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Molecular characterization of the immune microenvironment in melanoma

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About the Author

Rita started her studies in Health Sciences at the University of Lisbon, Portugal, where she completed her Master's in Molecular Biology and Genetics, in 2015. She spent the last year of the Master's in Rome, Italy, developing her thesis at the European Molecular Biology Laboratory (EMBL). Soon after, she was selected for a Marie Skłodowska-Curie fellowship, to be part of MELGEN European Training Network and thus, join a PhD position at Lund University, under the supervision of Göran Jönsson. Her PhD projects, now included in this thesis, involved the characterisation of different aspects of the tumour immune microenvironment in melanoma, representing a knowledge contribution that, in the future, might help improve the clinical management of patients in advanced stages of the disease.

In her spare time, Rita is passionate about cooking, travelling and spending time with her loved ones.

FACULTY OF MEDICINE

LUND UNIVERSITY

Molecular characterization of the immune microenvironment in melanoma

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Title and subtitle
Molecular characterization of the immune microenvironment in melanoma

Abstract

Malignant melanoma is the most lethal form of skin cancer and its incidence has increased drastically, with its clinical management lacking efficient options until recent years. The scenario changed dramatically in the last decade with the introduction of immunotherapy agents in the treatment of advanced melanoma, particularly immune checkpoint inhibitors. Antibodies inhibiting CTLA-4 and PD-1/PD-L1 signalling have been approved, as monotherapies or in combination, after showing great improvement in patient survival. Nevertheless, the response rates are still insufficient, with a high fraction of the patients showing resistance or developing severe immune adverse effects.

Due to the fact that melanoma is a highly immunogenic disease, it has been under the scope of research studies on the tumour immune microenvironment in order to discover predictive biomarkers for therapeutic agents. Despite these efforts, the mechanisms of sensitivity and resistance to treatment remain poorly understood.

The work included in this thesis explored some aspects of the tumour microenvironment and made important discoveries that could potentially be applied in the future to efficiently help predict responses to immune checkpoint inhibitors.

A specific type of lymphoid aggregates, tertiary lymphoid structures, known to play an important role in chronic inflammation, was documented to develop in several types of human tumours, including melanoma. In Paper I, we show that the presence of TLSs in the tumours, improves not only the patient survival and outcome, but it also confers better responses to immune checkpoint inhibitors.

Resistance to immunotherapy is developed due to the activation of mechanisms of immune evasion by the tumour. Loss of PTEN and low expression of MITF in melanoma tumours have a role in the initiation of these mechanisms. In Paper II, we show that the concomitant loss of PTEN and MITF, leads to a worse patient survival and development of resistance to therapy.

The immune landscape of a tumour is a fingerprint of its immune microenvironment. Thus, more effective methods to characterize it are currently being studied. In Papers III and IV, we show that two not extensively explored methods, are effective in distinguishing different tumour microenvironments. We were able to group tumours according to their level of DNA methylation and this grouping was differentially associated with the level of immune infiltration, activation of immune evasion mechanisms and prognosis. We also show that immune cell signatures derived based on single-cell RNA-seq data, have a prognostic value, both alone and when coupled with TMB, in treatment naïve and treated melanoma cohorts, showing their potential to be used as biomarkers.

Overall, our data represents a knowledge contribution that in the future, could allow the discovery of new biomarkers for the treatment of melanoma. This, combined with the discovery of technological tools, might improve the clinical management of patients in advanced stages, and allow an implementation of an optimized personalized medicine.

Key words
Melanoma, immune microenvironment, tertiary lymphoid structures, immunotherapy, immune evasion, methylation, immune gene signatures.

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Molecular characterization of the immune microenvironment in melanoma

Rita Cabrita

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To my magic three: Ilda, Francisco and Frederico

"If I have seen further, it is by standing upon the shoulders of giants."
Isaac Newton

"É o tempo da travessia:
E, se não *ousarmos fazê-la,*
teremos ficado,
para sempre,
à margem de nós mesmos."
Fernando Pessoa

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Nature. 2020, 577(561–565).

II. The role of PTEN loss in immune escape, melanoma prognosis and therapy response.

Cabrita R, Mitra S, Sanna A, Ekedahl H, Lövgren K, Olsson H, Ingvar C, Isaksson K, Lauss M, Carneiro A and Jönsson G.

Cancers. 2020, 12*(3), 742.*

III. Analysis of DNA methylation-based tumour immune microenvironment patterns in metastatic melanoma.

Mitra S, Lauss M, **Cabrita R**, Choi J, Zhang T, Isaksson K, Olsson H, Ingvar C, Carneiro A, Staaf J, Ringnér M, Nielsen K, Brown KM and Jönsson G.

Molecular Oncology. 2020, doi: 10.1002/1878-0261.12663 (Published online).

IV. Distinct transcriptional signatures derived from single-cell RNA sequencing data predict patient prognosis and response to immune checkpoint blockade in melanoma.

Mitra S, **Cabrita R**, Harbst K, Lauss M and Jönsson G.

Manuscript.

List of papers not included in the thesis

I. The X-linked DDX3X RNA helicase dictates translation reprogramming and metastasis in melanoma.

Phung B, Ciesla M, Sanna A, Guzzi N, Beneventi G, Cao Thi Ngoc P, Lauss M, **Cabrita R**, Cordero E, Bosch A, Rosengren F, Häkkinen J, Griewank K, Paschen A, Harbst K, Olsson H, Ingvar C, Carneiro A, Tsao H, Schadendorf D, Pietras K, Bellodi C and Jönsson G.

Cell Reports. 2019, 27(12), p. 3573-3586.e7.

II. Effects of promoter hypermethylation of melanocyte lineage genes on melanoma phenotype.

Sanna A, Mitra S, Phung B, Lauss M, Choi J, Zhang T, Njauw C, Cordero E, Harbst H, Rosengren F, **Cabrita R**, Johansson J, Isaksson K, Olsson H, Ingvar C, Carneiro A, Andersson M, Tsao H, Brown K, Pietras K and Jönsson G.

Manuscript.

Abbreviations

ACT	Adoptive cell transfer
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of variance
APC	Antigen-presenting cells
AKT	AKT serine/threonine kinase 1
BC	Before Christ
BRAFi	BRAF inhibitor
B2M	Beta-2-Microglobulin
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CDKN2A	CDK inhibitor 2A
cDNA	Complementary DNA
CNS	Central nervous system
CpG	5'—C—phosphate—G—3' (cytosine-guanine)
cRNA	Complementary RNA
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTNNB1	Catenin beta 1
CXCL13	C-X-C motif chemokine ligand 13
CXCR5	C-X-C motif chemokine receptor 5
DC	Dendritic cells
DC-LAMP	Dendritic cell lysosomal associated membrane glycoprotein
DMFS	Disease metastatic free survival
DNA	Deoxyribonucleic acid
EDNRB	Endothelin receptor type B
FDA	Food and Drug Administration

FDR	False Discovery Rate
FPKM	Fragments per kilobase of transcript per million mapped reads
GC	Germinal centre
GEO	Gene Expression Omnibus
GEX	Gene expression
HISAT	Hierarchical Indexing for Spliced Alignment of Transcripts
HLA-DR	Human leukocyte antigen – DR isotype
ICI	Immune checkpoint inhibitor
IGLL1/5	Immunoglobulin lambda like polypeptide 1/5
IHC	Immunohistochemistry
IL-2	Interleukin-2
IF	Immunofluorescence
IFN	Interferon
KIT	Receptor tyrosine kinase
MAPK	Mitogen-activated protein kinase
MPC	Microenvironment Cell Populations
MEK	Mitogen-activated protein kinase kinase
MEKi	MEK inhibitor
MHC	Major histocompatibility complex
MITF	Microphthalmia-associated transcription factor
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MYC	Master Regulator of Cell Cycle Entry and Proliferative Metabolism
NCBI	National Center for Biotechnology Information
NK	Natural killer
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PFS	Progression free survival
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
RB	Retinoblastoma

RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
ROI	Region of interest
RPKM	Reads per kilobase of transcript per million mapped reads
SAM	Significant analysis for microarray
SCNA	Somatic copy number aberration
SOX10	Sry-related HMg-Box gene 10
TCGA	The Cancer Genome Atlas
TCR	T-cell receptor
TERT	Telomerase reverse transcriptase
TIL	Tumour infiltrating lymphocyte
TLS	Tertiary lymphoid structure
TMA	Tissue microarray
TMB	Tumour mutational burden
TME	Tumour microenvironment
TIME	Tumour immune microenvironment
TPM	Transcripts per million
TP53	Tumour protein 53
Treg	Regulatory T-cells
SLN	Sentinel lymph node
UV	Ultraviolet
VST	Variance stabilizing transformation

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Every adventure begins with a big step. Sometimes it is not easy to take those steps with certainty. Four years ago, it took me some time to decide whether a PhD was the right step for me to take. When I got to know you, **Göran**, and the group you had built here in Lund, the certainty I lacked finally arrived and I knew I had to go for it and embrace this adventure. Thank you for believing in me (among so many other people), for teaching me so much and always being there for me.

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Popular science summary

Melanoma is, as any type of cancer, a complex disease in which different types of cells interact with each other to promote the growth of a tumour originated from the transformation of normal cells. Together with the cancer cells, non-transformed cells, such as immune cells, blood and lymphatic vessels and fibroblasts, are the main components of the tumour microenvironment. Among the many interactions that take place in this tumour microenvironment, the crosstalk between cancer and immune cells has been one of the main focuses of the scientific community for many years.

Melanoma is particularly rich in events involving the interaction of tumour and immune cells, meaning it is a highly "immunogenic" disease, and thus a good source for studying this topic. For this reason, melanoma is one of the types of cancer that responds positively to therapeutic strategies involving the empowerment of a patient's immune system, the so-called immunotherapy. Up to date, advanced melanoma had no treatment and patients would succumb shortly after diagnosis. Although immunotherapy treatment has been revolutionary in the field of cancer treatment and, as mentioned, in advanced melanoma, and it has shown great improvement and outcome in some of the patients, many others still suffer either from severe side effects or from resistance to the treatment. As such, there is a compelling need to develop tools to effectively identify and select the patients who may benefit from the different available immunotherapy treatment options.

A detailed knowledge about the mechanisms taking place at the tumour immune microenvironment will, in the future, open possibilities for the development of such tools.

This thesis explored some aspects and made some important discoveries related to the tumour microenvironment in melanoma.

Herein, it is shown that specific aggregates of immune cells which form in chronic inflammation sites in the body, and that have also been documented to develop in human cancer, are present in melanoma. More importantly, the presence of these

aggregates, called tertiary lymphoid structures, in the tumour is beneficial, since it improves not only the patient survival and outcome, but also confers better responses to immunotherapy agents. As such, the enhancement of TLS formation and function could be considered as a therapeutic approach in melanoma patients in the future.

During the crosstalk between cancer and immune cells, cancer cells have different strategies to evade the immune responses activated against them. These strategies are also partly responsible for the resistance to immunotherapy observed in a fraction of the patients. The loss of different genes, such as the PTEN gene, is known to be responsible for the initiation of these mechanisms of evasion and consequent resistance to therapy. Moreover, a decrease of MITF expression, an important gene for melanoma development, has also been shown to be initiating mechanisms of evasion of the immune system. In this work it is shown that the loss of PTEN and MITF proteins in combination leads to a worse patient survival and to the development of resistance to therapy. This stands for the possibility of including the reactivation of MITF in therapeutic approaches involving PTEN.

Having a detailed knowledge about the specific immune cells present in a tumour could help understand the crosstalk mechanisms happening in the tumour microenvironment. Herein, it is shown that two methods to distinguish different tumour microenvironments which have not been extensively studied could be used for this purpose. These methods were effective in both defining tumour groups with different immune characteristics and in predicting the prognosis of patients before and after being treated with immunotherapy agents.

Taken together, the work included in this thesis addresses some aspects of the tumour immune microenvironment in melanoma. Our data can then represent a knowledge contribution that in the future, combined with technological advancement, might improve the clinical management of patients in advanced stages of the disease, and reach an optimized personalized medicine.

Resumo em Português

O melanoma, tal como qualquer outro tipo de cancro, é uma doença complexa, na qual diferentes tipos de células interagem entre si. Esta interação tem como objetivo promover o crescimento de um tumor resultante da transformação de células do organismo. Em conjunto com as células cancerígenas, células não transformadas, tais como células do sistema imunitário, fibroblastos e vasos sanguíneos e linfáticos são os principais componentes do microambiente tumoral. Entre as muitas interações que ocorrem neste ambiente, a relação entre células tumorais e células imunes tem vindo a ser um dos maiores tópicos de investigação por parte da comunidade científica nos últimos anos.

O melanoma é particularmente rico em eventos que envolvem a interação entre células tumorais e células imunes, sendo por isso extremamente "imunogénico". Daí que os tumores de melanoma representem uma boa ferramenta para o estudo deste tópico. Por ter estas características, o melanoma é um dos tipos de cancro que responde de forma positiva a estratégias terapêuticas que têm como base o fortalecimento do sistema imunitário dos doentes, a já conhecida imunoterapia. Anteriormente, o melanoma em estadio avançado não tinha qualquer possibilidade de tratamento e os doentes sucumbiam pouco depois do diagnóstico.

Apesar de a imunoterapia ter sido revolucionária na área do tratamento de cancro, e em particular no tratamento do melanoma avançado, e de ter levado a uma melhoria considerável em muitos doentes, muitos outros ainda desenvolvem efeitos secundários severos ou mesmo resistência aos tratamentos, nunca chegando a responder aos mesmos. Como tal, há uma necessidade urgente de desenvolver ferramentas eficazes na identificação e seleção de doentes que beneficiem das diferentes opções terapêuticas disponíveis. No futuro, um conhecimento aprofundado sobre os mecanismos que ocorrem ao nível do microambiente tumoral poderá aumentar as possibilidades de um desenvolvimento dessas mesmas ferramentas.

Esta tese explora alguns aspetos relacionados com o microambiente tumoral em melanoma e fez descobertas que podem ser consideradas importantes nesta área.

Com este trabalho demonstra-se que agregados de células imunes que habitualmente se formam em locais de inflamação no organismo, e que ocorrem em diversos tumores humanos, estão presentes também no melanoma. A presença destes agregados, denominados estruturas linfóides terciárias, no tumor, é benéfica, uma vez que representa não só uma melhoria da sobrevivência e prognóstico do doente, mas também melhores respostas a agentes imunoterapêuticos. Como tal, futuramente, um aumento da formação de estruturas linfóides terciárias funcionais poderá ser considerado como uma abordagem terapêutica em doentes com melanoma.

Durante a interação entre células tumorais e células imunes, as células tumorais têm a capacidade de escapar às respostas imunes que são despoletadas contra si. Esta capacidade pode ocorrer de diversas formas e é responsável pela resistência aos agentes terapêuticos observada em muitos doentes. A perda de genes específicos, tal como o gene PTEN, é muitas vezes responsável pelo desencadeamento destes mecanismos de evasão do sistema imunitário e consequente resistência à terapêutica. Além disso, sabe-se que a diminuição da expressão de MITF, um importante gene no desenvolvimento de melanoma, também é responsável pelo despoletar desses mesmos mecanismos. Neste trabalho constata-se que a perda concomitante das proteínas PTEN e MITF representa um pior prognóstico e sobrevivência do doente, bem como um desenvolvimento da resistência à imunoterapia. Como tal, a reativação de MITF em abordagens terapêuticas que envolvem PTEN poderá constituir uma possibilidade futura.

Um conhecimento detalhado sobre o tipo de células imunes que estão presentes num tumor pode auxiliar no entendimento dos mecanismos de interação que ocorrem no microambiente tumoral. Neste trabalho comprova-se que dois métodos para distinguir diferentes microambientes tumorais, que até hoje não foram particularmente aprofundados, poderiam ser usados para este efeito. Estes métodos demonstraram ser eficazes na definição de grupos de tumores com diferentes características imunes, e também no prognóstico de doentes antes e depois de receberem tratamento com agentes imunoterapêuticos.

Genericamente, o trabalho incluído nesta tese explora alguns aspetos relativos ao microambiente tumoral. Os dados aqui apresentados representam um conhecimento que, no futuro, em conjunto com avanços ao nível tecnológico, pode contribuir para aperfeiçoar a gestão clínica de doentes em estadios avançados da doença, e conseguir uma medicina personalizada.

Abstract

Malignant melanoma is the most lethal form of skin cancer and its incidence has increased drastically, with its clinical management lacking efficient options until recent years. The scenario changed dramatically in the last decade with the introduction of immunotherapy agents in the treatment of advanced melanoma, particularly immune checkpoint inhibitors. Antibodies inhibiting CTLA-4 and PD-1/PD-L1 signalling have been approved, as monotherapies or in combination, after showing great improvement in patient survival. Nevertheless, the response rates are still insufficient, with a high fraction of the patients showing resistance or developing severe immune adverse effects.

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Overall, our data represents a knowledge contribution that in the future, could allow the discovery of new biomarkers for the treatment of melanoma. This, combined with the discovery of technological tools, might improve the clinical management of patients in advanced stages, and allow an implementation of an optimized personalized medicine.

The history of cancer

Cancer has afflicted humanity since pre-historic times. The oldest evidence of cancer in mammals consists of tumours found in fossils of dinosaurs and human bones. Human cancer was described for the first time in Egyptian manuscripts written between 1500 and 1600 BC and discovered in the 19th century. The Edwin Smith papyrus, as it was called later in time, is today thought to have been written by Imhotep, a physician-architect. This manuscript describes cases of breast neoplasms, mentioning that when such tumours had spread over the breast, no treatment could succeed. The earliest cancer cases in humans were found in Egyptian and Peruvian mummies dating back to 1500 BC.

Following the decline of the Egyptian and Greek empires, Roman medicine became dominant, especially with Hippocrates (460-370 BC), the well-known "Father of Medicine". Until today, he is credited with the origin of the word "cancer", by using the terms *carcinos* and *carcinoma* (in Greek, referring to crab) to describe different forms of tumours (non-ulcer or ulcer forms, respectively). The term *cancer* was established later on when the Roman physician Celsus (50-28 BC), an avid successor of Hippocrates, translated the word "crab" to Latin.

One of the first theories about the cause of cancer was proposed by Hippocrates. He believed health or disease were determined by the balance or imbalance of the four body fluids or *humours* (blood, black bile, yellow bile and phlegm) linked to different organs. The imbalance of these humours dictated a propensity towards certain diseases. He defended that for instance, cancer was a consequence of an imbalance and excess of black bile in the body.

Only in the 16th century did Hippocrates' black bile theory start to be questioned and new hypotheses were created. This happened as a consequence of a more extensive knowledge of the physical attributes of cancer, namely the distinction between benign and malignant tumours. With that, the era of cancer pathology had started. Giovanni Morgagni di Padua was the first one using autopsies to correlate patients' illness to pathological findings after death. Later on, the famous Scottish

surgeon John Hunter (1728-1793) defended that tumours that had not spread to nearby tissues, could be surgically removed.

The discovery of anaesthesia in 1842 by Crawford W. Long, together with refinements in surgical techniques and the development of antibiotics and anaesthetic agents, made surgery the early-stage cancer management primary choice and greatly increased the cure rates. The discovery of X-rays, and of chemical atoms like radium and polonium, by Pierre and Marie Curie, marked the era of modern diagnostic, therapeutic radiology and nuclear medicine, raising expectations that cancer treatment was soon to be achieved.

In the beginning of the 20th century, the establishment of innovative research tools enabled medical researchers to explore more efficiently the origin of cancer. This allowed, among other discoveries, the emergence of the connection between environmental agents, industrial products and consumer products, such as radon, asbestos and tobacco, respectively, and cancer. These started later being called *carcinogenic agents*.

During World War II, a compound called nitrogen mustard, commonly known as mustard gas, used during military actions, was by chance found to work against lymphoma. This agent served then as the model for alkylating agents that killed rapidly growing cancer cells by damaging their DNA. Not long after the discovery of nitrogen mustard, a paediatric pathologist in Sidney demonstrated that an agent called aminopterin produced remissions in acute leukaemia. This was then the predecessor of methotrexate, a cancer treatment drug commonly used today. Since then, other researchers developed many drugs specialized in blocking different functions in cell growth and replication. The era of chemotherapy had begun.**Fel! Hittar inte referenskälla.**

Melanocytes

Development

Melanocytes are specialized cells responsible for the production of the human organism's pigment - the melanin. These pigment producing cells originate from embryonic cells named neural crest cells. Melanocytes do not exist only in the outer layer of the skin, known as epidermis, in the hair and iris, but also in more unlikely locations, such as the inner ear, the nervous system and the heart[2,3]. Initially, melanocyte precursor cells, the melanoblasts, proliferate and migrate over large distances through the embryo. Simultaneously, these cells begin to express specific melanocytic markers, such as receptor tyrosine kinase (KIT), endothelin receptor type B (EDNRB) and the transcription factors Sry-related HMg-Box gene 10 (SOX-10) and microphthalmia-associated transcription factor (MITF). The expression of these markers dictates not only the survival, proliferation and migration of the embryonic melanoblasts, but more importantly their cell fate into mature melanocytes[4].

Function

Melanocytes reside in the deepest layer of the epidermis, the basal layer, in a symbiotic relationship with a pool of associated keratinocytes, the predominant type of cell in the epidermis. This symbiosis represents structures called epidermal-melanin units represented in Figure 1. These structures are responsible for the transfer of melanin into the keratinocytes, allowing them to perform two of their principal functions: thermoregulation and photoprotection[5]. The melanin is transported in organelles called melanosomes via the elongated dendrites of the melanocytes[6] and functions as a protective barrier against the harmful ultraviolet (UV) radiation, ultimately avoiding DNA damage[7]. Upon UV radiation exposure, a sequential response is activated in the keratinocytes in which several transcriptional

factors participate, stimulating the melanocytes to produce melanin and leading to the commonly known *tanning*[8].
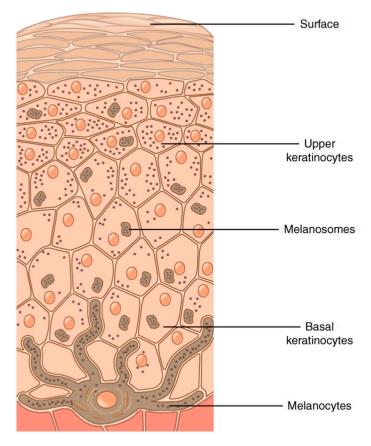

Figure 1. Schematic representation of an epidermal-melanin unit.
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Melanoma

Melanoma is a cancer arising from the malignant transformation of melanocytes. The majority of the cases derive from melanocytes localized in the skin. Behind the development of this cancer there are genetic factors, such as the presence of dysplastic nevi, familiar history of melanoma and skin phenotype, and environmental factors, such as UV exposure[9]. Regarding environmental factors, a higher risk is associated with intense intermittent ultraviolet radiation exposure, severe sunburns during the childhood[10,11] and the use of artificial tanning devices, such as sunbeds[12].

Melanoma is one of the most aggressive cancers, accounting for only 4% of all skin cancers, but being responsible for 75% of deaths caused by these malignancies[13]. This aggressiveness is due to a high invasive and metastatic potential.

Development

Initiation

Different genes and pathways involved in the regulation of melanocyte proliferation and differentiation are subverted, originating an over proliferation of these cells and resulting in the development of melanoma. For instance, *MITF* has been identified as a melanoma oncogene, and amplifications in this gene are present in metastasis, as well as in primary tumours[14]. Also, it seems like an optimal level of expression of this gene is essential for the proliferation and survival of melanoma cells, since a too low level leads to growth suppression, while a too high level induces differentiation[15,16,17].

However, the majority of the causes of the dysregulation of melanocyte proliferation and differentiation are linked to genetic factors originated in somatic mutations[18]. About 60% of melanomas have somatic mutations in B-Raf proto oncogene (*BRAF*), causing its overexpression. N-ras proto-oncogene (*NRAS*), on the other

hand, is mutated in 15% of the cases[19,20]. *KIT* proto-oncogene receptor tyrosine kinase (c-KIT) is also one of the most commonly mutated genes in melanoma[15]. These three genes share the characteristic of being involved in the mitogen-activated protein kinase (MAPK) pathway. Dysregulation of this pathway is the main source of the proliferation signal in melanoma initiation[21]. Activation of the pathway where BRAF is involved, results in the phosphorylation of the MAPK pathway, which phosphorylates and regulates the activities of transcription factors and kinases involved in the regulation of important cellular processes, such as cell survival, proliferation, differentiation and motility[22]. *BRAF* has been implicated in the development of melanoma, especially its most commonly mutant form, *BRAF^V600E*. This mutation causes the overactivation of BRAF kinase activity, promoting the oncogenic activation of downstream proliferative signalling pathways in melanoma cells[23,24]. Mutations is the *NRAS* gene have similar effects[25,26,21]. However, the fact that the frequency of these mutations in melanoma is similar to the one found in benign melanocytic lesions, shows that the activation of oncogenes is not sufficient to complete the transformation process[27,28]. This is when additional genetic changes take place. In the case of melanoma, the most common one is a mutation in the promoter of the telomerase reverse transcriptase *(TERT)* gene. TERT is responsible for maintaining telomere ends and preventing senescence of cancer cells. This mutation is present in approximately 70% of sporadic melanoma cases[29].

However, it is not only proliferation pathways that dictate the fate of the process. Mutations in tumour suppressor genes are also involved in malignant transformation.

Progression

Two closely related tumour suppressor genes take part in the progression of melanoma: retinoblastoma (*RB*) and tumour protein 53 (*TP53*). RB is a well-known negative regulator of the cell cycle and its impaired activity leads to dysregulated cell divisions. This tumour suppressor gene has as its activator protein p16, which is coded by the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene, mutated in 40-50% melanomas[30]. *CDKN2A* encodes for two important proteins: INK4A and ARF, both known to be CDK4 inhibitors. While ARF acts as a stabiliser of p53, INK4a directly inhibits CDK4 and CDK6, responsible for the phosphorylation of RB and consequent inactivation of the cell cycle progression. Therefore, upon loss of the *CDKN2A* gene in melanoma, there is no counterbalancing mechanism active and an over proliferation of melanoma cells occurs[31].

Simultaneously with these processes, the activation of the phosphoinositide 3-kinase-protein kinase B (PI3K/AKT) pathway also plays a role in the development and progression of melanoma. This pathway and associated components have been frequently observed to be deregulated in diverse cancers, including melanoma[32,33]. The two most common and studied events responsible for the activation of this pathway are activating mutations in the oncogene *NRAS* (15–20%) and loss of expression or function of the tumour suppressor *PTEN* (20–30%)[34]. These events are largely mutually exclusive. Nevertheless, additional genetic events can activate this pathway in melanoma, such as point mutations in the *PIK3CA*, which are detected in 2-6% of melanomas[35,36].

The concomitant activation of these pathways, MAPK and PI3K/AKT, ultimately guarantees uncontrolled cell divisions and immortality, two crucial features of the malignant cells[37].

Epidemiology

The scenario changed dramatically during the last 50 years. Melanoma went from being a rare type of cancer to having its incidence rising faster than almost any other cancer[38,39]. Current reports state melanoma as the 19th most common cancer worldwide[40]. The incidence rates vary drastically among countries, generally with higher rates seen in geographic areas closer to the equator and higher in altitude. The highest rates were reported in Australia (37 per 100,000) and the lowest in South-Central Asia (0.2 per 100,000)[41]. Within Europe incidence rates also vary widely, with Switzerland registering the highest rates and Greece the lowest. The Netherlands (19.4:100,000 cases per year), and Scandinavian countries, such as Denmark (19.2:100,000 cases per year), Norway (18.8:100,000 cases per year), and Sweden (18.0:100,000 cases per year) rated as having a high incidence[42].

The marked differences in the incidence rates are attributed to different pigmentation characteristics that predominate in the populations of different regions, but also to the discrepancy in frequency of recreational sun exposure among countries[39].

The incidence of cutaneous melanoma has been rising annually, at a rate faster than that of any other cancer. This is worrying, given that unlike other solid malignancies, in which the average age of diagnosis is around 65, melanoma affects a high proportion of younger patients, with a median age of diagnosis of 57. Regarding

gender, usually more women are diagnosed at a younger age, but the scenario drastically changes from the age of 55 onwards, with more men being diagnosed at that age range[42]. Overall, mortality rates are higher amongst men than women[44], probably because of a later presentation of the disease to dermatologists and consequent diagnosis[45].

Staging of the disease

Staging of cancer is fundamental to provide clinicians with prognostic information, help them define treatment strategies for the patients and evaluate survival statistics. This is of great importance, particularly in melanoma, due to the fairly different prognosis registered at different stages of the disease. Since 1998, the American Joint Committee on Cancer (AJCC) developed a melanoma staging system (TNM system) that has been the base for clinical classifications ever since, even after being refined throughout the years, as the biological understanding of the disease improved[46,47].

Localized Melanoma (Stage I and II)

Tumours at both these stages are exclusively described by tumour characteristics (T) in the TNM staging system. Currently, those characteristics divide in tumour Breslow thickness and level of ulceration. Both features, together with mitotic rate, have previously been associated with survival outcome[47,48]. However, in the latest update of the melanoma staging system of the AJCC, mitotic rate was discontinued and is no longer a T category criterion[49,57]. Localized tumours are categorized as T1, T2 or T3, according to whether their Breslow thickness falls into 1.0, 2.0 or 4.0-mm measurements, respectively. There is a significant decrease in survival as primary tumour thickness increases[47]. Ulceration has also been linked to a more aggressive disease and significant reduction of patient survival[50,51] and it has been, since then, considered as an adverse prognostic factor[47,52,53]. The absence or presence of ulceration is designated "a" or "b", respectively, in each T subcategory (e.g. T2a and T2b correspond to non-ulcerated and ulcerated stage II melanomas, respectively)[49].

At these two stages, the tumour is localized but invasive, since there is no dissemination of tumour cells to surrounding tissues (such as nearby lymph nodes

or distant parts of the body) but the tumour has penetrated beneath the epidermis into the dermis. In stage I, the tumour is smaller than 1 mm in Breslow thickness and may or may not be ulcerated. In stage II, tumours are deeper than 1 mm and as in stage I, may or may not be ulcerated. Although they are still localized, the risk of spreading is higher than in stage I, and physicians may recommend sentinel lymph node (SLN) biopsy, to assess whether melanoma cells have spread to local lymph nodes[53]. The prognosis for this type of patients is generally favourable, with a 5-year survival rate of 98.4%[47,48].

Regional Melanoma (Stage III)

The lymph nodes are the most common first site of metastasis in melanoma patients[55]. At this stage, the tumour has either spread to nearby lymph nodes, being classified as regional metastatic, or has not spread to nearby lymph nodes and classifies as non-nodal regional metastases, which can be satellite, microsatellite or in-transit metastases. Satellite metastases have been defined as clinically evident cutaneous and/or subcutaneous metastases occurring within 2 cm of the primary melanoma[56,57]. Microsatellites have been defined as microscopic cutaneous and/or subcutaneous metastases found adjacent or deep into a primary melanoma on a pathological examination. In-transit metastases have been defined as clinically evident cutaneous and/or subcutaneous metastases identified at a distance of more than 2 cm from the primary melanoma in the region between the primary and the nearest regional lymph nodes[56].

In stage III, Breslow thickness no longer plays a role in staging and the degree of disease advancement depends on whether the tumour has reached the nodes, the number of nodes involved, the number of cancer cells found in them and whether they are micro or macroscopic. If local lymph nodes are clinically evident, they are usually removed. However, melanoma cells can be present in not palpable lymph nodes, something that SLN biopsy can determine[53].

There is a large spectrum of prognosis for patients with stage III disease and resulting from the combination of the T and N categories, in the launching of the 8th Edition of the AJCC Staging System, four groups were recently created: stage IIIA-D.

The 5-year survival rate for stage III patients ranges from 93% in patients with stage IIIA disease to 32% for those with stage IIID disease[47,49]. These differences have implications for clinical decision making, particularly for patients with stage IIIA disease, since they can be eligible for adjuvant therapy immunotherapy[49].

Metastatic Melanoma (Stage IV)

At this stage, the disease has spread beyond the regional tissues through the form of metastases, and has reached distant sites, such as distant lymph nodes or organs. These tumours are then classifiable according to the formation of metastases (M). There are four M subcategories (a-d), depending on the anatomic site(s) of metastasis. In the 8th Edition of the AJCC Staging System, a new M subcategory ("d") was created, which considers patients with metastases in the central nervous system (CNS)[49]. Another adverse predictor of survival is the level of serum LDH in patients with stage IV disease[58]. This level can be classified as either "0" for not elevated and "1" for elevated for each M1 subcategory[49]. The most important prognostic factor is still the site of metastasis, with patients with metastases on distant skin (or distant lymph nodes or subcutaneous tissue) having the best survival rate, in contrast with patients with pulmonary metastases, with an intermediate survival and non-pulmonary visceral metastases and CNS metastases, having the worst survival[47,59]. The 5-year survival rate at this stage used to be less than 10%[60] but with the introduction of immunotherapy agents in the treatment of advanced melanoma, this number increased to 15-20%[61,62].
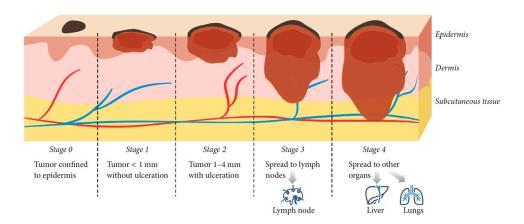

Figure 2. Five stages of malignant melanoma evolution process.
Image courtesy of Joana Jaworek-Korjakowska and Pawel Kleczek[63].

Treatment modalities in melanoma

Chemotherapy was the earliest treatment option for advanced-stage melanoma. Yet, it has proven to be insufficient to improve the overall survival of patients, even when administered in combination with other drugs[64]. Nowadays, for patients with stage I-IIIb melanoma, the primary treatment option is surgical resection[65]. However, surgical procedures differ according to the features of the tumour, namely the safety margins with which the tumour should be excised[66] and whether SLN biopsy is recommended[53]. Despite surgical resection being the primary treatment option in most malignant melanoma tumours, it is not always enough to reduce the risk of resistance and improve survival. Thus, administering adjuvant therapies, such as targeted therapy or immunotherapy, particularly in some stage III cases is usually an option, in order to reduce the risk of recurrence and consequently improve prognosis[67].

Targeted therapy

The development of targeted therapies was a big step in the field, since melanoma treatment went from attacking all dividing cells, with standard chemotherapy regimens, to being directed against specific molecular alterations. This type of therapy works particularly well in melanoma because about 70% of the patients harbour mutations in genes of key signalling pathways, such as MAPK, crucial for melanoma proliferation[68]. In 2011 and 2013, vemurafenib and dabrafenib, respectively, were approved by the U.S Food and Drug Administration (FDA) for the treatment of unresectable or metastatic melanomas harbouring activating $BRAF^{V600E}$ mutations. At the time, studies reported that 90% of the patients treated with these agents showed tumour regression and improved survival[69,70]. Most of the clinical trials with both vemurafenib and dabrafenib in monotherapy are now completed[71].

Despite bringing great advances to the treatment of the disease, the clinical benefit of these therapies was limited due to the rapid development of multiple mechanisms of resistance. Thus, there was the need to develop alternative drugs to BRAFi. As a result of the complexity of the MAPK pathway, several proteins could be targeted, and the mitogen-activated protein kinase kinase (MEK) was a candidate. In addition to BRAFi, in 2013 and 2015, two MEK inhibitors (MEKi) were approved by the FDA: trametinib and cobimetinib, respectively. These were approved for the treatment of advanced melanomas with a $BRAF^{V600E}$ or a $BRAF^{V600K}$ mutation[73,74].

Although single therapy of BRAFi and MEKi showed fairly good results in patients, the improvement in survival was still not satisfactory. Combination treatment of BRAFi with MEKi started then being used, having resulted in a much better patient outcome, conferring an improvement of from 3 to 6 months progression free survival (PFS) when compared with single therapy with BRAFi. Nowadays, the combination of BRAFi/MEKi is commonly used in the clinic to treat two specific types of tumours: stage III and stage IV $BRAF$ mutated melanomas[75].

Nevertheless, the development of resistance is still the main obstacle to overcome when it comes to administration of targeted therapies. One of the common strategies of the tumours to develop resistance is to activate the parallel signalling pathway PI3K-AKT-mTOR by activating mutations in $PI3KCA$ or loss of $PTEN$[76]. Another downside of these therapies is the fact that they can only be used in tumours that harbour the specific mutations to be targeted. To overcome these challenges, targeting distinct pathways by combining treatment of chemotherapy, targeted therapy and, more recently, immunotherapy, seems to be the most efficient option[70] and trials are ongoing[71].

Immunotherapy

Cancer and the immune system were for the first time associated to each other in the 19th century. It happened because tumours were frequently observed at sites of chronic inflammation and because immune cells were often present in tumour tissues[77]. In melanoma, this association was reported in studies that observed spontaneous regression. During the last decade, the increasing knowledge about the role of the immune system in tumour progression allowed the development of many different immunotherapies.

The cytokines were the first immunotherapy treatment option to be developed. In 1995, interferon alfa 2b (IFNalfa-2b) was approved as an adjuvant therapy for the treatment of resected melanomas[78]. This drug showed an immunomodulatory anti-tumour effect, inhibiting the proliferation of melanoma cells and inducing apoptosis[79]. In 1998, the FDA approved the first immunotherapy drug – the high-dose interleukin 2 (IL-2)[80]. Reports state that this cytokine has a complete response rate of 4%, partial response rate of 12.5% and an overall response of 19.7%[81]. Due to their high toxicity and low response rates, these two regimens are still included in clinical trials in combination with other immunotherapies and targeted therapies, but have a quite limited use in the clinic[82].

Another developed immunotherapy approach is vaccination against specific antigenic tumour epitopes. Although this type of treatment was very promising due to its specificity against certain tumour antigens and capacity of eliminating the associated cancer cells, favourable clinical responses were infrequent in melanoma[83].

Later on, adoptive cell transfer (ACT) started to be developed. In this type of treatment, patients are infused with a large number of melanoma-specific T-cells that are expanded and activated in culture[84]. Several studies have reported this type of immunotherapy to be associated with complete regression and durable responses in metastatic melanoma[85,86]. However, while this treatment seems to be quite effective in suppressing regulatory T-cells (Tregs) and eradicating host immunosuppressive factors, a considerable disadvantage is the fact that these cells are still difficult and time consuming to generate[87].

The treatment of advanced melanoma was revolutionized with the development and establishment of immune checkpoint inhibitors (ICIs). Tumours have the capacity of evading the immune system through different strategies. One of these strategies is to activate immune checkpoint pathways that suppress anti-tumour immune responses. ICIs block these anti-tumour immune responses by interrupting the inhibitory signalling pathways and promote immune-mediated elimination of tumour cells[88].

The first approved ICI was a monoclonal antibody targeting the cytotoxic T-lymphocyte antigen-4 (CTLA-4). Ipilimumab was approved in 2011 after it proved to be effective in preventing T-cell inhibition and promoting the activation and proliferation of effector T-cells[88]. Moreover, it showed to mediate improved survival in advanced melanoma with durable responses[89].

In 2014, two other monoclonal antibodies targeting programmed death-1 (PD-1) were approved by the FDA. PD-1 suppresses T-cell activation by binding to its ligands, programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2), expressed in antigen-presenting cells but also in many human tumours. Pembrolizumab and nivolumab have shown higher response rates with less toxicity than ipilimumab. These drugs were approved for the treatment of unresectable or metastatic melanoma, and of ipilimumab refractory melanomas[90].

Several clinical trials are ongoing using ICIs in monotherapy, in combination with each other or in combination with other therapies. Despite the great advancement immunotherapy brought to the treatment of melanoma, big challenges are still to be faced. Only a fraction of patients benefits from ICIs, and severe immune-related adverse events are frequently seen in some of these patients. Therefore, the development of predictive biomarkers is of great importance to allow an effective differentiation between responders and non-responders. Predictive biomarkers would reveal the outcome of a therapy before the initiation of that therapy. In order to establish these predictive biomarkers, a better understanding of the tumour immune microenvironment and the processes leading to the cancer-related inflammation is needed and is currently being intensely pursued[88].

The tumour immune microenvironment

Decades of cancer research have allowed the scientific community to understand most characteristics of cancer. However, much is yet to be described regarding tumour formation. In general terms, it is accepted that a tumour is an aggregate of transformed cells that, as a result of genetic mutations has gained the capacity of growing indefinitely[91]. However, a tumour mass has a lot more complexity in it, with a high amount of non-transformed cells being recruited by it, in order to ensure the growth of the malignant cells. It is, therefore, a communicating organ where interactions between malignant and non-transformed cells create the tumour microenvironment (TME). The major non-malignant cell types that are found in the TME are endothelial cells and pericytes that compose the vasculature, cancer-associated fibroblasts, the extracellular matrix and cells of the immune system[92].

Many different crosstalk mechanisms can be encountered in a tumour. They are responsible for ensuring the success of the three main stages of cancer: initiation, progression and metastasis[94]. One of those mechanisms is angiogenesis, which is the establishment of blood vessels in the tumour, as they are required for the delivery of oxygen and nutrients, and represent a route for tumour cells to migrate from the primary site and disseminate to distant organs, forming metastases. Another example of crosstalk mechanism is mesenchymal fibroblasts depositing extracellular matrix, ensuring a scaffold that allows physical and biochemical stability to the cancer cells[95]. Possibly one of the mechanisms of higher importance is the crosstalk between cancer cells and cells of the immune system, also subcategorized as tumour immune microenvironment (TIME). This complex interaction of malignant cells with cells of the immune system, including T-cells, B-cells, natural killer (NK) cells, macrophages, dendritic cells (DCs), etc., is the basis of one of the hallmarks of cancer: immune escape. Tumours have numerous strategies of escaping the immune system and reducing the risk of being rejected by the host, either by reducing their immune recognition or by making the immune system tolerant to them[96]. Although there is heterogeneity in the composition of the

TME and of the TIME, both intertumoural and intratumoural, many studies are now showing that targeting cells composing these systems can be an efficient approach for new therapeutic strategies. Immunotherapy agents targeting CTLA-4 and PD-1 in the clinical management of metastatic melanoma is one example[93,96,97].
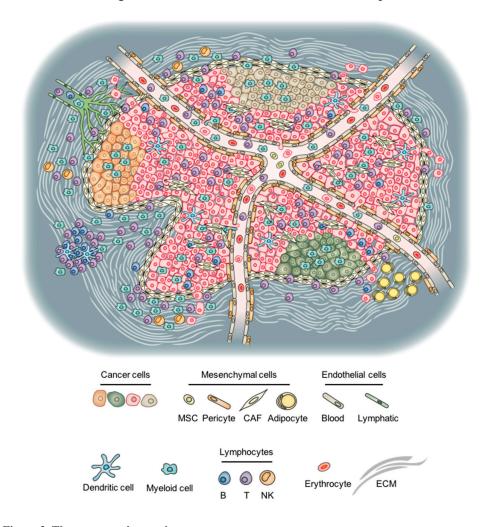

Figure 3. The tumour microenvironment.
In the complex tumour ecosystem, cancer cells co-exist with a variety of non-transformed cells, from mesenchymal cells (MSC: mesenchymal stem cell; CAF: cancer associated fibroblast) to endothelial cells and immune cells, such as lymphocytes, dendritic cells and myeloid cells. The tumour is surrounded by a three-dimensional network called extracellular matrix (ECM). Original image courtesy of Professor Frances Balkwill[93], modified version kindly provided by Matteo Bocci.

The immune system

The overall function of the immune system is to protect the organism from foreign antigens, such as microbes, viruses, toxins or cancer cells. In the course of an immune response, cells interact with each other and with tissues, so that the outcome increases the chances of eliminating an infection or cancer. Despite being a complex system, immunity can be simplistically viewed as having two "lines of defence": innate immunity and adaptive immunity. Innate immunity is the first line of defence and a non-specific mechanism, used by the host immediately after encountering an antigen. The adaptive immunity is antigen-specific and therefore, it takes longer to be fully established after exposure to the antigen.

Several features distinguish the innate immune response and the adaptive immune response. In the innate immunity, the rapid recruitment of immune cells to sites of inflammation/infection is achieved through the production of cytokines and chemokines. In the adaptive immunity, the response is initiated by the actions of the innate immune system, namely through the antigen-presenting cells (APCs). Another difference between the two types of immunity is the fact that in case the organism encounters the same pathogen it once eliminated, while the adaptive immunity is capable of recognizing it, due to its ability of developing immunologic memory, the innate immunity is unable to do it. On the other hand, the type of cells that both types of immune responses recruit are also quite distinct. In the innate immune response, phagocytes (macrophages and neutrophils), DCs, mast cells, basophils, eosinophils, NK cells and innate lymphoid cells, are the ones being recruited. In general, these cells perform three main actions: phagocyting microbes, eliminating those pathogenic microbes and inducing apoptosis. In contrast, in the adaptive immune response, antigen-specific T-cells and B-cells are the ones that act against pathogens. Both types of immunity are complementary, with defects in either system resulting in host vulnerability to external threats, or inappropriate responses[98,99,100].

T-cells

T-cells harbour an antigen-binding receptor, the T-cell receptor (TCR). Upon binding of a specific antigen to the TCR, allowed by the presentation of that antigen on the surface of APCs (such as dendritic cells, macrophages or B-cells) the T-cell rapidly proliferates and differentiates. On the surface of APCs is expressed a group of proteins called the major histocompatibility complex (MHC). These are either classified as class I, which are found on the surface of all nucleated cells, or class II, which are present only on certain cells of the immune system, including APCs. MHC class I is responsible for the presentation of endogenous peptides, while MHC class II presents only exogenous peptides to T-cells.

T-cells circulate in the body via the lymphatic system and blood stream and generally encounter APCs in the lymph nodes. When an antigen binds to its specific TCR, a stimulus is initiated for the differentiation of the T-cell into either a cytotoxic T-cell (CD8$^+$) or a T-helper (Th) cell (CD4$^+$). These two have different functions. CD8$^+$ cytotoxic T-cells are destructive cells – their main function is to eliminate cells infected by foreign agents or cancer cells expressing appropriate antigens. Thus, they are activated through the binding of their TCR with a peptide bound to an MCH class I molecule. The activation of these cells leads to the production of substances that induce apoptosis of the target cells.

CD4$^+$ T-helper cells help other cells by directing them to perform their functions. They are activated through the binding of their TCR with a peptide bound to an MCH class II molecule, expressed on the surface of APCs. After activation, Th cells release cytokines that regulate the activity of several cells, including the APCs that activate them.

Upon the resolution of the infection, most of these cells die and are phagocytized, while others remain as memory cells to ensure an effective response in possible future encounters with the same pathogen[99,100,101].

B-cells

In contrast to T-cells, B-cells can recognize antigens directly, without the need of an APC presenting it. They express clonally diverse cell surface immunoglobulin (Ig) receptors recognizing specific antigenic epitopes. Although they can function

as APCs, their main function is the production of antibodies. After being activated by a foreign antigen, B-cells undergo proliferation and differentiation into antibody-secreting plasma cells or memory B-cells. Immature B-cells migrate from the bone marrow to the spleen and there generate rapid antibody responses in the absence of MHC class II-restricted T-cell help, meaning without the presence of APCs assisted by $CD4^+$ Th cells[102]. On the other hand, mature B-cells are mainly located in lymphoid follicles of the spleen and lymph nodes, and here they encounter and respond to T-cell-dependent antigens bound to APCs, particularly follicular DCs. Then, activated mature B-cells proliferate and can differentiate into plasma cells, to produce antibodies or get recruited into germinal centres (GCs), in order to continue proliferating and differentiating into either long-lived plasma cells or memory B-cells[103].

Tertiary lymphoid structures (TLS)

TLSs are lymphoid aggregates that frequently develop at chronic infection sites, autoimmune diseases and allograft rejection to shape local adaptive immune responses[105,106,107].These structures are morphologically, cellularly and molecularly similar to secondary lymphoid organs, in particular lymph nodes. They contain three main types of cells: B and T-cells and DCs, which are normally organized into two compartments: a B-cell follicle mainly composed of naïve B-cells, surrounding a GC composed of highly proliferative B-cells, and an area composed of T-cells and DCs[105,108].

Many studies have already documented these structures in several tumour types in humans. Some of these reported a typical heterogeneity in these structures, not only among tumour types, anatomical locations, but also between primary tumours and metastatic lesions[109]. For instance, a study showed that colorectal metastases to the lung were associated with well-developed TLSs in humans, while renal cell carcinoma metastases to the same organ were associated with poorly developed TLSs[110]. In melanoma in particular, it was found that TLSs are much more common in metastatic lesions, as compared to primary tumours, where they are largely absent[111].

Although a limited number of studies have reported that tumour-associated TLS is a negative prognostic factor for cancer patient survival[110,112], most of the studies suggest positive associations with survival in a large number of different human

cancers[109]. Generally, the studies that report TLSs as a positive prognostic factor, interpret tumour-associated TLSs to be sites for newly tumour entering naïve T and B-cells for generating useful anti-tumour immune responses. They can also serve as more robust sites for intratumoural CD8$^+$ T-cell effector activity. One of the possible explanations for the differences in prognostic significance of tumour-associated TLSs might be due to the distinct functional features of the B-cell compartment in different tumours[113]. While some studies suggest that intratumoural B-cells can present tumour antigens to T-cells[114,115], others have shown that B-cells can play a suppressive role in anti-tumour immunity[116,117].

A substantial amount of discoveries still needs to be achieved in order to fully understand all the mechanisms behind these structures. Among other things, this knowledge would allow a clearer characterization of mechanisms of immune evasion and open up a future pathway where TLSs can be used as targets to efficiently design therapeutic strategies.
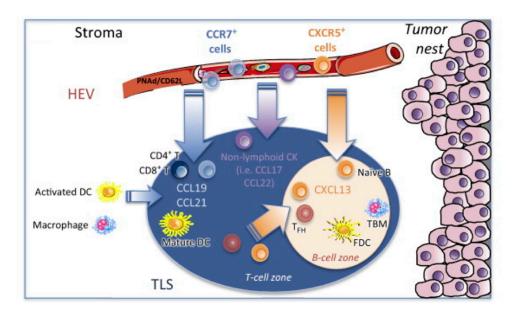
Figure 4. Immune recruitment occurring within TLSs.
Upon signals from lymphoid chemokine CCR7 ligands (CCL19 and CCL21) and CXCR5 ligand (CXCL13) sent through the blood stream in high endothelial venules (HEV), a recruitment of immune cells in the T- and B-cell rich areas of the TLS takes place. Among these immune cells are CD4$^+$ and CD8$^+$ T-cells, DCs, macrophages follicular helper T-cells (Tfh) and naïve B-cells.
Image courtesy of Marie-Caroline Dieu-Nosjean[118].

Immune Evasion

Although cancer immunotherapies have a major impact on patient outcomes, about 60% of patients develop primary resistance, while others experience initial clinical benefit and later on develop secondary resistance[89,119]. There are different mechanisms that lead to immune evasion and result in resistance to ICIs. These mechanisms are essentially divided in innate evasion, when tumours lack a baseline infiltrate of activated T-cells and there is no immune editing, and adaptive evasion, when immunogenic tumour cells are eliminated and mechanisms of adaptive immune resistance, such as PD-L1 are upregulated[120]. Innate evasion and subsequent lack of T-cell activation or recruitment into the tumour microenvironment can derive from alterations in signalling pathways, such as activation of the WNT/β-catenin pathway[121], activation of the PI3K pathway through loss of *PTEN*[121,122], activation of c-*Myc* signalling[123], and loss of *LKB1*[124]. Despite still being extensively studied, adaptive evasion has as base the loss of antigenicity through either loss of the antigenic peptide-MHC complex for T-cell recognition, or blunted IFN signalling[120,125].

The first two innate evasion mechanisms are rather important due to the extensive research developed on them, which led the scientific community to believe that inhibitors of these pathways could represent a future strategy to reverse innate evasion mechanisms, restore immune infiltration in melanoma tumours and enable checkpoint blockade efficacy[121]. Essentially, alterations in these signalling pathways blunt tumour-antigen cross presentation, leading to T-cell exclusion, mainly through failed activation of DCs and macrophages[120,122]. Interestingly, these tumours were also shown to be resistant to ICIs, consistent with previous studies showing that these agents' activity is dependent on pre-existing tumour-reactive T-cells[121,126]. Overall, this highlights the importance of both developing alternative therapeutic strategies to ICIs and also identifying better prognostic targets to effectively select patients to undergo a specific type of therapy.

Main Methods

Population-based retrospective melanoma cohort

Cohort study designs are often described as "observational" because, unlike in clinical studies, there is no intervention. Population-based cohort studies are a category of epidemiologic cohort studies, in which a sample, or even the totality of a defined population is followed up and observed through the course of a period of time to assess the relationship between exposure to risk factors and outcome[127]. The answers obtained in the study should be generalizable to the whole population. Therefore, a reliable and valid identification of populations in which research questions are to be applied, is one of the most important prerequisites in population-based studies[128]. On the other hand, cohort studies can also be retrospective. This type of study, often called historical study, considers events that have already occurred, meaning the follow-up of the subjects involved in the study has already been completed[129].

In a previous study[130] performed by our group with a focus on gene expression-based analysis, a sample cohort, representing a population-based retrospective collection (n=214), was obtained at the Department of Surgery at Skåne University Hospital. This cohort comprised a small subset of primary melanoma tumours (n=16) and a larger fraction of different types of metastases, including regional lymph node metastases (n=139), distant metastases (n=23), local recurrences (n=11) and in-transit metastases (n=15). This is an historic cohort collected between 2000 and 2012. Overall, 95 patients (59%) were untreated and 67 patients (41%) were treated. The treatment was initiated only when patients had developed distant metastatic disease, which made this cohort suitable for prognostic studies. 13 patients of the 67 treated cases received neo-adjuvant treatment (9 received chemotherapy and 4 immunotherapy). Only 7 cases received targeted therapy, of which 4 received BRAF inhibitor treatment. Two cases were treated with a vaccine, 24 cases with immunotherapy (mainly interferon treatment) and 23 cases received chemotherapy[130]. This cohort was used in the four papers included in this thesis.

Microarray – Global GEX and TMA

Once the human genome was sequenced in 2001, a new era of research started[131]. From then on, researchers were able to study a gene function through the expression measurements of a genome – a field that was then called functional genomics. DNA microarrays are one of the tools used in this field. There are two types of this technique: gene expression (GEX) microarrays and tissue microarrays (TMA)[132]. Data derived from both types of this technique were used in the papers included in this thesis.

Global Gene Expression (GEX)

The principle behind microarrays is that complementary sequences will bind to each other. GEX microarray stands on the evaluation of messenger RNA (mRNA). mRNA is an intermediary molecule which carries the genetic information during transcription. These molecules synthesize the corresponding protein by translation. So, indirectly by assessing the various mRNAs, it is possible to assess the genetic information or the gene expression. After harvesting a mRNA sample from the source of interest, due to its degradability, it is necessary to convert it into a more stable form, single-stranded complementary DNA (cDNA). This is achieved by using a reverse transcriptase and subsequent amplification using PCR[133]. During this process, nonlinear amplification between the initial mRNA and the amplification product (cDNA) can occur. As such, in order to avoid it, another approach can be used where cDNA, before amplification, is rendered double-stranded and transcribed into antisense RNA copies. This is often called complementary RNA (cRNA), since it is complementary with cDNA. cDNA is then labelled with two commonly used fluorescent dyes, Cy3 and Cy5. cDNA fragments hybridize simultaneously with DNA probes of a DNA chip and the fluorescence emission is measured with a laser beam. Ultimately, this sensitive and specific method, allows the analysis of the abundance of a particular sequence in the target population[132]. The relative abundance among samples is obtained in subsequent gene expression analysis, explained in detail in the "Gene expression analysis of Lund cohort" further ahead in this section.

Tissue Microarrays (TMA)

A great part of the work included in this thesis derives from subsequent analysis of previously obtained TMAs. TMAs are constructed by transferring cores of paraffin-embedded tissues to pre-cored holes in a recipient paraffin block. Subsequently, sections are cut from these TMA blocks and can undergo, for instance, immunohistochemistry or high-plex proteomic analysis, as done in papers included in this thesis. Each core on the TMA slide represents a single patient sample, allowing multiple patient samples to be analysed for a single molecular marker[132,134]. In this work, TMAs were constructed using, on average, three 1 mm cores per tumour sample. This allowed the obtainment of a representative picture of a single tumour.

Immunohistochemistry (IHC)

In the four papers included in this thesis, data obtained from subsequent IHC staining was used. The principle of IHC is based on an antigen-antibody binding reaction in biological tissues, to detect antigens in cells of a tissue section. This technique demands the use of a primary antigen-specific antibody, a secondary enzyme-linked antibody and a substrate. Upon binding of the primary antibody to the tissue antigen and consequent binding of the secondary antibody, the enzyme linked to it catalyses to generate coloured deposits at the sites where this reaction occurred in the tissue. Nuclei are then counterstained with haematoxylin, a deep blue-purple colour. This is often combined with another staining, eosin, in order to obtain an overview of the cell structures, such as nuclei, membranes and cytosol[135].

In Paper I, IHC staining was performed in the melanoma sample cohort using antibodies against CD3 (A0452), CD8 (M7103), CD20 (M07555), MITF (Clone C5), Ki67 (MIB-1), B2M (A0072) and SOX10 (Clone BC34). All the primary antibodies used were from Agilent/DAKO, except SOX10, whose staining was performed in the clinical routine laboratory of Clinical Pathology at Skåne University Hospital, using the mouse monoclonal IgG1 antibody from Biocare Medical.

In Paper II, tumours stained for PTEN (138G6, monoclonal, Cell Signaling) and MITF (same staining as in Paper I) were scored and considered for the analysis performed.

In Papers III and IV, an Agilent/DAKO antibody against CD68 (M0718) and CD163 (Clone 10D6) from Novocastra were used to stain for myeloid cells. Haematoxylin and eosin (HE) staining was used in the four papers.

Immunofluorescence (IF)

Immunofluorescence is an antigen-detection test used to detect antigens in cellular contexts. Antigens are detected through the binding of specially modified antigen-specific antibodies. These antibodies are modified due to their tagging with a fluorochrome, which absorbs ultraviolet light of a defined wavelength and emits light at a higher wavelength. The emitted light is then detected optically with a special microscope equipped with specific filters for the emission wavelength of the fluorochromes used. There are two types of IF, direct and indirect. In the direct type, the fluorochrome is bound directly to the antigen-specific antibody. In the indirect type, the fluorochrome is bound to an anti-immunoglobulin molecule that recognizes the antibody itself. The indirect IF has the particularity of increasing the sensitivity of the assay, but it may also increase the background. This technique allows researchers to evaluate whether cells in a sample express a specific antigen or not. When immunopositivity is found in the sample, it is possible to determine which subcellular compartment expresses the antigen, meaning that it is possible to analyse the distribution of proteins, glycans and small biological molecules in a sample[135].

In Paper I, initially, in order to address the features of the B-cell aggregates and whether they were TLSs, antibodies against CD20 (1:200, 00064779, DAKO), CXCR5 (1:200, 3180237-9, Abcam) and CXCL13 (1:200, NBP2-1604155, Novus Biologicals) were applied. These antibodies were detected at the wavelength of 488 and 546 nm and fluorescence images were acquired with an Olympus BX63 microscope, DP80 camera and CellSens Dimension v.1.12 software (Olympus).

High-plex proteomic analysis

IHC and IF have always been used to assess spatial distribution and heterogeneity of proteins and nucleic acids in tissue slices. However, these techniques have limitations, including difficult quantitation and limited multiplexing capability.

In Paper I, we used the Nanostring GeoMx Digital Spatial Profiler platform, a novel technique that unlike IHC or IF, performs a proteomic analysis with spatial resolution of a specific marker. The principle of this assay is based on antibodies or RNA probes coupled to photocleavable oligonucleotide tags. Following the binding of these probes to the sections being used, which in the case of our study were TMA sections, in the specific regions of interest (ROIs) the oligonucleotide tags are released from the tissue upon UV exposure. These tags are then aspirated, quantitated and the counts are mapped back to the corresponding tissue location[137].
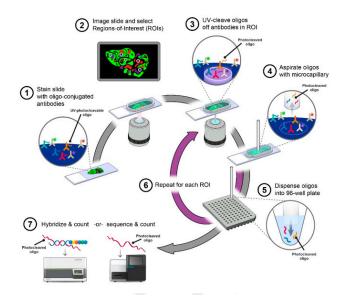

Figure 5. Digital Spatial Profiler workflow.
Image courtesy of Nanostring Technologies.

For study I, CD3, CD20, PMEL/S100B images of the TMA were used to select ROIs enriched for T-cells, B-cells and tumour cells, respectively. This allowed a characterization of protein expression in the TLSs, but also addressing their specific location in the melanoma tumours analysed.

Data analyses

Gene expression analysis of Lund cohort

In Cirenajwis et al.[130], RNA from the population based retrospective cohort previously described was extracted and isolated. Then, microarray expression data were generated using Illumina Human-HT12v4.0 BeadChip arrays for single-channel detection. This multi-sample format of array (12 samples per chip) comprised 47231 probes (oligonucleotides), covering content from National Center for Biotechnology Information (NCBI) RefSeq Release 38. These probes were then binded to beads expressing a specific address tag that enables localizing the bead on the array. Each probe-bead pair is replicated about 30 times on each array. Total RNA from the subjected specimens is then converted to cDNA and biotin-labelled cRNA. Finally, hybridization of the 50-mer probes on the beads occurs, before scanning of the BeadChips.

For the processing of the data, the GenomeStudio software from Illumina was used, which included removal of outlier beads and calculation of average bead signals and detection p-values. The data was then normalized using the algorithm for cubic spline quantile-normalization. R statistical software was used for all the additional processing of the data. Initially, the data was log2 transformed and probes with a detection p-value <0.01 were kept if present in a minimum of 80% of the samples. 13955 probes were retained and 13744 of these corresponded to RefSeq features. These features mapped to 11051 unique genes and the most varying probe for each gene was kept in the data. In order to compare gene expression levels across samples, the 11051 probes were mean centered. The gene expression data developed in this study by Cirenajwis et al.[130], were used in all the four papers included in this thesis.

Analyses of public gene expression datasets

Increasingly complex statistical models are being used for the analysis of biological data. However, applying a novel data analysis to an empirical dataset is usually not reliable, due to the uncertainty of generation of true or false positives. Thus, these methods need to be challenged in order to capture possible biological processes that could have created patterns in the data, a process called validation. Recently, researchers started making use of public datasets to perform validation studies[138]. In

the papers included in this thesis, several different publicly available datasets were used to validate our bioinformatics and statistical analyses.

In Paper I, RNA-sequencing (RNA-seq) data of metastatic melanomas from The Cancer Genome Atlas (TCGA) (level 3, release 3.1.14.0) were downloaded from the data portal, quantile-normalized and log-transformed as log2(data + 1).

Furthermore, different cohorts of patients treated with ICIs were used. In Paper I and II, we used two PD-1 inhibitor treatment RNA-seq datasets, from Gide, T. et al.[139] and Riaz, N. et al.[140]. The processing of these datasets was similar in both studies. Moreover, two more datasets were used in Paper I: one PD-1 inhibitor treatment NanoString gene expression dataset from Roh, W. et al.[141] and a CTLA-4 inhibitor treatment dataset from Van Allen, E. et al.[142].

Fastq files including the PD1-treatment RNA-seq data from Gide, T et al.[139] were downloaded from the European Nucleotide Archive (PRJEB23709) and fragments per kilobase of transcript per million mapped reads (FPKM) values were retrieved using two different programs that allow the assembling of RNA-seq alignments: Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT) and Stringtie. We then reduced the data to protein-coding genes, quantile-normalized the samples and log-transformed the data as log2(data+1).

The PD-1 inhibitor-treatment RNA-seq data from Riaz, N. et al.[140] were downloaded as count data ('CountData.BMS038.txt') with annotations retrieved from https://github.com/riazn/bms038_analysis/tree/master/data. By making use of exon annotations from the R package TxDb.Hsapiens.UCSC.hg19.knownGene, we reduced the data to protein-coding genes and normalized for transcript lengths. These were subsequently transformed to transcripts per million (TPM) data, quantile-normalized and log-transformed as log2(data+2)-1.

The PD-1 inhibitor treatment NanoString gene-expression data from Roh, W. et al.[141] were downloaded from the supplementary table made available in this publication.

The authors from Van Allen, E. et al.[142] provided us with the CTLA4 inhibitor treatment data as reads per kilobase of transcript per million mapped reads (RPKM) values. Subsequently, the data were quantile-normalized and log-transformed as log2(data+1).

The single-cell RNA-seq data used in Paper I were retrieved from the public functional genomics repository Gene Expression Omnibus (GEO). The protein-

coding genes were kept and cells with less than 1,700 or 1,000 genes expressed >0 were removed, respectively.

Similarly to Papers I and II, in paper IV gene expression and clinical information from immunotherapy treated cohorts from Gide, T. et al.[139], Riaz et al.[140], and Van-Allen et al[142] were used and processed as described above. Furthermore, RNA-seq based gene expression data of a cohort of PD-1 blockade treated patients from Liu et al.[143] were used. These were downloaded as TPM data and protein-coding genes were kept while four outlier samples with more than 5000 unexpressed genes were removed. The data were quantile-normalized and log-transformed as log2(data+1).

Furthermore, in Paper IV, the list of marker genes obtained from the single-cell gene expression-based immune cell signatures, was validated in the publicly available scRNA-seq dataset from Sade-Feldman et al.[144]. This dataset was obtained from GEO and both protein-coding genes and cell type annotations from this publication were kept for analysis. Furthermore, similar immune cell subsets were considered, in order to simplify the analysis. The RNA-seq based gene expression from the TCGA dataset for metastatic melanoma was downloaded from the TCGA PanCanAtlas Publications.

Gene signatures

Many studies exploring gene expression data generate one or more gene signatures. A gene signature is a list of genes with a common pattern of expression that is developed with the aim of classifying groups of samples in an independent dataset. In recent years, gene signatures have been increasingly used to interpret the results of gene expression data analyses. The number of studies developing new signatures and making them available is increasing and with that, users can benefit from a more thorough coverage of the extensive number of existing biological processes[145].

Methylation-based immune cell signatures

In Paper III, in order to explore DNA methylation as a determining factor of the immune landscape in the melanoma microenvironment, we created DNA methylation signatures, reflecting a wide range of tumour associated immune cell subsets. First, to derive an immune cell-type specific CpG (cytosine-guanine sites) set for metastatic melanoma, two immune cell type-associated gene signatures were used to identify immune cell types in melanoma tumours. These gene signatures were retrieved from Angelova et al.[146] and Tirosh et al.[147]. Next, we selected corresponding promoter CpGs. The CpGs were then further filtered using the following criteria:

1. *Selection of the most differentially methylated CpGs across reference immune cells:* β-values were dichotomized into robust methylation bins, as unmethylated ($\beta < 0.3$) and methylated ($\beta \geq 0.3$). Then, CpGs that had significantly different proportions of methylated and unmethylated signals among the reference immune cells were selected using Fisher's exact test and a False Discovery Rate (FDR) < 0.01.

2. *Non-immune cells with a high methylation:* To ensure that methylation in other cells present in the microenvironment would not interfere in our observations, and that any methylation difference was deriving from immune cells, we shortlisted CpGs with a high representation among the samples and a high level of methylation ($\beta > 0.7$ in > 98% of samples). The samples used were from non-immune normal cells and melanoma cell lines.

3. *Forming gene–CpG pairs:* To ensure that a single gene would not be over-represented, meaning the presence of multiple CpGs for that gene, we selected the most significant CpG for each single gene from Fisher's exact test in step 1.

This extensive selection process resulted in 67 gene–CpG pairs representing 21 immune cell populations. The same procedure was followed when deriving a CpG set for a non-melanoma TCGA pan cancer cohort, in order to compare metastatic melanoma to other cancer types.

Single-cell gene expression-based immune cell signatures

In Paper IV, in order to derive new transcriptional signatures describing immune cell subsets present in melanoma tumours, we used the single cell RNA-seq dataset from Jerby-Arnon et al.[147]. This dataset was obtained from GEO as both raw and

TPM data. The cell type annotations from the authors were used in order to distinguish immune cells from non-immune cells. Immune cells were then filtered using the expression of the cellular lineage markers and only genes with a mean expression >=2 in one or more cell types were kept for further analyses. The resulting dataset was then subjected to variance stabilizing transformation (VST). After VST, the data was clustered using the Seurat pipeline, to identify immune cells groups in an unsupervised way, and differentially expressed genes were determined for each immune cell group.

Publicly available immune cell signatures

In Paper I, II and III we made use of immune cell signatures derived in other studies and that were made available by the respective authors.

In paper I, previously described immunological gene signatures by Bindea et al.[149] representing different immune cell subsets, helped us in the characterization of the three CD8/CD20 melanoma tumour groups. In this study, after selecting B-cells for their further characterization, gene signatures derived by Angelova et al.[146] were used to define different types of B-cells: activated, immature, memory B-cells and plasma cells. Furthermore, we confirmed the correlation of the TLS signature with other signatures of T-cells and other types of immune cells. For that we used data from both Tirosh et al.[147] and Becht et al.[150].

In Paper III, immune gene expression signatures described by Angelova et al.[146] and Tirosh et al.[147] were again used. In order to explore the DNA methylation immune landscape in metastatic melanoma tumours, the corresponding gene promoter CpGs were identified in the two publicly available gene expression signatures.

Finally, in Paper II, the Microenvironment Cell Populations-counter (MPC-counter) method, described by Becht et al.[150], was used. This tool is available as an R package and it allows the quantification of the absolute abundance of immune infiltrates in a heterogeneous sample, by the production of an abundance score for different immune cell types.

TLS and MITF^{low}PTEN^{negative} signatures

In Papers I and II, significant analysis for microarray (SAM) analyses were performed in order to identify genes differentially overexpressed in different groups. In Paper I, this analysis was used to derive the TLS signature, by identifying genes overexpressed in CD8$^+$CD20$^+$ versus CD8$^+$ groups and by subsequently subtracting the genes overexpressed in CD8$^+$ versus double negative groups.

In Paper II, SAM analysis was used to determine genes with significantly different expression between MITF^{low}/PTEN^{negative} tumours and other melanomas.

Statistical analyses

When dealing with high throughput techniques, data handling/processing, and final bioinformatics and statistical analysis of the data output are needed. In the papers included in this thesis, all bioinformatical analyses were performed in R and different two-sided statistical tests were applied. Generally, Fisher's exact test was used for comparison of categorical variables, while Pearson correlation was used for comparison of numerical variables. Except in Paper III and IV, where numerical variables were compared using Spearman and Kendall correlation. When comparing groups, three tests were used: t-test, Wilcoxon test and analysis of variance (ANOVA). T-test and Wilcoxon were used when comparing only two groups, while ANOVA was used when comparing two or more than two groups. In Papers I and II, Kruskal-Wallis test was used for the association between numerical and categorical variables, such as mutational load and the immunohistochemical groups. In Papers III and IV, this association was explored using Mann-Whitney U test/Wilcoxon rank sum test. For univariate and multivariate survival analyses, Cox regression included in the survival R package was used across all studies.

Present Investigation

Paper I

Tertiary lymphoid structures improve immunotherapy and survival in melanoma

Aim:

To investigate the role of B-cells in anti-tumour responses in melanoma.

Results and Discussion:

When analysing the immunostaining of a B-cell marker, CD20, 25% of the tumour cases showed these cells to be organized in clusters. These clusters consisted of $Ki67^+$ and $Ki67^-$ B-cells, suggesting that some of these cells were active and proliferating. Also, the clusters were surrounded mainly by $CD4^+$ T-cells, which led us to hypothesize that these structures could resemble the formation of TLSs. Moreover, the expression of molecular markers of TLS formation, such as CXCL13, CXCR5 and DC-LAMP were upregulated in transcriptomic data from matched tumour tissue. We then confirmed these structures to be TLSs in an immunofluorescence staining, where we noted concomitant presence of B-cells (stained with CD20) and known TLS markers (CXCR5 and CXCL13) in the cluster. This supported that the $CD20^+$ B-cell clusters have molecular properties that have been described as necessary for TLS formation. Another evidence for the formation of TLSs was the fact that although $CD8^+$ T-cells were always present in the clusters' surroundings, they were localized mainly outside the clusters. The presence of these T-cells and of the TLSs themselves, was associated with improved patient outcome. More importantly, the combination of both TLSs and $CD8^+$ T-cells was associated with the best survival outcome, while the presence of $CD8^+$ T-cells alone was linked to an intermediate survival and the absence of both was linked to the worst survival outcome. This was then confirmed while analysing the location of the TLSs, where

we concluded that tumours with infiltrative TLSs had a significantly higher frequency of melanomas with a tumour-infiltrative CD8$^+$ T-cell pattern.

The spatial location of the TLSs in the tumour, proved to be independent of metastatic site. Also, when counting the number of TLSs, 44% of cases with TLSs had multiple TLSs per square millimetre. More importantly, these multiple TLSs were only found in lymph-node metastases, supporting the previous finding that these metastases represent the most prevalent sample site containing TLSs. While counting the number of TLSs, we also assessed the presence of GC-like structures within TLSs, and found cases in which canonical GC-like structures coexisted with non-GC-like TLSs in the same tumour. This supported the concept that different types of TLSs exist in a single tumour and this is independent of spatial location.

A high-plex proteomic analysis with spatial resolution was performed with Nanostring GeoMx digital spatial profiler. The data revealed CD20$^+$ B-cell populations in TLSs to be organized in two groups: with high or low expression of Ki67. Moreover, TLSs with Ki67^{high} B-cells had a higher proportion of CD4$^+$ cells and increased expression of the pro-survival anti-apoptotic molecule BCL-2. The data provided by the digital spatial profiler indicated that TLSs can be at different stages in the same tumour and that the T-cells co-existing with these structures have undergone antigen activation, belonging to mature TLSs. T-cells had different properties depending on whether they were T-cells obtained from within or in close proximity to TLSs, infiltrating T-cells in tumours with TLSs, or T-cells from tumours without TLSs. More specifically, T-cells obtained from within or in close proximity to TLSs had an increased expression of CD4 and decreased expression of CD8, as previously confirmed by IHC. After addressing the effect of TLSs on the intratumoural T-cell landscape, we concluded that distinct patterns of intratumoural adaptive immune activation exist and these might be in part driven by TLSs.

When investigating the expression of immune markers on tumour cell populations, as expected, the loss of antigen presentation via B2M and HLA-DR and decreased expression of PD-L1 expression, was found in tumours without an immune cell presence. However, there was no difference in PD-L1 expression in tumour cells between tumours with TLSs and tumours only with T-cells.

When analysing B-cells using single cell RNA-seq data, MHC class I and II molecules displayed a uniform high expression pattern across all B-cells, supporting that these cells within TLSs are generally capable of antigen presentation.

Transcriptional evidence showed that, in melanoma tumours, there is presence of a mixture of activated and immature B-cells, and only a small fraction of plasma cells, supporting the existence of TLSs. Moreover, this data was consistent with the heterogeneous states of TLSs observed previously, since it showed a wide range of B-cell-derived immature-to-mature GC signals. While MHC class I and II molecules displayed a uniform high expression pattern across all B-cells, the same was not observed with the expression of a component of the B-cell receptor in pre-B-cells, IGLL1. The expression of this protein displayed three groups: plasma cells, cells positive for IGLL1 and IGLL5 and cells negative for IGLL1 and IGLL5. These groups were further subdivided according to CD69 expression and IGLL5$^-$ CD69$^+$ cells were associated with response to ICIs. The different IGLL1/5 CD69 groups could not only reflect the maturation state of the GC reaction occurring in TLSs, but also support the presence of distinct subsets of B-cells at different stages of B-cell development, and their role in the response to ICIs.

When exploring whether the immune microenvironment adapts according to the presence of B-cells, the single-cell data showed that samples with a lot of B-cells contained more CD4$^+$ and CD8$^+$ T-cells with a naïve and/or memory-like features (expressing TCF7 and IL7R), compared to B-cell-poor samples, suggesting an influx of naïve and memory-like cells to TLSs. Moreover, this was consistent with the GeoMx data, that showed that T-cells in tumours without presence of TLSs had an exhausted-like molecular phenotype.

A gene signature representative of TLSs was created, containing known B-cell genes and genes expressed by other types of immune cells. When trichotomizing RNA-sequencing (RNA-seq) data from TCGA on the basis of our TLS signature, an association with patient survival was obtained, confirming the prognostic role of this signature. Applying the TLS signature to different cohorts of treated patients with ICIs (both in monotherapy and in combination) showed that it has a predictive effect, since it was once again, correlated to patient survival. Furthermore, the TLS signature was independent of mutational load in all the treatment cohorts analysed, consistent with previous studies showing that immune gene signatures are not correlated with mutational load. Also, adding to its predictive effect regarding immunotherapy treatment, this signature performed best in a Cox regression analysis across all cohorts treated with ICIs, compared to other immune signatures, supporting the robustness of the TLS signature and its potential for becoming a predictive biomarker for response to ICIs. By exploring further the biological relevance of the TLS signature, we could confirm not only that its functionality is

induced by ICIs in patients showing a clinical response, but also its ability to predict samples with TLSs.

Overall, this study allowed to evidence that TLSs may have a role in sustaining an active tumour immune microenvironment. In the future, this finding could be considered in the development of a therapeutic approach involving enhancement of TLS formation and function, to improve both clinical outcome and response to cancer immunotherapy.

Paper II

The role of PTEN loss in immune escape, melanoma prognosis and therapy response

Aim:

To explore the role of PTEN in prognosis, therapy response and immune escape in the context of MITF expression in melanoma.

Results and Discussion:

In the past decade, a deeper understanding of the crosstalk between the immune system and cancer establishment and progression, has elucidated how a dysfunctional immune system is one of the major ways by which tumours evade immune surveillance. More importantly, this increased understanding has changed profoundly the cancer treatment, in particular melanoma, with the introduction of immunotherapy in the clinic. The activation of the PI3K pathway through loss of PTEN is one of the most frequently altered processes in cancer, and has been described as a driver factor of an immune evasion mechanism that results in a lack of T-cell tumour infiltration.

In our study, with no difference in gender or age at diagnosis, PTEN was found to be differentially expressed according to the melanoma stage, as more advanced melanomas were PTEN negative, whereas the majority of the primary tumours were PTEN positive. Although it does not act independently, the expression of the PTEN protein in tumours is associated with a better patient outcome. Moreover, more *PTEN* mutations were found in PTEN negative tumours, than in positive cases and these were more frequently somatic mutations in the PI3K pathway. The tumour mutational load was, however, identical between PTEN positive and negative cases, suggesting that these tumours evolve independently of tumour genetic mechanisms. Overall, this supports the importance of this tumour suppressor gene.

One of the evidences that PTEN may act as a regulator of an immune escape mechanism, was the fact that tumours positive for PTEN expression, were more frequently found in tumours with T-cells. Moreover, tumours lacking PTEN expression had downregulation of genes associated to immune signatures. When investigating further the transcriptional features of PTEN negative tumours, we found that not only T-cells and cytotoxic T-cells were downregulated, but also a B-

cell signature, showing that PTEN loss might influence the function of other types of immune cells.

The loss of MITF and consequent dedifferentiation of melanoma tumour cells has previously been indicated as an immune evasion mechanism. To analyse the role of PTEN in MITF^{low} and MITF^{high} melanomas, we first confirmed that MITF status was independent of PTEN protein expression and then checked for their concomitant expression in melanoma tumours. After combining MITF and PTEN in four different biological subgroups, we observed that a smaller percentage (31%) of MITF^{low}/PTEN^{negative} melanomas had tumour-associated CD8+ T-cells, in contrast with a higher percentage (72%) of MITF^{high}/PTEN^{positive} tumours. Moreover, MITF^{high}/PTEN^{positive} scored the highest for the immune gene signatures characteristic of several immune cell populations, proving that these tumours are more immunologically inflamed than tumours with low expression of both proteins. These MITF/PTEN groups showed to be independent of mutations in the MAPK pathway, but the two PTEN deficient groups had more mutations in the PI3K pathway. On the other hand, these groups proved to be independent of tumour genetic mechanisms, since the tumour mutational load was not statistically significantly different between them. Not surprisingly, the group showing the worst survival was the MITF^{low}/PTEN^{negative} group, since it showed a lower counting score for immune cell subsets and was characterized by upregulation of the Wnt signalling and down regulation of immune related genes, in particular genes involved in antigen presentation, such as MHC. Overall, we could corroborate our hypothesis that loss of both proteins simultaneously could correspond to an extreme immune poor subtype.

When determining a gene signature characterizing the MITF^{low}/PTEN^{negative} tumours, characteristic genes of immune related pathways were enriched among down regulated genes and up regulated genes were enriched for pathways involved in cell adhesion and migration. More importantly, up regulated genes were enriched in the Wnt signalling pathway, supporting previous studies showing that aberrations in the Wnt signaling are commonly found in a variety of malignancies and elevated Wnt signalling shows a strong correlation with overall immune suppression. Moreover, the activation of the Wnt signalling pathway has been shown to be closely connected to the MITF "rheostat" model, which shows that the level of MITF expression and activity plays a role in the promotion of melanoma growth. Thus, a low expression of MITF denotes a high expression of the Wnt signalling pathway, leading to an overall immune suppression and worse survival, as we observed in the MITF^{low}/PTEN^{negative} tumours.

This gene signature characterizing the MITF^{low}/PTEN^{negative} melanomas was used to create a centroid that was applied on gene expression data derived from patients administered with immune checkpoint blockage agents or BRAF/MEK inhibitors. MITF^{low}/PTEN^{negative} tumours were more likely to have progressive disease under the treatment of BRAF/MEK inhibitors, which supports previous findings describing that MITF^{low} melanoma cells are intrinsically resistant to BRAF inhibitors. Moreover, the MITF^{low}/PTEN^{negative} group of tumours also displayed inferior survival following treatment with ICIs, supporting that inactivation of PTEN leads to immune evasion.

Overall, this study shows that loss of PTEN in conjunction with loss of melanocyte differentiation features, through the loss of MITF, leads to immune evasion mechanisms, rendering the tumours resistant to targeted- and immunotherapy.

Paper III

Analysis of DNA methylation-based tumour immune microenvironment patterns in metastatic melanoma

Aim:

To find immune lineages associated with the microenvironment in melanoma, determined by DNA methylation.

Results and Discussion:

DNA methylation is a major epigenetic driver in cancer progression and development. However, its role in the TME has only started being studied in the past few years, with some studies showing that hypomethylation promotes immune evasion and it could predict response to ICI treatment. Thus, DNA methylation could help in the distinction of different TMEs.

In order to explore the DNA methylation immune landscape in metastatic melanoma tumours, the corresponding gene promoter CpGs were identified in two previously described immune gene expression signatures (Angelova et al.[146], Tirosh et al.[147]). Among these, the CpGs that were most differentially methylated across seven immune cell subsets as well as highly methylated in non-immune cell types, were selected and identified as DNA methylation signatures representative of different immunological cell types. Three immune methylation clusters were identified when running consensus clustering of Illumina EPIC methylation array-based profiles from 180 metastatic melanoma tumours. These clusters showed a crescent gradient level of methylation of the immune CpGs, with Cluster 1 having the lowest, Cluster 3 the highest and Cluster 2 showing an intermediate level. These immune methylation clusters proved to be associated with patient survival, with Cluster 2 showing significantly decreased DMFS (distant metastasis-free survival) compared to Cluster 1. Moreover, both Cluster 2 and Cluster 3 proved to be of higher risk when compared to Cluster 1.

When confirming the differences in immune cell-infiltration between the clusters, not surprisingly, Cluster 1 had the highest percentage of samples with strong and localized infiltration of TILs, CD3$^+$ and CD8$^+$ T-cells, and fewer absent/low infiltrating samples of myeloid cells compared to Cluster 3. Also, Cluster 1 showed a significant enrichment of TLSs, emphasizing the possibility of a successful anti-

tumour immunity in the tumours belonging to this cluster. Thus, immune cell type-specific methylation is inversely correlated with enrichment of the corresponding immune cell types in the TME.

The immune-methylation clusters were not associated with tumour mutational burden (TMB). This could be due to the characteristic high immunogenicity of melanoma cells, allowing immune evasion to be achieved by transcriptional changes. Although *PTEN* is one of the most frequently mutated tumour suppressor genes in various types of cancer, leading to an immune evasion mechanism, the same has not been observed in melanoma. Thus, previously proposed alternative mechanisms that block *PTEN* functions, such as somatic copy number aberrations (SCNAs) and promoter hyper-methylation, might be involved in melanoma. We could confirm this hypothesis in Cluster 3 tumours.

After hypothesizing that the differentiation state of the melanoma tumour, defined by the presence or absence of expression of *MITF*, may determine the ability of Cluster 3 tumours to evade the immune system, and matching staining information for MITF protein, fewer MITF negative tumours were found in the immune-rich methylation Cluster 1. Thus, MITF also proved to be important in the distinction of the immune evasion mechanisms.

Immune exclusion-associated genes were differentially expressed across clusters. Catenin beta 1 (*CTNNB1*) and Master Regulator of Cell Cycle Entry and Proliferative Metabolism (*MYC*) were enriched in Cluster 3 compared to the other two clusters. However, this difference was dependent on MITF status and could only be observed in MITF positive tumours. The same did not happen with PI3K-Akt pathway genes, such as *PTEN* and *PI3K3R1*, which showed significant gene expression differences across clusters, irrespective of MITF status. Cluster 3 was the one showing the highest enrichment for PTEN.

The immune-methylation clusters could be validated in the TCGA cohort and similar features across the clusters, such as survival and gene expression scores, TMB and mutational patterns, were observed. The analyses performed on a pan-cancer cohort revealed similarities in the immune microenvironments of different tumour types in terms of immune-methylation. Samples correspondent to brain, lymph node and other metastases from distant sites were enriched for Cluster 3, irrespective of the metastasis location. Moreover, immune-methylation scores grouped metastatic melanoma tumours belonging to the low immune-methylation cluster, together with tumours from lung and gastro-intestinal tract, showing that these immune-rich microenvironments are similar among each other. Such

observation might explain why non-small cell lung cancer (NSCLC) has received significant attention regarding ICI treatment. Also, this reinforces the concept that an immune-rich environment may confer improved outcome to immunotherapy agents.

Overall, the immune-methylation clusters proved to be informative of prognosis not only in melanoma, but also in several solid tumour cohorts.

Paper IV

Distinct transcriptional signatures derived from single-cell RNA sequencing data predict patient prognosis and response to immune checkpoint blockade in melanoma

Aim:

To describe immune signatures derived from single-cell RNA sequencing data of tumour-associated immune cells from melanoma patients.

Results and Discussion:

Single-cell genomics has been revolutionary in the characterization of the TME heterogeneity and in describing novel immune subgroups. In this study, known immune cell subsets were clearly discerned when using unsupervised analyses of single-cell RNA-seq data from 33 melanoma tumours – B-cells, T-cells and macrophages. These immune cell subsets were characterized by different genes. CD8$^+$ T-cells were characterized by increased expression of OX40, TIGIT, PD1 and IFNG, suggesting an exhausted phenotype of these cells. On the other hand, another subset consisting of CD4$^+$ and CD8$^+$ T-cells were characterized by increased expression of IL7R, TCF7 and CCR7, corresponding to a memory T-cell phenotype. The B-cell subsets were distinguished according to the expression of IGLL5, a protein part of the pre-B-cell receptor.

After calculating immune cell type scores of these immune cell subsets, not only were they well associated with the immunostaining of several immune cells, and could validate the single-cell derived immune signatures, but were also linked to a better patient survival in an historic cohort of untreated melanoma patients, suggesting that they harbour a prognostic value in metastatic melanoma.

When applying the immune signatures to ICI treated cohorts, we concluded that there were elevated levels of the marker gene expression scores across the different single-cell derived immune cell subsets in on-therapy biopsies from responders to ICIs but not in non-responders. This suggests that immune cell subsets suffer alterations after ICI treatment administration in patients with a clinical response.

When investigating the association of the single-cell derived immune marker scores to the clinical response to ICIs, we registered that increased levels of most

lymphocyte marker scores were linked to improved overall survival of patients after receiving ICI treatment. Thus, single-cell derived immune marker scores may have the capacity to predict response to ICIs, similarly to what was observed in the treatment-naïve cohort.

After combining multiple immune signatures into one single immune inflammation signature, and comparing it with TMB in three datasets, a clear grouping of High/Low TMB and lymphocyte score was obtained. These groups proved to be significantly associated with patient survival, with the highly inflamed samples with high TMB to have the best survival. The fact that an association between TMB and the immune signatures and an association between TMB and patient survival in the treatment-naïve cohorts, could not be observed, suggested that the derived immune cell signatures were prognostic in these cohorts.

Overall, we identified immune cell type specific marker genes for metastatic melanoma TME, reflecting transcriptomic signatures of different immune cell subsets. These signatures showed prognostic implications in both treatment-naïve and immunotherapy treated melanoma cohorts. Also, they could have a potential role to serve as biomarkers along with TMB.

Conclusions and future perspectives

Melanoma is a highly complex disease comprised of different layers of molecular information, with many different crosstalk mechanisms involved in the maintenance of the tumour. In the past decade, a lot has changed when the details behind these mechanisms started being studied. Soon after the understanding about concepts such as tumour microenvironment started growing, the clinical management of metastatic melanoma went through profound changes. With the great improvement(s) of therapeutic approaches, such as immunotherapy, came other challenges. Only a small fraction of patients benefit from immune checkpoint inhibitors and severe adverse effects are frequently experienced by some of these patients. For this reason, clinical management of metastatic melanoma demands the tumour immune microenvironment field to be further explored, in order for an efficient selection of patients for specific treatments to become a reality rather than simply a wish. This thesis made some important discoveries related to the tumour microenvironment in melanoma and further clarified some aspects of this field.

While exploring the role of B-cells in anti-tumour responses in melanoma, due to the fact that this is less known in comparison with the role of T-cells, B-cells were seen to form clusters together with T-helper cells in tumours. Tertiary lymphoid structures were known to be an important type of lymphoid aggregate, already documented to develop in several tumour types in humans. In Paper I we show that the presence of these structures is beneficial in a tumour, as they play a key role in the tumour immune microenvironment, improving patient survival and outcome. It has been previously shown that an active immune microenvironment is determinant in the development of a response to immunotherapy agents. Not surprisingly, the presence of TLSs in melanoma tumours, conferred better outcomes upon immunotherapy administration. Thus, in the future, therapeutic approaches to enhance TLS formation and function should be considered for improved clinical outcome and in responses to cancer immunotherapy.

Finding ways to combat immunotherapy resistance has been one of the main topics in melanoma research. It is known that the cause of resistance to immune checkpoint

inhibitors is the activation of immune evasion mechanisms by the tumour. Among these mechanisms, there is the activation of the PI3K pathway through loss of *PTEN*. MITF^{low} cells have also been shown to have the ability to survive harsh conditions, such as the effect of targeted and/or immunotherapy agents, being involved in an immune evasion mechanism. In Paper II, we confirmed the role of PTEN in prognosis, therapy response and immune escape. More importantly, loss of PTEN in conjunction with loss of melanocyte differentiation features, by loss of MITF expression, leads to a worse survival, due to the establishment of an immune poor microenvironment, and the activation of immune evasion mechanisms, rendering the tumours resistant to targeted- and immunotherapy. Although inhibitors of the PI3K pathway are being extensively studied and considered as a therapeutic approach to reverse immune evasion mechanisms and enable checkpoint blockade efficacy, the concomitant reactivation of MITF, with a return of differentiation melanoma features, could be considered in this approach.

Deciphering the immune landscape in tumours is crucial to achieve a better knowledge about the tumour immune microenvironment. Current methods to do it are based on defining the transcriptome of tumours. In Paper III, we show that DNA methylation, a method that has not been extensively studied to distinguish different tumour microenvironments, particularly in melanoma, offers advantages compared to transcriptomic characterization of mixed cellular environments. DNA methylation shows more differences between cellular lineages than gene expression. More importantly, we show that tumours can be grouped according to their level of DNA methylation and this grouping is differentially associated with the level of immune infiltration, activation of immune evasion mechanisms and prognosis. For future considerations, DNA methylation could be used as a biomarker to help in the characterization of the immune landscape of a melanoma tumour, prior to a treatment administration.

Single-cell RNA-seq data of tumour-associated immune cells can be another useful tool to characterize the immune microenvironment of melanoma tumours, particularly by achieving a single-cell resolution of the transcriptional landscape of such tumours. In Paper IV, we derived immune cell signatures based on single-cell RNA-seq data and confirmed their robustness and their prognostic value both in treatment naïve and in treated melanoma cohorts. Also, these immune signatures proved to be successful in their prognostic effect when coupled with TMB, showing their potential to serve as biomarkers, not only individually but together with others, and thus, facilitate future patient selection for treatment.

Collectively, the work included in this thesis addresses some aspects of the tumour immune microenvironment in melanoma. It is, thus, a contribution to a rather long path that still needs to be completed in the field, in order to improve the clinical management of patients in advanced stages of the disease. Finally, this type of knowledge at the molecular level will need to be combined with technological advancement and development of tools that allow the implementation of a personalized medicine.

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