 172 participants further agreed to donate a blood sample. A phlebotomist obtained written consents from these participants while performing the blood draw[501]. Participation rate

after phone eligibility screening was approximately 78%. Population-based controls were frequency-matched to cases with respect to sex and age (Table 2.1).

2.2.3 DNA extraction

Buccal cells from melanoma patients and whole blood cells from healthy participants were re-suspended in a phosphate-buffered saline system. Ten microliters of the cell suspension were used directly as a template for whole genome amplification (WGA). The WGA procedure was conducted following the manufacturer's instruction from Sigma. In brief, a cell suspension (10 µL each) was heated to 95 °C for 5 min in a PCR machine in a strip of PCR tubes and cooled down on ice. One microliter of 10× Fragmentation Buffer was added to each tube. Tubes were then heated again in a PCR machine at 95 °C for exactly 4 min. Samples were cooled down on ice immediately and then centrifuged briefly to consolidate the contents. Out of 70 µL of the amplified sample, 6 µL was mixed with 1 µL of 6× loading buffer and directly used to load on an agarose DNA gel containing ethidium bromide. DNA was visualized under a UV lamp and water was used as a non-DNA negative control to compare with the presence of the visualized DNA product. Participants with little to no whole genome amplified DNA product were excluded from SNP genotyping (7 patients and 20 healthy controls were excluded, Figure 2.2).

2.2.4 SNP candidates

Functional SNPs were selected from a publicly available SNP database (dbSNP, NCBI) that have been found correlated with other diseases, based on the three criteria listed in the introduction (Table 2.2). In brief, 6 SNPs in the coding region of *NOX1* appeared in dbSNP. We were interested in D360N (rs34688635) and R315H (rs2071756) variants for the following reasons: (1) D360 is shared in *NOX1*, *-2*, *-3*, and *-4* [508], and conserved in various species including fish, mouse, bird, amphibian, and man[490]; and (2) 315H allele was found associated with diabetic patients, suggesting

that this is a functional allele and may be associated with other disease risks[509]. SNPs rs585197 and rs2164521 in *NOX4* have been linked to a protective effect on Hepatopulmonary Syndrome[510], and rs11018628 has a possible effect on plasma homocysteine level[511]. −930A>G in the *CYBA* promoter region (rs9932581) affects gene transcription activity and has been found to be associated with coronary heart disease due to ROS involvement in the pathogenesis of atherosclerosis[512]. Similarly, increased or decreased risks of hypertension[513] and coronary heart disease[36], respectively, have been found in *CYBA* alleles rs4673, rs13306296, and rs1049255. *CYBA* rs3180279 has been related to non-Hodgkin lymphoma prognosis[493]. Three SNPs, rs10951982, rs4720672, and rs836478 in *RAC1*, have been associated with risks in hypertension, inflammatory bowel disease, and end-stage renal disease[499,514-516]. Although these loci in *RAC1* have not yet been discussed in ROS-related malignancies, *RAC1* is a well-known melanoma oncogene with constantly activating mutations in some melanoma tumors[517,518].

SNPs in the three subtypes of *SOD* and *catalase* genes have been widely studied with various disease associations. For instance, rs7277748 and rs4998557 variants in *SOD1* (Cu-ZnSOD) were found to cause amyotrophic lateral sclerosis. Ile58Thr (rs1141718) in *SOD2* (MnSOD) severally impaired SOD2 enzymatic activity[519], while a variant of rs8031 increased oxidative stress[520]. V16A variant rs4880 in *SOD2* impaired mitochondrial importing and was found to be a risk factor for prostate cancer[492], whereas rs2758330 showed a protective effect on prostate cancer[521]. Variants rs2536512 and rs699473 in *SOD3* were associated with brain diseases, including cerebral infarction[494] and brain tumor[495]. The rs1001179 in *catalase* was also correlated to brain malignancy[495]. Additionally, −262C>T (rs1049982) variant in *catalase* showed a decreased interaction with HIF1α upon oxidative stress stimulation[522,523] (Table 2.2).

2.2.5 SNP genotyping

SNP genotyping polymerase chain reaction (PCR) assay kit was purchased from Life Technologies™ (Carlsbad, CA, USA). Allele-specific primers and probe sets for each SNP were also purchased from Life Technologies™, either custom-designed or from the library. DNA sample per participant was genotyped for every SNP in duplicates to ensure accuracy. About 97% of the SNPs were replicable. By definition, if one allele was amplified during PCR reaction, the call for that SNP assay was homozygous alleles (inherited the same alleles from both parents); if both alleles were amplified, the call for that SNP assay was heterozygous alleles (inherited different alleles from the parents). However, if no significant PCR amplification for either allele was observed, then the SNP assay was defined as N/A (genotyping failure) due to no reaction to the designed allele primers and probe. SNPs with a genotyping rate <75% were excluded from statistical analysis (SNPs rs13306296 and rs585197 were excluded from further analysis, Table 2.3). SNPs with inconsistent duplicated results were validated manually by reading the raw real-time PCR amplification plots, or through additional genotyping reactions.

2.2.6 SNP quality control

The raw PCR amplification data was analyzed by QuantStudio™ (Thermo Fisher Scientific Inc, Huntington Beach, CA, USA) Real-Time PCR software (v1.2). Those duplicated samples presenting identical calls were automatically determined by the software. However, if the calls were made differently between duplicates, or, in some rare cases, if the calls were "undetermined" by the software, then the individual PCR amplification plots were read manually and subjectively. Any amplification curve appearing after 20 cycles of PCR and being at least two-fold elevated from the threshold was determined as presenting a positive PCR amplification curve. Genotyping failure was assigned as N/A if no clear PCR amplification curve was observed.

2.2.7 Statistics

Allele frequency was determined by making counts of the participants based on different SNP conditions: genotypic, allelic, recessive, and dominant models. Chi-square or Fisher's exact test of independence was performed to examine the associations between SNP conditions and melanoma case-control status. Two-sided statistical significance level by default was set to be 0.05 (5%), and, to justify for multiple comparisons among the SNP candidates, the universal significance level was further adjusted to 0.05 divided by the number of final SNP candidates being tested, which was 0.05/21 = 0.00238, applying the most stringent Bonferroni approach[524,525]. Participant numbers varied among SNPs due to different genotyping rates, and only complete data were used for statistical analysis (participants with N/A data were excluded per SNP analysis). Bivariate simple logistic regression models showing the unadjusted associations between the binary response variable (melanoma cases vs. controls) and primary study variables of interest (SNPs) were conducted separately based on additive, recessive, and dominant allele models. Dummy variables of the SNPs in the three allele models were created by default, making genotype with homozygous major alleles as the reference to compare with. Odds ratios and 95% confidence intervals were calculated accordingly in RStudio (v0.99.893). Adjusted associations between SNPs and melanoma status were analyzed by fitting multivariate logistic regression models with the three allele models separately, controlling for known melanoma risk factors, including gender[526], age at diagnosis[527], family history of melanoma[114], and ever sunburned[528]. Genotypic Hardy–Weinberg equilibrium (HWE) exact test, which examines the expected frequencies of genotypes if mating is non-assortative and there are no mutations from one allele to another, was carried out by using R package "HardyWeinberg". In brief, a two-sided test was performed on genotype counts, whether

an excess or a dearth of heterozygotes counts as evidence ($p < 0.05$) against Hardy-Weinberg equilibrium.

2.3 Results

2.3.1 Study participants

Gender and age distributions of melanoma patients and healthy controls are listed in Table 2.1. In total, 177 retrieved cases and 172 recruited controls were approximately matched for age groups and gender. Overall, there are higher percentages of female patients aged 19–39 (55.4%) and 40–59 (26.5%), while, at age 60 and older, there is a higher percentage of male patients (47.9%). This may reflect the actual sex ratios of melanoma incidence at different age groups[114]. Of particular note, cases were retrieved from the international Genes, Environment, and Melanoma (GEM) study, which may not be strictly generalizable to a broader melanoma patient population.

Table 2.1 Characteristics of the study participants.

Study participant	Gender		
	Male ***n*** **(%)** [1]	**Female** ***n*** **(%)**	**Total** ***n*** **(%)**
Patients (n = 177)			
Age (years)			
19–39	5 (5.32%)	15 (18.1%)	20 (11.3%)
40–59	44 (46.8%)	46 (55.4%)	90 (50.8%)
≥60	45 (47.9%)	22 (26.5%)	67 (37.9%)
Controls (n = 172)			
Age (years)			
19–39	7 (7.1%)	15 (20.3%)	22 (12.8%)
40–59	45 (45.9%)	41 (55.4%)	86 (50.0%)
≥60	46 (46.9%)	18 (24.3%)	64 (37.2%)

[1] The percentage may not add up to 100% due to rounding.

SNP candidates and their currently known disease associations are listed in Table 2.2. Whole genome DNA amplification was successfully carried out in 322 study participants including 170 (96%) melanoma patients and 152 (88.4%) healthy controls (Figure 2.2). However, for each SNP, there were different numbers of failed genotyping samples due to poor PCR reaction, and the overall successful genotyping rates were between 66.4% and 98.7% in the controls, and between 78.8% and 99.4% in the cases. SNPs with a genotyping rate less than 75% on either arm (case or control group) of the participants were thus excluded from further analyses (SNPs rs13306296 and

rs585197 were excluded, Table 2.3). Ultimately, 161–169 melanoma patients, and 116–150 healthy controls remained to be further analyzed (Figure 2.2).

Table 2.2 Twenty-three SNP candidates.

Gene	SNP	location	dbSNP ID	Disease Association	Reference
NOX1	944G>A	R315H	rs2071756	Diabetes	509
	1284G>A	D360N	rs34688635	Severe pancolitis	508
NOX4	T>C	Intron	rs11018628	Increased plasma homocysteine level (risk in cardiovascular diseases)	511
	−114 C>T	5'UTR	rs585197	Decreased risk of the hepatic-pulmonary syndrome	510
	C>T	Intron	rs2164521	Decreased risk of the hepatic-pulmonary syndrome	510
CYBA^{p22phox}	−930A>G	Promoter	rs9932581	Modulates CYBA promoter activity	512,529
	242C>T	Y72H	rs4673	Decreased NOX activity; protective role in coronary heart disease	530-532
	−675A>T	Promoter	rs13306296	Related to hypertension	513
	C>G	Intron	rs3180279	Associated with non-Hodgkin lymphoma prognosis	493
	640A>G	3'UTR	rs1049255	Associated with coronary heart disease	533
RAC1	G>A	Intron	rs10951982	Risks in ulcerative colitis, hypertension, inflammatory bowel disease, end-stage renal disease	499
	T>C	Exon	rs4720672	Risks in inflammatory bowel disease, ulcerative colitis	514,515
	C>T	Intron	rs836478	Hypertension risk factor	499
SOD1	A>G	5'UTR	rs7277748	Familial amyotrophic lateral sclerosis	534
	7958G>A	Intron	rs4998557	Caused amyotrophic lateral sclerosis	534-536
SOD2	399T>C	Ile58Thr	rs1141718	Reduced enzyme activity	519
	T>C,A,G	V16A,D,G	rs4880	Mitochondrial importing, diabetes and prostate cancer	492,537-539
	T>A	Intron	rs8031	Oxidative stress	520
	C>A	Intron	rs2758330	Protective role in prostate cancer	521
SOD3	C>T	Promoter	rs699473	Brain tumor	495
	G>A	A377T	rs2536512	Cerebral infarction	494
Catalase	−262C>T	5'UTR	rs1049982	Down-regulated transcription upon oxidative stimulation	522,523
	C>T	5'UTR	rs1001179	Brain tumor	495

Table 2.3 Descriptive statistics of the 23 SNP candidates.

SNP [1]	Gene	Genotyping Rate [2]		Minor Allele Frequency (MAF)		Association (p-Value) [4]				HWE [5] (p-Value)	dbSNP MAF [6]
		Cases (n = 170)[3]	Controls (n = 152) [3]	Cases	Controls	Genotypic	Allelic	Recessive	Dominant		
rs10951982	*RAC1*	96.5%	83.0%	47.3%	23.6%	<0.001	<0.001	0.333	<0.001	0.459	16.6%
rs11018628	*NOX4*	99.4%	94.1%	50.0%	33.9%	<0.001	<0.001	0.458	<0.001	<0.001	16.7%
rs8031	*SOD2*	95.9%	87.5%	38.3%	49.6%	<0.001	0.008	<0.001	0.576	0.605	36.7%
rs2536512	*SOD3*	97.1%	75.8%	27.6%	37.5%	<0.001	0.016	<0.001	0.168	0.431	40.1%
rs4720672	*RAC1*	96.5%	92.2%	23.8%	17.6%	0.009	0.076	0.582	0.014	0.043	12.5%
rs4673	*CYBA*	98.8%	93.5%	36.6%	30.6%	0.014	0.132	0.561	0.013	0.238	33.6%
rs3180279	*CYBA*	98.8%	94.7%	48.2%	48.6%	0.022	0.985	0.154	0.127	0.030	44.5%
rs1049255	*CYBA*	97.1%	90.3%	48.2%	37.1%	0.027	0.007	0.034	0.041	0.719	46.9%
rs1001179	*Catalase*	96.5%	95.4%	16.8%	24.1%	0.062	0.030	0.610	0.025	1.000	12.6%
rs4880	*SOD2*	98.2%	93.5%	53.3%	55.2%	0.074	0.685	0.133	0.402	0.175	41.1%
rs9932581	*CYBA*	99.4%	90.9%	45.9%	42.5%	0.357	0.450	1.000	0.212	0.168	41.7%
rs699473	*SOD3*	97.1%	90.9%	63.0%	59.3%	0.461	0.388	0.745	0.280	0.861	44.1%
rs7277748	*SOD1*	95.9%	85.6%	4.0%	2.7%	0.486	0.518	N/A	0.511	1.000	3.9%
rs2164521	*NOX4*	98.2%	86.8%	9.3%	10.6%	0.646	0.688	0.442	0.792	1.000	26.2%
rs836478	*RAC1*	97.1%	94.8%	51.8%	50.0%	0.740	0.710	0.551	1.000	1.000	30.9%
rs4998557	*SOD1*	98.8%	94.1%	10.4%	11.5%	0.749	0.774	1.000	0.675	0.695	32.9%
rs1049982	*Catalase*	94.7%	88.8%	35.7%	36.3%	0.962	0.951	1.000	0.904	0.710	47.1%
rs34688635	*NOX1*	97.6%	90.2%	1.5%	1.1%	1.000	0.734	1.000	1.000	1.000	0.5%
rs1141718	*SOD2*	96.5%	98.7%	49.4%	49.0%	1.000	0.988	1.000	1.000	<0.001	N/A
rs2758330	*SOD2*	96.5%	96.1%	20.7%	20.6%	1.000	1.000	1.000	1.000	0.010	26.5%
rs2071756	*NOX1*	98.2%	92.2%	0%	0%	N/A	1.000	N/A	N/A	1.000	0.1%
rs585197	*NOX4*	73.5%	70.4%			Excluded from further analysis due to low genotyping rate (≤75.0%)					
rs13306296	*CYBA*	78.8%	66.4%			Excluded from further analysis due to low genotyping rate (≤75.0%)					

[1] Ordered according to smallest to largest genotypic p-values.
[2] Percentage of participants with SNP genotyping success.
[3] Participant number = n * %.
[4] Chi-square or Fisher's exact test of independence between SNP models and melanoma status (case and control).
[5] An exact test for Hardy–Weinberg equilibrium (HWE) on the controls, $p < 0.05$ counts as evidence against HWE. HWE is a test of genotype balance in a given population.
[6] Reference minor allele frequencies documented in the dbSNP database. N/A: not available.

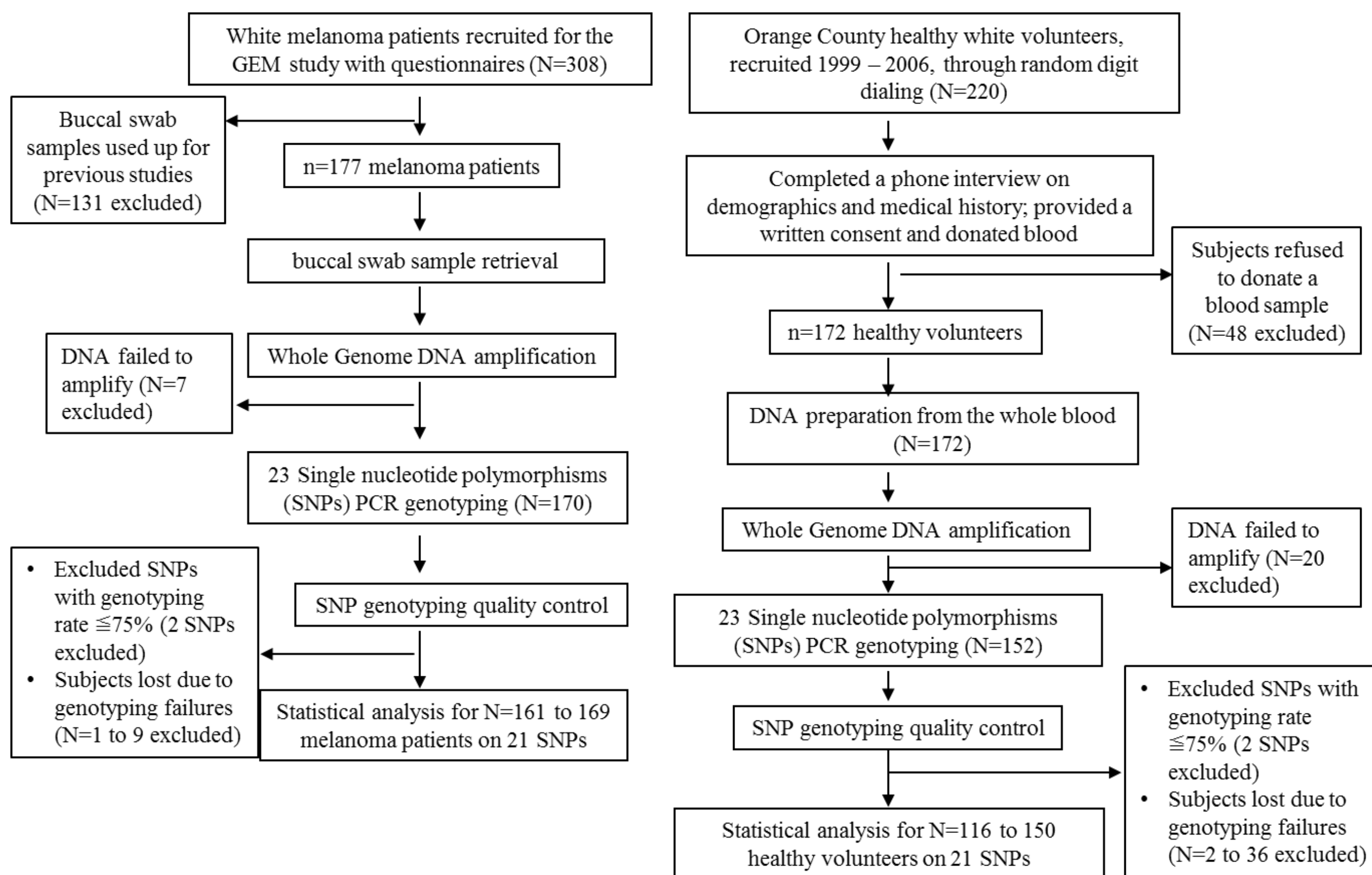


Figure 2.2 The inclusion and exclusion criteria of the participants in this study.

2.3.2 SNP associations

Chi-square or Fisher's exact test of independence was performed to identify SNP frequency differences between melanoma patients and healthy controls under genotypic, allelic, recessive, and dominant SNP models (Table 2.3). An exact test of genotype counts on the Hardy–Weinberg equilibrium (HWE) was conducted to identify and exclude SNPs, not in genotype balance in our study sample. Under the genotypic model, five SNPs exhibited statistically significant ($p < 0.05$) frequency differences between cases and controls: rs10951982 (*RAC1*), rs8031 (*SOD2*), rs2536512 (*SOD3*), rs4673 (*CYBA*), and rs1049255 (*CYBA*) (Table 3). The allelic model only determined three of them as being significant: rs10951982 (*RAC1*), rs8031 (*SOD2*), and rs2536512 (*SOD3*). These three alleles exhibited significance in the recessive model as well. In the dominant model, rs10951982 (*RAC1*), rs4673 (*CYBA*), and rs1049255 (*CYBA*) showed significance. The rs1001179 (*catalase*) showed a significant difference between cases and controls in the dominant and recessive models but the significance disappeared in the other two models.

2.3.3 Bivariate logistic regression analyses

The top five SNPs identified from the genotypic model without HWE violations were fitted into bivariate logistic regressions with additive, recessive, and dominant allele models, respectively. The odds ratios of melanoma risk were calculated using the homozygous major allele genotype as the reference (Table 2.4). Odds ratios derived from the regression models were compared to a corrected significance level at 0.00238 (0.05/21) to justify for multiple comparisons among the remaining 21 SNP candidates. Odds ratios with *p*-values < 0.00238 were considered having statistical significance in the results.

Table 2.4 Crude associations between the top five SNPs and melanoma risk.

SNP/Model	Allele	Cases (n = 170) n (%) [1]	Controls (n = 152) n (%)	OR (95% CI)	p-Value [2]
rs10951982 (*RAC1*)					
Additive	GG	21 (12.4%)	72 (47.4%)	Reference	--
	GA	131 (77.1%)	50 (32.9%)	8.98 (5.08, 16.44)	<0.001
	AA	12 (7.1%)	5 (3.3%)	8.23 (2.73, 28.39)	<0.001
Recessive	GG+GA	152 (89.4%)	122 (80.3%)	Reference	--
	AA	12 (7.1%)	5 (3.3%)	1.93 (0.69, 6.19)	0.230
Dominant	GG	21 (12.4%)	72 (47.4%)	Reference	--
	GA+AA	143 (84.1%)	55 (36.2%)	8.91 (5.09, 16.19)	<0.001
rs1049255 (*CYBA*)					
Additive	CC	47 (27.6%)	56 (36.8%)	Reference	--
	CT	77 (45.3%)	63 (41.4%)	1.46 (0.88, 2.44)	0.149
	TT	41 (24.1%)	20 (13.2%)	2.44 (1.27, 4.79)	0.008
Recessive	CC+CT	124 (72.9%)	119 (78.3%)	Reference	--
	TT	41 (24.1%)	20 (13.2%)	1.97 (1.10, 3.61)	0.022
Dominant	CC	47 (27.6%)	56 (36.8%)	Reference	--
	CT+TT	118 (69.4%)	83 (54.6%)	1.69 (1.05, 2.74)	0.031
rs4673 (*CYBA*)					
Additive	GG	53 (31.2%)	66 (43.4%)	Reference	--
	GA	107 (62.9%)	68 (44.7%)	1.96 (1.23, 3.15)	0.005
	AA	8 (4.7%)	10 (6.6%)	1.00 (0.36, 2.70)	0.994
Recessive	GG+GA	160 (94.1%)	134 (88.2%)	Reference	--
	AA	8 (4.7%)	10 (6.6%)	0.67 (0.25, 1.75)	0.412
Dominant	GG	53 (31.2%)	66 (43.4%)	Reference	--
	GA+AA	115 (67.6%)	78 (51.3%)	1.84 (1.16, 2.92)	0.010
rs8031 (*SOD2*)					
Additive	AA	45 (26.5%)	32 (21.1%)	Reference	--
	AT	111 (65.3%)	70 (46.1%)	1.13 (0.65, 1.94)	0.665
	TT	7 (4.1%)	31 (20.4%)	0.16 (0.06, 0.39)	<0.001
Recessive	AA+AT	156 (91.8%)	102 (67.1%)	Reference	--
	TT	7 (4.1%)	31 (20.4%)	0.15 (0.06, 0.33)	<0.001
Dominant	AA	45 (26.5%)	32 (21.1%)	Reference	--
	AT+TT	118 (69.4%)	101 (66.4%)	0.83 (0.49, 1.40)	0.489
rs2536512 (*SOD3*)					
Additive	GG	76 (44.7%)	43 (28.3%)	Reference	--
	GA	87 (51.2%)	59 (38.8%)	0.83 (0.51, 1.37)	0.477
	AA	2 (1.2%)	14 (9.2%)	0.08 (0.01, 0.31)	0.001
Recessive	GG+GA	163 (95.9%)	102 (67.1%)	Reference	--
	AA	2 (1.2%)	14 (9.2%)	0.09 (0.01, 0.33)	0.002
Dominant	GG	76 (44.7%)	43 (28.3%)	Reference	--
	GA+AA	89 (52.4%)	73 (48.0%)	0.69 (0.42, 1.12)	0.134

[1] Participants lost due to genotyping failure.

[2] p-value of the coefficient from the regression model. p-value was compared to a Bonferroni corrected significance level at 0.05/21 = 0.00238 to determine statistical significance. --: no p-value in the reference group.

In the additive allele model, carrying one copy of minor allele A in rs10951982 (*RAC1*) was significantly associated with a higher risk of melanoma (OR 8.98, 95% CI: 5.08, 16.44, $p < 0.001$), as compared to those who carried homozygous minor alleles AA (OR 8.23, 95% CI: 2.73, 28.39, $p < 0.001$). Dominant allele model further showed that combined minor allele copies (GA+AA) as compared to homozygous major alleles GG exhibited the highest risk of melanoma (OR 8.91, 95%CI: 5.09, 16.19, $p < 0.001$). A similar result was observed in rs4673 (*CYBA*), with one copy of the minor allele A exhibiting a higher risk of melanoma (OR 1.96, 95% CI: 1.23, 3.15, $p = 0.005$), and further confirmed in a dominant allele model (OR 1.84, 95% CI: 1.16, 2.92, $p = 0.010$). However, the p-values did not reach the corrected significance level of 0.00238.

The unadjusted odds of melanoma increased with homozygous minor allele T in rs1049255 (*CYBA*). TT exhibited an OR of 2.44 (95% CI: 1.27, 4.79, $p = 0.008$) in the additive model and an OR of 1.97 (95% CI: 1.10, 3.61, $p = 0.022$) in the recessive model. In both scenarios, *p*-values were greater than 0.00238, thus were non-significant because of the stringent Bonferroni correction for multiple comparisons.

In contrast, homozygous minor allele genotypes at both rs8031 (*SOD2*) and rs2536512 (*SOD3*) exhibited significant association with a reduced risk of melanoma in the additive allele model, with 84% reduction in odds of melanoma (OR 0.16, 95% CI: 0.06, 0.39, $p < 0.001$) for rs8031 (*SOD2*), and 92% reduction in odds of melanoma (OR 0.08, 95% CI: 0.01, 0.31, $p = 0.001$) for rs2536512 (*SOD3*). Similar results were also observed in the recessive model, where an 85% reduction in the odds of melanoma (OR 0.15, 95% CI: 0.06, 0.33, $p < 0.001$) was observed for rs8031 (*SOD2*) with TT minor alleles, and a 91% reduction (OR 0.09, 95% CI: 0.01, 0.33, $p = 0.002$ with marginal significance) for rs2536512 (*SOD3*) with AA minor alleles.

2.3.4 Multivariate logistic regression analyses

We continued to fit these top five SNPs into multivariate logistic regression under the three SNP models, controlling for major melanoma risk factors including gender, age at diagnosis, family history of melanoma, and lifetime ever-sunburned (Table 2.5). After adjusting for these risk factors, rs1049255 (*CYBA*), rs4673 (*CYBA*), rs8031 (*SOD2*), and rs2536512 (*SOD3*) were no longer associated with melanoma risk in all three models ($p > 0.00238$).

Table 2.5 Adjusted [1] associations between the top five SNPs and melanoma risk.

SNP/Model	Allele	Cases (n = 170) n (%) [2]	Controls (n = 152) n (%)	OR (95% CI)	p-Value [3]
rs10951982 (*RAC1*)					
Additive	GG	21 (12.4%)	72 (47.4%)	Reference	--
	GA	131 (77.1%)	50 (32.9%)	6.15 (2.98, 13.41)	<0.001
	AA	12 (7.1%)	5 (3.3%)	2.88 (0.68, 12.56)	0.149
Recessive	GG+GA	152 (89.4%)	122 (80.3%)	Reference	--
	AA	12 (7.1%)	5 (3.3%)	0.79 (0.21, 3.03)	0.719
Dominant	GG	21 (12.4%)	72 (47.4%)	Reference	--
	GA+AA	143 (84.1%)	55 (36.2%)	5.79 (2.84, 12.51)	<0.001
rs1049255 (*CYBA*)					
Additive	CC	47 (27.6%)	56 (36.8%)	Reference	--
	CT	77 (45.3%)	63 (41.4%)	1.20 (0.63, 2.30)	0.574
	TT	41 (24.1%)	20 (13.2%)	1.42 (0.61, 3.38)	0.420
Recessive	CC+CT	124 (72.9%)	119 (78.3%)	Reference	--
	TT	41 (24.1%)	20 (13.2%)	1.28 (0.59, 2.83)	0.531
Dominant	CC	47 (27.6%)	56 (36.8%)	Reference	--
	CT+TT	118 (69.4%)	83 (54.6%)	1.26 (0.69, 2.31)	0.456
rs4673 (*CYBA*)					
Additive	GG	53 (31.2%)	66 (43.4%)	Reference	--
	GA	107 (62.9%)	68 (44.7%)	2.17 (1.17, 4.07)	0.015
	AA	8 (4.7%)	10 (6.6%)	0.50 (0.11, 1.82)	0.315
Recessive	GG+GA	160 (94.1%)	134 (88.2%)	Reference	--
	AA	8 (4.7%)	10 (6.6%)	0.31 (0.07, 1.07)	0.080
Dominant	GG	53 (31.2%)	66 (43.4%)	Reference	--
	GA+AA	115 (67.6%)	78 (51.3%)	1.88 (1.03, 3.47)	0.042
rs8031 (*SOD2*)					
Additive	AA	45 (26.5%)	32 (21.1%)	Reference	--
	AT	111 (65.3%)	70 (46.1%)	1.33 (0.66, 2.65)	0.421
	TT	7 (4.1%)	31 (20.4%)	0.32 (0.09, 0.94)	0.047
Recessive	AA+AT	156 (91.8%)	102 (67.1%)	Reference	--
	TT	7 (4.1%)	31 (20.4%)	0.26 (0.08, 0.70)	0.011
Dominant	AA	45 (26.5%)	32 (21.1%)	Reference	--
	AT+TT	118 (69.4%)	101 (66.4%)	1.06 (0.54, 2.08)	0.864
rs2536512 (*SOD3*)					
Additive	GG	76 (44.7%)	43 (28.3%)	Reference	--
	GA	87 (51.2%)	59 (38.8%)	0.68 (0.35, 1.28)	0.232
	AA	2 (1.2%)	14 (9.2%)	0.26 (0.03, 1.50)	0.144
Recessive	GG+GA	163 (95.9%)	102 (67.1%)	Reference	--
	AA	2 (1.2%)	14 (9.2%)	0.33 (0.04, 1.83)	0.218
Dominant	GG	76 (44.7%)	43 (28.3%)	Reference	--
	GA+AA	89 (52.4%)	73 (48.0%)	0.65 (0.34, 1.21)	0.175

[1] Adjusted for gender, age at diagnosis/interview, family history of melanoma, and ever sunburned. [2] Participants lost due to genotyping failure.

[3] p-value of the coefficient from the regression model. p-value was compared to a Bonferroni corrected significance level at 0.05/21 = 0.00238 to determine statistical significance. --: no p-value in the reference group.

Consistent with what we have found in Table 2.4, the most significant genotype was heterozygous GA genotype in rs10951982 (*RAC1*), which exhibited an OR of 6.15 (95% CI: 2.98, 13.44, $p < 0.001$) after controlling for other risk factors. This minor allele also showed a significant association with melanoma risk in the dominant model (OR 5.79, 95% CI: 2.84, 12.51, $p < 0.001$). Similar results were also found for rs4673 (*CYBA*) but with only marginal significance. Heterozygous GA genotype was associated with an increased risk of melanoma (OR 2.17, 95% CI: 1.17, 4.07, $p = 0.015$), which was further confirmed in the dominant allele model (OR 1.88, 95% CI: 1.03, 3.47, $p = 0.042$), although the *p*-values did not reach the corrected significance level of 0.00238.

The homozygous minor allele TT genotype in rs8031 (*SOD2*) was found associated with a decreased risk of melanoma, with an OR of 0.32 (95% CI: 0.09, 0.94, $p = 0.047$) in the additive model, and an OR of 0.26 (95% CI: 0.08, 0.70, $p = 0.011$) in the recessive allele model, which indicated that homozygous minor alleles TT reduced the odds of melanoma by 74%, but neither of these results reached the universal significance level of 0.00238.

2.4 Discussion

After removal of SNP markers with high error rates during the assessment of genotyping quality, 21 SNP candidates remained to be eligible for the genetic association analysis. Eight SNPs showed significant association with melanoma but three of them were not in Hardy–Weinberg equilibrium (HWE), which may suggest that there are multiple alleles in the same locus, and we missed genotyping of other alleles. Therefore, only five SNP candidates showed genotypic significance and were further analyzed in regression models, including rs10951982 (*RAC1*), rs1049255 (*CYBA*), rs4673 (*CYBA*), rs8031 (*SOD2*), and rs2536512 (*SOD3*). We corrected the universal *p*-value to be compared with at 0.00238 (0.05/21, 21 SNPs being tested) to justify the multiple comparison issues in genetic association studies, using a Bonferroni approach[524,525]. The rs10951982 (*RAC1*) and rs4673 (*CYBA*) exhibited the highest increased risk of melanoma when presenting one copy of the minor allele in the unadjusted regression model, but rs4673 did not reach the universal significance level at 0.00238 in the multivariate regression model with adjustments for melanoma risk factors including age, sex, family history of melanoma, and lifetime ever-sunburned. Of particular note, a homozygous minor allele TT genotype in rs8031 (*SOD2*) was found to be associated with reduced risk of melanoma in the bivariate regression, however, significance was lost in the multivariate regression analyses.

SOD2 is known to be a major superoxide detoxifying enzyme of cells, and therefore an altered function or expression of this enzyme may lead to unbalanced redox homeostasis and thus potentially increase or decrease the risk of melanoma[519]. Since SOD2 converts superoxide to hydrogen peroxide (Figure 2.1), which belongs to a type of ROS, the function of SOD2 is thus double-edged. Our multivariate analysis indicated that homozygous TT allele in rs8031 reduced the risk of melanoma, but little is currently known about the molecular function of this variant. We

suggest a lab-based functional molecular biology study to unravel the discrepancy between zygote expression and enzymatic activity in this particular SNP.

SNPs rs1049255 and rs4673 in *CYBA* showed genotypic frequency differences between cases and controls in the unadjusted model (Table 2.4), with more patients carrying higher copies of minor alleles in rs1049255. Variant rs4673 changes the amino acid at position 72 from tyrosine to histidine (Y72H) of the CYBA (p22phox) protein, which is frequently referred to a C242T variant in the literature[540]. The T allele exhibited decreased dimerization with NOX and therefore may potentially reduce NOX activity and cellular ROS level[531]. In fact, the CT and TT genotype showed lower NADPH oxidase activity in hypertensive patients as compared with CC genotype[529]. However, the opposite observation was also reported, where the CT genotype and T allele are associated with higher risk of coronary artery disease[541]. In our study, the CT and TT (GA and AA) showed a higher risk for melanoma as compared to CC (GG) allele (the dominant model in Tables 2.4 and 2.5). This observation needs further validation. Variant rs1049255 is located in the 3' untranslated region (3' UTR) of the *CYBA* gene. Although the molecular function of this SNP is unknown, current understanding of 3' UTR is an important miRNA binding site, and SNPs located in this region might have the potential to regulate mRNA stability and translation efficiency[542,543].

RAC1-GTPase is a NOX1 activator which promotes binding of NOX1 with its subunits and forms the complete enzyme complex[544-546]. NOX1 was one of the first cellular molecules found to be directly regulated by RAC1 in the phagocytic process[547-549]. However, SNP rs10951982 in *RAC1* alone has not been reported in any ROS-related activities thus far. Information on the function of this locus and its association with any malignancy is limited in the current literature. Nevertheless, this variant has been reported to be associated with over-reactive immune diseases

and an increased risk of hypertension[499,514-516]. Considering that *CYBA* variants have been widely studied in cardiovascular diseases, including coronary heart disease[533] and hypertension[513], which are tightly associated with increased levels of ROS, *RAC1* rs10951982 may also play a part in inducing oxidative stress. Since rs10951982 is the most significant variant in our current study, and in lieu of its function in immune diseases as well as a potential role in NOX1-induced oxidative stress, our discovery might not only suggest an inflammatory microenvironment created by RAC1 that is in favor of melanoma progression[550] but also indicate an elevation of ROS level via RAC1 in melanoma etiology. In addition, RAC1 is also a crucial kinase in the NRAS and PI3K pathway[551], both of which are key melanoma oncogenic pathways. Therefore, it is possible that RAC1 plays a non-ROS role and impacts these other oncogenic pathways.

Overall, of the three significant SNPs after adjustment against age, sex, family history, and lifetime sunburn history, the minor allele of *RAC1* rs10951982 (the A allele) showed a consistent role with an increase ROS and thus increased melanoma risk. The minor allele of rs4673 (the A allele) was reported a controversial role in ROS association[529,552], it may exhibit certain cell-specific effects. In our study, the minor allele showed a higher risk of melanoma in a dominant model. The minor allele of rs8031 (the T allele) exhibited a protective role against melanoma risk in a recessive model. It is unclear how this allele modifies ROS levels. Based on our results, the T allele can be associated with either increased or decreased SOD activities as SOD2 is double-edged and can play dual roles in ROS metabolism.

Of particular note, in our regression models, we applied the most common ways of disease transmission, namely additive, recessive, and dominant modes, in our analyses. This was because we did not want to make any assumptions about the disease transmission modes. According to Sham and Purcell[525], a test that assumed additive effects would have greater power than a test that

also allowed dominance if the true effects at the locus were indeed additive and did not show dominance. Conversely, if the underlying causal variant was recessive, then power would be lost by carrying out an analysis that assumed additively. If there was uncertainty regarding the true pattern of effects at a locus, then it might be appropriate to use several statistical tests to ensure adequate statistical power for all possible scenarios. We, therefore, included results from these additional models that may provide more information and maintain statistical power as well. Although the covariates were not presented as part of the results in our tables, family history of melanoma and lifetime ever-sunburned controlled in the multivariate models consistently showed statistical significance, whereas sex and age did not. The family history of melanoma[114], along with fair skin, light hair and eye color are known melanoma genetic risk factors, whereas the levels of sun exposure including sunburns and moles or freckles are important environmental risk factors for melanoma[553]. The statistical significance of the covariates might indicate a mediating role in our primary study interest, from the susceptible familial genetic makeup of these participants, as well as the behavior or attitude towards sun exposure that resulted in getting sunburns or freckles.

Our study had a few limitations. First, the small sample size does not always provide sufficient power[554]. Second, by the experimental design, we could only genotype two alleles. Therefore, loci with multiple alleles may not show HWE and must be excluded for analysis. Third, our study participants included only those white individuals from the southern California area, and therefore a loss of generalizability to the broader white population might be expected. Last, a common limitation of case-control studies is that the results provide only an association with risk, but they are not necessarily connected to causality. Replicating findings from another dataset is a common strategy to validate the results identified in our current study. However, even with the most stringent statistical design, SNP findings are usually hard to replicate[524,525]. Multiple reasons are

considered, such as there are still unknown and uncontrolled confounders, multiple comparisons only lead to chance findings, the gene and environment interaction is not easy to account for, and the target allele is in linkage disequilibrium with the identified allele, but the chance finding failed to locate the target allele and thus make replication difficult to achieve. Nevertheless, we will still validate our findings in a separate dataset in our next study, as our ultimate goal is to develop useful markers in prevention.

To conclude, our initial analyses revealed an increased risk of melanoma associated with rs10951982 (*RAC1*), and a decreased risk associated with rs8031 (*SOD2*). Multivariate analyses further confirmed the association of an increased risk of melanoma with rs10951982 (*RAC1*). Our results highlighted the importance of RAC1 enzyme and cellular oxidation-metabolizing efficiency controlled by SOD2 in association with ROS-mediated risk of melanoma. We suggest that these results shall be further validated with the goal of designing novel screening targets to identify highly UV-susceptible individuals, particularly in the *RAC1* and *SOD2* genes, in order to take the melanoma primary prevention strategy to a precision level.

CHAPTER 3 THE ROLE OF FEMALE SEX IN CUTANEOUS MELANOMA

3.1 Introduction

Cutaneous melanoma has been considered as a UV radiation-driven malignancy[44,553,555-557]. Therefore, deciphering the mechanisms involved in UV-mediated melanoma formation and development has been the top priority in the field of melanoma prevention[469]. Because the white population exhibits the highest incidence rate compared with other ethnic groups[296], related etiological mechanisms have been extensively studied in this population. For example, the rich pheomelanin in the whites is found not only less effective in protecting skin stem cells[558] but also more prone to produce reactive oxygen species (ROS) upon UV stimulation[212]. The UV-broken melanin fragments are also found to diffuse along with ROS into the nucleus to induce DNA mutations, immunosuppression, and photoaging[44,559]. All these factors had led to the importance of melanin-related oxidative stress in the photobiological cause of melanoma in the whites. Fewer studies were based on other ethnic groups.

Recently, new epidemiology study on melanoma gender differences discovered that sex might play an independent role in early-onset melanoma, which may add expanded levels of understanding to our current UV-based melanomagenesis mechanism[92]. Overall incidence rates of men in all races in the US were higher than that of women[560]. However, when stratified by age, women under age 50 showed higher incidence rates as compared to men of the same age; and the incidence rates switched after age 50[561]. The highest female to male incidence rate ratio was found to be between age 20 and 24 years in the whites[114]. Our previous study suggested that other ethnic groups should have shown a similar age-dependent gender differences pattern[114]. Therefore, in this study, we focused on the gender difference in the non-white ethnic populations

based on a hypothesis that young women are at higher risk of melanoma than men in the same age range regardless of their race or skin color. This hypothesis argued for the importance of gender-related factors in early onset melanoma based on two reasons: 1) skin from the non-whites usually contains higher eumelanin levels, which are more protective against UV radiation; 2) while tanning bed use is popular in the whites, there is no such popularity in the non-whites, thus it is presumed that the often-blamed tanning device use should be excluded from the risks of the observed gender difference for early-onset melanoma. In order to address these questions and provide evidence for our hypothesis, data from the US Surveillance, Epidemiology, and End Results (SEER) Program, National Cancer Institute were used to analyze the incidence rate and rate ratio by age in the non-Hispanic blacks, Hispanics, Asians/Pacific Islanders, and American Indians/Alaska Natives. Furthermore, data from the Cancer Incidence in Five Continents, International Agency for Research on Cancer (IARC)/World Health Organization were used to validate the SEER findings.

The intrinsic sex impact on melanoma development has been understudied because, in the past, most of the sex disparity of melanoma incidence rates at a younger age was attributed to differential artificial and solar UV exposure[232,562-564]. Therefore, the emphasis on prevention and molecular studies has been focused on the UV effect. This is partially due to the early incorrect conclusion that estrogen receptors were absent in melanoma[371]. All three estrogen receptors (ERα, ERβ, and GPER1) were recently reported in melanoma tissues and cell lines[565-568]. These results provide molecular and cellular supports for our epidemiological observations, in which the female sex is independent of UV radiation and serves as a risk factor at younger age[93]. Naturally, the high estrogen levels in pre-menopausal women become potential driving forces for melanoma. Our previous studies showed that the female-to-male

rate ratios approach 1 around 45–54 years of age[114], which is THE approximate age for menopause in women in the US[569]. Therefore, young or old ages in this study refer to ages younger or older than this menopause-approximated age. This study aims to show that the melanoma incidence rates are also higher in young women as compared to young men in non-white ethnic groups, thus potentially excluding the indoor tanning bed use as a major attributor for the observed sex difference at young ages.

Our results may open discussions in melanoma research field on non-UV risk factors, thus novel prevention strategies can be initiated. As a large effort of public education on sunscreen use has been in place for many years, the incidence rate of melanoma continues to increase. The understudied non-UV risk factors may, therefore, be an explanation for the observed ineffectiveness of sun protection behaviors.

3.2 Materials and Methods

3.2.1 Study population

SEER13 database (Incidence - SEER13 Regs Research Data + Hurricane Katrina Impacted Louisiana Cases, Nov 2016 Sub (1992-2014) <Katrina/Rita Population Adjustment>) was downloaded through SEER*Stat software (version 8.3.4). Melanoma cases were collected from 13 SEER registries, including San Francisco-Oakland SMSA, Connecticut, Detroit (Metropolitan), Hawaii, Iowa, New Mexico, Seattle (Puget Sound), Utah, Atlanta (Metropolitan), San Jose-Monterey, Los Angeles, Alaska Natives, and Rural Georgia. Melanoma cases from 5 new registries collected between the year 2000 and 2014 in the SEER18 database, including California (excluding SF/SJM/LA), Kentucky, Louisiana, New Jersey, and Greater Georgia were combined with SEER13 data. Non-Hispanic black (B, n=1,533), Hispanic (Spanish-Hispanic-Latino, n=9,122), non-Hispanic Asian or Pacific Islander (API, n=1,968), and non-Hispanic American Indian or Alaska Native (AI, n=585) melanoma cases were included in this study.

The Cancer Incidence in Five Continents database (CI5) is the result of a long collaboration between the International Agency for Research on Cancer (IARC)/World Health Organization and the International Association of Cancer Registries. The CI5-Plus (1978-2007) database, which contains updated annual incidence rates for 118 selected populations from 102 cancer registries published in CI5 volumes I to X, was downloaded for analysis to validate the findings from SEER. Cancer cases from registries of the US SEER were excluded (registry codes 84001-84099). Black (registry code 80002 “Uganda”, n=90), Hispanic (registry codes 7602 “Brazil”, 17001 ”Colombia”, 18800 “Costa Rica”, 21801 “Ecuador”, 72401, 72404, 72406, 72407, 72408, 72410, 72413 “Spain”, n=10,645), and Asian (registry codes 35604, 35606, 35607 “India”, 39203, 39204, 39206 “Japan", 60801 “Philippines”, 70200 “Singapore”, 76401, 76404, 76405

“Thailand”, n=4,491, no melanoma cases documented in China registries up to 2007) melanoma cases were included for validation analysis.

3.2.2 Definition of melanoma

Melanoma was defined in the SEER database based on the Site Recode ICD-O-3/WHO 2008 as “Melanoma of the skin” and having AYA site recode/WHO 2008 category “7.1 Melanoma”. The Primary Sites “C00.0-C80.9” were all included. ICD-O-3 Hist/behave, malignant categories of invasive melanoma “8720/3-8723/3, 8726/3, 8727/3, 8730/3, 8740/3-8746/3, 8761/3, 8770/3-8774/3, and 8780/3” were included, with the exclusion of those in situ and non-cutaneous morphologies. Malignant melanoma was defined as “C43 Melanoma of the skin” using the ICD-10 site code in the CI5 database with the exclusion of in situ melanomas, as well as having the cancer code of “65 Melanoma of the skin”. Therefore, melanoma in this study refers to invasive malignant melanoma and does not include in situ melanoma.

3.2.3 Statistics

Age-adjusted incidence per 100,000 person-years was derived using US 2000 Census Standard Population in 19 five-year age groups (0, 0–4, 5–9, …, 80–84, and 85+) in SEER[570], and using World (WHO 2000–2025) Standard Million in 19 age groups in CI5. All further analysis and data management were carried out in RStudio (version 3.2.2), Stata or Microsoft Excel 2010, if not specified. Incidence rate ratio was calculated using female age-specific incidence rate divided by male age-specific incidence rate. The 95% confidence intervals of rate ratios were calculated by Stata software using standard errors calculated from natural log rate ratios[571]. The *p*-value of a trend for rate ratio change over different time periods was calculated by Chi-square test for trend according to the Cochran–Armitage method[572].

3.3 Results

3.3.1 Patient characteristics

Cancer cases of all cause documented in the SEER13 database (1992-2014), as well as the 5 new registries in the SEER18 database (2000-2014), encompassed approximately 27.8% of the entire US population[573]. Melanoma cases extracted from SEER13 and 18 databases included 1,533 (12%) non-Hispanic blacks, 9,122 (69%) Hispanics, 1,968 (15%) non-Hispanic Asians or Pacific Islanders, and 585 (4%) non-Hispanic American Indians or Alaska Natives (Table 3.1).

Table 3.1 Cutaneous melanoma patient characteristics of the SEER and CI5 databases.

Registry		**SEER**	**IARC/WHO CI5-Plus** [1]
Year		1992-2014	1978-2007
Melanoma (N, Total)		13,208	15,226
Sex	Male (%)	5,953 (45%)	7,271 (48%)
	Female (%)	7,255 (55%)	7,973 (52%)
Ethnicity	Black (%)	1,533 (12%)	90 (0.6%) [2]
	Hispanic (%)	9,122 (69%)	10,645 (70%) [3]
	Asian or Pacific Islander (%)	1,968 (15%)	4,491 (29%) [4]
	American Indian or Alaska Native (%)	585 (4%)	N/A
Age	Mean	56	58
	Standard deviation	59	60
	Median	57	57

[1] Excluding melanoma cases from the US SEER registries.

[2] Source available only from the Uganda registry.

[3] Including registries from Brazil, Colombia, Costa Rica, Ecuador, and Spain.

[4] Including registries from India, Japan, Philippines, Singapore, and Thailand.

The Cancer Incidence in Five Continents database (CI5-Plus, 1978-2007), downloaded from the International Agency for Research on Cancer (IARC), World Health Organization, included 90 (0.6%) black melanoma cases extracted from the Uganda registry, 10,645 (70%) Hispanic cases extracted from the Brazil, Colombia, Costa Rica, Ecuador, and the Spain registries, and 4,491 (29%) Asian cases extracted from the India, Japan, Philippines, Singapore, and the Thailand registries. In both the SEER and CI5 databases, the female case numbers are higher than the case numbers of the male.

3.3.2 Age-specific incidence rate patterns

In both the SEER and CI5 databases, the age-adjusted incidence per 100,000 person-years of all ages shows that men have a higher overall incidence rate as compared to women in the Asian or Pacific Islander and the American Indian or Alaska Native populations. However, in the non-Hispanic black (black for short) and Hispanic groups, women have overall higher incidence rates than men (Table 3.2).

Table 3.2 Summary of the SEER and CI5 data.

	SEER [1]		**CI5** [2]	
	Male	**Female**	**Male**	**Female**
No. of melanoma in study (%)	5,953 (45%)	7,255 (55%)	7,271 (48%)	7,973 (52%)
Age-adjusted incidence per 100,000 person-years of all ages				
Black	2.27	1.81	13.05	22.46
Hispanic	8.49	7.99	69.96	72.08
Asian or Pacific Islander	2.24	1.75	9.07	7.44
American Indian or Alaska Native	6.74	4.79	N/A	N/A

[1] Age-adjusted incidence per 100,000 person-years was done by using the US 2000 Census Standard Population in 19 age groups in SEER.
[2] Age-adjusted incidence per 100,000 person-years was done by using World (WHO 2000-2025) Standard Million in 19 age groups in CI5.

As reported before, melanoma incidence rates increase with age, with age > 50 years showing higher incidence rates than age < 50 years in almost all ethnic groups (Figure 3.1A-

3.1F). One exception is that the highest age-specific rate is found in the group of 80-84 years in the Hispanic males and the American Indians or Alaska Natives (both male and females) (Figure 3.1B, 3.1D). The oldest group (85+ group) in fact shows a slightly decreased rate (Figure 3.1B, 3.1D). Among the ethnic groups in the CI5 database, the highest age-specific incidence rate is observed in the oldest men (85+ group).

Figure 3.1 Age-specific incidence rates of melanoma by gender. **A** Non-Hispanic blacks (SEER data). **B** Hispanics (SEER data). **C** Asians or Pacific Islanders (SEER data). **D** American Indians or Alaska Natives (SEER data). **E** Hispanics (CI5 data). **F** Asians (CI5 data).

In the SEER database, black women show higher incidence rates at the age of 15-49 years. No incidence was reported before age 15. Men, on the other hand, exhibit higher risk after age 50. In the Hispanics, women exhibit higher incidence rates when younger than 59 years and men present higher risks after age 60 (Figure 3.1B). A similar trend is also observed in the Asian or Pacific Islander (Figure 3.1C) and the American Indian or Alaska Native groups (Figure 3.1D). Higher incidence rates are found between the age of 20 to 54 years in the females, and the trend reverses after age 55, with males showing higher incidence rates in the Asian or Pacific Islander group. However, among American Indians or Alaska Natives, women show higher rates at the age of 20-44 years, while men show higher incidences after age 45.

3.3.3 Age-specific incidence rate ratio pattern

Age- and gender-specific incidence rate ratios (RRs) were calculated and are listed in Table 3.3. A higher risk of melanoma is evident in younger women in the non-Hispanic blacks, Hispanics, Asians or Pacific Islanders, and the American Indians or Alaska Natives in the SEER database (Table 3.3). The highest female-to-male RRs is 3.85 (95% CI: 1.04, 21.2), 2.51 (95% CI: 1.11, 6.19), and 4.36 (95% CI: 0.87, 42.2, not significant) at the 20–24 age group in the non-Hispanic blacks, Asians or Pacific Islanders, and the American Indians or Alaska Natives, respectively, similar to what was observed in the white population[114]. Overall the pooled population showed RR of 2.67 (2.17–3.30) at 20–24 years of age category (Table 3.3). Men present higher incidence rates of melanoma after age 60 in all four ethnic groups.

Table 3.3 Age-specific incidence rate ratios by ethnicity (SEER 1992-2014).

Age	Black			Hispanic			Asian			American Indians			All non-whites		
	RR (F/M)	95% CI		RR (F/M)	95% CI		RR (F/M)	95% CI		RR (F/M)	95% CI		RR (F/M)	95% CI	
		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper		Lower	Upper
0-4	0.91	0.07	12.52	1.04	0.37	2.97	3.69	0.70	36.40	N/A	N/A	N/A	1.44	0.67	3.21
5-9	N/A	N/A[2]	N/A	1.21	0.52	2.88	0.70	0.14	2.94	N/A	N/A	N/A	1.26	0.74	2.16
10-14	0	0	1.57	**1.95**	1.03	3.84	0.60	0.13	2.36	N/A	N/A	N/A	**1.70**	1.13	2.59
15-19	3.42	0.88	19.35	**1.96**	1.19	3.29	0.58	0.18	1.70	N/A	N/A	N/A	**2.17**	1.66	2.85
20-24	**3.85**[1]	1.04	21.24	**2.10**	1.56	2.85	**2.51**	1.11	6.19	4.36	0.87	42.19	**2.67**	2.17	3.30
25-29	1.78	0.80	4.27	**3.31**	2.59	4.25	1.35	0.75	2.49	**3.36**	1.39	9.31	**1.93**	1.65	2.25
30-34	1.68	0.97	2.99	**2.22**	1.86	2.65	**2.09**	1.32	3.39	0.83	0.41	1.65	**1.96**	1.71	2.25
35-39	1.10	0.69	1.77	**2.40**	2.04	2.82	**1.46**	1.00	2.16	1.46	0.68	3.21	**1.61**	1.43	1.82
40-44	1.33	0.83	2.17	**1.81**	1.57	2.09	1.39	0.99	1.99	1.53	0.85	2.84	**1.42**	1.26	1.60
45-49	1.20	0.77	1.89	**1.67**	1.45	1.92	1.05	0.76	1.47	0.88	0.48	1.62	1.27	0.65	2.51
50-54	0.80	0.55	1.15	**1.33**	1.17	1.52	1.23	0.90	1.69	0.79	0.49	1.28	1.18	1.05	1.32
55-59	0.86	0.61	1.21	1.06	0.92	1.22	0.58	0.42	0.80	0.37	0.21	0.65	0.88	0.78	0.98
60-64	0.54	0.39	0.75	0.86	0.74	0.98	0.69	0.51	0.94	0.47	0.26	0.83	0.75	0.67	0.84
65-69	0.57	0.41	0.79	0.83	0.72	0.96	0.50	0.37	0.68	0.88	0.48	1.61	0.72	0.64	0.81
70-74	0.89	0.65	1.22	0.68	0.58	0.79	0.48	0.35	0.64	0.69	0.35	1.36	0.66	0.59	0.75
75-79	0.56	0.40	0.78	0.54	0.46	0.63	0.61	0.44	0.84	0.28	0.11	0.63	0.55	0.48	0.62
80-84	0.81	0.55	1.19	0.42	0.35	0.51	0.69	0.47	1.00	0.61	0.28	1.32	0.53	0.45	0.61
85+	0.79	0.53	1.19	0.61	0.50	0.75	0.54	0.38	0.77	0.76	0.30	2.02	0.63	0.54	0.74
All age	1.02	0.92	1.13	1.36	1.31	1.42	0.92	0.84	1.00	0.90	0.76	1.06	1.19	1.15	1.23

[1] Bolded: significant numbers for higher F/M ratio.
[2] N/A: not applicable.

In the CI5 database, the case numbers for blacks are too small when stratified by age and sex (each age group has less than 10 cases and many groups have 0 cases), and thus blacks are excluded for further age-specific rate ratio analysis.

In accordance with what we have found in the US SEER database, the Hispanics (Figure 3.1E) and the Asians (Figure 3.1F) in the CI5 database show similar trend to the SEER results, in which younger Hispanic women (< 55 years) and younger Asian women (< 40 years) exhibit higher incidence rates than men, whereas men have higher risks of melanoma at older ages.

Again, the age-specific female-to-male incidence rate ratios were calculated and are listed in Table 3.4. The Hispanic females start to show a higher rate ratio of 1.81 (95% CI: 1.18, 2.81) at the age of 15–19 years up until the age of 45–49 years (RR = 1.23, 95% CI: 1.07, 1.41), similar to the trend observed in the Hispanic population documented in the US SEER database (Table 3.3). The highest rate ratio of 1.81 (95% CI: 1.18, 2.81) is found in the 15–19 age group (Table 3.4), somewhat deviating from 25 to 29 age group in the US SEER Hispanic population where the rate ratio is the highest at 3.31 (95% CI: 2.59, 4.25) (Table 3.3). Incidence rates in the Hispanic men exceed that in women after age 55, comparable to age 60 in the US SEER data. In Southeast Asia, females under age 39 exhibit higher risk of melanoma than males and males tend to have a higher risk of melanoma after age 40 (Table 3.4). The significance levels are compromised in most of the young age group as the RR intervals containing 1, except for age group 30–34 (RR = 1.44, 95% CI: 1.04, 2.00). The highest rate ratio was observed at the youngest age categories with non-significant 95% confidence intervals (0–4 and 5–9, Table 3.4).

Table 3.4 Age-specific incidence rate ratios by ethnicity (CI5 1978–2007).

Age	Hispanic			Asian			All CI5 [1]		
	RR (F/M)	95% CI		RR (F/M)	95% CI		RR (F/M)	95% CI	
		Lower	Upper		Lower	Upper		Lower	Upper
0-4	0.32	0.08	1.04	2.92	0.87	12.59	0.94	0.44	1.99
5-9	0.47	0.13	1.48	1.70	0.49	6.59	0.92	0.42	2.02
10-14	1.77	0.88	3.70	0.45	0.17	1.07	1.06	0.63	1.77
15-19	**1.81** [2]	1.18	2.81	1.27	0.64	2.52	**1.71**	1.20	2.46
20-24	**1.58**	1.19	2.11	1.25	0.79	2.00	**1.61**	1.27	2.05
25-29	**1.71**	1.39	2.10	0.97	0.69	1.37	**1.53**	1.29	1.82
30-34	**1.61**	1.35	1.92	**1.44**	1.04	2.00	**1.67**	1.43	1.95
35-39	**1.52**	1.29	1.79	1.26	0.94	1.70	**1.53**	1.33	1.76
40-44	**1.25**	1.09	1.45	0.94	0.73	1.20	**1.24**	1.09	1.40
45-49	**1.23**	1.07	1.41	1.03	0.83	1.28	**1.23**	1.10	1.38
50-54	1.08	0.95	1.23	0.87	0.72	1.06	1.07	0.96	1.19
55-59	0.89	0.78	1.01	0.85	0.70	1.03	0.91	0.82	1.02
60-64	0.94	0.83	1.07	0.70	0.58	0.84	0.88	0.79	.098
65-69	0.97	0.86	1.10	0.70	0.59	0.83	0.88	0.80	0.97
70-74	0.83	0.73	0.94	0.71	0.58	0.86	0.79	0.72	0.88
75-79	0.75	0.66	0.85	0.59	0.48	0.73	0.69	0.62	0.77
80-84	0.84	0.72	0.98	0.68	0.53	0.87	0.74	0.65	0.84
85+	0.54	0.46	0.64	0.66	0.50	0.87	0.52	0.45	0.60
All age	1.14	1.10	1.19	0.96	0.91	1.02	1.13	1.09	1.17

[1] All CI5: including Hispanics, Asians, and Blacks.

[2] Bolded: significant numbers for higher F/M ratio.

As shown in Figure 3.2, age-specific incidence rate ratios were plotted for both the US SEER (Figure 3.2A) and WHO CI5 (Figure 3.2B) databases. Younger women across all races show a higher incidence than men in the same age range, with a female to male rate ratio greater than 1. In contrast, older men (> 60 years) of all races show higher incidence rates than women in the same age range, with a rate ratio of less than 1. Data from the US SEER and WHO CI5 show similar trends, but the difference is manifested at slightly different levels, with CI5 data showing lower rate ratios (i.e. showing fewer gender differences) than the US SEER data. Overall, the same gender difference pattern is observed across all races between the two databases.

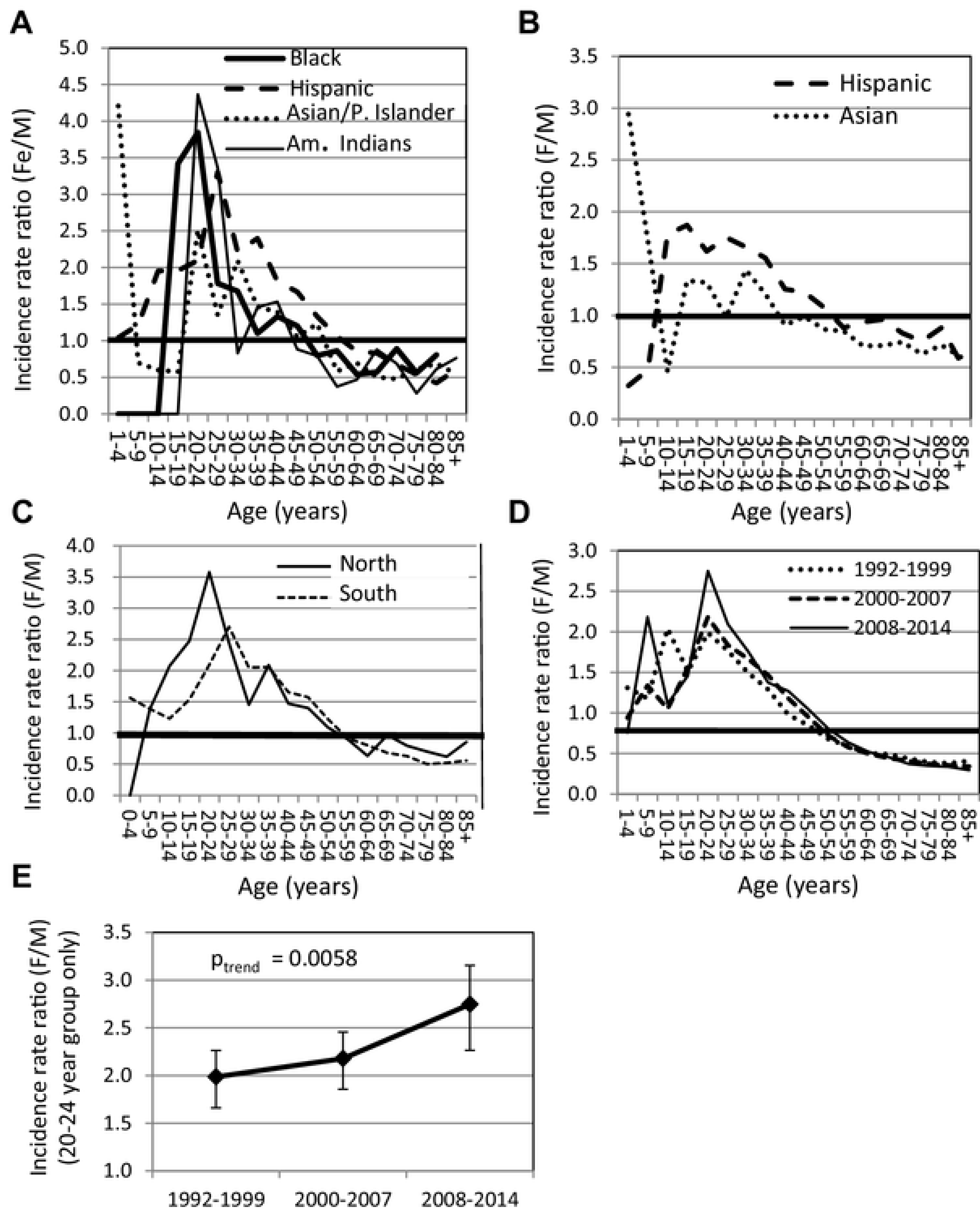


Figure 3.2 Age-specific female-to-male incidence rate ratios by race, age, and years of diagnosis. **A** SEER data by race and age. **B** CI5 data by race and age. **C** SEER data with all races stratified by geographical location (South and North are divided by latitude of 40°N). **D** SEER data with all races stratified by diagnosis years. **E** the trend of female-to-male rate ratio at 20–24 years of age in different periods of time. Thick black horizontal line: RR = 1.

UV is the most important environmental risk factor for melanoma. In order to examine whether geographical UV radiation impacts the female to male rate ratios, we separated the US SEER registries by latitude of 40°N, resulting in two strata: South (California, Utah, New Mexico, Louisiana, Georgia, and Kentucky and Hawaii) and North (Alaska, Washington, Iowa, Illinois, Connecticut, and New Jersey). The North and South age-specific melanoma rate ratios were calculated and are plotted in Figure 3.2C. Overall, the rate ratio patterns are similar in the North and South. Incidence rates are higher in young women than in young men. The peak difference shows a slight age shift – peak at 20–24 in the North group and 25–29 in the South group.

To find out whether this gender ratio changes over time, we combined all non-white populations for every 8-year period in the US SEER data, namely years 1992–1999, 2000–2007, and 2008–2014 (the last period only contains 7 years of data) (Figure 3.2D). Over these different periods of time, there are the same patterns of the female to male rate ratio, in which females show higher incidence rates at a younger age and lower rates at an older age as compared to males. The peak difference is at age 20–24 for all three periods, exactly as we reported before in the Caucasian population[114]. When we selectively examined the 20–24 years age group, it is quite interesting that the female to male rate ratios increased over time, from 1.99 (95% CI: 1.71, 2.31) in 1992–1999, to 2.17 (95% CI: 1.90, 2.50) in 2000–2007, and 2.74 (95% CI: 2.34, 3.23) in 2008 to 2014 (Figure 3.2E). The trend in this ratio change is significant ($P_{trend} = 0.0058$).

3.4 Discussion

In this cancer registry-based study, we show that there is a coherent age-dependent gender difference in the risk of melanoma across all ethnic groups. Specifically, in the SEER database, black women under the age of 50 exhibits higher incidence rates of cutaneous melanoma; above that aged women showed lower incidence rates. The switching age is 60 in the Hispanics, 55 in the Asians, and 45 in the American Indians. In the WHO CI5 database, Hispanics women under age 55 and Asian women under age 40 have higher incidence rates of cutaneous melanoma. Men over the age of 60 years, in general, unanimously exhibit a higher risk for melanoma than the same age range women in the above-mentioned ethnic groups. The switching age shows a slight shift among these populations. The female to male rate ratios also varies from slightly over 1 to nearly 4 (with significant 95% confidence intervals, Tables 3.3, 3.4; Figure 3.2). The highest age-specific female to male incidence rate ratios in the SEER database is found in the 20–24 years age group in the blacks, Asians, and American Indians, and 25–29 years in the Hispanics. In the WHO CI5 database, Asians also show general higher female to male rate ratios at the reproductive age, but the trend is less consistent. The highest rate ratio is observed at 0–4 (RR = 2.92, 95% CI: 0.87, 12.59) and 5–9 (RR = 1.70, 95% CI: 0.49, 6.59), neither reached significance level of 0.05. In the WHO CI5 Hispanics population, the highest age-specific female-to-male incidence rate ratios (RR = 1.81, 95% CI: 1.18, 2.81) are observed in the 15–19 years pubescent age group, similar to that in the SEER dataset. Previously, this gender difference pattern was reported in the Caucasian population[92,114]. Now we demonstrate that the age-dependent gender differences in melanoma risk are shared across ethnic groups, which may suggest gender as one of the melanoma risk factors in addition to the traditional UV radiation.

Higher incidence rates in younger females in the Caucasians have been speculated to be associated with their lifestyle and tanning bed use[214,350]. In other words, women of the younger age are less covered in the sun and use tanning devices more frequently[214,350]; hence they are more exposed to solar and/or artificial UV radiation. Ultimately, UV radiation is assumed responsible for the gender difference at a younger age. However, it was reported that indoor tanning only accounted for 2.6–9.4% of melanoma incidence[563] and the overall UV radiation contribution was approximately 50% to melanoma etiology[574]. Also, females are reported to use more sun protection strategies than males[355,575]. Therefore, the lifestyle and UV device use does not seem to completely explain the over twofold of incidence rate ratio (female/male) in the peak age (20–24 years) in the Caucasians[114]. Our previous results have demonstrated that the female sex is independent of ambient UV radiation as a risk factor for early-onset melanoma[93]. However, tanning device use may still be a confounder for the female sex. We could not rule out the alternative explanation from tanning device use for a higher incidence of early-onset melanoma in women. Tanning device use is a popular habit in the white population as tanned skin color is considered fashionable and desired. On the other hand, despite the possibility of tanning among some non-white populations, such behavior might be unpopular in these ethnicity groups. Findings from the present study reveal that darker skin females under the age of 40–55 still have a higher risk of melanoma than their male peers, which indicates that tanning device use may not be a common determinant for the observed higher risk of early-onset melanoma in women.

A better health consciousness in younger women may be a partial reason for the observed gender differences due to early detection that explains the higher incidence in this age range. In a retrospective case-control study in the non-Hispanic whites, it was found that women were

more likely to notice melanomas on their partners, as well as to their own bodies compared with men[354]. This resulted in women having statistically significant smaller and thinner melanomas and better outcomes than men. Women at all ages also showed fewer melanoma metastases and fewer melanoma-related deaths than men. In addition, a survey conducted in Texas found that women were more likely to limit outdoor activity, and seek shade when being outdoors as compared to men[355]. Socioeconomic factors such as income, educational level, and access to healthcare might be the background determinants in shaping the health consciousness[576]. How socioeconomic factors contribute to the observed sex difference in incidence rates requires further exploration.

A pathophysiological factor may provide an alternative explanation for the observed gender differences. Melanoma is the most frequently diagnosed cancer during pregnancy and also in the 25–29 age group[577]. It is well documented that pregnancy impacts cutaneous melanocyte homeostasis[9]. In our recent study, cutaneous melanoma cases from 31 European cancer registries and SEER18 database were extracted and correlated to geographical UV radiation[92]. Results showed that local ambient UV differentially affected cutaneous melanoma incidence rates between genders. No correlation between female cutaneous melanoma incidence rates and UV index was found, but men showed a significant correlation to ambient UV index[92]. Assuming the female sex exhibits a significant impact on early-onset melanoma risk, the lack of significant association between female rates and ambient UV index can be explained. The current study provides further evidence that it may be the female sex itself that promotes melanoma risk under the age of 50, although indirect.

On the other hand, old men show higher incidence rates than old women in almost all ethnicity groups. In the US, men not only show overall higher melanoma incidence than

women, but they also show worse outcome[578]. The precise reason is not fully understood, but it is known that men use less sun protection and thus are likely to accumulative more UV damage at older age[579]. If our hypothesis of hormonal regulation is correct, the sharp decrease of testosterone levels and/or an increase of estrogen levels at an older age in men may also contribute to melanoma risk.

Recent publications suggested possible roles of estrogen and its receptors in melanoma etiology[565,580,581]. Estrogen can potentially induce melanocyte growth during pregnancy[582]. Although expression of estrogen receptors was found in melanoma cells, different subtypes (namely, ERα, ERβ, and GPER1) may exhibit different functions[565]. Genetic variations in ESR1 (ERα) were found to impact melanoma risk[583]. However, overall the role of estrogen in melanoma risk remains to be further elucidated. Through our current study, we hope to confirm an independent role of sex in melanoma development, which will help to build a base for further investigations on sex hormones and melanoma.

As shown in Table 3.2, the absolute age-adjusted incidence rates in the SEER database are much lower than the corresponding rates in CI5 for all races. In the CI5 database, the number of black cases is small because the only available source was the Uganda registry. The higher rate in this population may be associated with its high average annual UV index (UVI = 11–12, higher than the annual average UVI of 10 in Hawaii). Hispanic is a mixed heritage with much heterogeneity of skin color and genetic background. Their skin color can range from light (the "white Hispanics"[313]) to darker shades. It is unclear whether the Hispanics from CI5 datasets are "whiter" than their peers in the US. The dramatic difference of melanoma incidence in these two datasets for Hispanics may provide a good model for genetic epidemiological research and warrant further investigation[584]. As for the different rates in the two datasets in

Asians, we also do not understand the underlying reasons, but it is known that the cancer incidence patterns in immigrants change after the first generation, presumably due to diet and environmental changes[585].

To conclude, age-dependent gender differences in the incidence rates of melanoma are observed in all ethnic groups. Younger women at pubescent and reproductive ages show higher risks than men in the same age range, while older men exhibit a higher risk than their women peers. The switching age is close to women's menopause period (i.e. 40–55 years), suggesting a hormonal effect. These new findings may provide a base for promoting additional prevention strategies. The role of the female sex in melanoma risk and the underlying mechanisms warrant further investigation.

CHAPTER 4 ANATOMIC SITE-SPECIFIC GENDER DIFFERENCES IN CUTANEOUS MELANOMA

4.1 Introduction

Malignant cutaneous melanoma (CM) is the number one cause of death in skin cancer patients in the US[586] and the incidence rate continues to increase since the 1930s[587]. The risk factors have not been completely elucidated but a pathophysiological role of sex had been suggested in addition to UV radiation[114,588,589]. The sex differences in body site distribution of CM incidence rates had contributed to this assumption[81,590-592]. Of the sex differences, men have tumors predominantly on the trunk, especially on the back[593], while women have more tumors on the lower extremities[593]. In recent years, CMs in the head and neck area are rising in men, of which subtle distinctions were identified between sexes on the face[594]. UV radiation had long been considered the primary environmental risk factor for CM because of UV-induced DNA damages and mutations[595-597]. The distribution of CM on the body surface has been recognized to reflect UV impact as different body sites receive different levels of UV exposure[82,88]. To date, the main hypothesis to explain the bodily distributive differences in CM lies heavily in UV exposure and its associated behavioral factors such as clothing styles and use of cosmetics and occupation choice, of which may reflect differences between genders[594,598].

Additional melanoma risk factors to UV exposure have been suggested by recent epidemiological publications[92,114]. It had been reported since 1975 that women under the age of 50 showed higher incidence rates than men in the same age range[599]. The underlying etiological mechanisms had been largely attributed to UV exposure, including the impact of ambient UV radiation and indoor tanning popularity in younger women[214]. Nevertheless, this sex discrepancy of UV exposure did not result in a similar age-specific incidence difference in non-melanoma

skin cancers (NMSCs)[114], which are mostly found in sun-exposed body sites[82,429] and exhibit a straightforward causative relationship with UV exposure[600]. In support of a less straightforward role of UV exposure, melanoma is frequently found in less sun-exposed body areas (i.e. trunk)[601]. Moreover, the increased sunscreen use has not shown a preventive role in melanoma incidence rates in the sun-exposed body areas[602], by which raised a question whether the current UV-based primary prevention message was sound[603].

The goal of the current study was to understand melanoma disparity and differential etiology among different ages, genders, and race groups through analyzing melanoma tumor distributions in four major body regions – the head and neck region, the shoulder and upper extremities, the trunk, and the hip and lower extremities, using the US Surveillance, Epidemiology, and End Results Program (SEER) data. These results suggested that melanomas diagnosed at younger and older ages may show different risk profiles which warrant further investigation.

4.2 Materials and Methods

4.2.1 Study design

This is a retrospective cancer registry-based cohort analysis using the US Surveillance, Epidemiology, and End Results Program (SEER) data.

4.2.2 Study population

For body-site specific incidence rate analysis, SEER18 database (Incidence - SEER18 Regs Research Data + Hurricane Katrina Impacted Louisiana Cases, Nov 2017 Sub (2000-2015) <Katrina/Rita Population Adjustment>) was downloaded through SEER*Stat software (version 8.3.5). Melanoma cases were collected from 18 SEER registries, including San Francisco-Oakland SMSA, Connecticut, Detroit (Metropolitan), Hawaii, Iowa, New Mexico, Seattle (Puget Sound), Utah, Atlanta (Metropolitan), San Jose-Monterey, Los Angeles, Alaska Natives, Rural Georgia, California (excluding SF/SJM/LA), Kentucky, Louisiana, New Jersey, and Greater Georgia. Caucasian white melanoma cases (N=262,130) were retrieved in this study, including 152,666 men and 109,464 women. Non-white patients, including black Americans, Hispanics, American Indians/Alaska Natives, and Asian Americans/Pacific Islanders (N=11,295) of 5,088 men and 6,207 women were also retrieved to make comparisons with the white population. For calculating the annual percentage change of incidence rates, age- and sex-specific rates for each body site were downloaded similarly via SEER*Stat software and adjusted by the US 2000 standard population. For the incidence trend analysis, SEER data from 1973 to 2015 was used.

4.2.3 Definition of melanoma

Melanoma was defined based on the ICD-O-3/WHO 2008 site recode as “Melanoma of the skin” and having AYA site recode/WHO 2008 category of “7.1 Melanoma”. ICD-O-3/WHO 2008 primary site code “C44-Skin” includes “C44.0-Skin of lip (NOS, not otherwise specified),

C44.1-Eyelid, C44.2-External ear, C44.3-Skin of other and unspecified parts of face, C44.4-Skin of scalp and neck, C44.5-Skin of trunk, C44.6-Skin of upper limb and shoulder, C44.7-Skin of lower limb and hip, C44.8-Overlapping lesion of skin (overlapping with other malignant neoplasms of skin), and C44.9-Skin (NOS, not otherwise specified)" were categorized into 4 groups in the present study as follows: C44.0 – C44.4 as "Head/Neck" region, C44.5 as "Trunk" region, C44.6 as "Upper" region, and C44.7 as "Lower" region. C44.8 and C44.9 categories were showing zero number of cases in the database. ICD-O-3/WHO 2008 Hist/behave, malignant categories (excluding melanoma in situ) of melanoma "8720/3-8723/3, 8726/3, 8727/3, 8730/3, 8740/3-8746/3, 8761/3, 8770/3-8774/3, and 8780/3" were included in the present study.

4.2.4 Variables

The outcome of interest in the current study was the incidence rate of melanoma (definition above). We analyzed the incidence rates separately by the four major anatomic sites as abovementioned. Age (0-4, 5-9, …, 80-84, 85+, and all ages combined), gender (female and male), and race (Caucasian white and non-white combined) were the major exposures of interest. No other covariates were used in the current study besides these primary variables of interest.

4.2.5 Statistics

Age-specific incidence rates were calculated by case number divided by population (per 100,000 person-years) and presented in 19 age groups (0-4, 5-9, …, 80-84, 85+, and all ages combined). Age-standardized rates were adjusted to the US 2000 standard population. The rate ratios were calculated using female incidence rates divided by male rates as previously described[342]. The 95% confidence intervals of the rate ratios were calculated by Stata (version 13.1, StataCorp LLC, Texas, USA)[571]. The incidence rates were stratified by the four major

anatomic sites, two genders, and 19 age groups as crude unadjusted results without controlling for potential confounders. Incidence rates in different race groups were presented in separate tables. The average annual percentage changes of incidence rates were produced by the SEER Joinpoint Regression[604] Program (version 4.6.0), downloaded from the SEER website based on SEER*Stat data from 1973 to 2015 (age-adjusted). The mean diagnosed age comparison was made by the two-sample student t-test with no correction for multiple comparisons. The two-sided significance level was set at 0.05 by default.

4.3 Results

4.3.1 Patient characteristics and differences in the mean age of diagnosis

Caucasian white ("white" in short) melanoma cases (n=262,130) in the SEER18 database (2000-2015) were retrieved, including 109,464 (41.8%) women and 152,666 (58.2%) men (Table 4.1). Melanoma cases from the non-white races including black Americans, non-white Hispanics, American Indians/Alaska Natives, and Asian Americans/Pacific Islanders (SEER18 database, 2000-2015) were also retrieved (Table 4.1, total 5,088 men and 6,207 women). Case distribution in the head and neck region ("Head/Neck"), the upper limbs and shoulder ("Upper" in short), the trunk, and the lower limbs and hip region ("Lower") in each gender and race was listed in Table 4.1. The total white cases were 262,130 and the non-white cases were 11,295. The percentage of women was higher (55.0%) in the non-white populations but lower in the white population (41.8%). Overall, men were diagnosed at older ages than women in all body sites and in all race groups (Table 4.1). The mean age of diagnosis in the Head/Neck region was the oldest among all body sites, also in all race groups. The youngest mean age of diagnosis varied in both genders and all race groups (Table 4.1). While the mean age of diagnosis for all sites did not show a significant gender difference in the white population ($p=0.18$, two-sample t-test), the mean diagnosis ages in the Head/Neck, Trunk, and Lower areas were significantly different between the white men and white women. Only the Upper region did not show a significant gender difference ($p=0.51$). In the non-white populations, however, there was no significant gender difference in any body site or in all body sites combined. The mean age of diagnosis was significantly younger in the non-white groups than the whites in both genders.

Table 4.1 Cutaneous melanoma patient characteristics of the SEER18 database (2000-2015).

Body site			Head/Neck	Upper	Trunk	Lower	All
Whites	Number of cases (N, %)	Male	42,496 (16.2%)	36,560 (13.9%)	59,779 (22.8%)	13,831 (5.3%)	152,666 (58.2%)
		Female	15,534 (5.9%)	31,830 (12.1%)	29,017 (11.1%)	33,083 (12.6%)	109,464 (41.8%)
		Total	58,030 (22.1%)	68,390 (26.0%)	88,796 (33.9%)	46,914 (17.9%)	262,130 (100%)
	Mean age of diagnosis	Male	70	67	65	62	63
		Female	66	62	57	59	57
		p-value	0.03	0.51	0.02	0.01	0.18
Non-whites	Number of cases (N, %)	Male	1,176 (10.4%)	955 (8.5%)	1,572 (13.5%)	1,385 (12.3%)	5,088 (45.0%)
		Female	946 (8.4%)	1,441 (12.8%)	1,364 (12.1%)	2,456 (21.7%)	6,207 (55.0%)
		Total	2,122 (18.8%)	2,396 (21.2%)	2,936 (26.0%)	3,841 (34.0%)	11,295 (100%)
	Mean age of diagnosis	Male	63	57	59	60	59
		Female	57	50	53	55	54
		p-value	0.34	0.24	0.33	0.08	0.69
p-value (whites vs non-whites, age of diagnosis)		Male	<0.001	<0.001	<0.001	<0.001	<0.001
		Female	<0.001	<0.001	<0.001	<0.001	<0.001

4.3.2 The age-specific melanoma incidence rates in each body site in the white population

The crude site-specific melanoma incidence rates (unadjusted for other potential confounders such as hair/eye color and genetic background due to data limitations, same for the rest of the paper when rate is mentioned) in the white population were calculated (Table 4.2) and plotted in Figure 4.1 A-D. As published by us before, white men showed an overall higher incidence rate (8.94 per 100,000 person-years in men vs. 2.64 in women) as compared to women in the Head/Neck region, and the trend remained significant after the age of 30 (Table 4.2, Figure 4.1A). In the Upper region, younger women (< 55 years) and older men (≥ 55 years) showed higher rates than the opposite sex (Figure 4.1B). A similar pattern was observed in the Trunk region where younger women (<40 years) and older men (≥ 40 years) showed greater rates compared to the opposite sex (Figure 4.1C). At the age of 40 to 84, the rates stayed at a relatively stable plateau in women and dropped slightly after the age of 80. In contrast, there was a continuous increase in men's Trunk region with age. Similar to the elderly women, there was also a drop in the incidence rate at 85+ of age. Men exhibited higher rates in the Trunk region as compared to women, with an incidence rate ratio (IRR) of 0.45 (female to male). In the Lower region, a monotonously higher rate in women was observed as compared to men across all age groups (Table 4.2, Figure 4.1D). The rate in men also increased with age, but with a slope less steep than in women. Overall, the incidence rate ratio (female to male) was 2.18 in the Lower region.

Table 4.2 Age- and site-specific incidence rates (per 100,000 person-years) and rate ratios (F/M) in the Caucasian population.

	Head/Neck			Upper			Trunk			Lower			All body sites		
Age	F	M	Ratio (95% CI)	F	M	Ratio (95% CI)	F	M	Ratio (95% CI)	F	M	Ratio (95% CI)	F	M	Ratio (95% CI)
0-4	0.04	0.05	0.86 (0.43, 1.75)	0.02	0.01	1.57 (0.44, 5.57)	0	0	NA	0.03	0.03	0.87 (0.38, 2.02)	0.09	0.10	0.96 (0.59, 1.55)
5-9	0.05	0.04	1.20 (0.59, 2.46)	0.06	0.03	1.75 (0.86, 3.58)	0.05	0.03	1.72 (0.81, 3.64)	0.08	0.02	3.67 (1.67, 8.06)	0.25	0.13	1.91 (1.33, 2.75)
10-14	0.11	0.13	0.83 (0.54, 1.27)	0.08	0.08	1.05 (0.63, 1.76)	0.10	0.13	0.75 (0.48, 1.18)	0.14	0.05	3.03 (1.74, 5.26)	0.43	0.39	1.12 (0.89, 1.41)
15-19	0.28	0.38	0.74 (0.57, 0.96)	0.34	0.21	1.63 (1.22, 2.17)	0.84	0.55	1.53 (1.28, 1.84)	0.54	0.16	3.34 (2.49, 4.48)	2.00	1.30	1.54 (1.37, 1.74)
20-24	0.70	0.73	0.96 (0.80, 1.14)	**1.29**	**0.48**	**2.69 (2.26, 3.21)**	**2.71**	**1.16**	**2.33 (2.07, 2.61)**	**1.89**	**0.37**	**5.05 (4.20, 6.07)**	**6.59**	**2.75**	**2.40 (2.23, 2.58)**
25-29	1.14	1.22	0.93 (0.81, 1.07)	2.31	0.92	2.50 (2.20, 2.85)	4.44	2.46	1.81 (1.66, 1.96)	3.45	0.75	4.63 (2.06, 5.29)	11.33	5.35	2.12 (2.01, 2.24)
30-34	1.26	1.67	0.75 (0.67, 0.85)	3.40	1.65	2.07 (1.87, 2.28)	5.57	3.78	1.48 (1.38, 1.58)	4.98	1.28	3.87 (3.49, 4.29)	15.20	8.37	1.82 (1.74, 1.90)
35-39	1.54	2.09	0.74 (0.66, 0.83)	4.27	2.53	1.69 (1.55, 1.83)	6.48	5.77	1.12 (1.06, 1.19)	6.30	2.08	3.02 (2.78, 3.28)	18.60	12.48	1.49 (1.43, 1.55)
40-44	1.81	3.01	0.60 (0.55, 0.66)	5.86	3.81	1.54 (1.44, 1.64)	7.13	8.72	0.82 (0.78, 0.86)	8.10	2.52	3.21 (2.99, 3.46)	22.90	18.06	1.27 (1.23, 1.31)
45-49	2.23	4.51	0.49 (0.45, 0.54)	7.54	6.02	1.25 (1.18, 1.32)	8.52	12.41	0.69 (0.66, 0.72)	9.50	3.34	2.84 (2.67, 3.03)	27.78	26.29	1.06 (1.03, 1.09)
50-54	2.83	6.94	0.41 (0.38, 0.44)	9.17	8.85	1.04 (0.99, 1.09)	9.06	17.61	0.51 (0.49, 0.54)	10.25	4.36	2.35 (2.22, 2.50)	31.32	37.76	0.83 (0.81, 0.85)
55-59	3.54	11.13	0.32 (0.30, 0.34)	10.72	13.03	0.82 (0.79, 0.86)	9.39	24.11	0.39 (0.37, 0.41)	10.65	5.34	1.99 (1.88, 2.12)	34.29	53.62	0.64 (0.62, 0.66)
60-65	4.72	17.85	0.26 (0.25, 0.28)	12.94	18.50	0.70 (0.67, 0.73)	10.06	30.79	0.33 (0.31, 0.34)	11.62	6.40	1.81 (1.71, 1.93)	39.34	73.54	0.53 (0.52, 0.55)
65-69	7.03	27.78	0.25 (0.24, 0.27)	15.29	24.72	0.62 (0.59, 0.65)	10.69	39.82	0.27 (0.26, 0.28)	13.11	7.70	1.70 (1.60, 1.81)	46.12	100.02	0.46 (0.45, 0.47)
70-74	9.56	39.87	0.24 (0.23, 0.25)	16.17	32.14	0.50 (0.48, 0.53)	10.40	44.49	0.23 (0.22, 0.25)	13.69	8.47	1.62 (1.51, 1.73)	49.82	124.99	0.40 (0.39, 0.41)
75-79	12.08	52.09	0.23 (0.22, 0.24)	18.13	37.71	0.48 (0.46, 0.51)	10.23	49.77	0.21 (0.19, 0.22)	14.55	9.95	1.46 (1.36, 1.57)	54.98	149.52	0.37 (0.36, 0.38)
80-84	15.11	69.56	0.22 (0.21, 0.23)	18.62	42.05	0.44 (0.42, 0.47)	9.43	50.44	0.19 (0.17, 0.20)	14.64	10.38	1.41 (1.30, 1.53)	57.80	172.44	0.34 (0.33, 0.35)
85+	19.03	89.10	0.21 (0.20, 0.22)	17.01	43.47	0.39 (0.37, 0.41)	7.46	45.08	0.17 (0.15, 0.18)	13.72	11.01	1.25 (1.14, 1.36)	57.22	188.65	0.30 (0.29, 0.31)
All age [1]	2.64	8.94	0.30 (0.27, 0.32)	5.60	7.40	0.76 (0.71, 0.80)	5.33	11.82	0.45 (0.43, 0.48)	5.98	2.74	2.18 (2.02, 2.36)	19.55	30.90	0.63 (0.61, 0.65)

[1] Age-adjusted.

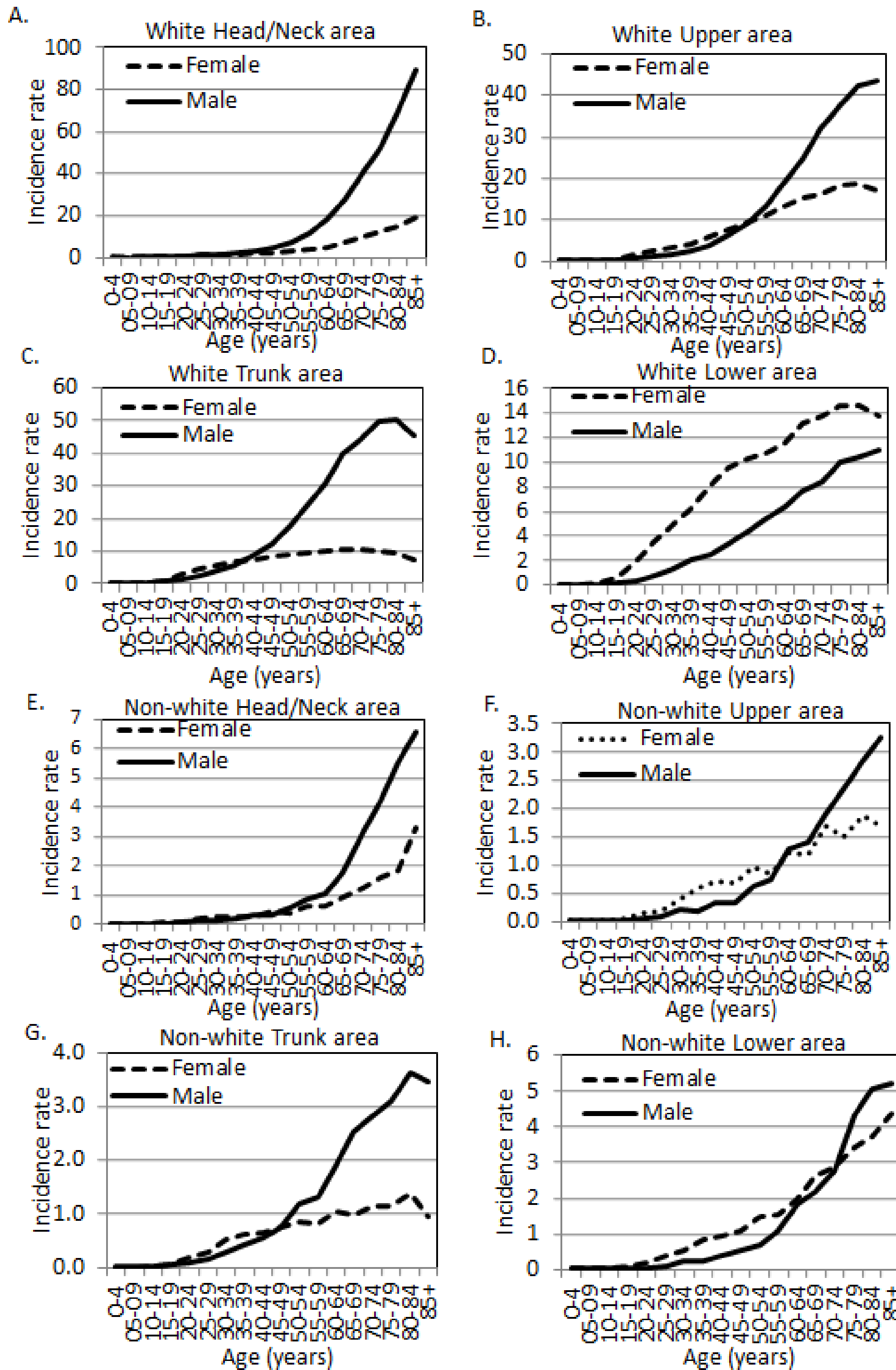


Figure 4.1 Melanoma incidence rates by age, sex, and anatomic site. **A-D** White population. **E-H** Non-white populations. **A** and **E** Head and neck area. **B** and **F** Upper limbs and shoulder area. **C** and **G** Trunk. **D** and **H** Lower limbs and hip area (SEER 18 data, 2000-2015).

When all body sites were combined, men's incidence rate was higher than women's. When stratified the incidence rate by age, there was a uniformly spiked difference at the age of 20-24 with women exhibiting higher incidence rates (Figure 4.2A). In this age category, the female to male IRRs in the Upper, Trunk, and Lower regions were 2.69 (95% CI: 2.26, 3.21), 2.33 (95% CI: 2.07, 2.61), and 5.05 (95% CI: 4.20, 6.07), respectively. There was a non-significant surge at the age of 20-24 (IRR 0.96, 95% CI: 0.80, 1.14) in the Head/Neck region (Table 4.2, Figure 4.2A).

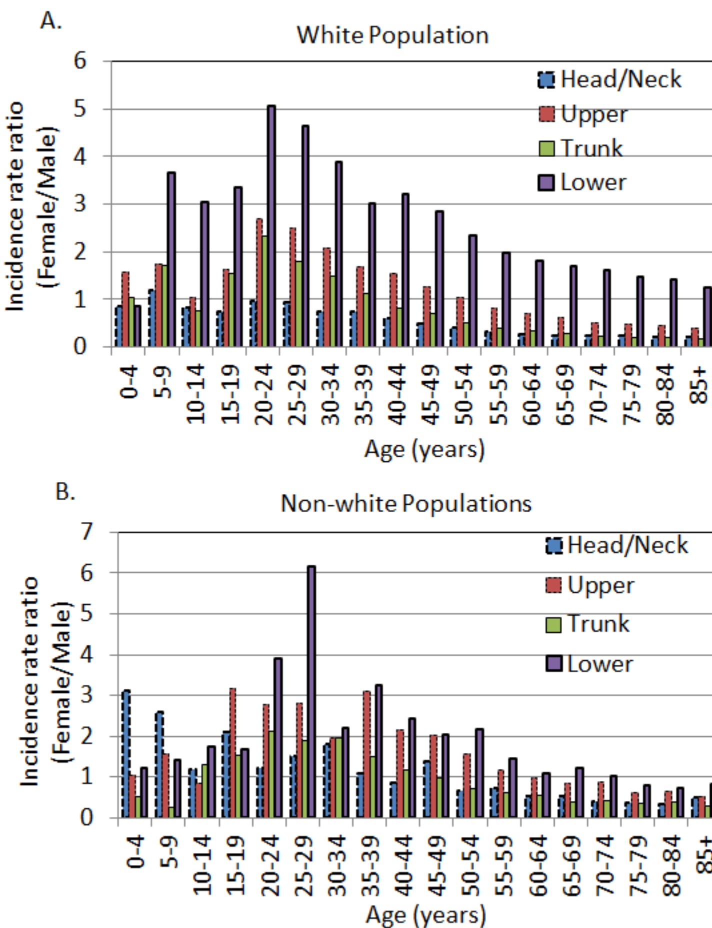


Figure 4.2 The age-specific female to male incidence rate ratios by anatomic site. **A** Caucasian whites. **B** Non-white populations, including black Americans, Hispanics, American Indians/Alaska Natives, and Asian Americans/Pacific Islanders (SEER 18 data, 2000-2015).

4.3.3 Sex differences in age- and body site-specific melanoma incidence rates in the non-white populations

The unadjusted age-, site-, and sex-specific incidence rates and female to male rate ratios in the non-white race groups were listed in Table 4.3. The rate ratios were plotted in Figure 4.1 E-H. The Head/Neck region in the non-whites exhibited a similar pattern to other body sites where there was a significantly higher rate in women at younger ages. The spiked difference was observed at the age of 30-34 years with a female to male IRR of 1.81 (95% CI: 1.21, 2.72). The Upper body, Trunk, and Lower regions each exhibited a spiked difference at the age of 35-39 (IRR 3.10, 95% CI: 2.20, 4.36), 20-24 (IRR 2.12, 95% CI: 1.29, 3.46), and 25-29 (IRR 6.17, 95% CI: 3.63, 10.50), respectively (Table 4.3, bolded). Overall, the non-white men also showed higher incidence rates in their Head/Neck, Trunk, and Lower body regions just like the Caucasian men. However, the magnitude of differences in the non-whites seemed to be not as dramatic as in the whites, as the IRRs were closer to 1 in the non-whites in each body site and in all sites combined (Table 4.2 and 4.3, Figure 4.2).

Table 4.3 Age- and site-specific incidence rates (per 100,000 person-years) and rate ratios (F/M) in the non-white populations.

	Head/Neck			Upper			Trunk			Lower			All body sites		
Age	F	M	Ratio (95% CI)	F	M	Ratio (95% CI)	F	M	Ratio (95% CI)	F	M	Ratio (95% CI)	F	M	Ratio (95% CI)
0-4	0.012	0.004	3.12 (0.32, 30.0)	0.012	0.011	1.04 (0.21, 5.16)	0.004	0.007	0.52 (0.05, 5.74)	0.027	0.022	1.21 (0.41, 3.61)	0.054	0.045	1.21 (0.56, 2.62)
5-9	0.020	0.008	2.60 (0.50, 13.4)	0.012	0.008	1.56 (0.26, 9.32)	0.004	0.015	0.26 (0.03, 2.32)	0.044	0.030	1.43 (0.57, 3.55)	0.079	0.061	1.30 (0.67, 2.51)
10-14	0.031	0.026	1.19 (0.43, 3.28)	0.016	0.019	0.83 (0.22, 3.10)	0.02	0.015	1.30 (0.35, 4.84)	0.059	0.034	1.73 (0.76, 3.96)	0.126	0.094	1.33 (0.79, 2.25)
15-19	0.040	0.019	2.12 (0.73, 6.21)	0.036	0.011	3.18 (0.86, 11.8)	0.064	0.042	1.54 (0.72, 3.33)	0.076	0.046	1.68 (0.82, 3.46)	0.217	0.118	1.85 (1.19, 2.88)
20-24	0.073	0.060	1.22 (0.62, 2.38)	0.146	0.053	2.78 (1.50, 5.15)	**0.191**	**0.090**	**2.12 (1.29, 3.46)**	0.191	0.049	3.91 (2.11, 7.22)	0.602	0.252	2.39 (1.79, 3.19)
25-29	0.172	0.114	1.50 (0.94, 2.41)	0.188	0.067	2.81 (1.61, 4.90)	0.274	0.146	1.88 (1.26, 2.81)	**0.388**	**0.063**	**6.17 (3.63, 10.5)**	**1.022**	**0.390**	**2.62 (2.08, 3.31)**
30-34	**0.268**	**0.148**	**1.81 (1.21, 2.72)**	0.404	0.206	1.97 (1.40, 2.76)	0.528	0.272	1.95 (1.45, 2.62)	0.536	0.243	2.21 (1.62, 3.00)	1.737	0.868	2.00 (1.70, 2.36)
35-39	0.234	0.213	1.10 (0.75, 1.61)	**0.579**	**0.187**	**3.10 (2.20, 4.36)**	0.63	0.417	1.51 (1.17, 1.95)	0.817	0.252	3.24 (2.42, 4.35)	2.259	1.069	2.11 (1.82, 2.46)
40-44	0.264	0.306	0.86 (0.61, 1.23)	0.716	0.334	2.14 (1.62, 2.83)	0.649	0.552	1.18 (0.92, 1.50)	0.945	0.39	2.42 (1.88, 3.12)	2.575	1.583	1.63 (1.42, 1.86)
45-49	0.400	0.287	1.39 (0.99, 1.96)	0.677	0.334	2.03 (1.51, 2.73)	0.732	0.757	0.97 (0.77, 1.22)	1.098	0.538	2.04 (1.62, 2.58)	2.907	1.916	1.52 (1.33, 1.73)
50-54	0.368	0.560	0.66 (0.48, 0.90)	0.974	0.628	1.55 (1.21, 1.98)	0.832	1.181	0.70 (0.57, 0.87)	1.489	0.689	2.16 (1.73, 2.70)	3.664	3.058	1.20 (1.07, 1.35)
55-59	0.603	0.838	0.72 (0.54, 0.96)	0.841	0.728	1.16 (0.75, 1.23)	0.806	1.297	0.62 (0.49, 0.79)	1.535	1.06	1.45 (1.17, 1.80)	3.785	3.923	0.96 (0.85, 1.09)
60-65	0.579	1.048	0.55 (0.40, 0.76)	1.214	1.265	0.96 (0.75, 1.23)	1.049	1.892	0.55 (0.44, 0.70)	1.959	1.816	1.08 (0.88, 1.32)	4.801	6.021	0.80 (0.71, 0.90)
65-69	0.927	1.733	0.54 (0.40, 0.72)	1.171	1.401	0.84 (0.63, 1.11)	0.988	2.531	0.39 (0.30, 0.51)	2.635	2.154	1.22 (0.99, 1.51)	5.722	7.819	0.73 (0.65, 0.83)
70-74	1.199	3.085	0.39 (0.29, 0.51)	1.678	1.923	0.87 (0.66, 1.16)	1.151	2.832	0.41 (0.31, 0.54)	2.861	2.789	1.03 (0.82, 1.28)	6.890	10.63	0.65 (0.57, 0.74)
75-79	1.597	4.188	0.38 (0.29, 0.50)	1.492	2.35	0.63 (0.46, 0.88)	1.156	3.103	0.37 (0.27, 0.52)	3.404	4.308	0.79 (0.63, 0.99)	7.647	13.95	0.55 (0.48, 0.63)
80-84	1.812	5.539	0.33 (0.24, 0.45)	1.873	2.842	0.66 (0.46, 0.94)	1.359	3.613	0.38 (0.26, 0.54)	3.715	5.058	0.73 (0.57, 0.95)	8.760	17.05	0.51 (0.44, 0.60)
85+	3.279	6.564	0.50 (0.38, 0.66)	1.689	3.25	0.52 (0.35, 0.77)	0.96	3.445	0.28 (0.18, 0.44)	4.372	5.20	0.84 (0.64, 1.11)	10.30	18.46	0.56 (0.48, 0.66)
All age [1]	0.383	0.676	0.57 (0.39, 0.82)	0.553	0.49	1.13 (0.80, 1.58)	0.504	0.763	0.66 (0.49, 0.90)	0.964	0.74	1.30 (1.00, 1.70)	2.403	2.669	0.90 (0.77, 1.05)

[1] Age-adjusted.

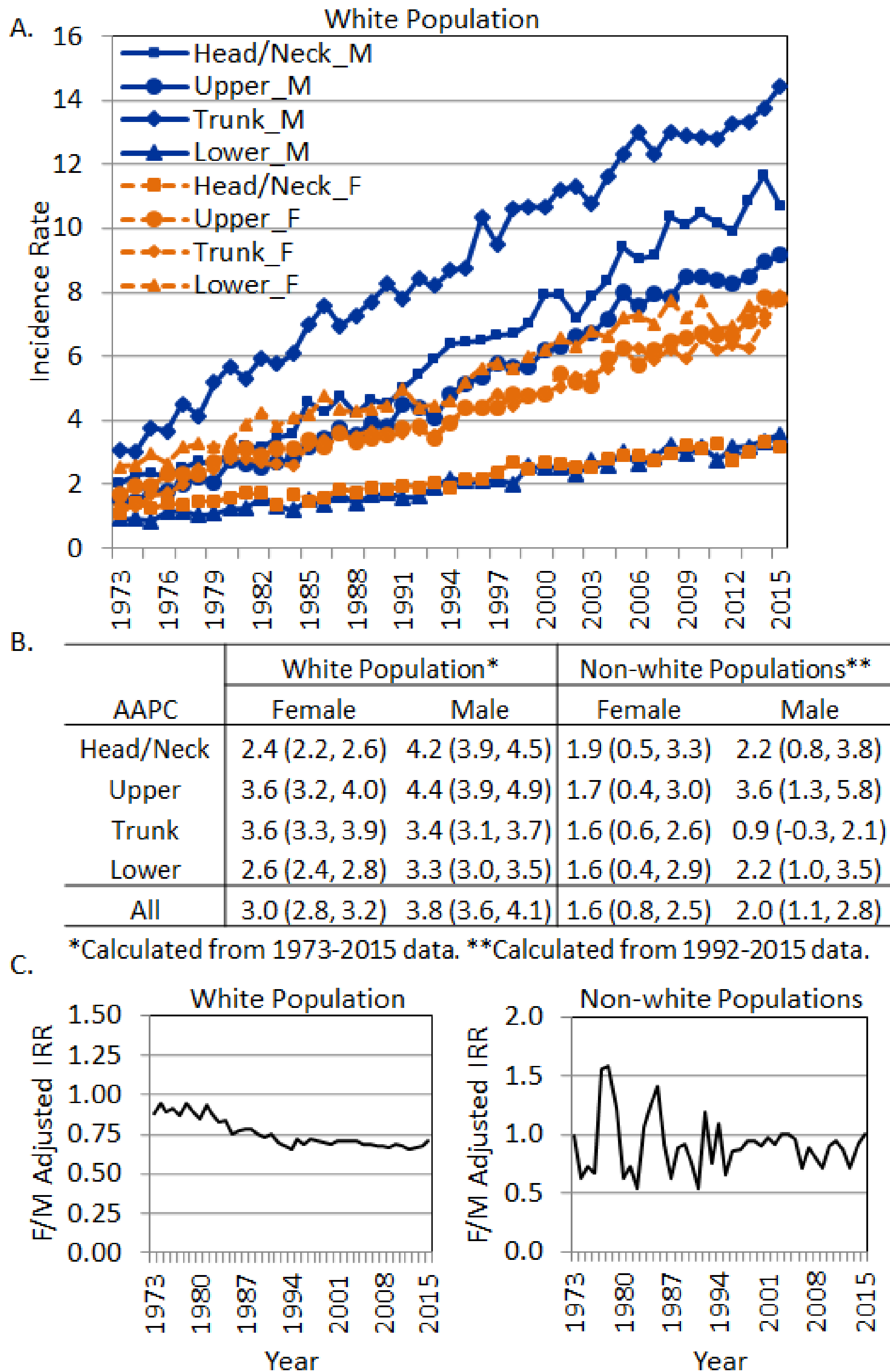
4.3.4 The age-dependent sex differences showed younger women and older men were more susceptible in comparison to their opposite sex of the same age

We and the others have previously reported that the unadjusted age-specific female to male IRRs exhibited a spiked difference around the age of 20-24 years in both the white and non-white populatations[114,342], with the non-whites showing a slight shift of age from 20 to 39 years[342]. This trend was, again, observed in three out of four major body sites in the whites, and all four body sites in the non-whites (Figure 4.2A and 4.2B). The only outlier was the Head/Neck area in the whites which did not show a significant difference at the 20-24 years of age, and the point estimate of IRR was close to 1 (Figure 4.2A). Regardless of the body sites, after the age of 20-24 years, the female to male IRR decreased with increasing age in all body sites in the white population (Figure 4.2A). This trend remained the same in the non-white populations but with some fluctuations and shifting of age (Figure 4.2B).

4.3.5 The yearly trend of melanoma incidence rates in each body site

In order to examine whether each body site exhibited different annual percentage change in the adjusted incidence rates, we further retrieved SEER*Stat data from 1973 to 2015 for each of the four major body sites. The yearly trend of the incidence rates in the whites was plotted in Figure 4.3A and the average annual percentage changes (AAPCs) were produced by the SEER Joinpoint Regression[604] Program. All AAPCs were significantly positive, indicating an increased trend of the incidence rates over the period of 1973-2015. Interestingly, in the white population, the highest AAPC was in the shoulder and upper extremity region in men (AAPC 4.4, 95% CI: 3.9, 4.9), followed by the Head/Neck area in men (AAPC 4.2, 95% CI: 3.9, 4.5). The lowest AAPC was in women’s Head/Neck region (AAPC 2.4, 95% CI: 2.2, 2.6), followed by the hip and lower extremity region in women (AAPC 2.6, 95% CI: 2.4, 2.8). Overall, men exhibited

higher AAPCs than women (3.8% vs. 3.0%) (Figure 4.3B). The overall annual female to male adjusted IRRs were plotted in Figure 4.3C (left panel). Apparently, there was a decreased trend of the female to male incidence rate ratios from 1973 to 1994, then the ratio stabilized at around 0.7 in the white population (Figure 4.3C, left panel).



AAPC	White Population*		Non-white Populations**	
	Female	Male	Female	Male
Head/Neck	2.4 (2.2, 2.6)	4.2 (3.9, 4.5)	1.9 (0.5, 3.3)	2.2 (0.8, 3.8)
Upper	3.6 (3.2, 4.0)	4.4 (3.9, 4.9)	1.7 (0.4, 3.0)	3.6 (1.3, 5.8)
Trunk	3.6 (3.3, 3.9)	3.4 (3.1, 3.7)	1.6 (0.6, 2.6)	0.9 (-0.3, 2.1)
Lower	2.6 (2.4, 2.8)	3.3 (3.0, 3.5)	1.6 (0.4, 2.9)	2.2 (1.0, 3.5)
All	3.0 (2.8, 3.2)	3.8 (3.6, 4.1)	1.6 (0.8, 2.5)	2.0 (1.1, 2.8)

*Calculated from 1973-2015 data. **Calculated from 1992-2015 data.

Figure 4.3 The trend of site-specific melanoma incidence rates by sex. **A** Incidence rates in the white population. **B** Average annual percentage change of site-specific incidence rates in each sex. **C** Overall female to male incidence rate ratios (SEER 9 data, 1973-2015). Left, white population; right, non-white populations.

In the non-white populations, the AAPCs were produced using SEER*Stat data from 1992 to 2015 due to zero cases in some body sites from 1973 to 1991. AAPCs were significantly positive in all body sites in both genders except for men's trunk which showed a non-significant increase (AAPC 0.9, 95% CI: -0.3, 2.1). Overall, men showed a higher AAPC of increase than women. In the non-white women, the AAPCs were similar among different body sites, but in men, the AAPCs varied from 1.6 (in the Lower region) to 3.6 (in the Upper region). The annual female to male adjusted IRRs in the non-white populations showed fluctuations around 1, with females showing slightly lower overall incidence rates over the years (Figure 4.3C, right panel).

4.4 Discussion

In this current study, we compared the gender differences between the whites and the non-whites in terms of their mean age of diagnosis, unadjusted age-specific body-site incidence rates, and AAPCs over the years. We first observed that the previously reported gender differences in the white population in different anatomic sites were confirmed in our data[81,88,592,593]. In other words, the most notable gender differences in melanoma incidence rates were in the head and neck region (higher in men) and hip and lower extremities region (higher in women). This was also observed in the non-white populations in the current study. Secondly, the age-specific differences in each anatomic site were similar between the white and non-white populations, with females universally exhibiting higher rates at younger ages and males exhibiting higher rates at older ages, except for the head and neck region in the whites. However, the mean age of diagnosis was significantly younger in the non-white populations than in the whites. The gender differences in the mean age of diagnosis were significant among the body sites in the whites but not in the non-whites. The overall gender differences in the non-white populations were not as dramatic as in the whites (female to male IRR was 0.63 in the whites and 0.90 in the non-whites, Tables 4.2 and 4.3). Lastly, all body sites from both genders and all race groups showed positive AAPCs but with varying magnitudes. The shoulder and upper extremities showed the fastest increase in AAPCs in men in all race groups. Overall, the whites showed a faster increase than the non-whites.

In terms of the increase in the incidence rate of melanoma (AAPC) over the past 43 years (1973-2015), all race groups showed increased incidence rates, with the whites showing a faster increase than the non-whites overall and in each body site. It has been hypothesized that the increased ambient UV radiation as a result of depleted ozone levels might have caused the

continued increase in melanoma incidence rates[605,606]. If this hypothesis had proven to be true, we would expect a higher AAPC in the sun-exposed body regions such as the head and neck areas. However, the fastest increase in men was observed in the current study in the Upper regions in all races, in the Trunk and Upper regions in the white women, and in the Head/Neck region in the non-white women. Therefore, an increase in the ambient UV dose cannot fully explain these observed phenomena. Increased ambient UV radiation seemed only to explain the observed differences in the Head/Neck region where white men showed a faster increase than white women, who usually use more cosmetics and sunscreen. Women's long hair may also play a protective role. However, in both the white men and women, the Lower region that was occasionally sun-exposed showed a slower increase in AAPC than the Trunk region which was often non-exposed. The ambient UV dose theory apparently could not be the best explanation for this observation.

Cancer is often a result of the interaction between the environment and genetics. The solar UV radiation is the most important risk factor for melanoma. We proposed a hypothesis that when the UV radiation does not provide a good explanation, the genetics, and the related pathophysiological factors may play more important roles in melanoma transformation. This hypothesis is supported by the body site distribution of melanoma incidence rate as observed in the current study. The age-specific incidence rate ratios of gender and race-specific melanoma statistics also support this hypothesis as discussed in the following paragraphs.

For the unadjusted age-specific incidence rate ratios of gender, we showed in the current study again that there was a universally higher incidence rate in young women in all body sites and among all races. The ratio trends reversed after about 40 to 50 years of age, with men exhibiting higher rates at older ages. In experimental mice, a single dose of UV treated on the

newborns led to melanoma development after 6 months[607], suggesting that the UV-induced mutations required a latent time for normal cells to progress to tumors. It is thus understandable that UV-induced melanomas are frequently diagnosed at an older age, as most of the UV radiation received during a lifetime is after the age of 18[608]. In contrast, it is also perceivable that the pathophysiological factors (largely genetics) may highly likely be associated with melanomas diagnosed at younger ages. We previously reported that the melanoma incidence rate was associated with geographical UV only in men[92] also supported this hypothesis. If our hypothesis is proven true, the higher melanoma incidence rate in young women perhaps is not completely due to their UV behaviors, as widely assumed.

Race-specific melanoma statistics also support our hypothesis. The non-whites are less impacted by UV exposure due to their darker skin pigmentation which provides additional UV protection[609]. However, the mean age of diagnosis of melanoma in the non-whites was significantly younger than the whites for both genders. The mean age of diagnosis was also much younger for trunk melanomas (less UV-exposed) than the head and neck melanomas (more UV-exposed) for both genders and all race groups.

On the hip and lower extremities, the overall age-adjusted IRRs were quite different among race groups (2.18 in the whites vs. 1.30 in the non-whites). However, at the age of 20-24 years, the magnitude of sex differences in the whites was similar to that in the non-whites (IRR 5.05 in the whites, age 20-24 vs. 6.17 in the non-whites, age 25-29), which represented the highest IRRs among all age groups and all body sites. If the higher rates in the white women were attributed to clothing style-associated UV behaviors, then why were the relatively UV-protected non-white women showed an even higher IRR on the lower extremities? Therefore, these results suggested that: 1) it is highly likely that the melanomas grew on the hip and lower extremities have an

additional site-specific pathophysiological risk factor(s) and 2) melanomas grew at younger ages are less influenced by UV radiation.

Our results in the current study strongly suggested that melanomas developed at younger ages are attributed more to genetic and/or pathophysiological factors than the environmental factor, while later-onset melanomas are associated more with UV radiation. To the best of our knowledge, this hypothesis that melanomas exhibit an age-specific causal factor has not been explicitly proposed to date. Further evidence can also be found in the literature. For example, germline MC1R variants (loss of function) have been linked to melanomagenesis, and a major pathway is through altering melanin synthesis[610]. Many MC1R variants are known to be the "red hair" variants because these individuals synthesize a large amount of pheomelanin which exacerbates their vulnerability to sun exposure[611]. In this case, both germline variants and UV exposure are responsible for the end result of melanoma. However, because of the research and public education of melanoma awareness, people carrying these variants may be more careful in avoiding sun exposure and in seeking dermatology screening, resulting in early diagnosis. Under these conditions, genetics, rather than the UV exposure, may be more influential in earlier diagnosis. Interestingly, recently it was found that the red hair variants of MC1R differentially affected melanoma outcome between men and women[612] further supporting the gender differences in melanoma arising from genetic background.

Nevertheless, there were a few limitations in the current study. First, the coarse categorization of melanomas by wide body area groupings in the SEER database limited our ability to differentiate more refined differences in tumor distributions between genders and among age strata. For example, we grouped the head and neck regions together but in fact, due to hair protection, scalp and face may receive different levels of UV exposure. Secondly, although

the US SEER database provides population-based cancer information and personal UV exposure levels are well-documented at the individual level, there is no information on UV-associated behaviors, hairstyles, clothing, and hair-removal preferences that we could have used to sort out confounding effects and strengthen our hypothesis. Therefore, due to data limitations, our results could not control for these potential confounders and are considered to be unadjusted. Whenever possible, we used known confounders for our adjustment, such as age in calculating the overall incidence rates. Lastly, this type of data analyses was reported in similar studies in the white population by many others previously[88,592,593,601]. One strength of the current study was that we included different race groups in comparison to validate the observed tumor rates in different body areas and stratified by age to generate our new hypothesis.

It is well documented that UV radiation and oxidative stress are linked[311], both of which can be differentially impacted by sex hormones. As redox biology is unique in melanocytes (evidenced by its regulation via melanocytic master regulator MiTF[613,614]), melanocytes may undergo a lineage-specific UV-induced DNA damage repair which is highly likely impacted by hormones and thus resulting in differential mutation rates. In fact, a gender disparity in mutation burden in melanoma samples from The Cancer Genome Atlas (TCGA) dataset was observed[358], providing additional evidence to our hypothesis.

In conclusion, our results showed various gender differences in age- and body site-specific melanoma incidence rates and the trend of incidence rates over the years. Based on these data we postulated that melanomas at younger ages are less impacted by UV radiation and a total net increase of solar UV radiation may not completely explain the various sex differences in melanoma tumor distribution in different body sites. Further research in gender-related and/or body site-specific pathophysiological factors as a unique determinant in cutaneous melanoma is

warranted.

CHAPTER 5 ESTROGEN RECEPTORS SIGNALING NETWORK-ASSOCIATED RISK OF CUTANEOUS MELANOMA

5.1 Introduction

According to the Surveillance, Epidemiology, and End Results Program (SEER, National Cancer Institute) in their 2019 Annual Report[615], the incidence of cutaneous melanoma (CM) increased in both men and women in the US between 2011 and 2015. CM ranked second in the national trends in rates of new cancer cases in both genders[615]. We and others have shown that there is a distinct age- and sex-dependent incidence pattern in CM[92,118,430,589,616]. Specifically, the incidence rates are higher in older men (> ~ 50 years) but lower in younger men as compared to women of the same ages[118,617]. Younger women (< 50 years) showed a faster increase in incidence rates of CM[618].

The gender disparity in CM was reported in the 1970s. A number of hypotheses have been proposed, ranging from biological explanations to sun-protective behaviors. The higher incidence rate in men after the age of 50 was attributed to a lack of awareness in this senior population[345]. Steroid hormones, especially estrogens, have been continuously suggested in CM development in younger women (<50 years)[351]. Pregnant women with a CM diagnosis usually showed poorer prognoses[352]. However, it is still controversial whether oral contraceptives were associated with an increased risk of melanoma or not[619-621].

Estrogen initiates its effects mainly through binding to the two major estrogen receptors ERα (encoded by ESR1 gene) and ERβ (encoded by ESR2 gene) and an additional non-canonical receptor G protein-coupled estrogen receptor (GPER)[622]. The ER genomic pathway refers to the transcriptional activation of ER targeting genes triggered by estrogen/ER binding in the nucleus[399]. In the non-genomic pathway, estrogen binds to

membrane-bound ER to initiate signaling transduction such as the MAPK and PI3K pathways in the target cells[399]. Functions of the major ERs vary in different cancer types. ERα variants were associated with breast cancer risk in different racial groups[623-626]. Loss of ERα was found to be associated with advanced endometrial cancer[627]. In contrast, ERβ overexpression restored the protective role of estrogen, inhibiting cell proliferation in colorectal cancer[628,629]. In CM lesions where melanoma cells had spread to the sentinel lymph nodes, ERα was the predominant receptor[630], while ERβ was predominant in benign melanomas and normal skin[631]. ERα was not found in any benign melanocytic lesions[418].

Moreover, ERs can be activated independently of estrogen when it is coupled to the IGF/IGFR signaling. For instance, ER in the nucleus can be induced by insulin-like growth factor 1 (IGF1) and lead to MAPK signaling activity in the neuroblastoma cells via the genomic pathway[632]. Additionally, estrogen is able to initiate the IGF1 signaling pathway by inducing the expression of IGF1R and its downstream signaling messenger insulin receptor substrate (IRS)[633], which further activates the downstream PI3K signaling pathway[404]. Hence, ER and IGF1R respond to shared ligands (17β-estradiol and IGF1), activate the same downstream signal pathways and lead to cell proliferation[634]. These molecular events are, however, poorly understood in CM.

The present study used a gene prioritization approach and aimed to explore the association between single nucleotide polymorphisms (SNPs) in ER/IGF1R-related genes and the risk of melanoma, with a special focus on the gender differences. We first genotyped the selected SNPs in cases and controls from the International Genes, Environment, and Melanoma (GEM) Study, which served as our exploratory dataset to prioritize the top SNPs. We later attempted to validate the top SNPs in a second large dataset, the Gene Environment Association Studies Initiative

(GENEVA) dataset. Of the thirteen selected SNPs examined, IGF1 rs1520220 and IGF1R rs2229765 SNPs appeared to be significantly associated with melanoma risk in men but not in women.

5.2 Materials and Methods

5.2.1 Ethics statement

We obtained approval from the Institutional Review Board of the University of California Irvine Office of Research (protocol number 2011-8238, approved June 27th, 2011) for the use of the samples from the International Genes, Environment, and Melanoma (GEM) study. An Institutional Review Board was not required for the use of the data provided by U.T. M.D. Anderson Cancer Center, in which the data were part of the Gene Environment Association Studies Initiative (GENEVA, http://www.genevastudy.org) funded by the trans-NIH Genes, Environment, and Health Initiative (GEI). However, authorized access to the dataset was required by dbGaP.

5.2.2 Study population

The GEM study was described before[635] and served as the discovery set. The dbGaP GENEVA dataset contains samples of 2,054 European ancestry melanoma cases enrolled between 1994 and 2006 at the U.T. M.D. Anderson Cancer Center (Table 5.1). Friends or spouses of these enrolled cases (1,060 individuals) were recruited as controls. The exclusion criteria included a history of prior cancers (other than skin cancer). Only the Caucasian subjects from both datasets were included for analysis.

5.2.3 Genotyping and quality control

DNA was extracted from buccal cell samples using the Qiagen DNA extraction kit. PCR-based genotyping was performed using 384-well plates and the Applied Biosystems ViiA 7 system. Gene-specific primers were custom-designed by Qiagen. Genotyping calls were initially performed by the QuantStudio™ 6 software. Per-SNP quality control (QC) for the GEM samples was performed as previously described[213]. Quality control for the GENEVA dataset was

completed in two stages, including per-individual QC and per-SNP QC as described in the main text. Plink (v1.90b6.2) was used to examine missing data in sex and genotyping (<3%), as well as familial relationship. Overall, the genotyping call rate reached 99.887%. In terms of per-SNP QC, we performed SNP missingness (<5%), Hardy-Weinberg Equilibrium (> 1×10^{-4}, GENEVA recommendation), and minor allele frequency checks (MAF ≥ 5%). SNP number dropped from 1,012,904 to 739,936, in which rs827421 was included. Imputed SNPs were provided in the GENEVA dataset downloaded from dbGaP and thus imputed SNPs were included for analyses, such as rs1520220 and rs2229765.

5.2.4 Statistics

Computational analyses were performed by using Plink (v1.90beta6.2, June 12, 2018, http://zzz.bwh.harvard.edu/plink/), Plink2.00alpha (June 29, 2018, specifically for imputation analysis, https://www.cog-genomics.org/plink/2.0/) in the High-Performance Computing Cluster (University of California Irvine, https://hpc.oit.uci.edu/), and RStudio (v1.1.453). χ^2 test of independence was performed to examine the associations between SNP candidates and melanoma case-control status. Statistical significance was adjusted by the Benjamini–Hochberg procedure to correct multiple comparisons, using a false discovery rate of 25%[636,637]. Simple logistic regression models showing the crude odds ratios between the binary response variable (melanoma case-control status) and primary study variables of interest (top SNPs) were conducted separately based on additive, recessive, and dominant allele models. Dummy variables of the SNPs in the models were created by default, making the genotype with homozygous major alleles as the reference. The family history of melanoma – which was one of the confounders necessarily to be adjusted as previously described by us[213] – was controlled in the multiple logistic regression models. The gender

effect was evaluated by confounding adjustment and effect stratification in the logistic regression models. The lambda value[638] of population stratification was computed to be 1.01 (as close as to be 1.00) in the GENEVA dataset and thus population stratification was not considered necessary to be controlled in the regression models. Indeed, >99% of the patients in the GENEVA dataset were European ancestry whites[639] and we did not include any racial minority subjects in the present study.

5.3 Results

5.3.1 Study participants

Table 5.1 summarizes the 349 participants from the International Genes, Environment, and Melanoma Study (GEM)[501,635] collected in Southern California and 3,114 study subjects from the Gene Environment Association Studies Initiative (dbGaP GENEVA, details are in the materials and methods section 5.2.2) dataset with available phenotypes[640]. In the GEM dataset, cases and controls were approximately 1:1 matched by age and gender, whereas in the GENEVA dataset, patient numbers were roughly 2:1 to the healthy controls. There were more women cases in younger ages (< 50 years) in both datasets, similar to the age distribution of CM in the general population. The family history of melanoma was higher in the controls than in the cases in the GENEVA dataset (63.4% vs 32.5%, p<0.0001). However, a considerable percentage of patients (27.7% of men and 20.9% of women cases) declared an unknown status of their family history of melanoma. Nearly all of the control subjects submitted a known status of their family history (Table 5.1). In contrast, there was a significantly higher number of cases having a family history of melanoma as compared to the controls (16.8% vs 2.3%, p<0.0001) in the GEM dataset.

Table 5.1 Characteristics of the study participants.

	GEM (N=349)								GENEVA (N=3,114)							
	Cases (N=177)				Controls (N=172)				Cases (N=2,054)				Controls (N=1,060)			
	Men		Women		Men		Women		Men		Women		Men		Women	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Age (years)																
0-29	0	0%	2	1.1%	0	0.0%	0	0%	55	2.7%	100	4.9%	16	1.5%	46	4.3%
30-49	13	13.8%	28	15.8%	20	11.6%	37	21.5%	373	18.2%	352	17.1%	198	18.7%	184	17.4%
50-69	43	24.3%	37	20.9%	59	34.3%	29	16.9%	606	29.5%	336	16.4%	366	34.5%	186	17.5%
70+	33	18.6%	12	6.8%	19	11.0%	8	4.7%	153	7.4%	79	3.8%	48	4.5%	16	1.5%
Unknown	5	2.8%	4	2.3%	0	0.0%	0	0%	0	0%	0	0%	0	0%	0	0%
Family history of melanoma																
Yes	10	5.6%	18	10.2%	1	0.6%	3	1.7%	388	18.9%	280	13.6%	399	37.6%	273	25.8%
No	78	44.1%	60	33.9%	97	56.4%	71	41.3%	231	11.2%	157	7.6%	227	21.4%	159	15.0%
Unknown	6	3.4%	5	2.8%	0	0.0%	0	0.0%	568	27.7%	430	20.9%	2	0.2%	0	0.0%
Total	94	53.1%	83	46.9%	98	57.0%	74	43.0%	1187	57.8%	867	42.2%	628	59.2%	432	40.8%

5.3.2 SNP selection

Thirteen candidate SNPs were selected based on their associations with various diseases, particularly cancer (Table 5.2). SNPs rs12662670, rs2234693, rs2046210, rs3734805, and rs827421 in ESR1 were widely studied of their associations with breast cancer risk[623,625,626,641-647]. ESR2 was also found to be related to various cancer types. For instance, rs1255998 and rs1256061 were linked to lung tumors[648], and rs1256049 was correlated with increased risks of colorectal cancer[649] and prostate cancer[650]. SNPs rs1520220, rs2946834 and rs5742694 in IGF1 had continuously been linked to cancer prognosis[651] as well as coronary artery disease[652]. SNPs rs2229765 and rs8038415 in IGF1R had been linked to several cancer types including colorectal cancer, breast cancer, papillary thyroid carcinoma, and non-small cell lung cancer[653-656].

Table 5.2 Selection of the 13 candidate SNPs.

Gene	SNP	Location	dbSNP ID	Minor allele disease associations	References
ESR1	T>G	Intron	rs12662670	Common breast cancer locus	625
	-397T>C	Promoter	rs2234693	breast cancer susceptibility, prostate cancer risk	657,658
	G>A	Promoter	rs2046210	Breast cancer risk	644,645
	A>C	Promoter	rs3734805	Breast cancer risk	644
	A>G	Intron	rs827421	Breast cancer risk	659
ESR2	C>G	3'UTR	rs1255998	Endometrial cancer	660
	C>T	Exon	rs1256049	Risks of breast cancer and colorectal cancer	649,661
	G>A	Intron	rs1256061	Risks in lung tumors and ovarian cancer	648,662
IGF1	C>G	Intron	rs1520220	Obesity, poor breast cancer survival, pancreatic cancer risk	651,663,664
	A>G	Intron	rs2946834	Poor outcome in patients with breast cancer	651
	C>A	Intron	rs5742694	Colorectal cancer risk, poor breast cancer survival	651,665
IGF1R	G>A	Exon	rs2229765	Colorectal cancer risk, papillary thyroid carcinoma risk	653,654
	T>C	Intron	rs8038415	Risks in non-small cell lung cancer, breast cancer	655,656

5.3.3 Genotyping and SNP association in the GEM cohort

In order to examine the possible associations between the 13 selected SNPs and risk of melanoma, we used a high throughput PCR-based method to genotype these SNPs in the GEM cohort as an initial exploratory step. The GEM patient samples were originally acquired for the International Genes, Environment, and Melanoma (GEM) Study[501,556]. Genotyping calls were machine-validated and then manually examined, as described previously[213]. Four genetic models are considered: genotypic, allelic, recessive and dominant models. The genotypic model considers the number of minor alleles in a genotype (e.g. aa, Aa, and AA represents the highest, the medium, and the lowest risk, respectively), while the allelic model only considers the presence of one allele (i.e. the counts of A v.s. a in the cases and controls) in each group[666]. The dominant model assumes the minor allele (i.e. the presence of one minor allele in the genotype) leads to the disease, while the recessive model requires homozygous minor alleles to be associated with the disease. All models assume the minor allele is associated with the disease due to its lower frequency in the population[667].

The Hardy-Weinberg equilibrium (HWE) test was performed using the GEM control cohort to exclude SNPs significantly deviated from equilibrated genotypes ($p<0.05$). Three SNPs (rs12662670 and rs2234693 from ESR1, rs5742694 from IGF1) were thus excluded for further analyses, even though their χ^2 statistics showed significant association with melanoma risk (Table 5.3). The screening criterion was set at a p-value < a Benjamini–Hochberg critical value[637] to prioritize the SNPs with significant associations with melanoma. Benjamini–Hochberg multiple comparison correction was used at this step at a false discovery rate of 0.25 by default[636]. Association of alleles or genotypes with melanoma risk were tested by the χ^2 test of

independence based on genotypic, allelic, recessive, and dominant genetic models (Table 5.3). IGF1R rs2229765 (p=0.004) and IGF1 rs1520220 (p=0.005) showed significant genotypic and recessive differences between melanoma cases and healthy controls (Table 5.3).

Table 5.3 Association of the 13 SNP candidates with melanoma in the GEM dataset.

SNP	Gene	Genotyping rate [a]		Minor allele frequency (MAF)		Association (p-value | Benjamini–Hochberg critical value) [b]				HWE [c] (p-value)	dbSNP MAF [d]
		Cases (n=170)	Controls (n=152)	Cases (n=170)	Controls (n=152)	Genotypic	Allelic	Recessive	Dominant		
rs12662670	*ESR1*	97.1%	82.2%	6.7%	9.6%	0.083 | 0.077	0.255 | 0.115	0.034 | 0.077	0.638 | 0.173	0.014	10.7%
rs2046210	*ESR1*	94.7%	88.2%	30.4%	36.6%	0.247 | 0.115	0.137 | 0.058	0.650 | 0.212	0.120 | 0.058	0.458	41.2%
rs2234693	*ESR1*	98.2%	96.1%	38.3%	36.0%	0.254 | 0.135	0.598 | 0.212	0.588 | 0.173	0.238 | 0.077	0.007	44.6%
rs3734805	*ESR1*	98.2%	75.7%	6.3%	7.8%	0.575 | 0.212	0.590 | 0.192	N/A	0.575 | 0.154	1.000	10.5%
rs827421	*ESR1*	98.8%	86.8%	51.2%	59.1%	0.141 | 0.096	0.059| 0.038	0.214| 0.096	0.097 | 0.038	0.857	47.8%
rs1255998	*ESR2*	98.8%	96.1%	8.6%	11.3%	0.349 | 0.154	0.325 | 0.135	0.465 | 0.154	0.370 | 0.115	0.696	36.9%
rs1256049	*ESR2*	97.6%	98.0%	7.8%	7.7%	1.000 | 0.25	1.000 | 0.25	N/A	1.000 | 0.25	1.000	13.0%
rs1256061	*ESR2*	98.2%	88.8%	49.1%	47.4%	0.384 | 0.173	0.740 | 0.231	0.335 | 0.115	0.786 | 0.212	0.731	40.0%
rs1520220	***IGF1***	97.1%	88.8%	28.2%	24.8%	**0.005** | 0.058	0.404 | 0.154	**0.013** | 0.038	0.672 | 0.192	1.000	32.0%
rs2946834	*IGF1*	97.6%	74.3%	35.2%	40.7%	0.445 | 0.192	0.222 | 0.077	0.370 | 0.135	0.362| 0.096	0.435	40.0%
rs5742694	*IGF1*	98.8%	93.4%	50.6%	41.2%	< 0.001 | 0.019	0.024| 0.019	0.025 | 0.058	< 0.001 | 0.019	< 0.001	21.6%
rs2229765	***IGF1R***	97.6%	96.1%	35.8%	40.8%	**0.004** | 0.038	0.239 | 0.096	**0.003** | 0.019	0.918 | 0.231	1.000	33.6%
rs8038415	*IGF1R*	97.1%	98.1%	51.5%	54.3%	0.654 | 0.231	0.411 | 0.173	0.648 | 0.192	0.458 | 0.135	1.000	42.5%

[a] Percentage of participants with successful SNP genotyping.
[b] Chi-square test of independence between SNP models and melanoma case-control status. P-value < Benjamini–Hochberg critical value for multiple comparison correction counts as statistically significant. The false discovery rate for Benjamini–Hochberg procedure is at 0.25 by default.
[c] Exact test for Hardy-Weinberg equilibrium (HWE) using the control samples only. P-value < 0.05 counts as evidence of un-equilibrated genotypes.
[d] Reference minor allele frequencies documented in the NCBI dbSNP database.

5.3.4 An attempt to validate the top 2 SNPs in the GENEVA dataset

In order to validate our findings from the GEM dataset, we extracted genotyping data from the GENEVA dataset (Table 5.4). Quality control (QC) for the GENEVA dataset was performed in two stages, including per-individual QC and per-SNP QC. The initial per-individual QC report was attached to the GENEVA dataset (details are in the materials and methods section 5.2.3). Of the 3,114 study subjects (Table 5.1), genotyping data were available for 3,110 individuals. Per-individual QC reported 68 duplicated individuals and an additional 17 individuals with familial relationships. The rest of 3,025 individuals were further examined for genotyping intensity and chromosomal anomalies (i.e. genotyping errors). 3,003 (1,965 cases and 1,038 controls) study subjects passed this level of QC and were used for further analyses. The genotyping call rate reached 99.9%.

All 13 SNPs were analyzed in the GENEVA dataset in an attempt to gain a complete understanding of the ESR/IGF1 pathway. As shown in Table 5.4, the melanoma associations of IGF1R rs2229765 and IGF1 rs1520220 were not replicable in the GENEVA dataset. On the other hand, SNPs rs2234693 ($p=0.035 < 0.038$ critical value) and rs827421 ($p=0.018 <$ critical value 0.019) in ESR1 had only borderline Benjamini–Hochberg corrected significant genotypic differences between melanoma cases and healthy controls in the GENEVA dataset (Table 5.4). The minor alleles also fitted in a dominant genetic model ($p=0.010$ and 0.005, respectively). However, these two SNPs were not observed in the GEM discovery set. It was noted that rs2234693/ESR1 did not present a significant Benjamini–Hochberg corrected association with melanoma risk in the GEM set might be a result of HWE deviation.

Table 5.4 Association of the 13 SNP candidates with melanoma in the GENEVA dataset.

SNP [a]	Gene	Minor allele frequency (MAF)		Association (p-value | Benjamini–Hochberg critical value) [b]				HWE [c] (p-value)	dbSNP MAF [d]
		Cases (n=1,965)	Controls (n=1,038)	Genotypic	Allelic	Recessive	Dominant		
rs12662670 [e]	*ESR1*	14.1%	14.3%	0.995 | 0.231	0.446 | 0.212	0.961 | 0.231	0.069 | 0.077	0.997	10.7%
rs2046210 [e]	*ESR1*	54.2%	51.1%	0.640 | 0.135	0.248 | 0.135	0.936 | 0.212	0.155 | 0.154	0.984	41.2%
rs2234693	***ESR1***	47.2%	44.4%	**0.035** | 0.038	0.047 | 0.038	0.580 | 0.077	**0.010** | 0.038	0.059	44.6%
rs3734805	*ESR1*	8.0%	6.9%	0.181 | 0.096	0.129 | 0.115	0.660 | 0.115	0.090 | 0.115	0.624	10.5%
rs827421	***ESR1***	50.7%	47.6%	**0.018** | 0.019	0.027 | 0.019	0.440 | 0.058	**0.005** | 0.019	0.192	47.8%
rs1255998	*ESR2*	8.9%	10.5%	0.093 | 0.058	0.048 | 0.058	0.110 | 0.019	0.079 | 0.096	0.869	36.9%
rs1256049	*ESR2*	2.8%	3.5%	NA	0.112 | 0.096	NA	0.104 | 0.135	0.634	13.0%
rs1256061 [e]	*ESR2*	60.7%	62.6%	0.971 | 0.212	0.404 | 0.192	0.678 | 0.154	0.581 | 0.231	0.979	40.0%
rs1520220 [e]	*IGF1*	63.7%	62.6%	0.752 | 0.173	0.281 | 0.154	0.642 | 0.096	0.257 | 0.173	0.985	32.0%
rs2946834	*IGF1*	31.2%	33.2%	0.158 | 0.077	0.102 | 0.077	0.672 | 0.135	0.055 | 0.058	0.043	40.0%
rs5742694 [e]	*IGF1*	65.0%	65.5%	0.941 | 0.192	0.283 | 0.173	0.740 | 0.173	0.263 | 0.192	1.000	21.6%
rs2229765 [e]	*IGF1R*	59.7%	57.2%	0.315 | 0.115	0.950 | 0.25	0.403 | 0.038	0.417 | 0.212	1.000	33.6%
rs8038415 [e]	*IGF1R*	61.3%	60.0%	0.646 | 0.154	0.815 | 0.231	0.806 | 0.192	0.609 | 0.25	1.000	42.5%

[a] Ordered by genes according to smallest to largest rs numbering.

[b] Chi-square test of independence between SNP models and melanoma case-control status. P-value < Benjamini–Hochberg critical value for multiple comparison correction counts as statistically significant. The false discovery rate for Benjamini–Hochberg procedure is at 0.25.

[c] Exact test for Hardy-Weinberg equilibrium (HWE) on the controls. P-value < $1x10^{-4}$ counts as evidence of unbalanced genotypes (GENEVA recommendation).

[d] Reference minor allele frequencies documented in the NCBI dbSNP database.

[e] Imputed.

Since estrogen is able to initiate the IGF1 signaling pathway by inducing the expression of IGF1R and its downstream signaling that leads to cell proliferation[634], the IGF1 and IGF1R SNPs might still provide crucial information on the ER/IGF1R network in melanoma. Moreover, as described in one of our previous publications[213], all patients in the GEM set had multiple melanomas which might provide unique genetic information on melanoma risk. The IGF1 rs1520220 and IGF1R rs2229765 SNPs were thus further analyzed in the logistic regression models to determine the odds ratio (OR) of melanoma in individuals carrying minor alleles/genotypes in comparison with the reference alleles/genotypes (Table 5.5).

In the un-adjusted crude regression analyses (Model A), only the recessive genetic models showed significant associations with melanoma: the OR of GG v.s. CC+CG reference genotypes in rs1520220/IGF1 was 2.97 (95% CI: 1.35, 7.23, p=0.010, model likelihood p=0.006); the OR of AA v.s. GG+GA reference genotypes in rs2229765/IGF1R was 0.29 (95% CI: 0.12, 0.62, p=0.003, model likelihood p=0.001) (Table 5.5). Therefore, due to the complexity of the regression analyses based on different genetic models (i.e. additive/genotypic, recessive, and dominant models), hereafter we only presented the recessive genetic model results of IGF1 and IGF1R as indicated by the un-adjusted crude results in Table 5.5 and the $\chi 2$ test results in Table 5.3.

Additional regression analyses were adjusted by gender (Model B), family history of melanoma (Model C), or both (Model D). The odds ratio estimation from the gender-adjusted Model B: OR 2.86 (95% CI: 1.29, 6.98, p=0.014) was slightly reduced for rs1520220/IGF1 as compare to Model A. On the contrary, the odds ratio estimation remained the same for rs2229765/IGF1R in Model B: OR 0.29 (95% CI: 0.12, 0.63, p=0.003). It was noted that the coefficients of gender did not show significance for both SNPs. However, the overall model

significance remained significant ($p=0.009$ for rs1520220/IGF1 and $p=0.001$ for rs2229765/IGF1R).

When the family history of melanoma was taken into consideration, the odds ratio estimations were further reduced for both SNPs. In the family history of melanoma-adjusted recessive models, the OR for rs1520220/IGF1 was 2.79 (95% CI: 1.24, 6.92, $p=0.018$) in Model C and approximately the same in Model D. The OR for rs2229765/IGF1R was 0.25 (95% CI: 0.10, 0.57, $p=0.002$) in Model C and remained the same in Model D. This might be because of the coefficients of family history of melanoma were highly significant in both Models C and D for both SNPs and might confound the SNP results. However, this confounding effect was not considered severe because the OR differences were less than 10%[668] for both SNPs between models (rs1520220/IGF1 Crude OR: 2.97 → Model C OR: 2.79; rs2229765/IGF1R Crude OR: 0.29 → Model C OR: 0.25).

Table 5.5 Associations of IGF1 rs1520220 and IGF1R rs2229765 SNPs with melanoma risk in the GEM dataset.

Logistic Regression Models				Crude (Model A)		Model B [b]		Model C [c]		Model D [d]	
SNPs/Genetic Models	**Genotypes**	**Cases n (%)**	**Controls n (%)**	**OR (95% CI)**	**p-value [a]**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
rs1520220/IGF1 Recessive	CC+CG	139 (81.8%)	127 (83.6%)	Reference	--	Reference	--	Reference	--	Reference	--
	GG	26 (15.3%)	8 (5.3%)	2.97 (1.35, 7.23)	0.010	2.86 (1.29, 6.98)	0.014	2.79 (1.24, 6.92)	0.018	2.80 (1.24, 6.93)	0.018
	Sex	--	--	--	--	1.08 (0.68, 1.72)	0.743	--	--	0.95 (0.58, 1.53)	0.819
	Family History	--	--	--	--	--	--	6.60 (2.48, 22.86)	0.0006	6.68 (2.50, 23.26)	0.006
	Model	--	--	--	0.006	--	0.009	--	0.0117	--	0.0117
rs2229765/IGF1R Recessive	GG+GA	157 (92.4%)	122 (80.3%)	Reference	--	Reference	--	Reference	--	Reference	--
	AA	9 (5.3%)	24 (15.8%)	0.29 (0.12, 0.62)	0.003	0.29 (0.12, 0.63)	0.003	0.25 (0.10, 0.57)	0.002	0.25 (0.10, 0.56)	0.002
	Sex	--	--	--	--	0.98 (0.62, 1.56)	0.948	--	--	0.84 (0.52, 1.36)	0.484
	Family History	--	--	--	--	--	--	8.38 (3.08, 29.79)	0.00017	8.77 (3.19, 31.44)	0.00014
	Model	--	--	--	0.001	--	0.001	--	0.002	--	0.002

[a] p-value of the coefficient from the regression model. The overall model significance was derived from the likelihood ratio test (χ^2 statistic).
[b] Model B, adjusted for gender.
[c] Model C, adjusted for family history of melanoma.
[d] Model D, adjusted for gender and family history of melanoma.

5.3.5 Gender disparity of the association

Lastly, the GEM cohort was stratified by gender and logistic regression analyses were performed to measure the associations of rs1520220/IGF1 and rs2229765/IGF1R and melanoma risk in men and women, respectively. Surprisingly, the melanoma associations were only significant in men for both SNPs.

For rs1520220/IGF1 in the recessive model, men with GG genotype showed extremely high risk of melanoma (OR 8.11, 95% CI: 2.20, 52.50, p=0.006) while women with GG genotype showed a significant lower risk (OR 0.15, 95% CI: 0.018, 0.86, p =0.045). For rs2229765/IGF1R, men with AA genotype presented a significant lower risk (OR 0.24, 95% CI: 0.07, 0.64, p=0.008) while AA in women did not show a significant association (Table 5.6).

Table 5.6 Associations of IGF1 rs1520220 and IGF1R rs2229765 with melanoma risk in two gender strata in the GEM dataset.

Model		**Male OR (95% CI)**	**p-value [a]**	**Female OR (95% CI)**	**p-value**
rs1520220/IGF1 Recessive	CC+CG	Reference	--	Reference	--
	GG	8.11 (2.20, 52.5)	0.006	0.15 (0.018, 0.86)	0.045
rs2229765/IGF1R Recessive	GG+GA	Reference	--	Reference	--
	AA	0.24 (0.07, 0.64)	0.008	1.70 (0.32, 8.86)	0.526

[a] p-value of the coefficient from the regression model.

5.4 Discussion

In an attempt to understand the genetic predisposition in gender-biased risk in cutaneous melanoma, a gene prioritization approach and a case-control study design were used to measure melanoma associations with a group of 13 SNPs from the ESR/IGFR pathway. Genotyping of SNPs was carried out in the GEM cohort originated from Southern California. The most significant 2 SNPs (IGF1R SNP rs2229765 and IGF1 SNP rs1520220) were further examined in the large GENEVA cohort originally recruited by MD Anderson Cancer Center, Huston, Texas. After Benjamini–Hochberg multiple testing correction, IGF1R SNP rs2229765 and IGF1 SNP rs1520220 failed to be validated in the GENEVA cohort. On the other hand, ESR1 SNPs rs2234693 and rs827421 only slightly presented Benjamini–Hochberg significances of their genotypic and dominant genetic model differences between melanoma cases and controls in the GENEVA cohort. Nevertheless, as the GEM cohort included multiple melanoma patients[213] that might provide unique genetic information, the IGF1R SNP rs2229765 and IGF1 SNP rs1520220 were further analyzed in regression models. Multiple logistic regression models later revealed that the G allele in IGF1 rs1520220 carriers exhibited a higher risk of melanoma as compared to the reference C allele in a recessive genetic model, while the A allele in IGF1R rs2229765 showed a protective effect comparing to the reference G allele in the recessive genetic model. These effects were, however, only shown significances in the male cohort but not in females.

IGF1 SNP rs1520220 first appeared in the literature in 2005. The C allele of rs1520220 was found significantly associated with increased serum IGF1 levels and an increased risk of breast cancer in women[669]. Later, the rs1520220 C allele was widely discussed in increasing different types of cancer risks and IGF1 serum levels such as prostate cancer[670,671], ovarian cancer[672], stomach cancer[673], and more. Of these studies, none have been addressed in melanoma, and

hence the actual causal mechanism of IGF1 rs1520220 in melanoma remains unclear. Nevertheless, IGF1 is one of the essential growth factors known for its direct carcinogenesis effect by activating the PI3K/Akt mitogenesis, cell cycle protection, and anti-apoptosis pathway through binding to IGF1R[674]. Indirectly, IGF1 works with sex hormones to intensify these cancerous activities including cell proliferation, transformation, and metastasis[674]. Our current results also showed an increased risk of melanoma by IGF1 rs1520220 C alleles. This effect was further magnified in the male cohort, but an opposite association was found in females. Perhaps the indirect effect of IGF1 is playing a role in gender disparities in melanoma, which awaits further laboratory studies to reveal any gender-specific crosstalk between IGF1 rs1520220 and sex hormones in melanoma.

In contrast, the A alleles in IGF1R SNP rs2229765 presented a protective effect on melanoma in the current study. The current understanding of this SNP in the literature is controversial. For instance, the A allele was found associated with advanced colorectal cancer[675] and an increased risk of breast cancer[676]. On the contrary, the A alleles showed no association with non-small cell lung cancer survival[677], IGF1R expression, or breast cancer survival[678]. Interestingly, the A allele was discovered to be protective in papillary thyroid carcinoma[653]. rs2229765 SNP G>A is known to be a "silent" mutation, which means the nucleotide acid change from Guanine to Adenosine at this locus does not change the encoded protein and is thus not likely to be a functional alteration[678]. From experimental animal studies, IGF1R showed a pivotal role in the development of ovaries and fertility in female mice[679]. This gender-specific activity was also seen in breast cancer cells where estradiol (a female-sex hormone) interacts with IGF1R to adhere to extracellular matrices as a marker of cancer progression[680]. This perhaps could be a reason why our result in females

also showed an increased risk of melanoma by IGF1R rs2229765, although the result was nonsignificant and its association with breast cancer is still inconclusive in the literature. On the other hand, IGF1R usually co-expressed with androgen receptor in response to dihydrotestosterone (a male-sex hormone)-dependent prostate cancer cell proliferation[681]. While this silent mutation of rs2229765 polymorphism favored longevity in male carriers of the homozygous A alleles[682]. The protective effect of IGF1R rs2229765 in the current study in men remains an interesting focal point in future melanoma gender disparity studies.

The IGF1 SNP rs1520220 and IGF1R SNP rs2229765 were identified in the GEM cohort but failed validation in the GENEVA cohort. Similarly, ESR1 SNPs rs2234693 and rs827421 were not observed significantly in the GEM cohort. A possible explanation for this non-replication on genotype frequencies is perhaps because the patient characteristics in these two datasets are very different. In the GENEVA cohort, only newly diagnosed malignant melanoma cases were eligible and recruited. Of these patients, only 2.8% developed more than one primary tumors. However, the GEM cohort included a large portion of patients with multiple melanoma tumors (22.0%)[501,556]. The ESR1 SNPs are still likely to play a role in melanoma development. Indeed, we have found a universal expression of an ESR1 isoform ERα36 in melanocytes and melanoma cells (Liu-Smith et al., unpublished data), which may further explain the importance of ESR1 SNPs rs2234693 and rs827421. Therefore, these two SNPs also warrant further investigations.

A major limitation of this study was the high missing rate of family history of melanoma status in the GENEVA patients as shown in Table 5.1. Approximately 27.7% of men and 20.9% of women showed an unknown status of their family history, which may influence the results from the adjusted models and decrease the precision of OR estimation. On the other hand, almost

all controls reported a family history of melanoma (Table 5.1), which may also add biases to the results. A minor limitation was the small sample size in the discovery cohort of GEM study, which may not provide sufficient power for the targeted SNPs, although the primary reason for non-replication may likely be the patient composition differences between these two cohorts as described in the paragraph above. Nevertheless, as demonstrated in our series of publications[92,93,114,430], the gender difference in melanoma incidence is significant but not fully explained. Meanwhile, studies on hormone impact on melanoma are still ongoing in our lab and other research groups. Taken together, this current study will certainly help us form hypotheses that aid in future research.

In summary, our data suggest that the G allele in IGF1 rs1520220 is likely to be associated with melanoma risk, while the A allele in IGF1R rs2229765 may have a protective effect, especially in men, in recessive genetic models. The ESR1 SNPs rs2234693 and rs827421 may play a role in melanoma patients, but further analysis is needed. These findings may provide some understanding of gender-specific melanoma risks. However, the molecular mechanisms will require further investigation in order to completely dissect the role of ER/IGF1R pathway in cutaneous melanoma development.

CHAPTER 6 SUMMARY AND FUTURE DIRECTIONS

Because of the complexity of the current dissertation, here are the major conclusions in bullet points:

- Chapter 1 of the current dissertation reviewed the latest information on cutaneous melanoma, including clinical manifestations, risk factors, and current treatments
    - Despite that UV radiation has long been recognized as the primary and major cause of cutaneous melanoma, its involvement in anatomic tumor distributions, early-onset of the disease, and gender disparities has not yet provided a comprehensive explanation
    - UV radiation seems to only impose the first punch on melanoma via creating a DNA mutation burden. There are various additional secondary effectors joining the game to complete the disease progression
    - One of the chief secondary drivers is the reactive oxygen species. They are produced by NADPH oxidase complex 1 in melanocytes and exhibit enhanced production via NADPH oxidase complex 4 in melanoma cells
    - The tumorigenic responses elicit by reactive oxygen species are crucial to melanoma progression
- Chapter 2 of the dissertation conducted a case-control study of reactive oxygen species-associated genetic risk of melanoma
    - It was statistically significant that, RAC1-GTPase, the activator of NADPH oxidase complex 1, predisposed the fair-skinned population to melanoma
- Chapters 3 and 4 of the current dissertation explored a tendency of the early disease-onset in the darker-skinned populations

    - The results demonstrated a shared phenomenon of an early disease-onset in young females regardless of skin color variations
    - The darker-skinned populations demonstrated an even younger age of disease diagnosis than the fair-skinned population
    - The tumors that grow in the non-UV-exposed regions such as on the trunk and on the hips presented the highest increase in incidence rates over the years

- Chapter 5 of the dissertation attempted to explain the predisposed early melanoma-onset tendency introduced by female-sex, using a case-control study of estrogen receptors' signaling network-associated genetic risk of melanoma
    - Estrogen's downstream insulin-like growth factor 1 and its receptor may play a deciding role in gender disparity of melanoma in the fair-skinned population
    - This work requires further data analyses from additional datasets to validate the findings

Nevertheless, there are a few limitations in the current dissertation:

- The current dissertation cannot rule out the influence of tanning device usage in the fair-skinned population due to data limitation in the genetic datasets utilized in Chapters 2 and 5
- The current dissertation is lacking in the area of UV-associated behavioral influences due to data limitation in the datasets utilized in Chapters 3 and 4

Yet, the transformation from defective melanoblast development to malignant melanoma is a multifactorial disease progression process. There are countless biological, cultural, and geographic factors that are beyond human control. Therefore, the major future directions should include:

- Cutaneous melanoma is a different disease in the darker-skinned populations and the causal factors are currently unknown
- The melanoma tumor subtype is unique in the darker-skinned populations compared to the tumors that grow in the fair-skinned population which warrants further investigation of possible etiologies

The final takeaway message from the current dissertation:

- There is an age-dependent melanoma causal factor(s), regardless of UV radiation impact on different skin colors that predisposed women to develop melanoma at an earlier age than men
- Melanoma primary prevention strategy should be reshaped to incorporate female-sex-oriented message to educate the public in both the fair-skinned population and the darker-skinned populations in the US and worldwide

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