A study of the effect of incidental drug exposures, body mass index and diabetes on survival from melanoma

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“I confirm that the work submitted is my own, except where work which has formed part of jointly authored publications has been included. The contribution of myself and the other authors to this work has been explicitly indicated below. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others.”

Some of the data collected for chapters 3 and 4 in the thesis and the methodology in chapter 2 was used in the following publication. 25-Hydroxyvitamin D2/D3 levels and factors associated with systemic inflammation and melanoma survival in the Leeds Melanoma Cohort, *Newton-Bishop JA, Davies JR, Latheef F, Randerson-Moor J, Chan M, Gascoyne J, Waseem S, Haynes S, O'Donovan C, Bishop DT. Int J Cancer 2014 Nov 18*

My role was predominantly in data collection and in reviewing the draft publication. The paper above focused on vitamin D and inflammation rather than incidental drugs which is the main focus of my thesis. The first two authors Prof Newton-Bishop and John Davies led the analysis and write up for the paper above and I have also acknowledged their help in my thesis in the next section.

Abstract

Introduction: Drugs intended to treat incidental medical conditions could moderate host-tumour interaction and therefore melanoma survival.

Method: Drug exposure data were collected from the 2184 newly diagnosed melanoma patients in the Leeds Melanoma Cohort (recruited 2000-2012) and their primary care physicians. An ever-never analysis and drug usage at diagnosis of melanoma (including 12 months prior) were chosen as the most applicable analysis methods (overall and sex stratified). The effects of exposure to different classes of drugs on MSS and overall survival (OS) were then assessed using Cox Proportional Hazards models whilst adjusting for confounding variables including diabetes and BMI, firstly in unadjusted models followed by adjustment for known predictors of MSS in a multivariate model.

Results: For most drugs there were no statistically significant effects on MSS. The drugs that I ultimately chose to look at in detail were aspirin, simvastatin and metformin.

Whilst adjusting for age and Breslow thickness, women who had ever taken aspirin were significantly less likely to die from their melanoma compared with those who never used the drug at any point in their lifetime with hazard ratios (HR) for MSS of 0.51 (95% CI: 0.30-0.87, P= 0.014) compared to men with an HR (MSS) 0.99 (95% CI: 0.71-1.37, P= 0.948).

With both ever/never use of simvastatin and at diagnosis (including 12 months prior) analysis, when adjusting for age and Breslow thickness, men had a significantly reduced risk of death from melanoma with HRs of 0.54 (95% CI: 0.37-0.79, P=0.002) and 0.57 (95% CI: 0.38-0.85, P=0.006) respectively when compared to females who had HRs of 1.22 (95% CI: 0.82-1.83, P=0.327) and 1.21 (95% CI: 0.79-1.86, P = 0.379).

Metformin usage was negatively associated with MSS in individuals with primaries on the trunk, which was used here as a surrogate marker for *BRAF* mutated tumours with an HR of 3.87 (95% CI 1.29-11.57, P= 0.02) for chest primaries.

Conclusion: The associations seen in my thesis require further validation in larger international data sets as well as examination of biological models to assess if these represent real effects or whether confounding factors are responsible for these changes. I would propose that future studies looking at factors influencing melanoma survival should consider stratifying their findings by sex.

KEYWORDS: drugs, melanoma survival, chemoprevention, metformin, aspirin, statins

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Abbreviations

ACC	Acetyl-CoA carboxylase
AJCC	American Joint Committee on Cancer
ALT	Alanine transaminase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AMS	Atypical mole syndrome: presence of many naevi often
ASA	Acetyl salicylic acid
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BMI	Body Mass Index
BNF	British National Formulary
CAG	Confidentiality Advisory Group
CDKI	Cyclin-dependent kinase inhibitors
CI	Confidence Interval
CK	Creatinine kinase
CM	Cutaneous Melanoma
COX	Cyclooxygenas
CRC	Colorectal cancer
CRP	C-Reactive Protein
CRUK	Cancer Research UK
CSC	Cancer stem cells
CSS	Cancer-specific survival
CVD	Cardiovascular disease
DCSI	The Diabetes Complications Severity Index
DFS	Disease-free survival
DLT	Dose-limiting toxicities
DNA	Deoxyribonucleic acid
FAS	Fatty acid synthase
FNAC	Fine needle aspiration cytology
FPP	Farnesylpyrophosphate
GCP	Good Clinical Practice
GenoMEL	Melanoma Genetics Consortium - http://www.genomel.org
GI	Gastrointestinal
GLUT-1	Glucose transporter 1

GPP	Geranylpyrophosphate
GPRD	General Practitioners' Research Database
HES	Hospital Episode Statistics
HIF-1α	Hypoxia-inducible factor 1
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme-A
HR	Hazard Ratio
hsCRP	High-sensitivity C reactive protein
HSCIC	Health and Social Care Information Centre
HUVEC	Human umbilical vein endothelial cells
IGF-1 and 2	Insulin growth factor 1 and 2
IHD	Ischemic Heart Disease
IMD	Index of Multiple Deprivation
IT	Information Technology
LDL	Low-density lipoprotein
LFA1	Leukocyte function antigen-1
LMC	Leeds Melanoma Cohort
LOX-1	Lectin-like oxidized LDL receptor 1
LTHT	Leeds Teaching Hospitals NHS Trust
MC1R	Melanocortin-1 receptor gene
MetS	Metabolic Syndrome
MD	Mammographic density
MICE	Multiple Imputation with Chained Equations
MREC	Multi-Centre Research Ethics Committees
MSS	Melanoma Specific Survival
mLST8	Mammalian LST8/G-protein β-subunit like protein
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NCDR	National Cancer Data Repository
NCIN	National Cancer Intelligence Network
NCRS	National Cancer Registration Service
NF-κB	Nuclear factor kappa B
NHS	National Health Service
NICE	National Institute for health & Care Excellence
NRES	National Research Ethics Service
NSAIDs	Non-Steroidal Anti-inflammatory Drugs
NO	Nitric Oxide

OCT1	Organic cation transporter 1
OR	Odds Ratio
OS	Overall Survival
OTC	Over The Counter
PI3K	Phosphoinositide 3-kinase
PG	Prostaglandins
PHE	Public Health England
PI	Principal Investigator
PPM	Patient Pathway Manager
RCT	Randomized control trial
R&D	Research and Development
RFS	Recurrence-free survival
RGP	Radial Growth Phase
ROS	Reactive Oxygen Species
RR	Relative risk
SLSP	System Level Security Policy
T2DM	Type 2 Diabetes Mellitus
TNF-α	Tumor Necrosis Factor
TNM	Tumour, Node, Metastasis
TX	Thromboxanes
TXA2	Thromboxane A2
VEGF	Vascular Endothelial Growth Factor
VGP	Vertical Growth Phase
UK	United Kingdom
UV	Ultraviolet
WHO	World Health Organisation

Chapter 1
Introduction

1.1 Introduction to the thesis

In this thesis, I examine the association of incidental drug exposures associated with metabolic syndrome (as represented by body mass index (BMI) and diabetic status) on melanoma survival, in the Leeds Melanoma Cohort (LMC) of cutaneous melanoma (CM) patients. Incidental drugs in this context refer to any drugs the patients may have been exposed to during a specified period to treat any other conditions other than the melanoma itself, which may include the metabolic syndrome. The metabolic syndrome refers to a state characterised by the development of multiple cardiovascular risk factors including insulin resistance, obesity, dysplipidaemia and hypertension and associated with low-grade inflammation [1]. Although melanomas may arise in the eye or from mucosal surfaces, this thesis is concerned only with the most common type of melanoma, which arises in the skin.

This work was prompted by – firstly, the work reported in studies of other cancers, which suggested that use of commonly used drugs such as aspirin [2], statins [3] or metformin [4] may change cancer risk. Secondly, by laboratory data, which suggested biological mechanisms to support the hypothesis that drugs might modify the likelihood of surviving, e.g. metformin in *BRAF* mutated melanoma [5]. Thirdly, by the observation that although the American Joint Committee of Cancer (AJCC) predicts survival reasonably well, there is still a significant degree of variance which remains unexplained (30 to 40%). My hypothesis is that lifestyle or exposure to drugs e.g. metformin may moderate host/tumour interaction and therefore survival contributing to the unexplained variance, as also postulated by Chen and Mellman [6].

Studies designed to understand the effects of concurrent drug exposure should take account of the following complexities. Most drugs are more frequently used in older individuals with concurrent diseases associated with systemic inflammation and increased age is associated with poorer cancer survival. That reduced survival might have been reported as a result of confusion of between cancer and non-cancer related death, reduced access to health care in the infirm, reduced tolerance of effective drugs or biological effects of the systemic inflammation associated with the co-morbidities.

These drugs may also be biologically related themselves to risk, via their mechanism of action [7] either by reducing the mediators of systemic inflammation or as a result of as yet unrecognised effects.

1.2 Thesis Layout

The layout of my thesis is as follows:

In chapter 1, I have presented the background to the topic and arrived at the aims of the study.

In chapter 2, I have presented the materials and methods used in the study.

In chapter 3, I have described the Leeds Melanoma Cohort data (which was the study data set used in this thesis) and explored potential candidate drugs and arrived at my three chosen drugs.

In chapter 4, I have examined the association of aspirin exposure with melanoma survival including undertaking a literature review, reported materials and methods specific to aspirin, and presented my results with respect to aspirin and concluded with a discussion of my findings.

In chapter 5, I have examined the association of statin exposure with melanoma survival including undertaking a literature review, reported materials and methods specific to statins, and presented my results with respect to statins and concluded with a discussion of my findings.

In chapter 6, I have examined the association of metformin exposure with melanoma survival including undertaking a literature review, reported materials and methods specific to metformin, and presented my results with respect to metformin and concluded with a discussion of my findings.

In chapter 7, I have presented a general discussion of my thesis as well as reviewed the relevant literature in light of my findings, discussed the limitations of my approach and finally drawn conclusions and suggestions for future work based on my results.

1.3 What is Melanoma

Melanoma is a skin cancer that is derived from pigment cells or melanocytes. Melanocytes are found in the basal layer of the epidermis and are responsible for generating melanin, which is the pigment responsible for a “suntan” and offers protection for the skin against the harmful effects of ultraviolet radiation exposure.

1.4 Types of Melanoma

Four main clinicopathological subtypes of melanoma are recognised clinically and histologically: superficial spreading melanoma (SSMM); nodular melanoma (NMM); lentigo maligna melanoma (LMM), and acral lentiginous melanoma (ALMM) [8-11].

There are also several uncommon variants that constitute less than 5% of the cases, for example desmoplastic, spitzoid, and naevoid melanoma. The term melanoma *in situ* (MMIS) is used when melanoma cells are confined to the epidermis with no invasion into the dermis. Criteria for the histological diagnosis of melanoma are architectural and cytological [12].

1.4.1 Superficial spreading melanoma (SSMM)

This is the most frequently observed type of melanoma in white skinned peoples and accounts for 70% of cases seen within this group of people. It most commonly occurs at sites of intermittent, intense sun exposure (on the trunk in men, and on the legs and back in women) and is the most frequent type in individuals aged 30-50 years [13]. It can appear *de novo* or be associated with a naevus and slowly progresses to a plaque, often comprising multiple colours and pale areas of regression. The concept of radial growth phase was first introduced by Clark to describe a protracted phase of growth in which proliferation occurs first in the most superficial part of the skin (epidermis) [14]. SSMM has a radial growth phase (RGP), during which the lesion is predominantly in the epidermis but steadily increasing in diameter, followed by a vertical growth phase (VGP) in which the lesion extends downwards into the dermis and exhibits increased metastatic potential [14]. These melanomas are often found to have somatic mutations in the *BRAF* gene (most commonly V600E) [15]. It is hypothesised that these tumours result from sunburn associated sun bathing and occur on skin sites usually only exposed during recreational activities such as the back in men [13]. The term "*in situ*" may be used to describe this phase and whilst *in situ* lesions are typical of SSMM, they are also seen in LMMM and ALMM as will be discussed below. *In situ* disease progresses slowly, possibly taking many years to develop before genetic changes in the malignant cells occur which enable the malignant cells to invade and metastasise. During this period of relative quiescence these cells it might be argued, might be subject to host influences, which might promote tumour evolution or suppress it. My hypothesis is, therefore, that drug exposure in the period up to and including the period of diagnosis may modify stage at presentation.

1.4.2 Nodular melanoma (NMM)

It accounts for nearly 5% of all melanomas in pale skinned peoples. Such tumours usually appear as exophytic (protruding from the skin surface), brown-black and frequently eroded or ulcerated tumours, occurring most commonly on the legs and trunk of older men (greater than 60 years old) [16]. Since they have a predominantly VGP, devoid of RGP, NMM has a tendency toward greater depth of invasion and is associated with a worse prognosis than the other common subtypes [13]. There can also be the presence of an *NRAS* mutation, which has also been associated with a tendency to metastasis [17].

1.4.3 Lentigo maligna melanoma (LMM)

This accounts for 4-15% of melanomas in the UK. It is typically located on chronically sun-damaged skin (such as the head, neck, or arms) of pale-skinned older individuals [13]. Its benign precursor lesion (a form of *in situ* melanoma), lentigo maligna, is a tan macule that grows slowly in a radial fashion and may eventually display a palpable component of VGP clinically signalling progression to LMM [18]. Histologically, it is characterized by a lentiginous component [19]. There is proliferation of atypical melanocytes at the dermo-epidermal junction and features of chronic sun exposure (solar elastosis) [13]. In time these lesions may progress to a nodular invasive phase (LMM).

1.4.4 Acral lentiginous melanoma (ALMM)

ALMM occurs on the palms, soles, or beneath or around the nail plate (subungual variant) accounting for 2-8% of cases in pale-skinned persons. It accounts for 29-72% of melanoma in dark-skinned individuals, such as African-American, Asian, and Hispanic [20]. The incidence of this melanoma type is approximately similar in all ethnicities and there is no evidence that this form of melanoma is aetiologically related to skin colour or sun exposure [21].

1.5 Metastatic behaviour of Melanoma

Although a relatively uncommon form of cancer, melanoma is the primary cause of death due to skin cancer, and it has continued to increase in incidence. If the diagnosis is made at an early stage, a surgical excision is usually enough to cure > 90% of cases.

Although metastatic melanoma survival is changing rapidly with the advent of adjuvant and palliative immunotherapy, historically patients with metastasis (stage III and IV) survive for < 1 year, with a median survival of around 6-8 months. The 1-year survival rate was 45%, and for ≥ 5 years, the survival rate was < 10% [22]. While, a minority of newly diagnosed melanoma patients (4%), have distant metastasis at the time of diagnosis, the majority of the patients who are diagnosed at initial stage ultimately progress to metastatic disease as a result of disease advancement [23]. It is reported that approximately 33% of all melanoma patients will have recurrence of the disease [24].

In general, stage III metastatic melanoma patients (nodal metastases) have earlier recurrences than patients with negative lymph nodes. Additionally, age of the patient at the time of diagnosis also affects the timing of distant metastases i.e., patients aged > 50 years relapse earlier compared to younger patients [25].

Melanoma can metastasize to any tissue or organ, involving some of the sites seldom observed with various solid tumours [26]. However, there are sites, which are more likely to act as sanctuary for primary distant metastases. The most frequently observed primary sites of distant metastases include the skin, subcutaneous tissue, and lymph nodes, which are reported in 42 to 59% of melanoma patients. However, the primary sites of relapse seen in around 25% of all metastatic melanoma cases remains visceral organs and the most frequently involved sites of visceral metastases, in the decreasing order, are the lungs (18–36%), liver (14–20%), brain (12–20%), and bones (11–17%) [22].

Among the independent predictors of survival in patients with metastatic disease, the site of distant metastasis is an important predictor [26, 27]. It was reported that the patients with visceral metastasis have poor survival rates than the patients with loco-regional, distant nodal, and soft tissue metastasis [27, 28]. Moreover, it was reported that patients with lung as the only site of visceral metastasis had a superior 1-year survival than the patients with metastasis to other visceral organs. Median survival observed in patients with metastatic melanoma was 12 months and 7 months in those with metastasis to lungs and visceral organs other than the lung, respectively. Moreover, the median survival was 18 months in patients with metastasis to non-visceral sites (i.e., skin, subcutaneous tissue, and distant lymph nodes) [27, 29].

Another independent predictor of survival in patients with metastatic disease is the number of metastatic sites [30]. Patients with one distant metastatic site have a significantly improved outcome compared with those with two or more distant sites [27]. A study reported the 1-year survival rate of 36%, 13%, and < 1% in patients with one, two, and three or more metastatic sites, respectively [30]. Another study reported the median survival in patients with one and more than one metastases as 23 months and 8

months, respectively [31]. Additionally, the stage of melanoma prior to distant metastasis also acts as an important prognostic factor. In cases where patients progressed directly from stage I or II, a disease-free interval of ≥ 34 months was associated with prolonged survival, while in the case of patients with stage III melanoma, a disease-free interval of ≥ 18 months was shown to be associated with prolonged survival [32]. These differences according to number of metastatic sites or different sites are likely to reflect biological differences between tumours. Later progression does however imply that tumour cells may sit within niches in the body for some time after removal of the primary during which period they may be subject to changes in the host tumour environment.

For the purpose of this thesis it could be argued that the chances of a recurrence following the initial melanoma may be subject to host influences, including exposure to drugs or the presence of co-morbidities, which may promote tumour evolution or suppress it. For a majority of patients, prior to distant metastases, the time it takes for a recurrence or metastasis to occur appears to be inversely related to the stage of the tumour at presentation. In patients with thicker tumours, there is a higher risk of recurrence during the first year following the treatment and this decreases gradually with time. Following the initial diagnosis of the primary tumour, around 55 to 79% and 65 to 85% of the recurrences become evident in 2 and 3 years, respectively. Moreover, patients with ulcerated tumours have significantly shorter disease-free period [24, 26] and a previous study conducted by our group using the data in this cohort, has shown that ulceration (a validated prognostic factor in the AJCC criteria) may be a marker of inflammation that is in turn linked to other mediators of inflammation including smoking status and potentially the effects of drugs such as aspirin and the metabolic syndrome and vitamin D [33].

1.6 Epidemiology

1.6.1 Incidence and Future Trends

Melanoma is responsible for the majority of deaths due to skin cancer worldwide and 0.7% cancer-related deaths [34]. The global incidence of cancer has increased and so has the number of melanoma cases [20]. Melanoma was a relatively rare form of cancer, but in the last 5 decades its incidence has increased at a much faster pace compared to almost any other cancer in many countries in which the dominant skin colour is white. Its annual incidence has increased as rapidly as 4-6% in many pale-skinned populations that predominantly inhabit regions such as Northern Europe, North America, New Zealand, and Australia [34, 35]. In contrast, incidence rates are 10 to 20-fold lower in

non-white populations such as Hispanics, African Americans, American Indians, and Asians living in the US [36, 37]. Moreover, among the 195 countries studied, age-specific melanoma incidence rates and mortality rates are highest in New Zealand, Australia, Norway, Sweden, and the Netherlands [35].

It has been projected that based on the trends in melanoma incidence in the period from 1975-2004, incidence rates in many age groups will continue to increase until 2040 (Figure 1.1). Furthermore it has been projected that the incidence rates in people aged 60-79 years is likely to increase by a further third from current levels with the largest projected increase likely to be in people aged over 80 years [35, 38]. Although incidence rates do rise steadily with age, there is still a substantial number of cases affecting young adults with almost one third of all cases occurring in people aged less than 50 years, conferring a high burden of disease in terms of years lost and years living with a cancer diagnosis. However, despite the increase in the incidence of melanoma, mortality has been reported to have leveled off since the 1990s. This has been attributed to new measures for successful early detection of the condition [39]. Following the global trend, the incidence of melanoma has increased across Europe. Within Europe (2016), the UK has the 7th largest incidence rate of skin cancer, estimated at 19 per 100,000 people, compared to an average of 13 per 100,000 people [40]. In the UK, the incidence of skin cancer has increased by 45% in the last decade. Moreover, this rise in incidence has especially affected men, who have seen a 56% increase [41]. It is again worth considering whether this significant difference in risk between genders could be influenced by host factors and is something I will explore further in this thesis. In general, the number of localized thin melanomas is also increasing in white populations and especially, among younger women [42]. Recent epidemiological studies suggest that melanoma *in situ*, which is increasing by 9.5% annually, is responsible for a disproportionately higher percentage of the overall melanoma increase. But the total incidence of melanoma (i.e., invasive and *in situ*) was also found to rise by 2.6% each year [43].

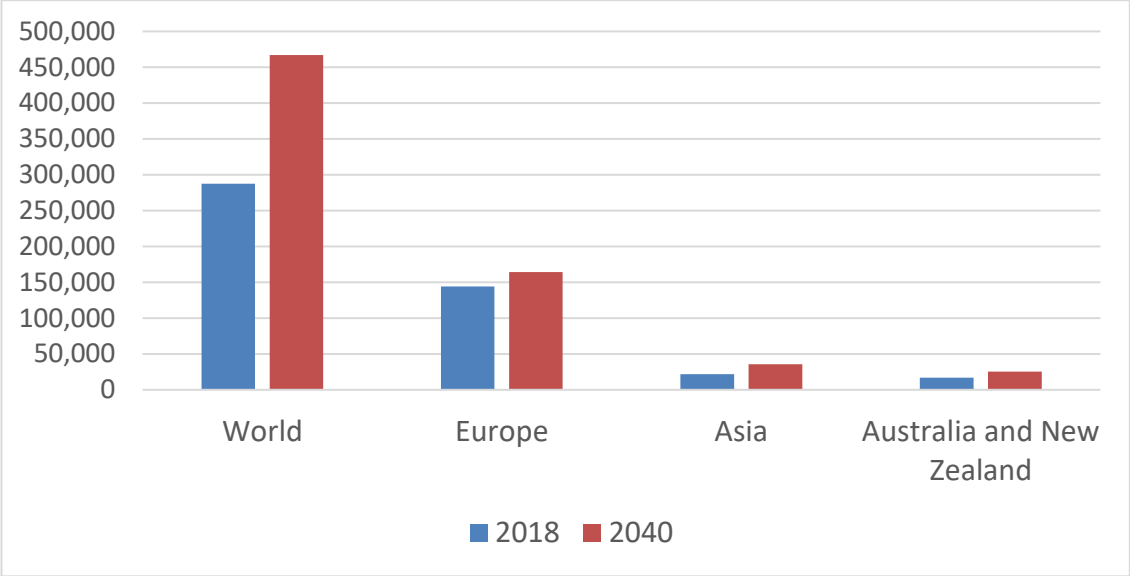


Figure 1.1: Projected incidence of melanoma of skin (on the basis of regions, both the genders and all age groups) per 100 000

Data source: GLOBOCAN 2018 [44]. Generated using an on-line tool at http://gco.iarc.fr/

1.6.2 Epidemiological data relating to melanoma aetiology

The marked difference in melanoma incidence in populations with pale skin compared with those with darker skin led to the hypothesis that sun exposure was an aetiological factor. The strongest behavioural risk factor for melanoma is sun bathing but reported sunburn overall irrespective of behaviours is the most robust non-phenotypic risk factor [45]. These observations further substantiated evidence that intense sun exposure causes melanoma.

Although partly attributable to increased surveillance and early detection, a proportion of the current trend of increased melanoma incidence is almost certainly due to lifestyle factors in terms of excessive recreational exposure to high-intensity sunlight and sunburns [45, 46]. Cheap flights from high-latitude countries in Europe to sunny holiday resorts are available all year round and it has been postulated that some of the increase in incidence noted could be explained by greater opportunities for burning of fair, non-acclimatised white skin [47].

1.6.3 Risk Factors

Melanoma is well described as being a malignancy that is complex and heterogeneous in nature [48]. The majority of risk factors are non-modifiable including pale skin that burns in the sun, blue eyes, red hair, older age, history of sunburns, clinically atypical

melanocytic naevi (moles), prior melanoma, and family history of melanoma. However, sunburn is one of the few modifiable risk factors for melanoma prevention. As a result, prevention of melanoma is primarily focused on sun protection measures and secondary prevention addressed to early detection of melanoma [10, 13, 47-49].

1.6.3.1 Race

As described above, people with type I and II skin have an increased risk of developing melanoma compared to people with darker skin colour (i.e., Africans, East Asians, and Hispanics). There are a number of pigmentation related risk factors, each of which is associated with an approximate doubling of risk, including pale skin, blue or green eyes, freckles, blonde or red hair, and sun sensitivity or inability to tan [46]. Inherited variants of the melanocortin-1 receptor (*MC1R*) gene are associated with the combination of red hair, freckling, and sun sensitivity. These are very common in populations living at high latitude, who are vulnerable to sunburn and therefore melanoma with access to sunny holidays.

1.6.3.2 Excessive exposure to sun

Excessive sun exposure is a major environmental factor for melanoma development. The sun is the principal source of ultraviolet (UV) radiation. Studies have shown that the risk of developing melanoma is most strongly linked to intermittent exposure to high-intensity sunlight, often resulting in sunburn, such as on holidays, rather than to the chronic exposure seen in occupations such as farming [45, 46]. Migration studies suggest that severe episodic sunburn in early life correlates most strongly with melanoma risk [50]. UV radiation can be sub-divided into 3 different types based on the respective wavelengths. These are UVA with the longest wavelength (315 to 400 nm), UVB (280 to 315 nm), and UVC (100 to 280nm). UVA and UVB are the main causes of the destruction of the skin's structure and the induction of melanocyte DNA damage, which leads to uncontrolled cell growth, as little UVC reaches the earth's surface. UVA exposure leads to the production of reactive oxygen species (ROS) that can cause DNA damage, including DNA breaks and oxidative modifications of nucleic acid bases. UVB radiation is genotoxic to DNA by photoproduct production. Failure to repair this DNA damage leads to DNA mutations in epidermis [51]. Although the major UV radiation source is the sun, other sources are tanning beds and UV lamps. Because of the modern aesthetic views, the general population considers "having a tan" as healthy and beautiful; therefore, many people expose themselves to excessive UV radiation exposure from the sun or from tanning beds. The data suggest that use of sunbeds, a source of artificial UVR, increases the risk of melanoma especially when used before the age of 35

although this was not shown to be the case in our Leeds melanoma cohort [52]. Changes in legislation that came into effect in 2010, banned the use of sunbeds in under-18s in England and Wales.

1.6.3.2.1 The evolution of SSMM from naevi

The development of melanoma from naevi is best understood as a multistep process with clinical and histological characteristics [53]. SSMM development may result from naevoid proliferations and this can be histologically divided into five stages, but some SSMM are thought to begin as *in situ* lesions. In the first stage of development from naevi, acquired naevi form due to an increase in melanocyte proliferation in response to UV radiation. Naevi are benign skin lesions, however, they can evolve into malignant melanomas [54]. Normally, proliferation of naevoid melanocytes ceases with time and the naevus involutes, but continued proliferation may result in a clinical entity called the dysplastic naevus, which has some potential to evolve into a melanoma if proliferation continues. In this third stage, dysplastic naevi continue to grow into the radial growth phase (RGP) primary melanoma. RGP melanomas, by definition, develop within the epidermis itself and do not possess the ability to invade through the basal membrane into the dermis. In the fourth stage, RGP melanomas go on to acquire invasive potential by virtue of genetic alterations and begin to invade through the basal membrane into the dermis. This is known as the vertical growth phase (VGP). At this point, a melanoma possesses the potential of self-sufficient growth and this signals its ability to invade, thus, making a curative excision at this point much less likely. In the fifth and final stage of melanoma development, the metastatic lesion itself is formed. In this stage, VGP melanomas possess the ability to grow larger and to invade surrounding tissues. The VGP melanoma becomes a metastatic melanoma once it invades into lymphatics and blood vessels and has the potential to colonize distant organs.

1.6.3.3 Dysplastic nevi

A naevus or a mole is a benign pigmented skin lesion. However, having many unusual types of moles, known as dysplastic or clinically atypical naevi, is associated with an increased risk of melanoma. These unusual types of moles can be identified by the ABCDE criteria: Asymmetric shape, Border irregularity, Colour mixture, Diameter size, and Evolution. The strongest phenotypic risk factor for melanoma is the presence of increased numbers of melanocytic naevi [45, 46]. There is a substantially increased risk (approximately seven-fold) associated with the presence of 101-120 naevi compared with less than 15. Some individuals are said to have the atypical mole syndrome (AMS), which has been defined as the presence of at least three of the following clinical features:

(a) 100 or more common naevi >2 mm in diameter; (b) two or more atypical naevi; (c) one or more naevi on the buttock and/or two or more naevi on the dorsum of the feet; (d) one or more naevi on the anterior scalp; (e) one or more pigmented lesion of the iris [55]. The AMS phenotype is a potent risk factor for melanoma. Twin studies have demonstrated that the number of naevi is predominantly genetically determined, with a smaller effect of sun exposure [56]. Genome-wide association studies have identified several loci associated with naevus number [57, 58].

1.6.3.4 Family history of melanoma

The risk of melanoma is greater if a person's parents or siblings have had melanoma. Individuals with a family history of melanoma have approximately double the risk of developing the disease [46]. This may be because families share similar genetic backgrounds or lifestyle (i.e., excessive sun exposure). Approximately 10% of cutaneous melanomas can be associated with a familial setting [59]. Rare families exist in which large numbers of melanoma cases arise and these families are more likely to have inherited highly penetrant melanoma susceptibility genes. Most of these families have hereditary mutations in the *CDKN2A* (cyclin dependent kinase inhibitor 2A) gene and some very rare families have mutations in the *CDK4* (cyclin dependent kinase 4) gene [60-62]. Mutations of *CDKN2A* and *CDK4* result in cell cycle dysregulation and promote melanoma development [63]. In the majority of *CDKN2A* mutation-positive families in the UK and Australia, family members have an increased risk of melanoma alone but in mutation-positive families in North America and some parts of Europe, there is also an increased risk of pancreatic cancer [64]. Hereditary mutations are responsible for a very small proportion of melanoma. The frequency of *CDKN2A* mutations is 20-40% in families where there are three or more affected first-degree relatives, and less than 5% if there are only two [65]. There are very rare additional inherited mutations behaving as high penetrance melanoma susceptibility genes in the *POT1* gene, other genes in the shelterin complex and one in the *TERT* gene.

1.6.3.5 Impaired immune system

People who have an impaired immune system have a higher risk of getting melanoma. This includes people carrying HIV or people taking immune suppressant drugs for organ transplantation or autoimmune disorders, again arguing for the possibility of incidental drugs and other host factors influencing risk of melanoma and progression of that cancer [66-68].

The effects of immune suppression were particularly highlighted by MacKie *et al.* who reported the development of fatal melanoma in two patients who had undergone kidney

transplantation [69]. This occurred 16 years following surgery for primary melanoma in the donor. However, primary melanoma was not identified in both the patients and secondary melanoma was diagnosed acquired from the kidney donor [69]. There are various cases melanoma reported in the literature that have been transferred through donor organs and this also illustrates that melanoma cells may survive in tissues for many years before proliferation and metastasis. The organs were transplanted six months to sixteen years after the donors had undergone melanoma surgery [69-72]. This represents variable time interval between melanoma surgery and organ donation resulting in fatal melanoma in the organ recipients, but notably is consistent with the view that melanoma cells may remain in the host symbiotically for many years. Thus, the usual advice is that patients with invasive melanoma should never donate an organ.

1.7 Determinant of survival outcome in Melanoma

1.7.1 AJCC staging system

In terms of prognosis or outcome for melanoma patients, the strongest prognostic factors are histological characteristics of the primary melanoma and the sentinel node status. The sentinel node is the hypothetical first lymph node or group of nodes draining a cancer and a biopsy from these can help with staging. These factors have been identified in large cohorts of patients and have been integrated to form a staging guideline, which provides general prognostic estimates in the form of the validated and internationally standardised American Joint Committee on Cancer (AJCC) guidelines. This staging is based on the TNM criteria; that is thickness of the tumour (T), extent to which it has spread to lymph nodes (N) and extent to which it has metastasised to other parts of the body. The staging system is continuously adjusted based upon ever maturing data sets. The latest version was recently published (8^{th} Edition) and I have summarised the system in Table 1.1 - Table 1.5 below.

The most important prognostic factors in the AJCC staging system are tumour (Breslow) thickness (mm) and ulceration (microscopic ulceration reported according to strict criteria by the reporting histopathologist) and which will be used in this thesis for the purposes of staging. Depth of tumour invasion (Breslow thickness) has been shown to be the factor that best single correlates with prognosis for primary disease [73]. It is measured vertically in millimetres from the top of the granular cell layer of the epidermis to the deepest point of tumour. Increased tumour thickness confers a higher metastatic potential and a poorer prognosis. Approximate five year survival rates are: 95-100% for tumours <1 mm thick; 80-96% for tumours 1-2 mm thick; 60-75% for tumours 2.1-4 mm

thick; and 50% for tumours >4 mm thick [74]. Presence and number of mitoses present within a stage I/II tumour strongly also correlates with prognosis [75] but are not part of the current AJCC staging system. It is likely that these histological factors are strongly associated with outcome because they correlate with the genetic landscape of the tumour and therefore its biological behaviour.

Despite taking into account the above factors there still exists some variance in outcome that remains unexplained. Currently, we are unable to explain why two patients exhibiting the same degree of invasion in their primary lesion as recorded by the Breslow thickness, the most powerful prognostic predictor of subsequent metastasis, can demonstrate such biological variability. One may be cured via a simple excisional surgery and yet another may present with, or go on to develop, widespread metastases [75].

Table 1.1: Definition of Primary Tumour (T)

Adapted from Eighth Edition of the American Joint Committee on Cancer (AJCC) Melanoma Staging System, Chicago, Illinois.] [76]

T Category	Thickness	Ulceration Status
TX: Primary tumour thickness cannot be assessed (e.g., diagnosis by curettage)	Not applicable	Not applicable
T0: No evidence of primary tumour (e.g., unknown primary or completely regressed melanoma)	Not applicable	Not applicable
Tis (melanoma in situ)	Not applicable	Not applicable
T1	≤1.0 mm	Unknown or unspecified
T1a	<0.8 mm	Without ulceration
T1b	<0.8 mm 0.8–1.0 mm	With ulceration With or without ulceration
T2	>1.0–2.0 mm	Unknown or unspecified
T2a	>1.0–2.0 mm	Without ulceration
T2b	>1.0–2.0 mm	With ulceration
T3	>2.0–4.0 mm	Unknown or unspecified
T3a	>2.0–4.0 mm	Without ulceration
T3b	>2.0–4.0 mm	With ulceration
T4	>4.0 mm	Unknown or unspecified
T4a	>4.0 mm	Without ulceration
T4b	>4.0 mm	With ulceration

Table 1.2: Definition of Regional Lymph Node (N)

Adapted from Eighth Edition of the American Joint Committee on Cancer (AJCC) Melanoma Staging System, Chicago, Illinois [76].

	Extent of regional lymph node and/or lymphatic metastasis	
N category	**No. of tumour-involved regional lymph nodes**	**Presence of in transit, satellite, and/or microsatellite metastases**
NX	Regional nodes not assessed (e.g., sentinel lymph node [SLN] biopsy not performed, regional nodes previously removed for another reason); Exception: pathological N category is not required for T1 melanomas, use clinical N information	No
N0	No regional metastases detected	No
N1	One tumour-involved node or any number of in-transit, satellite, and/or microsatellite metastases with no tumour-involved nodes	
N1a	One clinically occult (i.e., detected by SLN biopsy)	No
N1b	One clinically detected	No
N1c	No regional lymph node disease	Yes
N2	Two or 3 tumour-involved nodes or any number of in-transit, satellite, and/or micro satellite metastases with one tumour-involved node	
N2a	Two or 3 clinically occult (i.e., detected by SLN biopsy)	No
N2b	Two or 3, at least one of which was clinically detected	No
N2c	One clinically occult or clinically detected	Yes
N3	Four or more tumour-involved nodes or any number of in-transit, satellite, and/or microsatellite metastases with 2 or more tumour-involved nodes, or any number of matted nodes without or with in-transit, satellite, and/or microsatellite metastases	
N3a	Four or more clinically occult (i.e., detected by SLN biopsy)	No
N3b	Four or more, at least one of which was clinically detected, or the presence of any number of matted nodes	No
N3c	Two or more clinically occult or clinically detected and/or presence of any number of matted nodes	Yes

Table 1.3: Definition of Distant Metastasis (M)

Adapted from Eighth Edition of the American Joint Committee on Cancer (AJCC) Melanoma Staging System, Chicago, Illinois.] [76]. CNS indicates central nervous system; LDH, lactate dehydrogenase. *Suffixes for M category: (0) LDH not elevated, (1) LDH elevated. No suffix is used if LDH is not recorded or is unspecified.

	M Criteria	
M category*	**Anatomic Site**	**LDH Levels**
M0	No evidence of distant metastasis	Not applicable
M1	Evidence of distant metastasis	
M1a	Distant metastasis to skin, soft tissue including muscle, and/or nonregional lymph node	Not recorded or unspecified
M1a(0)		Not elevated
M1a(1)		Elevated
M1b	Distant metastasis to lung with or without M1a sites of disease	Not recorded or unspecified
M1b(0)		Not elevated
M1b(1)		Elevated
M1c	Distant metastasis to non-CNS visceral sites with or without M1a or M1b sites of disease	Not recorded or unspecified
M1c(0)		Not elevated
M1c(1)		Elevated
M1d	Distant metastasis to CNS with or without M1a, M1b, or M1c sites of disease	Not recorded or unspecified
M1d(0)		Not elevated
M1d(1)		Elevated

Table 1.4: AJCC Clinical Prognostic Stage Groups (cTNM)

Adapted from Eighth Edition of the American Joint Committee on Cancer (AJCC) Melanoma Staging System, Chicago, Illinois [76].

When T is…	And N is…	And M is…	Then The Clinical Stage Group is…
Tis	N0	M0	0
T1a	N0	M0	IA
T1b	N0	M0	IB
T2a	N0	M0	IB
T2b	N0	M0	IIA
T3a	N0	M0	IIA
T3b	N0	M0	IIB
T4a	N0	M0	IIB
T4b	N0	M0	IIC
Any T, Tis	≥N1	M0	III
Any T	Any N	M1	IV

Table 1.5: AJCC Pathological (pTNM) Prognostic Stage Groups

Adapted from Eighth Edition of the American Joint Committee on Cancer (AJCC) Melanoma Staging System, Chicago, Illinois [76]. *Pathological stage 0 (melanoma in situ) and T1 do not require pathological evaluation of lymph nodes to complete pathological staging; use clinical N information to assign their pathological stage.

When T is…	And N is…	And M is…	Then The Clinical Stage Group is…
Tis	N0*	M0	0
T1a	N0	M0	IA
T1b	N0	M0	IA
T2a	N0	M0	IB
T2b	N0	M0	IIA
T3a	N0	M0	IIA
T3b	N0	M0	IIB
T4a	N0	M0	IIB
T4b	N0	M0	IIC
T0	N1b, N1c	M0	IIIB
T0	N2b, N2c, N3b or N3c	M0	IIIC
T1a/b–T2a	N1a or N2a	M0	IIIA
T1a/b–T2a	N1b/c or N2b	M0	IIIB
T2b/T3a	N1a–N2b	M0	IIIB
T1a–T3a	N2c or N3a/b/c	M0	IIIC
T1a–T3a	Any N ≥N1	M0	IIIC
T4b	N1a–N2c	M0	IIIC
T4b	N3a/b/c	M0	IIID
Any T, Tis	Any N	M1	IV

1.8 Factors other than those captured in AJCC staging which predict outcome

In addition to the characteristics of the primary tumour and the draining nodes, host factors have been identified which are associated with outcome. These include increasing age [25], male sex [77], and tumour site (truncal or head/neck tumours) which all confer a poorer prognosis. However, these factors are currently not integrated into the AJCC staging. Furthermore, as has already been alluded to and which will also be

discussed in the ensuing sections, new immunotherapies now represent a fantastic development in positive treatment outcomes.

Prognosis worsens with increasing patient age at the time of diagnosis, in part because other factors known to worsen prognosis are more frequent in the melanomas seen in older patients. One study found that as age increased, so did the tumour thickness, presence of ulceration, presence of regression and proportion of men [78]. Age is however a predictor of poorer outcome independent of stage [79]. Both the evidence of thicker tumours in older people and increased death rates in older people independent of stage may be manifestations of an age-related decline in functioning of the immune system. These age effects are reported to affect the cellular, humoural, and innate immunity [80] but there may also be differences related to aging stroma [81]. T-cell mediated adaptive immunity is vital for the response of the host to cells undergoing malignant changes. However, available evidence suggests that reductions in cellular immunity results in ineffective immune responses against tumour cells in older age. For example, old mice have been reported to have an intrinsic age-related defect in naive T-cell responsiveness which is associated with cytokine secretion and gene expression profiles [82]. Memory CD8+ T-cells in humans have also been shown to undergo age-related changes [83]. Increasing evidence also suggests that the capacity of DCs to capture and process antigen is compromised with old age [84].

Female sex confers a better prognosis than male sex. Some of this protective effect is because women tend to tend to present with thinner tumours compared with men. This has been attributed to behavioural differences in that men are said to be less likely to visit a doctor and get suspicious-looking skin lesions examined [79, 85]. However, there may be biological differences between the sexes. It has been suggested that the prognosis is better because women present with melanoma at a younger age than men and tend to have more limb lesions than trunk lesions compared with men [79, 85, 86]. This may be due to the fact that truncal melanomas have been shown to spread more frequently to distant sites in comparison to lower extremity lesions, whilst melanomas on the lower extremities tend to metastasise more frequently to adjoining regions in comparison with the upper extremity lesions [85]. However, sex differences in tumour site have not been explained although it's thought to be behavioural in terms of sun exposure practices. The role of female sex hormone (oestrogen) and protective effect of pregnancy on melanoma survival has been explored but is not established [87]. Female sex is also an independent predictor of better outcome even in stage IV disease prior at least to the advent of immunotherapies [77] and this very interesting observation is not yet understood.

1.9 The immune system and tumorigenesis

It was initially postulated that the immune system has a protective role in the development of tumour but it has subsequently become more clear that the relationship between immune cells and tumour cells is a lot more complex: experimental studies have demonstrated that immune system itself can promote tumour development and progression, and functions to promote or select tumour variants with decreased immunogenicity [88].

1.9.1 "Good and Bad" Inflammation

The term "T cell inflammation" is used by Tom Gajewski to describe the sort of inflammation which can kill cancer cells [89]. I will refer to this in this thesis as "good inflammation". "Chronic inflammation" however is also thought to drive some cancers e.g. squamous cell carcinoma of the skin in epidermolysis bullosa and is being explored as a factor in melanoma as in other cancers such as lung cancer [90]. These observations point to a delicate balance between "good" immune responses which kill cancer cells and "bad" ones which drive cancers, and the hypothesis of the Leeds group is that environmental exposures can affect this balance, more recently summarised by Chen and Mellman [6].

1.9.2 The immune system and melanoma

Immune function in relation to melanoma have long been described both for pathogenesis and moderating progression. A possible role of sun induced immunosuppression in the aetiology of melanoma for example was mooted long ago in murine studies of melanoma pathogenesis [91].

Analysis of > 500 primary melanoma patients with > 7 years of follow-up demonstrated that patients with brisk tumour infiltrating lymphocytes (TILs) live two times longer than patients without TILs in their tumours. Following this, similar prognostic correlation has been demonstrated for the presence of TILs in melanomas that had spread to lymph nodes [92]. A retrospective analysis of data, over a period of 30 years, from the Transplant Tumour Registry revealed that patients with organ transplantation had twice the risk of developing melanoma over the general population [70] and this provides some evidence for a causal effect of immunosuppression. The development of vitiligo (auto-immune damage to normal melanocytes) was recognised as a marker of better responses of melanoma patients to chemotherapy and this observation is taken to reflect the development of immune responses to antigens shared by normal melanocytes and melanoma cells after tumour cell damage during chemotherapy. That the vitiligo was a

marker of better responses suggested that immune responses to melanoma were important even in terms of responses to chemotherapy. Uncommon dramatic therapeutic responses to interferon therapy and IL2 were reported, and now melanoma is in relative terms a good responder to immunotherapy. Taken together these observations strongly suggest a crucial role for immune responses to melanoma in survival and treatment responses. The complexity however of what is required for immune responses to kill melanoma was described by Chen and Mellman [6]. They postulated that hereditary differences in immune competence, environmental factors (including diet and the microbiome), tumour mutation rates, loss of key biological pathways mediating immune competence in the tumour all contribute. Although much is now understood of the interactions between immune cells and melanoma cells, much remains to be discovered and the rate of evolution of this knowledge is unexpectedly fast.

1.9.3 Immune mechanisms in melanoma development

Here I give a brief overview of what my reading of the literature reveals of what is known about the complexities of immune/cancer cell interaction in melanoma.

A number of components of the innate and adaptive immune system contribute to and defend against melanoma development (Figure 1.2). When tissue homeostasis is broken, components of the innate immune system including neutrophils, macrophages and mast cells release cytokines, chemokines, matrix remodelling proteases (MMP) and reactive oxygen species (ROS), which induce migration and infiltration of more leukocytes into damaged tissue, in a process known as inflammation. This response in the context of a tumour microenvironment such as melanoma can enhance its development [93].

In addition, local inflammation results in activation of adaptive anti-tumour immune responses. Antigen-presenting cells such as dendritic cells present tumour-associated antigens on major histocompatibility complex I molecules on their cell surface, resulting in the differentiation and expansion of tumour-specific cytotoxic CD8+ T cells. Interestingly, melanoma cells have been observed to down-regulate the MHC class I expression, thus preventing T cell activation and tumour elimination [94]. Melanoma similarly interrupts T cell co-stimulation and activation through modification of costimulatory molecule expression on the surface of melanoma cells including programmed death ligand 1 [95].

Other mechanism of immune evasion which melanoma employs include promoting up-regulation of immunosuppressive cytokines such as IL-6, IL-10, TNFα, TGFβ and VEGF which result in the inward migration of immunosuppressive cells such as myeloid-derived suppressive cells, tumour associated macrophages, or tolerogenic dendritic cells [88].

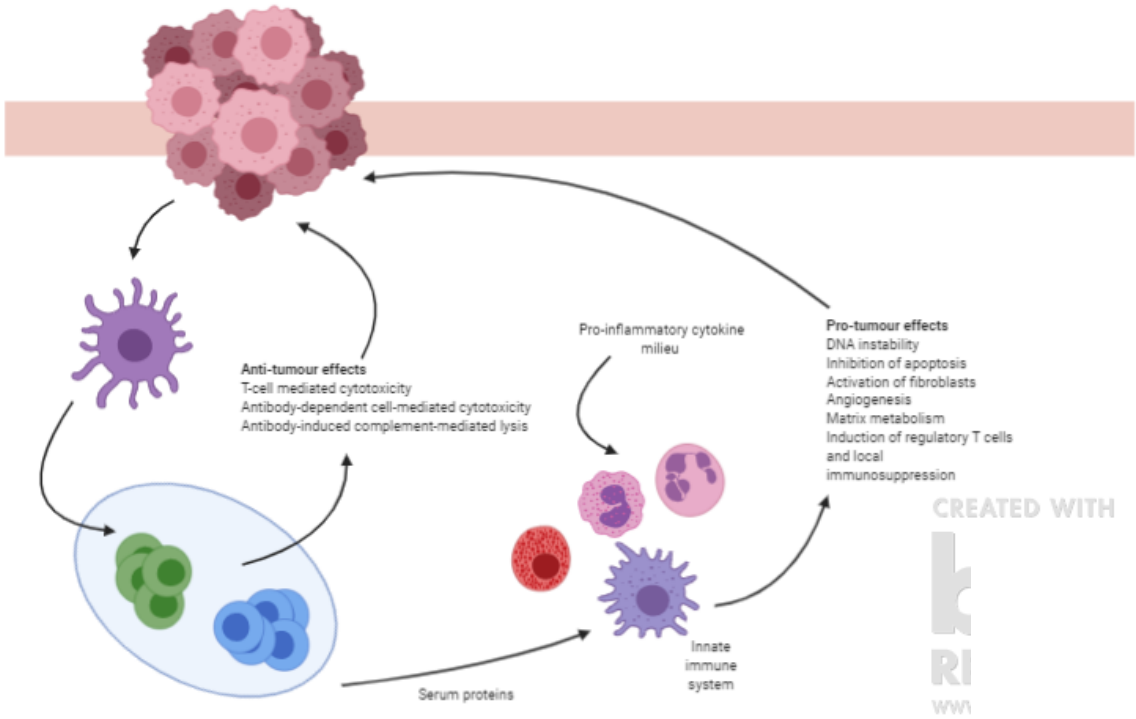


Figure 1.2: Graphic depiction of pro- and anti-tumour effects of the immune system in cancer and melanoma development.

Adapted from De Visser *et al* (2003) [96] and illustration created using www.biorender.com.

1.9.4 Evidence that pro-tumourigenic inflammation may play a role melanoma progression

As described above in Section 1.9, cytotoxic CD8+ T cell responses to melanoma play an important role in anti-melanoma responses. Infiltration of tumours with immune cells is however not held to be beneficial in all tumours, and the work of Lisa Coussens [97] and others support the view that some tumours are driven by pro-tumourigenic inflammation. The Coussens lab postulated that polarized M2 macrophages, resultant increased levels of proinflammatory cytokines, e.g. IL6 and IL8 in some tumours increase tumour growth and angiogenesis.

That chronic inflammation might drive cancer initiation is not a new concept. Chronic inflammation has been shown to be an aetiological factor in the onset of several cancers, particularly in those of an epithelial origin, and therefore may serve as a potential link between obesity and cancer. In the middle of the 19th century, Virchow first addressed the contribution of immune cells to tumourigenesis [98]. His conclusions were based on

the fact that tumours developed in the setting of chronic inflammation and that inflammatory cells were present in tumour biopsy specimens [99].

The Leeds Melanoma Group have reported evidence that microscopic ulceration (a biomarker of a poor outcome) has clinicopathological and transcriptomic features of a pro-tumourigenic inflammatory process. The clinicopathological features included increased vascularity and more macrophages [100] and transcriptomic features. Changes associated with ulceration were increased expression of pro-inflammatory cytokines IL6 and IL8 [101]. The cytokine IL6 is elevated in patients with systemic inflammation related to smoking, hypertension, obesity and the metabolic syndrome, which are risk factors of cardiovascular disease. The Leeds group therefore asked if there might be evidence that low-grade systemic inflammation which might be associated with ulceration of primary melanoma as a marker of a pro-tumourigenic environment as described below.

1.10 Can systemic low-grade inflammation also drive melanoma progression?

There has been increasing reported evidence for other cancers that host factors such as obesity may drive cancer incidence and progression. The Leeds group looked at data from the Leeds Melanoma Cohort and asked if factors related to low grade systemic inflammation (obesity, smoking, vitamin D deficiency and diabetes) might be associated with ulceration of primary melanomas [33]. It was shown that ulceration was associated with all of these factors in a univariate analysis lending support to the view that systemic inflammation may play a role in modulating host tumour interaction in melanoma and therefore survival. Below I report evidence from other cancers that obesity, and its associated systemic inflammation may be important in general and melanoma-specific cancer progression.

1.11 Obesity and cancer incidence

Obesity is a medical state defined usually as having a body mass index (BMI) > 30 kg/m^2. It is argued by many that the biological impact of “obesity” is related more to central (visceral) fat deposits than BMI, and therefore that waist/hip ratio may be more meaningful than BMI alone [102]. Indeed studies now increasingly use imaging to estimate visceral fat deposits in viscera hypothesised to better predict biological impact [103]. In the Leeds Melanoma Cohort reported height and weight were used to compute

BMI. Obesity is reported to be the sixth most important risk factor contributing to the overall global burden of disease [104]. As per the estimates of the CDC/National Centre for Health Statistics, around 70% of the adult population in the US are either overweight or obese and this has reached the level of an epidemic [105]. Similarly, in England, a third of people over the age of 35 are now deemed to be obese [106].

A review of the epidemiological data indicates that over 25% of all cancers are related to chronic inflammation and it is also estimated that 15% of cancer deaths are associated with inflammation [107, 108]. Acute inflammation is a physiological and vital healing process, which is generated by the body in response to either an injury, an infection, or some sort of irritation. However, the problem arises when this process begins to become chronic, and it may then contribute to a variety of diseases, including cancer.

1.11.1 Obesity and melanoma

A number of studies have shown a link between malignant melanoma and the presence of excess adiposity [109-111]. Despite this potential link, another large study demonstrated no such association [112, 113]. Furthermore some studies are suggestive of obesity being associated with an increased risk of malignant melanoma in males, but not in females [114-116]. One theory to explain this is that women who are obese may be less likely to expose their bodies to the sun as compared to obese men. Moreover, a number of studies have assessed the effect of various so called anthropometric characteristics such as height, weight, body mass index (BMI) and body surface area (BSA), on the risk of developing CM with conflicting results.

Studies that have demonstrated significant associations between some of these anthropometric characteristics and the risk of CM have included at least four different prospective studies as well as six separate case-control studies. One such prospective study conducted in Norway demonstrated a significantly higher risk of CM for the highest versus the lowest quintile of both height and body surface area (BSA) in both sexes. The effect of BMI on CM risk was positively associated in males and inversely in females [117]. A further cohort study conducted in Norway and which included 118 cases of CM, found a significant relative risk for greater height and BSA, but not for BMI [114]. Yet another cohort study looking at 187 women from Norway and Sweden with CM, demonstrated a direct association with BSA [118]. Another cohort study of more than 4000 male US veterans with CM,showed an excess risk for obese versus non-obese veterans in the case of both white and black men [111].

In terms of case–control studies, an Australian study demonstrated a borderline significant trend to increased risk of CM with increasing levels of BMI [119]. Similarly a US case-control study, demonstrated a two-fold excess risk of CM in subjects with high

BMI [120]. In contrast, a study of Canadian women with CM and with matched controls, showed significantly greater height, but not weight and BMI [121]. A further study carried it out in both Australia and Scotland, demonstrated a significantly higher risk of melanoma of the soles and palms in overweight and obese subjects (BMI >25 kg/m^2) in Scotland, but not in Australia [122]. Yet another US case–control study demonstrated a significant excess risk in men, but not in women, for both height, weight, as well as BSA [123]. Furthermore a case–control study from Italy reported that BSA, and weight, but mostly BMI were found to be directly associated with the risk of CM but with a particularly stronger association between BMI and CM in postmenopausal compared to premenopausal women [109]. This result would suggest that potentially differing levels of oestrogen may be playing an underlying role in mediating this difference.

In terms of meta-analysis, one study examining these cohort studies, demonstrated an elevated risk of CM with increasing BMI just among men [115]. Similarly, yet another meta-analysis reported an increased risk of melanoma among men with increasing BMI and BSA, but again this association was not evident in women. As discussed earlier, its possible the varying sunlight exposure in obese females may be a confounding variable in these studies [116]. The authors have suggested two possible mechanisms, which may explain this positive association between obesity and CM risk. Firstly, obesity is known to produce chronic insulin resistance, hyperinsulinemia, as well as downregulation of insulin-like growth factor (IGF) binding proteins 1 and 2 and an increase in activity of IGF-I. Studies have in fact shown that insulin is an independent risk factor for melanoma [124]. A further interesting observation is that the total circulating levels of IGF-I are higher in men compared to women [115] which may potentially explain the observations seen above in men. Secondly, a large body surface may simply be representing a larger area at risk for sunlight exposure, not to mention the larger number of exposed cells that would be at risk, thereby providing a more direct link to the incidence of melanoma [109]. It is also possible that obesity may moderate behaviours in the sun although it seems unlikely that obesity would increase sunbathing. It is also possible that controls may be less likely to participate in case-control studies if obese (bias of participation).

In contract to the studies presented above, there are also prospective and case control studies that have reported no associations between obesity and the risk of CM. Two such prospective Scandinavian studies including a a Danish record-linkage study [125] and a Swedish study [126] found no increase in risk when compared to the general population of the respective countries. A further US study demonstrated no increased mortality even for higher categories of BMI in males or females [127]. Similarly in the US Radiologic Technologists Study, there was no association found between CM risk and

the higher quartiles of height, weight as well as with BMI in both women or men in the study [128]. A further two case–control studies looking at women from Canada and Western Australia showed no association between the level of obesity or BMI and the risk of CM [129, 130]. Another study looking at just women where eight separate case control studies were pooled together again showed no association between BMI or body surface area (BSA) and CM risk [131].

In summary, although there appears to be some evidence for a relationship between obesity and melanoma risk judging from the studies there are still many conflicting results and this relationship remains unclear.

1.11.2 Obesity-related inflammation

As already alluded to, obesity, which is defined as an abnormal or excessive fat accumulation in adipose tissues, is considered to be associated with a chronic inflammatory disease state [132] and is also characterized by the presence of increased circulating fatty acids, and chemo-attraction of immune cells that play a role in the development of this inflammatory state[133]. Although the features of chronic inflammation seen in obese adipose tissue appear to be fairly clearly defined, the actual signals and mechanisms that eventually end up triggering chronic inflammation are not fully understood.

Obesity has also been shown to increase the risk for several chronic diseases including type 2 diabetes, cardiovascular disease, as well as fatty liver disease. Although it is variously defined, there is an international consensus that chronic systemic inflammation associated with obesity and/or insulin resistance is a recognised risk factor for cancer and for death from cancer, as well as for cardiovascular disease [134, 135]. By systemic, the Leeds Melanoma Group mean that as a result of this inflammation there are associated changes evident throughout the body: including in the blood, adipose tissue as well as the organs themselves. As already discussed there exists a strong relationship between metabolism and immunity and it has been postulated that this could potentially become harmful under conditions of metabolic stress.

The metabolic syndrome is associated with obesity and is defined by a combination of cardiovascular risk factors that include increased body mass index (or waist circumference), high blood pressure, hyperglycaemia, and raised serum triglycerides, as well as a decrease in high-density lipoprotein cholesterol (although definitions do vary between studies). Some require an additional measure of obesity (e.g., hip to waist measurement ratio and additional markers), while others (e.g., the WHO definition) require the presence of insulin resistance [136]. The metabolic syndrome has metabolic implications but is also a chronic inflammatory syndrome characterised by elevated

circulating inflammatory proteins e.g. TNF-α and IL-6, a consequent state of low grade inflammation and macrophage infiltration into adipose tissue [137].

The changes which are identified in response to obesity (e.g., which drive cardiovascular disease and cancer) are extremely complex, but my interpretation of the literature is that many changes described are associated with each other. The so-called metabolic inflammatory state is said to be orchestrated by metabolic cells in response to excess nutrients and energy [138]. The fat cells generate inflammatory responses (increased TNF-α, and many inflammatory cytokines such as IL-6), which sustain low-grade chronic inflammation in affected patients. Figure 1.3 below, illustrates a version of the hypothesis surrounding obesity related systemic inflammation. The recently published data by Ridker *et al*. [139] from the CANTOS trial suggest that the IL1β pathway is crucial in mediating cardiovascular disease, with evidence that it mediates cancer risk too. In this trial, more than 10,000 patients agreed to be randomised to placebo, 50, 150, and 300 mg 3 monthly of canakinumab, which blocks IL1β signalling, after a myocardial infarction. Eligibility required elevation of high sensitivity C reactive protein levels. The trial reports describe a dramatic reduction in serious cardiovascular events in the active treatment arms and notably, a reduction in death from lung cancer [139].

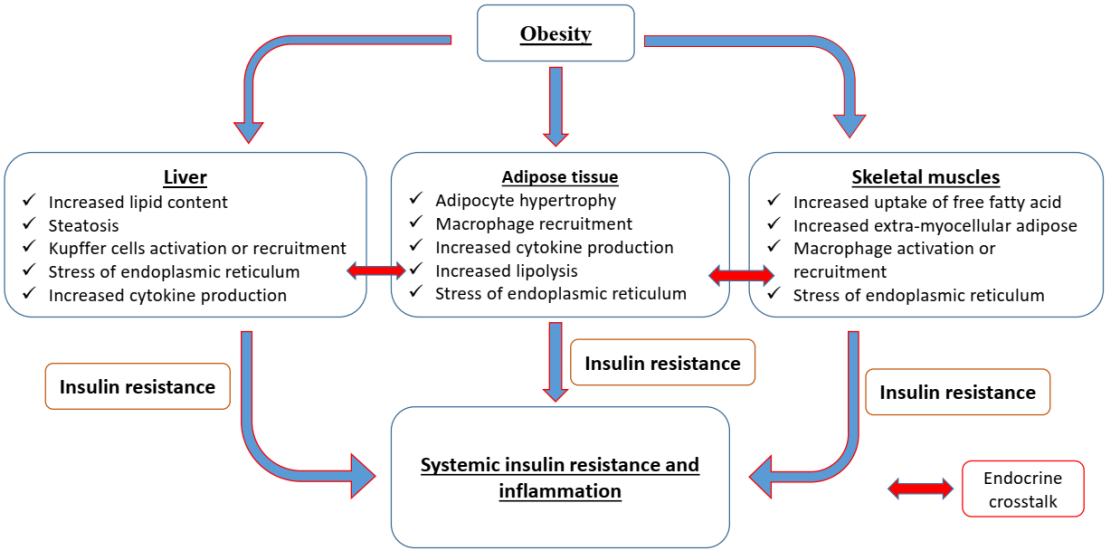


Figure 1.3: Diagram illustrating a version of the hypothesis surrounding obesity related systemic inflammation

Adapted from De Luca d'Alessandro et al. [136]

Interestingly, various components of the metabolic syndrome have been linked in some way to the development of cancer. Studies have demonstrated a direct association between diabetes and pancreatic and liver cancers which is thought to be due to an

excess of insulin which in turn promotes the development of cancer cells in the liver and pancreas [140]. Obesity is reported to be a major risk factor for cancer; prospective studies indicate that being overweight and obese are responsible for 14% of all cancer deaths in men and 20% in women. Obesity has been implicated in both the aetiology as well as the progression of cancer at various cancer sites by virtue of a variety of signalling pathways that regulate key functions, including proliferation, apoptosis, metastasis, and angiogenesis of cancer cells [141].

1.11.3 Markers of Inflammation

C reactive protein (CRP) or CRP as it is more commonly referred to, is an acute-phase protein released by the liver and is a non-specific marker for inflammation, infection, and tissue injury. In clinical practice it is often used to screen for evidence of infection or inflammation as part of a blood panel. However, given that as already established, adipose tissue secretes pro-inflammatory mediators, it comes as no surprise that CRP levels have been shown to correlate with the amount of adipose tissue. Elevated levels of CRP have been noted in 35% of obese men and 60% of obese women but also in animal models, showing a twofold increase in obese animals, compared to lean controls women [142] [143]. Interestingly CRP levels have also been used to predict onset of diabetes in both obese men and women [144]. CRP has also been reported to be associated with an increase in the risk of developing a number of cancers including colorectal, cervical, and ovarian cancer [145], which interestingly are also cancers that have been associated with obesity again suggesting a link between the two. Furthermore, a study examining the effect of obesity on survival after colon cancer found that high levels of CRP correlated with a higher likelihood of death from colon cancer. Furthermore, levels of CRP have been found to be inversely associated with survival in American Joint Committee on Cancer stage II patients, raising the possibility that CRP could eventually be used to help make treatment decisions in this subgroup of patients. The authors concluded that it is actually the obesity-related inflammation, rather than the obesity itself, that is, linked with poorer outcomes in colon cancer [146]. In summary, these data do however add more evidence for a link between obesity, inflammation and cancer, which warrants more investigation.

1.11.4 Obesity-related inflammation and cancer

In 2013, Maria E Ramos-Nino presented a study examining the links between obesity and inflammation, as well as between chronic inflammation and cancer [135]. The study suggested that as suspected, inflammation may be important in the obesity-cancer link. The changes that occur in the adipose tissue during the process of going from lean to

obese were described, including modulation of adipokine levels, hypoxia, increased reactive oxygen species (ROS), etc. which were postulated to lead to a chronic state of inflammation in the obese individual. It was postulated by the authors that this increased risk of obesity-related cancers could be mediated in part by these changes in the adipose tissue. A few of the key elements of this association are, amongst others, insulin resistance; overexpression of leptin, inflammatory cytokines, sex hormones, transcription factors like NF-κB, AP-1, STAT3, and oxidative stress; and down-regulation of the expression of anti-inflammatory factors like adiponectin and PPARγ, which disrupt the balance between cell proliferation and apoptosis [135].

Some authors such as Johanna Joyce have attempted to summarise some of the many ways in which these might impact on cancer cell [147] as represented in Figure 1.4 below.

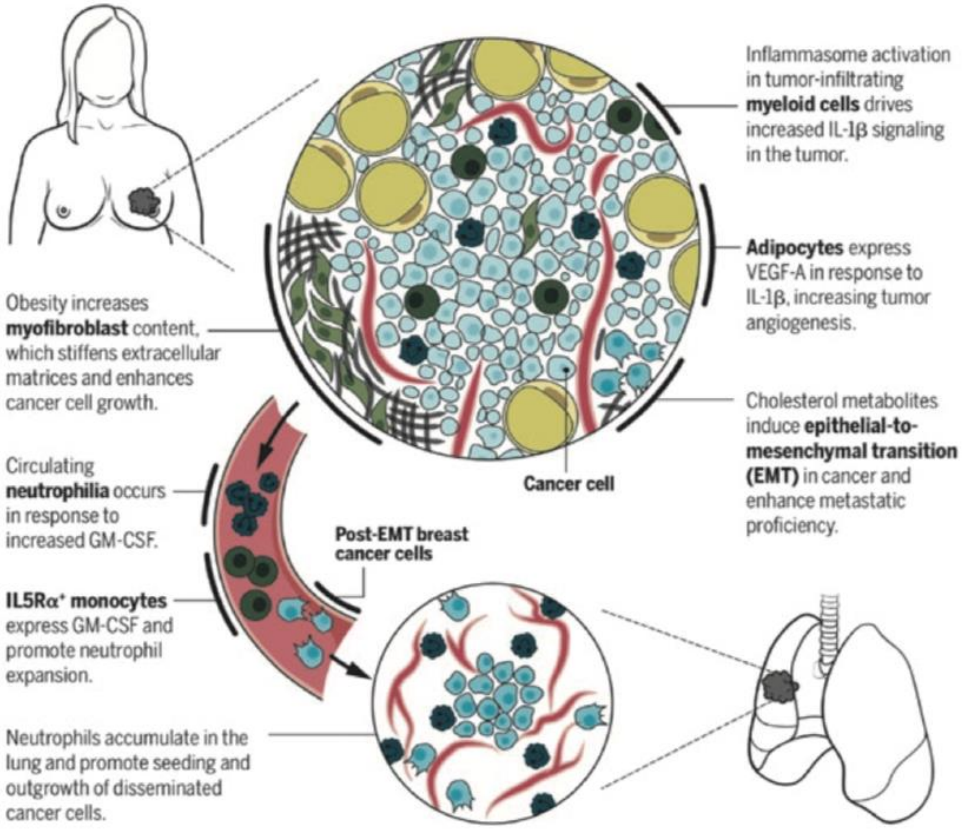


Figure 1.4: Interaction between obesity and cancer development

Adapted from Joyce *et al*. [147]

1.11.5 Mechanism of association

1.11.5.1 Increased leptin

Leptin causes activation of the leptin receptor long isoform (LEPRb) situated in the hypothalamus, which in turn acts on both POMC neurons and agouti related protein (AgRP) neurons [148]. This subsequently results in increased activation of melanocortin 3 (MC3R) and MC4R which leads to suppression of appetite [149] and in turn inhibits feeding behaviours [148, 150]. Leptin has a number of roles including causing inhibition of insulin secretion as well as lipogenesis [151], stimulating lipolysis and fatty acid oxidation, suppression of appetite, and promoting energy expenditure, ultimately resulting in a reduction in body weight [148]. However leptin resistance can develop which can result from defects with leptin transport, impaired LEPRb signalling, neuronal energy imbalance, or endoplasmic reticulum (ER) stress [148, 152, 153]. Leptin deficiency and leptin resistance are both known risk factors for obesity [148, 154] and can be associated with insulin resistance [155] as well as hypogonadism [154, 156]. Leptin resistance can promote ER stress and chronic inflammation that can then contribute to insulin resistance [157]. Interestingly, ER stress itself inhibits leptin signalling [158].

An increase in the circulating levels of leptin results in metabolic disturbances that are further compounded by inflammation [155]. Leptin has also been found to be structurally similar to pro-inflammatory cytokines and in this respect may also modulate CRP [159]. It is therefore plausible that the positive associations found between serum leptin levels and skin cancer may be due to inflammation secondary to leptin resistance and obesity. A further mechanism by which Leptin may result in growth of a melanoma is via increased nitric oxide (NO) production and increased levels of circulating endothelial progenitor cells (EPCs), which can ultimately promote angiogenesis and vasculogenesis also mediated by VEGF and endogenous fibroblast growth factor 2 (FGF-2) [160-162]. Leptin therefore behaves as a pro-inflammatory adipokine that in turn influences cytokine production, cellular immunity, and ultimately inflammation [163]. Furthermore it has been shown that leptin results in the decreased expression of the tumour suppressor p53 in order to promote cell cycle progression [164]. As melanoma cells, but not melanocytes, express the leptin protein, this results in the creation of a positive autocrine feedback loop that then stimulates uncontrolled proliferation of melanoma tumour cells [165].

1.11.5.2 Decreased adiponectin

Adiponectin has been found to exert anti-tumour effects by its ability to inhibit cell proliferation [166]. Interestingly, Adiponectin levels have been found to be approximately

50% lower in obese individuals compared to non-obese individuals [166]; resulting in increased cell proliferation and therefore potentially playing a role in carcinogenesis. However a study examining the relationship between serum Adiponectin levels and melanoma incidence showed no significant association between the two. [167].

1.11.5.3 Insulin resistance and insulin-like growth factor (IGF)

There is evidence to show that an increase in levels of insulin, IGF-1, and IGF-2 can induce tumourigenesis by virtue of its effects on the insulin receptor [168]. There is also evidence to show that inflammatory kinases can inhibit both insulin action as well as glucose uptake in obese individuals [169]. Therefore, it would seem that both hyperinsulinemia as well as increased levels of IGF-1 may be contributing to an increased cancer risk as well as cancer progression. It has similarly also been shown that undertaking calorie restriction, intentional weight loss measures, and treatment of diabetes can actually result in a reduction of the risk and rate of progression of skin cancer [170].

A further pathway that has been identified that induces insulin resistance is the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway whereby a PI3K inhibitor and an MTOR inhibitor have been shown to induce proliferation and prolonged survival of melanoma cells. Activation of this pathway is also thought to mediate melanoma cell resistance to chemotherapeutic drugs [171].

Abnormal levels of insulin and IGF are have been shown to be associated with inflammation, decreased physical activity, and impairment of the immune system. The evidence suggests that IGF-1, in particular, is associated with VEGF resulting in neovascularization and metastases. In addition, IGF-2 is also thought to play a role in tumorigenesis via both insulin and IGF receptors [172].

Studies have demonstrated that serum levels of IGF-1 can be significantly higher in patients with melanoma together with lower levels of insulin-like factor-binding-proteins 3 and 5 (IGFBP-3 and IGFBP-5) potentially resulting in melanoma cell proliferation, metastases, and reduced survival rates [173, 174].

1.12 The obesity paradox

Despite the reported relationship between obesity, inflammation and the risk of several cancers, perplexingly, recent studies have reported that obesity is associated with improved survival in cancers [175, 176] such as colorectal cancer [177], non-small cell lung cancer [178], and renal cell carcinoma [179]. Based on these findings, the term

"obesity paradox" has been introduced which suggests improved survival outcomes among overweight/obese patients relative to normal weight patients [180]. In a meta-analysis looking at the effects of increased BMI in patients with a number of different malignancies, excluding melanoma, who had received treatment with either cytotoxic chemotherapy or targeted therapy, results demonstrated variable benefit ranging from beneficial to adverse across tumour types, but interestingly were almost universally found to be beneficial in male patients [175]. A pooled meta-analysis, in patients with metastatic melanoma being treated with immune checkpoint inhibition as monotherapy (PD-1/PD-L1 or CTLA-4) and targeted therapy showed similar results [181]. The mechanism by which this phenomenon develops is unclear. Some concerns have been raised regarding bias in the epidemiological analyses, and this is an important area for future research [180].

1.13 Incidental drugs and effects on the skin

Cancer patients are often prescribed drugs intended to treat incidental medical conditions, which potentially could have effects on host-tumour interaction and therefore survival. These interactions are likely to be very complex and the effects might be positive or negative. Some of these effects may be directly attributed to the skin.

For instance, a potential association with the skin in seen in the effects of certain medications that can potentially contribute to both obesity as well as photosensitization. Obesity can result in a reduction in the rate of metabolism of a photosensitive drug, which in turn increases the intensity and duration of photosensitization [182], which theoretically could contribute toward the development of melanoma.

Diuretics or "water tablets" as they are often referred to are commonly used in the treatment of hypertension in elderly patients [183]. As discussed previously obesity and an increase in leptin levels can promote hypertension. Studies have demonstrated that people who are overweight and obese who are also on diuretics have an increased risk of a different type of skin cancer, called a basal cell carcinoma (BCC) [184, 185] and this is again thought to be as a result of diuretics increasing the risk of phototoxicity and photocarcinogenesis [186].

Patients who are obese are also likely to be treated with medications such as non-steroidal anti-inflammatory drugs (NSAIDs) either for cardiovascular benefit or potentially for analgesic benefit for joint pain and such like. These NSAIDs have also been shown to be associated with heightened photosensitivity [187, 188]. These cutaneous reactions

could be in addition to the drug's potential effects on inflammation and the immune system which will be discussed in more detail in the ensuing chapters.

1.14 Oncogenic Pathways involved in the development of Melanoma

Melanomas utilise a number of signalling pathways to regulate their activities, including proliferation, migration, differentiation, and apoptosis. De-regulated signalling pathways often lead to melanoma progression. Broadly speaking, these signalling pathways fall into two categories depending on how signalling pathways are activated. Signal pathways can be activated as a result of external stimuli which can result in a signalling cascade from the cell surface right down to the intracellular downstream effectors. Alternatively, signal pathways can also be activated as a result of constitutively activated internal oncogenes in the absence of external stimuli. The oncogenic signalling pathways at least in part promote carcinogenesis as a result of deregulated cell proliferation and effects on apoptosis.

The variation in outcome even within patients whose tumours have evidence of activation of these oncogenic signalling pathways may result from host/tumour interactions, genetic factors and environmental factors. We hypothesise that one such environmental factor may be incidental drug exposures that these patients are exposed to during this period.

As already alluded to, most patients over their lifetime are exposed to several different drugs, some of which may have incidental beneficial effects on disease states other than that for which they were originally prescribed. For instance, the cancer-preventative properties of NSAIDs are accepted in patients with colorectal cancer taking COX-2 inhibitors or aspirin and so far at least four studies have reported a similar effect in prevention of melanoma [189, 190]. Beta-blockers are another group of drugs that have also been shown to have a potential effect on melanoma incidence [191, 192]. Analysing such interactions could pave the way for the identification of drugs that could potentially be used for chemoprevention.

1.15 Vitamin D

Vitamin D is a fat-soluble steroid hormone, which is thought to exert its genetic effects through binding to the vitamin D receptor (VDR). Vitamin D exerts its effects via and although long known to be crucial to bone health, it has now been recognized to have

pleiotropic effects, and is postulated to play a role in cancer, cardiovascular disease, autoimmune diseases, as well as to susceptibility to infections and even in physiological ageing in animal models [193]. Vitamin D levels have also been reported to be lower in the elderly and in the obese and in the next section I will summarise what is known of the role of vitamin D and melanoma.

1.15.1 Vitamin D and melanoma

The published data on the association between vitamin D levels and melanoma risk are controversial. A few studies have prospectively measured serum vitamin D levels prior to the development of melanoma, whereas most investigations have looked at serum vitamin D levels close to the time of diagnosis, leading to concerns that low levels of vitamin D in people who later die of melanoma might reflect bias associated with poorer health. In further studies, as discussed below, vitamin D status at the time of diagnosis has been examined and patients were followed up looking at effects on survival. These studies appear to suggest that correlations between serum vitamin D levels and melanoma risk or serum vitamin D levels and survival from melanoma need to be accounted for whenever examining incidence and survival from melanoma [194].

There has been an increasing focus recently on understanding this link between vitamin D status and melanoma as well as with other cancers and chronic diseases. Despite this the relationship between serum levels of vitamin D and the genetic factors which govern melanoma risk and melanoma mortality remains unclear. There are however a number of fairly robust epidemiological studies which appear to confirm the hypothesis that higher vitamin D levels might protect from melanoma, with a number of cohort studies having addressed a potential protective effect of vitamin D [194]. The results of some studies do not indicate a statistically significant association between serum 25-(OH)D levels and melanoma [195]. In addition, others have stated that there remains insufficient evidence to be able to recommend vitamin D supplementation to decrease melanoma risk [194]).

In terms of laboratory studies however, there is clear evidence that vitamin D has anti-proliferative activity on melanoma cell lines in vitro. There is also evidence of a reduction in expression of the vitamin D receptor with progression from a naevus through to a primary and eventually a metastatic melanoma. These observations would suggest that if vitamin D shows anti-proliferative effects on melanoma cells in vivo, then those cells may be less likely to respond to the anti-proliferative effects of vitamin D as progression is happening [194]. Further studies indicate that high levels of vitamin D may correlate with the development of less aggressive tumours and reduced progression, with some studies suggesting that simply normal levels of vitamin D3 at the time of diagnosis are

associated with a better prognosis and yet other studies showing that patients with low Vitamin D levels tend to have thicker tumours. In addition to this reduced vitamin D levels have also been reported in patients with stage IV melanoma compared with those with stage I [196, 197].

1.16 Chemoprevention

Cancer prevention strategies can be broadly divided into three primary types [198, 199]:

- Primary prevention of cancer.
- Secondary prevention of invasive cancer in patients with premalignant conditions, or prevention of recurrence in which case the treatment is known as adjuvant therapy.
- Tertiary prevention of second and subsequent primary cancers.

In the case of melanoma, most primary prevention strategies have targeted sun protection, but with variable results. Chemoprevention has, therefore, been proposed as an alternative measure for the prevention of cutaneous melanoma. SSMs are relatively slow to progress, making this common type of melanoma ideally suited to chemopreventive interventions, by attempting to target the processes and molecular pathways that have been described to be involved in the progression of melanoma [199].

The term "chemoprevention" refers to efforts to prevent, delay or suppress the process of carcinogenesis with the help of dietary means, natural agents, synthetic agents, vitamins, etc. [199]. Furthermore chemoprevention can also refer to the use of the same types of agents to reduce the risk of reoccurrence of cancer in patients who have undergone successful primary cancer treatment and are in remission [199], which is the focus of this thesis. An ideal agent should have major additional health benefits, few adverse effects and be inexpensive. In has been postulated that in the ideal world, agents that are selected for the purpose of development as a chemopreventative should have evidence of potential activity based on data from a number of sources including experimental (mechanistic, *in vitro*, animal), epidemiologic (case-control, cohort, ecologic, secondary analyses), and clinical (phase I, IIA, IIB) trials [200].

Chemoprevention in cancer has become of interest following the success of drugs such as tamoxifen for breast cancer and celecoxib for familial adenomatous polyposis [201]. However, some potential candidate drugs such as rofecoxib have adverse thrombotic and cardiovascular safety profiles making them unsuitable as chemo-preventative agents [202], which is an important consideration when trying to identify potentially beneficial drugs. Similarly, some drugs may also produce unintended harm, and it’s

equally important to recognise this and consider stopping such drugs in potentially susceptible patients, if it is possible to do so.

Based on the above, we postulate that the effects of the drugs on the carcinogenic process may also be of relevance in preventing secondary relapse of cancer. Therefore commonly prescribed drugs might have an effect on survival and if so, there may be a complex relationship between the medical conditions for which such drugs are prescribed, incidental drugs, and survival from melanoma that requires careful investigation.

1.17 Management of Melanoma

Early detection of melanoma is vital, since the five-year survival rate of a patient without metastatic disease is 98%. Furthermore, patients with metastatic tumours until recently, have had a poor prognosis and have a five-year survival rate of around 23% and a limited overall median survival of 6 to 9 months [203]. However, early melanoma detection is hindered by late presentation (probably related to a lack of public education), the difficulties of educating the elderly in whom new benign skin lesions are common and the absence of clinically significant symptoms until the disease has reached an advanced stage [204]. The histopathological diagnosis of melanocytic lesions is also difficult.

Patients who have had one melanoma have been shown to be at higher risk of developing a further melanoma with this risk being elevated for up to 20 years and is found to be10 times greater than the risk of a first melanoma in the general population. In populations with genetic mutations, 12.7% developed a second primary melanoma within 2 years of the initial diagnosis and 19.1% by 5 years after diagnosis [202].

Currently, there are five types of standard treatment for melanoma patients including surgery, chemotherapy, radiation therapy, biologic (immuno-) therapy, and targeted therapy as summarised in the Table 1.6 below [205]. A detailed discussion of treatments for melanoma is beyond the scope of this thesis although some of the treatment options listed serve to highlight the immunogenic nature of melanoma and potential consequences of inadvertent exposure to drugs that modify this will be examined in this thesis.

Table 1.6: Summary of management of melanoma

Adapted from Domingues *et al* [205]

Treatment Type	Details
Surgery	First line treatment for all stage melanomas. Often (in advanced melanoma) combined with chemotherapy, radiation therapy, biologic therapy, and targeted therapy.
Chemotherapy	One of the earliest treatment options for advanced melanoma. **Dacarbazine** - the standard chemotherapy medication for metastatic melanoma. **Temozolomide (TMZ)** - an oral prodrug of the active metabolite of dacarbazine, used in advanced melanoma. Shown reduced improvement in median progression free survival when compared to dacarbazine. **Electrochemotherapy (ECT)** - a technique combining cytotoxic drugs, bleomycin and cisplatin, with high-intensity electric pulses, facilitating drug delivery into cells. Effective for the treatment of cutaneous and subcutaneous nodules of melanoma.
Radiation therapy	High-energy radiation (external and internal) to induce melanoma cell death.
Biological (immuno-therapy)	**Interferon (IFN) α-2b** - Used as adjuvant therapy for the resected stage IIB/III melanoma. Demonstrates an immunomodulatory antitumour effect with a dose-dependent pro-apoptotic effect. **Pegylated interferon (IFN) α-2b** - Used as adjuvant therapy for stage III melanomas. Combination of IFN α-2b with the molecule polyethylene glycol (Peg)- improves the therapeutic effect by facilitating the compound to stay in the blood for longer. **Interleukin -2** - Used as a treatment for metastatic melanomas. **Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade** - Ipilimumab (an anti-CTLA-4 antibody) used in the treatment of advanced melanomas. **Programmed cell death protein 1 (PD-1)/PD-1 ligand (PD-L1) blockade** - Used in the treatment of metastatic melanoma. Mediates immune responses inducing (preclinical) antitumour activity, which reduces tumour progression.

Treatment Type	Details
	• Pembrolizumab, an anti-PD-1 antibody, used in the treatment of advanced melanomas (treatment of ipilimumab refractory melanomas). **Oncolytic virus therapy** - The first oncolytic virus for the treatment of melanomas and leads to tumour cell lysis and the release of tumour-specific antigens in melanoma cells. • Coxsackievirus (CVA21) or CAVATAK is an oncolytic virus in late-stage clinical development where lytic activity against melanomas has been seen *in vitro* cultures and *in vivo.* **gp100 Peptide vaccine** - Limited clinical benefit as monotherapy can be used as adjuvant therapy **Toll-like receptor (TLR) agonists** - A potent adjuvant for vaccines and can activate the immune system **Adoptive T-cell therapy** - Infusion with melanoma specific T-cells. Involves inducing the formation of memory T-cells which can improve anti-tumoral functions.
Targeted Therapy	**BRAF inhibitors** - Vemurafenib, a selective oral BRAF-mutant inhibitor used for the treatment of unresectable or metastatic melanomas harboring activating $BRAF^{V600E}$ mutations. **MEK inhibitors** - Trametinib, a pharmacological MEK1/2 inhibitor with antitumoural activity, used for the treatment of unresectable or metastatic malignant melanomas with *BRAF* mutations. Decreases tumour cell proliferation. **CKIT inhibitors** - Imatinib is an oral c-KIT used in patients with metastatic melanoma harbouring *c-KIT* aberrations. **VEGF inhibitors** - Bevacizumab, an anti-VEGF monoclonal antibody, able to target and neutralise VEGF inhibiting tumour growth. **PI3K-AKT-mTOR pathway inhibitors** - Combination of PI-103, a PI3K inhibitor, with the mTOR inhibitor rapamycin may effectively block the growth of melanoma cells inducing autophagy. **Cyclin-dependent kinase (CDK) inhibitors** - Selective CDK4/6 inhibitors, including ribociclib, abemaciclib, and palbociclib. Abemaciclib may induce growth regression in vemurafenib-resistant melanoma models. **ErbB4 inhibitor** - *ErbB4* mutations identified in melanomas are associated with increased kinase activity and transformation ability.

1.18 Summary and aims

This introduction has explored the clinical presentation, epidemiology and known determinants of melanoma survival. I have outlined the complex role of the immune system in the promotion and prevention of cancer, in addition to specific mechanisms by which the immune system interacts with melanoma. I have described obesity as a physical characteristic which is independently associated with the development of multiple types of cancer and which is associated with a state of low-grade systemic inflammation. On the basis of these findings, I have hypothesized that drugs incidentally used to treat obesity and conditions related to metabolic syndrome may affect survival outcomes in melanoma.

The specific aims of my project are therefore as follows:

(i) To test the hypothesis that BMI and Diabetes, as components of the inflammation/metabolic syndrome may independently be associated with melanoma survival outcomes in the Leeds Melanoma Cohort (LMC).

(ii) To test the hypothesis that there are incidental drugs that the LMC may have been exposed to, having examined the literature, that is associated with survival outcomes independent of the effects on the inflammation/metabolic syndrome.

(iii) To test the hypothesis that my selected drugs, namely aspirin, statins and metformin are associated with survival outcomes in the LMC independent of the underlying medical problem for which these drugs have been prescribed or as moderators of the inflammation/metabolic syndrome which may also modify survival outcome as above.

Chapter 2
Materials and Methods

2.1 Aims

The aims of this chapter are:

- To describe the studies from which patient samples and clinical data have been derived for work presented in this thesis.
- To describe the sources and types of data collected including the process and strategies for ensuring the quality of the collected data and subsequent analysis
- To describe the methodology used throughout the thesis and describe methods considered and justify the chosen methods and outline any limitations

2.2 The Leeds Melanoma Cohort

The Leeds Melanoma Cohort is the largest cohort in the world of primary melanoma patients consisting of 2184 melanoma patients recruited in the period between 2001 and 2012 and who have consented to the use of their medical records and tissue samples (MREC 01/3/057). Participants also gave consent to allow researchers to access their medical records and flag them with the Office for National Statistics (ONS) to enable ascertainment of the date and cause of their death should they die. Patients were recruited with the help of specific pathology and clinical registers within a geographically defined area of the Northern part of the UK, as shown in Figure 2.1, with further recruitment from 32 other clinical centres that also undertake sentinel node biopsy (total 342 recruits) and of rare subtypes with melanomas arising in sun-protected sites (total 76 recruits). Patients were subsequently invited to participate at 3 months after diagnosis with the intention of interviewing and sampling them within a defined period of between 3 to 6 months after diagnosis. There was some variability in how quickly patients responded and the median time to interview was found to be 5.2 months. Each patient completed a series of detailed questionnaires, which included questions about their drug usage history, concurrent illnesses and smoking status at recruitment [33].

Cohort members were then followed up both directly (by annual re-contact of the majority of the participants) as well as passively by regular review of national registers and their medical records. For each patient responding to an annual follow up review, information regarding drugs and co-morbidities was supplemented: 1647 of the 2184 agreed to at

least one annual review. In cases were patients died, the death certificate (or cause of death data taken from the death certificate obtained from the office of national statistics (ONS)) and medical records were obtained. Melanoma specific survival (MSS) data was generated by research nurses and Professor Newton-Bishop who reviewed the evidence relating to cause of death from these sources and determined whether the cause of death for each case was melanoma related or non-melanoma related. Thus a cancer registry reported cause of death was assessed and compared with medical records (primary, secondary and tertiary care) and patient reports of disease progression to derive the best possible measure of melanoma specific death [33].

Figure 2.1: Leeds Melanoma Cohort and location of Leeds on a map of the UK

2.3 Data collected from cohort

At the point of recruitment into the cohort patients were asked to complete a questionnaire that included information regarding weight and height (from which BMI was calculated); usage of drugs and over the counter medication as discussed in detail below, diabetes at diagnosis; and smoking history. Variables for each of these measures were created from the Cohort questionnaire data. Seasonally adjusted serum vitamin D levels were generated by fitting levels in a linear model with test batch and season of blood draw (Jan-Mar, Apr-Jun, Jul-Sep, Oct-Dec) using Apr-Jun as the baseline. Vitamin D levels were classified as either deficient (less than 20 nmol/L), suboptimal (20–59.9 nmol/L) and three further levels of sufficient vitamin d levels – (60-84.9nmol/L), (85-99.9nmol/L), and (>100nmol/L). The 20–59.9 nmol/L category was used as the baseline

as this was the largest group. BMI (kg/m^2) was classified using the standard classification system defined by the World Health Organization of underweight (<18.5), normal (18.5–25), overweight (25–30) and obese (30+) [33].

In addition to this, cases were also interviewed about whether they had diabetes and also with regards to their smoking habits. Four smoking-related variables were generated from these data: patient ever regularly smoked (yes/no); patient current smoker (yes/no); duration for which patient smoked (in 5 year units); and an estimate of the quantity the patient smoked in pack years based upon self-reported consumption of commercial cigarettes, hand rolls, small cigars, large cigars and/or pipe tobacco (in units of 10 pack-years). Breslow thickness and ulceration status were derived from histopathology reports; in instances where no explicit mention of ulceration was made it was assumed that the primary had not been ulcerated. Survival time was defined as the period between the date of surgical excision of the primary and date of melanoma-specific death or last date of follow-up (at which point records were censored). Cases with multiple primaries and/or who had responded to the request to participate later and were therefore recruited more than 2 years after diagnosis, were excluded from survival analysis [33].

2.4 Drug Data Collection

Drug data provided by the patient at the time of recruitment in the form of completed questionnaires were further supplemented with data from annual follow up data collection in patients who participated in these. Further data was then obtained from GP records in cases where the GP did return data.

Once received, the GP records were firstly anonymised and then the data linked to the cohort via study numbers and then I scrutinised drug exposure data individually for each patient in the cohort. I then combined these data with the drug information from the initial questionnaire as well as the annual questionnaires for the patients participating in annual follow up (See Appendix A) which was a significant undertaking given the large numbers, accounting for much of the time on the project and this triangulation process helped to ensure optimal quality of data given the three sources as shown in the Figure 2.2 below. All patients provided drug data on recruitment into the cohort and 1647 of these agreed to at least one annual follow up. There were 1152 patients for whom we received GP returns and out of these 901 patients also had at least one annual follow up returned. 251 patients had GP returns only with no annual follow-up data at all, leaving 286 patients with just the data provided at the time of recruitment into the study as shown in Figure 2.2.

Total GP returns = 1152 (251+ 901)

At least one annual follow up = 1647 (901 +746)

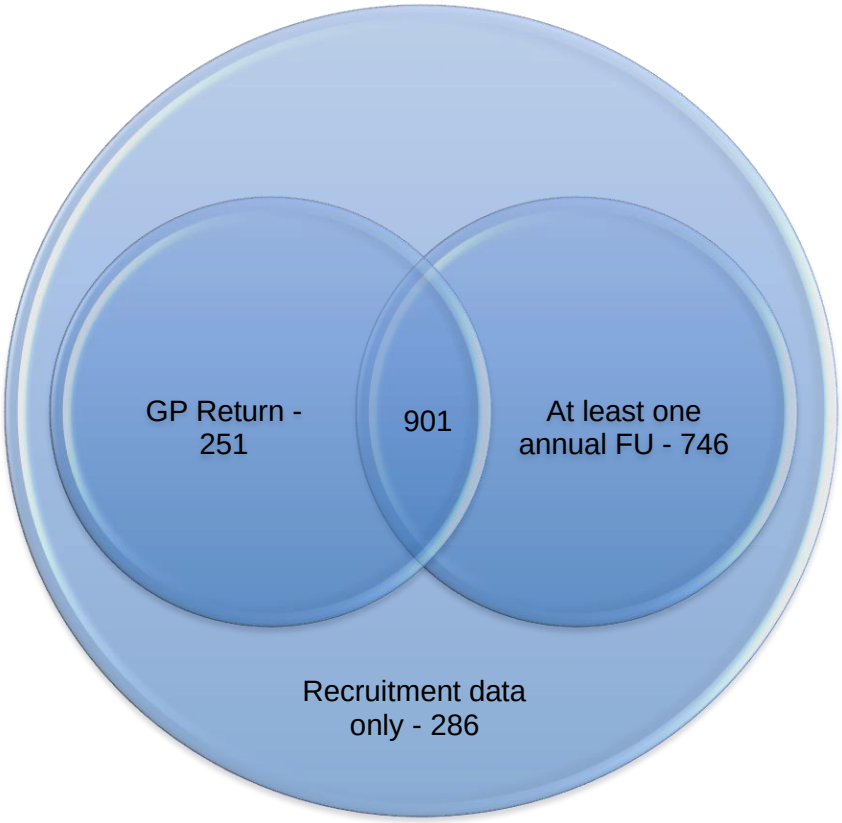


Figure 2.2: Sources of data including overlap between GP return data and annual follow up data

As seen in Table 2.1 and Table 2.2 there were 1647 patients who had at least one annual follow, 1140 with at least two and 249 patients who had 5 years of annual follow up.

Table 2.1: Breakdown of follow-up return data based on minimum number of returns per patient

No. of times followed-up	Cumulative number of patients
1	1647
2	1140
3	831
4	598
5	249

Table 2.2: Number of patients followed up per year

Year	No. of patients per follow-up year
1	512
2	306
3	234
4	346
5	249

Information regarding the name of the drug, the British National Formulary (BNF) code, dosage, frequency and start date/end date or last known date of being on the drug were also recorded. Only medications prescribed for more than a month were recorded as anything less than this was felt to be unlikely to result in a biological effect. Duration of drug usage was measured as the time between reported starting taking of the drug and stopping or until date of interview; cases for which no start date could be attributed were treated as missing data. Only drugs given systemically were included on the basis that it was postulated that their effects would be more consistent and measurable and therefore topical treatments, inhalers, and supplements were also excluded, which as will be discussed may be a limitation of the study. On occasions where no clear start/end date were available, the first known point at which a drug was started or the last available known point at which they were on a drug were recorded.

In most of the literature, researchers have classified duration of use of drugs in terms of short-term use versus long term use, but these studies have been concerned with investigating the effects of drugs on new cancer incidence whilst in our study we looked at relapse. As a result the time scales used in our study were considerably shorter and grouping of duration of drugs for the point of analysis had to take this into consideration. Furthermore, we considered comparing exposures at different dosages as suggested in the literature (low dose aspirin vs high dose aspirin for example) but given the short time scales as above, this led to further loss of power as well as further difficulties when trying to work out when dose changes occurred in the same patient and on discussion with the in-house statisticians I decided not to examine dosages in my analysis. Furthermore, as will be discussed in the results chapters, in most cases, patients were actually found to be on a standard dose of the drugs in question making it less likely that we would have missed a dose related effect.

Decisions about data analysis based upon biochemical groupings, functional analyses, and management of treatment over different time periods were made by:

- Examining the data and determining the distribution of exposures

- Consultation with Dr Barry Strickland-Hodge (Retired Senior Pharmacy Lecturer, University of Leeds)

2.5 Drug Grouping and Classification

All drugs recorded for the study were labelled with their designated British National Formulary (BNF) Code and this was considered to be the main reference for classifying and potentially grouping drugs with similar biochemical profiles and actions, with the intention of potentially increasing our statistical power for some of the rarer drugs. Common drugs were initially sub-classified as shown in Appendix B with the help of Senior Pharmacy Lecturer (Dr Strickland-Hodge). However, grouping of drugs with different BNF codes but presumed similar actions (Immunosuppressants for example) is fraught with high potential for error as the mechanism of action for the individual drugs is so varied. Furthermore, as can be seen from my literature analysis as well as the reviews specific to the individual drugs, the effects of drugs on melanoma and other cancers seem to be very specific to individual drugs within the same class of drugs, e.g. some effects are only seen with aspirin and simvastatin but not with the other cox inhibitors or other statins for instance. Additionally, many patients are switched between different drugs in the same class at different time periods, including being switched back to a previously taken drug. For instance, some patients would start out on simvastatin to control their hypercholesterolemia but then be switched to atorvastatin if they had for instance a stroke or a heart attack. A proportion of these, were subsequently found to have been switched back to simvastatin either as a result of side effects with atorvastatin, or due to cost saving measures. Having discussed this issue with our statisticians this was felt to be too complex for our proposed analysis methods as well as the fact that analysis of individual drugs would be a much cleaner approach in terms of looking for effects and any proposed underlying biological pathways.

2.6 BMI and Diabetes data as part of the Metabolic Syndrome

The data with regards to BMI and diabetes were readily available for the cohort and were used in analysing melanoma survival outcomes and together with the incidental drug exposures were the main focus of my thesis as my title suggests. However, ideally, I would have liked to investigate all the parameters of the metabolic syndrome including lipid profile and blood pressure. Unfortunately this was found to be inconsistently recorded in our cohort and although I looked at capturing this data by recording the use

of an anti-hypertensive as a marker that a patient has a diagnosis of hypertension, this was flawed by the fact that certain drugs like diltiazem or bisoprolol can be used both as an anti-hypertensive but also to treat arrhythmia and this approach was abandoned.

Also as previously mentioned the approach is complicated as both the drugs mentioned and the disease states for which they are prescribed could can have an effect on melanoma outcome.

2.7 Study Design

To achieve the aims of my thesis, several research designs could have been be considered. These include prospective cohort study, retrospective cohort study or a case control study potentially using telephone surveys or pharmacy databases. However, given access to this large cohort dataset as well as considering my aims and the agnostic approach adopted, a study design that allows one to consider multiple possible factors in the form of an observational longitudinal cohort study was felt to be an ideal starting point.

For the majority of the candidate drugs such as aspirin and statins, it is reasonable to assume that exposure allocation is unrelated to the outcome of interest, melanoma. Considering the fact that at the time of prescribing a drug, both the doctor and the patient are completely unaware of any potential effects on melanoma, the prescriber is effectively blind for the potential effect being investigated. It has been postulated that in such scenarios observational research may be as credible as randomised controlled trials [206].

2.8 Analysis of data

An issue considered in the early phases of the work described, was that many individuals in the study reported the use of multiple drugs and therefore I had to consider means of dealing with concurrent exposures in this observational study. Ideally then I would have considered exposure to combinations of therapies as well as individual exposures. However in discussion with my supervisors and the section statistical group, it was concluded that the study size was insufficient to allow this comparison and therefore that in this initial analysis, I would consider exposures to single drugs with an agnostic approach, accepting that this is a weakness of this approach.

Selection of an appropriate analysis method for investigating the effects of drugs on melanoma survival was one of the most challenging aspects of our study. We considered the following analysis methods and their limitations

2.8.2 Ever-never analysis

This is the simplest and most frequently used analysis method in the literature and divides the cohort into patients that have been exposed to the drug at some point (we only included patients into this group if we had evidence that they had been on the drug for at least 30 days) and those that had never been exposed to the drug in question. Although this type of analysis works well in studies looking at the effects on incidence of cancer, in studies like ours looking at survival, it does however introduce a potential bias referred to as the guarantee-time bias. This refers to the bias introduced by comparing survival across groups, which are defined by an event, which can occur at any time during the follow-up period. Patients experiencing the event may do so at any time during the study, with the likelihood being higher if the patient survives.

2.8.3 Duration Analysis

This analysis, which again is used routinely in studies looking at incidence of cancer, takes account of the effects of duration of usage of the drug in question. In a cohort study this however introduces a very obvious bias referred to as a survivorship bias whereby patients who have survived the longest would have in effect been on the drug for longer in which case it would be impossible to determine whether the drug allowed them to survive longer or whether they were on the drug longer because they survived longer for an unrelated reason. This bias, in addition to the lack of accurate drug use data over time in our cohort, precluded our use of this method of analysis.

2.8.4 Drug usage up until diagnosis

This method of analysis essentially replicates the traditional methods used in cancer incidence studies with no inherent bias but as the Leeds Melanoma Cohort data was not collected with this type of analysis in mind, we experienced problems with the poor quality data available before diagnosis and with some loss of power due to exclusion. Table 1 below demonstrates our power calculations and the likelihood of determining the degree of changes in hazard ratios proposed. Despite the limitations above, given the lack of any bias we included this type of analysis in our study.

2.8.5 Time-dependent or time-varying covariate analysis

This type of analysis is particularly suited for survival analysis and aims to minimise the biases discussed above. It reflects the phenomenon that a covariate such as drug use is not necessarily constant through the whole study. For instance, a patient may have been on and off a drug at different time points in the study and this could then be introduced in the statistical model as a time-varying covariate. In survival analysis, this would be done by splitting each study subject into several observations, one for each period of drug use/non-use. This method of analysis apart from being very complicated was not ideally suited to our data as we had to make some assumptions regarding continued use of drugs due to a lack of such specific drug usage data. The results from this type of analysis were therefore largely unchanged for any drug in our cohort and the approach was abandoned (See Appendix C).

2.9 Statistical Methods

The effects of exposure to different classes of drugs on melanoma specific (MS) and overall survival (OS) were assessed using Cox Proportional Hazards models. Data was first checked to ensure that the proportional hazards assumption was met. Hazard ratios (HR) and 95% Confidence intervals (CI) were estimated and Kaplan-Meier curves were plotted. Firstly, unadjusted models were examined and then adjustment for common confounders were applied; firstly gender and age at diagnosis, then Breslow thickness, ulceration, other comorbidity measures (smoking, body mass index (BMI), and serum level of vitamin D adjusted for seasonal variation. Analyses was conducted both with and without adjustment for stage (represented by Breslow thickness and ulceration), since a drug may influence outcome through an effect on the growth of the tumour, which may be captured by stage at diagnosis. No adjustment was made in the analyses for adjuvant cancer therapy since no therapy has been shown to influence melanoma survival until very recently. All the data was analysed using STATA 12.1 software (Stata Corp., 2003).

Multiple testing will be accounted for in these analyses by using Bonferroni correction to adjust the family wise error rate to 5%. The below power calculations have been run assuming the type I error rate is 0.1% which would be equivalent to a test at the 5% level adjusted using the Bonferroni correction for 50 simultaneous tests. Table 2.3 below shows the results of power calculations for our cohort and the resultant minimum detectable hazard ratios. The hypothesis-generating analysis looking for associations between other drugs and outcome will be conducted in a similar fashion using Cox

proportional hazard models. Significant findings would then need to be validated, by combining data from other large registries.

Table 2.3: Power Calculations

Prevalence of risk factor	Minimum detectable hazard ratio	
	80%	90%
331 melanoma specific deaths		
0.03	2.5	2.8
0.11	1.6	1.8
0.15	1.5	1.6
0.2	1.5	1.6
0.3	1.4	1.5
0.4	1.4	1.4
417 deaths from any cause		
0.03	2.2	2.5
0.11	1.6	1.7
0.15	1.5	1.6
0.2	1.4	1.5
0.3	1.3	1.4
0.4	1.3	1.4

As shown in Chapter 3, based on our power calculations above, I then determined for which drugs in the cohort I had sufficient statistical power. I also combined this with evidence from the literature of previous reports of effects in cancer and melanoma (see extract example in Appendix C) and finally ended up examining three drugs in more detail, namely aspirin (Chapter 4), statins (Chapter 5) and metformin (Chapter 6) followed by an overall discussion (Chapter 7) where I also discuss in detail the pros and cons of the methodology used.

Chapter 3
Descriptive analysis of cohort and results

3.1 Aims

The aims of this chapter are:

- To describe the characteristics of the Leeds Melanoma Cohort used in the thesis;
- To examine the effects of BMI and diabetes as part of the metabolic syndrome in our cohort;
- To summarise the literature survey approach and drug classes that have been reported to have effects on melanoma incidence and survival;
- To identify potential drug exposures of interest in our cohort and demonstrate survival analysis results for a selection of drugs examined;
- To describe how I arrived at the main drugs that will be covered in detail in the ensuing chapters of my thesis and to examine how commonly these are used together.

3.2 Characteristics of the Leeds Melanoma Cohort

As outlined in the last chapter, the Leeds Melanoma Cohort is the largest cohort in the world of primary melanoma patients consisting of 2184 melanoma patients recruited in the period between 2001 and 2012 from a geographically defined area of the Northern part of the UK, with additional recruitment from 32 other clinical centres carrying out sentinel node biopsy (total 342 recruits). To increase the data set of rare subtypes (melanomas arising in sun-protected sites) was the second reason for broadening recruitment to additional centres (total 76 recruits). Cases with multiple primaries and/or nodal primaries (patients with presenting with lymph node disease in the absence of a known skin primary) who had responded to the request to participate later and were therefore recruited more than 2 years after diagnosis, were excluded (67 patients) from the survival analysis as outlined in this chapter leaving 2117 patients for the purposes of my analysis.

As outlined in the methods chapter, each participant completed detailed questionnaires, which included questions about their drug usage history, concurrent illnesses, information regarding weight and height (from which BMI was estimated), diabetes status

and smoking history. Variables for each of these measures were created from the Cohort questionnaire data, as outlined below, in order to examine the components of the metabolic syndrome (BMI and Diabetes) as per my aims.

A variable for vitamin D levels was generated by fitting levels in a linear model with test batch and season of blood draw (Jan-Mar, Apr-Jun, Jul-Sep, Oct-Dec) using Apr-Jun as the baseline. This was necessary to allow for seasonal variation in vitamin D level. The justification for seasonal adjustment was that deficiency is known to predict bone health and higher summer levels might obscure winter nadirs if the data were not adjusted. Vitamin D levels were classified as either deficient (less than 20 nmol/L), suboptimal (20–59.9nmol/L) and three further levels of sufficient vitamin D levels – (60-84.9nmol/L), (85-99.9nmol/L), and (>100nmol/L). I used the 20–59.9nmol/L category as the baseline as this was the largest group.

Body mass index (BMI kg/m^2) was classified using the standard classification system defined by the World Health Organization of underweight (<18.5), normal (18.5–25), overweight (25–30) and obese (30+). The two main smoking-related variables used in my analysis below, generated from these data were: patient ever regularly smoked (yes/no); patient current smoker (yes/no). Breslow thickness and ulceration status were derived from histopathology reports; in instances where no explicit mention of ulceration was made it was assumed that the primary had not been ulcerated. Survival time was defined as the period between the date of surgical excision of the primary and date of death or last date of follow-up (at which point records were censored) and I also analysed overall survival in addition to melanoma specific survival for each drug.

The characteristics of the Leeds Melanoma Cohort are summarised in Table 3.1. Patients ranged from 17 to 90 years of age (mean age 54) as shown in the table and depicted in Figure 3.1. Of the 2117 patients 57% were female and 43% were male. In terms of risk factors for the metabolic syndrome within our cohort, 4% of patients were diabetic and 63% of patients were overweight or obese and with 22% being specifically classed as obese (Figure 3.2). In terms of smoking, 45% of patients had smoked at some point in their life whilst only 26% were current smokers. In terms of Breslow thickness the majority of patients (39%) had melanomas between 1.01-2.00mm in thickness with the thicker tumours (>4mm) only accounting for 11% with just 20% of tumours being ulcerated. In terms of Vitamin D levels, a large proportion (73%) had a less than normal Vitamin D (<60nmol/L). With regards to melanoma outcomes within the cohort, 427 patients (20%) of the cohort relapsed from their melanoma whilst 343 (16%) eventually died from their melanoma and 86 (4%) died from causes other than melanoma as shown in Table 3.1 below. Figure 3.3 below shows the various body sites of the primary melanomas in the cohort with the back being the most common site.

Table 3.1: Leeds Melanoma Cohort

For some variables, the summed total is less than the total number of patients because of missing values.

Baseline Characteristics	n (%)
Total no of patients	2,117(100)
Gender	
Female	1208 (57)
Male	909 (43)
Diabetes	
No	1948 (96)
Yes	81 (4)
Body Mass Index	
</=24.9kg/m^2	790 (38)
>24.9-29.9kg/m^2	854 (41)
>29.9kg/m^2	455 (22)
Vitamin D	
<20nmol/L	108 (6)
20-59.9nmol/L	1157 (67)
60-84.9nmol/L	391 (23)
85-99.9nmol/L	54 (3)
>100nmol/L	25 (1)
Ever-Never Smoked	
Ever Smoked	917 (45)
Never Smoked	1126 (55)
Current Smoking Status	
Not currently smoking	679 (74)
Currently smoking	239 (26)
Breslow thickness	
</=1mm	595 (28)
1.01-2mm	806 (39)
2.01-4mm	464 (22)
>4mm	228 (11)
Ulcerated Tumour	
Yes	429 (20)
No	1682 (80)
No of patients who relapsed	427 (20)
No of patients who died from Melanoma	343 (16)
No of patients who died from other causes	86 (4)

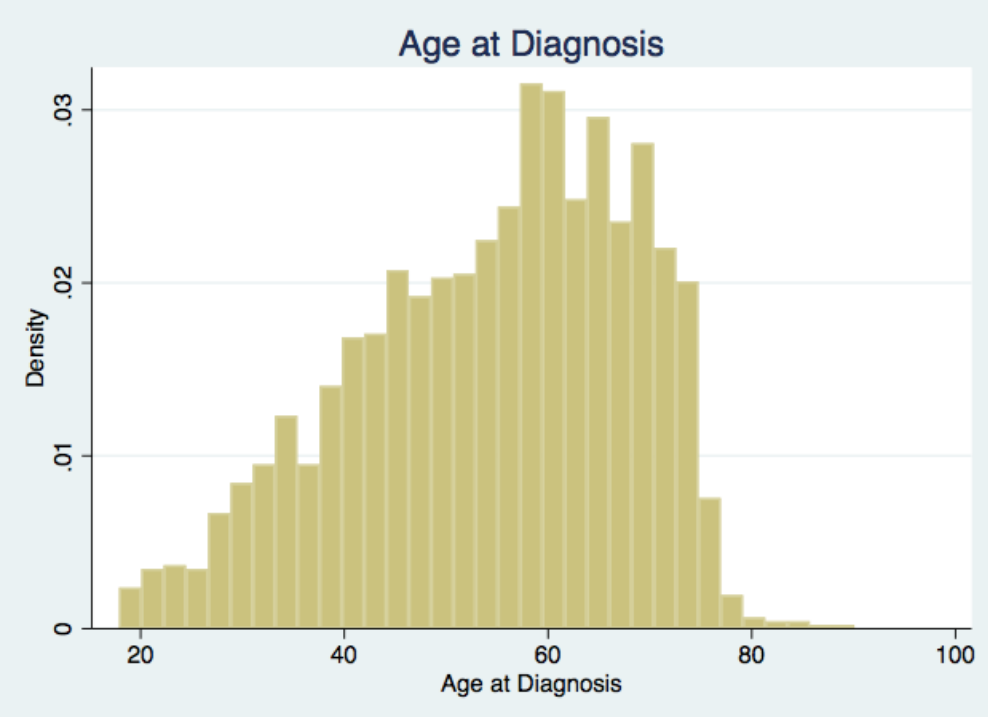


Figure 3.1: Distribution of age at diagnosis for LMC

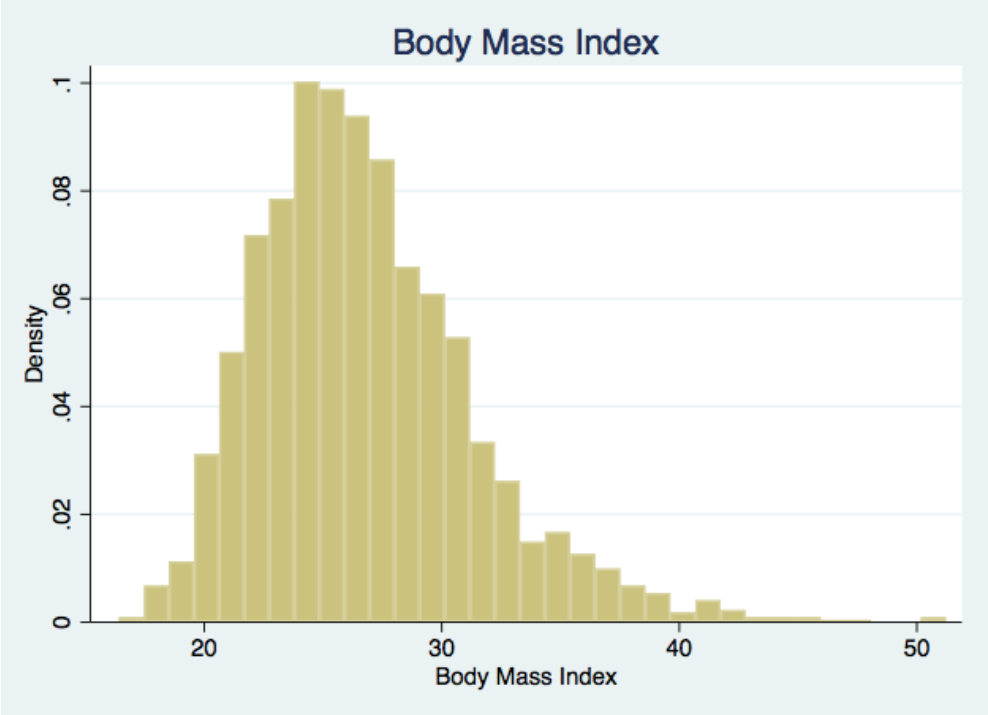


Figure 3.2: Distribution of BMI for patients in LMC

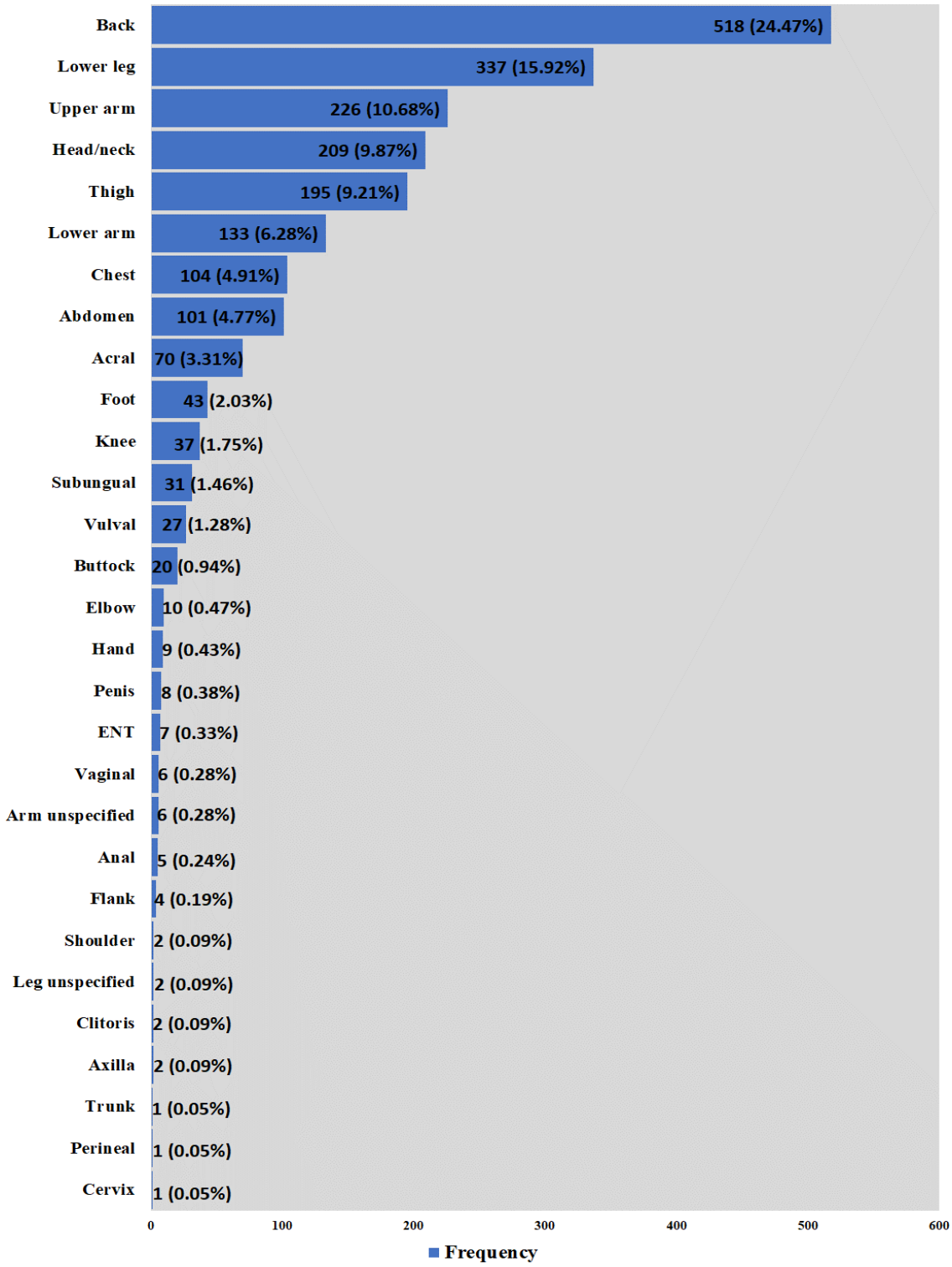


Figure 3.3: Sites of primary melanomas within the LMC

3.3 Effect of BMI and Diabetes on melanoma survival in the LMC

Before I examined the effects of incidental drug exposure, I needed to understand the relationship between the comorbidities being treated and melanoma survival. As per my aims I therefore assessed this by examining the association of BMI and Diabetes on melanoma survival in the LMC as outlined in the methods chapter by undertaking a Cox's proportional hazards regression analysis of melanoma-specific and overall survival dependent on BMI and diabetes separately. I first undertook an unadjusted analysis and then adjusted for known co-founders using the same approach I did with the incidental drug exposures.

3.3.1 BMI and melanoma survival in the LMC

As shown in Table 3.1 above, 63% of patients were classed as overweight or obese, with 22% being specifically classed as obese, which is less than what would be expected in the population as evidence suggests that (as discussed in Chapter 1), one third of the UK population are reported to be obese.

Table 3.2 below shows the results of the Cox's proportional hazards regression analysis of melanoma-specific and overall survival dependent on patients' body mass index. For both MSS and OS, the unadjusted model appears to show a statistically significant negative association with survival. However, increasing age and male sex are known predictors of melanoma survival but also for overall survival and when the model was adjusted for age at diagnosis and sex, these associations were no longer statistically significant and remained non-significant even in the multivariate analysis. This would therefore suggest that BMI in our cohort does not appear to have a directly significant association on survival for either MSS or OS. This could be a power effect if the risk is small, but the observation suggests that there was no strong association between obesity and MSS at least.

Table 3.2: Cox's proportional hazards regression analysis of melanoma-specific and overall survival dependent on patients' body mass index

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

	Melanoma-specific survival		Overall survival	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Unadjusted or crude model	1.01 (0.99-1.04)	0.074	1.02 (1.00-1.04)	0.055
Adjusted for age at diagnosis and sex	1.01 (0.98-1.04)	0.298	1.01 (0.99-1.03)	0.320
Adjusted for age at diagnosis, sex and Breslow	1.005 (0.98-1.02)	0.625	1.01 (0.98-1.02)	0.630
*Adjusted for other risk factors**	1.01 (0.99-1.04)	0.364	1.01 (0.98-1.04)	0.468

3.3.2 Diabetes and melanoma survival in the LMC

As shown in Table 3.1 above, 81 patients (4%) in the cohort were diabetic. Table 3.3 below shows the results of the Cox's proportional hazards regression analysis of melanoma-specific and overall survival dependent on diabetes. In both MSS and OS, the unadjusted model appears to show a statistically significant negative association with survival. However, as shown above and as will be shown with the same analysis for incidental drugs, increasing age and male sex are known predictors of melanoma survival and when the model is adjusted for age at diagnosis and sex, these associations were no longer statistically significant for MSS. Interestingly the negative association with overall survival remains significant despite adjusting for age and sex although the significance just disappears when adjusting for Breslow. The association remained statistically significant in the multivariable analysis suggesting that although diabetes does not appear to have a directly significant effect on melanoma specific survival it does predict overall survival. Given that a diagnosis of diabetes is associated with a greater mortality in the general population, the association with overall survival seen would be in keeping with this [207].

Table 3.3: Cox's proportional hazards regression analysis of melanoma-specific and overall survival dependent on diabetes status

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, body mass index, vitamin D levels, ulceration. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

	Melanoma-specific survival		Overall survival	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Unadjusted or crude model	1.85 (1.19-2.88)	**0.006**	2.16 (1.47-3.18)	**<0.001**
Adjusted for age at diagnosis and sex	1.37 (0.87-2.14)	0.164	1.52 (1.03-2.24)	**0.034**
Adjusted for age at diagnosis, sex and Breslow	1.24 (0.80-1.94)	0.336	1.43 (0.97-2.10)	0.073
*Adjusted for other risk factors**	1.51 (0.74-3.07)	0.250	2.37 (1.31-4.30)	**0.004**

In conclusion, from our analysis above it can be seen that in our cohort neither BMI nor diabetes alone appear to have a statistically significant association on melanoma specific survival although diabetes does appear to effect overall survival, and I cannot exclude a small association not detectable with confidence in this data set. This therefore would appear to reduce the complexity of any subsequent analysis of incidental drugs although I will continue to adjust for both BMI and diabetes in my drug analysis.

As discussed in Chapter 1, diabetic men have been shown to have inferior cancer outcomes and some studies have also demonstrated an elevated risk of CM with increasing BMI just among men [115]. Given this literature evidence of differential effects on sex in terms of diabetes and obesity and associations with cancer I therefore also undertook a stratification by sex analysis as shown in Table 3.4.

Table 3.4: Diabetes and Obesity – stratification by sex

* Adjusted for age at diagnosis and Breslow's thickness. Significant p-values are in bold figures

	Diabetes		BMI	
	Mortality HR (95% CI)	**p-value**	**Mortality HR (95% CI)**	**p-value**
Melanoma-specific				
Male	1.68 (1.00-2.82)	0.050	0.99 (0.96-1.03)	0.588
Female	0.71 (0.29-1.74)	0.449	1.02 (0.99-1.05)	0.205
Overall survival				
Male	1.77 (1.11-2.81)	**0.016**	1.01 (0.97-1.04)	0.740
Female	0.98 (0.48-2.00)	0.951	1.01 (0.98-1.04)	0.576

The table above shows an association of men with diabetes having a significantly poorer overall survival with a HR (OSS) of 1.77 (95% CI 1.11-2.81, p= 0.016) whilst diabetic men in the cohort also appear to have a negative association with melanoma survival which just falls short of being statistically significant with HR (MSS) of 1.68 (95% CI 1.00-2.82, p= 0.050). This would be in keeping with the literature evidence of such associations with diabetes in men for both for overall and melanoma specific survival as discussed in Chapter 1 [208].

As shown there appears to be no association between sex and obesity on either MSS or OS in our cohort which is contrary to the studies that have demonstrated an elevated risk of CM with increasing BMI particularly among men as referenced above. It is possible that this could be due to the fact that as pointed out earlier, only 22% of our cohort are classed as obese, which is less than what would be expected in the population as evidence suggests that (see Chapter 1), one third of the UK population are reported to be obese.

3.4 Summary of literature survey approach for drug selection

In order to gain insight into the current evidence for incidental drug exposures and chemoprevention in melanoma and cancer incidence and survival, a comprehensive literature review was undertaken (Figure 3.4).

An initial search was conducted on Pubmed using the terms melanoma and chemoprevention in the first instance to get an overview of the topic but then followed by separate searches with specific drug names and "cancer" and "melanoma" and any

reported effect on cancer in general and on melanoma specifically, were then recorded in a database along with the specific reference and article summary (see extract from database in Appendix C). In order to identify relevant drugs for analysis I then cross-referenced my findings with the most common drugs used in our cohort that would give me the best statistical power as described in the next section.

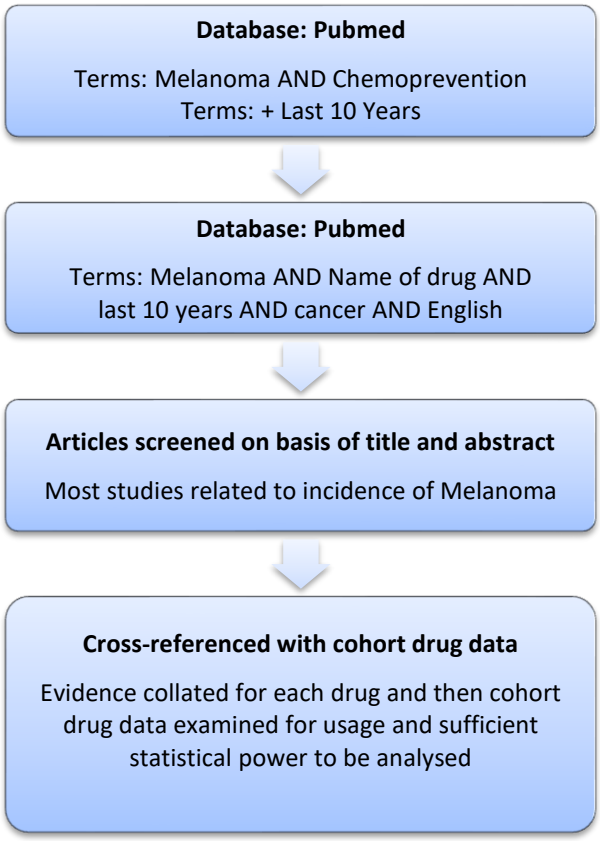


Figure 3.4: Search strategy to identify drugs affecting survival in melanoma

Examination of the results of my literature survey revealed some recurring drug groups with a body of evidence of reported effects on cancer and in melanoma but in most cases pertaining to melanoma incidence rather than survival. The main groups that emerged with evidence in human studies on melanoma included NSAIDs (including selective cyclooxygenase-2-inhibitors and in particular aspirin), statins, fibrates, and retinoids.

In addition to this, other drug groups like b-blockers, ace inhibitors, and calcium channel antagonists, which are commonly prescribed for high blood pressure, were also reported in the literature to have potential effects on cancers including in melanoma although there were also several reports showing no effect. As described in Chapter 1, melanoma is a very immunogenic tumour and is susceptible to the effects of immunosuppressants and

I was therefore also keen to see if any of these prescribed to patients in the cohort for treatment of conditions other than melanoma could be moderating survival.

Finally, metformin, a drug used in diabetes, has been previously shown in a smaller unpublished study looking at usage in our cohort, to have a trend towards a negative effect on melanoma survival. Although the literature in terms of epidemiological studies suggest a potentially positive association on melanoma outcome in patients some laboratory studies of melanoma cells with somatic *BRAF* mutations have shown that metformin has a negative effect on these cells as will be discussed in more detail in chapter 6. In addition, due to its potential link with chronic inflammation and the metabolic syndrome, metformin was therefore also of particular interest in this study.

3.5 Identification of relevant drug exposures within Leeds Melanoma Cohort

In order to identify relevant drug exposures, it was important to look at the prevalence of the relevant exposures and compare this with our power calculations and then tie it in with the results of the literature review above. I therefore firstly looked at the number of patients on medications as listed in Table 3.5 below by searching based on ever-never exposure and the specific BNF codes.

As discussed in the methodology section these BNF groups contain quite a heterogeneous group of drugs and cannot be analysed in this manner and need to be broken down into individual drugs. At this point it is worth re-visiting our power calculations as shown in Table 3.6 below. As can be seen a prevalence of 3% (0.03) (a drug being used in 3% of the cohort) gives us the ability only to identify a significant change in hazard ratio for MSS of 2.5 or 2.8 with 80% and 90% power respectively whilst at a prevalence of 15-20% (0.15-0.20) our power increases substantially as highlighted in the table. This sort of prevalence of 15-20% of a drug in our cohort would equate to between 317 to 423 exposures respectively to the particular drug. Although I am still able to analyse drugs below this threshold, I would only be able to pick up very significant changes in hazard ratios in terms of the effect of the drugs on MSS.

Table 3.5: Drug use by British National Formulary classification

BNF, British National Formulary

BNF Classification	No.	%
1: Gastrointestinal system	1,434	8.22
2: Cardiovascular system	7,215	41.38
3: Respiratory system	1079	6.19
4: Central nervous system	2,298	13.18
5: Infections	276	1.58
6: Endocrine	1480	8.49
7: Obstetric and genitourinary	717	4.11
8: Malignant disease and immunosuppression	216	1.24
9: Nutrition and blood	501	2.87
10: Musculoskeletal and joint disease	1,106	6.34
11: Eye	262	1.50
12: Ear, nose and throat	162	0.93
13: Skin	692	3.97
Total	17,438	100.00

Table 3.6: Power Calculations

Prevalence of risk factor	Minimum detectable hazard ratio	
	80%	90%
343 melanoma specific deaths		
0.03	2.5	2.8
0.11	1.6	1.8
0.15	**1.5**	**1.6**
0.2	**1.5**	**1.6**
0.3	1.4	1.5
0.4	1.4	1.4
429 deaths from any cause		
0.03	2.2	2.5
0.11	1.6	1.7
0.15	1.5	1.6
0.2	1.4	1.5
0.3	1.3	1.4
0.4	1.3	1.4

I therefore examined this further by looking at the power for the drugs in the literature above as having effects on cancer.

Table 3.7: Drugs from literature review

Drugs of interest from literature review	Sufficient number of individual drugs in cohort for meaningful analysis?
Aspirin	Yes- see analysis below
Statins	Yes- see analysis below
Fibrates	No- only 37 patients on bezofibrate, ezetimibe and fenofibrate together
Retinoids	No- only 2 patients on Acitretin noted
B blockers	Yes- see analysis below
Ace inhibitors	Yes- see analysis below
Calcium channel antagonists	Yes- see analysis below
Immunosuppressants	No- See table below for breakdown
Metformin	Yes- but power sufficient only to detect a significant change in HR - see analysis below

As can be seen, unfortunately there were not enough patients on fibrates, retinoids or individual immunosuppressants to carry out a meaningful analysis.

Next, I looked at individual drugs with the largest frequency of use (giving us the most power) together with the ones specifically reported in the literature (Table 3.8).

Table 3.8: Frequency of regular drugs ever taken by participants

NSAID, non-steroidal anti-inflammatory

Drug	Yes - No. (%)	No - No. (%)
Aspirin	347 (16.39)	1,770 (83.61)
Atorvastatin	116 (5.48)	2,001 (94.52)
Clopidogrel	34 (1.61)	2,083 (98.39)
Metformin	88 (4.14)	2,040 (95.86)
NSAID	255 (12.05)	1,862 (87.95)
Omeprazole	177 (8.31)	1,952 (91.69)
Simvastatin	345 (16.20)	1,785 (83.80)

Of the drugs reported in the literature, as can be seen aspirin and statins were the most commonly used drugs and therefore the ones for which the study was better powered. As metformin has previously been shown to have a possible negative association in an unpublished study from our cohort this was also selected for detailed analysis despite the low statistical power. These three drugs (aspirin, statins, and metformin) will be presented separately in the ensuing chapters although I will examine how many of the same patients are each of these groups at the end of this chapter.

Given the immunogenic nature of melanoma as discussed earlier, I was keen to examine the types of immunosuppressants our cohort had been exposed to and this is presented in Table 3.9 below. The 14 patients on Mycophenolate Mofetil were the largest group on an individual drug, which would give us insufficient power to carry out a meaningful analysis. Also, as can be seen, a number of these drugs were used to treat the melanoma itself and therefore this could not be explored further although this would be something that could be important to examine in any potentially larger validation cohorts for my study generally.

I did however examine a number of the other drugs in our cohort based on the literature evidence and prevalence in our cohort but none of this showed any statistically significant results as summarised in Table 3.10. Since I designed my study proton pump inhibitors such as omeprazole have attracted much attention as modifiers of the gut microbiome which is now understood to be related to host immune responses to melanoma [209].

Table 3.9: No. of patients receiving immunosuppressant drugs or chemotherapy

Immunosuppressant/Chemotherapy	No.	%
Azathioprine	3	0.10
Bevacizumab	5	0.16
Capecitabine	1	0.03
Chlorambucil	3	0.10
Ciclosporin	1	0.03
Cyclophosphamide	8	0.26
Dacarbazine	1	0.03
Estramustine	2	0.07
Fludarabine	2	0.07
Fludarabine	2	0.07
Hydroxycarbamide	3	0.10
Imatinib	1	0.03
Interferon alpha	1	0.03
Melphalan	1	0.03
Methotrexate	4	0.13
Miscellaneous	1	0.03
Mycophenolate mofetil	14	0.46
Prednisolone	1	0.03
Rituximab	3	0.10
Sirolimus	1	0.03
Sunitinib	2	0.07
Tacrolimus	6	0.20
Temozolomide	3	0.10
Thalidomide	1	0.03
Vemurafenib	7	0.23
Vindesine	1	0.03
Total	78	2.57

Table 3.10: Cox's proportional hazards regression analysis of melanoma-specific and overall survival in patients who received a given drug (ever-never)

Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and body mass index.

	Hazard Ratio	P-value	95% Confidence Intervals
Melanoma-Specific Survival			
Diclofenac	1.16	0.53	(0.73, 1.82)
Omeprazole	1.09	0.67	(0.73, 1.63)
Atorvastatin	0.67	0.12	(0.40, 1.11)
Overall Survival			
Diclofenac	0.97	0.91	(0.62, 1.53)
Omeprazole	1.17	0.40	(0.81, 1.68)
Atorvastatin	0.69	0.11	(0.44, 1.08)

As shown in Table 3.10 above, there is no evidence of any significant associations with ever use of diclofenac, omeprazole or atorvastatin on either overall survival or melanoma specific survival.

I have also shown the complete results in terms of our analysis subtypes as per our methodology below for a selection drugs after this for atenolol (Table 3.11 - Table 3.14), bisoprolol (Table 3.15 - Table 3.17) and diclofenac (Table 3.18 - Table 3.22).

3.5.1 Atenolol

As per Table 3.11 looking at the characteristics of patients on atenolol, the majority of these were women (95, 50.80%) although the difference between men and women was not statistically significant ($p<0.070$). In terms of BMI, patients on atenolol were more likely to be overweight or obese ($p<0.001$) which given that atenolol is used in patients with high blood pressure would be in keeping with this finding.

Table 3.12 demonstrates how there is no association of atenolol ever use with either MSS or OS in both unadjusted and adjusted models in our cohort. As mentioned previously this has been reported in the literature to have some associations in limited studies but this was not the case in my analysis.

Table 3.13 demonstrates how there is no association of atenolol use at diagnosis (or within 12 months) with either MSS or OS in both unadjusted and adjusted models in our cohort.

Table 3.14 demonstrates how there is no association of atenolol use when stratified by sex with either MSS or OS in both unadjusted and adjusted models in our cohort.

Table 3.11: Characteristics of patients ever treated with atenolol

Continuous variables are summarized as median (interquartile range) and p-values were generated using the Mann-Whitney U test. Categorical variables are represented as n (%) and p-values were generated using chi-squared test or Fisher's exact test if expected cell count was less than five. P-values were considered statistically significant if <0.05.

Ever Treated with Atenolol?	Yes - n (%)	No - n (%)	P-value
Total no of patients	**187 (100)**	**1,930 (100)**	
Gender			
Female	95 (50.80)	1,113 (57.67)	0.070
Male	92 (49.20)	817 (42.33)	
Diabetes			
No	170 (92.39)	1,778 (96.37)	0.009
Yes	14 (7.61)	67 (3.63)	
Body Mass Index			
</=24.9kg/m^2	29 (15.51)	761 (39.80)	
>24.9-29.9kg/m^2	92 (49.20)	762 (39.85)	<0.001
>29.9kg/m^2	66 (35.29)	389 (20.35)	
Smoking status			
Not currently smoking	68 (87.18)	611 (72.74)	0.005
Currently smoking	10 (12.82)	229 (27.26)	
Breslow thickness			
</=1mm	49 (26.34)	546 (28.63)	
1.01-2mm	62 (33.33)	744 (39.01)	0.144
2.01-4mm	48 (25.81)	416 (21.81)	
>4mm	27 (14.52)	201 (10.54)	
Vitamin D			
<20nmol/L	5 (3.03)	103 (6.56)	
20-59.9nmol/L	124 (75.15)	1,033 (65.80)	
60-84.9nmol/L	33 (20.00)	358 (22.80)	0.056
85-99.9nmol/L	1 (0.61)	53 (3.38)	
>100nmol/L	2 (1.21)	23 (1.46)	

Table 3.12: Cox's proportional hazards regression analysis of melanoma-specific and overall survival in patients who received atenolol (ever-never)

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and body mass index. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

Model	Parameter	Atenolol (ever-never)	
		Melanoma-specific	Overall survival
Unadjusted or crude model	*HR (95% CI for HR)*	1.29 (0.94-1.77)	1.21 (0.90-1.63)
	p-value	0.113	0.207
Age at diagnosis & sex-adjusted	*HR (95% CI for HR)*	0.92 (0.66-1.28)	0.87 (0.64-1.18)
	p-value	0.630	0.365
Adjusted for age, sex and Breslow	*HR (95% CI for HR)*	0.91 (0.65-1.26)	0.85 (0.62-1.15)
	p-value	0.555	0.287
*Adjusted for other risk factors**	*HR (95% CI for HR)*	0.86 (0.59-1.25)	0.86 (0.61-1.22)
	p-value	0.434	0.401

Table 3.13: Cox's proportional hazards regression analysis of melanoma-specific and overall survival in patients who received atenolol within 12 months of diagnosis

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and body mass index. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

Model	Parameter	Atenolol (use within 12 months of diagnosis)	
		Melanoma-specific	Overall survival
Unadjusted or crude model	HR (95% CI for HR)	1.27 (0.91-1.80)	1.22 (0.89-1.68)
	p-value	0.158	0.225
Age at diagnosis & sex-adjusted	HR (95% CI for HR)	0.91 (0.64-1.29)	0.86 (0.62-1.18)
	p-value	0.606	0.352
Adjusted for age, sex and Breslow	HR (95% CI for HR)	0.90 (0.64-1.28)	0.84 (0.61-1.17)
	p-value	0.569	0.304
*Adjusted for other risk factors**	HR (95% CI for HR)	0.87 (0.59-1.29)	0.86 (0.60-1.24)
	p-value	0.483	0.434

Table 3.14: Cox's proportional hazards regression analysis of melanoma-specific and overall survival stratified by sex in patients treated with atenolol

*Adjusted for age at diagnosis and Breslow's thickness. Significant p-values are in bold figures. HR, hazard ratio; 95% CI, 95% confidence interval.

Sex	Ever-never*		12 months of diagnosis*	
	Mortality HR (95% CI)	p-value	Mortality HR (95% CI)	p-value
Melanoma-specific				
Male	0.90 (0.58-1.39)	0.632	1.00 (0.64-1.59)	0.984
Female	0.94 (0.57-1.56)	0.825	0.81 (0.47-1.40)	0.452
Overall survival				
Male	0.81 (0.54-1.21)	0.298	0.85 (0.56-1.31)	0.468
Female	0.94 (0.60-1.50)	0.798	0.85 (0.52-1.39)	0.508

The above tables for atenolol have demonstrated the full range of analysis that I will carry out as per our methodology for our chosen drugs.

3.5.2 Bisoprolol

As per Table 3.15 looking at the characteristics of patients on bisoprolol, the majority of these were men (56, 66.67%) compared to women (28, 33.33%) and this difference was statistically significant (p<0.001). No other significant differences between users and non-users are shown in the table.

Table 3.16 demonstrates how there is no association of bisoprolol ever use with either MSS or OS in both unadjusted and adjusted models in our cohort. As mentioned previously with atenolol, these beta-blockers have been reported in the literature to have some associations in limited studies but this was not the case in my analysis.

Table 3.17 demonstrates how there is no association of bisoprolol use when stratified by sex with either MSS or OS in both unadjusted and adjusted models in our cohort.

Table 3.15: Characteristics of patients ever treated with bisoprolol

Continuous variables are summarized as median (interquartile range) and p-values were generated using the Mann-Whitney U test. Categorical variables are represented as n (%) and p-values were generated using chi-squared test or Fisher's exact test if expected cell count was less than five. P-values were considered statistically significant if <0.05.

Ever Treated with Bisoprolol?	Yes - n (%)	No - n (%)	P-value
Total no of patients	84 (100)	2,033 (100)	
Gender			
Female	28 (33.33)	1,180 (58.04)	<0.001
Male	56 (66.67)	853 (41.96)	
Diabetes			
No	74 (91.36)	1,874 (96.20)	0.029
Yes	7 (8.64)	74 (3.80)	
Body Mass Index			
</=24.9kg/m^2	22 (26.51)	768 (38.10)	
>24.9-29.9kg/m^2	38 (45.78)	816 (40.48)	0.089
>29.9kg/m^2	23 (27.71)	432 (21.43	
Smoking status			
Not currently smoking	38 (80.85)	641 (73.59)	0.269
Currently smoking	9 (19.15)	230 (26.41)	
Breslow thickness			
</=1mm	21 (25.30)	574 (28.56)	
1.01-2mm	32 (38.55)	774 (38.51)	0.764
2.01-4mm	22 (26.51)	442 (21.99)	
>4mm	8 (9.64)	220 (10.95)	
Vitamin D			
<20nmol/L	4 (5.63)	104 (6.25)	
20-59.9nmol/L	48 (67.61)	1,109 (66.65)	
60-84.9nmol/L	15 (21.13)	376 (22.60)	0.610
85-99.9nmol/L	4 (5.63)	50 (3.00)	
>100nmol/L	0 (0.00)	25 (1.50)	

Table 3.16: Cox's proportional hazards regression analysis of melanoma-specific and overall survival in patients who received bisoprolol (ever-never)

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and body mass index. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

Model	Parameter	Bisoprolol (ever-never)	
		Melanoma-specific	Overall survival
Unadjusted or crude model	HR (95% CI for HR)	0.65 (0.36-1.25)	1.12 (0.71-1.77)
	p-value	0.210	0.633
Age at diagnosis & sex-adjusted	HR (95% CI for HR)	0.44 (0.23-0.83)	0.68 (0.43-1.09)
	p-value	0.012	0.107
Adjusted for age, sex and Breslow	HR (95% CI for HR)	0.52 (0.27-0.98)	0.79 (0.50-1.25)
	p-value	0.045	0.315
*Adjusted for other risk factors**	HR (95% CI for HR)	0.49 (0.25-0.96)	0.78 (0.47-1.29)
	p-value	0.068	0.338

Table 3.17: Cox's proportional hazards regression analysis of melanoma-specific survival stratified by sex in patients treated with bisoprolol

*Adjusted for age at diagnosis and Breslow's thickness. Significant p-values are in bold figures. HR, hazard ratio; 95% CI, 95% confidence interval.

Sex	Ever-never*	
	Mortality HR (95% CI)	p-value
Melanoma-specific		
Male	0.63 (0.61-1.47)	0.376
Female	0.36 (0.16-0.82)	0.015

3.5.3 Diclofenac

As per Table 3.18 looking at the characteristics of patients on diclofenac, the majority of these were female (72, 54.55%) compared to males (60, 45.45%) although this difference was not statistically significant (p<0.546). No other significant differences between users and non-users were seen, although the association with Breslow thickness fell just short of being significant.

Table 3.18: Characteristics of patients ever treated with diclofenac

Continuous variables are summarized as median (interquartile range) and p-values were generated using the Mann-Whitney U test. Categorical variables are represented as n (%) and p-values were generated using chi-squared test or Fisher's exact test if expected cell count was less than five. P-values were considered statistically significant if <0.05

Ever Treated with Diclofenac?	Yes - n (%)	No - n (%)	P-value
Total no of patients	132 (100)	1,985 (100)	
Gender			
Female	72 (54.55)	1,136 (57.23)	0.546
Male	60 (45.45)	849 (42.77)	
Diabetes			
No	125 (96.90)	1,823 (95.95)	0.593
Yes	4 (3.10)	77 (4.05)	
Body Mass Index			
</=24.9kg/m^2	34 (26.15)	756 (38.40)	
>24.9-29.9kg/m^2	54 (41.54)	800 (40.63)	**0.002**
>29.9kg/m^2	42 (32.31)	413 (20.98)	
Smoking status			
Not currently smoking	42 (73.68)	637 (73.98)	0.960
Currently smoking	15 (26.32)	224 (26.02)	
Breslow thickness			
</=1mm	44 (33.33)	551 (28.10)	
1.01-2mm	61 (46.21)	745 (37.99)	**0.008**
2.01-4mm	22 (16.67)	442 (22.54)	
>4mm	5 (3.79)	223 (11.37)	
Vitamin D			
<20nmol/L	4 (3.39)	104 (6.43)	
20-59.9nmol/L	78 (66.10)	1,079 (66.73)	
60-84.9nmol/L	30 (25.42)	361 (22.33)	0.699
85-99.9nmol/L	4 (3.39)	50 (3.09)	
>100nmol/L	2 (1.69)	23 (1.42)	

Given that Breslow thickness is an independent predictor of melanoma survival, I explored the association between diclofenac use and Breslow thickness by performing a univariable analysis modelling the association of diclofenac use with age at diagnosis, gender, diabetes status, BMI, Breslow thickness and vitamin D status (Table 3.19). From this, it was observed that age at diagnosis, BMI >29.9kg/m^2 and Breslow thickness (>4mm category only) were associated with a statistically significant higher odds of diclofenac use. I then performed a multivariable regression analysis to explore if any of the association of diclofenac use with higher Breslow thickness was accounted for by these other associated variables (Table 3.20). Importantly, this analysis demonstrated that higher Breslow thickness category was not associated with a higher odds of diclofenac use following adjustment for age, male gender, diabetes and BMI.

Table 3.19: Univariable analysis of diclofenac use (ever-never)

Regression analyses were performed using a logistic regression approach with diclofenac use as a dependent variable and age, gender, diabetes status, body mass index, Breslow thickness, and vitamin D as independent variables in each univariable analysis.

	Odds Ratio	95% Confidence Interval	P-value
Age at diagnosis	1.02	1.00-1.04	**0.011**
Male gender	1.09	0.76-1.56	0.648
Diabetes	1.26	0.54-2.96	0.591
Body mass index			
>24.9-29.9kg/m^2	1.44	0.03-2.25	0.106
>29.9kg/m^2	2.08	1.29-3.35	**0.002**
Breslow thickness			
1.01-2mm	0.96	0.63-1.45	0.840
2.01-4mm	0.67	0.40-1.13	0.132
>4mm	0.23	0.08-0.65	**0.005**
Vitamin D	1.00	1.00-1.01	0.335

Table 3.20: Multivariable analysis of diclofenac use (ever-never)

Regression analyses were performed using a logistic regression approach with diclofenac use as a dependent variable and age, male gender, body mass index and Breslow thickness as independent variables.

	Odds Ratio	95% Confidence Interval	P-value
Age at diagnosis	1.02	1.01- 1.04	0.004
Male gender	0.93	0.64-1.36	0.700
Body mass index			
>24.9-29.9kg/m^2	1.37	0.87-2.17	0.175
>29.9kg/m^2	2.11	1.30-3.44	0.002
Breslow thickness			
1.01-2mm	0.87	0.57-1.32	0.518
2.01-4mm	0.56	0.33-0.96	0.034
>4mm	0.19	0.07-0.53	0.002

Table 3.21 demonstrates how there is no association of diclofenac ever use with either MSS or OS in both unadjusted and adjusted models in our cohort.

Table 3.21: Cox's proportional hazards regression analysis of melanoma-specific and overall survival in patients who received diclofenac (ever-never)

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and body mass index. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

Model	Parameter	Diclofenac (ever-never)	
		Melanoma-specific	Overall survival
Unadjusted or crude model	*HR (95% CI for HR)*	0.90 (0.60-1.35)	0.90 (0.61-1.32)
	p-value	0.608	0.597
Age at diagnosis & sex-adjusted	*HR (95% CI for HR)*	0.84 (0.56-1.26)	0.82 (0.55-1.20)
	p-value	0.392	0.298
Adjusted for age, sex and Breslow	*HR (95% CI for HR)*	1.15 (0.76-1.73)	1.03 (0.70-1.52)
	p-value	0.513	0.863
*Adjusted for other risk factors**	*HR (95% CI for HR)*	1.11 (0.70-1.75)	0.97 (0.61-1.52)
	p-value	0.657	0.883

Table 3.22 demonstrates how there is no association of diclofenac use when stratified by sex with MSS when adjusted for age at diagnosis and Breslow's thickness..

Table 3.22: Cox's proportional hazards regression analysis of melanoma-specific survival stratified by sex in patients treated with diclofenac

*Adjusted for age at diagnosis and Breslow's thickness. Significant p-values are in bold figures. HR, hazard ratio; 95% CI, 95% confidence interval.

Sex	Ever-never*	
	Mortality HR (95% CI)	p-value
Melanoma-specific		
Male	0.60 (0.32-1.13)	0.116
Female	1.13 (0.65-1.95)	0.672

3.6 Drug combinations of selected drugs for analysis within LMC

Of the drugs reported in the literature, as can be seen aspirin and statins were the most commonly used drugs and therefore the ones for which the study was best powered to study. As metformin has previously been shown to have a potentially negative association with melanoma survival in a small unpublished study in our cohort this was also selected for detailed analysis despite the low statistical power. These three drugs (aspirin, statins, and metformin) will be presented separately in detail in the ensuing chapters but I will present the number of patients on combinations of these drugs in Table 3.23 and Table 3.24 below as background prior to going through them in detail.

Table 3.23: Patients receiving two drug combinations

Drug 1	Drug 2	
	No - n (%)	Yes - n (%)
Aspirin	**Metformin**	
No	1,723 (84.84)	47 (54.65)
Yes	308 (15.16)	39 (45.35)
Aspirin	**Simvastatin**	
No	1,589 (89.2)	181 (53.87)
Yes	192 (10.78)	155 (46.13)
Aspirin	**Atorvastatin**	
No	1,734 (86.66)	36 (31.03)
Yes	267 (13.34)	80 (68.97)
Metformin	**Simvastatin**	
No	1,749 (97.78)	291 (84.84)
Yes	36 (2.02)	52 (15.16)
Metformin	**Atorvastatin**	
No	1,933 (96.60)	98 (84.84)
Yes	68 (3.4)	18 (15.52)

Table 3.24: No. patients receiving a combination of aspirin, metformin and simvastatin

	Simvastatin			
	No		Yes	
Aspirin	Metformin		Metformin	
	No	Yes	No	Yes
No	1570	19	153	28
Yes	175	17	133	22

As can be seen 155 patients were on both aspirin and simvastatin and 80 patients on both aspirin and atorvastatin. This would be in keeping with what we would expect in that these drugs are often prescribed together for primary and secondary prevention of ischaemic heart disease.

In terms of metformin, 39 patients were on this as well aspirin and 52 patients on metformin as well as simvastatin, and 18 patients on metformin and atorvastatin. As above diabetes is a risk for cardiovascular disease and therefore patients on metformin

are often prescribed a statin or aspirin as well. 22 patients were on all three medications (aspirin, simvastatin and metformin).

The above findings demonstrate the complexity of trying to analyse drug effects of our chosen three drugs, aspirin, statins and metformin and also highlights one of the drawbacks of our study approach, as we are only able to analyse one drug at a time as will be discussed in further detail in the ensuing chapters.

Ideally a population based study using public data bases could be used to address the relative associations of exposure to pairs of drugs but after discussion with the team and in particular Professor Jenny Barrett, I accepted that the data set was sufficient only to look at exposures independent of each.

3.7 Summary

In this chapter I have firstly, introduced the Leeds Melanoma Cohort and described the characteristics of the cohort highlighting aspects pertinent to my study aims. I have shown how I derived the different variables known to influence melanoma survival, such as Vitamin D status, BMI, and smoking status for instance and how they will be analysed.

Before examining the effects of incidental drug exposure I have then tried to accomplish at the outset one of my aims in terms of identifying any associations of BMI and diabetes as part of the metabolic syndrome in the cohort, as I needed to understand the relationship between the comorbidities being treated and melanoma survival. I therefore assessed this by examining the association of BMI and Diabetes on melanoma survival in the LMC as outlined in the methods chapter by undertaking a Cox's proportional hazards regression analysis of melanoma-specific and overall survival dependent on BMI and diabetes separately. I first undertook an unadjusted analysis and then adjusted for known confounders and found that in our cohort neither BMI nor diabetes alone appear to have a statistically significant association on melanoma specific survival although diabetes does appear to be negatively associated with overall survival, and I cannot exclude a small association not detectable with confidence in this data set. This therefore would appear to reduce the complexity of any subsequent analysis of incidental drugs although I will continue to adjust for both BMI and diabetes in my drug analysis. Given the literature evidence of differential effects on sex in terms of diabetes and obesity and associations with cancer I therefore also undertook a stratification by sex analysis for diabetes and obesity. This showed an association of diabetic men having a significantly poorer overall survival with a HR (OSS) of 1.77 (95% CI 1.11-2.81, p= 0.016) whilst diabetic men in the cohort also appear to have a negative association with

melanoma survival which just falls short of being statistically significant with HR (MSS) of 1.68 (95% CI 1.00-2.82, p= 0.050). This would be in keeping with the literature evidence of such associations with diabetes in men for both for overall and melanoma specific survival as discussed in Chapter 1 [208]. There was however no association seen between sex and obesity on either MSS or OS in our cohort which is contrary to the studies that have demonstrated an elevated risk of CM with increasing BMI particularly among men as discussed in chapter 1. It is possible that this could be due to the fact that only 22% of our cohort are classed as obese, which is less than what would be expected in the population as evidence suggests that (see chapter 1), one third of the UK population are reported to be obese.

Following this I then went on to summarise the literature survey approach undertaken and drug classes that have been reported to have effects on melanoma incidence and survival. The main groups that emerged with evidence in human studies on melanoma included NSAIDs (including selective cyclooxygenase-2-inhibitors and in particular aspirin), statins, fibrates, and retinoids. In order to identify relevant drug exposures, it was important to look at the prevalence of the relevant exposures and compare this with our power calculations and then tie it in with the results of the literature review above. Although there were specific drug groups such as immunosuppressants which would have been of interest in terms of their biological effects, I had insufficient numbers of patients on these drugs to be able to carry out an analysis. I have also presented some sample analysis of other drugs that were examined in this chapter. Ultimately I chose to examine aspirin, statins and metformin which will be covered in detail in the ensuing chapters and as a preliminary check I also examined how many patients were receiving a combination of these drugs as that could potentially have a bearing on our results going forward.

Chapter 4
Aspirin and Melanoma Survival

4.1 Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are traditionally prescribed because of their analgesic, antipyretic, and anti-inflammatory effects. NSAIDs inhibit the cyclooxygenase (COX) enzyme reversibly leading to reduced synthesis of prostaglandins (PGs), and thromboxanes (TXs).

Based upon their pharmacological effects, NSAIDs can be subdivided in three groups:

- First, traditional NSAIDs, e.g. diclofenac, naproxen, sulindac, indomethacin, and piroxicam, reversibly inhibit both the constitutively expressed COX-1 and the inducible COX-2 isoforms of the enzyme (i.e., nonselective COX-inhibitors).
- Secondly, the selective COX-2-inhibitors, e.g. celecoxib, etoricoxib, and rofecoxib, in regular doses, inhibit only the COX-2 isoform.
- Aspirin forms the third group, because it irreversibly inactivates COX-1 by acetylating a serine residue in its active site and, therefore, reduces thromboxane A2 (TXA2) in platelets. Due to the fact that platelets cannot synthesize new enzyme, TXA2 synthesis does not recover until new platelets arise after 7-10 days.

4.1.1 Biological effects of NSAIDs

The conversion of arachidonic acid to PGs and TXs is dependent on the enzyme cyclooxygenase (COX). The non-selective NSAIDs, including aspirin, act by inhibiting the activity of COX. Their *capacity* to decrease the inflammation is mainly due to the inhibition of COX activity, thus, decreasing the formation of pro-inflammatory PGs [192].

Two isoforms of COX are recognised i.e., COX-1 and COX-2 [210]. COX-1 is thought to be responsible for homeostatic or maintenance levels of PGs; although high levels are reported in some cancers. Whereas, COX-2, responsible for various inflammatory actions, is activated by a range of pro-inflammatory cytokines and growth factors in specific pathophysiologic conditions, and is overexpressed in many premalignant and malignant conditions, including Barrett's oesophagus, oesophageal cancer, gastric cancer, colorectal adenomas and cancer, and a wide range of other malignant conditions[191, 211, 212].

Overexpression of COX, especially COX-2, has been demonstrated in human cancer cells of several tumour types. Based upon these observations, the COX-pathway was

identified as a possible mediator of carcinogenesis. Indeed, the *ras* oncogene stimulates and *p53*, a tumour suppressor, down-regulates COX-2 expression. Moreover, COX-2 expression also seems to enhance metastatic potential of colon cancer cells and may be involved in resistance to chemotherapeutic drugs [213]. Thus, the primary potential mechanism of action of NSAIDs in cancer chemoprevention is considered to be COX inhibition [214].

Increased COX-2 expression has been noted in the majority, but not all, melanoma cell lines [215, 216]. Denkert *et al.* showed that five melanoma cell lines (A375, MeWo, SK-Mel-13, SK-Mel-28, and IGR-37) and 26 out of 28 (93%) patient derived primary melanomas showed COX-2 expression, whereas benign nevi (n=4) and epithelial cells were negative. After introduction of a COX-2 blocking agent, NS-398, cell line growth and invasive potential were inhibited [215]. Similarly, in a series of 101 *ex vivo* melanoma cell cultures, 96 (95%) showed COX-2 expression. More importantly, in this study, the level of COX-2 expression was also negatively associated with disease-specific survival (p = 0.046) [216]. Increasing evidence suggests that NSAIDs inhibit tumour growth and invasion [215, 217, 218] and can induce apoptosis [218, 219]. Roh *et al.* demonstrated an inhibitory effect of both celecoxib and indomethacin on melanoma cell growth in a murine B16F10 melanoma model [220]. Also, in a study of human A-375 melanoma cells, incubations for 72-hour of 50 and 100 micromolar (μM)of celecoxib showed reduced proliferation. Additionally, in a Toxilight TU-cytotoxicity assay, 100 (μM) celecoxib was toxic to the cancer cells. In this experiment, indomethacin (240 and 480 μM) also inhibited cell proliferation, but was only slightly toxic. Neither aspirin nor piroxicam exhibited cytostatic or cytotoxic effects. Thus, of the tested NSAIDs (aspirin, indomethacin, piroxicam, and celecoxib), only celecoxib and indomethacin reduced proliferation. Because these NSAIDs inhibit COX-2 in these concentrations, the authors suggested that the growth inhibitory effect of celecoxib cannot be explained solely by its COX-inhibitory activity [217].

Additional COX-independent pathways have also been suggested in other cancer types [221, 222]. Numerous possible targets, such as lipoxygenase metabolism (ALOX15), the pro-apoptotic gene *PAWR*, the anti-apoptotic gene *BCL2L1*, activation of caspases, activation of *p38* MAP kinase, release of mitochondrial cytochrome c, and activation of the ceramide pathway have been suggested to be involved [223-229]. These COX-independent pathways, however, need further study. For example, some investigators have suggested that only higher aspirin doses lead to these COX-independent molecular mechanisms [230]. Moreover, aspirin may have additional anticancer pathways as compared to other NSAIDs, such as inhibition of thrombocyte-aggregation [231], NF-κB, DNA-repair systems, apoptosis, oxidative stress, or mitochondrial calcium uptake [221].

Recently, Thyagarajan *et al.* [232] assessed the mechanism of action of aspirin in a highly aggressive melanoma and reported that aspirin acts by inhibiting the survival of murine melanoma cells via inducing apoptosis, suppresses the *in-vivo* growth of melanoma tumours, sry-related high-mobility box-2 (SOX2) mediates ASA-induced decreased growth of melanoma tumours *in-vivo,* PGF2α modulates SOX2 in mediating ASA-induced effects, SOX-2 up-regulation blocks ASA-induced effects, and SOX-2 up-regulation blocks PGF2α mimetic-induced effects. These findings suggest that the SOX2 signalling pathway mediates aspirin-induced decreased growth of highly aggressive melanoma [232].

4.1.2 Review of literature of associations of aspirin usage with cancer

Various studies have highlighted that the use of NSAIDS, especially Aspirin is associated with the reduced incidence of cancer. Moreover, there is extensive experimental evidence on how platelets and the coagulation system protect tumour cells within the circulation from immune elimination, enable cancer cells to adhere to vascular endothelium and thereby, enhance the growth of the metastatic cells [233]. Therefore, a reduction in metastatic spread by Aspirin is a highly plausible explanation.

Moreover, several trials have suggested protective effect of Aspirin in various cancers. A study by Holmes *et al.* demonstrated an association between use of anti-platelet drugs and reduced prevalence of cancer in patients with diabetes [234]. In another trial, Shebl *et al.* concluded that daily use of aspirin, but not ibuprofen, is liked with lower risk of prostate cancer [235]. Similarly, Soriano *et al.* observed that amongst majority of individuals without prior CVD, commencing low-dose aspirin is associated with a decreased incidence of CRC [190].

A possible role for aspirin in reducing the mortality associated with cancer has been best explored in colorectal cancer (CRC). Epidemiologic studies, *o*ver the last two decades, have reported that patients receiving non-selective NSAIDs such as Aspirin, experience around 40-50% decrease in mortality from colorectal cancer (CRC), than those not receiving Aspirin [191]. As discussed earlier, the evidence suggests, however, that there may be different levels of benefit in a variety of cancer types. Thus, there appears to be about a 25% reduction with Aspirin in the mortality of colon cancer (HR = 0.75; 95% CI = 0.68–0.83), about 20% reduction in breast cancer mortality (HR = 0.80; 95% CI = 0.66, 0.97), and a probable 15% reduction in prostate cancer deaths (HR = 0.86 (95% CI = 0.78, 0.95). There is also evidence of a substantial reduction in the incidence of metastatic spread of these cancers, together with a reduction in all-cause mortality across all the cancers [236]. However, various studies have reported conflicting results and are described below.

Aspirin and other non-aspirin NSAIDs are recognised for the prophylactic effect against CRC [221, 237-241]. Previously, it was thought that only high dose Aspirin exerts these effects, but recent findings support that prophylactic doses of Aspirin (75 mg per day) may be equally efficacious [242, 243]. Moreover, other findings suggest that patients receiving NSAIDs prior to the diagnosis have improved survival following the diagnosis of CRC [241, 244]. Additionally, in these studies, the greatest effects were seen in patients who commenced the use of Aspirin following diagnosis. Another study reported that CRC patients with high levels of COX-2 benefited the most from Aspirin [245].

Din *et al.* performed the first study to demonstrate a protective effect against CRC associated with the lowest dose of Aspirin (75 mg per day) after only 5 years use in the general population. Low-dose Aspirin use was associated with decreased CRC risk ($p = 0.004$), evident after 1 year and increasing with duration of use ($p_{trend} = 0.004$). NA-NSAID and any NSAID use were also inversely associated with CRC. There was no demonstrable effect of NSAIDS on all-cause ($p = 0.22$) or CRC-specific survival ($p = 0.93$). Additionally, the use of NSAID prior to CRC diagnosis did not influence the survival [242]. In another study, Walker *et al.* concluded that the use of Aspirin during the first 5 years may be beneficial in reducing the mortality in CRC patients. However, the same is not true for other NSAIDs, where a small rise in mortality was seen [246]. Regarding the survival in patients with *o*esophageal or gastric cancer, Spence *et al.* observed that use of low-dose Aspirin was not associated with increased survival [247]. Similarly, McMenamin *et al.* reported little evidence of a protective association between low-dose Aspirin use and cancer-specific mortality in a large population-based lung cancer cohort [248]. Another study by Verdoodt *et al.* concluded that l*ow-dose* Aspirin does not result in reduced mortality among women with ovarian cancer [249]. However, in another study, Flahavan *et al.* observed that the use of Aspirin was associated with a non-signi*fi*cant reduced risk of prostate cancer-speci*fi*c mortality in men with localised prostate cancer. However, men receiving higher doses of Aspirin had a statistically signi*fi*cant reduced risk of prostate cancer-speci*fi*c mortality [250].

Contrary to these findings, McNeil *et al.* [251]reported a higher all-cause mortality amongst apparently healthy older adults receiving daily Aspirin than among those who were receiving placebo and this was attributed primarily to cancer-related death [251].

4.1.3 Associations in melanoma

Healthy cohort studies are the best means of identifying the effects of concurrent drug use on cancer risks, however, conflicting results exist on NSAIDs in melanoma prevention. Initially, Harris *et al.* [252] reported a small case control study (110 cases, 609 controls, all females) in which regular NSAID use showed a significantly decreased

relative risk (RR) of melanoma (RR = 0.45; 95% CI = 0.22-0.95). With increasing NSAID use, melanoma risk further decreased ($p < 0.05$). Estimates for daily use of aspirin were similar (RR = 0.55) [252].

Subsequently, in a small retrospective cohort study (83 melanoma patients), users of NSAIDs or COX-2-inhibitors, as compared with nonusers, had a lower incidence of new melanoma, recurrence, and metastasis (combined end point; odds ratio (OR) = 0.08, 95% CI = 0.01-0.77) [253]. However, it is possible that a bias referred to as the guarantee-time bias may have influenced these results. In explanation, NSAID exposure, in this study, was defined as any prescription after first diagnosis of melanoma and prior to development of a new melanoma, a recurrence or metastatic lesion. Consequently, patients with longer survival are more likely to be categorized as a NSAID user, due to the simple fact that their follow-up period was longer, referred to as the guaranteed-time bias. More complex study designs and statistical analyses are required to prevent such a bias as explored in our methodology chapter, although we have found that they are difficult to implement and interpret [254].

In a secondary analysis of the Women's Health Study, Cook *et al.* [255] studied low-dose aspirin (100 mg every other day) versus placebo. Among the 39,885 women included in this RCT, low-dose aspirin was not associated with melanoma risk (RR = 0.97, 95% CI = 0.70-1.36) [255]. Similar results were obtained in a secondary analysis of the Cancer Prevention Study II Nutrition Cohort. Although long-term adult-strength aspirin (≥325 mg for ≥5 years) was associated with lower overall cancer incidence in men and a non-statistically significant lower overall cancer incidence was observed in women, melanoma incidence was not reduced (current daily use, ≥5 years: RR = 1.15, 95% CI = 0.83-1.59; <5 years: RR = 0.99, 95% CI = 0.79-1.25) [256].

Recently, in the Vitamins and Lifestyle (VITAL) cohort study, Asgari *et al.* [257] examined the association between NSAID use and melanoma risk. Among 63,809 men and women, during a 10-year follow-up period, 349 patients with incident melanomas were identified including 157 *in situ* melanomas. Use of any NSAID for at least 4 days per week as compared to non-use, did not seem to reduce the melanoma hazard rate (HR = 1.12, 95% CI = 0.84-1.48). Similar results were obtained for any NSAID excluding low-dose aspirin (HR = 1.03, 95% CI = 0.74-1.43), for regular- or extra-strength aspirin (HR = 1.10, 95% CI = 0.76-1.58), and for non-aspirin NSAIDs (HR = 1.22, 95% CI = 0.75-1.99). Additionally, NSAID use was not associated with tumour invasion (p-interaction = 0.38), tumour thickness (p-linear trend = 0.98), or risk of metastasis (HR = 1.09, 95% CI = 0.32-3.62) [257].

In a large population-based case control study of groups including 1,318 patients with invasive melanoma and 6,786 controls, incident melanoma was not associated with

aspirin use (OR = 0.92, 95% CI = 0.76-1.12) or non-aspirin NSAID use (OR = 1.10, 95% CI = 0.97-1.24). However, continuous use of low-dose aspirin was associated with a significant reduction of melanoma risk in women (OR = 0.54, 95% CI = 0.30-0.99), but not in men (OR = 1.01, 95% CI = 0.69-1.47). A significant linear trend (p = 0.04) from non-use, non-continuous use, to continuous use was observed in women [258].

In summary, due to heterogeneity in study design (ascertainment and definition of exposure, type of NSAID, dose, duration, patterns of use, drug adherence, study population, etc.), conflicting results and the limited number of epidemiological studies, the efficacy of NSAIDs and aspirin for melanoma prevention remains unclear. The results of *in vitro* and animal studies as discussed above, however, are promising. A pivotal unresolved problem is the definition of the temporal and dose-response cause effect relationships between NSAIDs use and incident invasive melanoma. Thus, additional experimental and observational research is warranted, particularly on required dosages and duration.

4.1.4 Safety and Compliance

Side effects of NSAIDs are gastrointestinal (GI) such as nausea, vomiting, dyspepsia (10-20%), diarrhoea, duodenal or gastric ulcers (10-30%), sometimes even leading to GI bleedings or perforation (± 2%) [259]. In addition, skin reactions, cardiovascular (CV) and cerebrovascular events, and decreases in renal function also occur. Rare, but serious, side effects are bone marrow disturbances and hepatotoxicity. The prevalence of GI related side effects differs substantially between several traditional NSAIDs, being less pronounced for aspirin and diclofenac compared to piroxicam.

COX-2-inhibitors have been developed to selectively inhibit COX-2 and thus, to reduce side effects related to COX-1-inhibition, most importantly duodenal and gastric ulcers. Indeed, duodenal or gastric ulcers are less prevalent (± 2%) for this class of NSAIDs [259]. However, thrombotic CV events observed in the APPROVe trial, a chemopreventive trial in which patients with a history of colorectal adenomas were randomized to receive rofecoxib or placebo [260], have raised safety concerns regarding the risk-benefit ratio of COX-2-inhibitors in cancer chemoprevention [261]. Subsequent epidemiological studies have suggested that these events are also associated with traditional NSAIDs, such as ibuprofen or diclofenac [262]. In these studies, naproxen, as an exception, was reported to be associated with a reduced CV event rate [262]. To prevent GI ulcers and bleeds, additional interventions such as *Helicobacter pylori* eradication and concomitant use of a proton pump inhibitor to the chemopreventive strategy could be considered, but this introduces new adverse effects and additional costs. Thus, in the AspECT trial, a combination of aspirin plus proton pump inhibitor was

studied for the chemopreventive activity on cancer among patients with Barrett's oesophagus [238]. And, it was observed that high-dose (40 mg twice-daily) PPI (omeprazole) with aspirin significantly and safely improved outcomes in patients with Barrett's oesophagus [68].

Aspirin may also cause bleeding through inhibition of thrombocyte aggregation. Due to this feature, however, aspirin does not cause an excess of CV events and actually has the advantage of protection against CV disease and apart from its use as an anti-pyretic and painkiller, it is used in both primary and secondary prevention of CV disease as well as in patients who have had coronary stents inserted. Moreover, aspirin may have additional chemopreventive effects as compared to other COX-inhibitors [221, 231].

4.2 Materials and Methods

Having collected the drug data for all drugs used in our cohort, which is the largest cohort of melanoma patients as detailed in Chapter 2, and having identified Aspirin as drug of interest with sufficient power to potentially demonstrate a significant effect, various further drug specific considerations were considered as detailed below.

Firstly, I had to ensure all entries for Aspirin were identified accurately by accounting for trade names, BNF codes, misspellings in data entry, and missing data by cross-checking all data sources. The most common dose for aspirin within our cohort was 75 mg with over 90% of patients on this, although there were some entries without a dosage specified. As discussed in Chapter 2, I, therefore, did not examine the effects of drug dosages given the standard doses used (which are generally the same in studies which looked at CRC and aspirin as discussed), additional complexity of the analysis as well as the perceived lack of a significant biological effect of dosage over duration, which was felt to be more significant from the review of the literature, as demonstrated above.

I then interrogated the data further by looking at the demographics of the population taking Aspirin by examining the number of males and females on the drug in our cohort, their smoking status, their diabetic status, vitamin D levels, and the distribution based on Breslow thickness. As Aspirin is prescribed to the same group of patients who are likely to have features of the metabolic syndrome with increased BMI and Diabetes and ischaemic heart disease (IHD), we expected to see some common trends within our analysis.

Given that men have a higher risk of these conditions, we would expect to see more men being prescribed Aspirin than women and we would also expect to see more smokers and diabetics having been prescribed Aspirin, as these are independent risk factor for

IHD. We would also expect this group of patients to be overweight compared to the rest of the cohort population (BMI > 25 Kg/m^2).

As detailed in the methodology chapter, I then undertook a survival analysis, based on firstly, the ever vs never approach with the accepted guarantee-time bias, first examining the adjusted model and then, adjusting for known confounders.

The second approach was a survival analysis looking at the effects of aspirin up to diagnosis or within 12 months of diagnosis. This method of analysis, used frequently in cancer incidence studies has no inherent bias, but as discussed in the methodology section our study was not geared up for this, as we experienced problems with some loss of power due to exclusion as well as the issue of poorer quality data before diagnosis. As with the previous approach, we first examine the unadjusted model and then, adjusted for the known confounders.

Finally, given the significant literature evidence of potential varying effects of drugs on survival based on sex, as demonstrated in our literature survey with the large Dutch population based study on melanoma incidence showing that continuous use of low-dose aspirin was associated with a significant reduction of melanoma risk in women (OR = 0.54, 95% CI = 0.30-0.99) but not in men (OR = 1.01, 95% CI = 0.69-1.47) as described above. We also carried out a survival analysis, whereby, we stratified study population by sex to identify any sex specific trends. As with the previous approaches, I first examined the unadjusted model and then adjusted for known confounders including age and Breslow thickness.

4.3 Results

Having implemented the methodology as detailed above and in Chapter 2, the results indicate that 347 (16%) of the 2158 participants in the Leeds Melanoma Cohort had ever taken aspirin at some point.

As per Table 4.1 below, the majority of these were men, with nearly twice as many men (226, 65.13%) as women (121, 34.87%) taking aspirin ($p<0.001$). This would be expected given that studies suggest that men are twice as likely as females to have IHD and as discussed, aspirin is often prescribed to reduce this risk.

Similarly analysis of the cohort showed that a higher proportion of people on Aspirin were diabetic (37, 10.95% vs. 44, 2.6%; $p<0.001$) or ever smokers (186, 54.87% vs. 731, 42.9%; $p<0.001$) compared to those not on Aspirin. This would be expected given that both diabetes and smoking are risk factors for IHD and this population is, therefore, more likely to be prescribed Aspirin. Interestingly people who had ever used Aspirin were more

likely to be current non-smokers (153, 82.26% vs. 526, 71.86%; p=0.004), and may have stopped smoking possibly due to cessation advice. In terms of BMI, patients on Aspirin were more likely to be overweight or obese (p<0.001), as this is a risk factor for IHD. As discussed earlier, vitamin D is one of the factors thought to influence melanoma survival and we, therefore, wanted to analyse our cohort to see if there was a statistical difference in vitamin D levels between users and non-users by defining different ranges of vitamin D and comparing with the base group and we observed no statistical difference as shown (p=0.273).

As a result of the observed higher proportion of participants ever treated with aspirin who had Breslow thickness of 2.01-4mm (90, 26.24% vs. 374, 21.37%) and >4mm (48, 13.99% vs. 180, 10.29%) in participants who had ever received aspirin compared to those never treated, I explored the association between aspirin use and Breslow thickness by performing a univariable analysis modelling the association of aspirin use with age at diagnosis, gender, diabetes status, BMI, Breslow thickness and vitamin D status (Table 4.4). From this, it was observed that age at diagnosis, male gender, presence of diabetes, BMI and Breslow thickness (2.01-4mm and >4mm categories only) were associated with a statistically significant higher odds of aspirin use. I then performed a multivariable regression analysis to explore if any of the association of aspirin use with higher Breslow thickness was accounted for by these other associated variables (Table 4.5). Importantly, this analysis demonstrated that higher Breslow thickness category was not associated with a higher odds of aspirin use following adjusted for age, male gender, diabetes and BMI.

Table 4.1: Characteristics of patients ever treated with aspirin

Continuous variables are summarized as median (interquartile range) and p-values were generated using the Mann-Whitney U test. Categorical variables are represented as n (%) and p-values were generated using chi-squared test or Fisher's exact test if expected cell count was less than five. P-values were considered statistically significant if <0.05.

Ever Treated with Aspirin?	Yes	No	P-value
Age at diagnosis	66 (11.5)	54 (20.1)	<0.001
Sex			
Female	121 (34.87)	1,087 (61.41)	<0.001
Male	226 (65.13)	683 (38.59)	
Diabetes			
No	301 (89.05)	1,647 (97.4)	<0.001
Yes	37 (10.95)	44 (2.6)	
Body Mass Index			
</=24.9kg/m^2	89 (25.8)	701 (39.97)	
>24.9-29.9kg/m^2	163 (47.25)	691 (39.4)	<0.001
>29.9kg/m^2	93 (26.96)	362 (20.64)	
Smoking status			
Never	153 (45.13)	973 (57.1)	<0.001
Ever	186 (54.87)	731 (42.9)	
Smoking status			
Not currently smoking	153 (82.26)	526 (71.86)	0.004
Currently smoking	33 (17.74)	206 (28.14)	
Breslow thickness			
</=1mm	79 (23.03)	516 (29.49)	
1.01-2mm	126 (36.73)	680 (38.86)	0.011
2.01-4mm	90 (26.24)	374 (21.37)	
>4mm	48 (13.99)	180 (10.29)	
Vitamin D			
<20nmol/L	22 (7.72)	86 (5.93)	
20-59.9nmol/L	176 (61.75)	981 (67.66)	
60-84.9nmol/L	70 (24.56)	321 (22.14)	0.273
85-99.9nmol/L	12 (4.21)	42 (2.9)	
>100nmol/L	5 (1.75)	20 (1.38)	

Table 4.2: Univariable regression analysis of aspirin use (ever-never)

Regression analyses were performed using a logistic regression approach with aspirin use as a dependent variable and age, gender, diabetes status, body mass index, Breslow thickness, and vitamin D as independent variables in each univariable analysis.

	Odds Ratio	95% Confidence Interval	P-value
Age at diagnosis	1.10	1.08-1.11	**<0.001**
Male sex	2.97	2.34-3.78	**<0.001**
Diabetes	4.60	2.92-7.25	**<0.001**
Body mass index			
>24.9-29.9kg/m^2	1.86	1.41-2.46	**<0.001**
>29.9kg/m^2	2.02	1.47-2.78	**<0.001**
Breslow thickness			
1.01-2mm	1.21	0.89-1.64	0.218
2.01-4mm	1.57	1.13-2.19	**0.007**
>4mm	1.74	1.17-2.59	**0.006**
Vitamin D	1.00	1.00-1.01	0.265

Table 4.3: Multivariable analysis of aspirin use (ever-never)

Regression analyses were performed using a logistic regression approach with aspirin use as a dependent variable and age, male gender, diabetes, body mass index and Breslow thickness as independent variables.

	Odds Ratio	95% Confidence Interval	P-value
Age at diagnosis	1.10	1.08- 1.12	<0.001
Male sex	2.27	1.74- 3.00	<0.001
Diabetes	2.69	1.61-4.49	<0.001
Body mass index			
>24.9-29.9kg/m^2	1.40	1.00- 1.88	0.052
>29.9kg/m^2	1.80	1.25-2.60	0.002
Breslow thickness			
1.01-2mm	0.98	0.70-1.38	0.911
2.01-4mm	0.97	0.67-1.41	0.866
>4mm	0.91	0.58-1.41	0.665

4.3.1 Effects of aspirin use on survival outcomes

As described in the methodology chapter, a comparison of melanoma-specific and overall survival distributions amongst participants who had ever used aspirin and those who used aspirin within 12 months of diagnosis was performed. I have discussed the results for aspirin ever-use and aspirin use within 12 months separately below.

4.3.1.1 Ever-never aspirin use

Unadjusted survival curves comparing melanoma-specific survival in participants who did or did ever use Aspirin is shown in Figure 4.1. This figure showed that melanoma-specific survival was significant reduced in patients who had ever used aspirin (logrank test p=0.045).

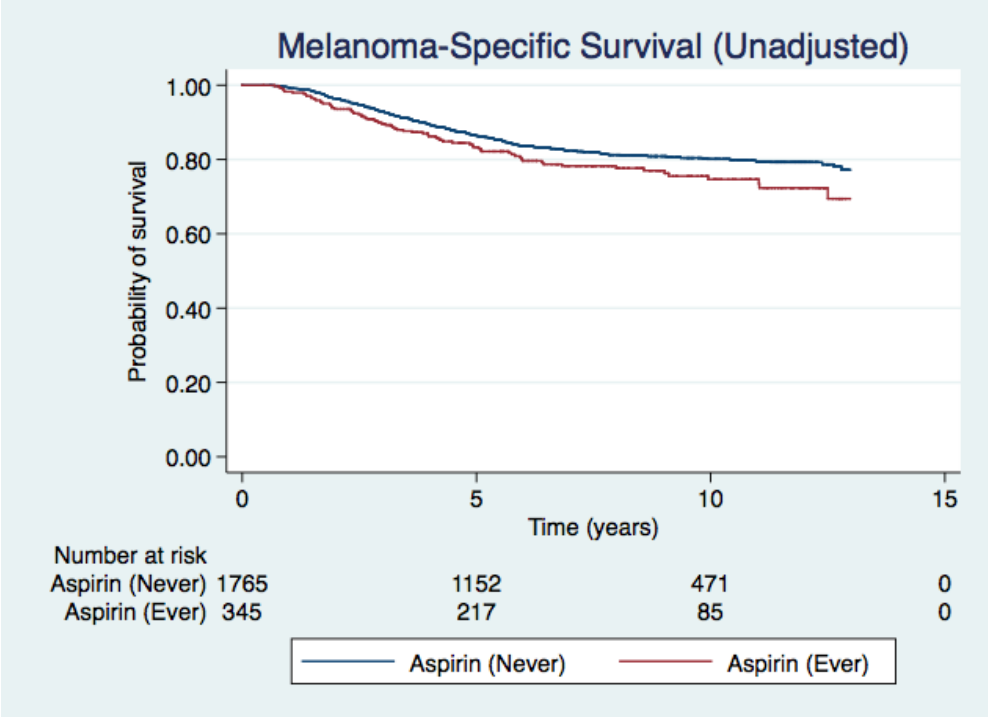


Figure 4.1: Kaplan-Meier survival plot comparing melanoma-specific survival in participants receiving aspirin (ever-never use).

A similar observation was made in the unadjusted Cox regression analysis (Table 4.4) and there was a significant increase in the risk of death in melanoma participants who reported having ever taken aspirin as compared to participants who had never used aspirin (HR = 1.30; 95% CI = 1.01-1.68; p-value = 0.046). A similar finding was observed for overall survival (HR = 1.53; 95% CI = 1.22-1.93; p-value < 0.001), which was statistically significant. However, increasing age and the male sex are known predictors of melanoma and overall survival. When the model was adjusted for age at diagnosis

and sex, this effect disappeared for melanoma specific (HR MSS = 0.81; 95% CI = 0.61–1.06; p-value = 0.120) and for overall survival (HR = 0.88; 95% CI = 0.69-1.12; p-value = 0.308) and in fact, suggested a non-significant protective effect. The same was true in the model adjusted for age at diagnosis, sex, and the other known confounding factors in a multivariable analysis as shown in the table below with melanoma specific (HR = 0.78; 95% CI = 0.56–1.08; p-value = 0.131) and for overall survival (HR = 0.88; 95% CI = 0.66-1.18; p-value = 0.398). However, as discussed in the methodology section, this analysis was subject to the guarantee time bias, by virtue of the fact that, the longer someone lives, the higher the likelihood of them becoming part of the aspirin-ever category.

Table 4.4: Cox's proportional hazards regression analysis on the association between ever having taken aspirin regularly (ever-never) with survival

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and BMI. Significant p-values are in bold figures

Model	Parameter	Aspirin (ever-never)	
		Melanoma-specific	Overall survival
Unadjusted or crude model	**HR (95% CI for HR)**	1.30 (1.01-1.68)	1.53 (1.22-1.93)
	p-value	**0.046**	**< 0.001**
Age at diagnosis & sex-adjusted	**HR (95% CI for HR)**	0.81 (0.61-1.06)	0.88 (0.69-1.12)
	p-value	0.120	0.308
Adjusted for age, sex and Breslow	**HR (95% CI for HR)**	0.85 (0.64-1.10)	0.93 (0.73-1.18)
	p-value	0.225	0.557
Adjusted for other risk factors*	**HR (95% CI for HR)**	0.78 (0.56-1.08)	0.88 (0.66-1.18)
	p-value	0.131	0.398

4.3.1.2 Aspirin use up to or within 12 months of diagnosis

Similar to the findings in Figure 4.1 among participants who had ever or never used aspirin, Figure 4.2 suggests that participants who had used aspirin up to or within 12 months of diagnosis of melanoma, had a significantly poorer melanoma-specific survival compared with those who reported no use of aspirin (logrank test p=0.008).

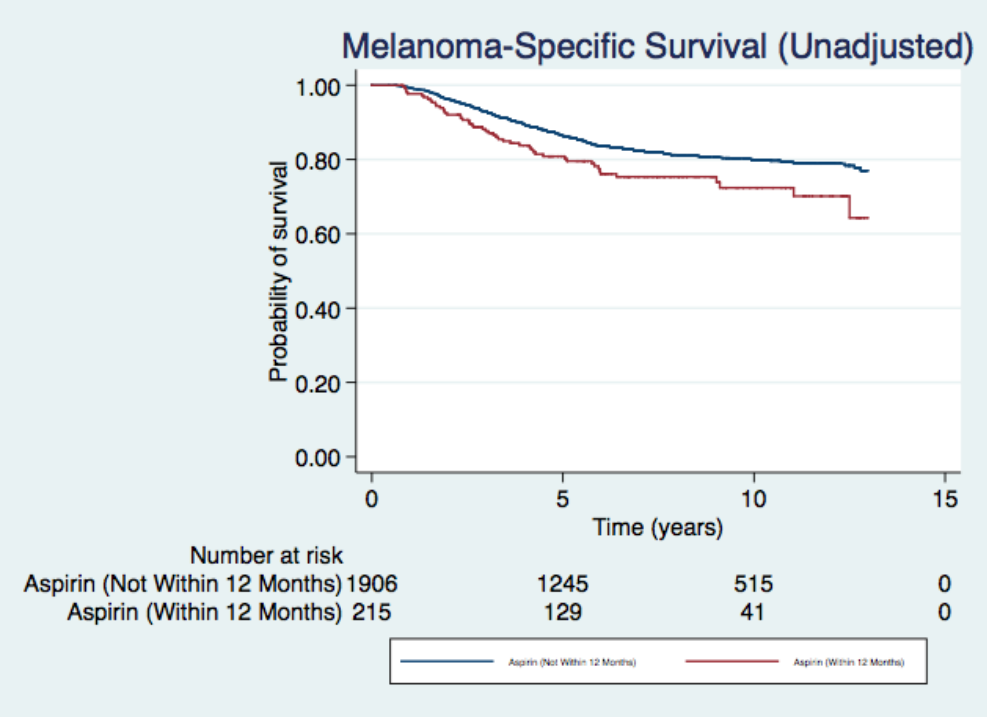


Figure 4.2: Kaplan-Meier survival plot comparing melanoma-specific survival in participants receiving aspirin within 12 months of diagnosis.

As seen with the previous analysis, Table 4.5 shows that in the unadjusted model, there appeared to be an increased risk of death from melanoma or all causes with HR of 1.49 (95% CI = 1.11-2.01, p-value = 0.009) and 1.58 (95% CI = 1.20-2.08, p-value = 0.001), respectively. This risk again was statistically significant, however when adjustment is made for age and sex, the HR was reversed, suggesting a non-significant protective effect with no statistically significant difference between melanoma specific survival and overall survival that was maintained in the multivariate analysis with HR of 0.93 (95% CI = 0.64-1.35, p-value = 0.706) and 0.87 (95% CI = 0.62-1.23, p-value = 0.442) respectively. A similar finding was found in the models adjusting for age, sex, Breslow thickness, and other risk factors. This analysis does not appear to have any inherent bias as with the previous analysis type, which is subject to the guarantee time bias. However the fact that the results are very similar would suggest the guarantee time bias didn't play such a large role in the previous analysis.

Table 4.5: Cox's proportional hazards regression analysis on the effect of aspirin use within 12 months of diagnosis on survival

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, microscopic ulceration, and BMI. Significant p-values are in bold figures

Model	Parameter	Aspirin (use within 12 months of diagnosis)	
		Melanoma-specific	Overall survival
Unadjusted or crude model	**HR (95% CI for HR)**	1.49 (1.11-2.01)	1.58 (1.20-2.08)
	p-value	**0.009**	**0.001**
Age at diagnosis & sex-adjusted	**HR (95% CI for HR)**	0.91 (0.66-1.25)	0.90 (0.68-1.20)
	p-value	0.573	0.491
Adjusted for age, sex and Breslow	**HR (95% CI for HR)**	0.91 (0.66-1.25)	0.91 (0.68-1.21)
	p-value	0.557	0.527
Adjusted for other risk factors*	**HR (95% CI for HR)**	0.93 (0.64-1.35)	0.87 (0.62-1.23)
	p-value	0.706	0.442

4.3.1.3 Stratification by sex

Based on literature evidence that women present with thinner melanoma and that women do better than men independent of thickness [86], I carried out a survival analysis stratified by sex. Concerning the effect of Aspirin use on survival when stratified by sex, the relative differences between these hazard ratios are tested as seen in Table 4.6. Sex did indeed confer a statistically significant influence on the risk of mortality in terms of both melanoma-specific and overall survival (p-value < 0.05). Female participants who had ever taken Aspirin appeared to be less likely to die from melanoma when compared with other females who never use the drug at any point in their lifetime with HR for melanoma specific survival of 0.51 (95% CI = 0.30-0.87, p-value = 0.014) and for overall survival of 0.61 (95% CI = 0.38-0.97, p-value = 0.035), suggesting that as compared to males, Aspirin use may have a preferentially protective effect in female participants.

Table 4.6: Cox's proportional hazards regression analysis of the association between aspirin use on survival stratified by sex

* Adjusted for age at diagnosis. Significant p-values are in bold figures

Sex	Ever-never*		12 months of diagnosis*	
	Mortality HR (95% CI)	p-value	Mortality HR (95% CI)	p-value
Melanoma-specific				
Male	0.99 (0.71-1.37)	0.948	1.07 (0.74-1.55)	0.717
Female	0.51 (0.30-0.87)	**0.014**	0.60 (0.30-1.18)	0.135
Overall survival				
Male	0.99 (0.71-1.37)	0.948	0.99 (0.70-1.38)	0.932
Female	0.61 (0.38-0.97)	**0.035**	0.68 (0.37-1.22)	0.197

4.4 Discussion

In this section I will discuss my findings in relation to the literature and what we already know about potential associations of aspirin on inflammation, cancer and melanoma and to determine whether these associations can explain my findings. I will also discuss the limitations of our study approach in examining these associations.

As already demonstrated, inflammation is driven by complex metabolic pathways, with arachidonic acid (AA) as one important molecule of origin in these pathways. The metabolism of AA is fundamental for both promotion and inhibition of inflammatory processes. As discussed in vitro studies demonstrate COX-2-expression in melanoma and suggest effects of NSAIDs on growth inhibition, invasiveness and apoptosis. COX independent pathways, however, may also be involved in these anti-tumour effects.

Initial reports on aspirin and cancer described a reduction in metastatic spread and focused primarily on the role of platelets, consistent with a treatment, rather than a preventive effect. Later, evidence emerged regarding the potential effects of aspirin on certain biological mechanisms relevant to cancer growth and to metastatic capacity which justified an expectation of benefit from aspirin treatment in cancer. Some of the long-term follow-up studies of early vascular trials gave evidence of reductions attributable to aspirin in the metastatic spread of a range of cancers in subjects who had been free of metastases at diagnosis, again suggesting a treatment effect of aspirin. Furthermore, while there is usually a delay before evidence of a reduction in incidence of cancer becomes apparent, typically with the need for large observational studies over

a long period, a reduction in mortality in patients with metastases appears to be easier to detect, strengthening a potential treatment effect of aspirin [263].

The role of low-dose aspirin prophylaxis however, has now become well accepted in the case of vascular disease and in the reduction of CRC, and probably other cancers, and it has even been predicted that 'prevention of cancer could become the main justification for aspirin use [263].

The results of my study demonstrate a non-significant protective effect of aspirin on both melanoma specific and overall survival, when adjusting for known confounders such as age and sex as well as in multivariate analysis accounting for multiple factors such as age, sex, diabetes, BMI, vitamin D, smoking status, and Breslow thickness. This was demonstrated by the ever-never analysis showing a HR (MSS) of 0.81 (95% CI = 0.61–1.06, p-value = 0.120) and for overall survival a HR (OS) of 0.88 (95% CI = 0.69-1.12, p-value = 0.308) in the multivariate model, with similar findings in the aspirin up to or within 12 months of diagnosis analysis, with the multivariate analysis showing a HR (MSS) of 0.93 (95% CI = 0.64-1.35, p-value = 0.706) and HR (OS) of 0.87 (95% CI = 0.62-1.23, p-value = 0.442) respectively. Therefore I was unable to show a significantly protective association with aspirin use and melanoma survival in our cohort. One limitation of our study, which may play a role in this result, particularly when comparing to associations seen with CRC as described before, is that I was unable to account for duration of aspirin use. Studies in CRC suggest protective effects of aspirin are seen only after 5 years with prophylactic doses of aspirin (75mg) [242]. Although we did not specifically look at dosage either, as mentioned in the results, the majority of patients in the cohort were prescribed this particular dose and therefore the absence of duration data appears to be the main limitation of our approach. Potentially stratifying patients based on duration of use would have helped to see if this could be playing a role in our results.

However, my significant finding from the study relates to when the analysis was stratified by sex, whilst still adjusting for age and Breslow thickness. As shown, female participants who had ever taken aspirin were significantly less likely to die from melanoma, when compared with others who had never used the drug at any point in their lifetime with hazard ratios for melanoma specific survival of 0.51 (95% CI = 0.30-0.87, p-value = 0.014) compared to men with an HR (MSS) 0.99 (95% CI: 0.71-1.37, P= 0.948), suggesting that aspirin use may have a preferentially protective effect in females.

There are two main studies that have reported a general survival benefit in melanoma patients receiving aspirin.

The first study performed by Famenini *et al*. was a cross-sectional retrospective study involving 39 patients with melanoma and aspirin use before the diagnosis of melanoma

and 109 patients with melanoma without prior aspirin use [264]. They reported a significant difference in Breslow thickness between aspirin users versus non-users (95% CI = 0.0297-0.8127, p-value = 0.03517). No significant difference was found in presence of ulceration or metastasis, Clark's stage, or mitotic activity between the 2 groups. Although the study was limited by sample size and as in our study, a lack of information regarding duration of aspirin treatment and exposure, it was concluded that aspirin may be associated with reduced Breslow thickness [264]. My initial findings also suggested a potential association with Breslow thickness as a result of the observed higher proportion of participants ever treated with aspirin who had Breslow thickness of 2.01-4mm (90, 26.24% vs. 374, 21.37%) and >4mm (48, 13.99% vs. 180, 10.29%) in participants who had ever received aspirin compared to those never treated. I therefore explored this association between aspirin use and Breslow thickness further by performing a univariable analysis, modelling the association of aspirin use with age at diagnosis, gender, diabetes status, BMI, Breslow thickness and vitamin D status (Table 5.2). From this, it was observed that age at diagnosis, male gender, presence of diabetes, BMI and Breslow thickness (2.01-4mm and >4mm categories only) were associated with a statistically significant higher odds of aspirin use. I then performed a multivariable regression analysis to explore if any of the association of aspirin use with higher Breslow thickness was accounted for by these other associated variables (Table 5.3). Significantly, this analysis demonstrated that higher Breslow thickness category was not associated with a higher odds of aspirin use following adjusted for age, male gender, diabetes and BMI.

The second study performed by Rachidi et al. was a retrospective cohort study involving 1,522 patients diagnosed with melanoma [265]. They reported that aspirin use was associated with longer overall survival in a similar univariate analysis as I performed above, with them adjusting for age, sex, stage, and treatment modalities (HR = 0.58, 95% CI = 0.45-0.75). Moreover, aspirin use was not associated with survival in patients with *in situ* and stage I melanoma, but was associated with better survival in stages II (HR = 0.45, 95% CI = 0.24-0.82) and III (HR = 0.57, 95% CI = 0.34-0.96). No statistical significance was observed in stage IV patients (HR = 0.55, 95% CI = 0.27-1.13). In turn, patients using Aspirin before diagnosis were less likely to be diagnosed in stages III or IV disease. Thus, authors concluded that aspirin could provide a survival advantage in melanoma [265].

However, neither of these studies reported any preferential survival advantage in female sex. Moreover, the only literature evidence of a potential survival advantage in women comes from studies looking at incidence of new melanomas and which was one of the reasons we considered an analysis looking at stratification by sex. A report by Gamba

et al. from the Women's Health Initiative demonstrated a 20% reduction in melanoma incidence in women taking aspirin suggesting a potential chemopreventive benefit of aspirin to reduce melanoma risk which may explain our findings and hint at a possible biological mechanism for this effect [266]. Similarly, a study by Joosse *et al.* looking at incidence of cutaneous melanoma in a large Dutch population based study demonstrated continuous use of low-dose aspirin was associated with a significant reduction of cutaneous melanoma (CM) risk in women (adjusted OR = 0.54, 95% CI = 0.30-0.99), but not in men (OR = 1.01, 95% CI = 0.69-1.47). A significant trend (p-value = 0.04) from no use, non-continuous use to continuous use was observed in women. Continuous use of low-dose aspirin may, therefore, be associated with a reduced incidence of CM in women, but not in men [258].

My study examined the largest cohort of melanoma patients in the world and to my knowledge is the first study showing that aspirin use may have a potentially preferential protective association in females in terms of melanoma survival as opposed to melanoma incidence as seen in the Dutch study, described above. This may therefore point towards a possible common biological pathway mediating this association in both incidence and survival. This discrepancy between men and women could potentially be explained by either pharmacological or melanoma differences. One consideration proposed by the Dutch group is that pharmacodynamics and pharmacokinetics of Aspirin differ between men and women. The effect on platelets differs across sexes and it seems that women achieve higher concentrations with equal doses being administrated [267]. As Aspirin may influence oxidative stress, the sex difference in antioxidant enzymes may also play a role. This is explained by the fact that certain disease states are linked with platelet oxidative stress, and it has been demonstrated that Aspirin inhibits the expression of lectin-like oxidized LDL receptor 1 (LOX-1) on platelets, in part by favourably affecting the ROS species and NO release from the activated platelets [69]. Interestingly, an RCT investigating antioxidant supplementation showed an increase in the incidence of CM in women, but not in men [268]. Another explanation may be that biology of melanoma itself may not be comparable in men and women, as CM survival differs significantly across the sexes when adjusted for other prognostic factors [79, 269]. Although behavioural differences such as compliance in taking the medication, with women being more likely to adhere to drug usage could have played a role in our study, studies suggest that differences in adherence to cardiovascular drugs are unlikely to explain the observed sex differences [270].

The main limitation of our approach in relation to this result is that of statistical power. When conducting the sex based stratification, I lose significant statistical power, as there

were nearly half the number of females on Aspirin when compared to males (as shown in Table 4.1).

Furthermore we were unable to adjust for associations related to patients being on a combination of drugs, which may be associated with inflammatory pathways as will be discussed in subsequent chapters. This could be significant in the case of aspirin, because as shown in table 3.20 in Chapter 3, in terms of the 347 patients ever on aspirin in our cohort, nearly a half were on simvastatin 155 (44%) 80 (23%) on atorvastatin, and 18 (5%) on metformin. Therefore it is difficult to ascertain if the associations could have been strengthened or weakened because of potential synergistic or opposing effects. The other main drawback, common to the whole thesis, is with problems of differentiating the effects of the co morbidities from these drugs used to treat them.

Further limitations include the retrospective approach and quality of drug data prior to diagnosis and the lack of a national cancer linked pharmacy database. Our study options were also limited from an analysis approach as we were unable to look at dose and duration given the potential biases in our methodology, as we were unable to perform the analyses with a time-dependent approach. These biases generally would result from not properly classifying exposure during the follow-up period as well as the guarantee time bias discussed in our methods section with our ever-never analysis. Although the guarantee time bias should have meant that it would be very difficult to look at exposure to the drugs after diagnosis interestingly my results were very similar to the at diagnosis or within 12 months of diagnosis analysis which does not have an inherent bias, suggesting that the guarantee time bias may not be such an issue in my analysis.

On the other hand, in terms of strengths of the study, we had the largest cohort of melanoma patients allowing us the ability to perform an epidemiological study on the effects of aspirin on melanoma survival given the shortage of such studies in the literature. I also had access to high-quality survival data given that it was a cohort study, which utilised multiple routes to obtaining those data. Given the significant amount of data collected at recruitment were also able to adjust for several known variables that can effect melanoma survival unlike other studies.

The next step following this would then be to try to determine the mechanism, whereby Aspirin exerts these effects. As discussed, various *in vitro* studies have demonstrated COX-2-expression in melanoma and suggested effects of NSAIDs on growth inhibition, invasiveness, and apoptosis. COX independent pathways, however, are also involved in these anti-tumour effects, as discussed. These pathways should be further investigated in order to disentangle dose-response and duration relationships, in terms of Aspirin. Although as discussed promising efficacy data were shown in other cancers, NSAIDs, especially Aspirin have yet to demonstrate sufficiently convincing evidence for

efficacious melanoma chemoprevention. Convincing evidence is lacking and comparing the conflicting results of the limited number of published studies discussed in our literature review is challenging due to heterogeneity in study design and uncertainties in temporal and dose-response relationships. Moreover, concerns over the long-term safety of COX-2 inhibitors, aspirin, and other NSAIDs have tempered the enthusiasm for their use in chemoprevention. Therefore, if sufficient data on efficacious drug dosages and temporal cause effect relationships become available, formal risk-benefit analyses should be performed on different scenarios of chemopreventive strategies.

A clinical trial is currently on-going to assess the impact of long term Aspirin intake on recurrence and survival in colorectal, gastro-esophageal, prostate, and breast cancers [70], and a similar trial in melanoma is warranted.

In conclusion, although my results in terms of sex stratification are interesting, and backed up by other studies, looking at incidence of melanoma rather than survival within a cohort they do require further validation in larger international data sets as well as an examination of biological models to assess if these represent real associations either related to pharmacological or melanoma differences or whether confounding factors are responsible for these changes. One possibility is to look at using Public Health England data as this is an increasingly common approach although it will be limited in terms of having access to other variables known to effect melanoma survival as was available in our cohort.

Chapter 5
Statins And Melanoma Survival

5.1 Introduction

Statins, or 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase inhibitors, are very frequently used group of drugs intended to reduce cholesterol levels aiming to prevent cardiovascular events. This drug class currently consists of atorvastatin, fluvastatin, lovastatin, mevastatin, simvastatin, pitavastin, pravastatin, and rosuvastatin. The various statins although grouped together, differ in several aspects. For instance, lovastatin, simvastatin, and pravastatin were originally derived from fungi, whereas atorvastatin and fluvastatin are synthetically derived. Additionally, some statins are prodrugs, e.g. simvastatin and lovastatin, and have a closed lactone ring that is converted by carboxyesterases to the open-ring acid form that inhibits HMG-CoA reductase. Atorvastatin, lovastatin, and simvastatin are lipophilic which implies they can cross the blood brain-barrier and cause central nervous system side effects (like insomnia) whereas pravastatin, rosuvastatin, and fluvastatin are more hydrophilic, which may also a play role in their biological effects [271].

Historically, an inverse association between cholesterol and the incidence of (smoking-related) cancers has been observed [272], suggesting a link between low cholesterol and cancer. In addition, lovastatin and gemfibrozil (a fibrate- another lipid lowering drug) were shown to promote development of liver cancer in rodents [273]. However, subsequent research demonstrated paradoxical results suggesting decreased cancer incidences with use of lipid-lowering drugs. In this chapter, I will explore this further, but it is first worth reviewing the biological effects of statins.

5.2 Biological effects of statins

As a class, statins (HMG-CoA reductase inhibitors) cause reduction in the serum cholesterol levels by inhibiting HMG-CoA reductase, a rate-limiting enzyme in the mevalonate synthesis pathway [274]. Increased use of statins over the last 30 years has been reported to be associated with a decrease in cholesterol levels and cardiovascular mortality [275].

The putative mechanism of action for both the cholesterol lowering and anticancer effects of statins is considered to be inhibition of HMG-CoA reductase, an enzyme upstream in the mevalonate biosynthetic pathway. Inhibition of HMG-CoA reductase

leads to reduced synthesis of mevalonate and its downstream products. Farnesylpyrophosphate (FPP), a C15-moiety, is one of these downstream products and is the precursor of both geranylpyrophosphate (GPP), a C20-moiety, and cholesterol. Thus, statins reduce cholesterol levels by reducing mevalonate levels.

Statins, however, also reduce the levels of additional intracellular proteins, such as ras, rho, nuclear lamins, transducin c, rhodopsin kinase, and G proteins. Consequently, statins lead to pleiotropic effects [276] which is consistent with the reported reduced mortality in takers of statins independent of their effects on cholesterol levels.

A number of authors have reported evidence of a relationship between the use of statin and the risk of cancer [61-66]. Furthermore, some experimental studies report that statins may have a promising function in cancer chemoprophylaxis [271, 277, 278].

Various preclinical *in vitro* studies using different cell lines have demonstrated the propensity of statins to restrain the growth and development of tumour. Statins have pro-apoptotic, anti-proliferative, and anti-invasive properties and this has been reported in different cancer cell lines and with different sensitivity. In humans, the anti-myeloma property of statins was first demonstrated with the simultaneous administration of simvastatin in patients with refractory multiple myeloma (MM), resulting in decreased drug resistance [67]. Atorvastatin-induced effects on tumor proliferation and HMGCR expression were studied in a pre-operative study involving patients with primary invasive breast carcinoma and it was concluded that, in breast cancer cells (*in vivo*), HMGCR is targeted by statins and statins may have an anti-proliferative effect in HMGCR-positive tumors [68]. Additionally, fluvastatin was evaluated in patients with invasive, high-grade, stage 0/1 breast cancer and it demonstrated reduced tumour proliferation and increased apoptotic activity [69].

Apart from *in vitro* efficacy, various animal models of cancer have demonstrated *in vivo* antitumor effects of statins as an efficacious chemopreventive agents and includes radiation-induced mammary tumorigenesis [70], chemical-induced colon tumorigenesis in rodent models [71], and chemical-induced lung tumour in mice [72]. Additionally, statins have also been reported to decrease metastasis in mouse mammary tumour [73], murine colon tumour [74], and mouse melanoma [75]. Moreover, three tumour models have demonstrated that statins result in increased *in vivo* antitumor effect of doxorubicin and this is accompanied by attenuation of its cardiotoxicity [76]. Similarly, a murine tumour model has demonstrated that statins lead to an increase in antitumor effect of tumour necrosis factor by inhibiting the tumour-induced angiogenesis [77].

5.2.2 Review of literature of associations of statin usage with cancer

5.2.2.1 Epidemiological data on statins and in cancer other than melanoma

The first suggestion of a possible decreased cancer incidence with statin use resulted from observations made in participants in randomised clinical trials of statins and cardiovascular disease [271]. Cancer incidence in these studies was included as a secondary safety outcome because of concerns that reducing cholesterol might actually increase cancer risk. In a meta-analysis, however, published in The Lancet, the Cholesterol Treatment Trialists' (CTT) Collaborators included 14 RCTs of statins and found no evidence for a decreased cancer incidence (RR = 1.00, 95% CI = 0.95-1.06) [279].Since then, a large number of meta-analyses and observational studies investigating statin use and cancer incidence have been performed.

Looking at cancer in general, two large population-based studies reported decreased incidences of cancer [280, 281]. While, one reported that statin use was associated with a 20% decrease in cancer incidence (OR = 0.80, 95% CI = 0.66-0.96) and this association was more pronounced with prolonged use (statin use ≥ 4 yrs, OR = 0.64, 95% CI = 0.44-0.93) [280], other reported a significantly reduced risk of CRC (OR = 0.50, 95% CI = 0.40-0.63) with the use of statins (≥ 5 years versus nonusers) [281]. Additionally, a Danish Registry based study reported that the cumulative incidence of death from any cause as a function of follow-up time from the date of the cancer diagnosis was significantly lower among statin users than among patients who had never used statins (p-value < 0.001). Also, absence of a dose–response relationship for statins and cancer-related mortality suggests that any statin dose will suffice in reducing mortality among patients with cancer [282]. Similarly, the PRIME study reported a reduced cancer mortality, although statistically non-significant, among dyslipidemic men using statins as compared to untreated dyslipidemic men (OR = 0.41, 95% CI = 0.19-1.06) [283]. These findings are also supported by the observation that use of statin is associated with the reduced cancer-related mortality among patients with advanced prostate cancer and a correspondingly reduced recurrence among patients with prostate or breast cancer [284-287]. Additionally, various recent epidemiologic studies have shown reductions in mortality risk among statin users with ovarian, prostate, and renal cell cancers compared with non-users [288-290].

A systematic review and meta-analysis demonstrated that statin exposure is associated with a 21%, 17%, and 15% reduced risk of all-cause mortality, lung cancer-specific mortality, and risk of recurrence, respectively [291]. Another systematic review and meta-analysis reported that statin use is associated with reduced overall mortality and CRC-specific mortality. Analyses stratified by statin use before and after CRC diagnosis

showed that post-diagnosis statin use led to a 30% reduction in CRC-specific mortality and a 24% reduction in overall mortality compared with non-users. However, their findings showed that pre-diagnosis statin use led to a 20% reduction in CRC-specific mortality and a 30% reduction in overall mortality compared with non-users. However, post-diagnosis statin use did not improve disease-free survival (DFS) and recurrence-free survival (RFS) [292].Thus, after reviewing all the above cited studies (i.e., both preclinical and clinical), I can conclude that compared to statin non-users, statin users have significantly lower cancer specific and overall mortality.

5.2.2.2 Laboratory data relating to statins and cancers other than melanoma

Various studies have evaluated the efficacy of atorvastatin in different cancers other than melanoma [293][23]. An *in-vitro* study evaluated the effects of atorvastatin on proliferation of cells in ovarian cancer and observed that atorvastatin inhibited the proliferation of both the Hey and SKOV3 ovarian cancer cells in a dose-dependent manner. This activity was linked with induction of apoptosis, autophagy, cellular stress, and cell cycle (G1) arrest through induction of the MAPK and blocking of AKT/mTOR pathways. Additionally, atorvastatin resulted in decreased expression of VEGF and MMP9 as well as inhibition of cell adhesion and invasion. Ovarian cancer cells exposed to atorvastatin had down regulation of c-Myc. JQ1 mediated inhibition of c-Myc synergistically enhanced the sensitivity of ovarian cancer cells to atorvastatin. Thus, the authors concluded that atorvastatin may have a role in the treatment of ovarian cancer and requires further exploration in clinical trials [293]. Another study assessed the effect of atorvastatin (40 mg) on biomarkers of risk in breast cancer in high-risk premenopausal women i.e., mammographic density (MD) and insulin growth factor 1 (IGF-1) and reported a significant reduction in the levels of cholesterol and low-density lipoprotein (LDL). After taking BMI into account, there was no observed difference in change in MD between the two groups. While, in the statin group, there was a significantly elevated level of serum IGF-1. Thus, the authors concluded that no change in MD and significant change in other biomarkers suggests that statins may not act via change in MD although the short duration of the study is a potential limitation [294]. Additionally, the effect of atorvastatin on biomarkers in breast tissue and serum of women at increased risk of breast cancer were studied in another study and a significant decrease in serum CRP, cholesterol, and low-density lipoprotein (LDL), and rise in atorvastatin metabolites in serum and breast FNACs was observed. Thus, authors concluded that atorvastatin and its metabolites are detectable in breast samples and may decrease serum CRP in women without hyperlipidemia [295].Similarly, some studies have evaluated the efficacy of simvastatin in different cancers other than melanoma. A prospective study was

undertaken to identify potential biomarkers of prophylactic activity of simvastatin. A high-risk model in the form of contralateral breast of women with a prior history of breast cancer was used. During the study, there was a significant decrease in total cholesterol, LDL cholesterol, triglyceride, and hsCRP (P values <0.001, <0.001, 0.003, and 0.05, respectively). Moreover, simvastatin treatment resulted in a significant decrease in concentration of estrone sulfate (P = 0.01 overall), especially among post-menopausal women (P = 0.006). Thus, authors concluded that this study depicts the feasibility of short-term biomarker modulation studies using the contralateral breast of high-risk women [296]. Another study explored the viability of nasopharyngeal carcinoma cell line, C666-1, after addition of simvastatin and assessed by the alamar Blue Cell Viability Assay. It was observed that simvastatin, in a concentration-dependent manner, resulted in a marked decline in cell viability, enhanced caspase 3 activity, and induction of apoptosis in C666-1 cells. Additionally, inhibition of the expression of cyclin D1 and cyclin-dependent kinase 4, and enhanced expression of p27 resulted in arrest of the cell cycle in the G1 phase. Thus, it was concluded that simvastatin is a potential chemotherapy agent in the treatment of nasopharyngeal carcinoma [297].

Various epidemiological studies have reported variable potential association between incident melanomas and statin use. A case-control study on cancer and statin use utilized data from the GPRD (General Practitioners' Research Database) in the UK [298]. In a sub analysis within this study, they observed a relative risk of 2.5 (95% CI = 0.78-7.3) for melanoma using records from 79 incident melanoma cases between 1990 and 2002 and up to five controls per case matched on year of birth, sex, general practice providing the data, year of entry into the GPRD, and index date. The follow-up in this study ranged between 3 and 13.7 years with a median of 6.4 years [298]. However, the number of melanoma cases in this study was very small as reflected in the wide confidence intervals. Another case-control study had 1,318 melanoma cases and 6,786 controls matched on sex, date of birth and geographic region, and they reported no association between statin use and melanoma incidence (OR = 0.98, 95% CI = 0.78-1.2). However, interestingly the Breslow thickness in melanomas was lower among statin users (–19%, 95% CI = –33% to –2.3%). In a pre-specified stratified analysis they observed that the difference in thickness was non-significant among women (–4.8%, 95% CI = –29.6% to 28.8%), but significant in men (–27.8%, 95% CI = –43.7% to –7.4%). The lack of an association with melanoma incidence in the study was potentially thought to have been explained by the relatively short follow-up period of 3 years for all individuals [299].

In a Cochrane review incident melanomas were assessed, included as a secondary outcome of RCTs with primary cardiovascular outcomes. In this review, 6 statin RCTs

providing data on incident melanomas were included. The resulting odd's ratio was 0.90 (95% CI = 0.56-1.44) indicating no statistically significant difference. However, due to the low numbers of incident melanomas, a (clinically relevant) association could not be excluded. More importantly, three of the included RCTs studied pravastatin, which may have, as *in vitro* studies have suggested, lower chemopreventive activity than other statins. Interestingly, a subgroup analysis by type of statin showed a reduced melanoma incidence for lovastatin (OR = 0.52, 95% CI = 0.27-0.99). This analysis was, however, importantly limited by the fact that there was only one trial with lovastatin. Thus, the authors concluded that they could not exclude the possibility that statin prevent melanoma [300].Additionally, a sub analysis among the trials for which melanoma incidence was available, the Cholesterol Treatment Triallists reported no statistically significant change in melanoma incidence (RR = 1.03, 95% CI = 0.71-1.50) in 14 RCT statin studies [279]. Other similar meta-analyses have reported melanoma incidence with estimates for melanoma incidence ranging from 0.84 to 1.5 [301, 302]. However, they mainly included the same RCTs.

These clinical trials, however, have several disadvantages which include small numbers of incident melanomas, relatively short follow-up for melanoma incidence (ranging from 3 to 6 years), and, generally, of being a retrospective reviews of cardiovascular trials, in which the design was not adapted for the analysis for melanoma incidence as they were not stratified for other factors known to influence melanoma survival as done in this study. Therefore, retrospective analyses on these trials will always be of limited value.

In terms of melanoma survival and statins, which is the main focus of this study, the main study of relevance is a Dutch population based study by Livingstone *et al.* [303] involving a cohort of 709 melanoma patients. Neither timing, nor duration or dosage of statin use changed the hazard of death significantly. Stratification on sex, however, demonstrated possible superior survival of statin users compared to nonusers in males only and I, therefore, also carried out this stratification in the cohort of this study. In keeping with the cohort, in this study, almost half of all the statins dispensed were for simvastatin (47.4%), followed by atorvastatin (28.7%).

Additionally, two abstracts appeared on a preliminary case control study comparing the use of statins among 74 melanoma cases and age, sex and race-matched controls. Preliminary results in this study were promising (OR = 0.55, p = 0.11) [304, 305]. However, to the best of my knowledge, the results of the final analysis have not been published.

In summary, the results of secondary analyses of cardiovascular trials and of observational research on the potential relation between statin use and incident melanomas are conflicting. Both these RCTs as well as the epidemiological studies have

some important limitations such as potential residual confounding, and small numbers of incident melanomas and thus, limited power. Furthermore, there are limited studies looking at melanoma survival and statin use making this study all the more important.

5.2.2.3 Laboratory data relating to statins and melanoma

Several of the proteins dependent on posttranslational prenylation, either farnesylation or geranylgeranylation, such as ras, rhoA and rhoC, have been linked to cancer pathogenesis. For example, *ras* is a known oncogene and ~30% of human tumours harbour *ras* mutations resulting in aberrant ras activity which is dependent on prenylation [276]. Specifically, N-*ras* and B-*raf* mutations are observed in ~30% and ~60% of melanomas, respectively. N-*ras* and B-*raf* mutations both result in activation of the so-called Ras/Raf/MEK/ERK signalling pathway [306]. Raf which is downstream of ras, however, does not require prenylation to achieve full biological activity [307]. Still, in melanomas with a B-*raf* mutation, but no *ras* mutation, possible antineoplastic effects may be mediated through for instance rhoA or rhoC. A potential chemopreventive agent that may interfere in this pathway are the statins [276, 306]. Specifically, one hypothesis is that some of the potential beneficial effects of statins in terms of cancer, would relate to reduced activation of key pathways involved in carcinogenesis such as MAP kinase signalling. Furthermore, the rho family is involved in signalling and regulation of cell differentiation and proliferation [308].

High-throughput screens for transcriptionally regulated targets involved in metastasis have shown that rhoC overexpression is strongly associated with the metastatic potential of inoculated melanoma in mice [309]. Indeed, *in vitro* and animal melanoma studies show a potentially chemopreventive activity of statins. More specifically, anti-tumour effects exerted by statins have been shown to be act in a number of different ways as listed below:

1) **Inhibition of tumour growth:** Lovastatin, mevastatin, and simvastatin, but not pravastatin, reduced tumour growth of human melanoma cell lines HT144, M14, and SK-MEL-28 *in vitro* with IC50 values between 0.8 and 2.1 μm [310].

2) **Induction of apoptosis:** Jani *et al.* observed induction of apoptosis by lovastatin in murine B16F10 melanoma cells through a geranylation-specific mechanism [311]; Additionally, increased apoptosis, in a dose-dependent manner, was observed in human M14 cells after 72-h incubations (4-8 μm) of lovastatin, mevastatin, and simvastatin [310]. In human A375 melanoma cells, Shellman *et al.* also showed induced apoptosis by lovastatin [312]. Interestingly, Shellman *et al.* also performed add back experiments showing that supplementation of GPP, but not FPP, blocked the apoptotic effect of

lovastatin which indicates apoptosis must involve proteins dependent on geranylgeranylation [312].

3) **Reduce invasiveness and metastasis:** Atorvastatin (1-3 µm) reduced invasiveness of A375M, CHL, SK-MEL-28 and WM 166-4 melanoma cells in an experiment performed by Collisson and colleagues [313]. In this experiment, atorvastatin (4 dd 10 mg/kg) orally also reduced metastasis of A375M melanocytes in severe combined immunodeficient (SCID) mice [313]. Likewise, Jani *et al.* showed reduced metastasis by lovastatin and simvastatin in murine B16F10 melanoma cells [311]. Experiments reported by Glynn *et al.* also showed decreased invasiveness by lovastatin, mevastatin, and simvastatin on HT144, M14, and SK-MEL-28 cells [310].

4) **Effects on angiogenesis**: Lovastatin (2-12.5 µm) exhibited a concentration-dependent pro-angiogenic influence on A375M and G361 cells in an angiogenesis model with a co-culture of HUVEC cells (human umbilical vein endothelial cells) and human diploid fibroblasts (HDF) [314]. However, in non-melanoma cells, some studies with low-dosed statins have suggested increased angiogenesis [276].

5) **Effects independent of HMG-CoA reductase and cholesterol lowering**: some experiments with statins in the closed ring form, which do not inhibit HMG-CoA reductase, do show *in vitro* anticancer effects [315]. Further investigations on these cholesterol-independent pathways are needed.

Examples of the cholesterol-independent pathways that have been suggested are:

- binding to the leukocyte function antigen-1 (LFA1) which has an important role in leukocyte migration and T-cell activation [316].
- inhibition of the proteasome [315] which could for instance account for effects on the cyclin-dependent kinase inhibitors (CDKIs) p21 and p27 [317], and increased fibrinolytic activity [318].
- altered membrane receptor function due to changes in membrane fluidity caused by cholesterol depletion. For example, melanocortin receptor (MC1R) [319] or insulin-like growth factor receptor function [320], both of which are involved in melanocyte and melanoma growth.

6) **Potential direct effects of cholesterol lowering**: Some investigators suggested that direct toxic effects of cholesterol lowering are involved [321]. Malignant cells metabolize cholesterol differently and, therefore, may be more sensitive. However, the evidence for this hypothesis is limited.

Although *in vitro* and animal experiments in general show promising results, some critical issues should be mentioned. E.g., pravastatin, the only hydrophilic statin, does not exhibit clear chemopreventive effects in most experiments. Moreover, most studies have

used statins at serum concentrations and dosages that exceed doses applied for the treatment of hypercholesterolemia. Lovastatin dosed at ~1 mg/kg/ day, for example, yields steady-state serum concentrations of 0.15–0.3 μm [322]. Often tumour cell lines were only sensitive to lovastatin at higher concentrations, e.g. 1.0-12.5 μm [310, 312, 314].

Additionally, the effects of various statins i.e., simvastatin, fluvastatin, or lovastatin on proliferation, apoptosis induction, cell cycle progression, autophagy, and migration of melanoma cell were evaluated in an *in vitro* study involving melanoma cell lines. It was observed that all the three statins exhibited a strong inhibitory effect on the growth of melanoma cells at submicromolar concentrations, in virtually all cell lines irrespective of genotype. Simvastatin induced apoptosis and autophagy, arrested cell cycle at G0/G1 phase, and inhibited cell migration. Analogous effects were also observed with other inhibitors of the mevalonate pathways. Importantly, it was observed that combinations of simvastatin or fluvastatin with vemurafenib, CI-1040, ZSTK474, or NVPBEZ-235 significantly enhanced the inhibitory effect toward melanoma cell growth *in vitro*. The *in vivo* effects of simvastatin were also investigated and it was found that simvastatin delayed the growth of NZM37 xenograft in athymic nude mice. Furthermore, they completed whole-genome, positive-selection CRISPR screens with the three statins and demonstrated evolution of marked resistance to these agents [323].

Apart from the above described mechanisms, statins have been reported to have anti-inflammatory effects, including decreasing the concentrations of C-reactive protein (CRP) [80]. As LDL cholesterol itself is a strong promoter of inflammation, The effects of lowering low-density lipoprotein (LDL) cholesterol with statins may lead to anti-inflammatory actions because [81]. Addition of statins to human hepatocytes reduces the levels of C-reactive protein induced by circulating interleukin 6 (IL-6), suggesting that the anti-inflammatory effects of statins are hepatic in nature [82]. Moreover, statins exert anti-inflammatory action by affecting mediators of inflammation such as IL-8, IL-1β, IL-12, tumour necrosis factor α (TNF-α), and nuclear factor κB (NF-κB) [83]. In a cohort study, Brewer *et al*. analysed the effect of statin on the primary inflammatory breast cancer and reported that weakly lipophilic to hydrophilic statins were associated with significantly improved progression-free survival compared with no statin (HR = 0.49; 95% CI = 0.28–0.84; $p < 0.01$) [84]. However, these results needs to be confirmed in a randomized study.

Interestingly, some agents may have synergistic chemopreventive action together with statins. For example, d-G-tocotrienol (5 μm) together with lovastatin (1 μm) totally blocked cell growth, whereas lovastatin (12%) and d-G-tocotrienol (8%) individually showed only limited growth inhibition in these concentrations [324]. Other agents that

have been suggested in combination with statins are NSAIDs, bisphosphonates, GGTIs, phosphoinositide 3-kinase (PI3K) inhibitors, CDKI, MEK inhibitors, and tyrosine kinase inhibitors [276]. The possible synergistic effects, alongside NSAID, is a particularly relevant to this study, but, as discussed in the methods chapter, is a limitation of the study as we cannot analyse both together. Although, I have looked at the number of patients who were on both drugs as shown in Chapter 3.

5.2.2.4 Looking at different statins separately

Although *in vitro* and animal experiments in general show promising results for statins as a group in cancer, there exists significant variability between the effects and potential mechanisms of actions of individual statins. The two main statins reported in the context of cancer and in our cohort are atorvastatin and simvastatin and I will compare these in order to determine how best to analyse them.

As noted previously based on *in vitro* studies involving melanoma cell lines, simvastatin primarily mediates its effects via a strong inhibitory effect on the growth of melanoma cells, in virtually all cell lines irrespective of genotype. Simvastatin has also been found to induce apoptosis and autophagy, arrested cell cycle at G0/G1 phase, and inhibit cell migration. Analogous effects were also observed with other inhibitors of the mevalonate pathways and most significantly it was observed that a combination of simvastatin and vemurafenib, significantly enhanced the inhibitory effect toward melanoma cell growth *in vitro*. The *in vivo* effects of simvastatin were also investigated and it was found that simvastatin delayed the growth of NZM37 xenograft in athymic nude mice [323].

Atorvastatin whilst also a lipophilic statin mediates its effects via preventing RhoC and preventing invasion and metastasis as opposed to growth of cells and atorvastatin treatment has been shown to inhibit the colonization and formation of lung metastases of melanoma cells overexpressing RhoC [313]It is also reported to differentially enhance endothelial cell proliferation, whereas high concentrations (2.5 mg/kg per day) have been shown to significantly inhibit angiogenesis [325].

There are reasons to consider analysing both drugs together such as clear overlapping mechanisms of action in terms of effects mediated via reduction in cholesterol and the fact that they are both lipophilic statins, which would help with statistical power. However, I decided to analyse them separately, accepting a loss of power, in order to make the analysis cleaner in terms of the differing biological effects as well as the issues outlined in the methodology in chapter 2 with switches between these drugs. For instance, some patients would start out on simvastatin to control their hypercholesterolemia but then be switched to atorvastatin if they had for instance a stroke or a heart attack. A proportion of these, were subsequently found to have been switched back to simvastatin either as

a result of side effects with atorvastatin, or due to cost saving measures with the same group of patients being switched between these statins making the statistical analysis practically very difficult to perform.

5.2.3 Safety and Compliance

In the cancer chemoprevention literature, the excellent safety profile of statins in cardiovascular disease has often been noted [306, 326]. Statins have demonstrated relatively mild side effects in the doses used to prevent cardiovascular events. The most prominent side effects of statins are the so-called statin-related myopathy (i.e., muscle pain and weakness), elevated creatinine kinase (CK) levels and as a rare but life-threatening side effect, rhabdomyolysis. In RCTs, the incidence of myopathy was 1.5-5%, whereas estimates in observational research indicated 5-10% [326]. Moreover, the US FDA Adverse Event Reporting System database reports rates of statin-induced rhabdomyolysis of 0.3–13.5 cases per 1,000,000 statin prescriptions [78]. In spite of the fact that the majority of side effects are thought to be mild, compliance with statin use cardiovascular disease has been poor, with only ~25% of patients still compliant 5 years after starting statin therapy [327].

In terms of cancer chemoprevention, higher day doses may be required and as such the tolerability of statins has been proven to be limited due to dose-dependent side effects such as myopathy. In phase I/II trials for cancer treatment, significant responses were only achieved with >25 mg/kg/day doses leading to dose-limiting toxicities (DLTs) including myalgia, muscle weakness, elevated CK activity, anorexia, ulcerative lesions, rhabdomyolysis, nausea, diarrhoea, and fatigue. With very high statin doses, cardiomyopathy may even be a side effect [328]. In the trials mentioned, among others cycled dosing with 3-4 week intervals was introduced to prevent DLTs [322].

For melanoma chemoprevention, given the limitations of the studies as discussed above, it remains uncertain what doses are required. However, since cell lines studies often indicate cytostatic rather than cytotoxic effects at achievable *in vivo* statin concentrations, continuous dosing is likely to be required [329]. Numerous risk factors for statin-related myopathy have been described [330]. Among these risk factors is using high statin doses which, as mentioned before, may be required for chemopreventive effects. The risk of myopathy or rhabdomyolysis with simvastatin alone is dose related; the incidence, determined from clinical trials, is approximately 0.03% at 20mg, 0.08% at 40mg and 0.4% at 80mg daily. This risk is increased with concomitant fibrates, as they alone can cause myopathy [79]. Some of the risk factors may be circumventable, such as excessive physical activity, use in the perioperative period, and concomitant use of drugs or grapefruit juice which precipitate drug interactions associated with elevated

serum statin levels. For atorvastatin, lovastatin, cerivastatin or simvastatin, concomitantly administered drugs resulting in drug interactions and subsequently, increased statin-induced myopathy are CYP3A4 inhibitors (fibrates, warfarin, macrolide antibiotics, azole antifungal, and others) and for fluvastatin these are CYP2C9 inhibitors (sulfaphenazole, valproic acid, flucaonazole, miconazole, amiodarone, and others) [330]. Avoiding the risk factor, temporary cessation of statin therapy, or drug alternatives for the inhibitors can be options in these cases. Non-preventable risk factors, such as advanced age, female sex, (relative) renal insufficiency, hypothyroidism, alcoholism or (family) history of myopathy or CK elevation [330], have been recommended for consideration as special subgroups in formal risk-benefit analyses. Some of the non-preventable risk factors might be considered contraindications for statin therapy, e.g. (relative) renal insufficiency.

The causal mechanism of statin-related myopathy is not entirely unravelled. Among the proposed mechanism is depletion of ubiquinone (also referred to as coenzyme Q10). Ubiquinone, a side-product in the mevalonate pathway, is widely used as a non-drug 'over the counter' (OTC) anti-aging agent, but studies on its long-term safety are sparse. Concomitant use of ubiquinone may, however, prove to be a good candidate to increase statins' tolerability. Indeed, Thibault *et al.* have used adding Q10 to lovastatin therapy for doses of 30 mg/kg/day as a strategy to prevent statin-related myopathy and increase tolerability. From these preliminary data, this strategy seems to be promising [322].Further research is needed to explore the precise mechanisms involved in statin-related myopathy, and after required statin doses have been established, to determine the long-term safety of this chemopreventive strategy.

In summary, long-term safety data for low dose statins is excellent, but may be less favourable for higher doses that are possibly likely to be required for chemoprevention of melanoma. Development of a chemopreventive strategy including risk factors for statin-related myopathy and preventive measures may ameliorate the risk-benefit ratio.

5.3 Materials and Methods

Simvastatin was the most frequently used statin in our cohort followed by atorvastatin in keeping with other studies, and as mentioned in the methodology, given the differing mechanisms of action of these, they were felt to be best analysed separately and I will discuss the limitations of this in the discussion.

Having collected the drug data for all drugs used in our cohort, which is the largest cohort of melanoma patients as detailed in Chapter 2, and having identified Simvastatin and

Atorvastatin as drugs of interest as presented in chapter 3,, various further drug specific considerations were considered as detailed below.

Firstly I had to ensure all entries for both statins were identified accurately by accounting for trade names, BNF codes, misspellings in data entry and missing data by cross-checking all data sources. As discussed in Chapter 2 due to limitations of the study approach I did not examine the effects of drug dosages but also given that “standard doses” are used to prevent cardiovascular disease with no patients on doses referred to in the phase I /II trials for cancer treatment discussed above, I would not expect there to be a significant variation in results due to a dose effect.

I then interrogated the data further by looking at the demographics of the population taking statins by examining the number of males and females on the drug in our cohort, their smoking status, their diabetic status, vitamin D levels and the distribution based on Breslow thickness. For statins, similar to aspirin, is prescribed to the same group of patients who are likely to have features of the metabolic syndrome with increased BMI and Diabetes and ischaemic heart disease. Therefore, we expected to see some common trends within our analysis.

The effects of exposure to statins on melanoma specific (MS) and overall survival (OS) were then assessed using Cox Proportional Hazards models. Data was first checked to ensure that the proportional hazards assumption was met. Hazard ratios (HR) and 95% Confidence intervals (CI) were estimated and Kaplan-Meier curves were plotted. Firstly, unadjusted models were examined and then adjustment for common confounders were applied; firstly, sex and age at diagnosis, then Breslow thickness, ulceration, other comorbidity measures (smoking, body mass index (BMI), and serum level of vitamin D adjusted for seasonal variation. Analyses were conducted both with and without adjustment for stage (represented by Breslow thickness and ulceration), since statins may influence outcome through an effect on the growth of the tumour, which may be captured by stage at diagnosis.

Given that men have a higher risk of these conditions we had expected to see more men being prescribed statins than women and we also expected to see more smokers and diabetics having been prescribed statin, as these are independent risk factors for ischaemic heart disease. We also expected this group of patients to be overweight compared to the rest of the cohort population (BMI > 25).

As detailed in the methodology chapter, I then undertook a survival analysis based on firstly the ever versus never approach with the recognised guarantee time bias, first examining the unadjusted model and then adjusting for known confounders.

The second approach was a survival analysis looking at the effects of statins up to diagnosis or within 12 months of diagnosis. This method of analysis essentially replicates the traditional methods used in cancer incidence studies with no inherent bias but as discussed in the methodology section our study was not geared up for this in terms of the data collection with potentially poorer quality data before diagnosis and with some loss of power due to exclusion. As with the previous approach we first examine the unadjusted model and then adjust for known confounders

Finally given the literature evidence of potential varying effects of drugs on survival based on sex as demonstrated in our literature survey with the large Dutch population based study on melanoma incidence as described above (ref), we also carried out a survival analysis whereby we stratified by sex to identify any sex specific trends whilst also adjusting for Breslow thickness given the possible effects of statins on this.

5.4 Results

In our cohort, simvastatin usage at any time was reported by 336 (15.87%) of melanoma participants in our cohort. Similarly, 126 patients (5.95%) reported ever using atorvastatin. Firstly I will present the results for simvastatin, which I will go though in some more detail but will then also present a summary of the results for atorvastatin.

We first looked at the characteristics of the simvastatin users and as per Table 5.1 there were significant differences noted between users and non-users in the majority of characteristics examined. The majority of users were male (198, 58.93%) compared to women (138, 41.07%) taking simvastatin ($p<0.001$), which would be expected given that studies suggest that men are twice as likely as females to have ischaemic heart disease and as discussed in the aspirin chapter they are often prescribed both a statin and aspirin to reduce this risk. Although the discrepancy was not as great as in the aspirin group it is important to bear in mind that as shown in Chapter 3, Table 3.23, 155 patients were on both aspirin and simvastatin and therefore there is a significant overlap of the same patients on both drugs.

Table 5.1: Characteristics of patients ever treated with simvastatin

Continuous variables are summarized as median (interquartile range) and p-values were generated using the Mann-Whitney U test. Categorical variables are represented as n (%) and p-values were generated using chi-squared test or Fisher's exact test if expected cell count was less than five. P-values were considered statistically significant if <0.05

Ever Treated with Simvastatin?	Yes - n (%)	No - n (%)	P-value
Total no of patients	**336 (100)**	**1,781 (100)**	
Age at diagnosis	65 (11.1)	54 (20.7)	<0.001
Gender			
Female	138 (41.07)	1,070 (60.08)	<0.001
Male	198 (58.93)	711 (39.92)	
Diabetes			
No	287 (86.97)	1,661 (97.76)	<0.001
Yes	43 (13.03)	38 (2.24)	
Body Mass Index			
</=24.9kg/m^2	59 (17.66)	731 (41.42)	
>24.9-29.9kg/m^2	173 (51.80)	681 (38.58)	<0.001
>29.9kg/m^2	102 (30.54)	353 (20.00)	
Smoking Status			
Never	147 (44.28)	979 (57.22)	<0.001
Ever Smoked	185 (55.72)	732 (42.78)	
Smoking status			
Not currently smoking	156 (83.87)	523 (71.45)	<0.001
Currently smoking	30 (16.13)	209 (28.55)	
Breslow thickness			
</=1mm	70 (21.02)	525 (29.83)	
1.01-2mm	143 (42.94)	663 (37.67)	**0.013**
2.01-4mm	82 (24.62)	382 (21.70)	
>4mm	38 (11.41)	190 (10.80)	
Vitamin D			
<20nmol/L	17 (5.92)	91 (6.28)	
20-59.9nmol/L	193 (67.25)	964 (66.57)	
60-84.9nmol/L	68 (23.69)	323 (22.31)	0.719
85-99.9nmol/L	7 (2.44)	47 (3.25)	
>100nmol/L	2 (0.70)	23 (1.59)	

Further analysis of the cohort showed that a higher proportion of people on simvastatin were diabetic (43, 13.03% vs. 38, 2.24%; p<0.001) or ever smokers (185, 55.72% vs. 732, 42.78%; p<0.001) compared to those not on simvastatin. This would be expected given that both diabetes and smoking are risk factors for IHD and this population is, therefore, more likely to be prescribed statins as in the case of aspirin. Like in the case of aspirin, people who had ever used simvastatin were more likely to be current non-smokers (30, 16.13% vs. 209, 28.55%; p<0.001), and may have stopped smoking possibly due to cessation advice. In terms of BMI, patients on simvastatin were more likely to be overweight or obese (p<0.001), and this would again be expected, as this is a risk factor for IHD. As discussed earlier, vitamin D is one of the factors thought to influence melanoma survival and we, therefore, wanted to analyse our cohort to see if there was a statistical difference in vitamin D levels between users and non-users by defining different ranges of vitamin D and comparing with the base group and we observed no statistical difference as shown (p=0.719).

Similar to aspirin, a tendency to higher Breslow thickness was observed in this case in users of simvastatin compared to non-users (p<0.013). I therefore explored this association by performing a univariable regression analysis of factors associated with simvastatin use followed by a multivariable analysis (Table 5.2 and Table 5.3). As in the case of aspirin, higher Breslow thickness category was not associated with a higher odds of aspirin use following adjustment for age, male gender, diabetes and BMI.

Table 5.2: Univariable analysis of simvastatin use (ever-never)

Regression analyses were performed using a logistic regression approach with simvastatin use as a dependent variable and age, gender, diabetes status, body mass index, Breslow thickness, and vitamin D as independent variables in each univariable analysis.

	Odds Ratio	95% Confidence Interval	P-value
Age at diagnosis	1.08	1.07-1.10	**<0.001**
Male gender	2.16	1.70-2.74	**<0.001**
Diabetes	6.55	4.16-10.31	**<0.001**
Body mass index			
>24.9-29.9kg/m^2	3.15	2.30-4.31	**<0.001**
>29.9kg/m^2	3.58	2.54-5.05	**<0.001**
Breslow thickness			
1.01-2mm	1.62	1.19-2.20	**0.002**
2.01-4mm	1.61	1.14-2.27	**0.007**
>4mm	1.50	0.98-2.30	0.064
Vitamin D	1.00	0.99-1.00	0.508

Table 5.3: Multivariable analysis of simvastatin use (ever-never)

Regression analyses were performed using a logistic regression approach with simvastatin use as a dependent variable and age, male gender, diabetes, body mass index and Breslow thickness as independent variables.

	Odds Ratio	95% Confidence Interval	P-value
Age at diagnosis	1.08	1.07-1.09	**<0.001**
Male gender	1.59	1.21-2.07	**0.001**
Diabetes	3.77	1.28-6.23	**<0.001**
Body mass index			
>24.9-29.9kg/m^2	2.52	1.80-3.54	**<0.001**
>29.9kg/m^2	2.99	2.04-4.39	**<0.001**
Breslow thickness			
1.01-2mm	1.32	0.94-1.85	0.111
2.01-4mm	1.09	0.74-1.60	0.655
>4mm	0.85	0.53-1.35	0.483

5.4.4 Effect of simvastatin ever use on survival outcomes

As shown in Figure 5.1 below the unadjusted survival distribution between participants who had ever used simvastatin and those who had never used the drug appear to be different for both MSS and OS as shown in Table 5.4 with participants who had used simvastatin appearing to have non-significant negative effect with hazard ratios of 1.14 (95% CI: 0.87-1.48, p = 0.342) and 1.15 (95% CI: 0.90-1.48, p = 0.263) respectively.

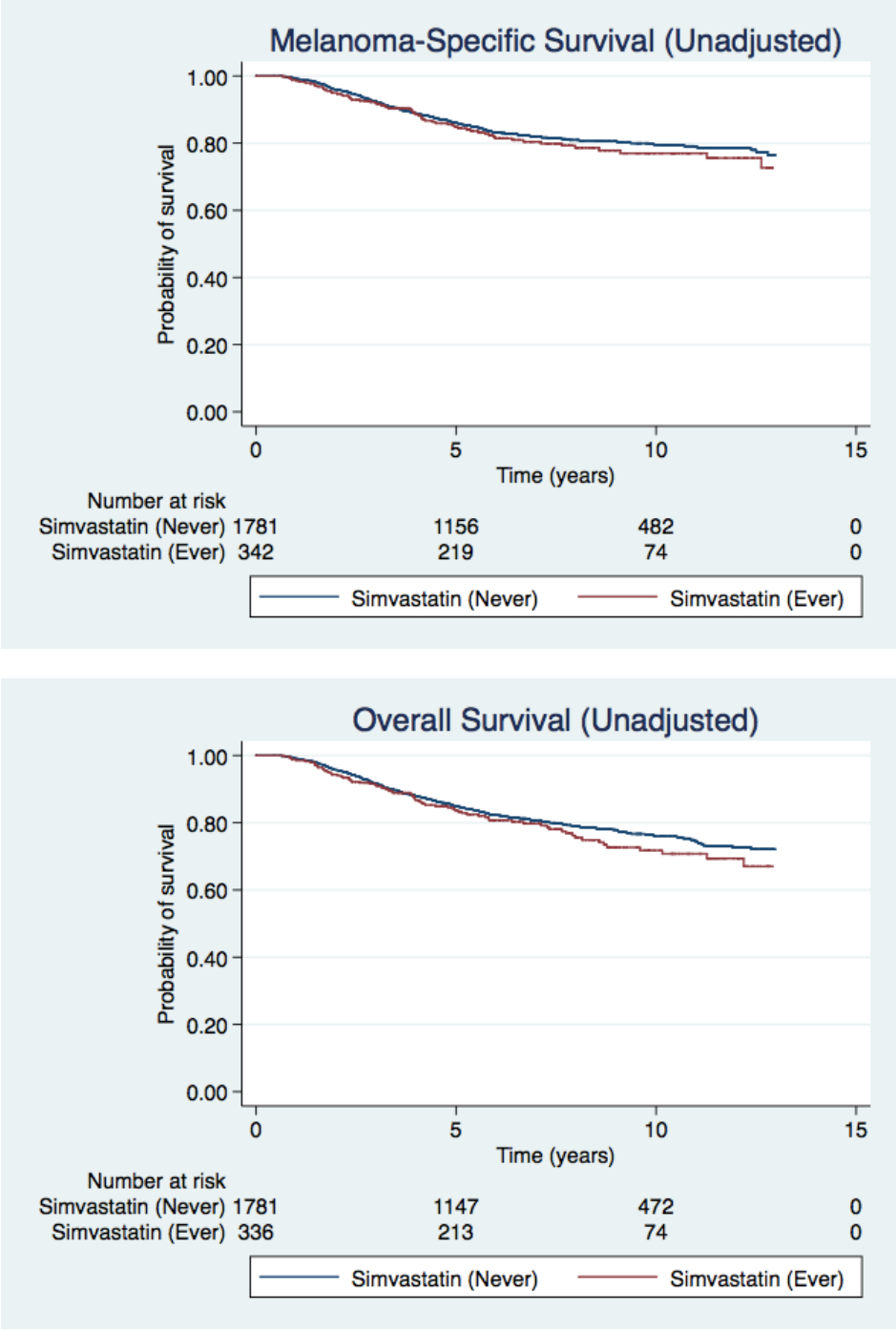


Figure 5.1: Unadjusted Kaplan-Meier survival plots for melanoma participants who had ever or never used simvastatin – MSS and OS

Table 5.4: Cox's proportional hazards regression analysis of melanoma-specific and overall survival in patients ever having taken simvastatin regularly (ever-never)

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and body mass index. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

	Melanoma-specific survival		Overall survival	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Unadjusted or crude model	1.14 (0.87-1.48)	0.342	1.15 (0.90-1.48))	0.263
Adjusted for age at diagnosis and sex	0.76 (0.57-1.00)	**0.048**	0.71 (0.55-0.92)	**0.010**
Adjusted for age at diagnosis, sex and Breslow thickness	0.79 (0.59-1.04)	0.096	0.75 (0.58-0.97)	0.028
Adjusted for other risk factors*	0.71 (0.50-0.99)	**0.043**	0.66 (0.48-0.90)	**0.009**

However, as previously established increasing age and the male sex are known predictors of melanoma and overall survival. Similar to the case of aspirin, when the model was adjusted for age at diagnosis and sex, this effect changed and ever taking simvastatin was associated with a lower risk of dying from melanoma HR (MSS) 0.76 (95% CI: 0.57–1.00; p = 0.048) and overall survival HR (OS) 0.71 (95% CI: 0.55-0.92; p = 0.010). The effect appeared to be maintained with significant protective effects seen in both MSS and OS even in our multivariable approach although it did appear to lose statistical significance for MSS when adjusting for Breslow (along with age and sex) (Figure 5.2) suggesting as per our literature review that simvastatin may be exerting some of this possible protective effect via a reduction in Breslow thickness. However as discussed in the methodology section this analysis is subject to the guarantee time bias, by virtue of the fact that, the longer someone lives, the higher the likelihood of them becoming part of the simvastatin-ever category.

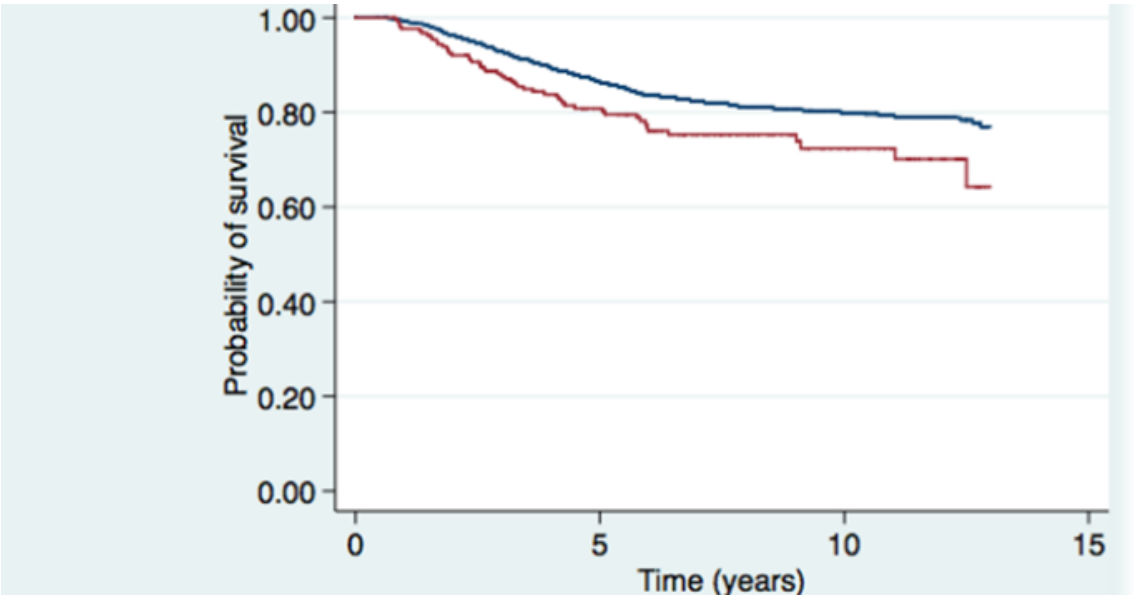
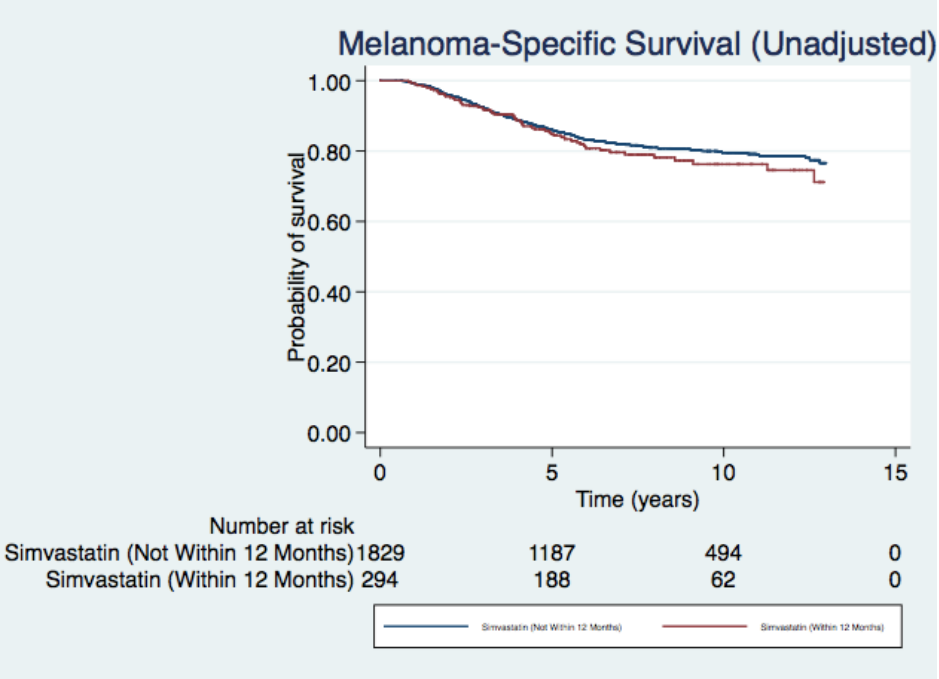


Figure 5.2: Adjusted Kaplan-Meier survival (MSS) plot for melanoma participants who had ever or never used simvastatin (adjusted for age, sex and Breslow)

Chart axes: x – years, y – probability of survival.

5.4.5 Simvastatin use within 12 months of diagnosis

The Kaplan Meier plot as shown in Figure 5.3 below again suggested that participants who used simvastatin at diagnosis or within 12 months of diagnosis, have a poorer chance of survival for both MSS and OSS as compared with those who did not use simvastatin in the unadjusted model although again non-significant with HR (MSS): 1.17 (0.88-1.55, p=0.278), and HR (OS) 1.18 (0.91-1.54, p=0.215). However as shown in Table 5.5, just as in the last analysis the hazard ratio is reversed when adjusting for the known predictors age and sex. However on this occasion the statistical significance was not maintained in the multivariate model, which as we discussed in the methods may be due to loss of power with this, analyses subtype.



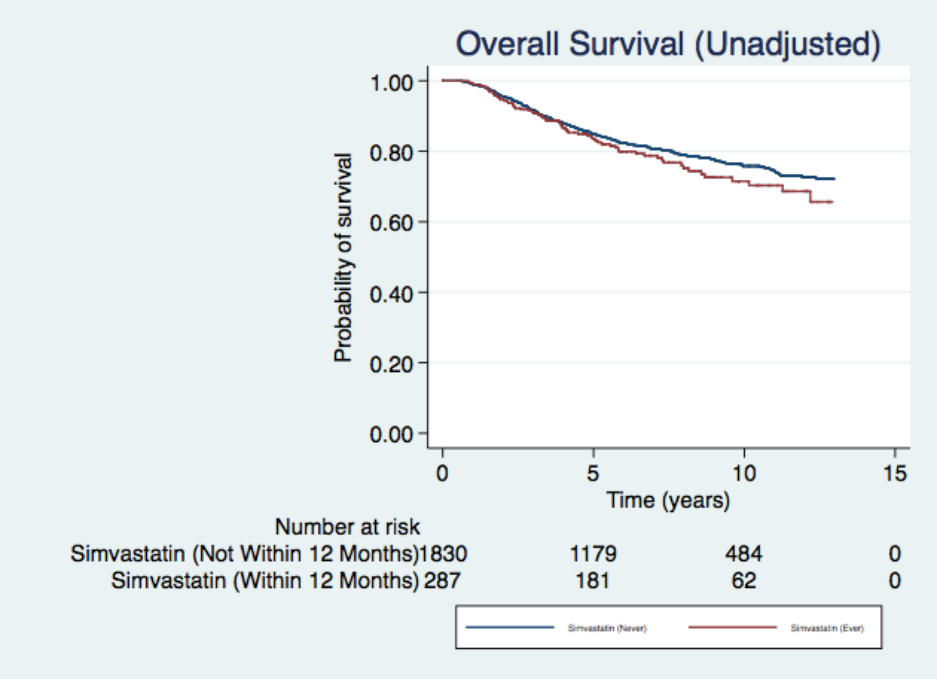


Figure 5.3: Unadjusted Kaplan-Meier survival plots for melanoma participants who had used simvastatin within 12 months of diagnosis.

Chart axes: x – years, y – probability of survival.

Table 5.5: Cox's proportional hazards regression analysis on the effect of simvastatin use within 12 months of diagnosis on survival

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and body mass index. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

	Melanoma-specific survival		Overall survival	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Unadjusted or crude model	1.17 (0.88-1.55)	0.278	1.18 (0.91-1.54)	0.215
Adjusted for age at diagnosis and sex	0.77 (0.58-1.04)	0.089	0.73 (0.56-0.96)	**0.023**
Adjusted for age at diagnosis, sex and Breslow thickness	0.82 (0.61-1.10)	0.184	0.77 (0.59-1.01)	0.059
Adjusted for other risk factors*	0.78 (0.55-1.11)	0.172	0.74 (0.53-1.02)	0.065

5.4.6 Stratification by sex

As discussed previously, based on literature evidence in other melanoma cohorts, an analysis was conducted and stratified by sex to explore if it was possible to identify any similar themes with this approach. Concerning the effect of simvastatin use on overall survival when stratified by sex i.e., males and females had different hazard ratios when the use of simvastatin was considered – whether ever-never or use within 12 months of diagnosis. In both cases of ever-never and 12 months of diagnosis analyses, males had hazard ratios less than 1, while females had hazard ratios greater than one globally. As presented in Table 5.6, exposure to simvastatin ever/never or within 12 months of diagnosis was associated with significantly reduced risk of death from melanoma with HRs of 0.54 (95% CI: 0.37-0.79) and 0.57 (95% CI: 0.38-0.85) respectively and with p-value < 0.05 in both cases when compared to females who had HRs of 1.03 (95% CI: 1.02-1.04, p-value < 0.001) and 1.21 (95% CI: 0.79-1.86, p-value = 0.379) in cases of ever/never and 12-months at diagnosis whilst also adjusting for age at diagnosis and Breslow's thickness, suggesting that simvastatin use may have a preferentially protective effect in male participants as compared to women.

Table 5.6: Simvastatin ever-never and 12 months survival analysis stratified by sex and adjusted for age and Breslow thickness

Adjusted for age at diagnosis and Breslow's thickness. Significant p-values are in bold figures. HR, hazard ratio; 95% CI, 95% confidence interval

Sex stratified	Ever-never		12 months of diagnosis	
	Mortality HR (95% CI)	p-value	Mortality HR (95% CI)	p-value

Melanoma-specific				
Male	0.54 (0.37-0.79)	**0.002**	0.57 (0.38-0.85)	**0.006**
Female	1.22 (0.82-1.83)	0.327	1.21 (0.79-1.86)	0.379
Overall survival				
Male	0.60 (0.40-0.88)	**0.009**	0.62 (0.41-0.92)	**0.019**
Female	1.16 (0.77-1.76)	0.471	1.21 (0.79-1.87)	0.384

5.4.1 Effect of atorvastatin on survival outcomes

We first examined the characteristics of the atorvastatin users and as per Table 5.7 and as for simvastatin there were significant differences noted between users and non-users in the majority of characteristics examined. As with simvastatin, the majority were male (68, 58.62%) compared to women (48, 41.38%) taking atorvastatin (p<0.001), which as discussed would be expected. In this case as shown in Chapter 3, Table 3.23, 80 patients were on both aspirin and simvastatin. Further analysis of the cohort showed that as in the case of simvastatin a higher proportion of people on atorvastatin were diabetic (18, 15.93% vs. 63, 3.29%; p<0.001) and users were also more likely to be overweight or obese compared to non-users (p<0.001).

Atorvastatin users were current smokers (9, 14.75% vs. 230, 26.84%; p<0.001) as compared to those not on atorvastatin As discussed earlier, vitamin D is one of the factors thought to influence melanoma survival and we, therefore, wanted to analyse our cohort to see if there was a statistical difference in vitamin D levels between users and non-users by defining different ranges of vitamin D and comparing with the base group and we observed no statistical difference as shown (p=0.719). Users were less likely to have ever smoked (p<0.057) or currently smoking (p<0.038)

As we have stablished Breslow thickness is an important predictor of survival and interestingly unlike in the case of aspirin and statin, there was no significant tendency to higher Breslow thickness in users of atorvastatin compared to non-users (p<0.013).

Table 5.7: Characteristics of patients ever treated with atorvastatin

Continuous variables are summarized as median (interquartile range) and p-values were generated using the Mann-Whitney U test. Categorical variables are represented as n (%) and p-values were generated using chi-squared test or Fisher's exact test if expected cell count was less than five. P-values were considered statistically significant if <0.05

Ever Treated with Atorvastatin?	Yes	No	P-value
Age at diagnosis	68 (10.7)	56 (20.4)	**<0.001**
Gender			
Female	48 (41.38)	1,160 (57.97)	**<0.001**
Male	68 (58.62)	841 (42.03)	
Diabetes			
No	95 (84.07)	1,853 (96.71)	**<0.001**
Yes	18 (15.93)	63 (3.29)	
Body Mass Index			
<=24.9kg/m^2	29 (25.44)	761 (38.34)	
>24.9-29.9kg/m^2	43 (37.72)	811 (40.86)	**<0.001**
>29.9kg/m^2	42 (36.84)	413 (20.81)	
Smoking status			
Never	53 (46.49)	1073 (55.62)	0.057
Ever	61 (53.51)	856 (53.51)	
Smoking Status			
Not currently smoking	52 (85.25)	627 (73.16)	**0.038**
Currently smoking	9 (14.75)	230 (26.84)	
Breslow thickness			
<=1mm	26 (22.81)	569 (28.75)	
1.01-2mm	47 (41.23)	759 (38.35)	0.218
2.01-4mm	23 (20.18)	441 (22.28)	
>4mm	18 (15.79)	210 (10.61)	
Vitamin D			
<20nmol/L	6 (6.0)	102 (6.24)	
20-59.9nmol/L	65 (65.0)	1,092 (66.79)	
60-84.9nmol/L	24 (24.0)	367 (22.45)	0.937
85-99.9nmol/L	3 (3.0)	51 (3.12)	
>100nmol/L	2 (2.0)	23 (1.41)	

5.4.2 Effect of atorvastatin ever use on survival outcomes

As shown in Table 5.8 below the unadjusted model again showed a poorer chance of survival for both MSS and OSS as compared with those who did not use atorvastatin and again non-significant with HR (MSS): 1.04 (0.69-1.56, p=0.858), and HR (OS) 1.05 (0.72-1.53, p=0.799). However as before the hazard ratio is reversed when adjusting for the known predictors age and sex and becomes significantly protective. The effect appears to remain statistically significant in both MSS and OS after further adjustment for Breslow, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and BMI - suggesting that atorvastatin use is protective.

Table 5.8: Cox's proportional hazards regression analysis of melanoma-specific and overall survival in patients ever having taken atorvastatin regularly (ever-never)

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and body mass index. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

Model	Parameter	Atorvastatin (ever-never)	
		Melanoma-specific	**Overall survival**
Unadjusted or crude model	*HR (95% CI for HR)*	1.04 (0.69-1.56)	1.05 (0.72-1.53)
	p-value	0.858	0.799
Age at diagnosis & sex-adjusted	*HR (95% CI for HR)*	0.64 (0.42-0.99)	0.65 (0.44-0.95)
	p-value	**0.046**	**0.026**
Adjusted for age, sex and Breslow	*HR (95% CI for HR)*	0.63 (0.41-0.97)	0.65 (0.44-0.95)
	p-value	**0.036**	**0.025**
*Adjusted for other risk factors**	*HR (95% CI for HR)*	0.56 (0.33-0.94)	0.55 (0.34-0.88)
	p-value	**0.028**	**0.013**

5.4.3 Atorvastatin use within 12 months of diagnosis

The survival analysis in this group as shown in Table 5.9 of patients who used atorvastatin at diagnosis or within 12 months of diagnosis, have a poorer chance of survival for both MSS and OSS as compared with those who did not use atorvastatin in the unadjusted model which on this occasion was significant. However as shown in previous analyses, the hazard ratio is reversed when adjusting for the known predictors age and sex. However, on this occasion there was no statistical significance seen in the

multivariable model, which as we discussed in the methods may be due to loss of power with this much smaller, analyses subtype.

Table 5.9: Atorvastatin use within 12 months of diagnosis survival analysis

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and body mass index. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

Model	Parameter	Atorvastatin (use within 12 months of diagnosis)	
		Melanoma-specific	Overall survival
Unadjusted or crude model	**HR (95% CI for HR)**	1.61 (1.03-2.53)	1.61 (1.03-2.53)
	p-value	**0.037**	**0.037**
Age at diagnosis & sex-adjusted	**HR (95% CI for HR)**	0.99 (0.63-1.55)	0.98 (0.62-1.55)
	p-value	0.952	0.938
Adjusted for age, sex and Breslow	**HR (95% CI for HR)**	0.97 (0.62-1.53)	0.97 (0.61-1.52)
	p-value	0.896	0.887
Adjusted for other risk factors*	**HR (95% CI for HR)**	0.91 (0.52-1.57)	0.91 (0.53-1.57)
	p-value	0.727	0.730

As discussed previously, based on literature evidence in other melanoma cohorts, an analysis was conducted and stratified by sex to explore if it was possible to identify any similar themes with atorvastatin, particularly in light of our other findings with simvastatin. As can be seen in Table 5.10 stratification by sex, adjusted for age at diagnosis and Breslow thickness on this occasion did not show any significant changes in hazard ratios in either ever-never or use within 12 months of diagnosis analysis types.

Table 5.10: Atorvastatin use stratified by sex

Adjusted for age at diagnosis and Breslow's thickness. Significant p-values are in bold figures

Sex	Ever-never*		12 months of diagnosis	
	Mortality HR (95% CI)	p-value	Mortality HR (95% CI)	p-value
Melanoma-specific				
Male	0.74 (0.45-1.22)	0.244	0.97 (0.52-1.79)	0.917
Female	0.49 (0.21-1.11)	0.086	0.91 (0.37-2.23)	0.837
Overall survival				
Male	0.68 (0.43-1.09)	0.108	1.08 (0.63-1.84)	0.771
Female	0.59 (0.30-1.16)	0.126	0.76 (0.31-1.85)	0.542

5.5 Discussion

In this section I will discuss my findings in relation to the literature and what we already know about potential associations of statins on melanoma survival and to determine whether these associations can explain my findings. I will also discuss the limitations of my study approach in examining these associations.

As discussed in the literature review, although preclinical data shows possible anticancer effects of statins in melanoma, meta-analyses could not demonstrate reduced melanoma incidence in statin users. However the studies looked at in the meta-analysis did however have limitations in that some of them were looking at cardiovascular benefit as a primary outcome and there are also inherent difficulties with interpreting case control studies with a different primary outcome without being able to adjust for known factors that influence melanoma survival. These limitations were highlighted by the Cholesterol Treatment Trialists' (CTT) collaborators who studied 14 RCTs of statins and reported that statins do not result in decreased incidence of cancer (RR = 1.00, 95% CI = 0.95-1.06) [279]. However, as discussed already, statins have been reported to decrease other cancer-specific [42,48-52,54,55], and overall mortality [48,54,55].

The results of my study showed that firstly some of the characteristics of the simvastatin users were significantly different to non-users in terms of the majority of users being male (198, 58.93%), diabetic (43, 13.03% vs. 38, 2.24%; p<0.001) ever smokers (185, 55.72% vs. 732, 42.78%; p<0.001) and more likely to be overweight or obese (p<0.001) compared to those not on simvastatin. Similar results were seen for atorvastatin with the majority being male (68, 58.62%) compared to women (48, 41.38%) taking atorvastatin

(p<0.001) and a higher proportion of people on atorvastatin were diabetic (18, 15.93% vs. 63, 3.29%; p<0.001) and users were also more likely to be overweight or obese compared to non-users (p<0.001). This would be expected given that these are risk factors for IHD and this population is, therefore, more likely to be prescribed statins as well as aspirin with 155 patients being on both aspirin and simvastatin and 80 patients on both aspirin and atorvastatin. It would therefore be difficult to exclude the possibility of both these drugs having a synergistic effect for instance on “bad inflammation” via cox pathways and IL6 respectively. Given the characteristics of the population taking simvastatin, with for instance more smokers or obese patients compared to non-users, this “bad inflammation” is very likely to be playing a significant role. Measuring serum levels of CRP in this group of patients would have been highly desirable as a marker of inflammation. For example the Leeds group has previously shown that smoking, another variable linked to inflammation, is an independent risk factor for melanoma [33]

As can be seen, the population characteristics of both simvastatin users and atorvastatin users are very similar and therefore if they had similar biological effects one might postulate that the results of the survival analysis should be similar taking into account the difference in power due to drug exposure. However based on literature evidence of different drug effects and given the difficulties and inaccuracies with the data collection process I chose to examine these separately and some differences in results were noted as discussed below.

In the simvastatin group a tendency to higher Breslow thickness was observed in users of simvastatin compared to non-users (p<0.013) with no such association seen with atorvastatin. Given the that from literature evidence we would have expected the Breslow thickness to be reduced, particularly as simvastatin primarily mediates its effects via a strong inhibitory effect on the growth of melanoma cells I explored this association further by performing a univariable regression analysis of factors associated with simvastatin use followed by a multivariable analysis (Table 5.2 and 5.3). A higher Breslow thickness category was not found to be associated with a higher odds of aspirin use following adjustment for age, male gender, diabetes and BMI. However analysis of ever use of simvastatin was associated with a lower risk of dying from melanoma with a HR (MSS) 0.76 (95% CI: 0.57–1.00; p = 0.048) and overall survival HR (OS) 0.71 (95% CI: 0.55-0.92; p = 0.010). The effect appeared to be maintained with significant protective effects seen in both MSS and OS even in our multivariable approach although it did appear to lose statistical significance for MSS when adjusting for Breslow (along with age and sex) (Figure 5.2) suggesting as per our literature review that simvastatin may be exerting some of this possible protective effect via a reduction in Breslow thickness. A similar association was seen for simvastatin use at diagnosis or within 12 months, however on

this occasion the statistical significance was not maintained in the multivariate model, which as we discussed in the methods may be due to loss of power with this, analyses subtype. In terms of atorvastatin use and the same analysis types interestingly for ever-never use atorvastatin use showed a significantly protective association when adjusting for age and sex, as well as when Breslow was adjusted for and finally even in our multivariate analysis with HR (MSS) 0.56 (95% CI: 0.33–0.94; p = 0.028) suggesting a much more protective association than with simvastatin for the same analysis showing HR (MSS) 0.71 (95% CI: 0.50–0.99; p = 0.043). This analysis could have been affected by the difference in power as a result of the difference in the number of patients on each drug as well as the potential of the guarantee time bias playing a role. Interestingly for both simvastatin and atorvastatin analysis of drug usage at diagnosis or within the last 12 months, the statistically significant association was lost which may reflect the presence of the bias in the previous analysis or be related to loss of power with fewer patients in this group.

None the less, our results point towards a potential protective effect of statins exposure on melanoma survival. The only other population-based study investigated statin use and cancer mortality in melanoma among several other cancer types in Danish patients, as discussed earlier with all-cause mortality among patients with cancer who were taking statins being reduced by 15%, but this was similar to the observed reduction in all-cause mortality of 10% among patients at risk of death from cardiovascular causes cancer [282]. In this study, the use of statin was calculated in terms of total defined daily doses and patients using statins were 15% less likely to die from any cause (HR = 0.85, 95% CI = 0.83–0.87) and from cancer (HR = 0.85, 95% CI = 0.82–0.87). Although a reduced cancer-related mortality for statin users was found in 13 different cancers including lung, colorectal, prostate and breast, it was not seen in melanoma (HR = 1.21, 95% CI = 0.95–1.52).

In my analysis of simvastatin and sex stratification in cases of both ever/never and 12-months at diagnosis analysis, whilst also adjusting for age at diagnosis and Breslow's thickness, demonstrated that simvastatin use may have a preferentially protective effect in male participants as compared to women with MSS showing HR of 0.54 (95% CI = 0.37–0.79; p-value = 0.002) in ever-never and MSS showing HR of 0.57(95% CI = 0.38–0.85; p-value = 0.006) in within 12 months of diagnosis analysis in males compared to females with an MSS showing HR of 1.22 (95% CI = 0.82-1.83; p-value = 0.327) in ever-never and MSS showing HR of 1.21 (95% CI = 0.79–1.86; p-value = 0.379) in the within 12 months of diagnosis approach. No such association was seen with the same stratification with atorvastatin again suggesting potentially a different mechanism of action, although the other possibility is related to loss of statistical power.

A sub-analysis for sex in melanoma patients was not performed in the Dutch registry study [60], but the HR for all cancer patients and cancer-related mortality showed that the HR of death in male patients using statins was reduced more than in female patients (HR = 0.82, 95% CI = 0.81–0.86 vs. HR = 0.92, 95% CI = 0.88–0.92). In terms of this preferentially protective effect in male participants as compared to women, this was also shown in the Dutch population based study by Livingstone *et al* [303]. Unlike in this study, they, however, were unable to demonstrate that statin usage on its own changed the hazard of death significantly in their cohort of 709 melanoma patients which may reflect loss of power with their smaller cohort, but also as they have indicated themselves a short follow up period of three years. Stratification based on sex, however, demonstrated possible superior survival among statin users, compared to nonusers in males only. The female survival advantage in melanoma in general was confirmed in this study, as was the case in the Dutch study [303] . The favourable results of statin use only in male statin users are, therefore, surprising. As discussed in the introduction, cancer survival has been shown to be generally better in females than in males for most cancers, especially, in melanoma. Even after adjustment for potential behavioural differences (primarily diagnostic delay and healthcare consumption), sex remains an independent prognostic factor for melanoma progression and survival. It would suggest that biological differences are, therefore, highly likely to be playing a role.

In our study as well as in the Dutch study, statin use somehow seems to negate the male survival disadvantage in melanoma. Therefore, the effects of statins on melanoma might be related to the underlying mechanism of the overall sex differences in melanoma survival. Two potential mechanisms were suggested by the Dutch group as moderating this sex difference in melanoma survival. As previously mentioned, somatic activating Rac1 mutations, ranking third after BRAF-and NRAS-mutations, in general, tend to occur significantly more often in men than in women. As statins have been shown to prevent Rac1 isoprenylation as explained earlier**,** and to inhibit the Rho-pathway it might be possible that males have worse survival rates than females due to a higher rate of Rac1 mutations leading to an increased activity of the Rho-pathway in male melanoma cells, which in turn might be counteracted by statin use. It was also proposed that, as has been shown, melanoma is a highly immunogenic tumour and males have a weaker immune system than females, it might be possible that males benefit more from the activating effect of statins on the anti-melanoma immune response than females, potentially explaining the differential effect of statin use across sex. Alternatively, we have seen that statins exert anti-inflammatory action by affecting mediators of inflammation such as IL-8, IL-1β, IL-12, tumour necrosis factor α (TNF-α), and nuclear factor κB (NF-κB) [83]. In the group there were more males who were smokers and obese suggesting high levels of bad inflammation and they may therefore benefit preferentially from these effects of

statins on inflammation. However, it was suggested that it is also possible that the sex differences might also only be an epiphenomena with the underlying cause being associated with sex but not caused by sex per se [303]. In terms of sex differences per se irrespective of exposure to drugs, as discussed in the literature review in Chapter 1, several studies demonstrate the fact that male melanoma patients are almost twice as likely to die compared with female patients. Although men are significantly more likely to have melanomas with unfavourable characteristics such as thicker and nodular melanomas located on the trunk, in accordance with previous observations [269], adjusting for these factors in our analysis do not explain the observed sex difference in melanoma survival with aspirin and statins.

Although the most common explanation for the sex difference in melanoma survival generally, is the better stage at diagnosis among women, our results could not be explained on this premise. A number of possibilities have been proposed to explain the difference in survival by sex. The thicker tumours reported in men have been attributed to delayed presentation. Recently however, the assumption that Breslow thickness is a surrogate marker for the time between development and diagnosis of melanoma, which has been applied in many epidemiological studies, has been challenged [269]. A large epidemiological study showed no positive association between melanoma thickness and time to diagnosis on a population basis and a histological study concluded that aggressive tumour growth, rather than delay in diagnosis, is responsible for the development of thick melanoma [331, 332]. One possibility therefore is that more aggressive melanoma growth in men may contribute to the differences observed and that potentially in our case the statin has had a preferentially protective effect on tumour growth [269]. A sex difference in the prevalence of lifestyle factors such as sun exposure [269] and dietary habits such as vitamin supplements including vitamin D, ethanol consumption, soy isoflavones, essential fatty acids and drug have also sometimes been used to part explain the difference in survival across the two sexes [269]. That assumes that survival in men may be worse because of co morbidities resulting from these lifestyle variables. The field of epigenetics is an emerging one and related effects on host tumour interaction may be mediated epigenetically. It is postulated that this could be the missing link between the environment and genes. Chemical substances that surround us may indirectly inflict permanent DNA-changes and account for the changes seen

The other possibility as alluded to is whether the effects of hormones such as oestrogen or testosterone may mediate differential survival per se and also differential response to different drugs. In terms of hormones, a few earlier studies indicated an increased melanoma risk in (long-term) oral contraceptive use [269], but meta-analyses did not confirm this association [333]. In randomised clinical trials, tamoxifen did not seem to

improve the survival rate in patients with metastatic melanoma either [269]. The likelihood of developing melanoma and its prognosis were also comparable in pregnant and non-pregnant females [334]. Furthermore a recent case–control study that focused on melanoma in women did not find reproductive, menstrual and hormonal factors that affected melanoma risk [335]. Dividing our cohort population into pre-menopausal and post-menopausal women and repeating the analysis would be an option to consider to try to elucidate this further.

The strengths of this study are the fact that it involves the largest cohort of melanoma patients, giving us comparably more power than the other studies and the fact that we had a longer study period compared to other studies. It was also population ascertained. Given the significant amount of data collected at recruitment, I was able to control several known variables that effect melanoma survival unlike the registry-based Dutch study where they felt that this was one of the drawbacks of their study, as they had no information on smoking status, for instance [60]. In particular, we were able to separate out melanoma specific death whilst in the Dutch population the cause of death was unknown and only all-cause rather than cancer-specific mortality could be assessed and therefore, any improvement could also be attributed to a decreased death risk due to cardiovascular comorbidities [60].

The main limitations of this study was the lack of a national cancer linked pharmacy database, as in the Dutch study, making our drug data less robust although, as shown in Chapter 2, every effort was made to optimise this by collecting information from multiple sources. We also undertook a retrospective approach as opposed to Livingstone et al who undertook a prospective approach as well as validating the data from two large, nationally representative and linked cancer-and pharmacy databases [60]. Our study options were also limited from analysis approach, as we were unable to look at dose and duration, given the potential biases in our methodology, as we were unable to perform the analyses with a time-dependent approach, as in the case of Livingstone *et al.* [60]. We also had insufficient data to investigate separately for lipophilic and hydrophilic statins, as this can have a variable improvement as shown in breast cancer, as discussed earlier. However, as in the other studies on melanoma, the majority of prescriptions in our cohort were for lipophilic statins and as we examined only these, our results are entirely attributable to lipophilic rather than hydrophilic statins. Other limitations of our study include the inability to look at the associations with combinations of drugs because as discussed in this chapter and as show in table 3.20 there is a significant overlap between these drugs used to treat similar conditions related to inflammation/metabolic syndrome and also the issue of differentiating the effects of the co morbidities from the drugs used to treat them. Not being able to examine duration of drug use was also a

significant limitation in this regard as was gaps regarding the recall of drug start or stop dates prior to diagnosis, adding to the difficulty in calculating the duration.

In conclusion, my study did appear to show a significant beneficial effect of simvastatin ever use on survival of melanoma patients. However, when we look at usage within 12 months of diagnosis, no significant effect was seen. The differential impact that statin use seems to have on male and female melanoma patients requires further research and the results as a whole require further validation in larger international data sets as well as an examination of biological models to assess if these represent real effects or whether confounding factors are responsible for these changes. One possibility is to look at using Public Health England data, as this is an increasingly common approach, although it will be limited in terms of having access to other confounding variables as in available in our cohort. Additionally, when *in vitro* experiments are conducted, both male and female melanoma cell lines should be used to see if sex differences can be noticed and I would that future studies looking at factors that may be associated with melanoma survival should consider stratifying their results by sex.. Future studies could also look at addressing the effects of lipophilic versus hydrophilic statins which was something we were unable to examine.

Chapter 6

Metformin and Melanoma survival

Introduction

Metformin (N,N-dimethylbiguanide) belongs to a group of drugs called the Biguanides and is the most prescribed anti-diabetic medication in the world, currently estimated to be used to treat more than 120 million people worldwide [336]. Other drugs in this group include phenformin, and buformin, which were widely used in diabetic treatments starting in 1920 until their high toxicity in patients was discovered and they were then withdrawn. It wasn't until a study by French chemist Jean Sterne in 1957, where he demonstrated metformin's effects on type 2 diabetes without any obvious toxicity or risk of hypoglycaemia, that metformin came back on the market [5].

After years of usage of metformin, retrospectives studies suggested that metformin treated diabetic patients had a decreased cancer incidence compared to those treated with another antidiabetic drug, with several subsequent studies confirming these results [4, 337, 338]. In this chapter I will examine the effects of metformin on melanoma survival specifically and I will begin by looking at the reported epidemiological associations and biological effects of metformin.

6.2 Biological effects of metformin

Metformin is able to exert its anti-diabetic function by reducing insulin resistance of glucose-intolerant patients as well as hepatic gluconeogenesis in type 2 diabetes, where it has been noted that hepatic gluconeogenesis is increased relative to healthy patients. In this sense, the liver is considered to be the principal site of action, where it can act on gluconeogenesis, glycolysis, and glycogen synthesis [5]. During treatment with metformin, glucose absorption and average glucose levels can decrease to 75%, which is also facilitated by increased absorption of glucose by skeletal muscles [339]. Furthermore, metformin also blocks the effects of glucagon, which normally enhances gluconeogenesis, by inhibiting essential enzymes in this process and stimulating glycolysis via the alteration of numerous enzymes in this signalling pathway [340].

In general, metformin increases glucose absorption by increasing the plasma membrane translocation of glucose receptors, such as glucose transporter 1 (GLUT-1), in both hepatic cells and skeletal muscle cells although we currently do not fully understand all the mechanisms of actions of metformin in these patients and consequently how these can impact on cancer [5].

In the next pages, I will summarise the reported epidemiological studies of the relationship between metformin and risk of cancers other than melanoma and then melanoma. I will then summarise laboratory data on metformin and cancers other than melanoma followed by specific experiments looking at melanoma.

6.3 Review of literature of associations of metformin usage with cancer

6.3.1 Epidemiological data on metformin in cancer other than melanoma

Epidemiological data on cancer risks in diabetes overall suggest that diabetes is associated with an increased risk.

In last few years, epidemiological studies involving type 2 diabetic (T2DM) patients have suggested around 2-times increased chances of developing cancers involving the endometrium, pancreas, and liver [341]. Similarly an increase in incidence of cancers involving breast, kidney, bladder, and colorectal region, has been reported with smaller associations (1.2-1.5 times) [342]. The prevalence of T2DM is higher in newly diagnosed cancer patients than non-diabetics and is estimated to be between 8 and 18% [343, 344]. An increased cancer risk is partly attributed to increasing levels of circulating growth factors, such as insulin or insulin growth factor 1 and 2 (IGF-1 and 2). An association between raised IGF-1 and diabetes risk has also been demonstrated in patients with acromegaly, characterized by a hypersecretion of growth hormone (GH) and consequently higher endogenous IGF, with studies showing a 2-fold increased risk of gastrointestinal cancers in these patients [345, 346].

Furthermore, studies have also shown a modest association between higher circulating IGF-1 and -2 levels and an increased risk for prostate, breast, colorectal, and ovarian cancers [347-353].

A number of retrospective studies showed that patients with T2DM treated with metformin however had lower cancer incidence and/or reduced cancer mortality [337, 354-356], compared to other diabetics not on metformin, creating interest in metformin as an anticancer agent and I explored some of these studies in more detail below.

Metformin was first linked to the prevention of cancer in a case-control study involving T2DM patients (n = 923) from the United Kingdom. The authors reported that use of metformin was associated with a reduction of the risk of developing cancer by 23% [337].

This finding resulted in a rapid rise in the number of observational studies assessing the link between use of metformin and risk of developing cancer, leading to several meta-analyses trying to quantify the emerging evidence. One such meta-analysis reported that use of metformin was linked with an overall 27% decrease in the risk of developing any malignancy [357]. Another study observed that 7.3% of type 2 diabetes patients treated with metformin developed cancers compared with 11.6% of patients treated with other anti-diabetics [354]. Similarly a recent study in a Korean population with type 2 diabetes showed a reduction in cancer development for patients treated with long-term metformin (5.8 years) with an incidence of 13.2 per 1000 compared with an incidence of 21.8 per 1000 in patients treated with other treatments [358].

Further cancer specific meta-analyses have suggested that diabetic patients have around 1.3 to 1.5-times increase in all-cause and cancer-specific mortality across certain types of cancer including CRC, endometrial, and breast [359, 360]. Though prostate cancer is less prevalent in men with T2DM, mortality was reported to be increased in patients who develop the disease, especially in patients with hyperinsulinemia and high body mass index (BMI), highlighting the need to assess this complex association which I am attempting to do in this thesis [361, 362]. Hyperinsulinemia on its own, can also result in activation of chronic inflammatory processes that can act as a triggering factor for initiation and progression of cancer [363]. Additionally, cancer cells typically have high levels of glucose uptake, and in this regard hyperglycemia may create a fuel-rich environment for cancer progression. One study compared cancer mortality in diabetics treated with three different treatments, metformin, insulin, or sulfonylureas, over 5 years in approximately 10,300 diabetes patients. The results demonstrated that patients treated with metformin had a lower cancer-related mortality rate than patients treated with other treatments [364]. Furthermore, a study by Currie et al. showed that patients treated with insulin developed more solid cancers than those treated with metformin [365].

6.3.2 Laboratory data on metformin in cancer other than melanoma

Given the suggestive epidemiological data, many research groups have tried to understand the mechanisms of action of metformin in different types of cancers, such as lung, prostate, and ovarian cancers. The *in vitro* effects of metformin, alone or in combination with other drugs, have been studied in many different cancers [366-369]. Furthermore, *in vivo* studies have demonstrated the efficacy of metformin in decreasing tumoural growth [370, 371].

The mechanisms through which metformin is postulated to promote anticancer effects can be broadly divided into indirect or direct effects. In this context, indirect refers to a

more systemic effect that modulates whole body physiology (such as by reduction of IGF levels) whilst I have used the term “direct” to refer to any mechanism acting directly on cancer cells inhibiting cancer progression. I will discuss the studies relating to each effect separately below.

6.3.2.1 Indirect effects of metformin

As discussed above, in the different studies looking at cancers, such as breast, colon, or prostate cancer, hyperinsulinemia and obesity induced by insulin and IGF1/2 are associated with a poorer prognosis. As already outlined, the liver, exposed to high levels of the drug after oral administration, is considered the main target organ of metformin. The cell surface organic cation transporter 1 (OCT1), necessary for the active transport of metformin (which is positively charged) into the cells is expressed in high quantities in the liver cells [372]. Within these cells, mitochondria appear to be the primary target of metformin whereby the drug inhibits the respiratory complex resulting in reductions of ATP as well as oxidation of NADH [373], This reduction in levels of ATP stimulates an increase in AMP levels inducing activation of the cell energy sensor 5’-AMP-activated protein kinase [230]. Once activated, AMPK seeks to down-regulate processes such as protein synthesis that require ATP resulting in catabolism and pathways that ultimately generate more ATP. This energy stress results in a reduction of hepatic gluconeogenesis [374] with a resulting reduction in circulating levels of glucose and insulin. This lowering of blood levels of glucose is also a result of increase of sugar uptake via skeletal muscles caused by the metformin-induced membrane translocation of the glucose transporter GLUT-4 specifically expressed in these tissues [375]. There is evidence to suggest that as elevated serum levels of insulin and insulin-like growth factor-1 (IGF-1) are frequently necessary to sustain the growth and survival of cells in different cancer types, the systemic reduction of these hormones can impair malignant growth inhibiting circulating insulin levels. Further evidence in mouse models shows that metformin inhibited lung cancer cell growth, induced by hyperinsulinemia and obesity, by decreasing the circulating level of insulin and by activating the AMPK pathway [376]. Furthermore, a study of non-diabetic woman with breast cancer, showed that metformin decreased circulating insulin levels by 22% and increased insulin sensitivity by 25% [377]. These results suggest that a decrease in insulin induced by metformin may mediate metformin inhibition of tumorigenesis.

Metformin can also inhibit the inflammatory signalling that promotes carcinogenesis by suppressing different pro-inflammatory cytokines such as tumour necrosis factor αTNF-α, nuclear factor kappa B (NF-κB) and interleukin 6 (IL-6) [378, 379]. It is possible therefore that metformin could have beneficial anti-inflammatory effects on cancer cells but additionally on systemic inflammation and risk of cardiovascular disease. There are

published papers discussed below in 7.4, which provide some evidence for a lower incidence of cardiovascular toxicity. The anti-angiogenetic effect of metformin is linked to reduction of the main factors involved in vascular remodelling such as VEGF and HIF-1α [380, 381]. Finally it was also observed that metformin treatment can selectively target cancer stem cells (CSCs), a tumour sub-population characterised by self-renewal capacity, resistance to chemotherapy and increase in cancer recurrence [379, 382-387].

A further indirect effect may be related to the fact that metformin seems to impact the microbiota which may be relevant in its action on T2DM patients but also in cancer development [388]. In a randomised study, woman with breast cancer were given routine doses of metformin and it was observed that testosterone, insulin levels and several indices of insulin resistance deceased significantly [389]. In another randomised study, compared to controls, metformin in a dose of 250 mg/day resulted in reduction of colorectal aberrant crypt foci (surrogate marker) by 40% in non-diabetic patients [390].

6.3.2.2 Direct effects of metformin

Despite the effects discussed above, the principal effects of metformin on cancer cells are reported to be direct effects, which predominantly induce inhibition of the mammalian target of rapamycin complex 1 (mTORC1). The protein mTORC1 is essentially complex composed of five different proteins: DEP domain-containing mTOR interacting protein (DEPTOR), mammalian LST8/G-protein β-subunit like protein (mLST8), regulatory-associated protein of mTOR (RAPTOR), proline-rich AKT substrate of 40 kDa (PRAS40), and a mammalian target of rapamycin (mTOR). This complex is primarily involved in regulation of protein synthesis, which is pivotal to cell growth and tends to be activated in cancer cells and can be implicated in resistance to cancer treatment. In addition to this mTORC1 plays a pivotal role in the growth and proliferation of normal stem cells as well as cancer stem cells. Its role in cancer stem cell proliferation has been in a number of different cancer types [5].

The direct anticancer effects of metformin can be broadly classified as AMPK dependent and AMPK-independent. AMPK has been shown to be directly activated by an increase of AMP/ATP ratio, or indirectly, through its upstream regulator serine/threonine liver kinase B1 [391]. Once activated, AMPK suppresses the mammalian target of rapamycin (mTOR) pathway either through the phosphorylation and activation of tuberous sclerosis complex 2 (TSC2) that in turn inhibits the mTOR activator Rheb [392] or by phosphorylation of Raptor, a positive regulator of mTOR [393]. The block of mTOR further inhibits the activation of its downstream target 40S ribosomal protein subunit S6 kinase (S6K or S6) and of the translational repressor 4E-binding protein 1 (4E-BP1), inactivated by mTOR mediated phosphorylation [394]. Metformin can also inhibit mTOR signalling independently from AMPK activation by suppressing the Ragulatory complex,

consisting of the RAG family of GTPases [395], or by activating the negative regulator of mTOR regulated in development and DNA damage responses 1 [396]. In non-diabetic women with breast cancer, metformin therapy not only led to reduction in the load of Ki67-positive cancer cells but also in changes in gene expression of molecules implicated in the mTOR and AMPK pathways [397].

The inhibition of protein synthesis via mTOR is only one of the mechanisms by which metformin can reduce cancer growth. Metformin exerts an inhibitory effect on glucose metabolism and has been shown to reverse the Warburg effect, present in most cancer cells. This term Warburg effect refers to the phenomenon by which, even in aerobic conditions, cancer cells tend to favour metabolism via glycolysis rather than the much more efficient oxidative phosphorylation pathway, which is the preference of most other cells of the body. Interestingly, melanoma appears to be one of the cancers that is the most dependent on and impacted by changes in metabolism as it requires glycolytic metabolism, which is mediated by mitochondrial activity [398, 399].

Metformin appears to do this either through the decrease of the glycolytic enzyme hexokinase 2 [400] or through the suppression of oncogenes such as c-Myc, Akt and hypoxia-inducible factor 1 α (HIF-1α that support the glycolytic phenotype [401]. The 32 metformin-induced AMPK activation can reduce enzymes involved in fatty acids biosynthesis as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), reducing energy supply and further counteracting tumour progression [402, 403]. Metformin can also promote cell cycle arrest through AMPK-mediated activation of TP53 and reduction of cyclin D1 expression [366, 404-406]. It has also been reported that metformin can reduce the risk of mutagenesis in cancer cells, either by the inhibition of reactive oxygen species (ROS) in the mitochondria production or through the activation of ataxia telangiectasia mutated (ATM) protein, a tumour suppressor involved in DNA repair [407, 408].

In terms of direct effects it appears therefore that metformin acts as a major metabolism disruptor in cancer cells and activates autophagy and apoptosis processes directly effecting cancer cells.

In summary, metformin has two proposed anti-neoplastic mechanisms of action. Firstly, an indirect route connected to its insulin-lowering activity, which may decrease the proliferation of tumour in individuals with hyperinsulinaemia and by effects on the stromal cells as above and secondly, a direct action in target tissues, preneoplastic and neoplastic cells, directed against respiratory complex I of the electron transport chain in mitochondria, thereby reducing the consumption of energy in the cells (118). Both these routes are associated with metformin mediated induction of AMP-activated protein kinase (AMPK), resulting in inhibition of the mammalian target of the rapamycin (mTOR)

pathway, reduction in cell proliferation, and induction of apoptosis and arrest of cell-cycle [409, 410]. Based on the target cell, the mechanism of action can be direct, indirect, or a combination of both.

6.3.3 Metformin and melanoma

In contrast to epidemiological studies looking at metformin and cancer in general there appears to be a real dearth of such studies looking at melanoma specifically with the majority of studies being laboratory based. A recent study by Tseng (2018) showed a reduced incidence of melanoma and non-melanoma skin cancer in Taiwanese patients with type T2DM although the study was limited by the number of patients with skin cancer in the study [411]. One large population cohort study in the US looked at rates of melanoma as well as colorectal, bladder, liver, and pancreatic cancers in patients treated with a number of anti-diabetics and reported no difference in incidence of any of these between treatment groups [412].

On the other hand there are quite an extensive number of number of laboratory studies that have looked at metformin and its effects on melanoma cells, showing that metformin or phenformin (another biguanide compound) can inhibit melanoma cell proliferation [370, 413-415].

As previously discussed, metformin can inhibit cancer cell proliferation and induce cancer cell death by many different mechanisms. Metformin induces melanoma cell death by both AMPK-dependent and AMPK-independent pathways. Through a mechanism that is not fully understood, metformin induces cell cycle arrest in melanoma cells, which is responsible for the activation of autophagy, and in turn the activation of apoptosis, leading to melanoma cell death. In initiating melanoma cells, metformin decreased cell transformation and proliferation by inhibiting the NF-κB pathway and the inflammatory pathway. It has been shown that metformin induces cell cycle arrest in melanoma cells in the G0-G1 phase after 24 h of treatment at 10 mM [5]. Studies have reported evidence that that this cell cycle arrest is responsible for autophagy (at 72 h) and apoptosis (at 96 h) induction by metformin in melanoma cells [413]. In this model, inhibition of AMPK (by siRNA) induces a partial restoration of melanoma cell viability under metformin treatment, suggesting that AMPK plays a partial role in metformin-induced melanoma cell death. This finding also suggests that another AMPK-independent mechanism is implicated in metformin-induced melanoma cell death. Studies in mouse models have shown that metformin decreases the tumoural volume of melanoma cells with no cell death being observed in normal human melanocytes even if endogenous AMPK is expressed [416, 417]. In another recent study, metformin induced autophagy activation in melanoma cells by inhibiting a potentially new

therapeutic target, tribbles pseudokinase 3 (TRIB3) [370]. In this study, the authors showed that metformin attenuated melanoma growth and metastasis by reducing TRIB3 expression in non-diabetic and diabetic mouse models.

A further recent study showed that metformin can act not only on melanoma cells to induce cell death but also on the tumour microenvironment, particularly in the context of an immune response [418]. As described in the introduction, the immune system is very important in melanoma therapies, and current immunotherapies have revolutionized the treatment of advanced melanoma. This study showed that metformin activated both autophagy and apoptosis in melanoma cancer cells *in vitro* and confirmed the results *in vivo* in mouse models challenged with B16 murine melanoma cells. The results showed that metformin activity on melanoma cells was partly due to the immune system and that the antitumor activity of metformin was lost on immunodeficient (NSG) mice. This group also showed that metformin interaction with the immune system was principally associated with T cells [5]. Studies like this showing the interactions between the immune system and metformin, has led to further studies looking at a combination of metformin treatment and immunotherapies, such as anti-PD1, to increase the effects of immunotherapies in melanoma cells which I will discuss further below.

These observations also suggest that it may be interesting to assess metformin in the context of other drugs or therapies that impact metabolism, such as targeted therapies (BRAF inhibitors) or immunotherapies (anti-PD1) in melanoma cells. Some studies have examined these effects of metformin in combination with BRAF inhibitors, such as vemurafenib and showed encouraging results with synergistic effects for inducing melanoma cell death [417]. Indeed, *in vitro* experiments show synergistic antiproliferative effects, particularly in *BRAF*V600E mutant cell lines. In other studies, metformin increased the toxicity of cisplatin, a chemotherapeutic drug, in melanoma cells [419].

6.3.3.1 Putative paradoxical effects of metformin in melanoma

Although I have described epidemiological and laboratory research which supports a view of metformin as a drug which reduces the incidence of cancer and reduces the growth of cancer cells, paradoxical effects have been described. Work by the Marais group suggested that metformin accelerates the growth of *BRAF*-V600E driven melanoma [420]. Martin et al showed that melanoma cells that are driven by oncogenic BRAF are resistant to the growth-inhibitory effects of metformin because RSK sustains TORC1 activity even when AMP-activated protein kinase (AMPK) is activated and therefore accelerate the growth of these cells *in vivo* [420]. Thus, metformin usage in diabetics might be beneficial in terms of survival in non-*BRAF* mutated melanoma but

deleterious in *BRAF* mutated melanoma. Data on *BRAF/NRAS* mutation status was available only for a proportion of the Leeds Melanoma Cohort.

It would therefore appear that the effect of metformin based on the mutation status of the tumour warrants more investigation. In this chapter I describe my analysis of the association of metformin use in melanoma patients with diabetes, and survival. In the majority of the Leeds Melanoma Cohort tumours, *BRAF* testing was not performed, so my analysis relates to an overall association between metformin usage and survival and an analysis comparing survival from melanoma on the trunk with that in other sites as the proportion of *BRAF* mutated tumours is reported to be higher in truncal primaries [17].

6.4 Safety and Tolerability

Metformin is one of the most widely prescribed oral blood glucose lowering drugs. It is associated with a very low risk of developing hypoglycemia but a high risk of gastrointestinal (GI) side-effects (affects an estimated 25% of patients) [421, 422]. The risk of GI side effects can be lowered by recommending patients to take metformin along with food. A further recommendation to patients suffering from GI side effects is to take metformin in a divided dose three times daily. Lower doses are to be used depending on renal function, and it is contra-indicated in severe renal insufficiency. Metformin has also been associated with an increased risk of lactic acidosis. This is a serious complication, and there is an increased risk of lactic acidosis in patients with renal insufficiency, liver disease, dehydration and excessive alcohol intake [422-424].

Two studies analysed the adverse events of lactic acidosis, and metformin was not associated with an increased risk. However, according to a systemic review [425] these studies had several methodological limitations. There are only a few studies that provide data on adverse effects other than hypoglycaemia and falls related to metformin use in the elderly population such as nausea and diarrhoea.

In summary, metformin is a safe, affordable and effective medication in the treatment of diabetes. The GI side effects do however affect compliance. Caution is recommended in patients with renal insufficiency and other conditions that can increase the risk of developing the rare, but serious complication of lactic acidosis.

6.4.1 Metformin and all-cause mortality/ non-cancer health

Observational studies have reported reduced all-cause mortality in diabetics taking metformin although others showed no such associations. Studies reported significantly

fewer deaths in patients on metformin compared to those taking, either no insulin [426], no other anti-diabetic drugs [427] or no metformin [428]. Another study reported reduced mortality in the group taking metformin as a mono-therapy (16% of 422 patients) compared to participants taking metformin and sulfonylureas as a combination therapy (32%) and participants on sulfonylurea as a monotherapy (51%) [429]. No significant difference in mortality was seen in patients aged over 80 years or for patients with a GFR ≤60 [425] however and in a large study conducted by Inzucchi et al [430], there was no significant difference in mortality in the metformin group.

Metformin compared to sulfonylureas showed fewer hospital admissions for acute myocardial infarction, stroke, hypoglycaemia or death. There were also fewer reports of non-fatal cardio-vascular disease and myocardial infarction, congestive heart failure, allcause mortality, and fewer fractures. Another study comparing metformin against thiazolidinediones showed a significant difference regarding mortality and all cause and heart failure readmissions. A comparison of metformin to rosiglitazone and pioglitazone showed no significant differences between the different treatments with regards to myocardial infarction, congestive heart failure as well as all-cause mortality [425] The UKPDS study showed cardio-protective effects while taking metformin doses as low as 500mg daily [421].

6.5 Materials and Methods

As discussed in Chapter 2, I first collected the drug data for all drugs used by participants in the cohort, which is the largest cohort of melanoma patients.

Firstly I had to ensure all entries for metformin were identified accurately by accounting for trade names, BNF codes, misspellings in data entry and missing data by cross-checking all data sources. As discussed in Chapter 2 due to limitations of the study approach and quality of the data I was unable to examine the effects of drug dosages on survival, which is a significant limitation given the evidence from particularly laboratory studies above that some effects seen are related to higher doses of metformin.

I then went on to interrogate the data further by looking at the demographics of the population taking metformin by examining the number of males and females on the drug in our cohort, their smoking status, their diabetic status, vitamin D levels and the distribution based on Breslow thickness. Metformin, much like aspirin and statins, is prescribed to similar groups of patients who are likely to have features of the metabolic syndrome with increased BMI and ischaemic heart disease, aside from of course having

diabetes for which the drug is specifically used. Therefore I expected to see some common trends within my analysis.

Given that men have a higher risk of these conditions we had expected to see more men being prescribed metformin than women and we also expected to see more smokers and diabetics having been prescribed metformin, as these are independent risk factors for ischaemic heart disease. We also expected this group of patients to be overweight compared to the rest of the cohort population (BMI > 25).

The effects of exposure to metformin on melanoma specific (MS) and overall survival (OS) were then assessed using Cox Proportional Hazards models. Data was first checked to ensure that the proportional hazards assumption was met. Hazard ratios (HR) and 95% Confidence intervals (CI) were estimated and Kaplan-Meier curves were plotted. Firstly unadjusted models were examined and then adjustment for common confounders were applied; firstly sex and age at diagnosis, then Breslow thickness, ulceration, other comorbidity measures (smoking, body mass index (BMI), and serum level of vitamin D adjusted for seasonal variation. Analyses were conducted both with and without adjustment for stage (represented by Breslow thickness and ulceration), since metformin may influence outcome through an effect on the growth of the tumour, which may be captured by stage at diagnosis. No adjustment was made in the analyses for adjuvant cancer therapy since no therapy has been shown to influence melanoma survival until very recently and the cohort stopped recruiting in 2012. Requests have recently been made to Public Health England for data on exposure to immunotherapies via SACT (Systemic Anti-Cancer Therapy) but I did not have access to those data.

As detailed in the methodology chapter, I then undertook a survival analysis, based on firstly, the ever vs never approach with the accepted guarantee-time bias, first examining the adjusted model and then, adjusting for known confounders. The second approach was an analysis examining the association of survival with reported exposure to metformin up to diagnosis or within 12 months of diagnosis. This method of analysis essentially replicates the traditional methods used in cancer incidence studies with no inherent bias, but as discussed in the methodology section our study was not specifically designed for this type of analysis, and we experienced some loss of power due to exclusion. As with the previous approach, we first examine the unadjusted model and then, adjusted for the known confounders.

I also examined any potential varying effects of metformin on survival based on sex, as in the case of aspirin and statins as described previously, although there were no similar reported associations with sex in the literature with metformin. I however carried out a survival analysis whereby I stratified by sex to identify any sex specific trends whilst also adjusting for age and Breslow thickness

Finally given the literature evidence above of potential varying associations of metformin depending on somatic *BRAF* mutation status, I also carried out a stratification via site of primary using truncal melanomas as a potential surrogate marker for *BRAF* mutated tumours although my statistical power for this type of sub-analysis was low [17].

6.6 Results

6.6.1 Characteristics of patients ever treated with metformin

Table 6.1 below shows the characteristics of patients who had ever been on metformin. Only 88 (4.4%) of the cohort had used metformin for any reason and at some point in their lives (ever-use). As expected a larger proportion of males (49, 56.98%) reported that they had used the drug compared to females (37, 43.02%) which fell just short of being significant (p= 0.07) and being an effective oral hypoglycemic agent, the drug was used by just over two-thirds (57, 68.67%) of diabetics in the study. Interestingly nearly a third of patients (26, 31.33%) reported having used metformin at some point in their life yet did not volunteer a diagnosis of diabetes. There are a number of possible explanations for this. As mentioned earlier in the chapter, although metformin is primarily used for frank diabetes it is also used in the case of pre-diabetes or impaired glucose tolerance where blood sugars haven't quite got to the diabetic range for the patient to be labeled as a diabetic. As mentioned earlier it can also be used in other conditions such as PCOS and Hidradenitis suppurativa, which is probably less likely to be the case in our cohort. It is also possible that either patients were not diabetic when filling in their recruitment questionnaire and subsequently became diabetic. Of note, there was also missing data for sex for two patients on metformin as well as data regarding diabetic status being missing for a small number of patients who have ever used metformin (5, 0.5%). Therefore, based on these assumptions all patients on metformin were assumed to be diabetic for the purposes of our analysis.

As expected the majority of patients taking metformin were obese (55, 65%) which was statistically significant (p = <0.001) and they were also more likely to have smoked (51, 61%, p= 0.02). As already discussed Breslow thickness is an important predictor of survival and there was again a statistically significant difference noted between the different categories. As discussed earlier, vitamin D is one of the factors thought to influence melanoma survival and we therefore wanted to analyse our cohort to see if there was a statistical difference in vitamin D levels between users and non-users by defining different ranges of vitamin D as outlined in Chapter 2 and 3, but no statistically significant difference was noted in this case.

6.6.2 Association of Metformin ever use on survival outcomes

As per my proposed methodology I then examined the association between patients having ever taken metformin and both melanoma specific and overall survival. As shown in Table 6.2 and the two Kaplan Meier plots in Figure 6.1, the unadjusted model in each case, suggested a statistically significant increase in hazard of death in patients taking metformin HR (MSS) 2.06 (95% CI: 1.40-3.02, p= <0.001) and HR (OS) 1.74 (95% CI: 1.18-2.57, p= 0.005).

However, as previously established increasing age and the male sex are known predictors of melanoma specific and overall survival. As in the case of aspirin and statins, when the model was adjusted for age at diagnosis and sex, the hazard ratios were attenuated as reflected in the two adjusted Kaplan Meier survival plots in Figure 6.2, but still showed a statistically significant negative association with metformin ever use and melanoma specific survival HR 1.59 (95% CI: 1.07-2.36, p value = 0.021). However once adjustment was made for Breslow thickness this became non-significant with an HR (adjusted for age, sex and Breslow) of 1.28 (95% CI: 0.86-1.91, p value =0.215 and completely disappeared in our multivariate analysis when the analysis was adjusted for the variables associated with metformin exposure e.g. smoking, ulceration reported diabetes and BMI.

Table 6.1: Characteristics of patients ever treated with metformin

Continuous variables are summarized as median (interquartile range) and p-values were generated using the Mann-Whitney U test. Categorical variables are represented as n (%) and p-values were generated using chi-squared test or Fisher's exact test if expected cell count was less than five. P-values were considered statistically significant if <0.05 and are displayed in bold. *For some variables, the summed total is less than the total number of patients because of missing values.

Ever Treated with Metformin?	Yes	No	P-value
Sex			
Female	37 (43.02)	1,171 (57.66)	**0.007**
Male	49 (56.98)	860 (42.34)	
Diabetes mellitus			
No	26 (31.33)	1,922 (98.77)	**<0.001**
Yes	57 (68.67)	24 (1.23)	
Body mass index			
<=24.9kg/m^2	3 (3.57)	787 (39.06)	
>24.9-29.9kg/m^2	26 (30.95)	828 (41.09)	**<0.001**
>29.9kg/m^2	55 (65.48)	400 (19.85)	
Smoking status			
Never	32 (38.55)	1,126 (55.12)	**0.002**
Ever	51 (61.45)	917 (44.88)	
Breslow thickness			
<=1mm	7 (8.24)	588 (29.28)	
1.01-2mm	38 (44.71)	768 (38.25)	**<0.001**
2.01-4mm	25 (29.41)	439 (21.86)	
>4mm	15 (17.65)	213 (10.61)	
Vitamin D			
<20nmol/L	6 (8.96)	102 (6.12)	
20-59.9nmol/L	49 (73.13)	1,108 (66.43)	
60-84.9nmol/L	11 (16.42)	380 (22.78)	0.514
85-99.9nmol/L	1 (1.49)	53 (3.18)	
>100nmol/L	0 (0)	25 (1.5)	

Table 6.2: Analysis of melanoma-specific and overall survival in patients ever having taken metformin regularly (ever-never)

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and body mass index. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

	Melanoma-specific survival		Overall survival	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Unadjusted or crude model	2.06 (1.40-3.02)	**<0.001**	1.74 (1.18-2.57)	**0.005**
Adjusted for age at diagnosis and sex	1.59 (1.07-2.36)	**0.021**	1.31 (0.88-1.95)	0.169
Adjusted for age at diagnosis, sex and Breslow thickness	1.28 (0.86-1.91)	0.215	1.09 (0.74-1.63)	0.641
Adjusted for other risk factors*	0.82 (0.42-1.63)	0.579	0.60 (0.32-1.13)	0.118

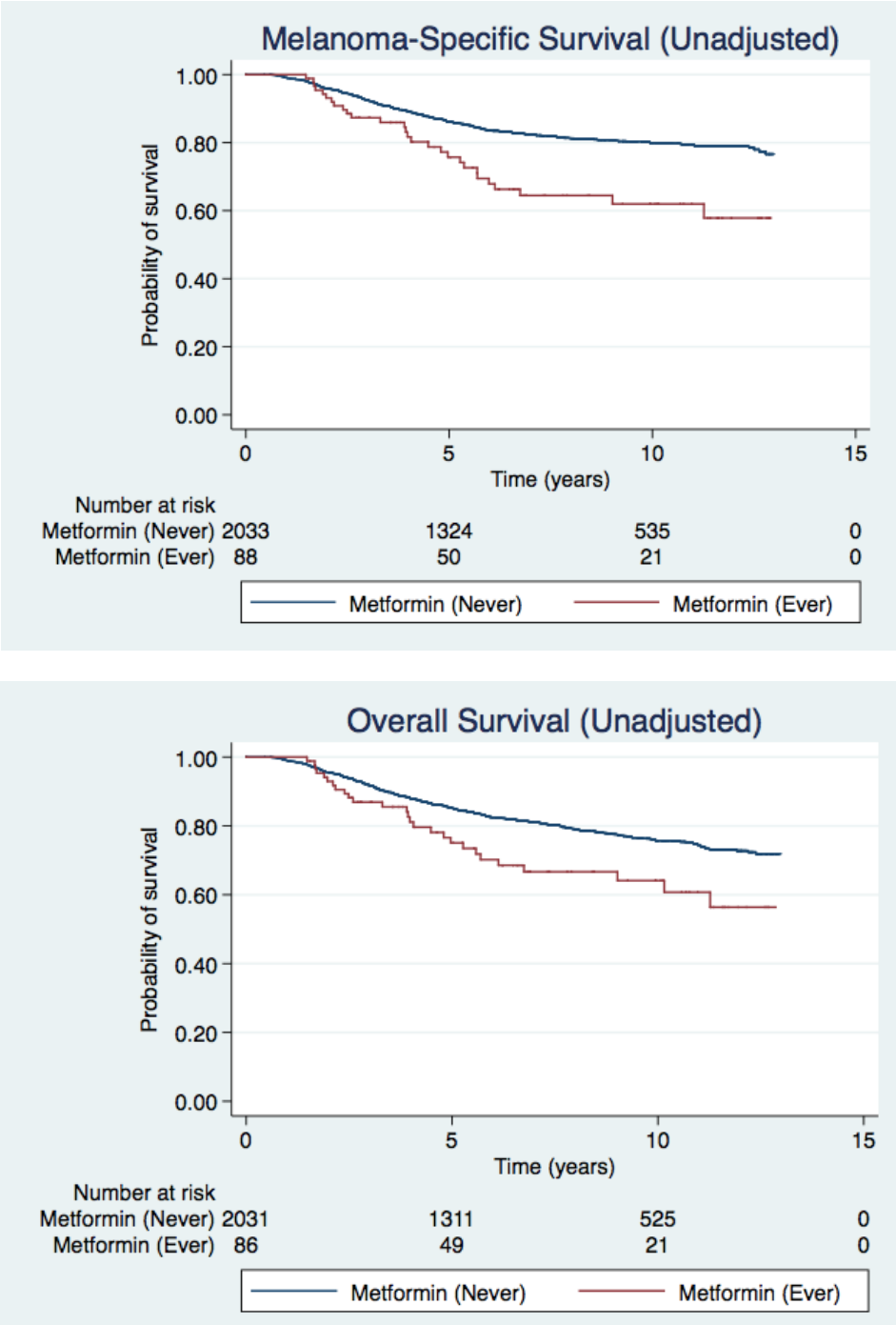


Figure 6.1: Unadjusted Kaplan-Meier survival plots for melanoma participants who had ever (n = 88) or never used metformin (melanoma-specific survival and overall survival).

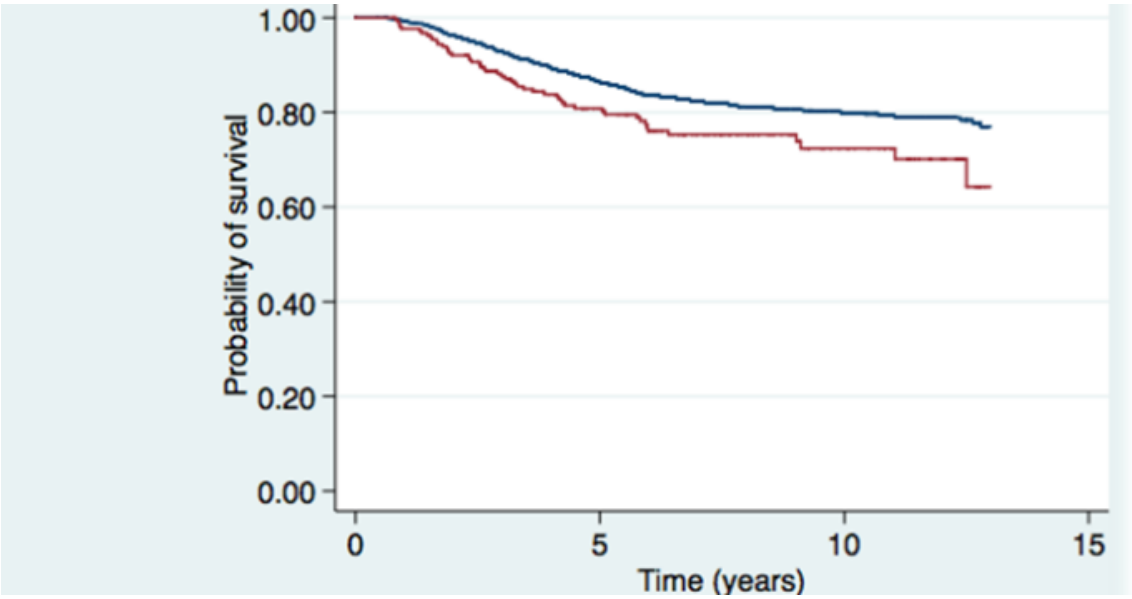


Figure 6.2: Age and sex adjusted Kaplan-Meier survival plot for melanoma participants who had ever (n = 88) or never used metformin (melanoma-specific survival).

6.6.3 Association of Metformin usage at diagnosis or within 12 months on survival outcomes

I next examined the association between metformin usage at diagnosis or within 12 months and survival outcomes (Table 6.3). There were 71 patients (3.34%) in this group as opposed to the 88 in the metformin ever category. Although the reduced number resulted in a modest loss of power this analysis, as discussed in the methodology section, does not have an inherent bias. Despite this, encouragingly the results are very similar to the results in the metformin ever group with an initial significant increase in hazard of death in patients taking metformin HR (MSS) 2.28 (95% CI: 1.51-3.45, p= <0.001) and HR(OS) 1.95 (95% CI: 1.28-2.96, p= 0.002).

Table 6.3: Analysis of melanoma-specific and overall survival in patients having taken metformin at diagnosis or within 12 months of diagnosis

*Adjusted for age at diagnosis, sex, Breslow thickness, number of pack years of smoking, vitamin D levels, diabetes, ulceration, and body mass index. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

	Melanoma-specific survival		Overall survival	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Unadjusted or crude model	2.28 (1.51-3.45)	**<0.001**	1.95 (1.28-2.96)	**0.002**
Adjusted for age at diagnosis and sex	1.71 (1.12-2.61)	**0.014**	1.42 (0.93-2.16)	0.107
Adjusted for age at diagnosis, sex and Breslow thickness	1.29 (0.84-1.97)	0.248	1.12 (0.73-1.71)	0.607
Adjusted for other risk factors*	1.00(0.51-1.95)	0.995	0.77 (0.41-1.46)	0.421

However, as previously seen increasing age and the male sex are known predictors of melanoma and overall survival, and when the model was adjusted for age at diagnosis and sex, the hazard ratios were attenuated as shown but still showing a statistically significant negative association with metformin ever use and melanoma specific survival HR (MSS) 1.71 (95% CI: 1.12-2.61; p= <0.001). However in the multivariate analysis as shown in Table 6.3 and like the previous analysis, this statistical significance was again lost once Breslow was adjusted for with HR (MSS) 1.29 (95% CI:0.84-1.97; p= 0.248).

6.6.4 Stratification by sex

As done previously with aspirin and statins, an analysis was conducted and stratified by sex to explore if it was possible to identify any similar themes with metformin, although in this case without previous literature evidence. When assessing the association of metformin in this manner as shown in Table 6.4 below, when adjusted for age at diagnosis males appear to have a statistically significant worse outcome in terms of melanoma specific survival when compared to females on metformin (HR=1.68, 95% CI 1.03-2.74, p=0.036). However once adjustment is made for age as well as Breslow thickness, the hazard ration drops and the statistical significance is just about lost suggesting that some of the hazard maybe attributed to thicker tumours in males (HR=1.52, 95% CI 0.94-2.48, p=0.090)

Table 6.4: Association of metformin use on melanoma specific survival stratified by sex

Significant p-values are in bold figures

	Metformin – Ever-Never			
	Adjusted for age at diagnosis		**Adjusted for age at diagnosis and Breslow thickness**	
Sex	**Mortality HR (95% CI)**	**p-value**	**Mortality HR (95% CI)**	**p-value**
Male	1.68 (1.03-2.74)	**0.036**	1.52 (0.94-2.48)	0.090
Female	1.44 (0.74-2.83)	0.286	0.96 (0.49-1.90)	0.913

6.6.5 Association of Metformin usage on survival when stratified by site of primary

Finally given the literature evidence discussed above of potential varying associations of metformin depending on somatic *BRAF* mutation status, I also carried out a stratification via site of primary using truncal melanomas as a potential surrogate marker for *BRAF* mutated tumours. As expected my statistical power for this type of sub-analysis was very low as shown in Table 6.5 below. As shown, there appeared to be a statistically significant negative effect with metformin use in melanomas on the chest which although could be used as a surrogate marker *BRAF* mutated tumours given the relatively small number of cases (n=6) it is difficult to be certain of a real association and in fact the tumours on the back which make up the largest number (n=27) did not show a statistically significant result.

Table 6.5: Association of metformin use on melanoma specific survival stratified by site of primary

Adjusted for age at diagnosis and Breslow. Significant p-values are in bold. HR, hazard ratio; 95% CI, 95% confidence interval.

Site	No of cases	Mortality HR (95% CI) (MSS)	P-Value
Abdomen	**5**		
Metformin ever use		1.28 (0.17 - 9.69)	0.81
Acral	**3**		
Metformin ever use		2.08 (0.47 - 9.25)	0.34
Back	**27**		
Metformin ever use		1.76 (0.89 - 3.49)	0.11
Chest	**6**		
Metformin ever use		**3.87** (1.29 - 11.57)	**0.02**
Other sites (no of cases) : flank (1), foot (3), head/neck (9), lower arm (3), shoulder (1), subungal(3), thigh(7), upper arm (6), vaginal(1), vulval (1)			

6.7 Discussion

As discussed in the literature review at the start of this chapter, a number of retrospective studies in cancers other than melanoma, showed that patients with T2DM treated with metformin have lower cancer incidence and/or reduced cancer mortality compared to other diabetics not on metformin, creating an interest in metformin as an anticancer agent. These findings also appear to be validated in meta-analysis of studies looking at metformin in these other cancers.

In terms of melanoma however there are very limited epidemiological studies showing similar associations. On the other hand there are several laboratory studies done on melanoma cells showing that metformin can inhibit melanoma cell proliferation. In summary, metformin has two proposed anti-neoplastic mechanisms of action. Firstly, an indirect route connected to its insulin-lowering activity as well as recued levels of pro-inflammatory cytokines such as IL6, which may decrease the proliferation of tumour in individuals with hyperinsulinaemia and secondly, a direct action in target tissues, pre-neoplastic and neoplastic cells, directed against respiratory complex I of the electron transport chain in mitochondria, thereby reducing the consumption of energy in the cells [373]. Both these routes are associated with metformin's action on mitochondria, which requires the activation of metabolic checkpoint AMP-activated protein kinase (AMPK), resulting in inhibition of the mammalian target of the rapamycin (mTOR) pathway,

reduction in cell proliferation, and induction of apoptosis and arrest of the cell-cycle [409, 410]. AMPK is implicated in several pathways, and following metformin activation, it decreases protein synthesis and cell proliferation. Many studies have demonstrated the role of metformin in the regulation of cancer cells, particularly its effects on cancer cell proliferation and cell death.

However contrary to the large body of evidence reviewed showing a beneficial effect of metformin in many cancers including melanoma, two papers reviewed suggested a somewhat paradoxical effect of oral metformin in *BRAF* mutated melanoma. It would therefore appear that the effect of metformin based on the mutation status of the tumour warranted more investigation.

In this chapter I attempted to investigate the association of metformin with melanoma survival in the LMC, the largest cohort of melanoma patients studied in an epidemiological study. As expected only a small number of patients in our cohort (n=88, 4.4%) had been on metformin for any reason and at some point in their lives (ever-use) reducing our statistical power to identify significant associations.

As expected from our literature review, a larger proportion of males (n=49, 56.98%) reported that they had used the drug compared to females (n=37, 43.02%) and it was the drug of choice in just over two-thirds (n=57, 68.67%) of diabetics in the study. Also as expected, because diabetes is associated with many co morbidities, the majority of patients taking metformin where obese (n=55, 65%) and more likely to have smoked (n=51, 61%) which we as we have identified previously are linked with inflammation. Given metformin's indirect effects on inflammation via cytokines and IL6 it would have been interesting to see if for instance CRP levels were elevated or not in this subgroup. As the severity of diabetes and end organ damage is dependent on glucose control and the length of time someone has had diabetes this would also have been a useful sub-analysis had this data been available. Surprisingly nearly a third of patients (n=26, 31.33%) reported to have used metformin in some point in their life yet did not volunteer a diagnosis of diabetes. As discussed there are a number of possible explanations for this, including the possibility that patients with pre-diabetes or impaired glucose tolerance are started on metformin, possible use in other conditions such as PCOS and Hidradenitis suppurativa, the possibility that either patients were not diabetic when filling in their recruitment questionnaire and subsequently became diabetic or that it may have been a data quality issue in that they did not fill in this part of the questionnaire and the discrepancy is as a result of missing data. Either way, this was an unexpected finding and depending on the actual reason for the discrepancy as above, this may have had an effect on our further analysis.

As per my proposed methodology I then examined the association between patients having ever taken metformin and both melanoma specific and overall survival. The unadjusted model in each case, suggested a statistically significant increase in hazard of death in patients taking metformin HR (MSS) 2.06 (95% CI: 1.40-3.02; p= <0.001) and HR (OS) 1.74 (95% CI: 1.18-2.57, p= 0.005). However, as previously established increasing age and the male sex are known predictors of higher melanoma and overall death rates and therefore need to be adjusted for in any representative model. Once the model was adjusted for age at diagnosis and sex, the hazard ratios were attenuated but still showed a statistically significant negative association with metformin ever use and melanoma specific survival HR (MSS) 1.59 (95% CI:1.07-2.36; p= <0.021). The statistical significance was lost once the model was adjusted for Breslow thickness, HR (MSS) 1.28 (95% CI:0.86-1.91; p= <0.215), suggesting that some of this negative effect was accounted for by thicker melanomas which warrants further examination as will be discussed below.

I next examined the association between metformin usage at diagnosis or within 12 months and survival outcomes. There were 71 patients (3.34%) in this group as opposed to the 88 in the metformin ever category. Although there was a slight loss of power, as discussed in the methods chapter, this type of analysis does not have any inherent survival bias and despite this, encouragingly the results are very similar to the results in the metformin ever group suggesting that the guarantee bias may not actually be playing such a significant role in our ever-never analysis. As with our previous analysis there was an initial significant increase in hazard of death in patients taking metformin in the unadjusted model with HR (MSS) 2.28 (95% CI: 1.51-3.45; p= <0.001) and HR (OS) 1.95 (95% CI: 1.28-2.96, p= 0.002). However, when the model was adjusted for age at diagnosis and sex, the hazard ratios were attenuated but still showing a statistically significant negative association with metformin use at diagnosis or within 12 months of diagnosis and melanoma specific survival HR (MSS) 1.71 (95% CI: 1.12-2.61; p= <0.001). However in the multivariate analysis once adjustment was made for Breslow thickness the statistical significance was again lost, HR (MSS) 1.29 (95% CI: 0.84-1.97; p= 0.248), although the trend towards a negative association was maintained as in the previous analysis which together with our results above does warrant more discussion.

A meta-analysis by Qi et al suggests that T2DM on its own may be an independent risk factor for melanoma and results in increased growth of the tumour [208]. Although we were not able to show a significant association of T2DM on melanoma survival in our cohort it was as shown in chapter 3 associated with a significant negative overall survival. A previous study from the Leeds group in this cohort has already shown that low vitamin D and smoking are associated with ulceration of primary melanomas and a poorer MSS

[33]. As shown above, patients in the cohort on metformin were more likely to have been smokers. We have seen in chapter 1 how all these factors are potentially related to inflammation and we accounted for all of these in our multivariable model where the hazard ratio dropped below 1 suggesting that the potential negative association first seen was mediated by these exposures rather than the metformin itself. Another possibility would be that diabetics or patients with multiple co-morbidities present later and therefore have thicker melanomas although I could find no evidence of this in the literature.

Accepting the low statistical power given the small numbers involved the results are still in contrast to the larger body of epidemiological and laboratory evidence suggesting a protective effect with melanoma in most studies. However evidence for a further explanation may be sought from the results of two laboratory studies with specific somatic mutations in the melanoma cells that did show a negative effect on survival and one would wonder if these somatic mutations (*BRAF* and *NRAS*) were potentially more prevalent in these patients in my cohort to potentially account for the negative trend in terms of an association between metformin use and melanoma survival in our cohort.

In order to be consistent with my methodology, as previously done with aspirin and statins, an analysis was conducted and stratified by sex to explore if it was possible to identify any similar themes with metformin, although in this case without previous literature evidence of such an association. When assessing the association of metformin in this manner when adjusted for age at diagnosis males appeared to have a statistically significant worse outcome in terms of melanoma specific survival when compared to females on metformin (HR=1.68, 95% CI 1.03-2.74, p=0.036). However once adjustment is made for age as well as Breslow thickness, the hazard ratio drops and the statistical significance is just about lost suggesting that most of the hazard maybe attributed to thicker tumours in males (HR=1.52, 95% CI 0.94-2.48, p=0.090), or potentially due to the fact that they are more likely to be smokers or obese and more likely to present late. In the absence of any epidemiological or laboratory evidence of a sex effect this is unlikely to be significant to metformin itself.

Finally given the laboratory evidence discussed above of potential varying associations of metformin depending on somatic *BRAF* mutation status, I also carried out a stratification via site of primary using truncal melanomas as a potential surrogate marker for *BRAF* mutated tumours. As expected my statistical power for this type of sub-analysis was very low and although there appeared to be a statistically significant negative effect with metformin use in melanomas on the chest specifically, given the relatively small number of cases (6) it is difficult to be certain of a real association and in fact the tumours on the back which make up the largest number (27) did not show a statistically significant

result. Combining the two groups may have been a better reflection of truncal melanomas but the way in which the data was coded, and limitations of the statistical software made me unable to perform this analysis.

Although our results are interesting, the main limitation of our study was the lack of statistical power given the relatively small number of patients on metformin and our power calculations done in chapter 2. Furthermore we had insufficient power to look at combinations of drugs because as show in table 3.20 in Chapter 3, in terms of patients on metformin 39 (48%) patients were also on aspirin, 52 (62%) patients were also on simvastatin, and 18 (35%) were also on atorvastatin, with 22 (27%) patients being on both aspirin and simvastatin together with the metformin. Therefore, it is difficult to ascertain how much of the associations seen are to do with metformin or indeed if the associations could have been strengthened or weakened because of potential synergistic or opposing effects. The other main drawback, common to the whole thesis, is with problems of differentiating the effects of the co morbidities from these drugs used to treat them.

Further limitations include the retrospective approach and quality of drug data prior to diagnosis and the lack of a national cancer linked pharmacy database. Our study options were also limited from an analysis approach as we were unable to look at dose and duration given the potential biases in our methodology, as we were unable to perform the analyses with a time-dependent approach. These biases generally would result from not properly classifying exposure during the follow-up period as well as the guarantee time bias discussed in our methods section with our ever-never analysis. Although the guarantee time bias should have meant that it would be very difficult to look at exposure to the drugs after diagnosis interestingly my results were very similar to the at diagnosis or within 12 months of diagnosis analysis which does not have an inherent bias, suggesting that the guarantee time bias may not be such an issue in my analysis.

On the other hand, in terms of strengths of the study, we had the largest cohort of melanoma patients allowing us the ability to perform an epidemiological study on the effects of metformin on melanoma survival given the real lack of such studies in the literature. I also had access to high-quality survival data given that it was a cohort study, which utilised multiple routes to obtaining those data. Given the significant amount of data collected at recruitment were also able to adjust for several known variables that can be associated with melanoma survival.

In conclusion, our study did appear to show a trend toward a significant harmful association of metformin use on melanoma survival in our cohort with a possible suggestion that this could be related to somatic mutations in *BRAF* by using truncal melanomas as a surrogate marker for this mutation, although there was a significant lack

of power to demonstrate this fully. Given the conflicting laboratory evidence this requires further research and the next step would be to repeat the analysis with data regarding mutation status (*BRAF* and *NRAS*), which has now been collected for the cohort.

Our results as a whole require further validation in larger international data sets as well as an examination of biological models to assess if these represent real associations or whether confounding factors are responsible for these changes. One possibility is to look at using Public Health England data as this is an increasingly common approach although it will be limited in terms of having access to other variables known to effect melanoma survival as was available in our cohort.

The link between diabetes and inflammation and the effects of multiple drugs is another point that needs further research in terms of developing an approach to adjust for these varying effects and potentially measuring markers of inflammation such as CRP and given the effects related to Breslow thickness potentially stratifying based on thickness would be further considerations. As mentioned, there is also a suggestion that metformin acts on the gut microbiome, which may have an effect on melanoma survival directly which also warrants more research.

Chapter 7
General Discussion

7.1 Aims

The aims of this chapter are:

- To review the rationale for undertaking this study and my proposed hypothesis;
- To summarise my findings;
- To discuss my findings in relation to existing literature;
- To discuss the limitations of my study;
- To draw final conclusions and suggestions for future work.

7.2 Introduction

Cancer patients are prescribed drugs intended to treat incidental medical conditions, which potentially could have effects on host-tumour interaction and therefore survival. These effects might be positive or negative. Evidence suggestive of beneficial effects for some drugs on cancer survival paved the way for the identification of drugs that can be used in chemoprevention, e.g. aspirin for colon cancer prevention. Yet, other drugs, e.g. immunosuppressants can have harmful effects leading to an increased death rate.

Several commonly prescribed drugs such as non-steroidal anti-inflammatories, lipid and blood sugar lowering medications used to treat conditions associated with the obesity and diabetes related metabolic syndrome are postulated to moderate cancer risks and survival. In this thesis I have addressed what the role of such drugs is in melanoma, by examining the association of these incidental drug exposures, whilst adjusting for the effects of the metabolic syndrome itself (as represented by BMI and diabetes status) and other known predictors on melanoma survival. This work was carried out in the largest cohort of melanoma patients represented by the Leeds Melanoma Cohort (LMC).

As outlined in Chapter 1, this study was prompted firstly, by epidemiological studies in other cancers suggesting that the use of commonly used drugs such as aspirin, statins or metformin may modify cancer risk. Secondly, laboratory data suggesting biological mechanisms to support the hypothesis that drugs might modify the likelihood of survival e.g., metformin in *BRAF* mutated melanoma. Thirdly, by the observation that although the American Joint Committee of Cancer (AJCC) predicts survival reasonably well, there is still a significant degree of variance which remains unexplained (30 to 40%). I would

therefore postulate that lifestyle or exposure to drugs, may moderate host/tumour interaction and therefore survival contributing to the unexplained variance.

In carrying out this study I had to be wary of the fact that most drugs, are more frequently used in older individuals with concurrent diseases associated with systemic inflammation and increased age is associated with poorer melanoma survival.

My specific aims were:

(i) To test the hypothesis that BMI and Diabetes, as components of the inflammation/metabolic syndrome may independently be associated with melanoma survival outcomes in the Leeds Melanoma Cohort (LMC).

(ii) To test the hypothesis that there are incidental drugs that the LMC may have been exposed to, having examined the literature, that are associated with survival outcomes independent of the effects on the inflammation/metabolic syndrome.

(iii) To test the hypothesis that my selected drugs, aspirin, statins and metformin are associated with survival outcomes in the LMC. The hypothesis is that these drugs may moderate outcomes independent of the underlying medical problem for which these drugs have been prescribed. or as moderators of the inflammation/metabolic syndrome which may also modify survival outcome as above.

7.3 Summary of findings and discussion

My findings in relation to my aims above were as follows:

(i) In terms of BMI, 63% of patients in the cohort were classed as overweight or obese, with 22% being specifically classed as obese which was less than expected in the general population based on the literature evidence. Our results suggested that BMI did not have a statistically significant independent association with melanoma survival in the LMC neither in the unadjusted or adjusted model with a hazard ratio (HR (MSS) of 1.01 (95% CI: 0.99-1.04; p= 0.364) seen in the multivariate model when adjusting for all our known cofounders. There was also no significant association when the analysis was stratified by sex. Although this does not exclude a deleterious effect of obesity: failure to show such could be a power effect if the risk is small, or due to the unrepresentative nature of the cohort population as above. The observation suggests that there was no strong association between obesity and MSS in the LMC.

In terms of diabetes, 81 patients (4%) in the cohort were diabetic. Our results suggested again that diabetes did not have a statistically significant independent

association with melanoma survival in the LMC neither in the unadjusted or adjusted model with a hazard ratio (HR (MSS) of 1.51 (95% CI: 0.74-3.07; p= 0.250) seen in the multivariate model when adjusting for all our known cofounders. There was also no significant association when the analysis was stratified by sex apart from a slight suggestion that males with diabetes may do worse. Although diabetes did not appear to have a directly significant association on melanoma specific survival, within our power constraints, it did appear to be significantly associated with overall survival in the LMC with a hazard ratio (HR (OS) of 2.37 (95% CI: 1.31-4.30; p= 0.004) seen in the multivariate model when adjusting for all our known cofounders. This was expected given its association with the inflammation/metabolic syndrome and organ damage as described in the literature.

(ii) Aside from our chosen drugs aspirin, statins and metformin the following drug categories were identified in the literature as having multiple reports of potential associations with melanoma survival: fibrates, retinoids, b-blockers, ace inhibitors, calcium channel antagonists and immunosuppressants. Unfortunately, there were not enough patients on fibrates, retinoids or individual immunosuppressants to carry out a meaningful analysis due to a lack of statistical power. With the other drugs examined including other drugs that could play a role in the metabolic syndrome and inflammation, including other non-steroidal anti-inflammatories, anti-hypertensive and other oral anti-diabetic drugs such as diclofenac, bisoprolol, and gliclazide there were no statistically significant effects on melanoma survival seen. However, I noted some interesting results with my chosen drugs as presented below.

(iii) **Aspirin** - when the analysis was stratified by sex, whilst adjusting for age and Breslow thickness, women who had ever taken aspirin were significantly less likely to die from their melanoma compared with those who never used the drug at any point in their lifetime with hazard ratios (HR) for MSS of 0.51 (95% CI: 0.30-0.87, P= 0.014) compared to men with an HR (MSS) 0.99 (95% CI: 0.71-1.37, P= 0.948). This suggests that aspirin use may have a preferentially protective effect in female participants as compared to males.

Statins - Similarly in the case of both ever/never use of simvastatin and at diagnosis (including 12 months prior) analysis, when adjusting for age and Breslow thickness, men had a significantly reduced risk of death from melanoma with HRs of 0.54 (95% CI: 0.37-0.79, P=0.002) and 0.57 (95% CI: 0.38-0.85, P=0.006) respectively when compared to females who had HRs of 1.22 (95% CI: 0.82-1.83, P=0.327) and 1.21 (95% CI: 0.79-1.86, P = 0.379) respectively. This would suggest that simvastatin use may have a preferentially protective effect in male participants as compared to women.

Metformin - In keeping with a previous smaller unpublished study done within our cohort, metformin usage appeared to have a trend towards a negative effect on melanoma survival in our cohort. I saw a significant association with ever-never metformin use when adjusting for age and sex with an HR 1.59 (95% CI: 1.07-2.36, p value = 0.021) but once adjustment was made for Breslow thickness this became non-significant with an HR (adjusted for age, sex and Breslow) of 1.28 (95% CI: 0.86-1.91, p value =0.215 and). In the at diagnosis (or within 12 months prior) analysis, metformin use showed a similar significant association when adjusting for age and sex with an HR 1.71 (95% CI: 1.12-2.61, p value = 0.014) but once adjustment was made for Breslow thickness this again became non-significant with an HR (adjusted for age, sex and Breslow) of 1.29 (95% CI: 0.84-1.97, p value =0.248). In view of the publications referred to previously suggesting that metformin has different effects on *BRAF* mutated tumours, such that patients on *BRAF* mutated tumours might do less well on metformin but those without *BRAF* mutations would benefit from metformin, I repeated the analysis, stratifying by tumour site, given reported evidence that truncal tumours are more likely to be BRAF mutated than tumours arising in other sites. On stratification for the site of primary, for melanomas on the chest, which as part of melanomas on the trunk could be used as a surrogate marker for *BRAF* mutated tumours, showed an HR of 3.87 (95% CI 1.29-11.57, p-value = 0.02) although this was not significant for tumours on the back HR of 1.76 (95% CI 0.89-3.49, p-value = 0.11).

7.4 Discussion of findings in relation to existing literature

As discussed in my literature review, the effects of BMI and Diabetes and the metabolic syndrome and inflammation on cancer incidence and survival are well documented. There has been increasing reported evidence particularly in cancers other than melanoma, that host factors such as obesity may drive cancer incidence and progression. The changes which are identified in response to the obesity related so-called metabolic inflammatory state is said to be orchestrated by metabolically active cells in response to excess nutrients and energy [431]. The fat cells generate inflammatory responses (increased TNF-α, and many inflammatory cytokines such as IL-6), which sustain low-grade chronic inflammation in affected patients, and this sustained inflammation is hypothesised to lead to the development of co morbidities such as cardiovascular disease.

Suppression of this pathway was recently explored as a means of prevention of significant cardiovascular disease in people at risk. This recently published data by

Ridker *et al* from the CANTOS trial suggests that the IL1β pathway is crucial in mediating cardiovascular disease, with evidence that it mediates cancer risk too. In this trial, more than 10,000 patients agreed to be randomised to placebo, 50, 150, and 300 mg 3 monthly of canakinumab, which blocks IL1β signalling, after a myocardial infarction. Eligibility required elevation of high sensitivity C reactive protein levels. The trial reports describe a dramatic reduction in serious cardiovascular events in the active treatment arms and notably, a reduction in death from lung cancer as a secondary outcome [139].

The Leeds group reported an investigation of microscopic ulceration of primary melanoma in 2015 (Jewell *et al*) in which the gene expression data suggested that ulcerated tumours have a pro-tumourigenic inflammatory environment with raised IL6 and IL8 gene expression [101]. The group then asked, if there is a role for IL6 in tumour progression locally, is there an association between conditions associated with systemic inflammation and ulceration? The group looked at data from the Leeds Melanoma Cohort and asked if factors related to low grade systemic inflammation (obesity, smoking, vitamin D deficiency and diabetes) might be associated with ulceration of primary melanomas [33]. It was shown that ulceration was associated with all of these factors in a univariate analysis lending support to the view that systemic inflammation may play a role in modulating host tumour interaction in melanoma and therefore survival. This was the justification for my examination of the role of drugs, which are reported to reduce systemic inflammation, in melanoma survival.

In observational studies such as mine, patients commonly use a multitude of drugs. For instance patients with a history or risk of cardiovascular disease may very easily be prescribed all of the drugs in question at the same time and co-administration of metformin, statins and aspirin might affect several different cellular pathways and inflammatory pathways at the same time. As shown in our cohort characteristics for the patients on these individual drugs they are often more likely to be obese or overweight and to have diabetes when compared to patients not on these drugs. Although in this study I was unable to demonstrate a statistically significant association in terms of BMI and Diabetes alone on melanoma survival that could be due to the limitations of my study that will be discussed in more detail below. Despite this, BMI and Diabetes as part of the metabolic syndrome would still be a very important consideration in any similar future studies.

In terms of my chosen drugs as discussed in the individual chapters there are already pre-clinical observational and laboratory studies suggesting potential effects of these drugs on melanoma incidence with a range of potential mechanisms of action for their anti-cancer effects having been proposed but with many fewer studies examining associations with survival. In most studies, only the effects of exposures prior to cancer

diagnosis were examined and in these as well as studies assessing survival, there was a lack of information on potential confounding factors, that I have been able to adjust for due to the presence of high-quality cancer data, which enabled detailed consideration of tumour staging and survival in particular.

My significant findings in terms of the aspirin and statin results when stratifying by sex, have not previously been reported in any studies of this size looking at melanoma survival outcomes as this was conducted in the largest cohort of melanoma patients reported in the literature. Interestingly similar significant protective effects in melanoma incidence, as opposed to survival, in woman taking aspirin and men taking statins have been shown in the same Dutch population-based study by Joosse *et al* [258]. Furthermore Livingstone *et al* in 2014 conducted a smaller but similar study to ours and again found that statins may reduce melanoma mortality, particularly in males [303]. Furthermore there are also other studies suggesting a similar association with Aspirin in females with melanoma incidence. [257].

The factors underlying the observed differences in association with drug exposure and melanoma survival, are not understood. In terms of sex differences per se irrespective of exposure to drugs, as discussed in the literature review in Chapter 1, several studies demonstrate the fact that male melanoma patients are almost twice as likely to die compared with female patients. Although men are significantly more likely to have melanomas with unfavourable characteristics such as thicker and nodular melanomas located on the trunk, in accordance with previous observations [269], adjusting for these factors in our analysis do not explain the observed sex difference in melanoma survival with aspirin and statins.

Although the most common explanation for the sex difference in melanoma survival generally, is the better stage at diagnosis among women, our results could not be explained by this argument. A number of theories have been developed to explain the difference in survival by sex. The thicker tumours reported in men have been attributed to delayed presentation. Recently however, the assumption that Breslow thickness is a surrogate marker for the time between development and diagnosis of melanoma, which has been applied in many epidemiological studies, has been challenged [269]. A large epidemiological study showed no positive association between melanoma thickness and time to diagnosis on a population basis and a histological study concluded that aggressive tumour growth, rather than delay in diagnosis, is responsible for the development of thick melanoma [331, 332]. One possibility therefore is that more aggressive melanoma growth in men may contribute to the differences observed [269]. A sex difference in the prevalence of lifestyle factors such as sun exposure [269] and dietary habits such as vitamin supplements including vitamin D, ethanol consumption,

soy isoflavones, essential fatty acids and drug have also sometimes been used to part explain the difference in survival across the two sexes [269]. That is that survival in men may be worse because of co morbidities resulting from these lifestyle variables. Lifestyle related effects on host tumour interaction may be mediated epigenetically. The field of epigenetics is currently emerging. It is theorised to be the link between the environment and genes. Chemical substances that surround us may indirectly inflict permanent DNA-changes and account for the changes seen

Could it be that the effects of hormones such as oestrogen or testosterone mediate differential survival per se and differential response to drugs? In terms of hormones, a few earlier studies indicated an increased melanoma risk in (long-term) oral contraceptive use [269], but meta-analyses did not confirm this association [333]. In randomised clinical trials, tamoxifen did not seem to improve the survival rate in patients with metastatic melanoma either [269]. The likelihood of developing melanoma and its prognosis were also comparable in pregnant and non-pregnant females [334]. Furthermore a recent case–control study that focused on melanoma in women did not find reproductive, menstrual and hormonal factors that affected melanoma risk [335]. Dividing our cohort population into pre-menopausal and post-menopausal women and repeating the analysis would be an option to consider to try to elucidate this further. Given the similarity in findings with Joosse *et al* [258] with these associations seen with aspirin and simvastatin on melanoma incidence our results may point to a common biological pathway mediating these effects via sex differences both in development of melanoma as well as influencing survival from melanoma, which warrants further investigation and I will propose some potential future study options in the next section.

With regards to metformin, as significantly fewer patients were on this drug in our cohort compared to aspirin and statins I had much less statistical power to undertake an analysis, although my results did appear to show a trend towards a non-significant harmful association of metformin use on melanoma survival in our cohort. This is in contrast to most epidemiological and laboratory studies as presented in the metformin chapter, in which in cancer overall, metformin exposure is reported to be of benefit in terms of survival. As I did not have access to somatic mutation status for cohort participants overall and I was therefore unable to explore the hypothesis that the results generated were driven by adverse effects in *BRAF* mutated tumour [420].

As alluded to previously, co-administration of multiple drugs at the same time are typical for many patients and in our study co-administration of metformin, statins and aspirin could well have influenced the individual effect on inflammation and tumorigenesis as well as effecting several different cellular pathways, at the same time. In terms of patients on metformin 39 (48%) patients were also on aspirin, 52 (62%) patients were also on

simvastatin, and 18 (35%) were also on atorvastatin, with 22 (27%) patients being on both aspirin and simvastatin together with the metformin. Therefore, it is difficult to ascertain how much of the associations seen are to do with metformin or indeed if the associations could have been strengthened or weakened because of potential synergistic or opposing effects. Furthermore, although metformin is currently the first-line treatment in type 2 diabetes its effectiveness may be impacted depending on whether patients have early or late stage diabetes for instance, which may also correlate to levels of chronic inflammation. This issue is related to the main challenge, common to the whole thesis, with the issue of differentiating the effects of the co morbidities from the drugs used to treat them.

7.5 Limitations of study

There were a number of limitations with my study that I will discuss in this section.

One of the main limitations was that despite the large cohort of melanoma patients in the study the study itself was observational only and indeed a retrospective one as the cohort had already been recruited prior to the setup of this particular study. I therefore did not have the benefit of setting specific inclusion and exclusion criteria specific to the aims of my study and to enable me to collect data specific to the study to try to limit potential biases. In the next section I will discuss some of these biases in more detail and assess how much of an impact they could have had on my results.

A bias is defined as a distortion of study results, which may occur when the study groups under investigation (exposed and unexposed) are identified, treated or evaluated differently to each other. There are a number of different forms of bias including selection bias, where exposed and unexposed patients are selected for study due to reasons, which may influence the outcomes, and information bias, where data is collected in an unbalanced manner between exposed and unexposed groups. The main selection bias with regards to my study would be with regard to whether the patients in the study were more or less likely to have co-morbidities that could influence survival outcomes irrespective of drug use and other known variables that we adjusted for in our analysis. This could lead to confounding, where the results of a study are not due to a true association between exposure and outcome, but may in fact be explained by another factor, e.g. age, which is associated with both the exposure and the outcome. The main confounders in our study are likely to be other mediators of inflammation and the metabolic syndrome that we have not accounted for such as hypertension and hyperlipidaemia.

This was a significant limitation of being able to assess an association of the metabolic syndrome with melanoma survival, as I was unable to adjust for all the components of this syndrome, as I did not have access to blood pressure recordings or measurements of lipid profile for instance. Although as discussed in the methodology I considered capturing this data by recording the use of an anti-hypertensive as a marker that a patient has a diagnosis of hypertension, this was flawed by the fact that certain drugs like diltiazem or bisoprolol can be used both as an anti-hypertensive but also to treat arrhythmia and this approach was abandoned. As discussed in Chapter 1.13, other measures such as hip to waist measurement ratio and presence of insulin resistance would potentially have been helpful adjuncts, particularly with defining obesity as calculating BMI is not always felt to be an accurate indicator. Related to this, it is also generally expected that in observational studies, exposure to the drug under investigation occurs in a non-random manner. A patient may receive a certain drug for a variety of reasons known or unknown to the researcher. For example, the age of a patient or the cost of a treatment may be a factor in deciding whether a particular drug is suitable for a patient. If these factors are also associated with the outcome under investigation, the exposure/outcome relationship is likely to be confounded. Various methods have been suggested to reduce confounding in observational studies and include both study design and analysis approaches. Study design approaches include matching exposed and control patients by a common potential confounding factor, or restriction of the analysis to only one level of a particular confounding factor, which given the complexity of my data set would be insufficient to analyse my data. Alternative proposed approaches in dealing with confounding at the analysis stage include stratification and multivariate analysis both of which I utilised in my study.

A further limitation is related to the issue of the use of multiple drugs at the same time as mentioned above where co-administration of metformin, statins and aspirin might affect several different cellular pathways, at the same time. Ideally a population based study using public data bases could be used to address the relative associations of exposure to pairs of drugs but after discussion with the team, I accepted that the current data set was sufficient only to look at exposures to single drugs with an agnostic approach, accepting that this is a weakness of this approach.

Another related issue is what is referred to as a Detection bias. Detection bias occurs in cohort studies where the follow-up procedures for detecting adverse events are different for patients with and without the drug exposure. Patients using a multitude of drugs or with a strong history of cardiovascular disease for instance, may visit the health services more frequently, and therefore increase their chance of having a melanoma relapse picked up earlier and better survival and which may introduce a bias whereby this is seen

as a potential protective effect falsely attributed to the medications in question. This form of bias may also play a role with regards to the recognised side effects of aspirin, statins and metformin, which may cause patients to visit health services. For instance, among diabetic patients, those using metformin, as compared to patients exposed to sulfonylureas, have been shown to have a slightly increased risk of undergoing a colonoscopy, (HR-1.12, 95% Cl 1.06-1.18) potentially due to patients experiencing gastrointestinal side effects following metformin treatment (ref) which led to earlier detection of colon cancer. In RCTs, the random assignment of a large number of participants into either the treatment or control group is expected to result in a balance of known and unknown risk factors/confounders for the outcome among the two groups. However, in observational studies, as discussed earlier, exposure to the drug under investigation occurs in a non-random manner.

An additional concern relating to the detection bias is the 'healthy user/adherer' effect. This form of bias is highly prevalent in epidemiological studies looking at drug effects and is also relevant to my study. The healthy adherer/user effect occurs as a result of patients who use a drug, or have high adherence to a drug therapy, being more likely to partake in healthy behaviours; for example, patients who adhere consistently to their drug therapy have been shown to be more likely to engage in cancer screening activities [432]. However, it is expected that adjustment for factors related to healthy choices (e.g. smoking status), or early detection of cancer (as represented by tumour stage), as done in my study, should reduce any potential healthy user bias.

A further bias that is likely to be present in my study is the concept of “recall bias.” The data obtained at recruitment to the cohort was based on questionnaire data, which is going to be influenced by what the patient accurately recalls in terms of drug usage and co-morbidities. Although I supplemented the drug data by including GP returns data this recall bias may explain why a proportion of the nearly a third of patients on metformin did not volunteer a diagnosis of diabetes on the questionnaire.

Another important limitation of the study was that I was unable to incorporate timing, duration or dosage of exposure to the drugs due to incomplete or missing data as result of the manner in which the data had been collected. Given the literature evidence in colorectal cancer for instance with aspirin, where effects on incidence are only seen after 5 years of use of aspirin, this would suggest that duration of drug use would have been a significant consideration to fully assess the associations with some drugs in the cohort. Another issue with the missing data and lack of detailed exposure data was our inability to study continuous versus infrequent drug exposure.

Selection of an appropriate analysis method for investigating the effects of drugs on melanoma survival was one of the most challenging aspects of our study. As discussed

in the methods (Chapter 2), we considered the following analysis methods and their limitations.

7.5.1 Ever-never analysis

This is the simplest and most frequently used analysis method in the epidemiology literature and divides the cohort into patients that have been exposed to the drug at some point (we only included patients into this group if we had evidence that they had been on the drug for at least 30 days) and those that had never been exposed to the drug in question. Although this type of analysis works well in studies looking at the effects on incidence of cancer, in studies like ours looking at survival, it does however introduce a potential bias referred to as the guarantee-time bias. This refers to the likelihood of someone who survives longer having a greater chance of being exposed to the drug in question and ending up in the ever user category. This longer survivorship could therefore be incorrectly interpreted as being as a result of having been exposed to the drug. Although we would have expected that due to this bias these results needed cautious interpretation, interestingly for most of our ever-never analysis the results were remarkably similar to our less biased drug use until within 12 months of diagnosis approach described in further detail below, suggesting that this bias played less of a role in our cohort.

7.5.2 Drug usage up until diagnosis

This method of analysis essentially replicates the traditional methods used in cancer incidence studies with no inherent bias but as my study was not specifically designed for this I did experience some loss of statistical power as well as the issue of poorer quality data before diagnosis.

7.5.3 Time-dependent or time-varying covariate analysis

This type of analysis is particularly suited for survival analysis and aims to minimise the biases discussed above and was used by Joosse *et al* in their study [258]. It reflects the phenomenon that a covariate such as drug use is not necessarily constant through the whole study. For instance, a patient may have been on and off a drug at different time points in the study and this could then be introduced in the statistical model as a time-varying covariate. In survival analysis, this would be done by splitting each study subject into several observations, one for each period of drug use/non-use. This method of analysis apart from being very complicated was not ideally suited to our data as we had to make some assumptions regarding continued use of drugs due to a lack of such

specific drug usage data as opposed to Joosse *et al* [258] who had very accurate drug data linked to a national pharmacy database. The results from this type of analysis were therefore largely unchanged for any drug in our cohort and the approach was abandoned.

An additional important concern is the risk of misclassifying patients who are exposed to drug as unexposed; this issue was highlighted in a recent review of studies examining associations between aspirin and improved colorectal cancer survival [433]. The quality of the exposure data in our study to aspirin for instance can be questioned due to the fact that low-dose aspirin is available over the counter in the UK for analgesic purposes. Practically however as we defined use as a minimum of one month, its unlikely that the majority of these patients eligible for NHS prescriptions would be likely to pay 'out-of-pocket' for aspirin in this way, at least over a long-term basis. Therefore, while some patients with infrequent exposure to analgesic doses of aspirin purchased over the counter may have been misclassified as 'unexposed', the vast majority of aspirin exposure would be expected to be correctly classified within my study.

When it comes to analysis of my results one of the significant limitations alluded to throughout the study is the lack of statistical power to examine several of the drugs of interest such as immunosuppressants to which the exposure in the cohort was very small. This is shown by the power calculations in Chapter 2 and the exposures to various drugs as demonstrated in Chapter 3. Further power issues relate to our stratified/sub-analysis and the number of melanoma specific deaths where the results do need to be interpreted with caution due to further loss of power.

7.6 Final conclusions and suggestions for future work

As discussed, the results from my study are broadly consistent with previous studies on associations between aspirin, and statin use on melanoma survival in the literature. The trend toward a negative association with metformin on melanoma survival differs from the majority of epidemiological studies as discussed although the proposed potential pathway of a negative effect via effects on *BRAF* mutated tumours has been reported in a laboratory study. My findings in terms of differential effects of aspirin and statins on melanoma survival within the same cohort when stratifying by sex is a significant finding although backed up by a large study on melanoma incidence in a Dutch population study as discussed. Although our study did not demonstrate an independent association of diabetes and BMI on melanoma survival, from our literature review it is clear that they play an important role in inflammation and cancer pathways.

The possibility of anti-cancer effects of drugs such as aspirin and statins, commonly used for other clinical indications is an exciting prospect particularly as these drugs are off-patent and relatively inexpensive, in contrast to agents considered in conventional cancer drug discovery. Although in contrast to our findings, some studies have examined the effects of metformin in combination with BRAF inhibitors, such as vemurafenib and showed encouraging results with synergistic effects for inducing melanoma cell death and represent yet another use of this commonly prescribed medication.

In considering the use of an existing drug towards a new therapeutic indication as proposed above, it is important to try to establish a strong evidence base to support testing of the drug in clinical trials. Evidence may be generated from preclinical studies (i.e. laboratory studies of in vitro or in vivo effects) or through analyses of data from patients who have already been taking the drug for other indications as in this study. Drug repurposing (also referred to as repositioning, redirecting, or reprofiling) is the investigation of existing drugs, such as aspirin, statins or metformin, for application outside the scope of their original therapeutic indications [434]. This concept represents a substantially faster route of drug development than what would conventionally be expected and some examples of its application previously include the repurposing of thalidomide for erythema nodosum laprosum and multiple myeloma, or the repurposing of sildenafil as a treatment for erectile dysfunction [435]. The additional advantage of this approach is that several phases of the process can be bypassed as the drug candidate will often have undergone these testing procedures for their original indication; the pharmacokinetic, pharmacodynamics and toxicity profiles of the drug candidates are already well established, meaning their progression to phase II and III clinical studies may be expedited. This is particularly relevant in the field of cancer therapeutics where toxicity and expense of new therapies often limit their application. Repurposing may also involve the consideration of drugs such as aspirin, statins or metformin as lead compounds; identification of anti-cancer effects of these drugs results in new drug development efforts to improve upon the original chemicals [434].

A further consideration would be the use of biomarkers to identify which patients may benefit from chemoprevention with information from a blood sample probably being the most optimal. It is estimated that only 30-50% of patients will experience a chemopreventive effect from aspirin which may reflect levels of inflammation, and so far, regular use of aspirin as chemoprevention is not recommended generally, notwithstanding the potential toxicities discussed previously [436]. Some studies have explored polymorphisms of the metabolising enzymes of aspirin, and it may be beneficial to have the slower metabolising variant. Others have seen different responsiveness of aspirin depending on sex and body-mass-index [436].

In general, drugs have different response between individuals and whether there are chemopreventive or carcinogenic properties seems to depend on the cancer subtype [437]. As already alluded to, when taking into account different mutations, patient gene polymorphism, and epigenetics this becomes a very complex field of study. This emphasizes the need for epidemiological studies and knowledge about genetics in order to establish a better picture. Biomarkers to identify the patients with potential benefits must be continued to be developed and evaluated in future studies.

In order to increase the robustness of further studies looking at the effects of incidental drugs, the use of healthcare databases should be encouraged. Examples of healthcare databases include pharmacy dispensing records, health insurance claims databases, general practice research databases, electronic hospital records, and disease registries; but crucially such databases should be linked in order to provide access to specific outcome related information, e.g. the linkage of pharmacy claims databases to disease registries in order to determine particular medications received by patients with a condition of interest. Simply having a healthcare database without cancer registry information is a flawed approach, as one is then unable to adjust for known predictors of cancer survival as in the case of Joosse *et al* [258] where they were unable to adjust for ulceration as in our study, which is an independent predictor of melanoma survival and used in the AJCC staging. Cancer registries are typically a comprehensive source of information in cancer research and usually include detailed tumour information such as stage, grade and morphology. They may also include details of cancer treatment and may include accurate outcome data. Medical record linkage of a cancer registry to pharmacy dispensing records can provide information on the type of regular medications patients were receiving, thus permitting the evaluation of associations between the drugs and tumour characteristics and/or cancer outcomes. This type of linked database represents a valuable resource as it comprises independently collected prospective data, reducing concerns regarding recall bias which may affect studies of cancer patients as discussed with our study. However, as these databases contain previously collected data, they often lack information on potential confounding factors relevant to the study being performed. Public Health England is working towards building such a data resource.

The associations seen in my thesis require further validation in larger international data sets as well as examination of biological models to assess if these represent real effects or whether confounding factors are responsible for these changes. In particular additional epidemiological and basic research is warranted to investigate this important sex differences in melanoma survival identified and therefore I would propose that future studies looking at factors influencing melanoma survival should consider stratifying their

findings by sex. It would also be important to repeat my analysis for metformin within the cohort when the complete somatic mutation (*BRAF* and *NRAS*) status is available for the cohort to see if there is a true negative association as a result of mutation status.

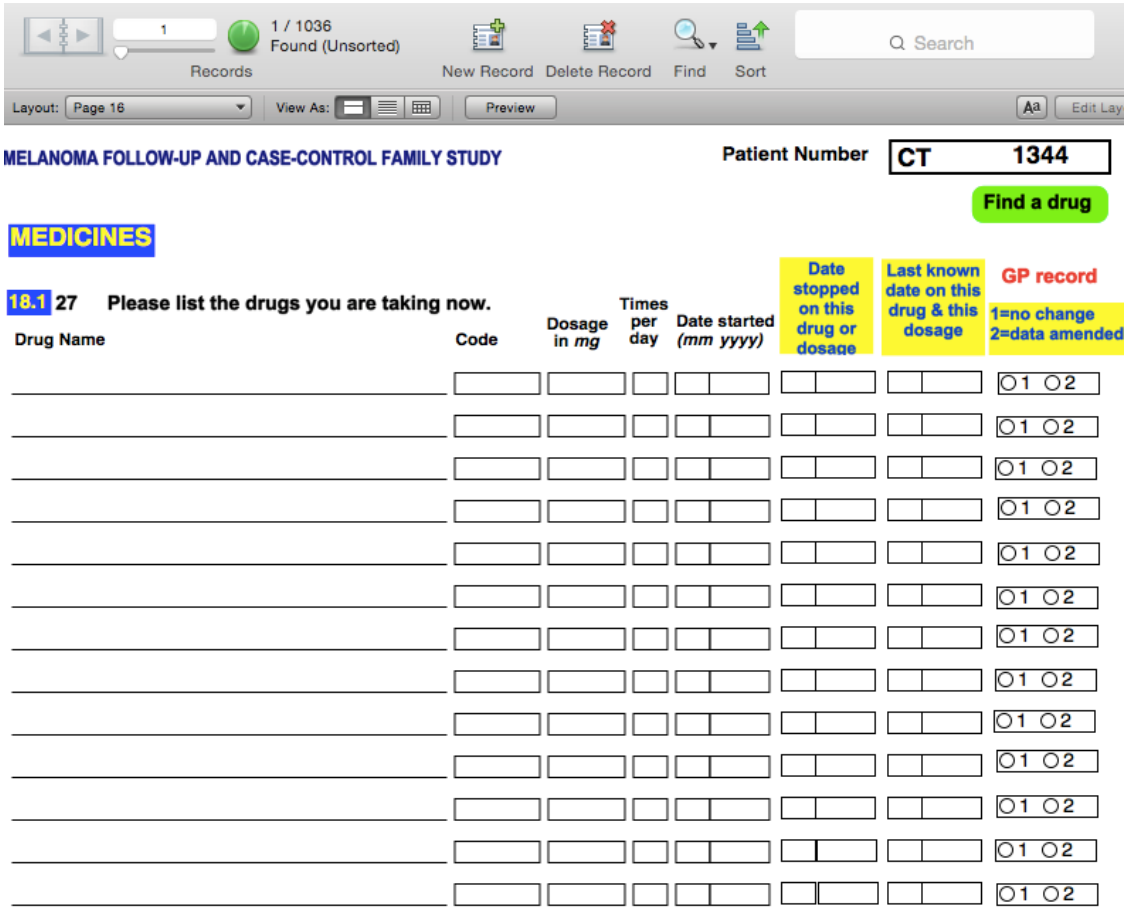
Chapter 8

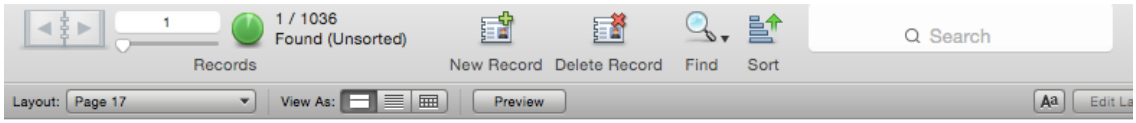
Appendices

Appendix A : Drug history information

A.1 Recording of drug history

Screen shots of drug history data recorded for the Leeds Melanoma Cohort.





MELANOMA FOLLOW-UP AND CASE-CONTROL FAMILY STUDY

Patient Number CT 1344

Find a drug

MEDICINES continued...

18.2 Were you taking any drug which was stopped in the last ten years? If so, could you please list those below?

Drug Name	Code	Dosage in *mg*	Times per day	Date started *(mm yyyy)*	Date stopped *(mm yyyy)*	GP record 1=no change 2=data amended
						○1 ○2
						○1 ○2
						○1 ○2
						○1 ○2
						○1 ○2
						○1 ○2
						○1 ○2
						○1 ○2
						○1 ○2
						○1 ○2
						○1 ○2
						○1 ○2
						○1 ○2

A.2 Extraction of specific drug information

Example of coding/searches needed to identify a drug in the cohort:

```
use "/Users/medfla/Documents/DRUG DATA/newest drug data/Faheem time dependent/masterdrug.dta", clear

tab code if regexm(code, "^2.1")

keep if regexm(code, "^2.1")

tab name

gen atorvastatinever = 1 if regexm(name, "[Aa]to") == 1 & regexm(name, "[Tt]in") == 1


tab atorvastatinever

*list atorvastatinever

replace atorvastatinever = 1 if name == "atvastatin"

replace atorvastatinever = 1 if name == "atovastatin"

replace atorvastatinever = 1 if name == "atrovastatin"

replace atorvastatinever = 1 if name == "artovastatin"

replace atorvastatinever = 1 if name == "atrovastatin"

replace atorvastatinever = 1 if name == "atorvastatn"

replace atorvastatinever = 1 if name == "atrovastatin"

replace atorvastatinever = 1 if name == "lipitor"

replace atorvastatinever = 1 if name == "lipator"


tab name atorvastatinever,m


drop if atorvastatinever == .


keep patientnumber atorvastatinever

duplicates drop

save "/Volumes/GED/Genetic-Epidemiology/John D_Faheem/updated data/atorvastatin.dta",replace
```

Appendix B : British National Formulation Classifications

B.1 Drug Grouping and Classification

B.1.1 Overview of Drug Groups (BNF)

1. Gastro-intestinal system
2. Cardiovascular system
3. Respiratory system
4. Central nervous system
5. Infections
6. Endocrine system
7. Obstetrics, gynaecology, and urinary-tract disorders
8. Malignant disease and immunosuppression
9. Nutrition and blood
10. Musculoskeletal and joint diseases
11. Eye
12. Ear, nose, and oropharynx
13. Skin
14. Immunological products and vaccines
15. Anaesthesia

B.1.2 Classification of drugs used

1 Sub-sections NSAIDs

2 Aspirin

3 Betablockers

4 Metformin

5 Immunosuppressants

6 Statins

7 ACE inhibitors

8 AIIR antagonists

9 Beta-adrenoceptor blocking drugs

10 Thiazides and related diuretics

11 Calcium-channel blockers

12 Thyroid hormones

13 Proton pump inhibitors

14 Loop diuretics

1 Sub-Sections NSAIDS

10.1.1 COX II selective and non-selective.

Selective Cox II inhibitors shown in bold

Aceclofenac

Acemetacin

Celecoxib

Dexibuprofen

Dexketoprofen

Diclofenac potassium

Diclofenac sodium

Etodolac

Etoricoxib

Fenoprofen

Flurbiprofen

Ibuprofen

Indometacin

Ketoprofen

Mefenamic acid

Meloxicam

Nabumetone

Naproxen

Piroxicam

Sulindac

Tenoxicam

Tiaprofenic acid

2 Aspirin

2.9 Antiplatelet drugs

2.10 Stable angina, acute coronary syndromes, and fibrinolysis

2.10.1 Management of stable angina and acute coronary syndromes

Central nervous system > **4.7** Analgesics > **4.7.1** Non-opioid analgesics and compound analgesic preparations >

Musculoskeletal and joint diseases > **10.1** Drugs used in rheumatic diseases and gout > **10.1.1** Non-steroidal anti-inflammatory drugs

3 Betablockers

SUB-SECTIONS- Cardio-selective and non-selective

Selective B blockers shown in bold

Propranolol hydrochloride

Acebutolol

Atenolol

Bisoprolol fumarate

Carvedilol

Celiprolol hydrochloride

Co-tenidone

Esmolol hydrochloride

Labetalol hydrochloride

Metoprolol tartrate

Nadolol

Nebivolol

Oxprenolol hydrochloride

Pindolol

Sotalol hydrochloride

Timolol maleate

4 Metformin

6.1.2.2

5 Immunosuppressants

8.2.1 Antiproliferative immunosuppressants

Azathioprine

Mycophenolate Mofetil

8.2.2 Corticosteroids and other immunosuppressants

Antithymocyte immunoglobulin (rabbit)

Basiliximab

Belatacept

Ciclosporin

Sirolimus

Tacrolimus

6 2.12

Statins

Atorvastatin

Fluvastatin

Pravastatin sodium

Rosuvastatin

Simvastatin

7 2.2.5.1

ACE inhibitors

Captopril

Cilazapril

Enalapril maleate

Fosinopril sodium

Imidapril hydrochloride

Lisinopril

Moexipril hydrochloride

Perindopril erbumine

Perindopril arginine

Quinapril

Ramipril

Ramipril with felodipine

Trandolapril

8 2.5.5.2

Angiotensin-II receptor antagonists

Azilsartan medoxomil

Candesartan cilexetil

Eprosartan

Irbesartan

Losartan potassium

Olmesartan medoxomil

Telmisartan

Valsartan

9 2.4

Beta-adrenoceptor blocking drugs

Propranolol hydrochloride

Acebutolol

Atenolol

Bisoprolol fumarate

Carvedilol

Celiprolol hydrochloride

Co-tenidone

Esmolol Hydrochloride

Labetalol hydrochloride

Metoprolol tartrate

Nadolol

Nebivolol

Oxprenolol hydrochloride

Pindolol

Sotalol hydrochloride

Timolol maleate

10 2.2.1

Thiazides and related diuretics

Bendroflumethiazide

Chlortalidone

Cyclopenthiazide

Indapamide

Metolazone

Xipamide

11 2.6.2

Calcium-channel blockers

Amlodipine

Diltiazem hydrochloride

Felodipine

Isradipine

Lacidipine

Lercanidipine hydrochloride

Nicardipine hydrochloride

Nifedipine

Nimodipine

Verapamil hydrochloride

12 6.2.1

Thyroid hormones

Levothyroxine sodium

Liothyronine sodium

13 1.3.5

Proton pump inhibitors

Esomeprazole

Lansoprazole

Omeprazole

Pantoprazole

Rabeprazole sodium

14 2.2.2

Loop diuretics

Bumetanide

Furosemide

Torasemide

Appendix C : Drug Exposure Analyses

Extract of results obtained with time dependent analysis that was subsequently abandoned

C.1 Aspirin

Cumulative aspirin exposure: Unadjusted; adjusted for age and sex; adjusted for age, sex and Breslow

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
cumdrug	1.002869	.0013732	2.09	0.036	1.000181	1.005563

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
age	1.037958	.0055979	6.91	0.000	1.027044	1.048987
_Igender_2	1.468308	.1804819	3.12	0.002	1.153955	1.868295
cumdrug	.9991061	.0014791	-0.60	0.546	.9962114	1.002009

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
age	1.03384	.0056364	6.10	0.000	1.022852	1.044947
_Igender_2	1.497203	.1859453	3.25	0.001	1.173723	1.909835
breslow	1.243474	.0180753	14.99	0.000	1.208547	1.27941
cumdrug	.9995861	.0014776	-0.28	0.779	.9966943	1.002486

Adjusted for age, sex, Breslow and smoking; adjusted for age, sex, Breslow, smoking and serum Vitamin D.

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
cumdrug	.9992314	.0015051	-0.51	0.610	.9962859	1.002186
age	1.033625	.0056476	6.05	0.000	1.022615	1.044753
_Igender_2	1.490869	.1863447	3.20	0.001	1.166937	1.904723
breslow	1.240322	.0181413	14.72	0.000	1.20527	1.276393
smoke	1.139143	.1421975	1.04	0.297	.8919167	1.454898

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
cumdrug	.9992929	.0015776	-0.45	0.654	.9962056	1.00239
age	1.032445	.0057853	5.70	0.000	1.021168	1.043847
_Igender_2	1.55264	.2018645	3.38	0.001	1.20338	2.003266
breslow	1.230433	.0190312	13.41	0.000	1.193693	1.268305
smoke	1.183003	.154141	1.29	0.197	.9163842	1.527195
_Ivitgrp2_2	1.700398	.3971338	2.27	0.023	1.075844	2.687519
_Ivitgrp2_3	.8884383	.1440265	-0.73	0.466	.6466023	1.220723
_Ivitgrp2_4	.6634449	.2761461	-0.99	0.324	.2934328	1.500034
_Ivitgrp2_5	.6020845	.4291659	-0.71	0.477	.148909	2.434412

Adjusted for age, sex, Breslow, smoking and ulceration; adjusted for age, sex, Breslow, smoking, serum Vitamin D and presence of diabetes

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
cumdrug	.9994642	.0015074	-0.36	0.722	.9965141	1.002423
age	1.029503	.0056722	5.28	0.000	1.018446	1.040681
_Igender_2	1.414238	.1778769	2.76	0.006	1.105253	1.809602
breslow	1.186658	.020469	9.92	0.000	1.14721	1.227462
smoke	1.134085	.1415172	1.01	0.313	.8880315	1.448315
_Iprimary_u_2	4.770951	4.793008	1.56	0.120	.6659907	34.17761
_Iprimary_u_3	.6064809	.1466097	-2.07	0.039	.3776149	.9740587
_Iprimary_u_4	2.018528	.2894234	4.90	0.000	1.524006	2.673517

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
cumdrug	.9993194	.001593	-0.43	0.669	.9962022	1.002446
age	1.032954	.0058754	5.70	0.000	1.021503	1.044534
_Igender_2	1.569176	.2040601	3.46	0.001	1.216127	2.024717
breslow	1.231558	.0190957	13.43	0.000	1.194694	1.269559
_Ivitgrp2_2	1.674107	.3981014	2.17	0.030	1.050433	2.668077
_Ivitgrp2_3	.9034783	.1469597	-0.62	0.533	.6568427	1.242722
_Ivitgrp2_4	.6652947	.2770603	-0.98	0.328	.2941259	1.504856
_Ivitgrp2_5	.6234577	.4444607	-0.66	0.507	.154166	2.521305
diabetes_secD	1.378707	.3543874	1.25	0.212	.8330599	2.281748

Adjusted for age, sex, Breslow, smoking, serum Vitamin D, presence of diabetes and BMI

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
cumdrug	.9994516	.0015926	-0.34	0.731	.9963351	1.002578
age	1.031288	.0060117	5.29	0.000	1.019572	1.043138
_Igender_2	1.589049	.2085173	3.53	0.000	1.228688	2.055101
breslow	1.237591	.0196066	13.46	0.000	1.199753	1.276622
_Ivitgrp2_2	1.667671	.3969493	2.15	0.032	1.045929	2.659002
_Ivitgrp2_3	.8956659	.1469853	-0.67	0.502	.6493184	1.235476
_Ivitgrp2_4	.6933351	.2887615	-0.88	0.379	.3065019	1.568387
_Ivitgrp2_5	.6198538	.4422072	-0.67	0.503	.1531219	2.509234
diabetes_secD	1.41735	.3764407	1.31	0.189	.8421755	2.385349
bmi	.9935757	.0133469	-0.48	0.631	.9677577	1.020082

5 year aspirin exposure: Unadjusted; adjusted for age and sex

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
catdrug5yr	1.619454	.3951735	1.98	0.048	1.003832	2.612617

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
age	1.036967	.0054622	6.89	0.000	1.026316	1.047728
_Igender_2	1.453161	.1784737	3.04	0.002	1.142276	1.848656
catdrug5yr	1.03979	.2580813	0.16	0.875	.6392536	1.691291

10 year aspirin exposure: Unadjusted; adjusted for age and sex

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
catdrug10yr	1.592564	.719186	1.03	0.303	.6572082	3.859144

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
age	1.037071	.0054262	6.96	0.000	1.02649	1.04776
_Igender_2	1.455519	.178109	3.07	0.002	1.145139	1.850026
catdrug10yr	1.017031	.4615439	0.04	0.970	.4178747	2.475268

C.2 Simvastatin

Cumulative simvastatin exposure: Unadjusted; adjusted for age and sex; adjusted for age, sex, Breslow and smoking.

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
cumdrug	1.003676	.0020211	1.82	0.068	.9997224	1.007645

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
age	1.037344	.0054851	6.93	0.000	1.026649	1.048151
_Igender_2	1.461155	.1795945	3.09	0.002	1.148346	1.859173
cumdrug	.9993923	.002189	-0.28	0.781	.9951112	1.003692

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
cumdrug	.9997185	.0022131	-0.13	0.899	.9953903	1.004066
age	1.033023	.0055483	6.05	0.000	1.022206	1.043955
_Igender_2	1.48374	.1854597	3.16	0.002	1.161347	1.89563
breslow	1.240736	.0181483	14.75	0.000	1.205671	1.276821
smoke	1.136093	.1418061	1.02	0.307	.889544	1.450975

Adjusted for age, sex, Breslow, smoking and serum Vitamin D.

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
cumdrug	.9992936	.0023682	-0.30	0.766	.9946627	1.003946
age	1.032093	.0056847	5.74	0.000	1.021011	1.043295
_Igender_2	1.549567	.201483	3.37	0.001	1.200971	1.999347
breslow	1.230791	.0190062	13.45	0.000	1.194097	1.268612
smoke	1.181658	.1539599	1.28	0.200	.9153512	1.525444
_Ivitgrp2_2	1.697801	.3964968	2.27	0.023	1.074239	2.683321
_Ivitgrp2_3	.8860594	.1435344	-0.75	0.455	.6450229	1.217168
_Ivitgrp2_4	.6627992	.2758976	-0.99	0.323	.2931297	1.498664
_Ivitgrp2_5	.5976682	.4260147	-0.72	0.470	.1478183	2.416529

5 year simvastatin exposure: Unadjusted; adjusted for age and sex

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
catdrug5yr	1.619454	.3951735	1.98	0.048	1.003832	2.612617

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
age	1.036967	.0054622	6.89	0.000	1.026316	1.047728
_Igender_2	1.453161	.1784737	3.04	0.002	1.142276	1.848656
catdrug5yr	1.03979	.2580813	0.16	0.875	.6392536	1.691291

Adjusted for age, sex and Breslow

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
age	1.033188	.0055153	6.12	0.000	1.022434	1.044054
_Igender_2	1.487382	.1845834	3.20	0.001	1.166243	1.896952
breslow	1.243945	.0180845	15.02	0.000	1.209001	1.2799
catdrug5yr	1.079883	.2687548	0.31	0.757	.663032	1.758809

10 year aspirin exposure: Unadjusted; adjusted for age and sex

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
catdrug10yr	1.592564	.719186	1.03	0.303	.6572082	3.859144

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
age	1.037071	.0054262	6.96	0.000	1.02649	1.04776
_Igender_2	1.455519	.178109	3.07	0.002	1.145139	1.850026
catdrug10yr	1.017031	.4615439	0.04	0.970	.4178747	2.475268

C.3 Effects of drugs on cancer and specifically melanoma

Example of literature evidence summary collected for each drug group based on previous reports of effects in cancer and melanoma

BNF Drug Groups	Cancer Effect	Melanoma Effect	Protective or Negative Effect	References
1 Gastro-intestinal system				
1.1 Dyspepsia and gastro-oesophageal reflux disease				
Antacids and simeticone	?Possible Weak effect	none reported	NA	Cruse, P., M. R. Lewin, et al. (1980). "Aluminium hydroxide and colon cancer." Lancet 2(8202): 1030. Cruse, J. P., M. R. Lewin, et al. (1981). "Experimental evidence against the bile salt theory of colon carcinogenesis." Eur Surg Res 13(2): 117-124. The results provide reassurance that Aludrox (aluminum hydroxide) does not promote colon cancer and tend to contradict the bile salt theory of colon carcinogenesis.
ALUMINIUM HYDROXIDE				
MAGNESIUM CARBONATE				
MAGNESIUM TRISILICATE				
1.2 Antispasmodics and other drugs altering gut motility	none reported	none reported	NA	
1.3 Antisecretory drugs and mucosal protectants				
H2-receptor antagonists	Effect reported	none reported	Protective effect	Hahm, K. B., I. S. Park, et al. (1996). "Comparison of antiproliferative effects of 1-histamine-2 receptor antagonists, cimetidine, ranitidine, and famotidine, in gastric cancer cells." Int J Immunopharmacol 18(6-7): 393-399.the immune system, histamine is known to suppress cytotoxic T-lymphocytes and nitrogen induced lymphocyte thymidine uptake, down-regulate some cytokines, and activate suppressor T-lymphocytes, and in the gastrointestinal system, histamine was reported to have trophic effects on gastrointestinal epithelial cells. Enhanced rates of cell proliferation by histamine are implicated in the pathogenesis. Beneficial clinical outcomes could be anticipated from cimetidine treatment in patients with gastric cancer by anti-proliferating effects against gastric cancer cells. These effects of H2-RA are likely to be mediated by specific interactions at the H2-receptor.
CIMETIDINE				
FAMOTIDINE				
NIZATIDINE				
RANITIDINE				
Prostaglandin analogues				Lawson, J. A., W. J. Adams, et al. (1994). "The effect of misoprostol on colon cancer." Aust N Z J Surg 64(3): 197-201. A synthetic prostaglandin E1 (PGE1) analogue, misoprostol, was investigated for its effects on the growth of colon cancer in two in vivo models. . Oral administration of misoprostol has an inhibitory effect on early tumour growth of some colonic cancers, but not on established tumours. Basso, N., A. Materia, et al. (1992). "Time-related interference of misoprostol with experimental gastric cancer formation induced by N-methyl-N'-nitro-N-nitrosoguanidine in the rat." J Cancer Res Clin Oncol 118(6): 441-446. -Exogenous prostaglandins are able to prevent the early MNNG-induced gastric mucosal lesions, thus interfering with gastric carcinogenesis.
MISOPROSTOL	Effect reported	none reported	Protective effect	
Proton pump inhibitors	Effect Reported	none reported	Protective and negative effects reported	Poulsen, A. H., S. Christensen, et al. (2009). "Proton pump inhibitors and risk of gastric cancer: a population-based cohort study." Br J Cancer 100(9): 1503-1507. - Proton pump inhibitor (PPI) use leads to hypergastrinaemia, which has been associated with gastrointestinal neoplasia. We evaluated the association between PPI use and risk of gastric cancer using population-based health-care registers in North Jutland, Denmark, during 1990-2003. . These estimates are in contrast to significant overall IRRs of 9.0 and 2.8, respectively, without the lag time. In lag time analyses, increased IRRs were observed among PPI users with the largest number of prescriptions or the longest follow-up compared with H2RA users or non-users. Although our results point to a major influence of reverse causation and confounding by indication on the association between PPI use and gastric cancer incidence, the finding of increased incidence among PPI users with most prescriptions and longest follow-up warrants further investigation. Ohta, T., H. Arakawa, et al. (1996). "[A new strategy for the therapy of pancreatic cancer by proton pump inhibitor]." Gan To Kagaku Ryoho 23(12): 1660-1664. Bafilomycin A1 is a specific inhibitor of vacuolar type proton pump (V-ATPase). This study was designed to examine the effect of bafilomycin A1 on the growth of Capan-1 human pancreatic cells which overexpress V-ATPase. Nude mice bearing a xenografted tumor of Capan-1 cell line were treated for 4 weeks with bafilomycin A1 (1.0 mg/kg/day). This treatment inhibited tumor growth, which was significantly reduced as compared with controls after 21 days ($p < 0.05$). However, there were no significant differences in body weights between groups. Microscopically, a large number of tumor cells in the treated group showed signs of apoptosis. These findings suggest that apoptosis induced by bafilomycin A1 was the event involved in suppression of tumor growth in vivo.
ESOMEPRAZOLE				
LANSOPRAZOLE				
OMEPRAZOLE				
PANTOPRAZOLE				
RABEPRAZOLE SODIUM				

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