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The Differential Contribution of Behavior and Biology to Breslow Thickness and Melanoma Survival in Males Compared to Females

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THE DIFFERENTIAL CONTRIBUTION OF BEHAVIOR AND BIOLOGY TO BRESLOW THICKNESS AND MELANOMA SURVIVAL IN MALES COMPARED TO FEMALES

by

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DISSERTATION

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To Jeff, Michelle, Sheré, and Alyson Lilyquist

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ABSTRACT

There are many well-documented differences between males and females regarding melanoma including incidence rates, presentation, markers of progression, and survival. A common hypothesis to explain the female survival advantage is that males are less aware of their skin, resulting in thicker lesions at diagnosis, and ultimately poorer survival. However, there are also multiple hypotheses attributing the female survival advantage to biological differences between males and females, mostly regarding sex-steroid hormones. Sex has been identified as an independent prognostic marker in multiple studies, supporting the hypothesis that melanoma progression varies between men and women. Despite these findings, stratifying by sex in melanoma studies is uncommon. Here we present four studies investigating both behavioral and biological differences as they relate to Breslow thickness and melanoma survival in analyses stratified by sex.

We found that different factors contribute to Breslow thickness and survival between males and females. Our results suggest that UV exposure is associated with increased male survival independent of Breslow thickness. UV exposure is associated with decreased Breslow thickness in females, but was not significant in the survival models suggesting that the effect was encompassed by Breslow thickness in the survival model. Furthermore, skin awareness was associated with increased survival and decreased Breslow thickness in females, but not in males.

We also identified multiple SNPs in DNA repair and immune response genes that were associated with Breslow thickness, and interacted with UV exposures to modify Breslow thickness. Importantly, there was only one SNP that overlapped in the male and female analyses, and the analysis of SNPs in the overall population was not representative of the analyses stratified by sex.

Our results may help explain previous inconsistencies in the literature regarding UV exposure impact on Breslow thickness and survival. Furthermore, these studies provide a good foundation for further investigating the role of UV exposures in the female survival advantage. Finally, we have demonstrated the importance of analyses stratified by sex in the study of melanoma.

List of Abbreviations:

Terminology
AJCC—American Joint Committee on Cancer
BER—Base excision repair
CPD—Cyclobutane pyrimidine dimer
E1—Estrone
E2—Estradial/17β-estradiol
E3—Estriol
FDR—False discovery rate
LD—Linkage disequilibrium
LDH—Lactose dehydrogenase
LMM—Lentigo maligna melanoma
MAF—Minor allele frequency
NER—Nucleotide excision repair
NM—Nodular melanoma
RGP—Radial growth phase
SEER—Surveillance, Epidemiology, and End Results
SNP—Single nucleotide polymorphism
SSE—Skin self-examination
SSM—Superficial spreading melanoma
TIL—Tumor infiltrating lymphocytes
TNM—Tumor, nodes, and metastasis
UV—Ultraviolet
VGP—Vertical growth phase

Gene names:
APEX1—APEX nuclease (multifunctional DNA repair enzyme) 1
BRAF—B-Raf proto-oncogene, serine/threonine kinase
CXCL8—Chemokine (C-X-C motif) ligand 8
EGFR—Epidermal growth factor receptor
ERCC4—Excision repair cross complementation group 4
ERCC5—Excision repair cross complementation group 5
ERCC6—Excision repair cross complementation group 6
ERα—Estrogen receptor alpha
ERβ—Estrogen receptor beta
FBRSL1—Fibrosin-like 1
GPER—G protein coupled estrogen receptor
IFNγ—Interferon gamma
IGFBP3—Insulin-like growth factor-binding protein 3
IKBKB—Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta
IL-10—Interleukin 10
IL-1β—Interleukin 1beta
IL-6—Interleukin 6
IL-17A—Interleukin 17A

LIG1—Ligase 1, DNA, ATP-dependent
MDM2—Mouse double minute 2 proto-oncogene, E3 ubiquitin protein ligase
MGMT—O-6-methylguanine DNA-methyltransferase
NRAS—Neuroblastoma RAS viral (V-Ras) oncogene homolog
PARP1—Poly (ADP-ribose) polymerase 1
PGE2—Prostaglandin E2
RANKL—Receptor activator of nuclear factor-kappaB ligand
RFC1—Replication factor C (activator 1) 1
SLC45A2—Solute carrier family 45, member 2
SMAD3—SMAD family member 3
TDG—Thymine-DNA glycosylase
TGFβ—Transforming growth factor beta
TNF—Tumor necrosis factor
VDR—Vitamin D receptor
XPC—Xeroderma pigmentosum, complementation group C

CHAPTER ONE:

INTRODUCTION

1.1 Melanoma background:

Melanoma is a cancer that arises from melanocytes, which are the melanin producing cells that reside in the basal layer of the epidermis [1]. Other skin cancers, such as basal cell carcinoma and squamous cell carcinoma, arise from the keratinocytes in the epidermis [1]. Of these different types of skin cancer, melanoma is the most aggressive and the most deadly—melanoma accounts for less than two percent of skin cancers, but more than 75% of skin cancer deaths [2]. The incidence of melanoma has been steadily increasing over the last ten years, perhaps partially due to the increase in indoor tanning, and is currently the most common cancer among young adults aged 25-29 [3]. For 2015, it is estimated that there will be 73,870 new cases of melanoma and 9,940 deaths from melanoma [2].

There are several host factors that have been associated with melanoma risk including number of nevi, phenotypic index (hair color/eye color/tannability), weakened immune system, older age, sex, and genetic factors such as Xeroderma Pigmentosum (which affects DNA repair capacity) [4].

While there are multiple known host risk factors melanoma, there are few known environmental risk factors. The only well-studied environmental risk factor is ultraviolet (UV) radiation, such as that during sun exposure or indoor tanning. UV exposure, including UVA and UVB wavelengths, is thought to lead to melanoma because of its ability to cause DNA damage [5]. While there is some overlap, UVA and UVB radiation largely cause different types of DNA damage and are consequently repaired by different DNA repair mechanisms: base excision repair (BER) and nucleotide excision repair (NER) respectively [5,6]. Interestingly, the

UVA/UVB ratio and dose is different in sunlight than in tanning beds, and in fact, varies from tanning bed to tanning bed [7]. Furthermore, biological consequences resulting from UV exposure, such as vitamin D production and immunosuppression, may also influence melanoma risk [8–10].

1.2 Melanoma progression:

Once a patient has been diagnosed with melanoma, the best prognostic indicator is Breslow thickness, which is the depth of the lesion in millimeters from the granular layer of the epidermis to the deepest point [11]. Breslow thickness, along with ulceration and mitoses, make up the "tumor" classification of the tumor, nodes, and metastasis (TNM) staging system developed by the American Joint Committee on Cancer (AJCC) [12]. Breslow thickness also correlates with metastases; that is, the thicker the lesion is, the more likely it is that the melanoma has broken through basal layer of the epidermis and spread to lymph nodes or a distant site [11,12]. The 5-year survival rate for localized melanoma is 98.3%, but the survival rate dramatically decreases for nodal metastasis (63%) and distant metastasis (16.6%) [12].

Other variables, besides those included in the TNM staging system, have been identified as prognostic indicators for melanoma. Histologic variables associated with better prognosis are low Clark level (measurement of melanoma thickness determined by the layer of skin the lesion has reached), absent tumor vascularity, absent vascular invasion, absent microsatellites, absent regression of the primary tumor, and present tumor infiltrating lymphocytes (TILs) [4]. Clinical variables

associated with better prognosis include younger age, female sex, lesions located on extremities, and normal serum lactose dehydrogenase (LDH) levels (140-280 units/L) [4].

It is unclear exactly how melanoma progresses, but one theory is that it progresses in a linear fashion, and there is variation in the rate of growth between individuals [13]. Clark and colleagues developed this theory in 1989, and it outlines six histological changes from development of a benign nevus to metastatic melanoma [14]. Step 4 is the onset of melanoma and is called the radial growth phase (RGP) [14]. RGP is associated with superficial spreading melanoma (SSM), the histologic subtype that accounts for approximately 65% of melanomas [13]. Step 5 is the vertical growth of the melanoma that will allow it to eventually break through the basal layer of the skin and metastasize—this is called the vertical growth phase (VGP) [14]. Vertical growth phase is associated with nodular melanoma (NM), the histologic subtype that accounts for approximately 20% of melanomas [13]. Recently, Greenwald et al. challenged the linear progression model by reviewing evidence that SSM and NM are actually distinct biological entities [15]. To date, histologic subtype has not been implemented into the AJCC staging system or clinical practices [12,15].

1.3 Sex differences in melanoma:

1.3.1 Incidence

There are well-documented differences between males and females in melanoma incidence. SEER reports that men are 1.7 times more likely to develop

melanoma [16]. The incidence rate is also affected by age [16]. Women are slightly more likely than men to develop melanoma before age 50. Following age 50, there is a crossover in incidence rates, and the incidence rate for men drastically increases [16]. Interestingly, one-third of melanomas diagnosed in women are during childbearing years, and there is an increased risk of developing melanoma during pregnancy, implicating estrogen in the development of melanoma [17]. Furthermore, in the Netherlands, use of oral contraceptives and hormone replacement therapy has been associated with an increase in melanoma incidence in women [18]. These findings remain controversial as other studies have reported that use of oral contraceptives and hormone replacement therapy does not impact melanoma risk [19].

1.3.2 Presentation

Melanoma presentation also varies between men and women. As discussed above, men are more likely to present with melanoma at an older age [16]. Furthermore, men are more likely to develop melanoma on their head, neck, or trunk, whereas the majority of melanomas in women are on their extremities, particularly their legs [20,21]. Nodular melanomas, which are the most aggressive histologic subtype, generally arise on the trunk and are more common in men [22]. Men also tend to have thicker lesions that are more frequently ulcerated compared to women [23].

1.3.3 Progression

While studying the specific progression of melanoma can prove difficult, there are multiple studies suggesting that melanomas in males are inherently more aggressive, and thus progress more quickly, than melanomas in females. Regarding hormones and melanoma progression, decreased Breslow thickness in women who use oral contraceptive or hormone replacement therapy has been observed in a univariable model, but was not significant in the multivariable model [24]. In 2009, Liu et al. showed that melanomas in males have an increased rate of growth compared to females [25]. Another study showed that melanomas in females are less likely to develop regional or distant metastases [26]. Furthermore, even following metastasis, women have significantly better survival compared to men [26]. Finally, women with stage III and IV melanoma also have better relapse-free survival compared to men [27]. Taken together, these results demonstrate that melanomas in males are more likely to progress, and more likely to cause death following progression, compared to females. Therefore, it is possible that there are different biological factors (potentially hormones and medications affecting hormones) mediating melanoma progression in males compared to females.

1.3.4 Survival

The female survival advantage was observed as early as 1959 when White et al. reported that females have an increased 5-year melanoma survival rate compared to males [28]. In 1980, Rampen showed that women lived longer following first evidence of metastasis than men [29]. Additionally, Shaw et al. showed that women

with thicker lesions had better survival than men with thick lesions [30]. More recently, Joosse et al. has showed that sex is an independent prognostic indicator in stage I/II melanoma, as well as in stage III/IV melanoma [27,31]. Finally, Khosrotehrani et al. reported that females have superior survival across all stages of melanoma, and that the female survival advantage dissipated with increasing age [32].

Age also affects survival; the median age at death is 69 years, and the highest percent of melanoma deaths are aged 75-84 [16]. While age impacts the female survival advantage and older men are more likely to develop melanoma, age does not appear to entirely account for the sex discrepancy in melanoma survival. In 2009, Gamba et al. investigated survival in young men aged 15-39 compared to age-matched women [33]. They found that young men are twice as likely to die from melanoma than young women, even when they had thin (<1.00mm) lesions [33].

The complicated role of age melanoma survival implicates sex steroid hormones, particularly estrogen and its receptors, in melanoma survival. As age increases, the female survival advantage dissipates [34]. Furthermore, post-menopausal women have poorer survival than pre-menopausal women [34]. Because levels of estrogen and its receptors decrease following menopause, it is possible that hormones drive the age-dependent changes in female survival, as well as the sex differences in survival [29,35].

1.4 Estrogen and its receptors in normal skin and melanoma:

1.4.1 Normal skin

There are three known estrogen receptors: ERα, ERβ, and GPER [36]. ERα and ERβ are the canonical estrogen receptors that act primarily as transcription factors [36]. GPER is a newly discovered estrogen receptor that resides in the endoplasmic reticulum and acts primarily through rapid response signaling [36]. There are three estrogens that can activate estrogen receptors: estrone (E1), estradiol (E2 or 17β-estradiol), and estriol (E3) [37]. For women, E2 is most common pre-menopause and E1 is most common post-menopause [37]. It is unclear which estrogen is the most common in males, although it is likely to be E2 since it can be synthesized from testosterone [38]. Both males and females can synthesize estrogens in their skin [37].

While both of the traditional estrogen receptors are expressed in the skin, ERβ is more prevalent [35]. Men also express ERα and ERβ in their skin, but the expression is lower compared to females [39]. To date, the role of GPER in skin, and melanoma in particular, has not been published. Preliminary studies in our group show that GPER is expressed in melanoma (unpublished work). As menopause occurs and estrogen levels decrease, there are multiple effects on the skin including dryness, wrinkling, decrease in collagen and skin thickness, and delayed wound healing [35].

1.4.2 Melanoma

The role of estrogen receptors in cancers is context-dependent; however, in general, ERα promotes proliferation, while ERβ inhibits proliferation [40,41]. The exact mechanism of estrogen and its receptors in melanoma is not well defined. Di Giorgi et al. showed that decreased ERβ protein expression was associated with thicker lesions [39]. Furthermore, they showed that a decrease in ERα and ERβ mRNA levels were also associated with thicker lesions [39]. Similarly, Richardson et al. showed that *in vitro* exposure to estrone or estradiol, two endogenous estrogens, inhibits invasion [42]. Interestingly, the same group also reported that circulating estrone decreases with melanoma progression in male mice, but not in female mice, further implicating estrogens in the female survival advantage [42].

With the discovery of estrogen receptor expression in melanomas, there has been much debate over whether Tamoxifen, an anti-estrogen therapeutic, would be effective in treating melanoma. The results, summarized in a meta-analysis by Beguerie et al, have been complex and controversial [43]. Overall, Beguerie et al. found that Tamoxifen is usually used as an adjuvant therapy in stage III/IV melanoma, and works better in females [43]. However, they did not report an improvement in mortality from melanoma [43]. Interestingly, in other cancers, Tamoxifen is used to treat estrogen-dependent tumors, such as in ERα positive breast cancer. The female survival advantage in melanoma suggests that estrogen has an anti-tumor role in melanoma; therefore, it is not surprising that Tamoxifen does not have an overwhelming effect in the treatment of melanoma.

1.5 Androgen and its receptors in normal skin and melanoma

1.5.1 Normal skin

Androgen receptors are also in the skin, including the epidermis and the dermis, although they are less prevalent than ERβ [44]. There are four androgens that can activate the androgen receptor including dehydroepiandrosterone sulfate, androstenedione, testosterone, and 5α-dihydrotestosterone [45]. Interestingly, testosterone (women only) and 5α-dihydrotestosterone are also synthesized in the skin [45]. Androgens and its receptor are involved in multiple normal skin processes including sebaceous gland growth and differentiation, hair growth, epidermal barrier homeostasis, and wound healing [45].

1.5.2 Melanoma

A role for androgens in progression of melanomas was hypothesized as early as 1980 [46]. However, to date, expression of androgen receptors in melanoma tissues has not been identified [47]. Interestingly, an androgen-dependent protein, Apolipoprotein D, is expressed in melanomas, particularly nodular melanomas, but not normal skin [48]. Additionally, one study showed that blocking androgen improved patient response to a melanoma vaccine [49]. Therefore, the possibility that androgens play a role in melanoma cannot be entirely ruled out.

1.6 Behavioral differences between men and women:

1.6.1 Skin awareness

Early detection of melanoma is important to survival [16]. In 2005, Berwick et al. showed that skin awareness is associated with increased survival from melanoma, possibly due to earlier diagnosis [50]. It has also been shown that skin self-examination (SSE) reduces the risk of having a thicker tumor at diagnosis [51]. Men are less likely to be aware of their skin or perform skin self-examinations compared to women [52]. This finding contributes to the common hypothesis that men have increased Breslow thickness at diagnosis, and therefore have poorer survival because they are less aware of their skin. However, there is controversy as another study showed that performing SSE is associated with thicker tumors when compared with spouse, general physician, or dermatologist examination [22]. Furthermore, it is likely that nodular melanoma, which has a poorer prognosis and occurs more frequently in men than in women, is harder to detect by SSE because it often does not adhere to the SSE guidelines of a suspicious lesion [22]. Men have also been shown to have an increased rate of growth in their melanoma lesions, indicating that their melanomas may be more aggressive by nature [25]. Finally, Joosse et al. demonstrated that sex is an independent prognostic indicator for melanoma survival in stage I/II and stage III/IV melanomas, even with Breslow thickness included in the multivariable model [27,31]. These results suggest that skin awareness and skin self-examination only partially explain the female survival advantage.

1.6.2 UV exposure

It is well documented that UV exposure is a causative factor of melanoma. UV exposure has also been associated with increased survival and thinner lesions, but has not been studied as thoroughly, and the associations have been inconsistent [50,53]. Men and women have different behaviors regarding UV exposure, which may influence the development and progression of their disease, and therefore survival from melanoma [54,55]. Specifically, women are more likely to indoor tan and sunbathe to acquire a tan, but they are also more likely to wear sun protection during ambient UV exposure [54,55]. Men are more likely to work outdoors and are less likely to protect their skin from the sun [54].

1.7 Biological impact from behaviors:

1.7.1 Relevance of behaviors regarding biology

Skin awareness and SSE have an impact Breslow thicknes and melanoma survival due to early detection, but the action itself is not likely to affect biological processes. However, UV exposure interacts with multiple biological systems and their downstream effectors including Vitamin D, immune response, and DNA damage/repair, which may impact outcomes for melanoma. Not only are there sex differences in UV exposure behaviors, there is evidence for sex differences in these biological systems.

1.7.2 Vitamin D

UV exposure induces production of the hormone Vitamin D [56]. 7-dehydrocholesterol, the pro-vitamin to Vitamin D, resides in the epidermis and requires photoactivation by UVB [56]. Classically, Vitamin D is known for its ability to increase calcium and phosphorus absorption and impact skeletal development [56]. Extreme Vitamin D deficiencies result in rickets, a childhood disease that causes the bones to soften, resulting in debilitating skeletal deformities [56]. Vitamin D has also has a role in immune response, blood cell formation, and cell growth regulation [8,9].

More recently, epidemiological studies have associated Vitamin D with other diseases, including autoimmune diseases, cardiovascular disease, susceptibility to infections, and cancer [9,56,57]. However, it has been postulated that Vitamin D is merely a biomarker for overall health, and therefore is correlated with diseases, but does not have a direct impact [9]. Nonetheless, Vitamin D in melanoma is particularly interesting because UV exposure, a risk factor for melanoma, induces Vitamin D production, a potentially protective factor.

Vitamin D has multiple cellular effects that appear to inhibit proliferation through both genomic and non-genomic effects [58]. The non-genomic effects of Vitamin D are not well understood; however, there are some insights to the genomic effects [58]. The Vitamin D receptor (VDR) is a nuclear receptor that, following binding by its ligand Vitamin D, binds to Vitamin D response elements in genes to modulate transcription [58]. VDR acts as a transcription factor for and increases

expression of multiple genes including: osteopontin, RANKL, Calbindin-9k, IGFBP and β3 integrin [57]. Furthermore, it is responsible for downregulation of EGFR, a pathway that is constitutively active in melanomas with a *BRAF* or *NRAS* mutation [9,59]. Concomitantly, epidemiological studies investigating melanoma outcomes have consistently associated Vitamin D with decreased Breslow thickness and improved survival [9,60].

Sex differences in the effects of Vitamin D have been observed as early as 1981, when Thomas and Forte reported differences in longevity of male and female rats that were on a Vitamin D deficient diet [61]. In 2012, a study in patients with multiple sclerosis showed that Vitamin D and estrogen (E2) have a functional synergy that improves outcomes for women [62]. Another study showed obese men are 40% more likely to have a Vitamin D deficiency than obese women [63]. Taken together, these results suggest that there are sex differences in consumption, synthesis, and effect of Vitamin D. Therefore, there may be an interaction between UV exposure and Vitamin D that contributes to the female survival advantage.

1.7.3 Immune response

Melanoma is known to be an immunogenic cancer, as evidenced by primary tumor regression and lymphocytic infiltration [64,65]. In fact, therapeutic development for melanoma often focuses on exploiting the immune response [65]. Unfortunately, melanoma often evades the immune response via downregulation of antigenic molecules or secretion of immunosuppressive cytokines [64,65].

UV exposure can also influence the immune response via Vitamin D, as discussed above, and other immune modulators [8]. In 1974, Kripke reported that tumors developed in mice exposed to UV due to immunosuppressive effects of UV exposure [66]. Since then, it has been discovered that many immune factors are induced by UV exposure including: PGE2, IL-10, IL-6, TNF, platelet activating factor, and nerve growth factor [8]. It has also been observed that responses to acute and chronic UV exposures are different [67]. That is, acute UV exposure causes immunosuppression, but chronic UV exposure induces photoadaptation and photoprotection, diminishing the responses observed in acute exposures [67]. The understanding of photoadaptation and photoprotection regarding immune response to UV exposures is limited, but these processes may contribute to explaining the role of UV exposure in melanoma progression and survival [8,67]. Importantly, behaviors leading to acute and chronic UV exposure vary between men and women [54,55].

There are also sex differences in the immune response. In 1985, Ansar et al. published a review stating that women have more vigorous immune responses, are more resistant to infections, and have a higher incidence of autoimmune disease [68]. Furthermore, it has become evident that estrogen, androgens, and progesterone influence both innate and adaptive immune responses in different ways [69]. For example, estrogen appears to have an anti-inflammatory effect on neutrophils, an innate immune cell, while progesterone appears to have a pro-inflammatory effect [69]. Overall, the effect of hormones on the immune response is complex and varies with hormones levels in individuals [69]. Because there is an effect of UV exposures and hormones on the immune system, and both of these vary

between the sexes, immune response is an important consideration in the female survival advantage, especially given the immunogenic nature of melanoma.

1.7.4 DNA damage and repair

As discussed previously, UV exposure induces oxidative damage, which is usually repaired by BER, along with cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts, which are usually repaired by NER [5,6]. DNA damage caused by UV exposures has been implicated in melanomagenesis for decades. However, recently, it has been hypothesized that DNA repair is also important for melanoma survival. In 2008, Kauffmann et al. reported that four NER genes and two BER genes were overexpressed in primary melanomas [70]. Furthermore, as Breslow thickness increases, so does the expression of DNA repair genes [70]. Additionally, in 2009, Emmert and Kraemer suggested that aggressive melanomas require the ability to repair DNA quickly to allow for replication [71]. Similar to NER, increased MGMT (O-6 methylguanine DNA-methyltransferase) repair, which is a direct reversal DNA repair pathway that repairs alkylation damage, was associated with melanoma survival [72]. Inhibition of MGMT via promoter methylation has been shown to increase response to chemotherapeutic temozolomide and prolong progression-free survival [72].

There are reported sex differences in DNA damage and repair. In 2011, Slyskova et al. reported that in a study of healthy participants, women had more DNA damage [73]. Furthermore, in 2014, Slyskova et al. demonstrated that women have

decreased BER and NER capacity compared to men [74]. To date, the cause of NER and BER variations between the sexes has not been discovered.

There are also sex differences in the MGMT repair pathway. In a study on non-small cell lung cancer, MGMT promoter methylation varied by sex and smoking status [75]. In colon cancer, MGMT promoter methylation was increased in the right (ascending) colon of women [76]. These differences between cancers indicate that MGMT methylation is context-dependent, and perhaps influenced by environmental factors. Finally, one study reported that MGMT is a negative regulator of ERα transcription following treatment with an alkylating agent [77]. Taken together, these studies suggest that MGMT may be important to sex differences in melanoma survival, especially in for patients treated with alkylating therapeutics.

There is also evidence that DNA damage and repair induces an immune response [8]. Since immune response varies between the sexes, as discussed above, it is possible that DNA damage and repair induces an immune response differently in males compared to females. Therefore, when evaluating the female survival advantage in melanoma, it is important to consider the potential role of DNA damage and repair.

1.8 Single Nucleotide Polymorphisms (SNPs):

1.8.1 Definition and functionality of SNPs

Single nucleotide polymorphisms (SNPs) are inherited nucleotide variations in the germline DNA and are the most common form of genetic variation. The majority

of SNPs are located in intronic regions of genes, which often makes defining the potential biological importance of a SNP difficult [78,79]. There are a few known ways that intronic SNPs may have a direct biological impact. First, the SNP could be located in a regulatory element such as a transcriptional promoter or enhancer [79]. Second, the SNP could be located in small non-coding RNA that affects transcription of another gene [79]. Third, the SNP could be located in or near a splice site, thereby affecting alternative splicing [79].

While it is possible that an intronic SNP has a functional impact on gene expression, it is more likely that the SNP is in linkage disequilibrium (LD) with a SNP that is functional [78,80,81]. That is, the two SNPs, one of which is functional, have a nonrandom association [81]. Unfortunately, it can be challenging to correctly identify which SNPs are in LD [81]. Because the functional SNP cannot always be identified, SNPs can be helpful in identifying genomic loci of interest in a particular disease [80,81].

Furthermore, when designing a candidate gene SNP study, including exonic SNPs can be limiting because they often have a minor allele frequency (MAF) less than 0.05, which is generally the inclusion criteria in a SNP analysis due to a need for adequate statistical power [78]. A solution to this problem is to include an intronic tagging SNP—a SNP in an intron that is known to be in LD with other SNPs within the gene [78,80]. Intronic tagging SNPs generally have a higher MAF, and allow for adequate coverage in the evaluation of the gene of interest [78]. While tagging SNPs

have high frequency in the genome, they are less likely to have a strong association with disease risk and/or outcomes [78].

1.8.2 Gene-environment interactions

Single, high penetrance mutations are rare, and cause only a small proportion of cancers [78,80,82]. Furthermore, genetic variation within disease is complex, as evidenced by multiple low penetrance variants influencing phenotypes and outcomes [78,80,82]. As such, it has become evident that focusing only on the genetic variation within diseases is a reductionist approach and does not entirely account for disease phenotype or outcome [82–84]. One way to address this complexity is to investigate interactions between genetic factors, such as SNPs, and environmental exposures [83,85]. Gene-environment interactions allow researchers to explore multifactorial diseases while taking into consideration low penetrance variants and environmental exposures [83,85].

1.8.3 Previous associations with melanoma

A plethora of SNP studies in melanoma have previously been published. Here we will summarize the most relevant findings. In 2012, Ward et al. summarized SNPs associated with melanoma risk and prognosis, and reported SNP associations in 8 immune response genes and 11 DNA repair genes [86].

The immune response SNPs summarized by Ward et al. were associated with Breslow thickness and melanoma survival [86]. One SNP in IL-10 (-1082AA) has

been consistently associated with increased Breslow thickness and poor survival outcomes. Another SNP in IFNγ (-874A→T) was associated with increased response to therapy and overall survival [86]. In 2003, Howell et al. found that a SNP in IL-1β was associated with thinner lesions [87]. The group also investigated SNPs in cytokines in association with melanoma susceptibility, but did not identify any significant SNPs [88].

SNPs in DNA repair genes have been associated with both melanoma risk and survival, particularly SNPs in NER and MGMT genes [71,89–96]. As Emmert and Kraemer suggested, it is possible that increased DNA repair capacity is protective against developing melanoma, but is detrimental to fighting the disease following development [71]. As such, SNPs may be an important marker of inter-individual variation in DNA repair capacity. Furthermore, DNA repair SNPs have been shown to interact with indoor tanning to modify melanoma risk [95]. To date, there are no studies investigating DNA repair SNP interactions with UV exposures impact on Breslow thickness or melanoma survival.

Regarding sex differences in SNP studies, a SNP in MDM2, an E3 ubiquitin ligase that targets p53 for proteasomal degradation, was associated with melanoma risk in women, but no association was found in men [97]. Kocarnik et al. reported that men with a SNP in SLC45A2, a gene involved in estrogen-induced expression of tyrosinase, have an increased risk of developing melanoma when compared to women with the same genotype [98].

1.9 Rationale, hypothesis, and specific aims:

1.9.1 Rationale

Multiple studies have been published identifying sex as an independent prognostic indicator, providing further evidence that melanomas diagnosed in males are inherently more aggressive, and perhaps biologically different, than melanomas diagnosed in females. Despite this, it is uncommon to stratify analyses by sex. Here we aimed to investigate behavioral and biological factors in sex-stratified analyses to provide insight to the female survival advantage. A secondary goal of this project was to demonstrate that analyses for the overall population adjusted by sex are not representative of sex-stratified analyses.

1.9.2 Hypothesis

Our hypothesis was that behavioral and biological factors would differentially impact survival and Breslow thickness in males compared to females. We tested our hypothesis through following specific aims:

1.9.3 Specific aim 1

Determine the contribution of behavioral and biological factors melanoma survival in males compared to females.

Hypothesis: Behavioral and biological variables will be differentially associated with melanoma survival in males compared to females, while histopathological variables will be comparable.

1.9.4 Specific Aim 2

Determine the contribution of behavioral and biological factors to Breslow thickness in males compared to females.

Hypothesis: UV exposures, histopathological, and biological variables will be comparable between sexes, while skin awareness will be associated with decrease Breslow thickness in females.

1.9.5 Specific Aim 3

Evaluate the association of SNPs in DNA repair and immune response genes with Breslow thickness, independently in males and females.

Hypothesis: Distinct SNPs in DNA repair and immune response genes will be associated with Breslow thickness in males compared to females, and the SNPs that overlap between sexes will have differential odds ratios.

CHAPTER TWO:

FACTORS CONTRIBUTING TO SURVIVAL FROM MELANOMA VARY BETWEEN MALES AND FEMALES

2.1 Abstract:

Background: A female survival advantage has been observed in melanoma for many decades. Despite this, investigating the role of sex in melanoma survival using a stratified analysis is uncommon. We sought to determine which behavioral and biological factors contributed to 5-year and 15-year melanoma survival in males compared to females.

Methods: Multivariable survival analyses and Cox regression were performed using cases from a population-based case-control study of melanoma patients (males n=283, females n=260). All analyses were stratified by sex.

Results: In our model including all cases, sex was not an independent prognostic indicator. We found that UV exposures are inversely associated with mortality in males, particularly in the 5-year models, but are not associated with female survival. We also found that skin awareness was inversely associated with melanoma survival in females, but was not associated with survival in males. Breslow thickness was the only consistent predictor of survival at both 5 and 15 years follow-up. However, men have poorer survival from thick lesions (>2.00mm) than women with thick lesions. Menopause also appears to pay a role in female survival.

Conclusions: Despite the fact that sex was not an independent prognostic indicator in the model for all cases, we found that varying behavioral and histopathological variables contribute to melanoma survival when analyses were stratified by sex.

Impact: These findings support the notion that males have more aggressive melanomas that are biologically distinct from female melanomas, and highlight the need for sex-stratification in future melanoma studies.

2.2 Introduction:

Melanoma accounts for less than two percent of skin cancers, but more than 75% of skin cancer deaths [2]. The best prognostic indicator for melanoma is Breslow thickness, but many other histopathological variables such as ulceration, mitoses, and anatomic site have also consistently been associated with melanoma prognosis [4]. Behavioral variables such as UV exposure, self-skin examination (SSE), skin awareness, and physician visits, have also been associated with survival although less consistently [50,99,100].

Interestingly, a female survival advantage in melanoma has been observed for many years [4]. Multiple studies report evidence that melanomas in males are more aggressive than melanomas in females [25–27,31,101]. Two European studies have shown that sex is an independent prognostic indicator for melanoma regardless of the stage at diagnosis [27,31]. Khosroteharani et al. (2015) also found that females have better survival than males across all tumor stages [32].Using data from the US Surveillance, Epidemiology, and End Results (SEER) study, Fisher and Geller (2013) reported that men between the ages of 15 and 39 are 55% more likely to die of melanoma than women in the same age group [101]. A study by Liu et al. (2006) reported an increased rate of growth in melanomas diagnosed in males when compared with females [25]. Finally, de Vries et al. (2011) found that men's melanomas are more likely to metastasize [26]. Taken together, these findings suggest that melanomas progress differently between males and females.

Despite these apparent differences in melanoma biology, stratifying survival analyses by sex is uncommon. There are multiple hypotheses regarding the female survival advantage including hormonal influences on the immune system, reactive oxygen species, vitamin D metabolism, and X and Y chromosome-specific oncogene expression [102]. Behaviors such as UV exposure can impact these biological differences, and may influence melanoma progression differently in males compared to females [101]. Therefore, we sought to determine factors that would predict short-term (5 year) and long-term (15 year) survival of melanoma patients, and hypothesized that different behavioral, histopathological, and hormonal factors would contribute to survival of males compared to females.

2.3 Methods:

2.3.1 Subjects

This population-based study has been described previously [99]. Briefly, cases were identified through a rapid case ascertainment system functioning as an agent of the Connecticut Tumor Registry, and consisted of 650 non-Hispanic whites residing in Connecticut when they were diagnosed with invasive cutaneous melanoma between January 15, 1987 and May 15, 1989. Follow-up data for vital status and cause of death were collected at approximately 5 and 15 years (1994 and 2006 respectively), via personal contact (mail/telephone/physicians), the Connecticut Tumor Registry, or the National Death Index. For our analysis, 107 participants with missing information were excluded, leaving 543 subjects for analysis.

2.3.2 Categorization of Variables

We developed several summary variables. Definitions of all summary variables can be found in **Supplementary Data 1**. Here we include the most salient summary variables.

Histopathology was reviewed by RLB. Breslow thickness was evaluated both continuously and also categorized as thin (<1.00mm), intermediate (1.00-1.99mm), and thick (2.00mm+), adhering to the AJCC staging guidelines for melanoma [12]. We also combined Breslow thickness and sex (Breslow-sex) into six categories representing sex by thin, intermediate, and thick lesions.

Reported number of sunburns was collapsed into ever/never categorization. Intermittent sun exposure was created and categorized as high/low and was previously described; this variable was also previously referred to as "lifetime sun exposure" and "recreational sun exposure" [50,99,103]. Skin awareness was reported as multiple levels including 'unaware', 'aware of skin cosmetically', 'aware of changes in skin', 'aware of abnormalities in skin', and 'aware of other factors'. We re-categorized this variable to represent unaware or aware. A skin examination index variable was created as either 'yes' if a participant's skin had ever been examined by themselves, a spouse, or a doctor, or 'no' if they had not been examined. Females participating in the study were asked to report their menopause status, which was collapsed into a two-category variable indicating pre-menopausal and post-menopausal. We also created a variable, for both males and females, based

on the national average age at menopause (51 years) to report whether participants were older or younger than 51 years [104].

2.3.3 Statistical Analysis

We used a Chi-squared test to characterize the distribution of categorical variables at diagnosis between males and females. Using Cox Proportional Hazard Ratio models, we investigated survival, where the censored variable was death from other causes or alive at last follow-up. All variables included in the chi-squared analysis were evaluated in the baseline models. Baseline models included one variable of interest and were adjusted for sex (for the overall population) and age. Multivariable hazard ratios were adjusted for sex (for the overall population) and age, and all other variables significant ($p \leq 0.05$) in the baseline model calculations. In the multivariable analyses, Breslow thickness and age were used as continuous variables. The Akaike Information Criterion (AIC) was used to determine the best model fit. Kaplan-Meier curves were generated to graphically represent the differences in survival between the sexes, and test the differences via the log-rank test. Because Breslow thickness was not normally distributed, we utilized the Mann-Whitney-Wilcoxon test to compare distributions. To compare self-reported menopause status with menopause status determined by the national average age at menopause, we used diagnostic odds ratios. Two-sided tests were used for all analyses and $p \leq 0.05$ was considered significant. All analyses were conducted in SAS 9.3 (SAS Institute, Cary, NC).

2.4 Results:

2.4.1 Differences between men and women in the Connecticut population

Subjects were evenly distributed between the sexes, with 283 males (52.1%) and 260 females (47.9%). We performed a chi-squared analysis to determine differences between males and females in our population, including behavioral and histopathological variables. Overall, the men in our study were older (p<0.01) and more educated (p=0.01) (**Table 2.1**). Consistent with previous studies, we found that males had an increased number of melanomas on their head and trunk, while the majority of melanomas in females were on the extremities (p<0.01) (**Table 2.1**). Fewer men had solar elastosis (p=0.04) (**Table 2.1**). Men also had slightly more nodular melanomas and fewer melanomas that were classified as 'other' (p=0.01) (**Table 2.1**). Men did not have thicker lesions than women (p=0.12); however, because men are known to present with thicker lesions, we further investigated the distribution of Breslow thickness by performing a Mann-Whitney-Wilcoxon test. We found no significant difference, although men did tend to have thicker lesions (p=0.08) (data not shown). Aside from anatomic site, solar elastosis, and histologic subtype there were no significant differences in melanoma presentation between men and women. Behavioral differences between men and women included a higher number of men working outdoors (p<0.01), along with fewer men performing SSE (p=0.02) and reporting skin awareness (p<0.01) (**Table 2.1**). Men also had significantly more spouse/partner skin examinations than women (p<0.01) (**Table 2.1**).

2.4.2 Deaths from melanoma at 5 and 15-year follow-up

In our population of melanoma cases, 96 participants died from melanoma. At the five-year follow-up, 53 participants had died from melanoma, and at the 15-year follow-up an additional 43 participants had died from melanoma. Of the total deaths (n=96), 60.4% of deaths from melanoma were male participants, while 39.6% percent were female participants. At the 5-year follow-up 54.7% (n=29) of melanoma deaths were males, and 45.3% (n=24) were females. At the 15-year follow-up, an additional 29 men had died from melanoma (67.4%) and only 14 women had died from melanoma (32.6%).

2.4.3 Factors contributing to 5-year survival in the Connecticut population (overall and sex-stratified models)

Five-year survival results for this study were previously published by Berwick et al (2005) [50]. Despite the different selection criteria for the two studies, the results for the 5-year survival were quite similar, with the exception of anatomic site. Anatomic site was not significant in our baseline model (p=0.38), and therefore, was not included in our final multivariable model. However, when we included it in a multivariable model for comparison with the previous study, anatomic site was borderline significant (p=0.09).

For the model including all cases (referred to as overall population), Breslow thickness was highly significant (adj$^+$ HR 1.31, 95% CI=1.18-1.47, continuous per mm) (**Table 2.2**). Mitoses were also a strong predictor of survival (adj$^+$ HR 8.60,

95% CI=1.75-42.26), as was solar elastosis (adj+ HR 0.49, 95% CI=0.25-0.94) and skin awareness (adj+ HR 0.45, 95% CI=0.25-0.82) (**Table 2.2**).

In survival models stratified by sex, both males and females had an increased hazard ratio for Breslow thickness (adj+ HR 1.31, 95% CI=1.14-1.51; adj+ HR 1.28, 95% CI=1.09-1.51, respectively) and a decreased hazard ratio for solar elastosis (adj+ HR 0.40, 95% CI=0.17-0.95; adj+ HR 0.27, 95% CI=0.11-0.70, respectively) (**Table 2.2**).

Regarding behavioral variables in our survival analyses, males also had a reduced hazard ratio for intermittent sun exposure (adj^ HR 0.38, 95% CI=0.17-0.82) (**Table 2.2**). Painful burns and skin awareness were not included in the multivariable model, but were borderline significant in the baseline model (HR 0.50, 95% CI=0.24-1.04; HR 0.51, 95% CI=0.24-1.09, respectively). The only behavioral variable associated with female survival was skin awareness (adj# HR 0.31, 95% CI=0.13-0.76) (**Table 2.2**).

We also modeled survival stratified by sex using the variables that were included in the overall population. To determine best model fit, we compared the AIC values for each model. For females, the sex-specific model was the superior model as demonstrated by lower AIC values (**Table 2.2**).

2.4.4 Factors contributing to 15-year survival in the Connecticut population (overall and sex-stratified models)

Similar to the 5-year follow-up data, Breslow thickness (adj+ HR 1.23, 95% CI=1.13-1.34) and mitoses (adj+ HR 3.71, 95% CI=1.40-9.83) were associated with survival in the overall 15-year follow-up models (**Table 2.3**). Skin awareness (adj+ HR 0.67, 95% CI=0.44-1.01) and solar elastosis (adj+ HR 0.64, 95% CI=0.40-1.01) were borderline significant, but had less of an impact on survival than it did in the 5-year survival models (**Table 2.3**).

In models stratified by sex, the only consistent predictor of survival was Breslow thickness (males: adj^ HR 1.21, 95% CI=1.08-1.35; females: adj# HR 1.35, 95% CI=1.13-1.60) (**Table 2.3**). Males had a significantly increased hazard ratio for ulceration (adj^ HR 2.02, 95% CI=1.08-3.77). Intermittent sun exposure was borderline significant (adj^ HR 0.63, 95% CI=0.36-1.09) (**Table 2.3**). Skin awareness was not associated with survival in the baseline model for males (HR 0.83, 95% CI=0.50-1.39), contrary to the 5-year baseline model. Females had a decreased hazard ratio for solar elastosis (adj# HR 0.38, 95% CI=0.18-0.78) and skin awareness (adj# HR 0.49, 95% CI=0.25-0.95) (**Table 2.3**). Using AIC values, we showed that the sex-specific model was the best fit for both males and females (**Table 2.3**).

2.4.5 Analyses stratified by Breslow thickness and sex

We generated Kaplan-Meier curves comparing survival between males and females, which was significant (p=0.05) (**Figure 2.1**). We further compared survival between men and women when stratifying by Breslow thickness. When comparing survival curves of men and women with thin lesions (<1.00mm) and intermediate

lesions (1.00-1.99mm) in **Figure 2.2** (upper two lines and middles two lines in graph, respectively), the curves are quite similar, and there is no significant difference (p=0.45 and 0.60, respectively). However, there is a statistically significant difference (p=0.03) in the survival curves of men and women diagnosed with thick lesions (2.00+mm) (**Figure 2.2**, bottom two lines in graph). Furthermore, men with intermediate lesions compared to men with thin lesions had a higher hazard ratio in the baseline model (HR 5.97, 95% CI= 2.48-14.41) than women with intermediate lesions compared to women with thin lesions (HR 3.54, 95% CI=1.59-7.91) (**Table 2.3**).

Given these findings, we hypothesized that a variable combining Breslow thickness and sex (Breslow-sex; defined in methods) would be a better predictor of survival than variables independently representing Breslow thickness and sex. The AIC values were nearly identical with a value of 1094.6 for Breslow-sex, and 1094.8 for the model containing the individual Breslow thickness and sex variables. Furthermore, when the multivariable model was calculated using categorical variables for Breslow thickness, the Breslow-sex model was slightly preferred to the model including Breslow thickness and sex separately (AIC=1079.5 and 1080.3, respectively).

2.4.6 Menopause status and survival

Because age was significant only in the multivariable model for females (adj# HR 1.28, 95% CI=1.01-1.62), we investigated the effect of reported menopause status on survival. We found that women who self-reported as post-menopausal at

diagnosis had poorer survival than women who self-reported as pre-menopausal (p<0.01) (data not shown).

To allow for comparison between men and women, we also categorized participants based on national average age of menopause (51 years) [104]. We compared the number of women who reported having gone through menopause with the number of women age 51 and older, and found that average age at menopause positively predicted menopause 87% of the time, and was specific 92% of the time. Women older than 51 years had significantly worse survival than women younger than 51 years of age (p=0.04), while there was no significant difference in men (p=0.41) (**Figure 2.3**). Finally, women age 51 and older had survival comparable to both categories of men, while women younger than 51 had better survival (p=0.04) (**Figure 2.3**).

2.5 Discussion

Our findings suggest that there are different behavioral factors that contribute to survival in males compared to females. Skin awareness was inversely associated with mortality in females, but did not have an effect on survival in males. Similarly, UV exposure including intermittent sun exposure, indoor tanning, and painful burns, were inversely associated with mortality in males, but did not have an effect on survival in females. However, solar elastosis, which is a pathological indicator of sun exposure, was inversely associated with mortality in both males and females.

We also found that different histopathological variables contribute to survival in males compared to females. Ulceration and mitoses were associated with an

increased hazard ratio in males in the 15-year multivariable models, but did not have an effect on survival in females. Breslow thickness was the only consistent predictor of survival. However, the hazard ratio for Breslow thickness in males decreased in the 15-year follow-up compared to the 5-year follow-up, while the hazard ratio for females remained consistent. Interestingly, skin awareness was borderline significant for males in the 5-year baseline model and represented a protective effect, but this dissipated in the 15-year model. Taken together, these results indicate that Breslow thickness and skin awareness are more predictive of survival in females. Furthermore, processes independent of Breslow thickness may influence progression of melanoma in males.

Additionally, we looked at a variety of factors that represent a hormonal influence including age, number of children, number of pregnancies, ever use of estrogen replacement, self-reported menopause status, and predicted menopause status based on the average national age of menopause. We found that age was significantly associated with an increased hazard ratio for females, but had no effect on survival in males. Furthermore, women who were pre-menopausal when they were diagnosed with melanoma had significantly better survival than post-menopausal women. These results indicate that changes in estrogen-related variables impact melanoma progression. Interestingly, this is approximately the age at which the incidence rates also cross-over. Prior to age 50, women have a slightly increased risk of developing melanoma. After age 50, risk among males rises drastically and surpasses female risk [16].

Overall, we found that factors contributing to survival in the 5-year follow-up data were largely in agreement with factors contributing to survival in the 15-year follow-up data. In general, the confidence intervals narrowed in the 15-year data, representing increased power as expected. UV exposure variables that were borderline significant or significant in the 5-year follow-up data for males did not retain the same significance at the 15-year follow-up, indicating that UV exposures have a larger impact on male survival closer to the time of diagnosis.

One limitation of our study is that we did not remove participants with lentigo maligna melanoma, which is highly associated with solar elastosis and older age, in order to retain power. It is possible that this biased our findings that solar elastosis modified survival from melanoma; however, our findings are consistent with previous reports regarding solar elastosis, including those published by Berwick et al. (1996)[50]. We were unable to adjust for stage at diagnosis, which may have limited our survival analyses of Breslow thickness and sex. However, we have information on Breslow thickness, ulceration, and mitoses which are all of the factors use to calculate stage; therefore, we had a proxy for stage in our analyses. Furthermore, we do not have information about behavior or physiologic changes, other than age, after diagnosis, which could also impact melanoma survival.

Our study also has multiple strengths. To our knowledge, we are the first to report on survival regarding sex differences in melanoma with such extensive follow-up information. Additionally, to our knowledge, we are the first to compare the overall population to males and females separately, allowing the opportunity to

identify information that is lost when simply adjusting for sex in survival models. Furthermore, identification of the difference in the ability of certain factors to modify survival, especially UV exposures, may help explain previously inconsistent findings.

In summary, our study has identified that different factors contribute to survival from melanoma in males compared to females. Results showing that age, self-reported menopause status, and age-estimated menopause status affect survival in women, but not men suggest that sex differences in survival may be attributable to hormones. Additionally, men have poorer survival from thick lesions than women, suggesting that there are biological differences in the progression, and perhaps the initiation, of melanoma. Furthermore, skin awareness was borderline significant for male survival in the 5-year models, suggesting that men who are aware of their skin may present with thinner lesions. However, this trend dissipates in the 15-year survival models, reinforcing that men's melanomas may be inherently more aggressive. It appears that there are biological differences in melanomas in males compared to females, which may be due to inherent sex differences such as hormones. Behaviors such as UV exposure and skin awareness may also interact with inherent biological differences to drive the progression of melanoma.

While Breslow thickness was consistently significant for survival, we did identify differences between males and females. As the best prognostic indicator for melanoma, investigating Breslow thickness in a sex-stratified analysis may provide more insight to the female survival advantage. Overall, this study further highlights

the need for sex-stratified investigations to identify differences between melanomas in males and females.

2.6 Supplementary Data 1

Education status was categorized as completing college or not. Variables including hair color, eye color, and tannability were combined to create a "phenotype index" associated with melanoma risk as outlined by Kanetsky et al. (2006) [105]. Nevi were counted and categorized as previously reported [99]. A comorbidity index was created using eight of the ten categories proposed by Charlson et al (1987)[106]. Our weighted co-morbidity score had a range of 0-11, which was further categorized into three groups: none (0), mild (>0 and <3), and severe (>3) [107].

Histopathology was reviewed by RLB. Breslow thickness was evaluated both continuously and also categorized as thin (<1.00mm), intermediate (1.00-1.99mm), and thick (2.00mm+), adhering to the AJCC staging guidelines for melanoma [12]. We also combined Breslow thickness and sex (Breslow-sex) into six categories representing sex by thin, intermediate, and thick lesions. Histology was originally recorded in multiple categories (superficial spreading, nodular, lentigo maligna, acral, desmoplastic, neurotropic, other, and unclassified) and was re-categorized into four groups: superficial spreading, nodular, lentigo maligna, and other. Mitoses were reported as a continuous variable, and categorized as present or absent. Vertical growth phase (VGP) was reported as no, yes, early, possible, and indeterminate. We re-categorized early as yes, and possible/indeterminate as missing. Tumor infiltrating lymphocytes (TILs) were reported as absent, non-brisk, and brisk, and were re-categorized as absent or present. The anatomic site of the melanoma lesion was reported in multiple categories (head/face/neck, upper

shoulders/back, lower back, legs, arms, and other) and was collapsed into four groups: head/neck, trunk, extremities, and other. Solar elastosis was originally reported in multiple categories (none, slight, moderate, and marked), and was collapsed to represent absent or present.

Reported number of sunburns was reported in multiple categories (0, 1-2, 3-4, 5-9, 10-14, 15-19, and 20+) and was collapsed into four groups: 0, 1-5, 6-10, and >10, along with an ever/never categorization. Intermittent sun exposure was created and categorized as high/low and was previously described; this variable was also previously referred to as "lifetime sun exposure" and "recreational sun exposure" [50,99,103]. Skin awareness was reported as multiple levels including 'unaware', 'aware of skin cosmetically', 'aware of changes in skin', 'aware of abnormalities in skin', and 'aware of other factors'. We re-categorized this variable to represent 'unaware' or 'aware'. Spouse examination was categorized as 'no examination', 'yes- spouse examined', and 'yes- other examined'. We re-categorized this variable to represent never or ever examined by a spouse/partner/other. Doctor examination was categorized as 'yes', 'no', 'don't know but assumed yes', and 'don't know'. We combined 'don't know but assumed yes' with 'don't know', and retained the categories 'yes' and 'no'. A skin examination index variable was created. If a participant's skin had ever been examined by themselves, a spouse, or a doctor, the skin examination index was recorded as 'yes.' If a participant's skin had not been examined by themselves, a spouse, or a doctor, the skin examination index was recorded as 'no.' Females participating in the study were asked to report their menopause status, which was reported in multiple categories to include medical

considerations, pregnancies, and breastfeeding. This variable was collapsed into a two-category variable including 'pre-menopausal' and 'post-menopausal'. We also created a variable, for both males and females, based on the national average age at menopause (51 years) to report whether participants were older or younger than 51 years (17). Finally, we categorized the number of children each participant had (0, 1, 2, 3+), which was originally reported as a continuous variable

2.7 Tables and figures:

Table 2.1 Analysis of sex differences in Connecticut population					
	Males (n=283; 52.1%)		Female (n=260; 47.9%)		*p*-value
	n	%	n	%	
Age					
<30	8	2.8	15	5.8	
30-39	34	12.0	42	16.2	
40-49	44	15.6	61	23.5	
50-59	58	20.5	55	21.2	
60-69	68	24.0	40	15.4	
≥70	71	25.1	47	18.1	**<0.01**
Phenotype index					
1	29	10.3	18	7.4	
2	65	23.1	48	19.6	
3	117	41.6	92	37.6	
4	54	19.2	65	26.5	
5	16	5.7	22	9.0	0.10
Completed college					
No	164	58.0	178	68.5	
Yes	119	42.1	82	31.5	**0.01**
5-year follow-up Status					
Alive	242	85.5	226	86.9	
Died from other causes	12	4.2	10	3.9	
Died from melanoma	29	10.3	24	9.2	0.89
15-year follow-up Status					
Alive	147	51.9	172	66.2	
Died from other causes	78	27.6	50	19.2	
Died from melanoma	58	20.5	38	14.6	**<0.01**
Breslow thickness					
Thin (<1.00mm)	147	51.9	156	60.0	
Intermediate (1.00-1.99mm)	65	23.0	55	21.2	
Thick (2.00+mm)	71	35.1	49	18.9	0.12
Anatomic site					
Head/neck	42	14.8	35	13.5	
Trunk/pelvis	181	64.0	83	31.9	
Extremities	52	18.4	135	51.9	
Other	8	2.8	7	2.7	**<0.01**
Ulceration					
No	240	84.8	224	86.2	
Yes	43	15.2	36	13.9	0.66
Mitoses					
No	111	39.2	107	41.2	
Yes	172	60.8	153	58.9	0.65
Histology					
Superficial spreading	169	59.7	180	69.2	
Nodular	29	10.3	22	8.5	
Lentigo maligna	42	14.8	41	15.8	
Other	43	15.2	17	6.5	**<0.01**
Vertical phase					
No	73	25.8	80	30.8	
Yes	210	74.2	180	69.2	0.21
TILs					
Absent	171	61.3	170	66.7	
Present	108	38.7	85	33.3	0.20
Missing	9				
Solar elastosis					

No	123	43.5	91	35.0	
Yes	160	56.5	169	65.0	**0.04**
Number of nevi					
0	22	9.4	16	7.1	
1 to 10	82	35.2	90	40.2	
11 to 30	80	34.3	80	35.7	
31 to 50	26	11.2	24	10.7	
Greater than 51	23	9.9	14	6.3	0.50
Missing	86				
Intermittent sun exposure					
Low	96	33.9	101	38.9	
High	187	66.1	159	61.2	0.23
Ever had a painful burn					
Never	91	32.2	86	33.1	
Ever	192	67.8	174	66.9	0.82
Number of painful sunburns					
0	91	32.2	86	33.5	
1 to 5	149	52.7	130	50.6	
6 to 10	18	6.4	16	6.2	
Greater than 10	25	8.8	25	9.7	0.96
Missing	3				
Outdoor job					
No	92	32.5	201	77.3	
Yes	191	67.5	59	22.7	**<0.01**
Indoor tanning					
Never	226	79.9	197	75.8	
Ever	57	20.1	63	24.2	0.25
Skin awareness					
Unaware	146	51.6	81	31.2	
Aware	137	48.4	179	68.9	**<0.01**
Self-skin examination					
No	225	90.1	217	83.5	
Yes	28	9.9	43	16.5	**0.02**
Spouse-skin examination					
No	221	78.1	230	88.5	
Yes	62	21.9	30	11.5	**<0.01**
Doctor-skin examination					
No	150	53.0	153	58.9	
Yes	113	39.9	90	34.6	
Don't know	20	7.1	17	6.5	0.39
Skin examination index					
No	118	44.2	123	50.6	
Yes	149	55.8	120	49.4	0.15
Missing	33				
Comorbidities					
None	88	31.1	82	31.5	
Mild	129	45.6	110	42.3	
Severe	66	23.3	68	26.2	0.68
Number of children					
0	56	19.8	49	18.9	
1	33	11.7	39	15.0	
2	72	25.4	75	28.9	
3+	122	43.1	97	37.3	0.41
Estimated menopause (51 years)					
Below 51 years	94	33.2	125	48.1	
51 years and older	189	66.8	135	51.9	**<0.01**

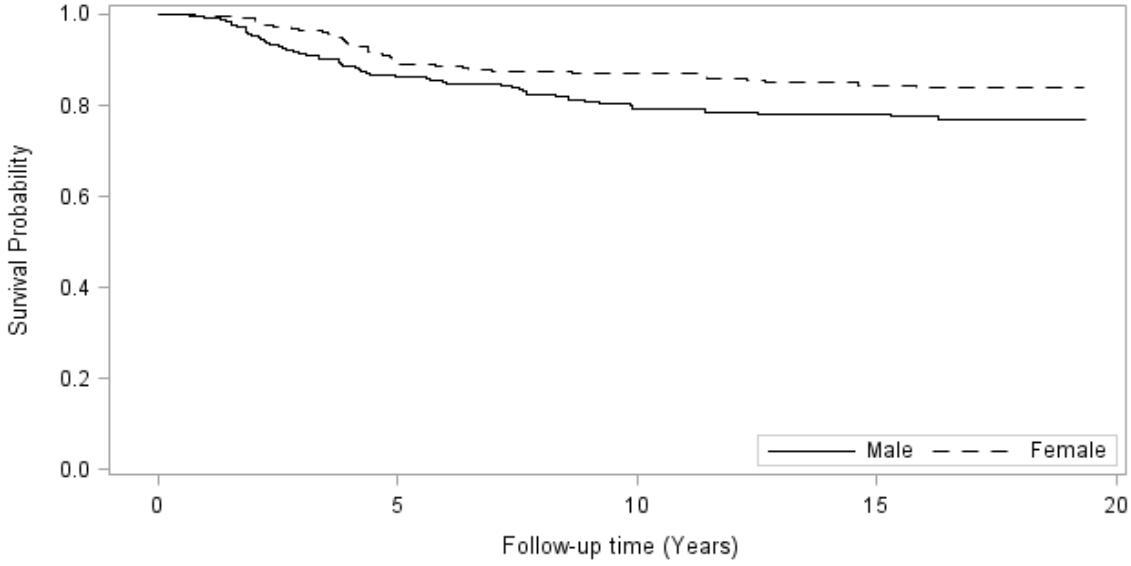
Table 2. 5-year Survival Models: Overall, Males, and Females								
	Overall (n=543)		**Males (n=283)**			**Females (n=260)**		
Factor	Baseline HR(95% CI)*	Adjusted HR(95% CI)+	Baseline HR(95% CI)*	Adjusted HR (95% CI)^	Adjusted HR(95% CI)+	Baseline HR(95% CI)*	Adjusted HR(95% CI)#	Adjusted HR(95% CI)+
Age (10years)	1.09(0.92-1.30)	1.04(0.85-1.27)	0.96(0.75-1.22)	0.88(0.67-1.16)	0.8(0.60-1.05)	1.24(0.97-1.59)	1.24(0.93-1.66)	1.32(0.96-1.81)
Sex	1.16(0.67-2.01)	0.99(0.55-1.80)	NA	NA	NA	NA	NA	NA
Completed college	0.56(0.29-1.07)	-	0.82(0.38-1.76)	-	-	0.22(0.05-0.97)	0.37(0.08-1.63)	-
Breslow thickness								
Thin	1.00(ref)	-	1.00(ref)	-	-	1.00(ref)	-	-
Intermediate	3.16(1.31-7.64)	-	5.91(1.15-30.50)	-	-	2.56(0.86-7.63)	-	-
Thick	11.74(5.59-24.64)	-	39.98(7.03-127.79)	-	-	5.53(2.12-14.42)	-	-
Breslow thickness (continuous)	1.45(1.33-1.57)	**1.31(1.18-1.47)**	1.41(1.28-1.55)	**1.31(1.14-1.51)**	**1.31(1.14-1.51)**	1.52(1.30-1.78)	**1.28(1.09-1.51)**	**1.52(1.17-1.96)**
Ulceration	3.44(1.91-6.21)	1.13(0.54-2.34)	4.87(2.27-10.46)	1.69(0.69-4.14)	2.19(0.88-5.46)	1.98(0.77-5.08)	-	0.67(0.15-2.96)
Mitoses	11.95(3.73-38.34)	**8.60(1.75-42.26)**	19.61(2.67-144.28)	15.3(0.68-346.18)	**23.75(1.06-530.92)**	7.99(1.88-34.04)	3.51(0.54-22.77)	4.01(0.62-25.58)
Vertical growth phase	6.90(2.15-22.14)	0.69(0.14-3.41)	10.49(1.43-77.18)	0.45(0.02-10.20)	0.29(0.01-6.76)	4.97(1.17-21.19)	1.31(0.20-8.76)	1.28(0.20-8.40)
Histologic subtype								
Superficial spreading	1.00(ref)	1.00(ref)	1.00(ref)	1.00(ref)	1.00(ref)	1.00(ref)	-	1.00(ref)
Nodular	2.68(1.29-5.56)	0.79(0.35-1.80)	5.08(2.08-12.40)	1.40(0.48-4.06)	1.52(0.52-4.44)	0.58(0.08-4.38)	-	0.14(0.02-1.26)
Lentigo maligna	0.67(0.25-1.80)	0.94(0.31-2.88)	0.81(0.17-3.81)	1.85(0.35-9.83)	2.43(0.43-13.84)	0.53(0.14-1.91)	-	0.60(0.12-3.04)
Other	1.85(0.86-3.98)	0.99(0.43-2.27)	2.17(0.80-5.86)	1.57(0.57-4.30)	1.73(0.59-5.01)	1.89(0.55-6.46)	-	0.44(0.08-2.37)
Solar elastosis	0.28(0.15-0.52)	**0.49(0.25-0.94)**	0.36(0.16-0.83)	**0.40(0.17-0.95)**	0.60(0.25-1.45)	0.19(0.07-0.48)	**0.27(0.11-0.70)**	**0.26(0.09-0.72)**
Indoor tanning	0.29(0.10-0.80)	0.41(0.14-1.17)	0.30(0.07-1.24)	-	0.38(0.09-1.70)	0.32(0.07-1.39)	-	0.36(0.08-1.61)
Intermittent sun exposure	0.63(0.36-1.09)	-	0.44(0.21-0.92)	**0.38(0.17-0.82)**	-	0.70(0.31-1.60)	-	-
Painful burn	0.57(0.33-0.98)	0.68(0.38-1.21)	0.50(0.24-1.04)	-	**0.29(0.13-0.66)**	1.05(0.45-2.45)	-	1.23(0.46-3.30)
Skin awareness	0.34(0.19-0.62)	**0.45(0.25-0.82)**	0.51(0.24-1.09)	-	0.66(0.28-1.55)	0.23(0.10-0.55)	**0.31(0.13-0.74)**	**0.27(0.11-0.69)**
AIC values with covariates				282.4	278.8		225.8	229.2

Variables not significant (NS) in baseline models and therefore not included in adjusted models are listed as -
* Adjusted for sex (only in overall population) and age
+ Adjusted for all variables except those listed as - in overall population
^Adjusted for all variables except those listed as - in males
#Adjusted for all variables except those listed as - in females
NA=Not Applicable
Bold=Significant in adjusted models
Note: All variables included in the Chi-Square analysis (Table 2.1) were evaluated in the baseline models; significant variables are shown in the table

Table 2.3 15-year Survival Models for the Overall Population, Males, and Females								
	Overall (n=543)		Males (n=283)			Females (n=260)		
Factor	Baseline HR(95% CI)*	Adjusted HR(95% CI)+	Baseline HR(95% CI)*	Adjusted HR(95% CI)^	Adjusted HR(95% CI)+	Baseline HR(95% CI)*	Adjusted HR(95% CI)#	Adjusted HR(95% CI)+
Age (10years)	1.17(1.02-1.33)	1.09(0.94-1.27)	1.97(0.89-1.27)	0.94(0.77-1.14)	0.97(0.80-1.17)	1.31(1.07-1.59)	**1.28(1.01-1.62)**	1.31(1.03-1.67)
Male sex	1.42(0.94-2.15)	1.12(0.73-1.73)		NA	NA	NA	NA	NA
Completed college	0.74(0.74-1.16)	-	1.07(0.63-1.80)	-	-	0.28(0.10-0.80)	0.39(0.13-1.13)	-
Breslow thickness								
Thin	1.00(ref)	-	1.00(ref)	-	-	1.00(ref)	-	-
Intermediate	4.41(2.46-7.92)	-	5.97(2.48-14.41)	-	-	3.54(1.59-7.91)	-	-
Thick	8.89(5.15-15.34)	-	14.70(6.49-33.26)	-	-	4.79(2.16-10.16)	-	-
Breslow thickness (cont.)	1.34(1.26-1.43)	**1.23(1.13-1.34)**	1.31(1.21-1.41)	**1.21(1.08-1.35)**	1.21(1.08-1.34)	1.53(1.33-1.76)	**1.35(1.13-1.60)**	1.41(1.16-1.72)
Ulceration	3.39(2.18-5.28)	1.41(0.86-2.32)	4.31(2.47-7.55)	**2.02(1.08-3.77)**	1.87(1.01-3.48)	2.36(1.14-4.89)	0.75(0.31-1.83)	0.87(0.33-2.33)
Mitoses	7.47(3.76-14.86)	**3.71(1.40-9.83)**	8.12(3.24-20.34)	3.62(0.94-13.93)	3.60(0.95-13.96)	6.61(2.34-18.66)	2.94(0.74-11.61)	3.20(0.82-12.52)
Vertical growth phase	6.44(2.82-14.72)	1.16(0.37-3.68)	7.30(2.28-23.35)	1.13(0.21-6.02)	1.19(0.23-6.29)	5.49(1.69-17.89)	1.49(0.31-7.10)	1.34(0.29-6.33)
Histologic subtype								
Superficial spreading	1.00(ref)	1.00(ref)	1.00(ref)	1.00(ref)	1.00(ref)	1.00(ref)	-	1.00(ref)
Nodular	2.83(1.60-5.02)	1.18(0.64-2.20)	4.10(2.01-8.34)	1.66(0.76-3.59)	1.74(0.81-3.75)	1.58(0.55-4.58)	-	0.42(0.13-1.40)
Lentigo maligna	0.80(0.38-1.67)	1.28(0.58-2.81)	0.84(0.28-2.47)	1.80(0.57-5.69)	1.73(0.55-5.45)	0.69(0.25-1.90)	-	0.82(0.26-2.63)
Other	3.18(1.92-5.27)	2.16(1.29-3.61)	3.74(2.00-6.98)	**2.85(1.51-5.37)**	2.82(1.48-5.36)	2.84(1.15-7.01)	-	1.11(0.39-3.12)
Solar elastosis	0.44(0.28-0.68)	**0.64(0.40-1.01)**	0.56(0.32-0.99)	0.75(0.41-1.36)	0.80(0.44-1.45)	0.28(0.14-0.59)	**0.38(0.18-0.78)**	0.41(0.19-0.90)
Indoor tanning	0.40(0.21-0.76)	**0.49(0.25-0.96)**	0.42(0.18-0.97)	0.45(0.19-1.08)	0.43(0.18-1.04)	0.41(0.14-1.17)	-	0.52(0.18-1.51)
Intermittent sun exposure	0.67(0.44-1.01)	-	0.59(0.35-1.00)	0.63(0.36-1.09)	-	0.84(0.43-1.65)	-	-
Painful burn	0.81(0.54-1.24)	-	0.87(0.51-1.50)	-	-	0.75(0.39-1.45)	-	-
Skin awareness	0.59(0.39-0.89)	**0.67(0.44-1.01)**	0.83(0.50-1.39)	-	0.88(0.51-1.52)	0.36(0.19-0.69)	**0.49(0.25-0.95)**	0.44(0.23-0.88)
AIC values with covariates				578.05	580.50		368.57	373.78

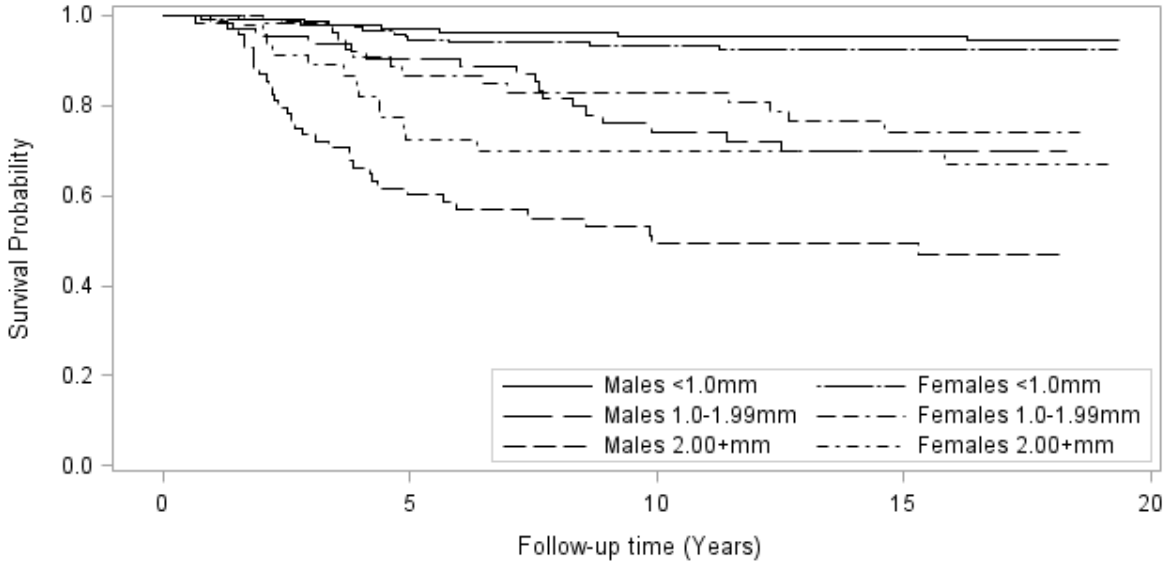
Variables not significant (NS) in baseline models and therefore not included in adjusted models are listed as -
* Adjusted for sex (only in overall population) and age
+ Adjusted for all variables except those listed as - in overall population
^Adjusted for all variables except those listed as - in males
#Adjusted for all variables except those listed as - in females
NA=Not Applicable
Bold=Significant in adjusted models
Note: All variables included in the Chi-Square analysis (Table 2.1) were evaluated in the baseline models; significant variables are shown in the table

Figure 2.1 Survival by Sex



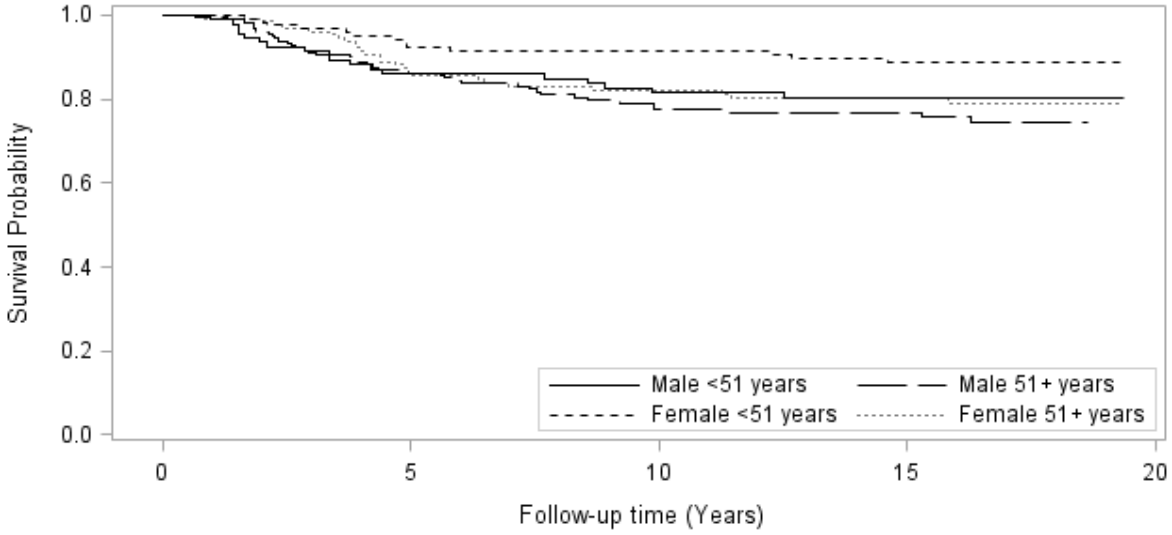
	Died from Melanoma	Total	p-value
Male	58	283	
Female	38	260	0.05

Figure 2.2 Survival by Sex and Breslow Thickness



Males	Died from Melanoma	Total	Females	Died from Melanoma	Total	p-value
<1.0mm	7	147	<1.0mm	11	156	0.45
1-1.99mm	17	65	1-1.99mm	13	55	0.60
2.0+mm	34	71	2.0+mm	14	49	0.03

Figure 2.3 Survival by Sex and Average Age of Menopause



	Died from Melanoma	Total	p-value
Male <51 years	18	94	
Male 51+ years	40	189	
Female <51 years	13	125	
Female 51+ years	25	135	0.04

CHAPTER THREE:

BEHAVIOR AND BRESLOW THICKNESS: A SEX-STRATIFIED POPULATION-BASED STUDY OF MELANOMA

3.1 Abstract:

Background: Men have poorer survival from melanoma than women, perhaps in part due to having thicker lesions at diagnosis. We sought to determine different behavioral factors that contribute to Breslow thickness in males compared to females

Methods: Multivariable linear regressions of log-transformed Breslow thickness were performed using cases from a population-based case-control study of melanoma patients (males n=283, females n=260). We modeled Breslow thickness for all of the cases, as well as stratified by sex.

Results: In females, we found that UV exposures and skin awareness were associated with decreased Breslow thickness. The association of UV exposure with Breslow thickness in females remained significant in baseline models for women who were unaware of their skin. In males, we did not identify any behavioral factors that associated with Breslow thickness. Histopathological variables associated with Breslow thickness were consistent across sexes. Skin awareness was not associated with Breslow thickness in males; however, other variables that are associated with skin awareness such as college education, indoor tanning, and painful burns were comparable across males and females.

Conclusions: Skin awareness, while comparable in males and females, does not contribute to Breslow thickness in males. UV exposures also modify Breslow thickness in females, which is not due to its correlation with skin awareness.

Impact: These findings suggest that thicker lesions at diagnosis cannot be attributable to skin awareness in men. UV exposure association with Breslow thickness in females but not males may explain previous inconsistencies in UV exposure and melanoma survival. Future studies investigating UV exposure should control for confounding by skin awareness.

3.2 Introduction:

Melanoma is the most deadly form of skin cancer, and a female survival advantage has been observed for decades [2,27,30,108]. There are multiple theories regarding the female survival advantage including sex differences in behavioral factors such as skin awareness, skin self-examination (SSE), and UV exposures [100,102]. Others speculate that the female survival advantage is related to hormonal differences between males and females, and the impact of sex steroid hormones on both the immune system and reactive oxygen species [39,102].

Interestingly, there are differences in the presentation of melanoma between males and females [20,21,109,110]. Men are more likely to develop melanomas on their trunks, whereas females are more likely to develop melanomas on their extremities [20,21,109,110]. Men are also more likely to develop nodular melanomas, which are the most aggressive histopathological subtype, whereas females are more likely to develop superficial spreading melanomas (SSM) [20,21,109,110]. Finally, males are more likely to have a thicker lesion at diagnosis, as measured by Breslow thickness in millimeters (mm), and an increased rate of growth compared to females, suggesting that male melanomas are more aggressive and develop differently [20,21,25,109,110].

Breslow thickness is the depth of a melanoma lesion from the granular layer of the skin to the deepest point, and is used in the current AJCC staging guidelines [12]. Importantly, Breslow thickness is the strongest and most consistent prognostic indicator for melanoma. That is, as Breslow thickness increases, the survival rate

decreases—a lesion <1mm has a 95-100% survival rate, a lesion 1.00-2.00mm has a 80-96% survival rate, a lesion 2.01-4.00mm has a 60-75% survival rate, and a lesion >4.01mm has a 37-50% survival rate [16].

In our previous study of melanoma patients ascertained from the Connecticut Tumor Registry, we found that males with thick lesions (>2.00mm) have poorer survival than women with thick lesions (p=0.03) [111]. Additionally, we found that the hazard ratio for Breslow thickness decreased in males from the 5-year follow-up to the 15-year follow-up. That is, Breslow thickness at diagnosis was more predictive of survival at the 5-year follow-up than the 15-year follow-up. However, the 5-year and 15-year hazard ratios for Breslow thickness at diagnosis remained consistent in females, indicating that Breslow thickness is a stronger long-term predictor of survival in females compared to males [111]. Since Breslow thickness is the best prognostic indicator for melanoma, and we previously observed differences in Breslow thickness between males and females, sex-stratification of analyses investigating Breslow thickness may provide more insights to the female survival advantage. Therefore, we sought to determine the behavioral and biological factors that contribute to Breslow thickness in males compared to females using the same cohort of melanoma patients.

3.3 Methods

3.3.1 Subjects

This population has been described previously [99,111]. Briefly, 650 non-Hispanic whites with cutaneous invasive melanoma were ascertained via the

Connecticut Tumor Registry from January 15, 1987 to May 15, 1989. We removed 107 participants with missing information from our analysis, so that 543 individuals make up the analysis dataset [111].

3.3.2 Statistical analysis

We used several summary variables (listed in **Table 3.1**) that were described previously [111]. Most relevant to our findings, we used self-reported data to determine skin awareness. To evaluate skin awareness, participants were asked, "Prior to your biopsy, did you ever think about your skin, how it looked or whether there were any changes; whether there were any abnormal marks?" Answers included 'No', 'Cosmetic changes', 'Abnormalities', and 'Other'. We created a dichotomous variable representing aware/unaware, where any reported awareness was considered aware, and the answer 'No' was considered unaware.

Chi-squared contingency tables were used to investigate differences between males and females in the population. Because Breslow thickness is not normally distributed, we used the nonparametric Mann Whitney Wilcoxon test to compare average Breslow thickness between males and females. Using linear regression, we built models for Breslow thickness for all of the cases (referred to as overall population), along with males and females separately. In the linear regressions, we used log-transformed Breslow thickness as the outcome to correct for the non-normal distribution of Breslow thickness. Baseline models included a variable of interest and were adjusted for sex (for the overall population) and age. Multivariable odds ratios included all variables significant in the baseline model

($p \leq 0.05$), and were adjusted for sex (for the overall population) and age. Output from the linear regressions modeling Breslow thickness was exponentiated, so odds ratios represent increases in Breslow thickness per 1mm. We also performed chi-squared contingency analyses to investigate associations of skin awareness with Breslow thickness, college education, indoor tanning, and 'ever had a painful sunburn' (referred to as painful burns). Each analysis was performed using two-sided test and $p \leq 0.05$ was considered significant. All analyses were performed in SAS 9.3 (SAS Institute, Cary, N.C.).

3.4 Results

3.4.1 Differences between men and women with melanoma in Connecticut

As previously reported, subjects were evenly distributed between the sexes, with 283 males (52.1%) and 260 females (47.9%) [111]. Significant differences were: men in our study were older ($p<0.01$) and more educated ($p=0.01$), had an increased number of melanomas on their head and trunk ($p<0.01$), and were less likely to have solar elastosis (**Table 3.1**). We performed a Mann Whitney Wilcoxon test to further investigate the distribution of Breslow thickness. There was a non-significant trend towards men having thicker lesions ($p=0.08$) (data not shown). Fewer men had performed SSE ($p=0.02$), and they were less likely to report skin awareness ($p<0.01$), yet were more likely to have skin examinations from a spouse/partner compared to women ($p<0.01$) (**Table 3.1**).

3.4.2 Breslow thickness models including other histopathological factors

For the overall population and both of the sex-stratified analyses, ulceration, mitoses, vertical growth phase, and histological subtype were significantly associated with increased Breslow thickness (**Table 3.2**). Sex-related differences included males having an increased Breslow thickness when mitoses were present compared to females (adj. OR 5.19, 95% CI=3.20-8.42 vs. adj. OR 3.18, 95% CI=2.02-5.01). Further, females had a slightly increased Breslow thickness for nodular melanomas (adj. OR 4.69, 95% CI=2.62-8.42) compared to males (adj. OR 3.80, 95% CI=2.14-6.73).

Regarding behavioral factors, skin awareness and indoor tanning were associated with Breslow thickness in the baseline model for the overall population. Similarly, skin awareness, indoor tanning, and painful sunburns were associated with Breslow thickness in the baseline model for females. However, none of these behavioral factors were significant in the baseline model for males, and therefore were not included in the adjusted model. In the adjusted model, skin awareness was the only behavioral variable that remained significant for the overall population (OR 0.67, 95% CI=0.47-0.97) and females (OR 0.72, 95% CI=0.52-1.00), but was not protective in males (**Table 3.2**). Additionally, indoor tanning and painful sunburns were borderline significant in the female model (OR 0.72, 95% CI=0.52-1.02; OR 0.77, 95% CI=0.56-1.07, respectively) **(Table 3.2)**.

3.4.3 Breslow thickness models excluding other histopathological factors

Because collinearity between Breslow thickness and other histopathological factors occurs and is difficult to correct for, we also modeled Breslow thickness using only behavioral variables. This was only done for the overall and female models given that we did not find any behavioral factors associated with Breslow thickness in the baseline model for males (**Table 3.2**).

For the overall population, age was significantly associated with increased Breslow thickness (OR 1.14, 95% CI=1.02-1.27) (**Table 3.2**). Skin awareness was significantly associated with a decreased Breslow thickness (OR 0.67, 95% CI=0.47-0.97), along with indoor tanning, which was borderline significant (OR 0.66, 95% CI=0.43-1.01) (**Table 3.2**).

In the analysis stratified by females, painful sunburns and skin awareness were significantly associated with decreased Breslow thickness (OR 0.61, 95% CI=0.37-1.00; OR 0.56, 95% CI=0.34-0.94, respectively) (**Table 3.2**). Additionally, age and indoor tanning were borderline significant (OR 1.11, 95% CI=0.96-1.29; OR 0.61, 95% CI=0.35-1.07, respectively) (**Table 3.2**).

3.4.4 Factors associated with skin awareness

Skin awareness was a self-reported variable, and could have differing results in males compared to females because of potential recall bias and variance in interpretation of the question. To account for these concerns, we performed a chi-

squared contingency analysis to compare factors that could be a proxy for skin awareness in the overall population, males, and females (**Table 3.3**). A higher percentage of participants who were college educated, indoor tanned, and painful sunburns also reported being aware of their skin for the overall population, as well as males, and females, indicating that the self-reported variable was comparable across the sex-stratification (**Table 3.3**). Furthermore, a higher percentage of participants with thin lesions reported being aware of their skin in the overall population and in females (62.4% and 75.0%, respectively) (**Table 3.3**). However, in the male stratification, a higher percentage of participants with intermediate lesions reported being aware of their skin (55.4%), while only 49% of participants with thin lesions reported being aware of their skin (**Table 3.3**). Taken together, these results show that males with comparable skin awareness are diagnosed with thicker lesions, suggesting that men have more aggressive melanomas than women.

3.4.5 Factors associated with Breslow thickness in participants who are unaware of their skin

To remove any confounding from correlations between skin awareness and UV exposures, we investigated the baseline linear regression model of Breslow thickness for participants who reported being unaware of their skin. In the overall population, 227 participants (41.8%) reported being unaware of their skin (data not shown). For the sex-stratified analyses, there were 146 (51.6%) male participants and 81 (31.2%) female participants (data not shown). In the baseline model, ulceration, mitoses, vertical growth phase, and histological subtype remained

significantly associated (p<0.01) with increased Breslow thickness for the overall population and both sex-stratified analyses (data not shown). Painful sunburns were not significant in the overall population or in males; however, it remained inversely associated with Breslow thickness in females (p=0.04) (data not shown). Indoor tanning was not significant in any of the models (data not shown).

3.5 Discussion

Our findings suggest that the histopathological factors that contribute to or are collinear with Breslow thickness are the same between males and females. However, we found that behavioral factors, such as skin awareness, indoor tanning, and painful sunburns are inversely associated with Breslow thickness in females. Conversely, we did not find any association with behavioral factors and Breslow thickness in males.

Although it is difficult to assess UV exposures due to potential confounding with skin awareness, our findings suggest that UV exposure association with Breslow thickness in females is a real effect. When we investigated UV exposures and Breslow thickness in participants who reported being unaware of their skin, painful sunburns were inversely associated with Breslow thickness in females, but not males or the overall population. We found that indoor tanning was no longer associated with Breslow thickness, but this may be due to the small number of females who reported being unaware of their skin and had indoor tanned (n=13, 16.0%).

Our previous study investigating melanoma survival revealed that UV exposure was inversely associated with mortality in males, but had no association in females [111]. Conversely, UV exposure was inversely associated with Breslow thickness in females, but had no association in males. Taken together, these findings suggest that UV exposure impacts male survival independent of Breslow thickness. We can also conclude that UV exposure was not significant in the female survival model because its effect was encompassed by Breslow thickness. Since anatomic site distributions are different in men and women, and UV exposure also varies by anatomic site, it could also play a complex role in melanoma sex differences.

One limitation of our study is that we did not remove participants with lentigo maligna melanoma (LMM) (n=83) to retain power. LMM is highly associated with solar elastosis, which is a histopathological marker of sun exposure that increases with age. Inclusion of participants with LMM could have affected our ability to accurately assess the impact of UV exposures on Breslow thickness. However, this is unlikely as solar elastosis was not significant in any of the models including histopathological factors. We were also limited in our ability to confirm the finding that indoor tanning was inversely associated with Breslow thickness in participants who were unaware of their skin.

Our study has multiple strengths. To our knowledge, this is the first study to investigate factors that contribute to Breslow thickness, especially in a sex-stratified manner. We are also the only study to consider the potential confounding between skin awareness and other variables that can be considered a proxy for skin

awareness, such as indoor tanning. Finally, our careful examination of UV exposure and Breslow thickness in a sex-stratified approach provides explanation for previously inconsistent results regarding UV exposure and Breslow thickness.

In summary, our study has shown the importance of behavioral factors in relation to Breslow thickness in females. Furthermore, we did not find any behavioral factors that contribute to Breslow thickness in males. Because UV exposures are inversely associated with mortality in males, we can conclude that UV exposures impact melanoma progression differently in males compared to females. Future studies distinguishing the role of sex steroid hormones in response to UV exposure and melanoma progression are merited. Additionally, we found that UV exposures such as painful sunburns and indoor tanning were associated with skin awareness suggesting that population-based studies investigating UV exposure and melanoma should control for skin awareness as a confounder. Finally, our findings highlight the importance of sex-stratified analyses in melanoma research.

3.6 Tables:

Table 3.1 Analysis of sex differences in Connecticut population Adapted from Lilyquist et al.*[111]					
	Males (n=283; 52.1%)		**Female (n=260; 47.9%)**		***p*-value**
	n	**%**	**n**	**%**	
Age					
<30	8	2.8	15	5.8	
30-39	34	12.0	42	16.2	
40-49	44	15.6	61	23.5	
50-59	58	20.5	55	21.2	
60-69	68	24.0	40	15.4	
≥70	71	25.1	47	18.1	**<0.01**
Completed college					
No	164	58.0	178	68.5	
Yes	119	42.1	82	31.5	**0.01**
15-year follow-up Status					
Alive	147	51.9	172	66.2	
Died from other causes	78	27.6	50	19.2	
Died from melanoma	58	20.5	38	14.6	**<0.01**
Breslow thickness					
Thin (<1.00mm)	147	51.9	156	60.0	
Intermediate (1.00-1.99mm)	65	23.0	55	21.2	
Thick (2.00+mm)	71	35.1	49	18.9	0.12
Anatomic site					
Head/neck	42	14.8	35	13.5	
Trunk/pelvis	181	64.0	83	31.9	
Extremities	52	18.4	135	51.9	
Other	8	2.8	7	2.7	**<0.01**
Histology					
Superficial spreading	169	59.7	180	69.2	
Nodular	29	10.3	22	8.5	
Lentigo maligna	42	14.8	41	15.8	
Other	43	15.2	17	6.5	**<0.01**
Solar elastosis					
No	123	43.5	91	35.0	
Yes	160	56.5	169	65.0	**0.04**
Outdoor job					
No	92	32.5	201	77.3	
Yes	191	67.5	59	22.7	**<0.01**
Skin awareness					
Unaware	146	51.6	81	31.2	
Aware	137	48.4	179	68.9	**<0.01**
Self-skin examination					
No	225	90.1	217	83.5	
Yes	28	9.9	43	16.5	**0.02**
Spouse-skin examination					
No	221	78.1	230	88.5	
Yes	62	21.9	30	11.5	**<0.01**
Average age at menopause (51 years)					
Below 51 years	94	33.2	125	48.1	
51 years and older	189	66.8	135	51.9	**<0.01**

*Phenotype index, 5-year follow-up status, ulceration, mitoses, number of nevi, intermittent sun exposure, ever had a painful burn, number of painful sunburns, indoor tanning, doctor skin-examination, skin exam index, comorbidities, and number of children were previously evaluated and reported as not significant between males and females in this population

Table 3.2 Breslow thickness models for the overall population, males, and females								
	Overall (n=543)			Males (n=283)		Females (n=260)		
Factor	Baseline* OR(95% CI)	Adjusted with Histolpath.^ OR(95% CI)	Adjusted Behavior Only^ OR(95% CI)	Baseline* OR(95% CI)	Adjusted with Histolpath.^ OR(95% CI)	Baseline* OR(95% CI)	Adjusted with Histolpath.^ OR(95% CI)	Adjusted Behavior Only^ OR(95% CI)
Age (10years)	1.17(1.04-1.30)	1.05(0.97-1.14)	**1.14(1.02-1.27)**	1.12(0.95-1.32)	1.02(0.91-1.16)	1.21(1.05-1.41)	1.08(0.97-1.21)	1.11(0.96-1.29)
Sex	1.26(0.89-1.79)	1.03(0.82-1.30)	1.16(0.81-1.65)	NA	NA	NA	NA	NA
Indoor tanning	0.62(0.41-0.95)	0.94(0.72-1.24)	0.66(0.43-1.01)	0.71(0.38-1.35)	-	0.56(0.32-0.98)	0.72(0.52-1.02)	0.61(0.35-1.07)
Ever had a painful burn	0.81(0.56-1.17)	-	-	1.15(0.67-1.99)	-	0.550.34-0.92)	0.77(0.56-1.07)	**0.61(0.37-1.00)**
Skin awareness	0.65(0.45-0.93)	0.81(0.64-1.02)	**0.67(0.47-0.97)**	0.78(0.47-1.30)	-	0.51(0.31-0.85)	**0.72(0.52-1.00)**	**0.56(0.34-0.94)**
Solar elastosis	0.48(0.33-0.71)	0.86(0.66-1.11)	-	0.50(0.29-0.88)	0.84(0.58-1.23)	0.47(0.28-0.78)	0.87(0.62-1.23)	-
Ulceration	13.83(8.86-21.58)	**3.38(2.41-4.74)**	-	13.66(7.16-26.03)	**3.18(1.97-5.14)**	13.94(7.57-25.70)	**3.66(2.26-5.91)**	-
Mitoses	17.23(13.25-22.41	**4.20(3.01-5.86)**	-	22.21(15.21-32.43)	**5.19(3.20-8.42)**	13.16(9.16-18.91)	**3.18(2.02-5.01)**	-
Vertical growth phase	19.22(14.28-25.88)	**3.86(2.66-5.59)**	-	26.17(16.83-40.68)	**4.68(2.70-8.09)**	14.14(9.50-21.04)	**3.42(2.08-5.62)**	-
TILs	3.70(2.60-5.25)	0.94(0.72-1.22)	-	3.91(2.35-6.50)	0.95(0.65-1.39)	3.49(2.16-5.66)	0.89(0.62-1.28)	-
Histologic subtype			-					-
Superficial spreading	1.00(ref)	1.00(ref)	-	1.00(ref)	1.00(ref)	1.00(ref)	1.00(ref)	-
Nodular	16.36(9.88-29.44)	**4.40(2.93-6.60)**	-	16.10(7.39-35.07)	**3.80(2.14-6.73)**	19.72(9.36-41.51)	**4.69(2.62-8.42)**	-
Lentigo maligna	0.61(0.38-0.98)	0.95(0.67-1.34)	-	0.53(0.27-1.04)	1.01(0.60-1.70)	0.76(0.41-1.39)	0.84(0.54-1.33)	-
Other	4.43(2.65-7.42)	**1.83(1.26-2.65)**	-	3.41(1.6-6.61)	1.45(0.90-2.35)	9.33(4.05-21.51)	2.83(1.51-5.29)	-

Variables not significant (NS) in baseline models and therefore not included in adjusted models are listed as -
* Adjusted for sex (only in overall population) and age
^Adjusted for all variables except those listed as – in adjusted models
NA=Not Applicable
Bold=Significant in adjusted models
Note: All variables included in the Chi-Square analysis (Table 3.1) were evaluated in the baseline models; significant variables are shown in the table

Table 3.3 Chi-squared: Associations with Skin Awareness						
	Overall n=543		Males n=283		Females n=260	
Factor	**Unaware n (%)**	**Aware n (%)**	**Unaware n (%)**	**Aware n (%)**	**Unaware n (%)**	**Aware n (%)**
Breslow thickness						
Thin (<1.0mm)	114 (37.6)	189 (62.4)	75 (51.0)	72 (49.0)	39 (25.0)	117 (75.0)
Intermediate (1.00-1.99mm)	51 (42.5)	69 (57.5)	29 (44.6)	36 (55.4)	22 (40.0)	33 (60.0)
Thick (2.00+mm)	62 (51.7)	58 (48.3)	42 (59.2)	29 (40.9)	20 (40.8)	29 (59.2)
p-value		0.03		0.23		0.03
Completed college						
No	148 (46.4)	63 (32.5)	163 (47.7)	179 (52.3)	65 (36.5)	113 (63.5)
Yes	171 (53.6)	131 (67.5)	64 (31.8)	137 (68.2)	16 (19.5)	66 (80.5)
p-value		<0.01		<0.01		<0.01
Indoor tanning						
Never	192 (45.4)	231 (54.6)	124 (54.9)	102 (45.1)	68 (34.5)	129 (65.5)
Ever	35 (29.2)	85 (70.8)	22 (38.6)	35 (61.4)	13 (20.6)	50 (79.4)
p-value		<0.01		0.03		0.03
Painful burn						
Never	93 (52.5)	84 (47.5)	58 (63.7)	33 (36.3)	35 (40.7)	51 (59.3)
Ever	134 (36.6)	232 (63.4)	88 (45.8)	104 (54.2)	46 (26.4)	128 (73.6)
p-value		<0.01		<0.01		0.02

CHAPTER FOUR:

DNA REPAIR VARIANTS, BRESLOW THICKNESS, AND UV EXPOSURES IN A SEX-STRATIFIED ANALYSIS OF MELANOMA

4.1 Abstract:

Background: SNPs in DNA repair genes have previously been associated with melanoma risk and survival, but not Breslow thickness. DNA repair SNPS have also been shown to interact with UV exposures, which vary between males and females.

Methods: Using cases from the Minnesota Skin Health study, we performed multiple logistic regressions stratified by sex to investigate SNP associations with Breslow thickness. We also investigated SNP interactions with UV exposures that modify Breslow thickness.

Results: We identified 3 SNPs associated with Breslow thickness in males, and 7 SNPs associated with Breslow thickness in females. Only 1 SNP was significant in both sexes. We identified 10 SNPs that interacted with UV exposures to modify Breslow thickness in males, and 13 SNPs in females. None of the SNPs in the interaction analysis overlapped between sexes. SNPs identified in males were largely associated with increased Breslow thickness, and SNPs identified in females were largely associated with decreased Breslow thickness. The SNP analyses for all of the cases was not representative of the results in the sex-stratified analyses.

Conclusions: Biological differences in DNA repair between the sexes may help explain the female survival advantage. Varying interactions with DNA repair SNPs and UV exposures between the sexes may help explain previous inconsistencies in UV exposure association with Breslow thickness and survival.

4.2 Introduction:

Ultraviolet (UV) radiation is the only well-established environmental risk factor for melanoma. It is thought that UV exposure causes melanoma by inducing DNA damage via reactive oxygen species, and formation of bulky adducts such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts [112]. The DNA damage induced by UV exposure is generally repaired by two major DNA repair mechanisms: base excision repair (BER) and nucleotide excision repair (NER) [112]. NER generally repairs CPDs and 6-4 photoproducts, while BER generally repairs oxidative damage; however, there is overlap between the two pathways [112,113]. Interestingly, there are inter-individual variations in the efficiency of DNA repair capacity that have been associated with single nucleotide polymorphisms (SNPs) in healthy individuals [73]. Therefore, investigating DNA repair SNPs in relation to diseases, especially cancers, may be important [73]. SNPs in multiple DNA repair genes and pathways have previously been associated with melanoma risk [89,94–96,114–117]. Furthermore, the effect of indoor tanning on melanoma risk appears to be modified by variants in DNA repair [95].

UV exposure has also been associated with melanoma survival, although inconsistently. In 2005, Berwick et al. reported a protective effect of UV exposure on melanoma survival with 5 years of follow-up information [50]. Using the same dataset, Lilyquist et al. (in preparation) found that UV exposure had a protective effect at 5-year follow-up, particularly in males, which dissipated at the 15-year follow-up [111]. In a validation study using 7-year follow-up information in a much larger population, Berwick et al. (2014) found that there was a weak protective

effect of UV exposure on melanoma survival [53]. Rosso et al. also reported increased survival for participants with intermittent sun exposure prior to diagnosis [118]. In contrast, Fortes et al. associated high UVB exposure with an increased mortality from melanoma located on the lower extremities, but found no associations with other anatomic sites [119]. Multiple SNPs in DNA repair genes have been associated with melanoma survival [71,120–122]. Therefore, it is possible that gene-environment interactions could explain the reported inconsistencies between UV exposure and survival.

UV exposure has also been associated with Breslow thickness—the best prognostic indicator for melanoma [53,123]. In 2013, Gandini and colleagues reported that taking sunny holidays was associated with thinner lesions in women, but not in males [123]. However, no studies have reported on associations between Breslow thickness and DNA repair SNPs to date.

Of note, there is a female survival advantage in melanoma, suggesting that there are biological differences in the progression of melanoma between males and females [26,27,31]. Previous studies investigating UV exposure in relation to melanoma survival and Breslow thickness have shown that UV exposure affects melanoma survival differently in males and females [111,123]. Furthermore, previous studies investigating DNA repair capacity have suggested differences between males and females [73,74]. Therefore, investigating associations between Breslow thickness and DNA repair SNPs in a sex-stratified manner may provide insight to the progression of melanoma in males compared to females. For that

reason, we sought to determine whether DNA repair SNPs are associated with Breslow thickness. We hypothesized that DNA repair SNPs differentially associate with Breslow thickness in males compared to females.

4.3 Methods:

4.3.1 Study Population

The Minnesota Skin Health Study has been previously described [124]. Briefly, cases were ascertained through the Minnesota State Cancer Registry. Patients aged 25 to 59 who were diagnosed with invasive cutaneous melanoma between 2004 and 2007 were enrolled. Controls were randomly selected from the state drivers' license list and frequency-matched based on age and sex. The State Cancer Registry and the University of Minnesota Institutional Review Boards approved the protocol for this study. Of the eligible participants, 1167 cases and 1101 controls completed a self-administered questionnaire designed to evaluate UV exposures, along with a telephone interview. Histopathology variables were obtained from the diagnostic pathology report. Of these subjects, 1755 (77.4%) submitted DNA samples for genotyping and 9 participants were removed for missing consents, leaving 1746 (929 cases, 817 controls) participants for genotyping.

4.3.2 Selection of SNPs

SNP selection for this study has been previously described [95]. Briefly, 154 SNPs from 28 DNA repair genes from multiple DNA repair pathways were chosen

based on 1) reported function in the literature and 2) tagging coverage using Haploview 4.1.

4.3.3 Genotyping platform

The genotyping platform for this study has been previously described [95]. Briefly, buccal cell DNA was collected using SCOPE® mouthwash, extracted using Qiagen® kits, quantitated, and genotyped on the Illumina BeadExpress GoldenGate® platform by the University of Utah Genotyping Core. All 1746 participants that submitted DNA samples were genotyped.

4.3.4 Quality control

From the genotyped population (n=1746), we removed 46 non-white participants and 7 participants with missing phenotypic index [95]. Using a 95% call rate as the cutoff, an additional 34 participants and 8 SNPs were removed from the analysis [95]. We also removed 7 monomorphic SNPs and 47 SNPs that had a minor allele frequency (MAF) less than 0.05 [95]. This resulted in a study consisting of 1659 participants (893 cases, 766 controls) and 92 SNPs in 20 DNA repair genes [95]. Finally, 4 SNPs (rs4253114, rs10764889, rs7075505, and rs574831) in the study were significant (p<0.01) for Hardy Weinberg Equilibrium (HWE) as described previously [95]. These SNPs were not removed from the analysis, but associations with these SNPs discovered in our study are noted and interpreted with caution.

For our analysis, we removed all participants with a missing Breslow thickness, including all of the controls, and missing age or sunburn information, resulting in 723 melanoma cases. Once again, we removed participants (n=0) and SNPs (n=1) with a call rate less than 95%, for a total of 723 participants and 91 SNPs in 20 DNA repair genes. We also removed any SNPs (n=1) and participants (n=0) with a call rate less then 95% in the sex-stratified analyses, which resulted in one SNP being removed from the male analysis. The final genotyping call rate, for all of the cases (referred to as overall population) and both of the sex-stratified analyses, was 99.8%. Participants with missing Breslow thickness were removed using SAS 9.3 (SAS Institute, Cary, NC), and all other steps in the quality control process were performed using PLINK 1.07 [125].

4.3.5 Statistical analysis

All analyses were performed using two-sided tests and $p \leq 0.05$ was considered significant.

We performed a chi-squared contingency analysis to compare differences between men and women in the population using SAS 9.3 (SAS Institute, Cary, NC).

Multiple logistic regression analyses in PLINK 1.07 were performed to investigate the association of each SNP with Breslow thickness using an additive genotype model [125]. Breslow thickness was split into two categories: thin (less than 1mm) and thick (greater than 1mm). We modeled Breslow thickness for all of the overall population, along with males and females separately. Odds ratios,

confidences intervals, and *p*-values were adjusted for age as a continuous variable (in all models) and sex (in the models for the overall population). We evaluated all associations for multiple comparisons using the False Discovery Rate.

We also assessed SNP interactions with UV exposures including indoor tanning status and number of painful sunburns (childhood, adult, and total) in PLINK 1.07 [125]. For these analyses, we investigated multiplicative interactions using multiple logistic regression that included the main effects and interaction term for the SNP and the UV exposure of interest. The *p*-values for the interactions on the multiplicative scale were calculated using Wald tests for the interaction term. The interaction analyses were adjusted for sex (in the overall population) and age.

4.4 Results:

4.4.1 Differences between males and females in the Minnesota population

The Minnesota Skin Health Population had more females than males (61.4% and 38.6%, respectively) (**Table 4.1**). We first calculated differences between males and females in the population using a chi-squared analysis. Males were older ($p<0.01$) and more educated ($p<0.01$) (**Table 4.1**). Regarding tumor characteristics, males had thicker lesions at diagnosis ($p<0.01$) and the majority of their melanomas on the trunk while females had thinner lesions at diagnosis and the majority of their melanomas on their extremities ($p<0.01$). Histological subtype was also distributed differently among males and females, with males having more nodular melanomas ($p<0.01$) (**Table 4.1**). Pertaining to behavioral factors, fewer males had indoor tanned ($p<0.01$) and more males reported having greater than ten painful burns as a

child, as an adult, and total number of lifetime sunburns (p=0.11, p<0.01, and p=0.02, respectively) (referred to as childhood burns, adulthood burns, and lifetime burns) (**Table 4.1**).

4.4.2 SNP associations with Breslow thickness in the overall population

In the overall population, we identified six SNPs that were associated with Breslow thickness at diagnosis after adjustment for age and sex (p≤0.05) (**Table 4.2**). Four of the SNPs are located in NER genes: RFC1 (rs2066786, rs2066782), ERCC4 (rs1800067), and ERCC6 (rs4253114) (**Table 4.2**). Both SNPs in RFC1 along with the SNP in ERCC4 had a decreased odds ratio of a thick lesion (>1mm). The SNP in ERCC6 was associated with an increased risk of having a thick lesion (**Table 4.2**).

Two of the SNPs (rs1574157 and rs12315756) were located in FBRSL1—a gene previously associated with DNA repair (**Table 4.2**). rs12315756 was inversely associated with Breslow thickness and rs1574157 was positively associated with Breslow thickness (**Table 4.2**).

4.4.3 SNP associations with Breslow thickness in males

In males, there were three SNPs that were associated with an increased risk of having a thick lesion after adjustment for age (p≤0.05) (**Table 4.2**). Two of the SNPs are located in NER genes: ERCC5 (rs876430) and ERCC6 (rs4253114) (**Table 4.2**). The remaining SNP was in another gene previously associated with DNA repair, FBRSL1 (rs1574157) (**Table 4.2**).

4.4.4 SNP associations with Breslow thickness in females

In females, seven SNPs were associated with Breslow thickness (**Table 4.2**). PARP1 (rs1805414), in the BER pathway, was associated with decreased odds of having a thick lesion (**Table 4.2**). We identified five SNPs in the NER pathway (**Table 4.2**). Three of the SNPs (rs2066786, RFC1; rs1800067, ERCC4; rs7325708, ERCC5) were inversely associated with Breslow thickness, and two of the SNPs (rs4253114, ERCC6; rs4150355, ERCC5) were associated with increased Breslow thickness (**Table 4.2**). There was also a SNP (rs12315756) in FBRSL1, which is involved in transcription, that was inversely associated with Breslow thickness (**Table 4.2**).

4.4.5 Comparison of SNP associations in the overall population, males, and females

There was not complete overlap between the SNP analyses for the overall population compared to the sex-stratified analysis (**Tables 4.2** and **4.3**). In fact, there was only one SNP, rs4253114 (ERCC6), that was significant in the overall population and both stratified models (**Tables 4.2** and **4.3**). For the 3 SNPs that were uniquely significant in the female analysis, the odds ratio was in the opposite direction to the male population (**Table 4.3**). Likewise, for the SNP that was uniquely significant in the male analysis, the odds ratio was in the opposite direction to the female population (**Table 4.3**).

4.4.6 Interactions with DNA repair SNPs and UV exposures in the overall population

For the overall population, 13 SNPs interacted with different measures of UV exposure; there were no interactions with SNPs and childhood burns (**Table 4.4**).

The two SNPs that interacted with indoor tanning (rs4253126) and lifetime burns (rs4253079) are both located in ERCC6 and are inversely associated with Breslow thickness (OR 0.42, 95% CI=0.19-0.92; OR 0.27, 95% CI=0.11-0.66, respectively) (**Table 4.4**). That is, someone with the minor allele for these two SNPs who has ever indoor tanned and had greater than 10 lifetime painful burns has a decreased risk of a lesion thicker than 1mm compared to someone who has either the minor allele or has ever indoor tanned/had greater than 10 lifetime painful burns.

Conversely, the 11 SNPs that interacted with adulthood burns were associated with an increased Breslow thickness (**Table 4.4**). Interestingly, these 11 SNPs spanned multiple DNA repair pathways including NER, BER, MGMT, and others (**Table 4.4**). Furthermore, rs4253114 in ERCC6 was also associated with increased Breslow thickness (as discussed above).

4.4.7 Interactions with DNA repair SNPs and UV exposures in males

In males, we found 1 SNP interacted with indoor tanning and 9 SNPs interacted with adulthood burns to modify Breslow thickness ($p \leq 0.05$) (**Table 4.5**). There were no SNPs that interacted with childhood burns or lifetime burns (**Table 4.5**). Interestingly, all 10 SNPs identified in this analysis interacted with UV exposures to increase Breslow thickness (**Table 4.5**). The SNPs were in genes involved in multiple DNA repair pathways including NER, BER, MGMT, and other (**Table 4.5**). Of note, five of the ten SNPs identified were in the MGMT gene/pathway (**Table 4.5**). Additionally, one SNP in ERCC5 (rs4150355) that was associated with increased

Breslow thickness in females (no interaction in females) also interacted with adulthood burns to increase Breslow thickness in males (**Table 4.3** and **Table 4.5**).

4.4.8 Interactions with DNA repair SNPs and UV exposures in females

In females we identified 6 SNPs that interacted with indoor tanning, 1 SNP that interacted with childhood burns, 1 SNP that interacted with adulthood burns, and 5 SNPs that interacted with lifetime burns ($p \leq 0.05$) (**Table 4.6**). The only SNP that was associated with increased Breslow thickness was rs2888805, which interacted with adulthood burns; rs2888805 was also one of two SNPs in the BER pathway that interacted with UV exposures in females (**Table 4.6**). All other SNPs that interacted with UV exposure inversely modified Breslow thickness (**Table 4.6**). Interestingly, we identified a SNP that interacted with childhood burns in the female analysis, whereas we did not identify any in the male stratification or overall population (**Table 4.6**). Additionally, one SNP in ERCC5 (rs7325708) was inversely associated with Breslow thickness in females, and also interacted with adulthood burns to inversely modify Breslow thickness (**Table 4.3** and **Table 4.6**).

4.4.9 Comparison of interactions with UV exposures in the overall population, males, and females

In the overall population, we identified 13 SNPs that interacted with UV exposure to modify Breslow thickness. Of these, 6 SNPs were unique to the overall population analysis, while 7 SNPs were also identified in either the male or female

stratification. There was no overlap in SNPs between the male and female stratification.

There were 10 SNPs identified in the interaction analysis for the male stratification, 5 of which were also identified in the interaction analysis for the overall population. All 5 of the SNPs overlapping in the male stratification and overall population interacted with adulthood burns to increase Breslow thickness.

There were 13 SNPs identified in the interaction analysis for the female stratification, 2 of which were also identified in the interaction analysis for the overall population. rs4253079 interacted with lifetime burns in the overall population to inversely modify Breslow thickness. Similarly, rs4253079 also interacted with childhood burns and lifetime burns in females to inversely modify Breslow thickness. rs2888805 interacted with adulthood burns in the overall population and in females to increase Breslow thickness.

4.4.10 SNPs significant for HWE

When evaluating the control population for HWE, we found 4 SNPs that were significantly out of HWE (p<0.01). rs4253114 (ERCC6) was significant in our SNP association analyses for the overall population, males, and females, as well as the interaction analysis for the overall population. rs10764889 (MGMT) was significant in the interaction analysis for females. rs7075505 (MGMT) was significant in the interaction analysis for the overall population.

4.5 Discussion:

DNA repair capacity appears to have a complex role in melanoma. Many SNPs in DNA repair genes have previously been associated with both melanoma risk and survival [89,94–96,114–117]. In 2013, Emmert and Kraemer issued a warning not to underestimate NER in regard to melanoma survival [71]. They introduced the idea that NER has a contextual role regarding melanoma; that is, diminished NER DNA repair capacity is associated with increasing melanoma risk, while increased NER DNA repair capacity is associated with decreased melanoma survival [71]. In the NER pathway, we found 7 SNPs associated with Breslow thickness, and 12 SNPs that interacted with UV exposures to modify Breslow thickness. There was only one SNP in the NER pathway that was identified in both males and females. A previous study showed that women have more DNA damage than men, and they have a decreased NER capacity [73].

The contextual role of NER suggested by Emmert and Kraemer has also been observed for the MGMT pathway [71]. The MGMT pathway repairs alkylation-induced DNA damage and has been associated with both melanoma risk and survival [93,95,126]. In 2009, Gu et al. found that participants with a decreased MGMT repair capacity have an increased risk for melanoma [93]. Furthermore, due to its ability to repair damage caused by chemotherapies, increased MGMT repair capacity is associated with chemotherapeutic resistance and lethal metastases [93].

Interestingly, one of the most consistent genes in our interaction analysis was MGMT. We found that 5 SNPs in MGMT interacted with adulthood burns in males to increase Breslow thickness. Conversely, we found that two different SNPs in MGMT

interacted with lifetime burns in females to decrease Breslow thickness. Furthermore, sex differences regarding the MGMT DNA repair pathway have previously been reported [75,76,127].

Additionally, in our previous publication investigating DNA repair SNPs, indoor tanning, and melanoma risk, we identified 7 SNPs that were also associated with Breslow thickness in this study [95]. Our previous analysis was not sex-stratified, so our ability to interpret the overlap between these two studies is limited. However, the overlap in results does reiterate the idea presented by Emmert and Kraemer that the role of DNA repair in melanoma is complex, and likely involved in etiology, progression, and survival [71].

Our study has some limitations. First, we recognize that there are over 120 genes involved in DNA repair, and our candidate gene study only included 20 of these genes. While we covered multiple pathways known to be important in melanoma, a more complete investigation of DNA repair SNPs in relation to Breslow thickness is warranted. Second, the majority of the SNPs that we investigated were intronic (>70%), which limits our ability to interpret the results in terms of functional impact. However, in our study design, we selected tagging SNPS, which allows for identification of a genomic region that may have functional significance. Therefore, it is possible that the significant SNPs are in linkage disequilibrium with functional SNPs, and should be further researched to determine functional impact. Additionally, none of our findings were statistically significant following FDR correction for multiple tests. However, functional evaluation of these SNPs may

reveal biological significance. Finally, 3 SNPs that were identified in this study were not in HWE in our control population. There are multiple reasons why SNP deviate from HWE, and it is difficult to determine the cause in our population. Therefore, the results for those SNPs should be interpreted with caution and validated in another study. Finally, age is a complicated factor that we adjusted for, but could not properly evaluate. Our study included participants aged 25-59, but the incidence of melanoma in males increases after age 50 surpasses female risk [16]. Furthermore, DNA repair capacity decreases with age. Taken together, we recognize that it is likely that age also plays an important role in DNA repair as it relates to sex differences in melanoma survival.

Our study also has multiple strengths. First, we have extensive UV exposure information on the participants allowing for investigation of interactions with multiple types of UV exposure. Second, the realization that UV exposure may affect Breslow thickness and melanoma progression differently in males compared to females may help to explain previous inconsistencies in the literature regarding UV exposure effects on Breslow thickness and survival. Furthermore, it may help explain the female survival advantage.

In summary, our study investigated SNP associations with Breslow thickness, as well as SNP interactions with UV exposures that modified Breslow thickness. Furthermore, we investigated these SNPs stratified by sex to identify factors that may contribute to melanoma progression differently in males compared to females. In the association analysis, we identified 10 SNPs that were associated with Breslow

thickness. Among the 10 SNPs identified, only one SNP was associated with Breslow thickness in both males and females. Furthermore, there was no overlap of the SNPs that interacted with UV exposures to modify Breslow thickness in males and females. Finally, for both the SNP associations and interactions, the majority of SNPs we identified in males increased the odds of a thick lesion. Conversely, in females, the majority of SNPS we identified decreased the odds of a thick lesion. These results suggest that different genotypes in DNA repair genes contribute to Breslow thickness, and potentially the progression of melanoma, in males compared to females. Therefore, there may be some inherent differences in DNA repair capacity between the sexes. Future studies investigating responses to UV exposure in males compared to females are warranted, including further investigation of differences in DNA repair and other pathways that have been associated with melanoma progression and survival.

4.6 Conclusions:

We have shown that different SNPs in DNA repair genes are associated with Breslow thickness in males compared to females. In the analysis of the overall population adjusted for sex, we did not identify all of the SNPs that were associated with Breslow thickness in males and females independently. Similarly, we found that different SNPs interacted with UV exposures to modify Breslow thickness in males compared to females. Our study suggests that there are biological differences between the sexes regarding UV exposure, DNA repair, and Breslow thickness. These findings may help explain the female survival advantage in melanoma and highlight the importance of sex stratification in melanoma research.

4.7 Tables

Table 4.1 Chi-squared analysis of sex differences in the Minnesota population					
	Males (n=279; 38.6%)		**Female (n=444; 61.4%)**		***p*-value**
	n	**%**	**n**	**%**	
Age					
<30	6	2.2	39	8.8	
30-39	36	12.9	76	17.2	
40-49	85	30.5	180	40.5	
50-59	152	54.5	149	33.6	**<0.01**
Completed college					
No	119	42.7	235	52.9	
Yes	160	57.3	209	47.1	**<0.01**
Income >$60,000					
No	71	25.6	138	31.7	
Yes	206	74.4	298	68.4	0.09
Missing					10
Breslow thickness					
Thin <1mm	197	70.6	354	79.7	
Thick	82	29.4	90	20.3	**<0.01**
Breslow thickness					
<1.0mm	197	70.6	354	79.7	
1.00-1.99mm	53	19	65	14.6	
2.00-3.99mm	23	8.2	18	4.1	
4.00+mm	6	2.2	7	1.6	**0.02**
Body site					
Scalp/Neck	22	7.9	13	2.9	
Face	29	10.4	20	4.5	
Trunk	126	45.2	109	24.6	
Upper extremities	67	24.0	127	28.6	
Lower extremities	30	10.8	170	38.3	
Unknown	5	1.8	5	1.1	**<0.01**
Ulceration					
No	255	93.7	396	94.3	
Yes	17	6.3	24	5.7	0.77
Missing					31
Histology					
Superficial spreading	117	41.9	186	41.9	
Nodular	26	9.3	23	5.2	
Lentigo maligna	15	5.4	9	2.0	
Other	121	43.4	226	50.9	**<0.01**
Missing					
Painful burns as child					
Less than 10	164	58.8	287	64.6	
Greater than or equal to 10	115	41.2	157	35.4	0.11
Painful burns as adult					
Less than 10	198	71.0	353	79.5	
Greater than or equal to 10	81	29.0	91	20.5	**<0.01**
Lifetime painful burns					
Less than 10	121	43.4	231	52.0	
Greater than or equal to 10	158	56.6	213	48.0	**0.02**
Indoor tanning					
Never	161	57.7	106	23.9	
Ever	118	42.3	338	76.1	**<0.01**

Bold=significant

Table 4.2 SNPs Associated with Breslow Thickness								
Overall Population								
Pathway	**Gene**	**SNP**	**Minor Allele**	**Major Allele**	**MAF**	**OR (95% CI)***	***p*-value***	**FDR Corrected *p*-value***
NER	RFC1	rs2066786	A	G	0.41	0.65 (0.50-0.85)	<0.01	0.12
	ERCC6	rs4253114	A	G	0.11	1.82 (1.23-2.67)	<0.01	0.12
	RFC1	rs2066782	G	A	0.13	0.61 (0.40-0.92)	0.02	0.45
	ERCC4	rs1800067	A	G	0.09	0.56 (0.33-0.95)	0.03	0.60
Other	FBRSL1	rs12315756	G	A	0.10	0.57 (0.36-0.90)	0.02	0.45
	FBRSL1	rs1574157	A	G	0.07	1.60 (1.02-2.51)	0.04	0.65
Males								
NER	ERCC6	***rs4253114***	A	G	0.09	2.15 (1.16-4.00)	0.02	0.9
	ERCC5	rs876430	A	G	0.29	1.48 (1.00-2.18)	0.05	0.9
Other	FBRSL1	**rs1574157**	A	G	0.05	2.06 (1.00-4.26)	0.05	0.9
Females								
BER	PARP1	rs1805414	G	A	0.32	0.68 (0.48-0.98)	0.04	0.68
NER	RFC1	**rs2066786**	A	G	0.42	0.56 (0.29-0.81)	<0.01	0.20
	ERCC5	rs4150355	A	G	0.37	1.52 (1.08-2.14)	0.02	0.67
	ERCC5	rs7325708	G	A	0.18	0.63 (0.39-1.01)	0.05	0.67
	ERCC4	**rs1800067**	A	G	0.08	0.37 (0.15-0.86)	0.02	0.70
	ERCC6	***rs4253114***	A	G	0.13	1.68 (1.01-2.78)	0.05	0.68
Other	FBRSL1	**rs12315756**	G	A	0.10	0.51 (0.26-0.97)	0.04	0.68

*Adjusted for sex (in overall population only) and age
Bold=Significant in overall population
Italicized=Significant in overall population, males, and females

Table 4.3 Comparison of Odds Ratios between Overall Population, Males, and Females						
Pathway	**Gene**	**SNP**	**Overall OR (95% CI)***	**Males OR (95% CI)***	**Females OR (95% CI)***	**OR Direction**
BER	PARP1	rs1805414	0.84 (0.65-1.10)	1.08 (0.74-1.60)	**0.68 (0.48-0.98)**	↓↑↓
NER	RFC1	rs2066786	**0.65 (0.50-0.85)**	0.76 (0.53-1.10)	**0.56 (0.29-0.81)**	↓↓↓
	ERCC6	rs4253114	**1.82 (1.23-2.67)**	**2.15 (1.16-4.00)**	**1.68 (1.01-2.78)**	↑↑↑
	RFC1	*rs2066782*	**0.61 (0.40-0.92)**	0.58 (0.30-1.10)	0.63 (0.36-1.10)	↓↓↓
	ERCC5	rs4150355	1.14 (0.89-1.46)	0.84 (0.58-1.21)	**1.52 (1.08-2.14)**	↑↓↑
	ERCC5	rs7325708	0.83 (0.60-1.14)	1.10 (0.69-1.73)	**0.63 (0.39-1.01)**	↓↑↓
	ERCC5	rs876430	1.05 (0.80-1.36)	**1.48 (1.00-2.18)**	0.77 (0.53-1.12)	↑↑↓
	ERCC4	rs1800067	**0.56 (0.33-0.95)**	0.80 (0.39-1.64)	**0.37 (0.15-0.86)**	↓↓↓
Other	FBRSL1	rs12315756	**0.57 (0.36-0.90)**	0.64 (0.33-1.24)	**0.51 (0.26-0.97)**	↓↓↓
	FBRSL1	rs1574157	**1.60 (1.02-2.51)**	**2.06 (1.00-4.26)**	1.37 (0.76-2.50)	↑↑↑

*Adjusted for sex (in overall population only) and age
Bold=significant
OR Direction: Direction of the odds ratio for overall population, males, and females respectively

Table 4.4 Multiplicative Interaction Analyses for SNPs and UV Exposures in the Overall Population						
UV Exposure	**Pathway**	**Gene**	**SNP**	**Minor Allele**	**OR(95% CI)**	***p*-value**
Ever Indoor Tanned	NER	ERCC6	rs4253126	A	0.42(0.19-0.92)	0.03
≥10 Adult Burns	BER	APEX1	**rs1130409**	A	1.44(1.05-1.98)	0.02
		TDG	*rs2888805*	A	2.16(1.09-4.30)	0.03
	NER	ERCC6	rs6537537	A	1.67(1.08-2.58)	0.02
		ERCC6	rs4253114	A	2.50(1.16-5.40)	0.02
		ERCC6	**rs4253226**	G	2.10(1.06-4.18)	0.03
		XPC	rs3731143	G	2.97(1.13-7.80)	0.03
	MGMT	MGMT	**rs7905095**	A	1.55(1.11-2.16)	0.01
		MGMT	**rs1008982**	G	1.53(1.07-2.19)	0.02
		MGMT	**rs532248**	T	1.38(1.01-1.87)	0.04
		MGMT	rs7075505	G	2.26(1.08-4.73)	0.03
	Other	IKBKB	rs10958713	A	1.54(1.03-2.29)	0.04
≥ 10 Lifetime Burns	NER	ERCC6	*rs4253079*	C	0.27(0.11-0.66)	<0.01

ORs, 95% CIs, and p-values adjusted for age and sex
Bold=Also identified in interaction analysis for males
Italics=Also identified in interaction analysis for females

Table 4.5 Multiplicative Interaction Analyses for SNPs and UV Exposures in Males						
UV Exposure	**Pathway**	**Gene**	**SNP**	**Minor Allele**	**OR(95% CI)**	***p*-value**
Ever Indoor Tanned	Other	FBRSL1	rs4883522	C	4.85(1.06-22.16)	0.04
≥10 Adult Burns	BER	APEX1	**rs1130409**	A	1.64(1.03-2.60)	0.04
	NER	ERCC5	rs4150355	A	2.09(1.19-3.67)	0.01
		ERCC5	rs4150386	C	4.02(1.21-13.35)	0.02
		ERCC6	**rs4253226**	G	3.25(1.10-8.86)	0.02
	MGMT	MGMT	**rs7905095**	A	1.91(1.16-3.14)	0.01
		MGMT	**rs1008982**	G	1.89(1.11-3.23)	0.02
		MGMT	rs4751118	A	1.84(1.10-3.09)	0.02
		MGMT	rs3793903	C	1.84(1.08-3.13)	0.02
		MGMT	**rs532248**	T	1.60(1.02-2.50)	0.04

ORs, 95% CIs, and *p*-values adjusted for age
Bold=Also identified in interaction analysis for the overall population

Table 4.6 Multiplicative Interaction Analyses for SNPs and UV Exposures in Females						
UV Exposure	**Pathway**	**Gene**	**SNP**	**Minor Allele**	**OR(95% CI)**	***p*-value**
Ever Use	BER	LIG1	rs13436	G	0.55(0.34-0.89)	0.02
	NER	XPC	rs3731068	A	0.36(0.15-0.84)	0.02
		ERCC4	rs1799800	A	0.48(0.24-0.93)	0.03
		ERCC4	rs744154	G	0.50(0.25-0.98)	0.04
		ERCC4	rs9302507	A	0.36(0.14-0.95)	0.04
	MGMT	MGMT	rs7897057	A	0.63(0.29-0.99)	0.05
≥ 10 Childhood Burns	NER	ERCC6	*rs4253079*	C	0.33(0.11-0.99)	0.05
≥ 10 Adulthood Burns	BER	TDG	*rs2888805*	A	3.10(1.26-7.65)	0.01
≥ 10 Lifetime Burns	NER	ERCC6	*rs4253079*	C	0.26(0.09-0.74)	0.01
		XPC	rs3731093	G	0.33(0.12-0.93)	0.04
		ERCC5	rs7325708	C	0.36(0.13-0.96)	0.04
	MGMT	MGMT	rs10764889	A	0.56(0.34-0.93)	0.02
		MGMT	rs6482744	A	0.57(0.34-0.93)	0.03

ORs, 95% CIs, and *p*-values adjusted for age
Italics=Also identified in interaction analysis for the overall population

CHAPTER FIVE:

IMMUNE REPONSE VARIANTS IN A SEX-STRATIFIED ANALYSIS OF BRESLOW THICKNES IN MELANOMA PATIENTS

5.1 Abstract:

Background: Melanoma is known to be an immunogenic tumor, and exposures such as UV and sex hormones can influence the immune system. Therefore, we investigated immune response SNPs in association with Breslow thickness in an attempt to further explain the female survival advantage in melanoma.

Methods: We investigated 22 immune response SNPs in cases from the Minnesota Skin Health study. Multiple logistic regressions, stratified by sex, were performed to determine SNP associated Breslow thickness in males and females.

Results: We identified two SNPs that were associated with Breslow thickness in females. We also identified 3 SNPs that interacted with UV exposures to modify Breslow thickness, 2 in females and 1 in males. There was no overlap among the SNPs identified in males and females. None of the SNPs were significant following FDR correction for multiple tests.

Conclusions: Different UV exposures and SNPs are important to Breslow thickness, and potentially melanoma progression, in males compared to females. These findings emphasize the importance of UV exposure and immune response, along with the impact of sex on these factors, in melanoma.

5.2 Introduction:

Melanoma is the most common cancer among young adults aged 25-29, and is the most aggressive form of skin cancer [1]. It is well established that melanoma is an immunogenic cancer [64]. Unfortunately, melanoma cells can evade the immune system and the host-response is not often sufficient to abrogate tumor growth [64].

Several factors can influence the immune system, including the only established environmental risk factor for melanoma, ultraviolet (UV) radiation [67,128]. Soluble mediators produced by cells in the skin after exposure to UV include immune modulators: tumor necrosis factor (TNF), IL-6, IL-10, and VDR [67,69,128]. It has also been suggested that UV source, wavelength, frequency, and duration can all impact the effect of UV on the immune response [67,128]. In addition, estrogen has been associated with protection against oxidative damage, which is induced by UV [129]. Induction of DNA repair, such as that caused by oxidative damage, is also known to influence the immune system [128].

Endogenous exposures, such as sex steroid hormones, can also affect the immune response [69]. The effects of estrogen, androgen, and testosterone on the immune system are context dependent; however, in many cases, each hormone induces its own unique response [69]. For example, estrogen increases IgG and IgM production, while testosterone inhibits it [130,131].

Hormonal influences on the immune system may account for the fact that females may have a more sensitive immune system than males [68]. This sensitivity is evidenced by more vigorous humoral reactions and a higher incidence of

autoimmune disorders in women [68]. Furthermore, estrogen is associated with increased T lymphocyte activation and proliferation [69]. Thus, in the case of an immunogenic tumor, a more sensitive immune system in females may be beneficial to survival. Specific effects of hormones in melanoma immunology have been reported. For example, estrogen, progesterone, and testosterone have the ability to inhibit IL-8 expression which has been shown to result in slowed melanoma cell growth [132].

Variation in UV exposures or sex steroid hormones may account for some of the inter-individual variation in immune responses, thereby impacting the immune response in melanoma, and potentially melanoma progression [102,111]. Importantly, UV exposures, such as indoor tanning and outdoor jobs, vary between men and women [55]. Genetic variation, or single nucleotide polymorphisms (SNPS), may also account for inter-individual variation in immune responses. Howell et al. identified several SNPs in immune response genes that are associated with Breslow thickness in melanomas [87,88,133].

Breslow thickness, which is a measurement of tumor depth in millimeters from the granular layer of the epidermis to the deepest point, is the best prognostic marker for melanoma [4,11]. That is, increased Breslow thickness is associated with more aggressive melanomas, an increased likelihood of metastasis, and ultimately increased mortality from melanoma [4,12].

Taken together, genetic variations, along with UV and endogenous exposures, may help explain why females often have thinner lesions and better survival

outcomes compared to males. Therefore, we hypothesized that immune response SNPs would differentially associate with Breslow thickness in males compared to females, and would also be influenced by UV exposures.

5.3 Methods

5.3.1 Study Population

Cases from the Minnesota Skin Healthy study, previously described, included individuals aged 25-59 years diagnosed with melanoma between 2004 and 2007 [95,124]. Briefly, controls were frequency matched on age and sex. Of the 2268 eligible participants (cases n=1167, controls =1101), 1755 (77.4%) submitted DNA samples for genotyping. Nine samples were removed for missing consent leaving n=1746 (cases n=929, controls n=817) samples for analysis. For each of these participants, we collected extensive UV exposure information from self-administered questionnaires and phone interviews. Histopathological information for cases was derived from the diagnostic pathology report.

5.3.2 Selection of SNPs

The selection of SNPs for this study has been previously described [95]. Briefly, we evaluated 25 SNPs in 15 immune response genes. We used a candidate gene approach, and the SNPs were selected based on 1) reported function in the literature, and 2) coverage of the gene of interest based on the tagging ability of the SNP using Haploview 4.1.

5.3.3 Genotyping Platform

Briefly, buccal cell DNA was obtained using SCOPE® mouthwash and was extracted using Qiagen® kits [95]. We quantitated the DNA and genotype was evaluated by using Illumina BeadExpress GoldenGate® platform.

5.3.4 Quality Control

We removed 46 non-White participants and 7 participants with missing phenotype index from the genotyped population (n=1693). Participants (n=261) and SNPs (n=1) with less than a 95% call rate were removed. Analysis of Hardy Weinberg Equilibrium (HWE) in the control population revealed that 5 SNPs were not in HWE ($p<0.01$); those SNPs were removed from the analysis.

We removed all controls and cases with missing Breslow thickness from our analysis (n=627). No additional SNPs or participants were removed due to a low call rate (<95%). Two SNPs were removed from the analysis for minor allele frequency (MAF) less than 0.05. The dataset for this analysis included 627 participants, leaving 252 males, 375 females, and 22 immune response SNPs. No additional SNPs or participants in the sex-stratified analyses were removed due to a low call rate or MAF (<95% and <0.05, respectively). The genotyping call rate for the all of the cases (referred to as overall population) and both sex-stratified analyses was 99.9%.

5.3.5 Statistical analysis

All analyses were performed using two-sided tests and $p\leq0.05$ was considered significant.

To compare differences between men and women in the population, we performed a chi-squared contingency analysis using SAS 9.3 (SAS Institute, Cary, NC).

We used multiple logistic regressions in PLINK 1.07 to determine which SNPs were associated with Breslow thickness [125]. The additive genotype model was used for these analyses. The outcome variable, Breslow thickness, was a dichotomous variable representing thin lesions (<1.0mm) and thick lesions (≥1.00mm). We adjusted all models for age (as a continuous variable) and, in the overall population, we adjusted for sex.

Multiplicative interactions between SNPs and UV exposures were determined using PLINK 1.07 [125]. We assessed interactions with indoor tanning and painful sunburns in childhood, adulthood, and lifetime. *P*-values for the interactions were determined using the likelihood ratio tests. That is, we compared the full model with the product term for the SNP and UV exposure to the model without the product term. We also adjusted the interaction analyses for sex (for the overall population) and age.

5.4 Results

5.4.1 Differences between males and females in the Minnesota Skin Healthy Study

For our analysis we had more females (n=375, 59.8%) than males (n=252, 40.2%) (**Table 5.1**). We also noted several differences between males and females in our population. The females in our study were younger (p<0.01) and less likely to

be college educated (p<0.01) (**Table 5.1**). Females had thinner lesions than men (p=0.02) and had more melanomas on their extremities (p<0.01). Females also had a different distribution of histologic subtypes; that is, they had fewer lentigo maligna melanomas (LMM) and nodular melanomas, and they had more melanomas that were classified as 'other' (p<0.01) (**Table 5.1**). For behavioral variables, more females had used tanning beds (p<0.01), but had less painful sunburns as an adult and in their lifetime (p=0.02 and p=0.04, respectively) (**Table 5.1**).

5.4.2 SNPs associated with Breslow thickness

We identified 2 SNPs (rs1065080 and rs35874463) located in SMAD3 that were associated with Breslow thickness in females (**Table 5.2**). rs1065080 was inversely associated with Breslow thickness (OR 0.37, 95% CI=0.18-0.78), and rs35874463 was positively associated with Breslow thickness (OR 2.13, 95% CI=1.08-4.22) (**Table 5.2**). There were no SNPs significantly associated with Breslow thickness in males or the overall population. Interestingly, the direction of the odds ratios for rs1065080 and rs35874463 seem to be opposite for males compared to females (**Table 5.2**).

5.4.3 Multiplicative interactions with UV exposures

We identified 5 SNPs that interacted with UV exposures to modify Breslow thickness. For the overall population, rs2227306 (CXCL8) interacted with childhood burns and was associated with decreased Breslow thickness (OR 0.68, 95% CI=0.47-0.98) (**Table 5.3**). That is, a person who has the minor allele and had greater than

10 burns as a child will have a decreased Breslow thickness compared to someone with either the minor allele or greater than 10 burns as a child independently. rs2227306 also interacted with lifetime burns in females and was associated with decreased Breslow thickness (OR 0.57, 95% CI=0.35-0.93) (**Table 5.3**). A SNP in IFNγ (rs2069705) interacted with indoor tanning use and was associated with decreased Breslow thickness in females (OR 0.55, 95% CI=0.31-0.99) (**Table 5.3**). The only SNP (rs3819035, IL-17A) that interacted with a UV exposure in males was associated with increased Breslow thickness (OR 10.47, 95% CI=1.31-83.43) (**Table 5.3**).

5.5 Discussion

Immune response has been recognized as an important factor in melanoma survival [64]. In fact, many of the current melanoma therapeutics exploit immune response pathways to combat the disease [65]. It is likely that the immune system has complicated interactions regarding sex differences in melanoma since both UV exposures (which vary between men and women) and sex steroid hormones can affect the immune response [7,55,128]. Our study identified 5 SNPs in 4 different immune response genes that may play a role in melanoma progression in a sex-dependent manner. Interestingly, each of the genes identified in our study have previously been associated with melanoma, estrogen, and/or UV exposures.

We identified 2 SNPs in SMAD3 that were associated with Breslow thickness in females. Inhibition of SMAD3 results in resistance to TGFβ-induced cell cycle arrest in melanoma cells [134]. Interestingly, it has been shown that estrogen promotes

SMAD3 degradation [135]. While estrogen appears to have a protective effect in melanoma, estrogen-induced SMAD3 degradation suggests there may be a more complicated role [136]. Furthermore, it has been shown that UV exposure decreases expression of SMAD3. These previous findings, along with the trend we identified in female melanoma patients, suggest a potential role for SMAD3 in a complicated and context-dependent manner.

Our analyses revealed a SNP in CXCL8 that interacted with childhood burns in the overall population and lifetime burns in females and decreased Breslow thickness. UV exposure has been shown to increase secretion of CXCL8 [137]. UV has been associated with melanoma survival; however, CXCL8 is involved in melanoma progression and metastasis [50,53,111,138]. Furthermore, estrogen has been shown to increase transcription of CXCL8 in breast cancer [139]. Together, these findings suggest a complex interaction between UV exposures, CXCL8, and melanoma progression that may be further complicated by sex-steroid hormones.

We also identified a SNP in IFNγ that interacted with indoor tanning use in females and decreased Breslow thickness. There is evidence that UV-induced IFNγ is involved in melanomagenesis, and promotes tumor survival [140]. However, it has also been shown that IFNγ is necessary for VDR expression, and Vitamin D deficiencies have been associated with increased Breslow thickness [60,128].

In males, we identified a SNP in IL-17A that interacted with adulthood burns to increase Breslow thickness. IL-17A has previously been shown to have a tumor-promoting role in melanoma [141]. Interestingly, UV has been shown to increase IL-

17A secretion and impact DNA repair capacity, which has been linked to both melanoma risk and survival [95,120,142].

We recognize that our study had limitations. First, there are many immune response genes that have been shown to play a role in melanoma, and we investigated only a small subset of SNPs in 15 genes. However, our results supported our hypothesis that different immune response SNPs contribute to Breslow thickness in males compared to females. Second, our study consisted of intronic SNPs, so it is difficult to determine the functional importance of our findings, especially since the effect of estrogen and UV on the immune response further complicates the interpretation. Furthermore, none of our SNPs were significant following FDR correction. However, identification of different SNPs in relation to Breslow thickness in males compared to females suggests there may be some biological significance to our findings.

Our study also had multiple strengths. First, we have extensive UV information on the participants in the study allowing us to uniquely contribute to the existing literature on immune response SNPs and Breslow thickness. Second, the participants in our study were ages 25-59. Since the average age at menopause is 51, it is less likely that our results were affected by decreasing estrogen levels following menopause compared to studies with older participants. Finally, our study may help to further explain the complicated role of UV exposures in melanoma progression and survival.

In summary, we identified 5 SNPs that were associated with Breslow thickness that differ in their effect between males and females. These results indicate that different genomic loci are important to melanoma progression in males compared to females. It is likely that the role of estrogen and UV exposure impact these differences. Therefore, future functional studies regarding the role of estrogen and UV in the progression of melanoma are warranted. Furthermore, our study highlights the importance of sex-stratified analyses in genetic epidemiology, particularly in relation to melanoma gene-environment interactions that vary between the sexes.

5.6 Tables

Table 5.1 Chi-squared analysis of sex differences in the Minnesota population					
	Males (n=252; 40.2%)		Females (n=375; 59.8%)		*p*-value
	n	%	n	%	
Age					
<30	6	2.4	34	9.1	
30-39	35	13.9	65	17.3	
40-49	77	30.6	149	39.3	
50-59	134	53.2	127	33.9	**<0.01**
Completed college					
No	107	42.5	201	53.6	
Yes	145	57.5	174	46.4	**<0.01**
Income >$60,000					
No	66	26.3	114	31.0	
Yes	185	73.7	254	69.0	0.21
Missing					
Breslow thickness					
Thin <1mm	183	72.6	301	80.3	
Thick	69	27.4	74	19.7	**0.02**
Body site					
Scalp/Neck	18	7.1	8	2.1	
Face	27	10.7	17	4.5	
Trunk	116	46.0	92	24.5	
Upper extremities	60	23.8	105	28.0	
Lower extremities	26	10.3	149	39.7	
Unknown	5	2.0	4	1.1	**<0.01**
Ulceration					
No	231	93.9	335	94.4	
Yes	15	6.1	20	5.6	0.81
Missing					26
Histology					
Superficial spreading	110	43.7	154	41.1	
Nodular	20	7.9	18	4.8	
Lentigo maligna	13	5.2	8	2.1	
Other	109	43.3	195	52.0	**0.03**
Missing					8
Painful burns as child					
Less than 10	145	57.5	242	64.5	
Greater than or equal to 10	107	42.5	133	35.5	0.08
Painful burns as adult					
Less than 10	181	71.8	296	78.9	
Greater than or equal to 10	71	28.2	79	21.1	**0.04**
Lifetime painful burns					
Less than 10	107	42.5	196	52.3	
Greater than or equal to 10	145	57.5	179	47.7	**0.02**
Indoor tanning					
Never	146	57.9	87	23.2	
Ever	106	42.1	288	76.8	**<0.01**

Bold=significant

Table 5.2 SNPs Associated with Breslow Thickness									
				Female				Male	Overall
Gene	**SNP**	**Minor Allele**	**Major Allele**	**MAF**	**OR (95% CI)***	***p*-value***	**FDR Corrected *p*-value***	**OR (95% CI)***	**OR (95% CI)**
SMAD3	rs1065080	A	G	0.13	**0.37 (0.18-0.78)**	<0.01	0.18	1.27 (0.72-2.25)	0.72 (0.47-1.11)
SMAD3	rs35874463	G	A	0.06	**2.13 (1.08-4.22)**	0.03	0.32	0.39 (0.11-1.38)	1.29 (0.72-2.30)

Table 5.3 Multiplicative Interaction Analyses for SNPs and UV Exposures

Stratificaton	UV Exposure	Gene	SNP	Minor Allele	OR(95% CI)	*p*-value
Overall	≥ 10 Childhood Burns	CXCL8	rs2227306	A	0.68(0.47-0.98)	0.04
Males	≥ 10 Adulthood Burns	IL-17A	rs3819025	A	10.47(1.31-83.43)	0.03
Females	Ever Use	IFNγ	rs2069705	G	0.55(0.31-0.99)	0.04
	≥ 10 Lifetime Burns	CXCL8	rs2227306	A	0.57(0.35-0.93)	0.02

CHAPTER SIX:

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions:

Despite the fact that a female survival advantage in melanoma has been observed for many decades, there is still no definitive explanation for why males have poorer survival than females [28]. A common hypothesis is that males have thicker lesions at diagnosis because they are less aware of their skin, and thicker lesions at diagnosis are associated with increased mortality [52]. However, we demonstrate that the female survival advantage is likely a complex phenomenon, and cannot be reduced entirely to skin awareness.

In survival models for melanoma, sex has previously been identified as an independent prognostic indicator for melanoma [27,31]. However, because sex is also tightly correlated with Breslow thickness, the sex effect does not appear as an independent prognostic indicator in some populations. This was the case in our investigation of melanoma survival at 5-year and 15-year follow-up time points. Despite the fact that sex was not an independent prognostic indicator in the survival models for all of the cases (referred to as overall population), we identified different factors contributing to melanoma survival in the sex-stratified analyses.

In particular, behavioral factors contributing to survival were different in males compared to females. UV exposures, such as high intermittent sun exposure, were associated with decreased hazards in males, especially in the 5-year follow-up model. UV exposures did not significantly predict survival in females. Skin awareness was significantly associated with increased survival for females in both the 5-year and 15-year follow-up models. Skin awareness was borderline significant

in the baseline model for males at 5-year follow-up, but was not included in the adjusted models. At the 15-year follow-up, skin awareness was not associated with male survival. Furthermore, while a higher percentage of females in the population reported being aware of their skin, nearly 50% of men reported being aware of their skin. Therefore, our finding that skin awareness is not predictive of survival in men is not due to lack of reported skin awareness in the male population.

Consistent with previous studies, Breslow thickness was the strongest predictor of survival. We also found that males with thick lesions had poorer survival than women with lesions of the same thickness. When comparing intermediate lesions to thin lesions and survival within sexes, we found that males had a higher hazard ratio than females. Furthermore, our results indicated that a variable combining Breslow thickness and sex to predict survival is a preferred model to one examining Breslow thickness and sex individually, as measured by AIC. Finally, we found that Breslow thickness at diagnosis was a stronger predictor of long-term survival in females than males. These results suggested that factors contributing to Breslow thickness might also vary between males and females.

To date, there are no other studies that investigate the multiple factors that may contribute to Breslow thickness, especially in a sex-stratified analysis. We evaluated other histopathological factors in the Breslow thickness models, and found the same associations in males and females. These results are difficult to interpret as all histopathological factors are markers of melanoma progression, and are likely collinear with Breslow thickness rather than explanative of Breslow thickness.

Opposite to the survival analysis, we found that UV exposures were associated with decreased odds of a thick lesion in females, but were not associated with Breslow thickness in males. Some of the UV exposure effect may be due to the correlation of skin awareness with UV behaviors; however, in a baseline analysis of females who reported being unaware of their skin, UV exposures were still inversely associated with Breslow thickness. This suggests that UV exposure effects on survival were encompassed by the Breslow thickness variable in our survival models, whereas UV exposure influenced male survival independent of Breslow thickness. Therefore, it is likely that UV exposure impacts melanoma progression and survival differently in males compared to females. Furthermore, skin awareness was associated with decreased Breslow thickness in females, but not in males. In fact, we did not identify any behavioral factors associated with Breslow thickness in males.

To evaluate the self-reporting of skin awareness, we performed a chi-squared contingency analysis, and found that skin awareness was similarly associated with college education, indoor tanning, and ever had a painful burn in both males and females. This finding suggests that the lack of association between Breslow thickness and skin awareness in males is not due to differences in reporting of skin awareness between the sexes. Overall, our findings suggest that male melanomas are inherently more aggressive and may be biologically distinct from female melanomas. The importance of skin awareness and SSE in early detection of melanoma should not be minimized; however, its limitations, particularly in males, should be recognized.

It is clear that UV exposure plays a complex role in melanoma initiation, progression, and survival, and it has impacts on multiple systems in the body that are also important to melanoma including the immune system and DNA repair [71,128]. One way to examine inter-individual variations in immune response and DNA repair is via investigation of SNPs located in genes associated with those processes [73]. Furthermore, it is evident that sex steroid hormones can influence UV exposure response, immune response, and DNA repair [68,69,73,128,143]. One way to control for sex-steroid hormone interactions with these systems is to stratify analyses by sex. Therefore, we investigated SNPs in DNA repair and immune response genes and their associations with Breslow thickness in a sex-stratified analysis. To factor in UV exposures, we investigated SNP interactions with UV exposures that modify Breslow thickness in a sex-stratified analysis.

In the analysis of DNA repair SNPs, we identified 3 SNPs associated with Breslow thickness in males and 7 SNPs associated with Breslow thickness in females. Of these SNPs, there was only one that overlapped between sexes. We also identified 10 SNPs in males that interacted with UV exposures to modify Breslow thickness, and 13 SNPs in females that interacted with UV exposures to modify Breslow thickness. Of these SNPs, there were no SNPs that overlapped between males and females. While none of the SNPs were significant following FDR correction, it is important to consider the biological relevance. The lack of overlap in SNPs between males and females, especially those that interacted with UV exposures, suggest that DNA repair responses to UV exposure may vary between men and women.

Importantly, these variations appear to affect Breslow thickness, which indicates that melanoma progression is impacted.

In the analysis of immune response SNPs, we identified 2 SNPs that were associated with Breslow thickness in females. We did not identify any SNPs that were associated with Breslow thickness in males. We identified 3 SNPs that interacted with UV exposures to modify Breslow thickness. Of these, 1 SNP modified Breslow thickness in males, and 2 SNPs modified Breslow thickness in females. There was no overlap in immune response SNP associations between the sexes. Similar to the DNA repair SNPs, none of the SNPs were significant following correction for FDR. However, these differences in males and females suggest a potential difference in the role of immune response in the progression of melanoma, especially following UV exposures.

6.2 Future studies:

We investigated the association of DNA repair and immune response SNPs with Breslow thickness, along with their interactions with UV exposures to modify Breslow thickness, in a sex-stratified analysis. DNA repair has been shown to play a role in melanoma from melanomagenesis to metastasis and survival [71]. Therefore, it would also be beneficial to investigate DNA repair SNP associations in melanoma risk and survival in a sex-stratified analysis. Similarly, melanoma is known to be an immunogenic tumor, highlighting the importance of investigating immune response SNP associations in melanoma risk and survival in a sex-specific manner [64,65]. Furthermore, investigating DNA repair and immune response SNP interactions that

modify melanoma risk and survival in males compared to females may provide more insight to the sex-specific role of UV exposure in melanoma.

To further investigate the SNPs we identified, they should be functionally evaluated. That is, we should determine whether the SNPs are in linkage disequilibrium with any functional SNPs. Furthermore, fine mapping studies investigating heritability and disease outcomes may provide functional clues. Finally, epigenetic changes, such as promoter methylation, may be associated with SNPs identified in our studies. In particular, MGMT promoter methylation is associated with increased therapeutic response, and we identified multiple SNPs in MGMT that were associated with Breslow thickness, especially in males [72,76,127]. Therefore, investigating MGMT promoter methylation and SNP associations would provide functional insight and perhaps identify sex differences in therapeutic response.

Interestingly, while ERβ is expressed in melanoma, and high expression has been associated with decreased Breslow thickness, there is very little known about the role of ERβ in melanoma etiology and progression [39]. Therefore, it is important to develop functional studies investigating ERβ in melanoma. We suggest that melanoma cells be treated with ERβ agonists and antagonists to evaluate the in vitro effects of ERβ on proliferation and invasion, along with evaluation of downstream effectors including genomic and non-genomic events. Furthermore, animal studies investigating the interaction between UV exposures and ERβ are important to learning about the role of UV exposures in the female survival advantage.

Similarly, the role of GPER in melanoma is unknown. Our preliminary data suggests that GPER is, in fact, expressed in melanoma tissues. However, future studies investigating its expression in males compared to females are needed. Following identification of GPER in melanoma tissues, the functional role of GPER in melanoma should be investigated.

6.3 Overall conclusions and perspectives:

Our study suggests that the explanation for the female survival advantage in melanoma is complex. Behavioral differences in males and females, such as UV exposure, appear to interact with biological differences to impact the progression of, and ultimately the survival from, melanoma. These findings have multiple implications in melanoma. With the importance of behavioral and biological interactions demonstrated, it is imperative that future studies of melanoma consider investigating melanoma risk, progression, and survival in a sex-stratified manner. Furthermore, it is necessary to determine the impact of UV exposures on melanoma risk, progression, and survival in males compared to females to fully understand the role of UV exposure in melanoma. Notably, these distinctions could be instrumental in personalized treatment of melanoma and therapeutic development.

Furthermore, these findings have implications for other diseases and epidemiological studies as a whole. First, there are many other diseases with evident sex differences that may benefit from sex-stratified analyses including cardiovascular diseases, lung cancer, and autoimmune disorders [36,68,144]. Second, epidemiological studies are generally stratified by population [145]. For

example, African Americans may be excluded from a study investigating cardiovascular disease limiting the study to Caucasians. This is because African Americans are known to be a genetically distinct population with different incidence and outcomes in cardiovascular disease [145]. Therefore, it is assumed that there are biological differences between the populations that would confound the results. Similarly, there are biological differences between males and females that confound results, as shown in our study. However, in the past, epidemiological studies adjust for sex in the model rather than stratifying by sex. Importantly, it is likely that the lack of sex-stratification is confounding results, even in epidemiological studies of diseases that do not have evident sex differences.

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