

**Cancer Immunology of Cutaneous Melanoma:
A Systems Biology Approach**

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**Imunologia do Câncer de Melanoma Cutâneo:
Uma abordagem de Biologia de Sistemas**

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Dedication

Dedicated to all affected by cutaneous melanoma and to the researchers, healthcare and all other professionals around the world who fight against cancer.

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...thank you, thank you, thank you

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Resumo

Muñoz Miranda, Mindy Stephania **Imunologia do Câncer de Melanoma Cutâneo: Uma abordagem de Biologia de Sistemas**. 2020. Tese (Doutorado) - Instituto de Matemática e Estatística, Universidade de São Paulo, São Paulo, 2020.

O melanoma cutâneo é um câncer de pele dos melanócitos e é um dos tumores mais agressivos em humanos. Causa um grande número de mortes em todo o mundo e no Brasil morrem aproximadamente 1.500 pacientes com melanoma cada ano. O repositório Gene Expression Omnibus (GEO) contém dados de expressão gênica de sequenciamento de nova geração de diferentes amostras de melanoma cutâneo. A biologia de sistemas parece ser o melhor abordagem na investigação dos mecanismos moleculares do sistema imunológico no melanoma e poderia explicar o escape do tumor, a proliferação, e crescimento em outros tecidos. Propomos uma análise de integração ômica com um abordagem de biologia de sistemas em dados de melanoma disponíveis no GEO. Encontramos genes relacionados à comunicação do sistema imunológico e melanoma. Usamos redes regulatórias de genes expressos, combinando análise de enriquecimento de fator de transcrição, interação proteína-proteína e análise de quinase, para prever marcadores de genes relacionados ao sistema imunológico que podem atuar como reguladores na progressão do melanoma. Distinguimos a interação de *CD74*, em células *CD14+* de pacientes com melanoma, em pacientes com melanoma com *BRAF V600E*, e também em linhagens celulares de melanoma que são resistentes a inibidores de *BRAF V600E*: indicando a presença desta molécula como um dos principais moduladores de comunicação entre células imunes e melanoma, junto com *ENO1*, *S100A6*, *SERPINE2*, *GAPDH* e *UBB*. Todos esses genes estão envolvidos na resposta do Microambiente tumoral (TME) à progressão e tratamentos no melanoma cutâneo. Identificamos *TNFAIP3* tendo um papel exclusivo no melanoma em células *CD14+* e *CD8+*, sugerindo que fatores de transcrição comuns envolvidos em *TNFAIP3* ou aqueles relacionados a este gene poderiam ser alvos de desenho de drogas no melanoma. Propusemos que o gene *FN1* seja modulado pela mutação *BRAF V600E* e tres genes *NQO1*, *ALDOA* e *ATCG1*, que poderiam estar modulados pelo gene *BRAF* com outra mutação ou pelo gene *NRAS* com mutação G13R em linhagens de células de melanoma. Associamos *IFNGR2* como um tipo de receptor para células de melanoma que pode interferir na vias de sinalização do melanoma metastático, e que poderia explicar a resistência aos medicamentos em imunoterapias. Esperamos que isto revele ligações novas e não apreciadas entre o sistema imunológico e a progressão do melanoma.

Palavras-chave: BIOINFORMÁTICA, MELANOMA, IMUNOLOGIA.

Abstract

Munoz Miranda, Mindy Stephania **Cancer Immunology of Cutaneous Melanoma: A Systems Biology Approach**. 2020. Thesis (D.Sc.) - Institute of Mathematics and Statistics, University of Sao Paulo, Sao Paulo, 2020.

Cutaneous melanoma is a melanocyte skin cancer, and it is one of the most aggressive tumours in humans. It causes a significant number of deaths worldwide, and approximately 1,500 melanoma patients die each year in Brazil. The Gene Expression Omnibus (GEO) repository contains high throughput gene expression data, from different samples of cutaneous melanoma. Systems biology seems to be the best approach to investigate the molecular mechanisms of the immune system in melanoma and could explain the tumour escape, proliferation, and growth in other tissues. We proposed an analysis of omic integration with a systems biology approach on melanoma data available from GEO. We found genes related to immune system communication and melanoma. We used regulatory networks from expressed gene data combining transcription factor, protein-protein interaction, and kinase enrichment analysis, to predict markers of immune-related genes that may act as regulators in melanoma progression. We distinguished the interplay of *CD74*, in CD14+ cells of melanoma patients, in melanoma patients with *BRAF V600E*, and also in melanoma cell lines that are resistant to *BRAF V600E* inhibitors: indicating the presence of this molecule as one of the principal modulators of communication between immune cells and melanoma, along with *ENO1*, *S100A6*, *SERPINE2*, *GAPDH*, and *UBB*. All of these genes are involved in the Tumour micro-environment (TME) response to progression and treatments in cutaneous melanoma. We identify *TNFAIP3* as having an exclusive role in melanoma in CD14+ and CD8+ cells, suggesting that common transcription factors involved in *TNFAIP3* or those related to this gene could be drug design targets in melanoma. We proposed that the *FN1* gene is being modulated by the *BRAF V600E* mutation and three genes *NQO1*, *ALDOA*, and *ATCG1*, which could be modulated by *BRAF* with another mutation or by *NRAS G13R* mutation in melanoma cell lines. We associated *IFNGR2* as a type of receptor for melanoma cells that may interfere with the signalling cascade of metastasis melanoma, and that could explain drug resistance to immunotherapies. We hope that this will reveal new and unappreciated links between the immune system and melanoma progression.

Keywords: BIOINFORMATICS, MELANOMA, IMMUNOLOGY.

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Abbreviations

BN	Benign nevi
CAF	Cancer-associated Fibroblast
CCL	Chemokine (C-C motif) ligand
CSD	cumulative sun-induced damage
DCs	Dendritic cells
DEG	Differentially expressed genes
EGF	Epidermal growth factor
FDR	False discovery rate
FEM	Fixed effects models
FPKM	Fragments per kilobase of exon model per million mapped reads
GEO	Gene Expression Omnibus
IFN- γ	Interferon-gamma
KGF	Keratinocyte growth factor
MDN	Melanocytic dysplastic nevi
NK	Natural Killer
PBL	Peripheral blood
PCA	Principal component analysis
PPI	Protein-protein interaction
REM	Random effects models
RGP	Radial-growth phase
RNA-Seq	RNA-sequencing
RPKM	Reads per kilobase of exon model per million reads
ROS	Reactive oxygen species
scRNA-Seq	Single-cell RNA-Seq
RNA	Ribonucleic acid
TAMs	Tumour-associated macrophages
TME	Tumour micro-environment
Tregs	Regulatory T-cells
TSGs	Tumour suppressor genes
t-SNE	t-distributed stochastic neighbour embedding
UV	Ultraviolet
UVA	Ultraviolet A
VGP	Vertical-growth phase

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Chapter 1

Introduction

Melanoma is the most aggressive and therapy-resistant human skin cancer, causing a high number of deaths worldwide [Mai12]. Early detection followed by local excision of the area is critical to prevent metastasis. Patients with metastatic melanoma have an overall 2-year survival rate of only 10%, with a median survival time of 6.2 months [KLL⁺08].

In Brazil, approximately 1,300 melanoma patients died each year [VSV⁺15] until 2015 and it was estimated more than 6,000 new cases were diagnosed in 2018 [VCM⁺19]. According to the Brazilian National Cancer Institute (INCA) the projection for 2018 was 6,260 cases of melanoma, with 1,547 related deaths ¹.

It must be also considered that in Brazil, it is recommended but not mandatory to register all cancer cases. In Brazil, after 2000, both incidences in men and women started to climb and an additional doubling of incidence rates happened moving from 2.52 to 4.84 in men and from 1.93 to 3.22 per 100,000 inhabitants in women. The number of new melanoma cases will rise around the world because of aging populations and high specific melanoma rates in the elderly. It is happening in Brazil, which has one of the youngest populations in the world ². predicting that most of the melanoma cases are to come [dMWB18].

The major risk factor for melanoma is continuous unprotected sun exposure [GG10]. Other contributing factors include family history, genetic susceptibility, the state of the immune system, and other environmental factors [SFG⁺15]. Melanoma is a heterogeneous disease highlighted by reversible changes within lineages of tumourigenic cells. While some cancer cells can reversibly gain competence to form a tumour, others may lose this competence [MM13]. In metastasis, the melanomas grow and can eventually spread to other areas of the body, such as lymph nodes, lung, or brain [SB16].

Tumour cells can attract immune cells that suppress the activity of other immune cells: a process known as evasion. Macrophages, neutrophils, dendritic and other cells respond to different cytokines, so they can become pro-tumours, and suppress the regulatory T cells and certain types of myeloid cells leaving the tumour microenvironment (TME) with two opposing immune responses on one side attacking the tumour, while on the other it is helping it to grow [GHW18].

Understanding the complex interplay between tumour cells, genetic drivers, and microenvironment factors can provide new and effective therapeutic targets [PKJ⁺16]. In this way, it is crucial to monitor the effectiveness of strategies against melanoma progression.

Computational models can then be used to predict the behaviour of the system to different experimental perturbations or biological conditions [KPP13]. When applied to cancer research, systems biology can provide a comprehensive view of dynamic systems, revealing the aberrations of signalling networks within cancer cells and the local microenvironment [WMR14], aid the development of better immunotherapies, helping elucidate the causes of cancer therapy resistance and

¹The Brazilian National Cancer Institute - INCA: <https://www.inca.gov.br/estimativa/taxas-ajustadas/melanoma-maligno-da-pele>

²The Brazilian Institute of Geography and Statistics - IBGE: <https://www.ibge.gov.br/estatisticas/sociais/populacao>

improving drug design.

1.1 Classification of melanoma

Normal skin consists of two layers; the dermis and epidermis. The epidermis layer is abundant in keratinocytes, and has melanocytes and Langerhans cells too. Keratinocytes produce the major structural protein of the skin, keratin, and regulate melanocyte proliferation through E-cadherin, desmoglein-1, and connexins [LSM⁺03].

The melanocytes are surrounded by 5-8 keratinocytes forming the “epidermal-melanocytes unit”. Melanocytes produce melanosome organelles which contain melanin (Figure 1.1), a dark pigment that produces the colouration of the skin, hair, and eyes. These organelles are transferred from melanocytes to keratinocytes, providing protective mechanisms against DNA damage produced by ultraviolet (UV) radiation [KH11].

Primary melanoma cells respond to different stimuli and in the TME generate a heterogeneous tumour. Melanocytic neoplasms can become benign lesions, called melanocytic naevi or nevi (plural of naevus or nevus, respectively, from *nævus*, Latin for "birthmark"), to malignant ones or melanomas.

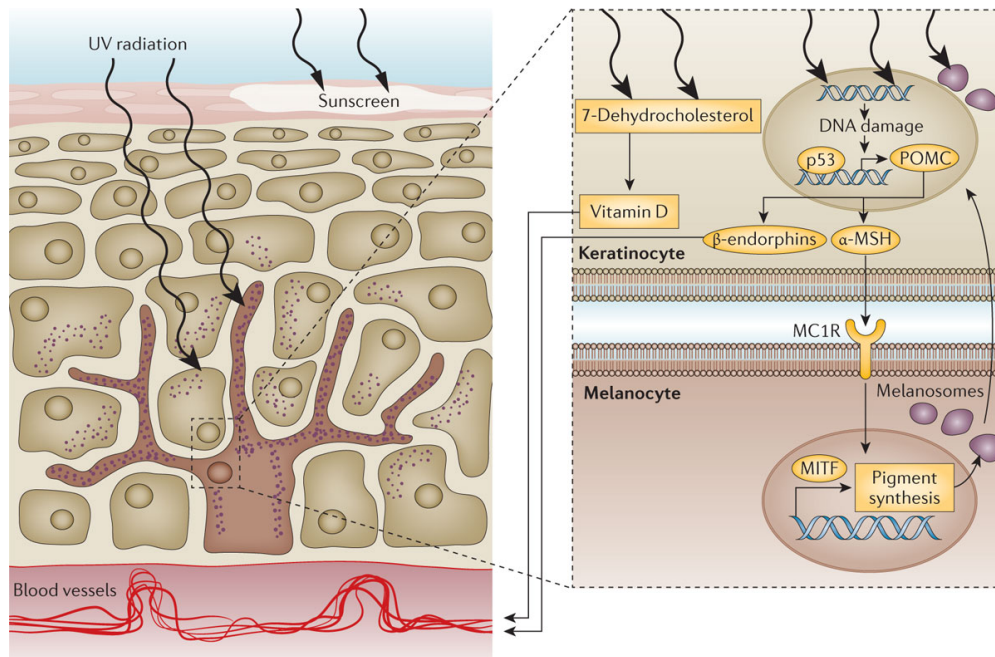
Epithelium-associated Melanomas have four categories:

1. Lentigo and Desmoplastic melanomas, from areas with high UV exposure.
2. Nos-CSD and Spitzoid Melanoma, from areas with low UV exposure.
3. Mucosal Melanoma, a rare variant of cutaneous melanoma that originates in the head and neck region (oral, nasal, and sinus mucosa) and anal/genital mucosal surfaces, from rare sites of origin, which can also include the lower urinary tract, esophagus, small intestine, and gallbladder.
4. Acral melanoma, the least common subtype which occurs on the soles, palms, and nails [SFG⁺15, KM15].

Clark et al., in 1984 recorded lesional steps during melanoma tumorigenesis, together with the clinical classification according to the total thickness in millimeters, mitotic rate, presence of ulceration, penetration depth, and location of existing metastases. In this way, melanoma progression can be distinguished by benign nevi without dysplastic changes (BN), melanocytic dysplastic nevi (MDN) by lentiginous melanocytic hyperplasia or aberrant differentiation or melanocytic nuclear atypia, radial-growth phase (RGP), vertical-growth phase (VGP), and metastatic melanoma [CEG⁺84, Chi03].

The progression represents the first stage of cancer in the melanocytic system, in melanoma, RGP cells can grow indefinitely and independently in contrast to the preceding lesional steps (BN and MDN) with limited and self-controlled growth [HMH02]. The autonomy acquired by RGP cells develops into metastatic melanoma. The modulator gene of the transition into metastatic melanoma is *AKT*, which can induce the expression of the ROS-generating enzyme NOX4, which is sufficient to convert radial growth into vertical growth in melanoma cells. The tumour cells grow vertically and invade the dermis and hypodermis in advanced human melanomas [GSV⁺07].

Therefore, the category of cutaneous melanoma besides of depends on the site where it is located according to the level of UV exposure also depends of the classification which indicate the stage of the disease and the location or site (known as M classification), such as levels in the skin or in nodes, the number of metastatic nodes (N) and size (T) in thickness, and if the ulcer is present, according to the American Joint Committee on Cancer these three types of classification M, N, and T can also be grouped clinically or pathologically according to the stages [GSH⁺17].



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Figure 1.1: *Epidermis surface and molecular interaction between melanocyte and keratinocyte*
In purple melanosomes, organoids which containing melanin, that cross from the melanocytes to keratinocytes to provide protection against UV radiation damage. From Schadendorf et al., 2015 [SFG⁺15].

1.2 Genetics of melanoma

The transformation of normal melanocytes into melanoma cells can involve several dominant genetic alterations [LFKLH14]. The sequential accumulation of genetic alterations include; inactivation of tumour suppressor genes (TSGs) in several chromosomal regions, such as *P16* manifested as a loss of heterozygosity (LOH); hypermutability; activation of oncogenes; and defects in house-keeping genes such as mismatch repair (MMR) genes [HRS⁺01, HST⁺01, Pit01, HRT⁺03, Hus04]. In patients with melanoma, germ line alterations came from two or more close relatives affected by melanoma. Most of them have mutations in the *CDKN2A* locus [FHSA⁺96, GCH⁺06].

Under normal conditions, signaling allows balanced control of cell cycle regulation, survival, motility and metabolism. Through different transcriptional initiation sites, the *CDKN2A* gene encodes two proteins, *P16* or *p16INK4a* and *P14* or *p14ARF*. *p14ARF* predicts the ubiquitination of *MDM2* and the subsequent degradation of *P53* [CGF06]. Thus, inactivating mutations in *CDKN2A* promote the loss of regulators of cellular homeostasis [SFG⁺15]. Specifically these common event in melanoma is the loss of the tumour suppressor protein *p16INK4a* through deletion, mutation, or promoter methylation [CZPS⁺02, YWM⁺14], which normally functions by binding to *CDK4* [BRKM01, YWM⁺14]. And *p14ARF* inhibits *MDM2*, which generally inhibits *P53* function that regulates the DNA damage response and apoptosis [GD14].

Genetic changes are often observed in the mitogen-activated protein kinase (*MAPK*) signaling pathway - currently the highest oncogenic and therapeutic relevance for melanoma - that make changes leaving the constitutive activation, and loss of homeostasis. Although more than 50% of all familial melanomas have an unknown genetic basis [Bas14].

MAPK pathway is depicted as a canonical signalling cascade composed of the small GTPase RAS (*HRAS*, *KRAS*, or the tumour suppressor and negative regulator of RAS; also known as *NRAS*) and the downstream activated kinases *RAF* (*ARAF*, *BRAF* or *CRAF*), MAP/ERK kinase (*MEK1* and *MEK2*; also known as *MAP2K1* or *MAP2K2*) and *MAPK* (*MAPK1* or *MAPK3*; also known as *ERK2* and *ERK1*) [GBC⁺03].

Malignant transformation requires genetic defects sometimes combined [GST⁺14]. Mutations in

NRAS and *BRAF* or other *MAPK* effectors are the most frequent in melanoma, but rarely occur in the same melanoma cell, and are mutually exclusive with only a few exceptions. Also, aberrations in cell-cycle control genes, such as *CDKN2A*, *CDK4* and *CCND1*, coexist rarely [HWK⁺12]. While combinations of mutations in *MAPK* signalling and cell-cycle control cooperate efficiently, coexistence of genetic alterations combined like *BRAF* and *PTEN* (phosphatase and tensin homologue) are seen in 40% of melanomas [HWK⁺12]. Melanomas with *BRAF* or *NRAS* wild type frequently increase in the number of copies of genes for cyclin-dependent kinase-4 *CDK4* and *CCND1* [CPCL05].

Activation of the *CDK4* pathway potentially cooperates with mutant *BRAF* or *NRAS* in the transformation of melanocytes [CAR⁺05, MRK⁺10]. Most mutations in *MAPK* cascade are not attributable to direct UV damage [HWK⁺12]. The somatic mutations that cause malignant melanoma include *BRAF*, *PTEN*, *STK11*, *TRRAP*, *DCC*, *GRIN2A*, *ZNF831*, *BAP1*, and *RASA2*. A large percentage of melanomas (40-60%) carry an activating somatic mutation in the *BRAF* gene, most often V600E [DBC⁺02, PHH⁺03]. Mutations in *BRAF* (50%), *NRAS* (25%), and neurofibromin 1 (*NF1*) (14%), are the most prominent mutations in melanoma and lead to increased proliferation [TWC⁺13]. Other mutations in the genome can include the deletion of the *CDKN2A* locus, the amplification/alteration of *MITF*, and the disruption of phosphatase and tensin homolog *PTEN* gene [HWK⁺12]. While *BRAF*, *NRAS* and *NF1* are common in acral melanoma, *SF3B1*, *CDK4* and *CCND1* are more common in mucosal melanoma [HTW⁺06, XCC⁺19]. However, mutations affecting the *TERT* (Telomerase Reverse Transcriptase) promoter is the most common of all melanomas [HWW⁺17].

In Brazilian melanoma patients the gene mutation rate is as follows: *TERT* 34.3%, *BRAF* 34.1%, *NRAS* 7.9%, together with other genes that have not been previously mentioned; *KIT* 6.2%, and *PDGFRA* 2.9% [VCM⁺19].

Potential high-penetrance melanoma susceptibility genes were identified, *BAP1*, *POT1*, *ACD*, *TERF2IP*, and *TERT*, while two well-established high-penetrance melanoma genes are *CDKN2A* and *CDK4* [SZZ⁺16]. *NRAS* led to the development of highly selective kinase inhibitors that target the *MAPK* pathway [TCGF12] and is capable of activating *AKT* by itself, while *BRAF* needs *PTEN/MMAC1* inactivation [GBC⁺03, TYG⁺04]. The *BRAF* mutation alone is not sufficient to cause melanoma [HTW⁺06], and tumours lacking malignant behaviour presenting only the *BRAF* V600E mutation [CLBM⁺18].

The mutation load increases with progression, intermediate lesions are enriched with *NRAS* mutations. Progression was marked by the appearance of *TERT* promoter mutations in invasive lesions. *TP53* and loss of *PTEN* (deletion of *PTEN*) which is a tumour suppressor responsible for the negative regulation of PI3K/*AKT* signalling, appears in advanced lesions [HTW⁺06, LXB⁺06, SSC⁺04].

Loss of *PTEN* plays an important role in tumorigenesis and angiogenesis [JL09] and promotes tumour development in malignant melanoma [SSC⁺04]. The *RAS-RAF-MAPK* and *PI3K/AKT* signalling pathways are usually strongly upregulated or activated in melanoma patients [LFKLH14]. The inhibition of *BRAF* activity in healthy cells by *RKIP* prevents uncontrolled proliferation and invasion by the deregulation of cellular differentiation-related processes [PAM⁺20]. *RKIP* is responsible for the regulation of the *MAPK* and *NF-κB* pathway, another hyperactivated pathway in melanoma caused by *BRAF* mutations [LBM⁺10].

MAPK and *AKT* pathways are frequently activated in parallel, by genetic means, to promote melanoma development [TYG⁺04], involving *BRAF*, *NRAS*, or growth factor receptors [BVF⁺02, DBC⁺02, FPK⁺10]. However, many other genes and pathways are involved in melanoma development, such as; phosphatidylinositol-3-OH kinase (*PI3K*) signalling pathway [ZGH⁺00]; *STAT* pathways [MYR⁺08, NBH⁺02]; *Notch* signalling pathway [MTF⁺06, LXB⁺06, HMP⁺13, HYS⁺17]; *Wnt* signalling pathway [MMJ⁺16] and *β-catenin* [LD06, DBM⁺07]; *SOX* proteins [BMB⁺10, PVN⁺09]; and vasoactive peptides named endothelins [DDWPD⁺01].

Cutaneous melanoma follows different steps in the metastasis cascade, first melanocytes grow horizontally or radially by proliferating and escaping immune system surveillance [LFKLH14]. Once the endothelium is eventually broken, metastatic melanoma cells can travel to distant sites

[HTB⁺85] a crucial step for melanoma tumorigenesis and further progression [HW03]. Of the different steps in the metastasis cascade, the post-dissemination phase is perhaps one of the least understood, and metastasis is responsible for the majority of deaths in cancer patients [LFKLH14]. During the process of invasion and metastasis formation melanocytes can dedifferentiate acquiring stem cell features that may contribute to their survival [GM11] and make them more resistant to therapy [KMR⁺19].

Dedifferentiated cells are characterized by low pigmentation and can switch to a differentiated phenotype induced by factors from the microenvironment [KHK⁺17]. The four phenotypes of melanoma cell plasticity were recently identified as undifferentiated, neural crest-like, transitory and melanocytic [TRP⁺18]. These stages can be defined by the expression of a set of genes: *MITF*, *AXL*, receptor tyrosine kinases (*RTKs*), paired-box transcription factor (*PAX3*), *TEAD*, *TYR*, *MLANA*, *NF- κ B*, *YAP* and *EDN* signalling [KMR⁺19]. A diagnostic biomarker signature for melanoma has been proposed, comprising the following genes: *EGFR*, *FGFR2*, *FGFR3*, *IL-8*, *PTPRF*, *TNC*, *CXCL13*, *COL11A1*, *CHP2*, *SHC4*, *PPP2R2C*, and *WNT4* [LPT13]. Recent publications describe *DNMT1* as a promising biomarker for cutaneous melanoma-specific survival [LWX⁺18] also a mediator of DNA methylation changes and contributor to malignant transformation [MTB17]. Transcription factors of these genes are mostly associated with the *Jak/STAT* signalling pathway.

Although these key genetic drivers are often required for transforming normal melanocytes into tumour cells, the surrounding microenvironment of the tumour is another essential factor for melanoma progression [SB16]. A wide range of microenvironmental factors, including hypoxia, inflammation, cell composition of tumour stroma, different concentrations of gases, nutrients, growth factors, and especially in melanoma the ultraviolet radiation, collectively contribute to malignant progression [LH07].

1.3 The damage stressor in the skin

UV irradiation directly activates specific transmembrane receptors, such as the epidermal growth factor (EGF) receptor and the keratinocyte growth factor (KGF) receptor, which go on to activate downstream pathways initiating the production of reactive oxygen species (ROS) [LS10]. The cells of the immune system react to ROS stress by secreting lipids or lipid oxidases. When stressed, the cells undergo apoptosis and release lipids onto the microenvironment which act as signals to the immune system. Melanoma cells take up palmitic acid lipids and may promote melanoma cell growth, and that lipid stimulates *AKT* phosphorylation by increasing *PI3K* expression [KFL⁺14].

The UVA stress produced in melanocytes, keratinocytes, or fibroblasts induces oxidative effects. Healthy cell activity products can also form ROS in various cellular compartments and in the TME [SKK⁺19] which might inhibit *PTEN* [CB09].

The sun-damaged dermis may thus contribute directly to hyper melanosis, a hyperpigmentation disorder. Skin hyperpigmentation usually results from an increased number, or activity, of melanocytes. The process starts with the oxidation of arachidonic acid that generates the leukotrienes LTC₄ and LTD₄, the thromboxane TXB₂, and the prostaglandins PGE₁, PGE₂ and PGF₂- α . These factors stimulate melanocytes which transfer more melanin to surrounding keratinocytes. Various messengers (cAMP, cGMP, diacylglycerol) and effectors (*MAPK*, *PKC*, *PKA*) are mobilised [LRM13, CH07].

Melanogenesis is stimulated by local damage to the skin resulting in the overproduction and upregulation of *ET-1* (endothelin-1), *SCF* (stem cell factor), or KGF and induces DNA adduct formation leading to the *P53* mutation [Gas00, KHA⁺02]. At organelle level, mitochondrial cell dynamics are significantly increased when malignant amelanotic melanoma A375 cells are exposed to Simulated Sunlight Irradiation (SSI), which can be inhibited by glutamine, suggesting that damage to mitochondrial dynamics could play an essential role in skin cancer development [ZGL⁺11].

1.4 Immune cell interaction in melanoma

Immune cells can infiltrate the TME and play a key role in the host response to cancer. Malignant cells frequently express antigens that can be recognised by host immune cells, allowing the stimulation and expansion of tumour-specific T cells [BL07]. For instance, analysis of the TME in patients with melanoma has identified CD8+ T cells (also known as TC, cytotoxic T lymphocyte, CTL, T-killer cell, cytolytic T cell, CD8+ T-cell or killer T cell) whose cytokine production revealed low amounts of IFN- γ and perforins production when analysed ex vivo [HKP⁺06, MPM⁺03]. However, those T cells can restore their properties in vitro through cytokine stimulation, suggesting that a major component of this dysfunction is reversible [GSF13].

In melanoma, T cells that infiltrated tumours express chemokines and *CCL21* which then recruit naive T cells and activate Dendritic cells (DCs) [MFE⁺12, HMP⁺09]. The expression of *LIGHT* or the *TNF* superfamily 14 protein (TNFSF14) has also been detected, and in mouse models, they induce lymph nodes-like structures in vivo [YLL⁺04, MSN⁺05]. Also, in the melanoma microenvironment the presence of CD8+ T cells is associated with the production of CCL2, CCL3, CCL4, CCL5, CXCL9 and CXCL10 [HMP⁺09], these last two compromise CXCR3 on the surface of CD8+ T cells effectors [KTG⁺99, DNG⁺10].

CD4+ T cell helper T lymphocytes or Th lymphocytes that are a subset of lymphocytes have a crucial role in establishing and maximizing the defense capabilities of the immune system and play a key role in tumour immunity [STG⁺16]. This T cell subset is critical for orchestrating immunological anti-tumour responses, which involve the secretion of effector cytokines such as IL-4 [TCL92], and IFN- γ [XAM⁺10], the signals for the recruitment of eosinophils and macrophages [HHLW⁺98, CSL⁺05], as well as helping CD8+ T cells [STG⁺16]. In addition, regulatory T cells (Tregs), or other types of suppressive cells, as well as immunosuppressive cytokines (e.g. TGF- β , TNF- α , IL-1, IL-6, CSF-1, IL-8, IL-10) can be part of the mechanism by which tumours evade immune surveillance [VRP⁺15].

The lack of function of T cells in the TME melanoma allows tumour outgrowth, showing high expression of PD-L1 and indoleamine-2,3-dioxygenase (IDO), and display high infiltration of CD4⁺Foxp3⁺ cells [Gaj07]. The upregulation of PD-L1 and IDO is driven by IFN- γ produced by CD8+ T cells in vivo that produce CCL22 via CCR4 and CCL1 to recruit Tregs cells [SSZ⁺13, KTO⁺16].

CD14+ monocytes have the potential to transform into macrophages, and DCs also are capable of trans differentiation into multipotential cells and endothelial cells [SKN⁺16]. Nonetheless, genetic instability (“immunoediting”) of proliferating tumour cells can eventually produce antigens with reduced immunogenicity, allowing immune system evasion [VRP⁺15].

Monocyte populations in the blood are distinguished in classical “inflammatory” monocytes (IMo, CCR2High CD14⁺⁺CD16⁻) and nonclassical “patrolling” monocytes (PMo, CX3CR1High CD14⁺CD16⁺) [CP16]. CX3CR1⁺ patrolling monocytes rarely extravasate into the tissue and differentiate into macrophages [AFG⁺07], but inhibit metastatic progression through the recruitment of NK cells to the metastatic site. Macrophages that differentiate from circulating classical monocytes after extravasation into tissues, respond to infections and tissue injuries, playing a pivotal role in tissue homeostasis and repair [LMRM15].

Melanoma high-grade tumour-associated macrophages (TAMs) correlate with C-C chemokine ligand (CCL) recruitment [TKdH⁺16, ST15], and promotes the defective cytotoxicity of T cells [TPM⁺19]. TAMs also directly stimulate cancer cell proliferation through the secretion of epidermal growth factor EGF, VEGF and metalloproteinases (MMPs) [OLMH93, SZW⁺08, KPW10] and corrupts the extracellular matrix a known event during tumour formation [CW02] being correlated with a poor prognosis [ZLG⁺12, NP14].

1.5 Cancer immunology in cutaneous melanoma

Cancer immunology studies interactions between the immune system and tumours, malignancies, or cancer cells. This field of research aims to discover cancer immunotherapies to treat and retard the progression of the disease. The immune response, including the recognition of cancer-specific antigens, has helped us understand the target to develop new treatments (such as new vaccines and antibody therapies) and tumour marker-based diagnostic tests.

Over the past 30 years, there has been notable progress and accumulation of scientific evidence for the concept of cancer immunosurveillance and immunoediting [DOS04, Rib16]. Within the new RNA-Seq technologies, the transcriptome of single cells isolated from melanoma patients has allowed us to identify malignant cells in distinct transcriptional cell states [BAvH⁺], as well as exhausting tumour-infiltrating T cells, to provide novel insights into signalling pathways implicated in metastasis [TPM⁺19]. The interaction between the tumour and the immune system, with stimulatory and inhibitory signalling pathways, limit the T-cell antitumor response and cancer cell detection in the metastatic spread to distant sites [SA15].

Since 2010, cancer immunotherapies consist of a wide range of strategies for novel drug design and new therapies both to directly modify the tumour or indirectly enhance immunogenicity by altering the microenvironment [MGWW20]. Depending on the features of the tumour the standard treatment in melanoma patients consists in surgical resection, chemotherapy combinations, radiotherapy, photodynamic therapy (PDT), immunotherapy, or targeted therapy [DLSP18].

Immunotherapy appears the favourable option for patients with advanced malignant melanomas when compared to previous standard therapies [GLC17]. Melanoma immunotherapies approved by the FDA or in trials include interferon α -2b/peginterferon α -2b, interleukin-2, onk, ipilimumab, nivolumab, pembrolizumab, talimogene laherparepvec, durvalumab, CK-301, avelumab, atezolizumab, gp100 vaccine, resiquimod, and CAR-T cells [DLSP18].

The majority of these are immune checkpoint inhibitors (ICIs) that target cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1/programmed cell death ligand 1 (PD-1/PD-L1) [SOS11]. Even so were reported cases of patients with increased tumor progression from immunotherapy with CTLA-4 and PD1 inhibitors, suggesting genetic tests in patients with a planned anti-PD1/PDL1 monotherapy [KGW⁺17], and where proposed outstanding questions to identify patients with Hyperprogressive disease (HPD), but the universal criteria is still needed [ASM⁺20]. Viral vaccines developed for melanoma show promising results in clinical trials; for example, a weakened version of the herpes simplex virus modified to produce an immune-stimulating factor [ST18]. Primary and acquired resistance to immunotherapy is common, maybe due to the lack of recognition by T cells and it can involve the interaction, preventing immune cell infiltration of regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and M2 macrophages [SHLWR17].

Immunotherapies involve different types of immune cells and heterogeneous tumour cells, interacting in a dynamic TME. Although cancer immunotherapies are proliferating, there is still a lot to be done to improve cell targeting, reduce the toxicity, and increase the knowledge of cell products [MGWW20]. Understanding the complex interplay between tumour cells, genetic drivers, and microenvironment factors can provide new and useful therapeutic targets [PKJ⁺16].

1.6 Goals

We perform an integrative omics analysis on melanoma samples in order to discover new potential immune regulators related to tumour evasion and progression.

1.6.1 Specific goals:

- To extract and process transcriptomic data from melanoma samples

- To identify signatures of melanoma progression, focusing on immune-related genes and pathways
- To integrate layers of biological information
- To generate novel insights into how melanoma tumour cells can evade immune surveillance

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Hardware

The analyses were run on 64 cores and 254 GB of RAM server, located in the Clinical Analysis Laboratory based at the School of Pharmaceutical Sciences at the University of São Paulo, belonging to the CSBL (Computational Systems Biology Laboratory).

2.1.2 Software

The server operating system (OS) used was Ubuntu 16.04.1 LTS (GNU/Linux 4.4.0-38-generic x86_64). The bioinformatics packages were obtained from Bioconductor [GCB⁺04], an open-source software project for the analysis and comprehension of high-throughput genomic data. Bioconductor is based on R statistical computing environment and graphical visualization environment <https://www.bioconductor.org/>.

We also used our packages developed in the laboratory already deposited in Bioconductor, as well as scripts stored in GitHub <https://github.com/csbl-usp/csbl> open-source platform to host and review code, manage projects, and build software used by more than 35 million developers. We used open-source online tools, such as X2K [CKS⁺18] that infers upstream regulatory networks from signatures of differentially expressed genes (DEG) <https://amp.pharm.mssm.edu/X2K>, NetworkAnalyst [ZSE⁺19, XGH15] a visual analytics platform for comprehensive gene expression profiling and meta-analysis <https://www.networkanalyst.ca/>.

We used CEMiTool [RFC⁺18] R package. This tool aims to perform a comprehensive modular co-expression analysis, adding key information about the whole gene set, and in subnetworks hereinafter referred to as modules. Also, the tool enriches the module by specific pathways. The modules are grouped by expression genes profiles, identifying genes with most connections and close to another in the same module, highlighting and labeling the genes with a higher degree and higher betweenness, referred as Hubs. The CEMiTool package uses the WGCNA [LH08] R package, calculating essential input parameters of WGCNA and adding them automatically like new features <https://cemitool.sysbio.tools/>.

We used the Cell Illustrator (Genomic Object Net: I. 2004) v5.0 Trial License software tool that enables biologists to draw, model, elucidate, and simulate complex biological processes and systems. GeneMANIA [WFDC⁺10] was used to predict the function of gene sets of expression genes tables results <https://genemania.org/>, Enrich [CTK⁺13, KJR⁺16] to enrich the lists of cells of the immune system from DEG lists <https://amp.pharm.mssm.edu/Enrichr/>, and for the detailed search of the functions of most genes, the tool GeneCards [SRP⁺16] Version 4.14 from GeneCardsSuite www.genecards.org.

2.1.3 Datasets

Single-cell RNA-Seq samples were obtained from Gene Expression Omnibus(GEO) [BWL⁺13], from the studies made public until October 31st, 2018. In order to obtain a list of publications for melanoma-related single-cell RNA-Seq technology, we made a query search for studies in NCBI PubMed.

We performed a query of PubMed using the combinations of the following keywords: (melanoma [Title] OR cutaneous skin melanoma [Title] OR metastatic melanoma [Title]) AND (Single-cell RNA-Seq [Title/Abstract] OR scRNA-Seq [Title/Abstract]), we did not find publications with raw data available, until the date mentioned above, but we find bulk transcriptomes of individual immune system cells added to the dataset. Bulk RNA-Seq samples comes from blood samples from patients with melanoma and healthy donors presented in the last line of the Table 2.1.

The query of this last search include: (melanoma [Title] OR skin cutaneous melanoma [Title] OR metastatic melanoma [Title/Abstract]) AND ("Immune cell" [Title/Abstract] OR "Immune System" [Title/Abstract]) AND (RNA-Seq [Title/Abstract] OR RNA sequencing [Title/Abstract] OR Single cell [Title/Abstract] OR single-cell RNA-Seq [Title/Abstract] OR scRNA-Seq [Title/Abstract]) AND ("2015/09/01" [Date - Publication]: "2018/10/31" [Date - Publication]).

The ID series GSE72056 belong to 19 patients with melanoma the samples come from freshly resected melanoma tumours, containing a total of 4,645 cells, classified in malignant, non-malignant cells or unresolved cells based on inferred CNVs (Copy Number Variations). The inferred cell types for non-malignant cells include T cells, B cells, Macrophages, Endothelial cells, and CAFs. We select 9 patients with malignant and no malignant cells (n=2,395), we separate all the groups of cells into malignant, T cells, B cells, Macrophages, Endothelial, Cancer-associated Fibroblast (CAF) and Natural Killer cells (NK), in order to obtain the same groups of immune cell-based annotation generated by Tirosh and collaborators.

We first analyzed the expression profile in a general way, with the 4645 cells all together, and then we we analyze separately by cell type of the 9 patients in order to obtain relatively homogeneous samples of cell types of the immune system and also we choose patients with different phenotypic and clinical features, leaving the characteristics of the patients as heterogeneous as possible.

The series ID GSE108397 have 327 melanoma cells, these cells belong to 451Lu (n=197) and A375 (n=130), each one with treatment to *BRAF V600E* inhibitor (1 μ M PLX-4720) and without treatment classified as parental. The treated cells were gradually increasing the dosage of *BRAF V600E* inhibitor (BRAFi) from 0.05 to 1 μ M on both cell lines and selecting for cells that survived after each round of treatment, derived a distinct population of resistant cells classified as BRAFi-resistant cells.

We selected the expression data of the A375 cells (n=130), since this cells lines shows gene expression data according to the experimental design [MCS⁺16], And PLX-4720 significantly inhibits the growth of tumor cell lines A375 (bearing the *BRAF V600E*) with GI50 of 0.50 μ M [TLW⁺08]. GI50 is the concentration for 50% of maximal inhibition of cell proliferation.

The series ID sample GSE81383 comes from three patients with genetic conditions: double-negative *BRAF V600E* /*NRAS* wild type, *BRAF V600E* mutant and *NRAS* wild type, *BRAF V600E* wild type and *NRAS* G13R mutant, the biopsies were used to sequenced with scRNA-Seq applied to 307 single-cells. To use these samples in the meta-analysis, we put together the double wild type *BRAF V600E* /*NRAS* with *BRAF V600E* wild type and *NRAS* mutant, and in the other group *BRAF V600E* mutant/*NRAS* wild type.

The dataset with GEO accession GSE104744 contains three kinds of immune cell types: CD4+ T lymphocytes, CD8+ cytotoxic T cell, and CD14+ monocytes from 132 patients in the IV melanoma stage and healthy donors, 64 and 68 respectively.

2.2 Methods

Were separated the methodology into three parts according to the type of analysis and data available as follows (Figure 2.1):

Serie ID	Publication date	Samples	Metastatic melanoma	Health	Source	Reference
GSE108397	Jul 01, 2018	327	327	0	Melanoma cell line	Wang L. et al., 2018 [WFK ⁺ 18]
GSE81383	Jan 12, 2017	307	307	0	Subcutaneous metastasis	Gerber T. et al., 2016 [GWLW ⁺ 16]
GSE72056	Apr 05, 2016	4645	1258	3257	Melanoma tumors	Tirosh I. et al., 2016 [TIP ⁺ 16]
GSE104744	Oct 10, 2018	132	64	68	PBL (CD4+, CD8+, CD14+)	Ho YJ. et al., 2018 [HAM ⁺ 18]

Table 2.1: Transcriptomic melanoma samples used for the analysis from the GEO database until October 31th, 2018. The table shows four datasets were used in this work, and the results of the query used to search melanoma-related single-cell sequencing detailed in the Datasets subsection, the first three lines was ordered by the best match, and the last line including the raw data of bulk RNA-Seq of the individual cells from patients with melanoma and healthy donors, results of the second query related.

- Co-expression analysis and enrichment pathways from the data of all cells of melanoma patients, we performed co-expression networks separated by modules. We did enrichment pathways analysis to the immune-related pathways. We selected co-expression modules. We separated the entire gene expression matrix by immune cell type. We selected patients by clinical information. We shown the modules most relevant in the analysis.
- Differential expression analysis was done on the T cells and monocytes from patients with melanoma versus healthy donors. we visually saw the genetic expression using tSNE plot, violin plot and Heatmap, as well as the differential expression with Dot plot and Venn diagram. Finally we showed regulatory networks of the immune system pathways with selected upregulated genes.
- Meta-analysis of melanoma cells with *BRAF V600E* and *NRAS* mutation, were we group in *BRAF V600E* versus *BRAF V600E* Wild type giving more relevance to the *BRAF V600E* gene, but also if the exercise were seen from the other side, the *NRAS* genes versus Wild type could be appreciated. For the meta-analysis purposes, the priority was *BRAF V600E* versus Wild type combining with the BRAFi-resistant versus Parental (no treated) melanoma cell lines.

2.2.1 Transcriptomic analysis

After download, the raw data were processed with the new Tuxedo pipeline [PPA⁺15]. The paired-end reads were mapped to the UCSC hg38 human reference [SGLH⁺17] using TopHat2 [KPT⁺13] with the default setting. Transcripts levels were quantified by Cufflinks [TRG⁺12] and Stringtie [PPA⁺15] to get the fragments per kilobase of mapped reads (FPKM) to obtain an expression matrix and calculate the DEG. We compared the results of correctly assembled isoforms, and we chose the Stringtie outputs to make the differential expression analysis (data not shown). We filtered the expression data removing the rows with FPKM = 0. To place similar cells together in low-dimensional space, we perform nonlinear dimensionality reduction techniques with the Seurat R package [BHS⁺18].

The pipelines and tools in the bulk and single-cell RNA-seq analysis can be used for the same different stages [STM15]. Seurat performance was compared versus ComBat [JLR07] and limma [RPW⁺15] widely used batch correction tools for both bulk and single-cell data [CMT⁺16]. Seurat gave the best results about the score used in the analysis [BHS⁺18].

We visualized into clusters with Barnes-Hut implementation of the t-distributed stochastic neighbour embedding (t-SNE) algorithm [Maa14] a shared-nearest neighbour (SNN) graph based on distance matrix for modularity-based clustering [WvE13]. We used the FindVariableFeatures

function with 2000 features and two methods: vst and mvp used to generate the clusters and to search markers (respectively).

Both methods explained below:

- vst: calculate local polynomial regression (loess), standardise and calculate the variance of the values of the observed mean and expected features and finally clips to a maximum.
- mean.var.plot (mvp): calculate average expression and dispersion for each feature. Divide features into 20 bins and calculates z-scores for distribution within each bin. The purpose of this is to identify variable features while controlling for the strong relationship between variability and average expression.

The bulk of Seurat’s differential expression features was performed through the FindMarkers function. We used default parameters based on the non-parametric Wilcoxon rank-sum test, filtering with a p-value less than 0.05 for each cell type.

We analyzed differential expression genes of T cells and monocytes (n=132) from patients with melanoma versus healthy donors. The data came from series ID GSE104744 [WFK⁺18].

2.2.2 Melanoma gene network construction and pathway enrichment analysis

Data analysis is related to the immune system, and we focus on the skin immunological defense and pathways. We use the immunologic signatures database (also called ImmuneSigDB) collection generated by manual curation of published studies in human and mouse immunology [GTL⁺16] at Dana-Farber Cancer Institute and the Human Immunology Project Consortium (HIPC) and Gene Ontology (GO) (C5, based on GO terms). The GO terms in the collection belong to one of three GO ontologies: molecular function (MF), cellular component (CC), or biological process (BP), and the collection is divided into sub-collections accordingly from Molecular Signatures Database (MSigDB).

To construct the networks with putative immune-related regulatory genes, interactions will be inferred from protein-protein interaction (PPI), composed by gene sets that represent cell types, states, and perturbations within the immune system through InnateDB [BFL⁺13].

We generate a co-expression network system-level functionality with CEMiTool [RFC⁺18] in order to obtain modules of co-expressed genes. The modules with a greater number of genes related to melanoma were selected and analyzed more carefully, we got from them the immune system pathways.

We got lists of genes from immune system pathways to use as input on the construction of upstream regulatory networks. This final network combined transcription factor, protein-protein interaction (PPI), and kinase enrichment analysis, which was created using X2K web tool [CXG⁺12]. CEMiTool and X2K were used for series ID GSE72056 data analysis, for the entire data set and for each of the malignant and non-malignant cells separately.

NetworkAnalyst was used for the meta-analysis together with series ID GSE108397 and GSE81383. The differential expression analysis of individual data sets was performed with limma. The cut-off p-values were adjusted using Benjamini-Hochberg’s False Discovery Rate (FDR) of 0.05. The purpose of a meta-analysis is used to combine different datasets for increased statistical power and usually refers to horizontal data integration to multiple expression data [XGH15].

We adjusted batch effects with Combat, option embedded in the same tool. And we used the Fisher’s method that combines the p-values from the studies for information integration ($-2 \times \sum \text{Log}(p)$) and Cochran’s Q tests that combine and compare across studies with two popular methods: fixed and random effects models (FEM and REM). In FEM, the estimated effect size in each study is assumed to come from an underlying actual effect size plus measurement error. In REM, each study further contains a random effect that can incorporate unknown cross-study heterogeneities in the model (i.e. due to different platforms).

Effect size is the difference between two group means divided by standard deviation. The method usually gives more conservative results (less DEG but more confident). We used different p-values

with Fisher to compare the results, but none of them could generate a visualisation. We applied Cochran's Q Test (FEM and REM) and we followed the protocol of the tool [XGH15], that procedure typically one big subnetwork with several smaller ones. The subnetworks have at least three nodes. The significant genes (seeds) were mapped to the molecular interaction literature-curated database InnateDB (Breuer et al., 2013). The networks for each study were enriched by KEGG [KFT⁺17] and Reactome [FSV⁺17] databases embedded within the tool.

GeneMANIA [WFD⁺10], Enrichr [KJR⁺16] and GeneCards [SRP⁺16] were used for identified function and perform the enrichment analysis of the DEG from the transcriptome analysis with Seurat on the data series ID GSE104744.

We generated gene networks of protein-protein interaction for the scRNA-Seq sets grouping them for the different types of analysis as follows:

- Co-expression analysis and enrichment pathways from the data of all cells of melanoma patients, containing the gene expression matrix of 4,645 cells of 19 patients. We filtered the matrix taking 9 patients of which we obtained 2,395 cells, we also separated the cell type leaving groups of 1,087 T cells, 328 B cells, 46 CAFs, 111 Macrophages, 35 NK cells and 727 malignant melanoma cells. The data came from series ID GSE72056 [TIP⁺16].
- A meta-analysis of melanoma cells, tissue samples came from 3 patients with melanoma with *BRAF V600E* and *NRAS* mutation (n=307), we grouped *BRAF V600E* (n=230) versus *BRAF V600E* wild type (n=77), and melanoma cell cultures with A375 "Resistant" to *BRAF V600E* inhibitors (BRAFi) cell lines (n=52) named BRAFi-resistant versus "Parental" or no treated A375 cell lines (n=78). The data came from the series ID GSE108397 and GSE81383 [HAM⁺18, GWLW⁺16].

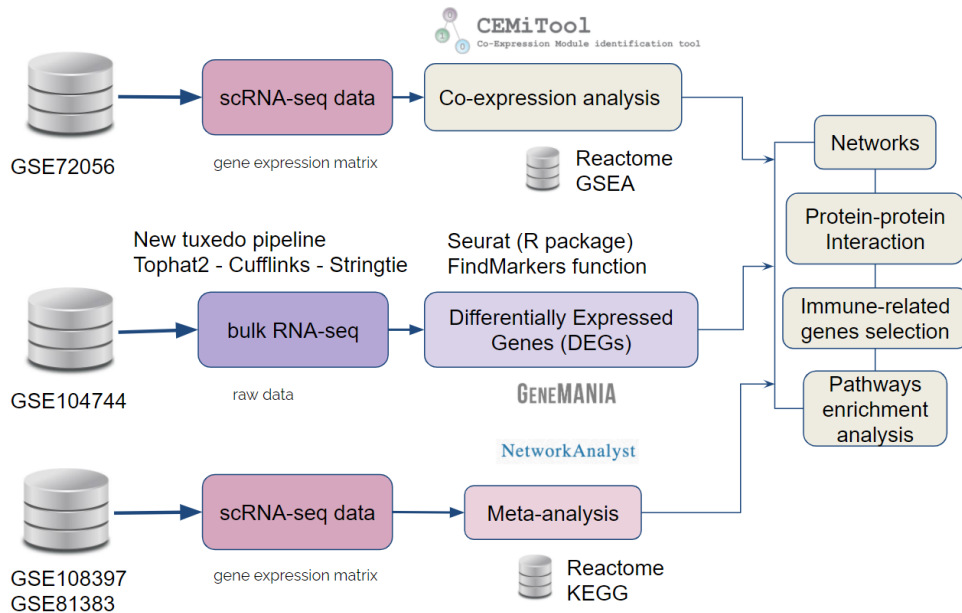


Figure 2.1: Workflow performed to the "Co-expression analysis, Differential expression analysis and Meta-analysis" of melanoma samples.

We generated PPI networks using Reactome, KEGG and GSEA databases. The software used has the logo as an image in each of the steps. Each datasets came from the series ID described in the materials.

Chapter 3

Results

The results were separated into three parts according to the time we were getting the data from the repository and according to the novel insights generated to complement what was already being published. And they will be presented according to the detailed structure as follows:

- Co-expression analysis and enrichment pathways from the data of all cells of melanoma patients: The result of the co-expression networks was separated by the modules with their respective pathways enrichment. We selected two co-expression modules of a total of 4 generated with the entire data set with 19 melanoma patients (n=4,645). We also analyzed separately by immune cell type, for that we selected 9 patients with 2,395 cells, we separated into 6 groups between malignant and non-malignant cells, from which we only selected 2 modules from 2 non-malignant cell types, macrophages (n=111) and natural killer (n=35). Which gave us crucial information for further analysis.
- Differential expression analysis of T cells and monocytes (n=132) from patients with melanoma versus healthy donors.
- Meta-analysis of melanoma cells with *BRAF* and *NRAS* mutation, where we group in *BRAF* *V600E* (n=230) versus *BRAF* wild type (n=77), and melanoma cell cultures with BRAFi-resistant (n=52) versus Parental (n=78) cell lines.

3.1 Data analysis in immune cells of single-cell RNA-Seq from melanoma patients

The single-cell RNA-seq data from Tirosh et al. (2016) GEO accession GSE72056 [TIP⁺16] contains the expression of 35,945 transcripts. The whole expression matrix was used to analyze the main pathways associated, among all immune cells, we analyzed 2 modules with the following associated enriched pathways: “Interferon Signaling”, “Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell”, “Cytokine Signaling in Immune system”, “PD-1 Signaling”, “MHC class II antigen presentation”, “Translocation of ZAP-70 to Immunological synapse” and “Phosphorylation of CD3 and TCR zeta chains” (Figure 3.1).

Some of these enriched pathways related to the immune system were carefully chosen to identify cytokines relating to melanoma. We highlight hubs found in these two modules, such as *CCL5* and *CD79A*. Wakefield group proposes *CD79A* as a novel target for cancer therapy demonstrating being upregulated on circulating myeloid cells from lung cancer patients [LYR⁺13]. *CD79A* is a known target gene of *PAX5*, which participates in B-cell tumorigenesis [METB11].

We performed a patient’s clinical data reduction-based within the most significant number of cells and filtering by alive patients (Table 3.1 3.2).

We found genes related to melanoma in almost all immune cells. We identified, for example, the *CDKN1A* gene as a hub in Natural Killer (NK) cells (Figure 3.2). We found in this module the maximum number of genes related to melanoma interacting within the other genes from the

<i>Patient</i>	<i>T cell</i>	<i>B cell</i>	<i>Macro</i>	<i>Endo</i>	<i>CAFs</i>	<i>NK</i>	<i>M</i>	<i>Unclass</i>	<i>Total</i>
Mel 67	65	19	0	0	0	1	0	10	95
Mel 72	117	35	0	0	0	1	0	28	181
Mel 74	118	13	5	0	0	1	0	10	147
Mel 94	129	65	2	19	16	1	10	122	364
Mel 84	61	25	25	1	1	7	11	28	159
Mel 53	72	0	12	11	4	10	16	18	143
Mel 89	201	106	26	1	0	1	98	42	475
Mel 88	112	16	41	1	2	9	112	59	352
Mel 80	212	49	0	29	23	4	125	38	480
Total	1087	328	111	62	46	35	372	355	2396

Table 3.1: Selected melanoma patient from the GSE72056 dataset. The table contains the number of cells of each patient selected for the analysis. The characteristics were carefully selected based on the greater variability (Table 3.1). The labels indicate Macro for macrophages cells; Endo, for endothelial cells; CAF, Cancer-associated Fibroblast cells; NK, Natural Killer cells; M, malignant cells; and unclass for cells that could not be identified.

Sample ID	Age/Sex	Mutation status	Pre-operative treatment	Site of resection
Melanoma 67	58/Male	<i>BRAF V600E</i>	None	Axillary lymph node
Melanoma 72	57/Female	<i>NRAS Q61R</i>	IL-2, Nivolumab, ipilimumab + anti-KIR-Ab	External iliac lymph node
Melanoma 74	63/Male	not available	Nivolumab	Terminal Ileum
Melanoma 94	54/Female	Wild type	INF, Nivolumab, ipilimumab	Iliac lymph node
Melanoma 84	67/Male	Wild type	None	Acral primary
Melanoma 53	77/Female	Wild type	None	Subcutaneous back lesion
Melanoma 89	67/Male	not available	None	Axillary lymph node
Melanoma 88	54/Female	<i>NRAS Q61R</i>	Tremelimumab + MEDI 3617	Cutaneous
Melanoma 80	86/Female	Wild type	None	Axillary lymph node

Table 3.2: Clinical features from melanoma patients selected. The table contains the characteristics of each patient carefully selected from the GSE72056 dataset based on; The mutation status; Site of resection; Pre-operative treatment; Age and sex.



Figure 3.1: Co-expression analysis with enrichment pathways from all cells of melanoma patients.

We generate networks of co-expression between all cells from the Tirosh et al., 2016 data set (GSE72056) with CEMiTool. Two modules related to the immune system are shown. The bar plots on the left display the enrichment pathways of each module on the right. The networks on the right illustrate the PPI of genes. Hub genes are labelled in blue to indicate co-expression and in brown indicate interaction, the size of the spot represent the degree of each gene.

expression matrix, also co-expressed genes. The size of the dot indicates the degree of each node or gene, the highest degree highlighted with a label mentioned as hub genes, the co-expression is labelled in blue and the interaction with genes enriched in the module pathways in brown.

In macrophages, we observed the same, the maximum number of genes related to melanoma in one module interacting with co-expression genes highlighting the enrichment pathway of the genes in the module corresponding to the “Inflammatory response” (Figure 3.3). Here we find the genes hub *IL1B*, *TNFAIP3*, *NFKBIA* and *PPP1R15A*. To understand the transcription factor and kinases involved in this module, we searched transcription factors and kinases in upstream regulatory networks, using the X2K web tool (Figure 3.4).

3.2 RNA-seq transcriptome analysis of T cells and monocytes, from melanoma patients and healthy donors

The results shown below come from the datasets based on T lymphocyte cells CD4+ and CD8+, and CD14+ monocytes, series ID GSE104744. The displayed t-SNE graph-based clusters were determined with the expression data co-localized T cells and monocytes (Figure 3.5) where we identify the group in green belongs to CD14+ cells, group in blue to CD4+ cells and group in red to CD8+. Also, we were able to identify the status of the 132 samples with the features coloured in pink for healthy donors and in light blue for melanoma patients.

The Seurat package defines the clusters by markers for specific cell populations, represented by one or two cells to classify them correctly. We graph distribution plots of gene expression of some marker genes of immune cells with violin plots (Figure 3.6). The markers genes: *CD8A*, *CD14*, *CCL5*, *S100A4*, *ANXA1*, *CCR7*, *CD74*, *CD3D*, and *ISG15* shows differences in the expression level for each cell, indicating a higher level of expression in *S100A4* and *CD74* in comparison with the other genes in all cells. We also perform scaled gene expression average values coloured from grey to

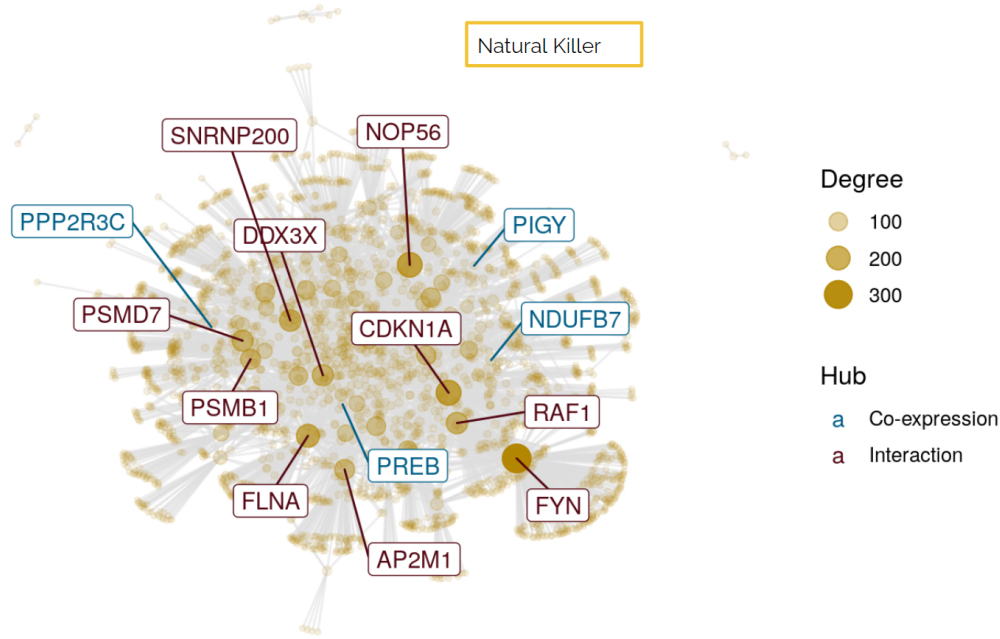


Figure 3.2: Immune-related module from Natural Killer cells from the co-expression analysis.

We identified *FYN*, *CDKN1A*, *NOP53*, *FLNA*, *RAF1*, *SNRNP200*, *DDX3X*, *AP2M1*, *PSMB1*, *PSMD7* labelled in brown, being hub genes with the maximum degree. This module contains the maximum number of genes related to melanoma interacting within the co-expression genes from the NK gene expression matrix. The co-expression genes hub in this module are labelled in blue; *PPP2R3C*, *PIGY*, *NDUFB7*, and *PREB*.

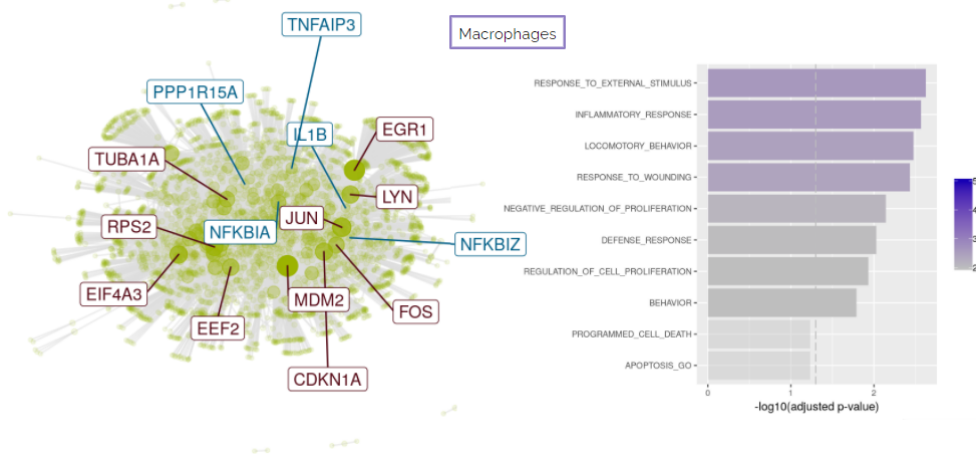


Figure 3.3: Immune-related modules from Macrophages and their enriched pathways from the co-expression analysis.

The enriched pathways with statistical significance in this module corresponding to: “Response to external stimulus” “Inflammatory response”, “Locomotory behaviour”, “Response to wounding”, “Negative regulation of cell proliferation”, “Defense response”, “Regulation of cell proliferation” and “Behavior”. The hub co-expressed genes labelled in blue *IL1B*, *TNFAIP3*, *NFKBIA*, *PPP1R15A*, and *NFKBIZ*. Genes with the highest degree in brown; *TUBA1A*, *RPS2*, *EIF4A3*, *EEF2*, *EGR1*, *LYN*, *JUN*, *FOS*, *MDM2* and *CDKN1A*.

blue in the different cell types based on the relative variance explained for each immune cell using tSNE visualization (Figure 3.7).

All of the markers of some cell types and also the last two seeing where they are mostly expressed. The *CD8A* gen mark CD8+ cells, *CD4* to CD4+ cells, and *CD14* is the CD14+ monocyte marker.

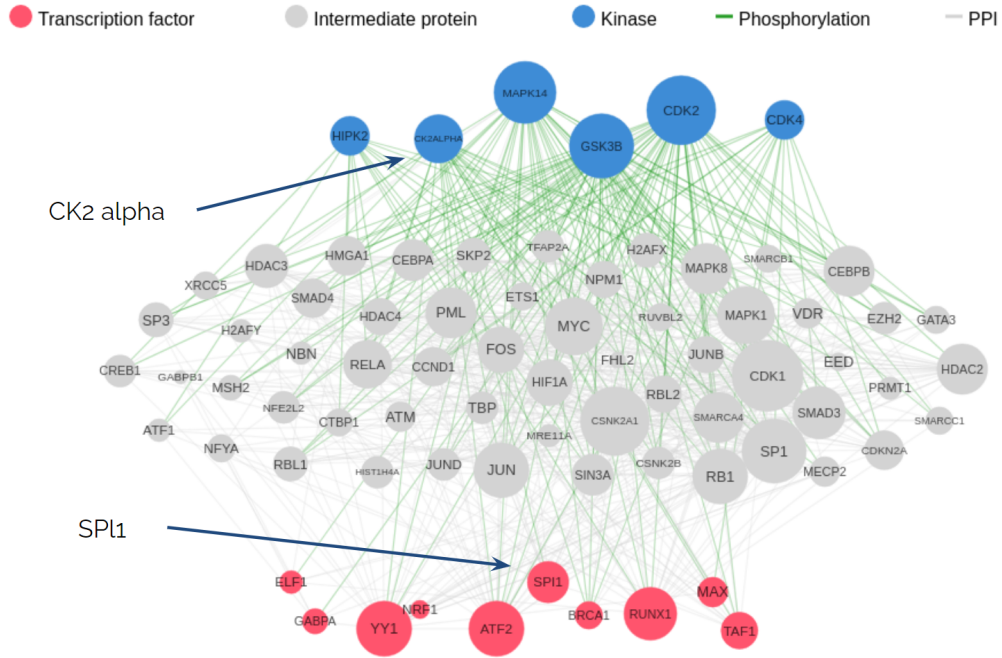


Figure 3.4: Upstream regulatory network applied to the genes from the “Inflammatory response” pathway of the selected module from Macrophages.

In the upstream network regulatory transcription factor (TF) are colored with pink, the searched genes are present in intermediate proteins in grey. Both TF and intermediate proteins are connected by phosphorylation (green lines) or protein-protein interaction (grey lines) between them or with kinases in blue, we highlight the CK2 alpha kinase and the *SPI1* transcription factor that in addition to being connected with intermediate proteins, they are directly connected by phosphorylation.

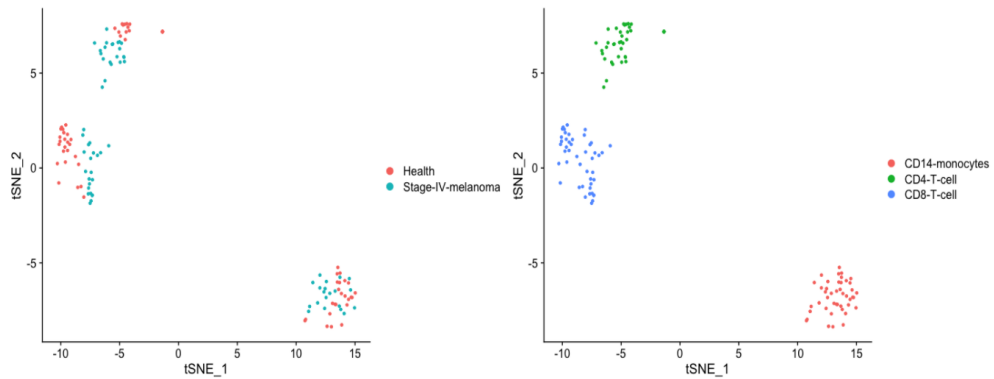


Figure 3.5: tSNE plot from expression data of RNA-seq from T cells and monocytes of melanoma patients and healthy donors.

Each dot in the figure represents 1 of the 132 RNA-Seq samples from GSE104744 dataset identified by categories. On the left pink dots correspond to healthy donors labeled as “Health” and with light blue patients with stage IV melanoma labeled as “Stage-IV-melanoma”, in the image of the right the same t-SNE but this time shows the identification of each immune cell type into the 3 clusters. In pink CD14+ labeled as “CD14-monocytes”, in green the CD4+ T cells labeled as “CD4-T-cell”, and with blue CD8+ T cells labelled as “CD8-T-cell”, these two last clusters mentioned belonging to T cells being closer to each other and separate from the one belonging to monocytes.

The *GNL* gene is associated with cytotoxic T lymphocytes and natural killer cells, *CD3E* mark T-lymphocyte cells, *LYZ* associated with the monocyte-macrophage system. *CD40* is a B-Cell Surface Antigen, *MIF* and *FCGR3A* the Fc Fragment of IgG Receptor IIIa that is involved in the removal of antigen-antibody complexes from the circulation. All the expression matrix was plotted on a Heatmap (Figure 3.8). We differentiated the cell types in the melanoma condition. We obtained 180

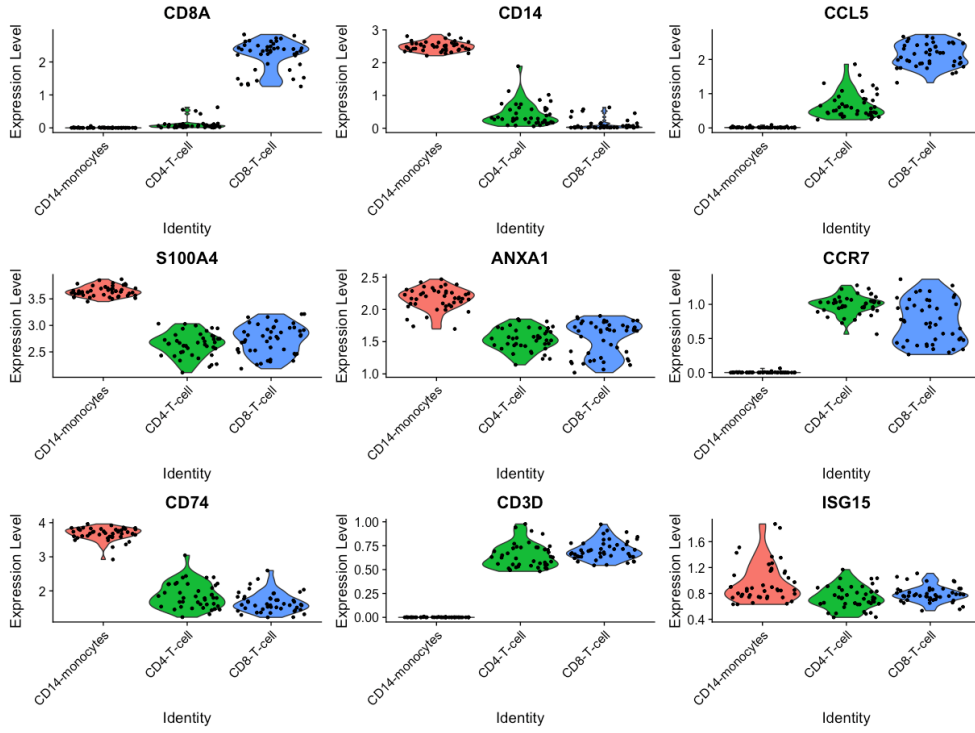


Figure 3.6: Violin plots showing the distribution of expression level of some immune marker genes. The markers genes: *CD8A*, *CD14*, *CCL5*, *S100A4*, *ANXA1*, *CCR7*, *CD74*, *CD3D*, and *ISG15*, grouped by identity cells; CD14+ in red; CD4+ in green; CD8+ T cells in blue.

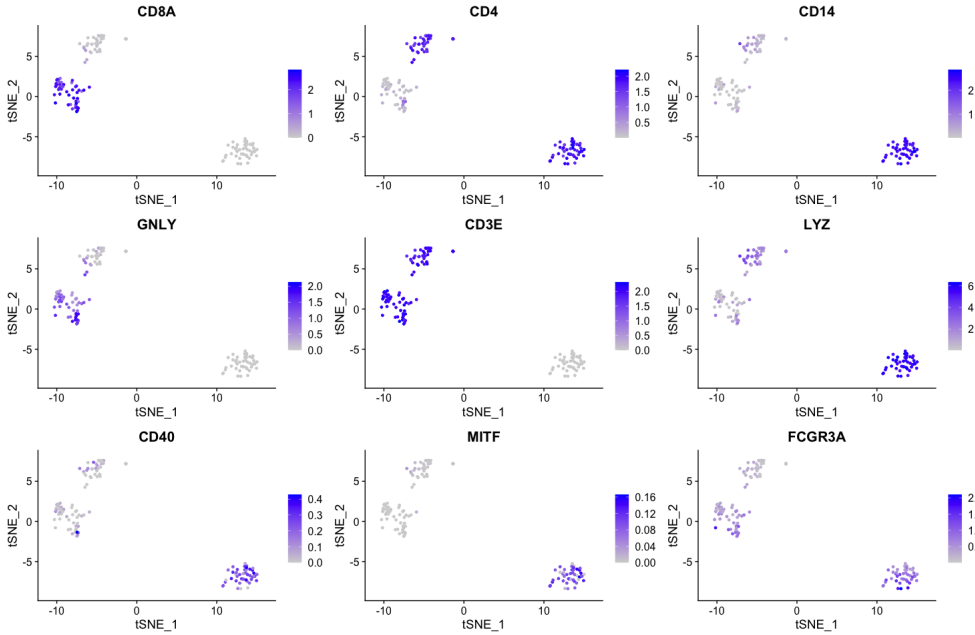


Figure 3.7: tSNE plots for marker genes showing the average expression values.

The scaled expression values show the population of immune cells (n=132 cells), of the following genes: *CD8A*, *CD4* and *CD14* in the first row of the figure. The *GNL*, *CD3E*, and *LYZ* in the second row of the picture. And in the third row, the first gene is mostly used as a B cell marker being *CD40* and the other two genes are related to the disease, *MITF* the Melanocyte Inducing Transcription Factor most expressed in monocytes and *FCGR3A* or the Fc Fragment Of IgG Receptor IIIa also expressed in monocytes and CD8+.

DEG for CD4+, 97 DEG for CD8+ and 21 DEG for CD14+ in total, detailed in Table 3.3. The lists of all the DEG between the cells were intersected (Figure 3.9).

<i>Cells</i>	<i>Upregulated</i>	<i>Downregulated</i>	<i>Total</i>
CD4+	40	140	180
CD8+	49	48	97
CD14+	13	8	21
Total	102	196	298

Table 3.3: Summary of DEGs from T cells and monocytes between melanoma patients and healthy donors. The table contains the total number of DEGs of each immune cell CD4+, CD8+, and CD14+. Among patients with melanoma in stage IV and healthy donors. We separated between upregulated (second column) and downregulated (third column) genes, the last column containing the total DEGs of each cell type.

Title	Serie ID	Matched Gene Symbol	Source	Sample condition 1	Sample condition 2	DEG
BRAF V600E v/s wild type	GSE81383	19019	Subcutaneous metastasis (n=307)	<i>BRAF V600E</i> / <i>NRAS</i> wild type	BRAF wild type/ <i>NRAS G13R</i> and BRAF wild type/ <i>NRAS</i> wild type	2,122 (701 Up and 1,421 Down)
BRAFi resistant v/s parental cell lines	GSE108397	27001	Melanoma cell line (n=130)	A375-Br (resistant)	A375-Par (parental)	6,126 (5,892 Up and 234 Down)

Table 3.4: Meta-analysis details of all samples used for each comparison to perform de DEG combining. The table contains the details of the data sets of the GSE81383 and GSE108397 series ID, in the first column the title of the study to distinguish, the third column contains the number of genes from each study, the fourth column has the number of total cells of each study, the last column the DEG obtained from each comparison between the conditions described in the columns "Sample condition 1" and "Sample condition 2".

We selected the 5 most upregulated genes and the 5 most downregulated ones to compare the expression of these genes in the cells (Figure 3.10).

The pathways enrichment analysis was generated for the upregulated and downregulated genes separately of each cell type with GeneMANIA, we were able to generate networks connected by physical interaction, co-expression, predicted genes, colocalization, pathways, genetic interactions, and shared proteins domains. Also, we identified in which pathways the genes are located colouring the node according to the enriched pathway. We generated networks from leukocyte differentiation genes, from up regulated lists of all the immune cells. The tool allows us to use known gene symbols, we did not manage to enrich the genes of CD14+ cells and this analysis was gendered only for T cells (Figures 3.11 - 3.12).

3.3 Meta-analysis of single-cell RNA-seq between "*BRAF V600E* versus Wild-type" and "BRAFi-resistant versus Parental" melanoma cells

The carried-out p-value combination in the meta-analysis must be done after the differential expression between different conditions or treatments within the cell groups. The meta-analysis revealed genes of cells BRAFi-resistant versus parental have a similar expression pattern with *BRAF V600E* vs wild type. The comparisons were carried on according to Table 3.4.

The meta-analysis matched 17,942 Gene Symbols of both datasets, with samples with the conditions that we specify in Table 3.4, columns five and six. Then we adjust the study batch effect

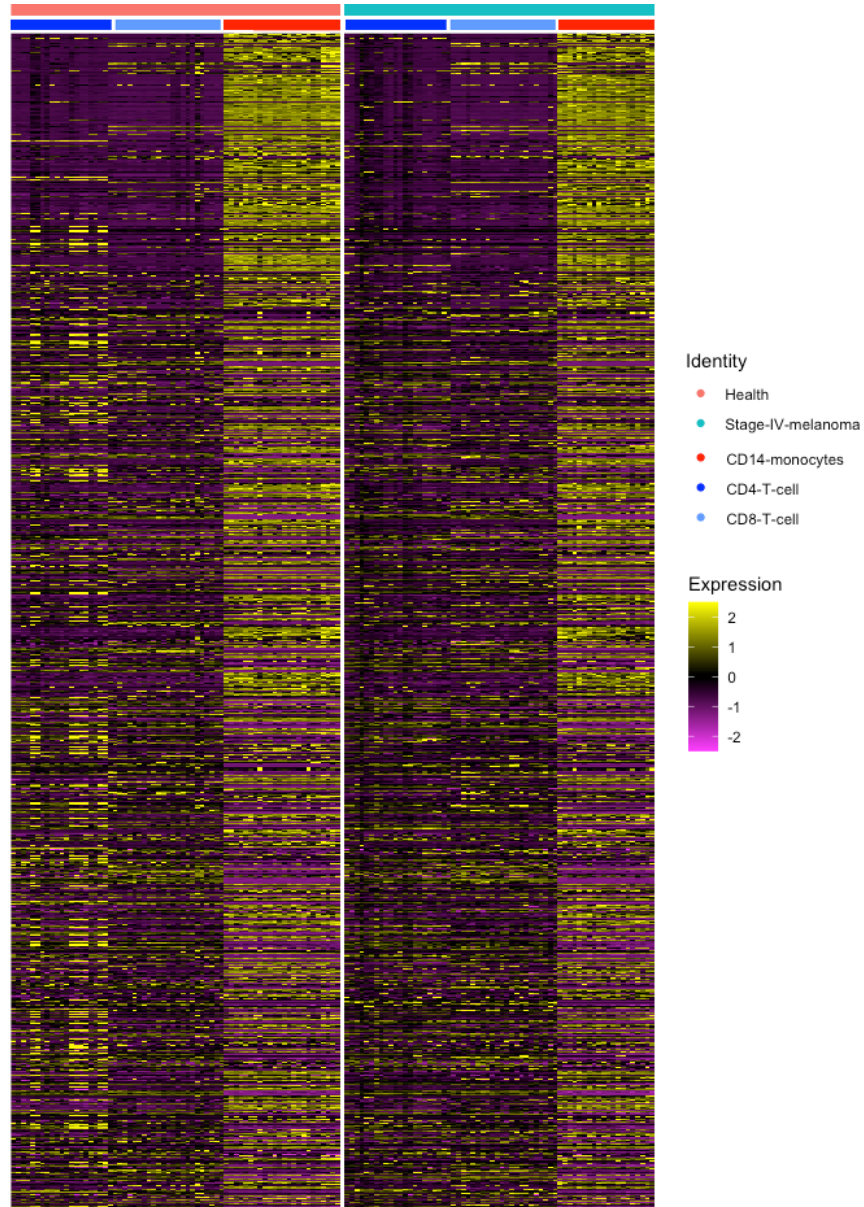


Figure 3.8: Heatmap of gene expression values of T cells and monocytes from melanoma patients and healthy donors.

The Heatmap contains the 132 cells expression values, the colour of the expression varies between violet and yellow, from the lowest to the highest expression respectively. We separated between tumour stages and immune cell types, with light blue and pink to identify the melanoma patients and healthy donors respectively and with red, blue, and light blue to identify CD14+, CD4+, and CD8+ cells respectively.

with Combat. We performed Fisher's Method combining p-values, setting by different significance levels, the results are shown in Table 3.5. Network visualisations can only be done with 10,000 nodes or less. We also performed Cochran's Q Test combining effect sizes, selecting fixed and random effects models (FEM and REM). The results of FEM are shown in Table 3.6, setting by different significance levels.

The first visualizations of the meta-analysis were performed with Cochran's Q Test of 0.05 significance value, showing in the first line of Table 3.6, we obtain; 3424 DEG, a big sub network with; 12,579 nodes, 75,724 edges, and 3,085 seeds; and 2 sub networks of 3 nodes, 2 edges and 1 seed. To reduce the number of nodes for the visualization (less than 10,000) was applied the "Strainer Forest Network" option getting a big sub network with 2,987 nodes, 2,986 edges and 2,773 seeds. With this method, we only obtained up regulated genes in the network (Figure 3.13). Even so, we

<i>Significance value</i>	<i>DEG</i>	<i>Nodes</i>	<i>Edges</i>	<i>Seeds</i>
0.05	7,932	14,066	125,299	7,530
0.01	5,729	13,485	110,130	5,218
0.005	4,960	13,295	102,936	4,538
0.001	3,6295	12,8165	89,3095	3,345
0.0005	3,170	12,671	84,399	2,940
0.0001	2,302	12,197	69,944	2,166

Table 3.5: Network details from meta-analysis using Fisher’s method. The meta-analysis contains varied features to network creation, depending on the significance value. With both dataset described in Table 3.4 was applied Fisher’s method combining p -values. The table show different significance value in the first column, descending from 0.05 to 0.0001, which made change in the number of DEGs, nodes, edges, and seeds in the succeeding columns. The software deploys networks with the seeds number which means the total DEGs used to create the subnetworks.

<i>Significance value</i>	<i>DEG</i>	<i>Nodes</i>	<i>Edges</i>	<i>Seeds</i>
0.05	3,424	12,579	75,724	3,085
0.01	1,927	11,748	53,691	1,774
0.005	1,507	11,401	47,548	1,395
0.001	1,005	10,950	36,358	943
0.0005	846	10,825	33,357	794
0.0001	575	10,548	27,397	545

Table 3.6: Network details from meta-analysis using Fixed effect model with Cochran’s Q Test method. Using the same dataset of Table 3.5, was applied the Fixed effect model option to combine transcriptomes underlying the effect size plus measurement error. The table show different significance value in the first column, descending from 0.05 to 0.0001, which made change in the number of DEGs, nodes, edges, and seeds in the succeeding columns. The software deploys networks with the seeds number which means the total DEGs used to create the subnetworks.

<i>Significance value</i>	<i>DEG</i>	<i>Nodes</i>	<i>Edges</i>	<i>Seeds</i>
0.05	830	6,606	20,217	776
0.01	516	5,149	13,441	480
0.005	457	4,864	12,174	426
0.001	289	3,960	8,694	273
0.0005	252	3,668	7,694	238
0.0001	178	2,921	5,470	167

Table 3.7: Network details from meta-analysis using Random effect model with Cochran’s Q Test method. Using the same data of Table 3.6, was applied the Random effect model option to combine transcriptomes, taking into account that each study contains a random effect that can incorporate unknown heterogeneity. The table show different significance value in the first column, descending from 0.05 to 0.0001, which made change in the number of DEGs, nodes, edges, and seeds in the succeeding columns. The software deploys networks with the seeds number which means the total DEGs used to create the subnetworks.

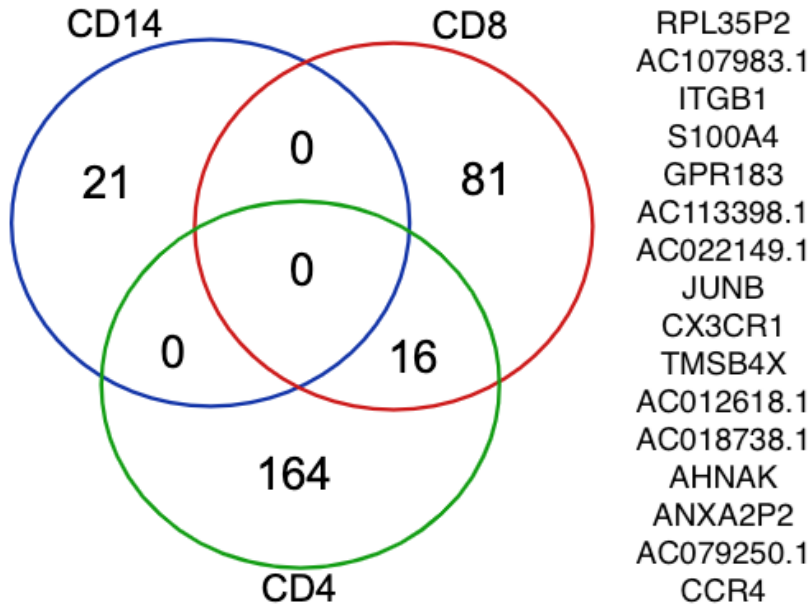


Figure 3.9: Venn Diagram of DEG intersection between T cells and monocytes from melanoma patients versus healthy donors.

The figure shows the number of differential expression genes of each cell, between melanoma patients versus healthy donors. CD14+ in blue with 21 unique DEG, CD8+ in red with 81 unique DEG and CD4+ in green with 164 unique DEG. The 16 genes belonging to the intersection between CD8+ and CD4+ would not serve as markers of each T cell, but could serve as a disease marker for both T cells in melanoma patients, their gene symbols were written to the right of the Venn diagram.

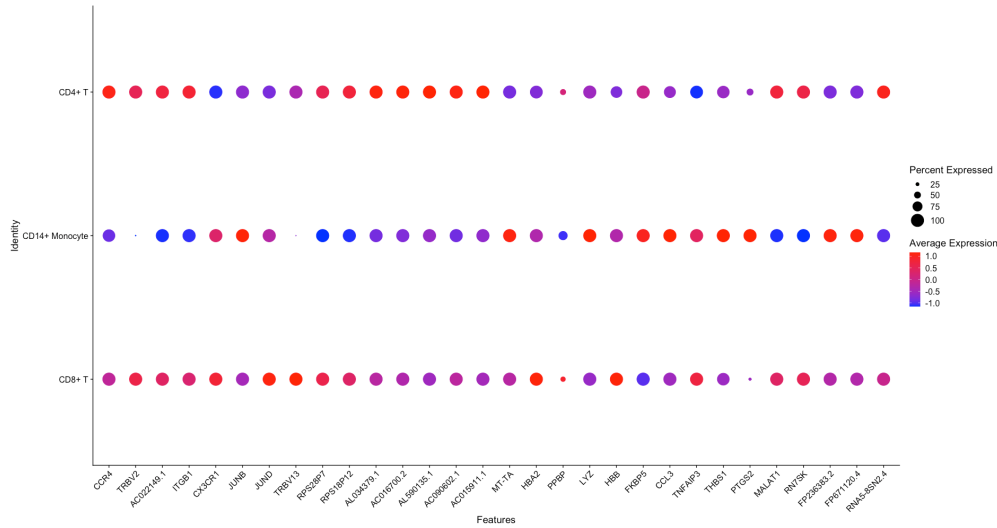


Figure 3.10: Dot plot of the first most upregulated and downregulated DEGs from melanoma patients versus healthy donors.

The average expression varies from blue to red, being blue the lowest expression. The size of each dot indicates the percentage expressed for each immune cell type, varying every 25 to 100%.

selected the most regulated genes, with greater betweenness and filtering by degree greater than 2 and logarithmic fold change (FC) greater than 0.5, because they could be positively responsible for the resistance to BRAF inhibitors (Figure 3.14).

We also performed Cochran's Q Test combining effect sizes with the Random effects models (REM), that as mentioned in the methods, generating less DEG in comparison with the previous methods, and this time including down regulated genes in the analysis. The results are shown in

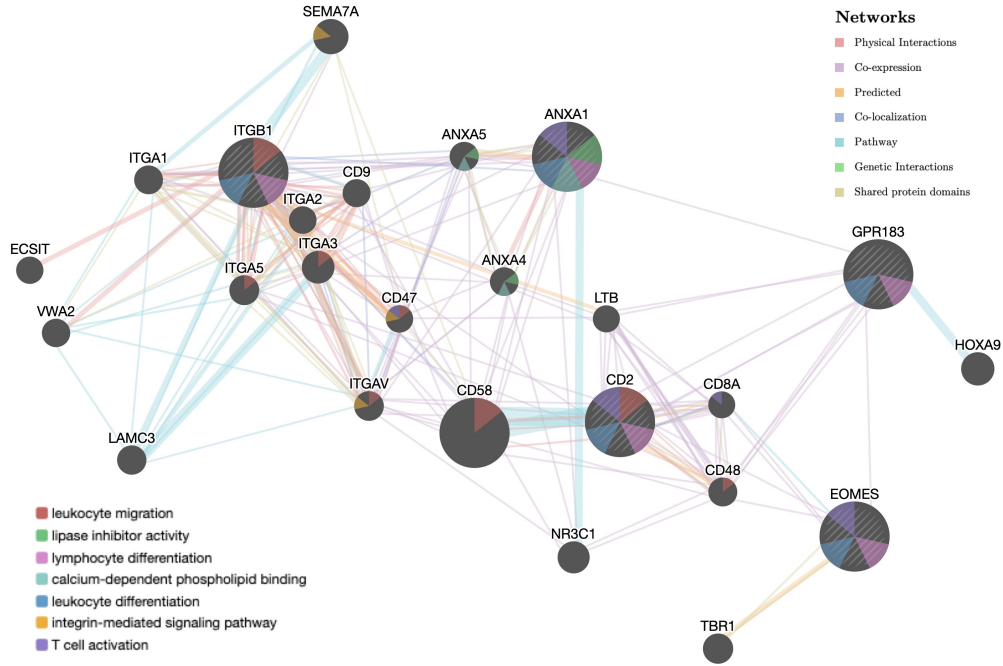


Figure 3.11: Networks and enriched analysis from lymphocyte differentiation genes of *CD8⁺ T cells*. The nodes with lines are the selected genes: *EOMES*, *CD2*, *GPR183*, *ANXA1*, and *ITGB1*. The pathways identified in most of these genes were integrin complex, leukocyte migration, extracellular matrix organization, lipase inhibitor activity, calcium-dependent phospholipid binding, leukocyte differentiation, and T cell activation.

Table 3.7, with different significance values for the model. The visualizations of the meta-analysis with Cochran’s Q Test of 0.05 with REM resulted in a big sub network with; 830 DEG; 6,606 nodes; 20,217 edges; and 776 seeds. Was not necessary to apply any reduction method to decrease the node numbers and visualize the network (Figure 3.15).

With REM and FEM, we obtained a greater number of upregulated nodes, and FEM had only upregulated genes, perhaps most coming from the study series ID GSE108397, indicating the REM method was best performed for our meta-analysis.

With this method we saw in detail a subnetwork looking in the DEG the most significant pathways. Embedded in the tool within the meta-analysis network, we looked for enriched pathways in upregulated genes with Reactome, highlighting; “Cell adhesion molecules (CAMs)”, “Th1 and Th2 cell differentiation”; “Toxoplasmosis”; “Tuberculosis”; “Epstein-Barr virus infection”; “Leishmaniasis” (Figure 3.16).

We marked with a blue border all the genes belonging to Th1 and Th2 cell differentiation and appeared: *IFNGR2*, *MAPK12*, *MAPK1*, *STAT6*, *RBPJ*, *MAML3*, *RUNX3*, *MAF* and several HLA class II histocompatibility antigens such as *HLA-DMB*, *HLA-DPB1*, *HLA-DRA*, *HLA-DRB1*, *HLA-DRB5*, *HLA-DQA1*, *HLA-DQA2* and *HLA-DPA1*.

We show in the following figures a more detailed analysis of the DEG shown in Table 5. We made a cross comparison of the upregulated lists with the downregulated lists between the datasets, interestingly, we found 908 genes that are downregulated in the first dataset (1,421) within the upregulated genes of the second dataset (5,892), within them we find *STAT3*, *CD63*, *CCL5*, and *TP53*.

On the other hand, the genes that are being upregulated within the first dataset (701) and downregulated in the second dataset (234), are possibly responding to *BRAF* inhibitor treatments and are exclusive regulated by the V600E mutation, these 17 genes from the intersection, where: *CCDC80*, *IRS1*, *IGFBP7*, *AEBP2*, *MGST1*, *FN1*, *PLAGL1*, *IL7R*, *NUAK1*, *SERPINE1*, *SOX9*, *CUL4B*, *GRIP1*, *ELL2*, *TGFBI*, *PDGFC*, *HMGA2*.

To identify if any of these genes are the most regulated in each list, we separated the networks by selecting the 30 most upregulated and the 30 most downregulated. The 30 most regulated in all

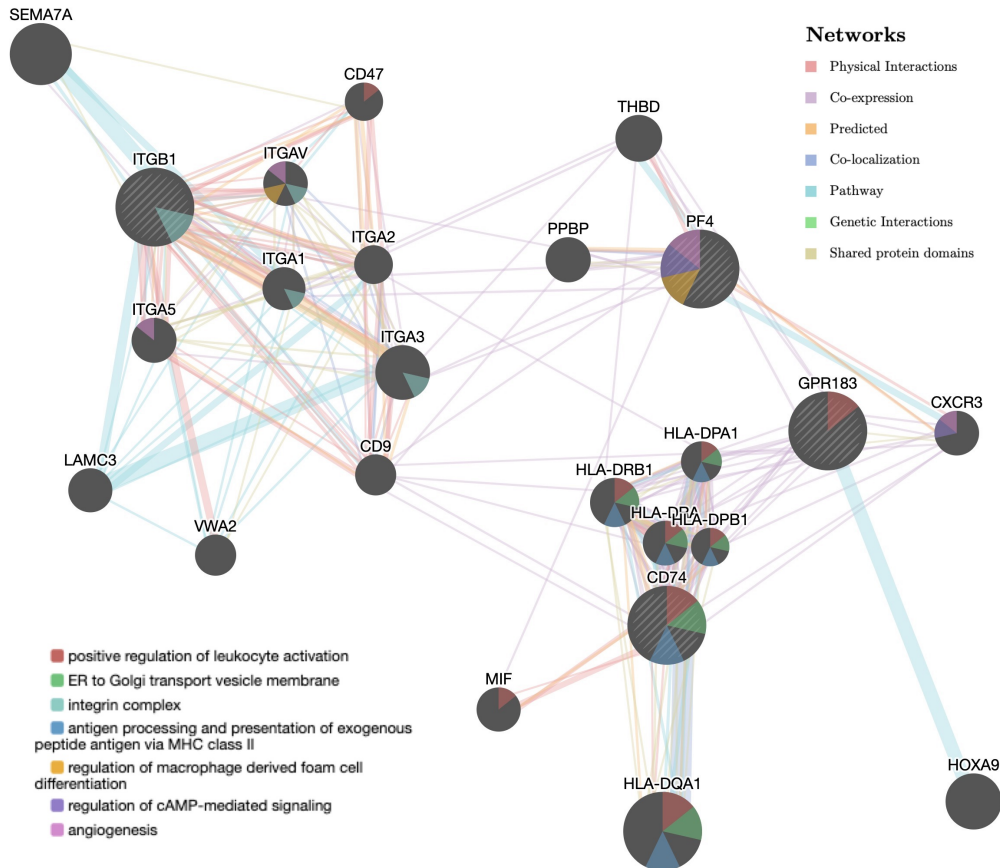


Figure 3.12: Networks and enriched analysis from lymphocyte differentiation genes of *CD4⁺ T cells*.

The nodes with grey lines are the selected genes: *CD74*, *GPR183*, *PF4*, and *ITGB1*. The pathways identified in most of these genes was positive regulation of leukocyte activation, ER to Golgi transport vesicle membrane, integrin complex, antigen processing and presentation of exogenous peptide via MHC class II, regulation of macrophage-derived foam cell differentiation, regulation of cAMP-mediated signalling, and angiogenesis.

comparisons were highlighted in the network interaction, keeping the position and distance of the nodes and hiding the other nodes in the network. In the advanced options we also selected views of Expression (Standard), Layout: Force Atlas, and we curved the lines for networks, in that way the labels of the nodes will be more visible. We highlight the genes that are found intersected in both comparisons of 30 most upregulated: *UBB*, *CD74*, *GAPDH*, *SERPINE2*, *S100A6*, *ENO1* (Figures 3.17-3.18). We perform the same analysis for the 30 most downregulated in all comparisons (Figures 3.19-3.20).

We found *FN1* most upregulated in *BRAF V600E* versus wild type but also most downregulated in cell lines with BRAFi-resistant versus parental. And we found three genes most downregulated in *BRAF V600E* versus wild type and most upregulated in BRAFi-resistant versus parental: *NQO1*, *ACTG1*, and *ALDOA*.

We made the most deregulated and overexpressed genes named with down- and up-regulated genes respectively to avoid confusion. Our overview, was showing up to 30 genes, but we also analyzed in-depth some pathways and genes as shown in Figure 3.16.

But, despite that we find important genes related to the disease, in *BRAF V600E* versus wild type, we highlight the up-regulated genes: *YWHAZ*, *MDM2*, and *FN1*. And the down-regulated genes: *MLANA*, *PMEL* and *CD63*. As also on the BRAFi-resistant versus parental, the up-regulated genes: *GAPDH*, *ENO1*, and *SERPINE2*. And the down-regulated genes: *TGFB1*, *FOS*, *FOXP1*, and *FN1*.

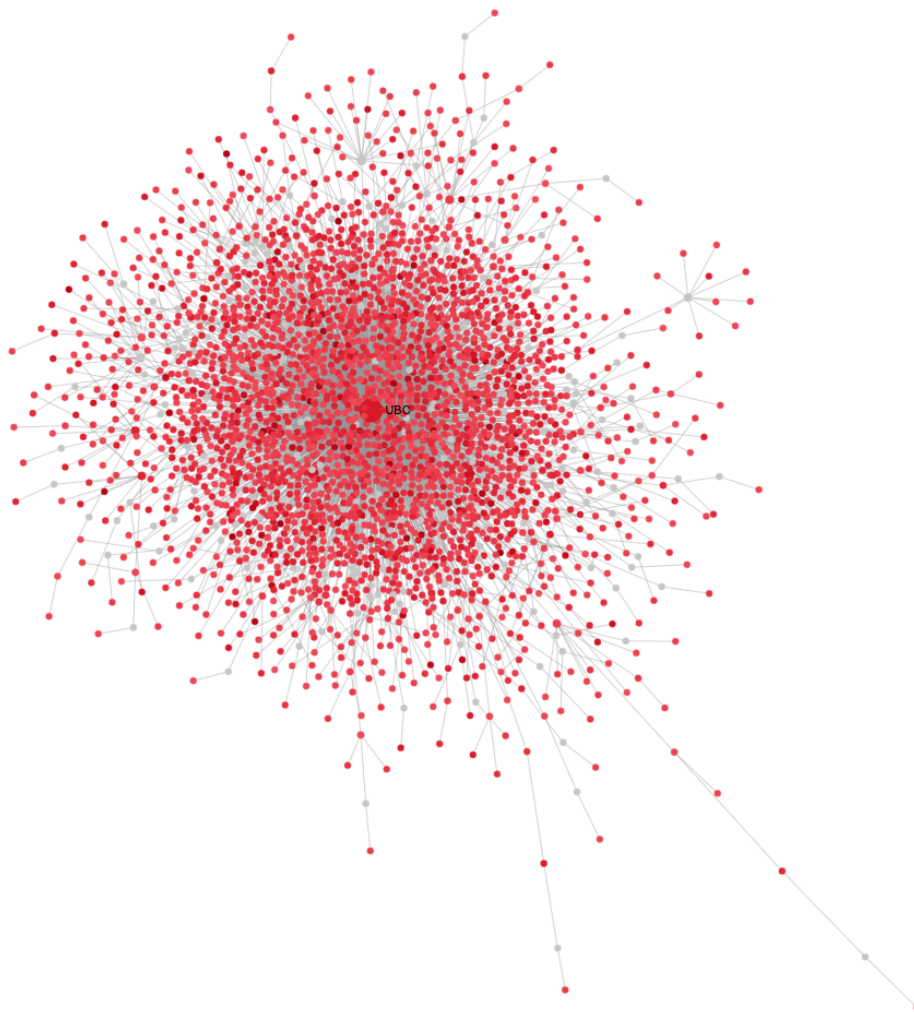


Figure 3.13: *Network from the meta-analysis using Cochran's Q Test Fixed-Effects Models (FEM) method.*
The figure shows the visualization of the big subnetwork generated by the Cochran's Q Test using FEM value of 0.05, all nodes in the network were upregulated.

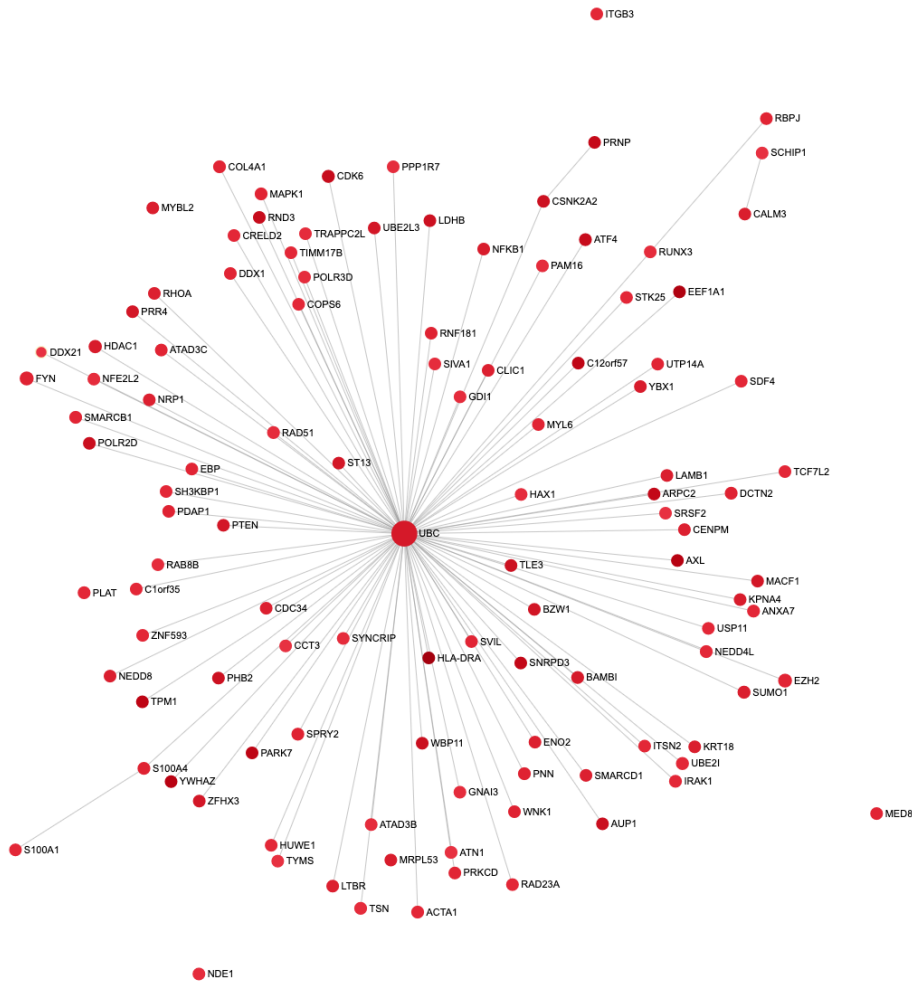


Figure 3.14: *Subnetwork from the meta-analysis using Cochran's Q Test FEM method.*

This network was generated from the subnetwork obtained with the Cochran's Q Test FEM, selecting the nodes with the highest betweenness, degree greater than 2 and differential expression greater than 0.5. The most interconnected gene in the center is the UBC gene in the network.

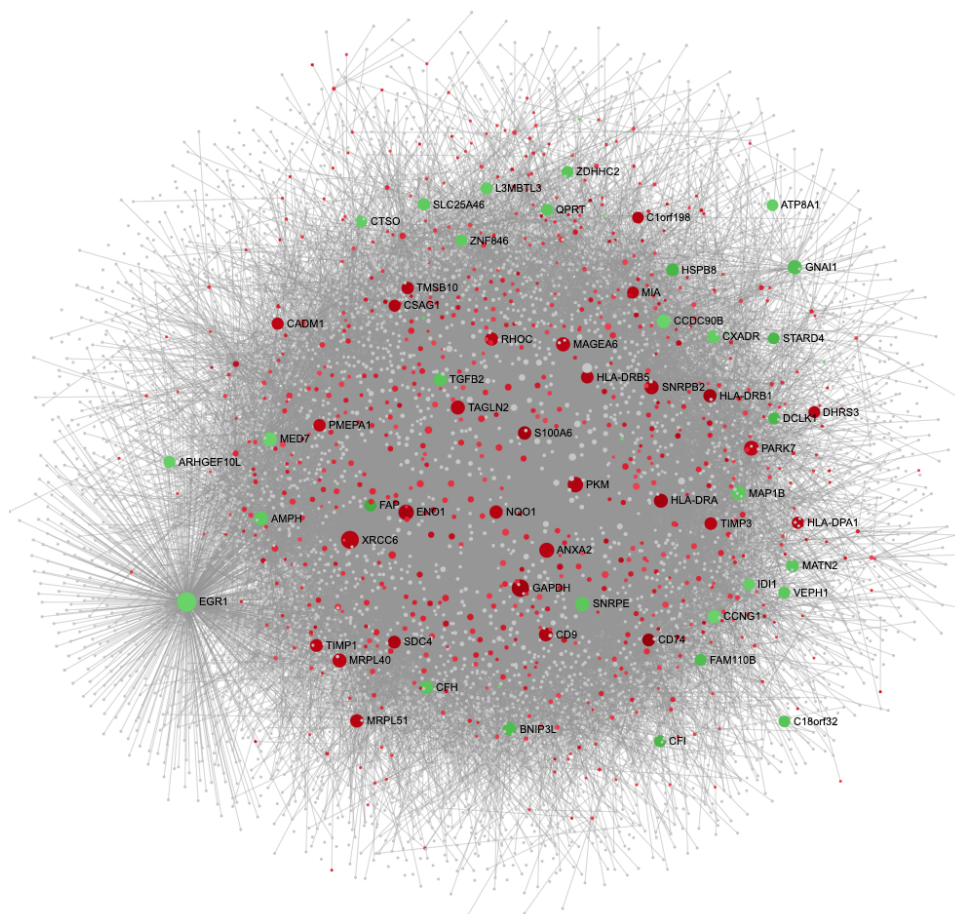


Figure 3.15: Network from the meta-analysis using Cochran's Q Test using Random-Effects Models (REM) method.

The figure shows the visualization of the big subnetwork generated by the Cochran's Q Test using REM significant value of 0.05. We highlight the 30 nodes most upregulated and 30 most downregulated and greater betweenness.

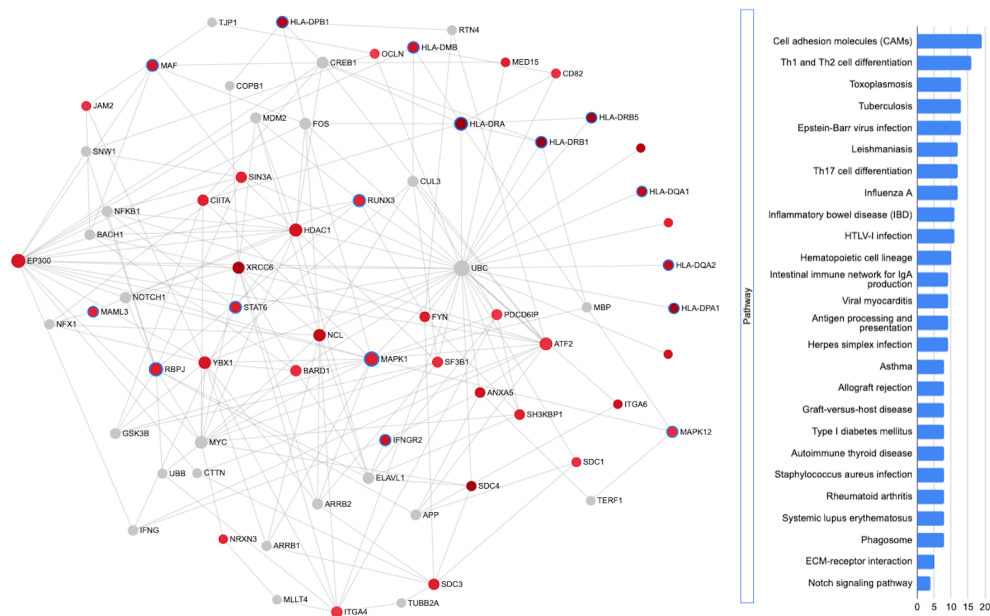


Figure 3.16: Subnetwork from the REM model highlighting the connection to IFNGR2 from the meta-analysis. The figure detailed on the right the most significant pathways (FDR < 0.0001), enriched with Reactome for all upregulated genes. The pathways ordered by hit found in the network. Nodes with light blue colour border indicate genes bellowing to the Th1 and Th2 (T CD4+) cell differentiation pathway.

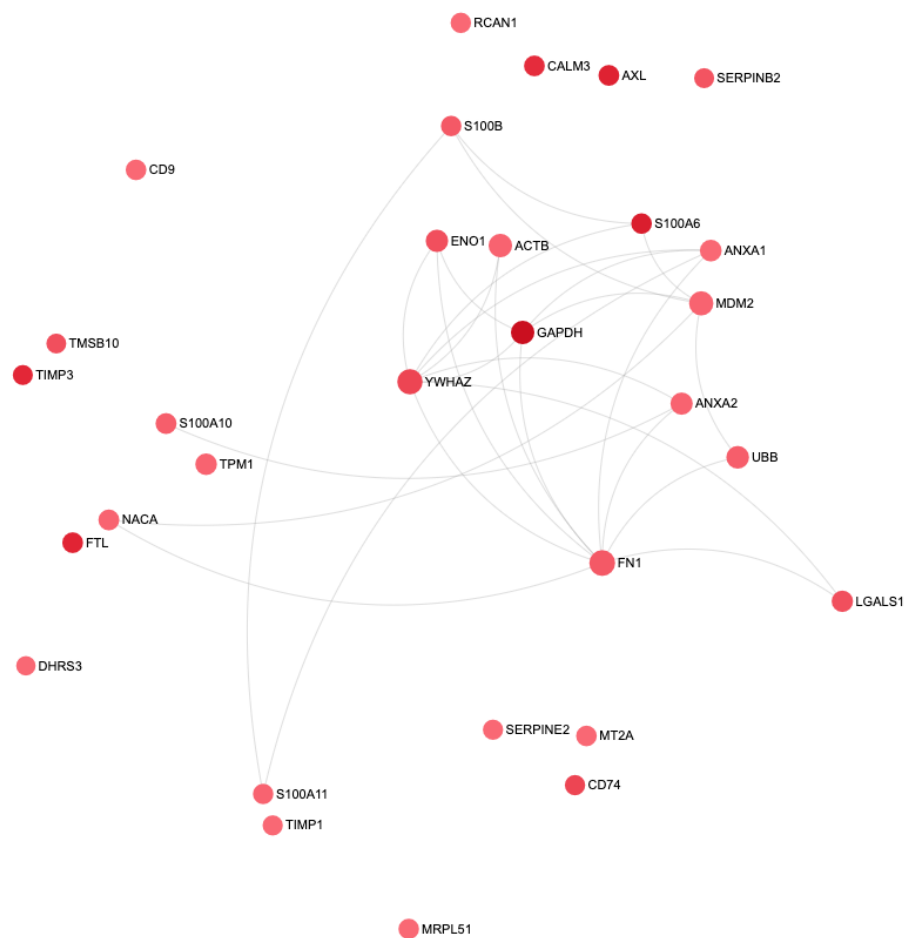


Figure 3.17: PPI subnetwork of the 30 most upregulated genes from *BRAF V600E* versus wild type comparison.

Genes *S100A10*, *NACA*, *S100A11*, *S100B*, *S100A6*, *MDM2*, *UBB*, *ANXA1*, *ANXA2*, *LGALS1*, *FN1*, *GAPDH*, *YWHAZ*, *ACTB*, *ENO1* are connected through protein-protein interaction. The expression level is according to the intensity of the coloured node.

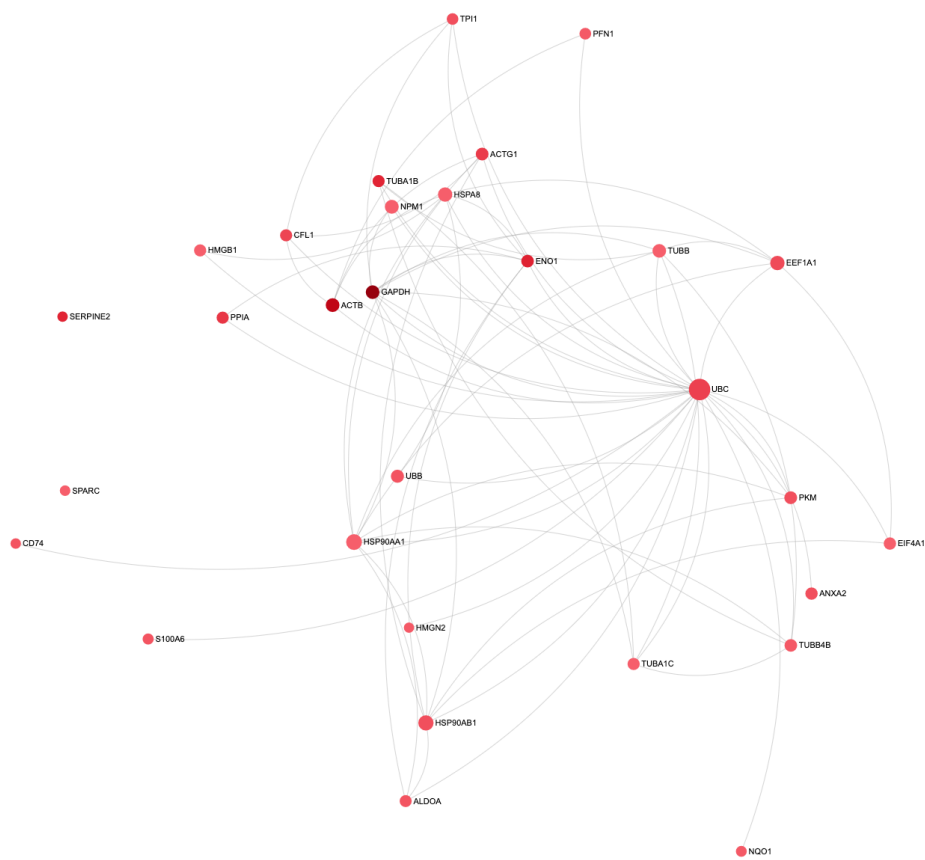


Figure 3.18: PPI subnetwork of the 30 most upregulated genes from the BRAFi-resistant vs parental cell line comparison.
Almost all the genes are connected through protein-protein interaction except for *SERPINE2* and *SPARC*.
The expression level is according to the intensity of the coloured node.

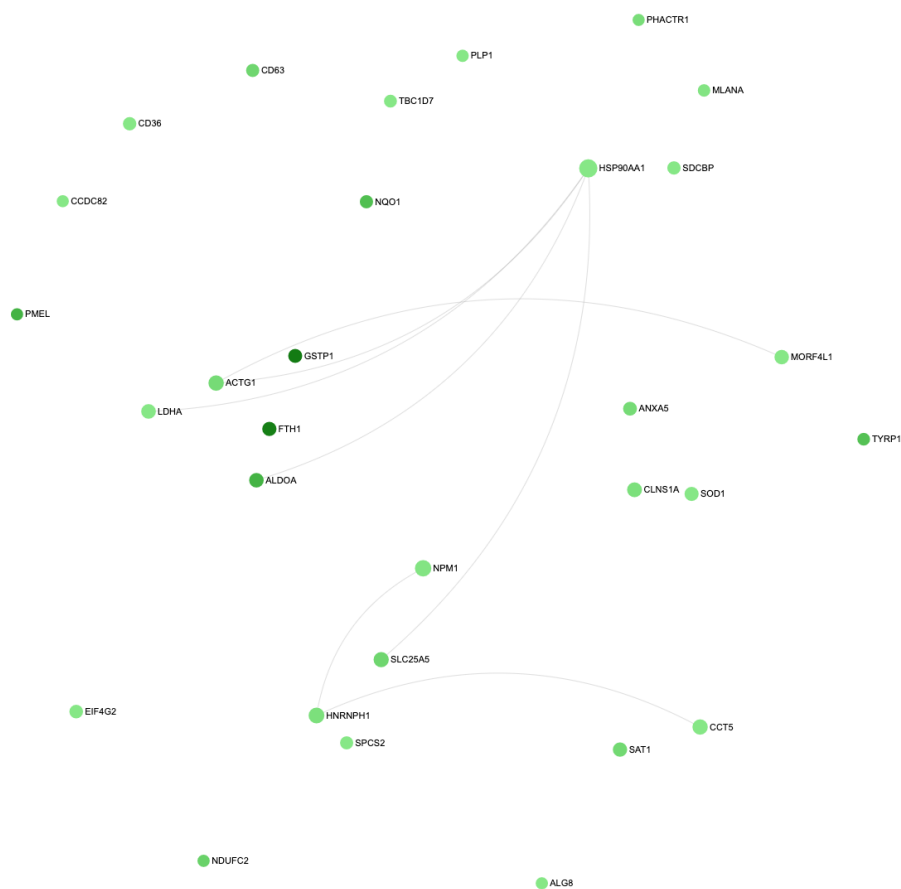


Figure 3.19: PPI subnetwork of the 30 most downregulated genes from *BRAF V600E* vs wild type comparison.

Genes *HSP90AA1*, *SLC25A5*, *ALDOA*, *LDHA*, *ATCG1*, and *MORF4L1* are connected between them, and *NPM1*, *CCT5* are connected with *HNRNPH1* separately, through protein-protein interaction. The expression level is according to the intensity of the coloured node.

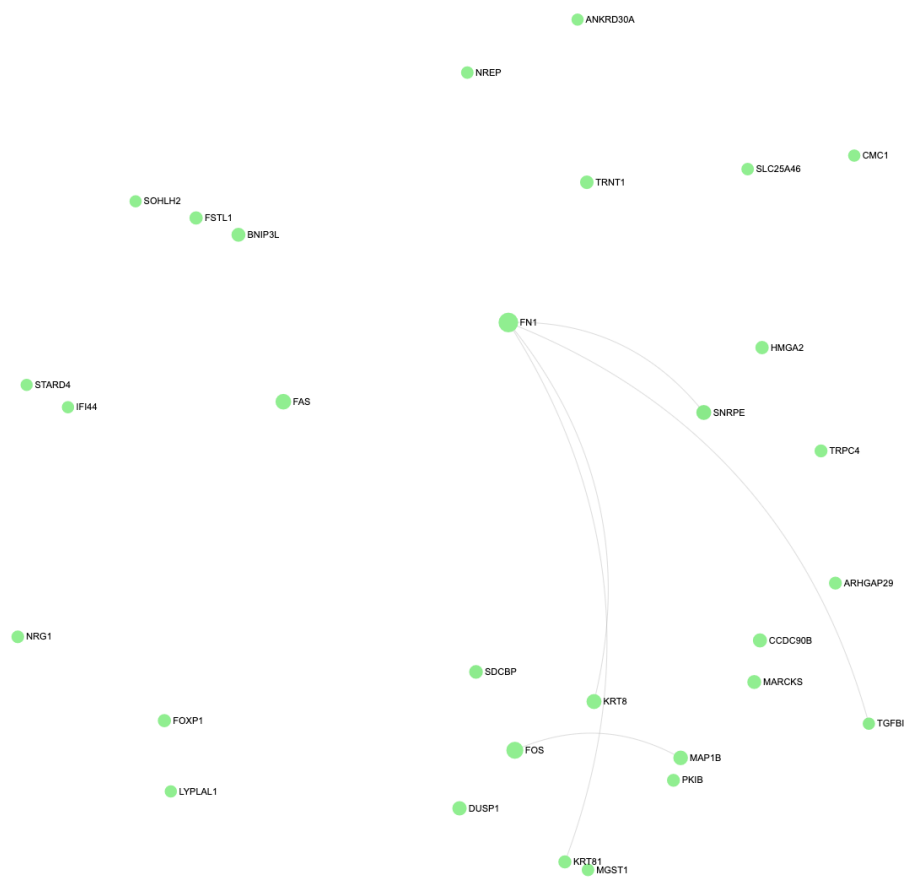


Figure 3.20: PPI subnetwork of the 30 most downregulated genes from the *BRAFi*-resistant versus parental cell lines comparison.
Genes *SNRPE*, *TGFBI*, *KRT8*, and *KRT81* are connected with *FN1*, and separately *FOS* and *MAP1B* are connected, all of them through protein-protein interaction. The expression level is according to the intensity of the coloured node.

Chapter 4

Discussion

Some genes appear several times in our analysis demonstrating their important role in the immune system, which could also harm immunosurveillance in melanoma. We highlighted the genes: *CCL3*, *CCL4* and *CCL5*. *CCL5* binds to *CCR5* activating the apoptosis of Tumor-infiltrating lymphocytes (TIL) cells [MdAM⁺01], which has been associated with higher expression in melanoma cells compared to normal melanocytes, and is associated with a higher malignancy state and increased tumour formation [MSM⁺99, MCM⁺94], which appears to be a prognostic immunological marker and an excellent target for immunotherapy [BML⁺17].

The secretion of *CCL5* recruitment Tregs to the tumour site, where they suppress the cytotoxic antitumor CD8⁺ T cells in squamous cell carcinoma [SLS⁺15]. They also recruit TAM, stimulating pro-tumour effects [HZB⁺16], and in recent studies, the same chemokine interaction was observed with single cells in melanoma [TIP⁺16, KDL⁺18]. In our data *CCL5* appears in the co-expression module of the complete expression matrix from Tirosh and collaborators dataset Figure 3.1. This appears too in DEG from CD8⁺ T cells in melanoma patients versus healthy donors, visualized in the violin plot 3.6.

The expression of *CCR7* in CD4⁺ samples indicates that they are in the naive (TN) state. While T cells from Tirosh and cols., seem to be more memory T cells (TM). TN gradually progressed towards TM accompanied by different levels of expression of chemokines. At the beginning of this trajectory, high levels of *SELL*, *CCR7* and *LRRN3* were expressed, while at the end of the trajectory they expressed more *IL32*, *CCL4*, and *CCL5* [CGSR⁺20].

Although important genes were found in the significant DEG lists such as *CX3CR1* in CD8⁺. Among the most upregulated genes from CD8⁺ cells 4 of them also appeared as the most upregulated in CD4⁺ DEG, including *CX3CR1*, *ITGB1*, *AC022149.1* or *FLT3* and *CCR4*.

A recent scRNA-seq transcriptomic analysis revealed the Tregs tissue-specific signature of CD4⁺ regulatory on the skin, the authors show evidence that CD4⁺ Tregs are being recruited by non-lymphoid tissue (NLTs) following a path from brachial lymph nodes (bLN) to the skin, adjusting their gene expression with substitute paralogs, inferring that the cell-cell communication pathways of these cells owe some plasticity [MGC⁺19]. In line with our data CD4⁺ T cells in melanoma patients upregulate the *ITGB1* follow the bLN-to-skin trajectory.

T cells that infiltrate the tumour express chemokines to recruit more effector T cells, expressing *CCL21* to recruit NK and DCs cells. But it has been observed that melanoma cells in the presence of interleukin-2 stimulate TNF (tumour necrosis factor), contributing to regulating T-cell responses to tumour cells [MSN⁺05], in B-cell controls, co-stimulatory molecule expression, and IL-6 production [DCHK18]. From the *TNF* superfamily, we observed *TNFAIP3* mostly expressed in CD14⁺, CD8⁺ cells in Figure 3.10 and hub gene in macrophages from Figure 3.3.

Normally *TNFAIP3* is expressed in small amounts, however, its expression is increased by the activation of *TNF-α* and *NFκB* in case of inflammation, and for that reason, *TNFAIP3* could serve as a potential molecular marker to anticipate the treatment response with *TNF-α*-inhibitors in psoriasis patients [CVBL14]. In CD8⁺ T cells, *TNFAIP3* regulates necroptosis, *IL-2*, and *IFN-γ* release [DCHK18].

IFN- γ or IFNG cytokine activates PD-1 receptor activity [CGSR⁺20] and also induces the expression of the PD-L1 molecule that binds to PD-1, this leads to increased *STAT1* signalling and decreasing *STAT3* activation in U937 monocytes and *HEK293-NF κ B-GFP* cells [ZBN⁺17]. Rosell and collaborators demonstrated in melanoma the correlation between *IFNG* and *STAT1* through *CTLA-4* activating *STAT3* and the nuclear factor of activated T cells c1 (*NFATc1*) complex, might be a particular immune cell mechanism of melanoma and lung cancer patients who were treated with nivolumab and pembrolizumab [KGCC⁺18].

IL-2 family cytokines quickly activate the signal cascade of *JAK-Stat5* pathway with transcriptional regulation, and signalling in T cells. In mice *STAT5* double knockout (*Stat5a*^{-/-}/*Stat5b*^{-/-}), T cells are deficient in proliferation and fail to undergo in cell cycle progression [LL00]. Increased activity of Tregs cells during IL-2 based immunotherapy has been shown in metastatic melanoma patients, indicating that in some patients it leads to immune tolerance rather than immunity against tumours [CDC⁺06]. STAT phosphorylation was studied in advanced melanoma, stages III to IV, in a defective response to IL-2, which has been attributed to impaired phosphorylation of *STAT1* and *STAT5* [MVM⁺09]. More details in Figure 4.1.

The presence of a T cell-inflamed tumour microenvironment may serve as a predictive biomarker for immunotherapies with therapeutic cancer vaccines, monoclonal antibody ipilimumab (anti-CTLA-4) and IL-2 in high doses. The scientific community is trying to design better immune effector responses by changing the effects of IL-2 in favour of immune activation before administration of IL-2 [Ros14] or CTLA-4 antibodies using other chemotherapeutic agents [RZL⁺17]. This could be accomplished with the identification of immunosuppressive pathways that are present in the tumour microenvironment in a subset of T cells, these include the PD-1/PD-L1, IDO, Tregs cells and T cell-intrinsic anergy [SF14, GSF13, JWN⁺09].

Blocking monoclonal antibodies against PD-1 or PD-L1 have been tested in patients with melanoma with a 30% response rate [THB⁺12, TTAP16]. Combination of immunotherapies including tumour antigen-antibody, a recombinant interleukin-2, anti-PD-1, and a T-cell vaccine was established for melanoma, but the efficacy depends on the response of both innate and adaptive immune cells [MOS⁺16].

As in T cells, *TNFAIP3* was found co-expressed in macrophages in patients with melanoma. All the genes co-expressed in one of the modules of the analysis led us to the upstream regulatory networks, where we found *CK2 α* . Casein Kinase 2 or CK2, is a protein kinase that consists of two catalytic subunits (α and α' ; gene IDs *CSNK2A1* and *CSNK2A2*, respectively) and two regulatory subunits (both CK2 β ; gene ID *CSNK2B*) is ubiquitously expressed in both healthy and cancerous cells [Mon16].

We found the kinase CK2 α which phosphorylates SP11 directly. CK2 interact with signaling pathways responsible for aberrant growth and proliferation in tumors including *JAK/STAT*, *NF κ B*, fibroblast growth factor (*FGF*), and *Wnt* signaling [BFT⁺96, DSS09, ZQF⁺11], being also an intermediary effector in the network of *EGFR/ERK*, that has been shown to stimulate *WNT/ β -catenin* through *CK2 α* [JWN⁺09].

It was reported that abnormally elevated expression of the *CK2 α* is sufficient to cause resistance to each of the three inhibitors approved for melanoma treatment: vemurafenib, dabrafenib and trametinib [ZRM⁺16]. To better understand the events of melanoma cells that are resistant to the immune checkpoint blockade we take the example of Karachaliou et al., 2018 [KGCC⁺18] (Figure 4.2).

Recently, the activity of protein kinase CK2 has been linked to epithelial-to-mesenchymal transition (EMT) induced by *TGF β* or *TGFB1*. *TGF β* signaling can also induce apoptosis through other members of the mitochondrial *Bcl-2* family, as well as via *NF κ B*, *AKT*, and *MAPK*. This activity is associated with the repression of epithelial genes such as *E-cadherin* [SSM07, XLD09]. In addition, other pathways downstream of *TGF β* activation contribute to EMT including *MAPK-ERK*, *PI3K/AKT*, *Rho/ROCK*, Hedgehog, and *WNT* signaling pathways [DMS14, ZTX16, LHH⁺17].

Melanoma cells undergo a change reminiscent of an EMT, which has recently been linked to chemoresistance [FDL⁺15, ZCK⁺15]. Recently, the Jia 2019 group detected a diminished EMT-

related gene signature including increased expression of *E-cadherin* and decreased expression of *N-cadherin* and *Vimentin*. Downregulation of *FN1* also increased *Bax/Bcl-2* ratio which might result in apoptosis of melanoma cells. Concluding that the role of *FN1* is important in melanoma metastasis by inhibiting apoptosis and regulating EMT [LSP⁺19]. Of the genes that change their expression in the meta-analysis among the melanoma cells with *BRAF V600E* inhibitors, we highlight *FN1* that seems to be unique to *BRAF* and of this mutation, the other genes that change its expression between the studies, being repressed in *BRAF V600E* mutants; *ACTG1*, *NQO1*, and *ALDOA* probably depends to another *BRAF* mutation position or respond exclusively to the *NRAS G13R* mutation.

More than 50% of all melanoma patients have to activate somatic mutations in *BRAF* involving L597 (Leucine in the position 597), T599, V600, and K601, also *BRAF V600* have the mutation to E or G or K or M or R, where Valine is replaced by Glutamic acid and so on, switching into a constitutively active protein kinase and cancer driver ¹[TBJ⁺19]. Diagnosis tests in driver mutations such as *BRAF* and *NRAS*, could improve the treatment, in patients misdiagnosed [AGC⁺19].

It should be taken into consideration that in our analysis we used A375 melanoma cell lines data expression treated with PLX4720, a potent and selective inhibitor of *BRAF V600E* with IC50 of 13 nM [TLW⁺08]. The IC50 represents the concentration of a drug that is required for 50% inhibition in vitro ². Several of the publications that study melanoma resistance use PLX4032 which is a *BRAF* inhibitor with IC50 of 31nM for *RAF V600E* and 48nM for *cRAF-1* [BHT⁺10, YHK⁺10]. The selection is also a crucial step since cells can be fully-resistant to the treatment or partially resistant with the treatment of BRAFi.

We believe that transcriptomic analysis revealed and complements the finding of mechanism in how the disease is progressing and becoming resistant. It would be ideal if analysis with a vertical integration was carried out for each patient. Personalised diagnosis performed in this disease could substantially improve the treatment due the several mutations and differences between the type of mutation that may exist.

Personalised medicine can range from small cheap tests to the most expensive and advanced test. Nevertheless, the kind of analysis seems to be the essential event giving us significant results on this approach and improve the treatments.

Multi-omics integration seems to be a challenge, due to the new molecular techniques, the discovery of new genes and genetic regulators, and especially the statistical method that we must apply for the data sets, maybe even creating new models where we first discriminate the essential genes in complex diseases.

The biggest problems we faced was the correspondence gene name or gene symbol between datasets, which appeared to be the a great challenge in the integration analysis, even when we analyzed multiple gene expression data, the different annotation could generate misinformation.

¹Catalogue Somatic Mutations in Cancer - COSMIC: <http://cancer.sanger.ac.uk/cosmic>

²NCBI glossary: <https://pubchemdocs.ncbi.nlm.nih.gov/glossary>

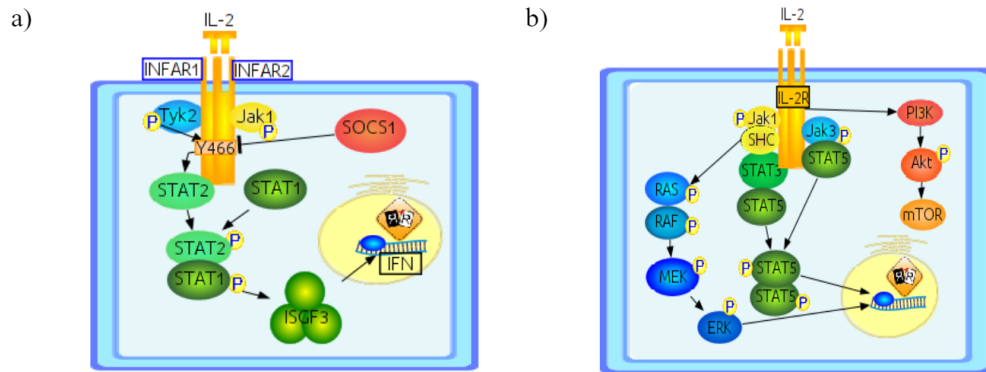


Figure 4.1: Signalling events following the activation of the IFN- α receptor.

a) Activation of IFNAR receptor results in phosphorylation of Tyk2 and Jak1. Phosphorylation of STAT2 as well as STAT1 results in STAT1/STAT2 that forms the ISGF3 complex. ISGF3 translocates to the nucleus and activates the transcription of *IFN* genes. b) IL-2 signal transduction. IL-2R heterodimer initiates the signals for the recruitment of Jak1 and Jak3. Jak1 associates with IL-2R β and Jak3 associates with IL-2R γ , starting the cascade for the recruitment of STAT3 and STAT5. Phosphorylated IL-2R β leads to the association of the SHC protein which in turn activates the *Ras*-MAP kinase pathway. STAT5 are phosphorylated, dimerized and translocated to the nucleus, where it regulates the transcription of essential genes for T cell growth and function. IL-2 also activates the phosphoinositol 3-kinase-Akt (*PI3K-AKT*) pathway, which further activates *mTOR* [Pla05, SFG⁺15].

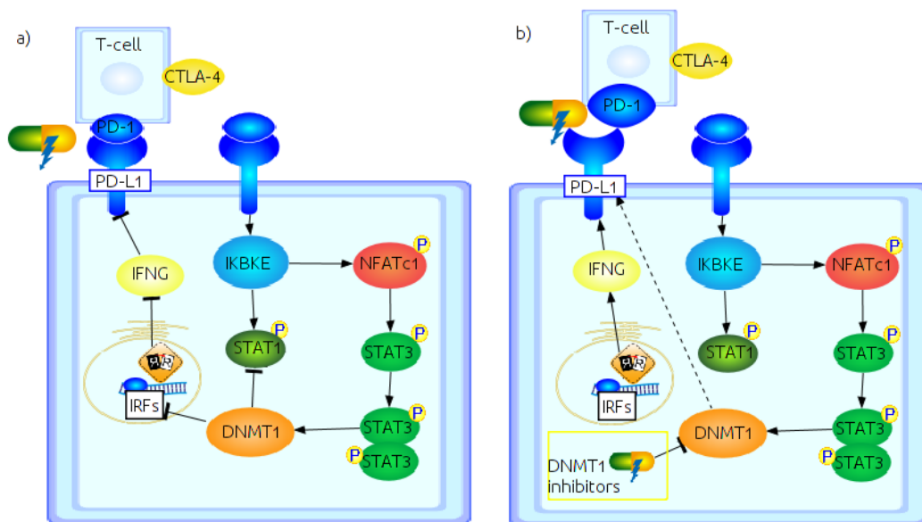


Figure 4.2: Immune cell mechanism in melanoma patients.

a) Melanoma cells that are resistant to the immune checkpoint blockade. IKBKE ($\text{I}\kappa\text{B}$ kinase family member; also called IKK ϵ or IKKi), induced by tobacco components phosphorylate the nuclear factor of activated T cells c1 (NFATc1) and STAT1. NFATc1 activates STAT3 enabled to form a complex. STAT3 can also indirectly inhibit the expression and activation of STAT1, through the activation of the DNA methyltransferase 1 (DNMT1) which interferes with transcriptional regulation silenced by the expression of interferon regulatory factors (IRFs), transcriptional regulators of the type I interferon system [MAAK⁺12], lead to interferon ‘insensitivity’. b) Melanoma cells with DNMT1 inhibitors can sensitize cells to the immune checkpoint blockade indirectly. Adapted from Karachaliou et al., 2018. [KGCC⁺18].

Chapter 5

Conclusion

Understanding the role of the immune system in tumour formation, growth and progression have crucial implications for cancer therapies. Thus, it is essential to identify the major players driving these processes. Using this approach, we explored important interactions in each cell of the immune system which was not observed by the authors in the original publication within melanoma tumours.

We saw large and new findings in the re-analysis of public scRNA-Seq and bulk RNA-Seq single-cell data. Our approach allowed us to understand a little more about the pathways and genes involved in melanoma.

We were able to observe in several pathways how distinguishing the transcription of genes leading by certain gene mutations could be a guide to the inhibitor mechanism of the resistance activation in melanoma. Data integration has recently begun, and some parameters and different normalization methods are still being tested. Even so, we were able to integrate omic data into a meta-analysis revealing interesting modulators in signalling pathways affecting the disease.

We distinguish the interplay of *CD74*, in CD14+ of melanoma patients, in melanoma patients with *BRAF V600E*, and also in melanoma cell lines resistant to *BRAF V600E* inhibitors, indicating the presence of this molecule as one of the principal modulators of communication between cells of the immune system and melanoma, along with *ENO1*, *S100A6*, *SERPINE2*, *GAPDH*, and *UBB*. All of these genes are involved in the TME response to progression and treatments in cutaneous melanoma.

We identify *TNFAIP3* as having an exclusive role in melanoma in CD14+ and CD8+ cells, suggesting that common transcription factors involved in *TNFAIP3* or those related to this gene could be drug design targets in melanoma.

We proposed that the *FN1* gene is being modulated by the *BRAF V600E* mutation and three genes *NQO1*, *ALDOA*, and *ATCG1*, which could be modulated by *BRAF* with another mutation or by *NRAS G13R* mutation in melanoma cells.

We associated *IFNGR2* as a type of receptor for melanoma cells that may interfere with the signalling cascade of metastasis melanoma, and that could explain drug resistance to immunotherapies. We hope that this will reveal new and unappreciated links between the immune system and melanoma progression.

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