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Biomarkers of prognosis and toxicity for metastatic melanoma patients treated with ipilimumab.

Tesi di Dottorato

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PhD Thesis

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Index

Abstract

Background

Metastatic melanoma (MM) has a dismal prognosis, as a consequence of its intrinsic aggressiveness and the lack of effective treatment options: in fact, until recently, systemic therapies were numbered. Ipilimumab is a fully humanized monoclonal anti-Cytotoxic T-Lymphocyte Antigen 4 antibody that demonstrated a significant improvement of metastatic melanoma patient survival, however toxicity may be severe and life threatening. Clinicians lack reliable prognostic factors for prognosis and toxicity and this makes treatment decisions difficult.

Methods

An observational prospective study was performed at the Veneto Institute of Oncology (IOV), the main inclusion criteria being the administration of ipilimumab 3mg/kg every 3 weeks for metastatic melanoma. A total of 140 patients were included, clinical features and circulating biomarkers were evaluated for an association with prognosis or adverse events. Out of 140 patients, 113 were evaluable for prognostic factors, and the full cohort was included in a toxicity study. A prognostic model was derived and data from 97 patients from two other Italian Institutes were used to validate this prognostic model.

Results

Baseline serum lactic dehydrogenase (LDH) concentration and neutrophil count were significantly associated with prognosis. In particular, patients with higher circulating levels of LDH and higher neutrophils before treatment had a shorter survival and increased HR of death (HR=1.36, 95% CI 1.16-1.58, P<.001 and HR=1.76, 95% CI 1.41-2.10, P<.001, respectively). Data were validated on the external cohort and the prognostic model was confirmed.

Female patients and patients with lower baseline serum levels of interleukin-6 had a higher risk of developing severe toxicity (OR=1.5, 95% CI 1.06-2.16 and OR=2.84 for 1ng/L variation, 95% CI 1.34-6.03, respectively).

Conclusions

We demonstrated that baseline levels of neutrophils and serum LDH could help clinicians to predict the outcome of melanoma patients treated with ipilimumab and that ipilimumab may not be the best treatment in patients with higher neutrophil count and LDH. Only comparative and translational studies could define if patients with high LDH and neutrophil are refractory to immunotherapy or have a more aggressive variant of melanoma independent from the treatment. Serum baseline IL6 could help in identifying patients with a greater risk of toxicity from ipilimumab and in planning a more specific monitoring during and after the treatment, with the purpose of increasing its safety. In particular, females with low IL6 serum levels should be carefully monitored for AEs.

1 Introduction

1.1 Aim of the project

The purpose of the present study was the identification of easily accessible independent prognostic factors for survival and toxicity for metastatic melanoma patients treated with ipilimumab, to provide a reliable tool for patient stratification and clinical decisions. In addition, the identification of patients who have a higher likelihood to develop severe adverse events could help personalize the safety survey, for example by means of telephonic interviews between visits or biochemical monitoring (e.g., thyroid or hypophysis hormone levels) also after treatment conclusion.

1.2 Brief introduction to melanoma

Malignant melanoma is a dismal cancer arising from skin, mucosal, meningeal or uveal melanocytes. It has been associated with a dismal prognosis, as a consequence of its intrinsic aggressiveness and the lack of effective treatment options. Until recently, the effect of systemic treatment was really disappointing, with poor response rates and virtually no survival benefit, as underscored by the meta-analysis of Korn et al. that indicated a median survival of 6.2 months for patients with metastatic disease and a 1-year life expectancy of 25.5%[1].

Melanoma is an immunogenic tumor, its immunogenicity being possibly a consequence of the high rate of somatic mutations and expression of neoantigens[2, 3]. Elucidation of the cellular and molecular mechanisms underlying the activating and suppressive immunological checkpoints has led to paradigm-changing results[4, 5].

1.3 History of immunotherapy

The first connection between cancer and immune system dates back in the nineteenth century, when Rudolf Virchow observed the white blood cell infiltrate in his cancer histological samples. No more than sixty years later, a New York surgeon, dr. Coley, observed tumor regression following bacterial infections, and tried the first immunotherapeutic approach, by administering, intra-tumourally, a mixture of denatured S. pyogenes and S. marcescens to advanced cancer patients who were ineligible for surgery[6]. Thomas and Burnet proposed the first formal elaboration of the immunosurveillance paradigm during the second half of the twentieth century [7, 8]. Immunosurveillance was described as the processes, conjugate of the defence system against external pathogens like bacteria or viruses, by which cells of the immune system looked for and recognized pre-cancerous or neoplastic cells in the body. This concept received several criticisms and revisions during the following decades, up to the modern concept of immunoediting.

Immunoediting defines the relationship between cancer cells and immune system as complex interactions in which the immune system operates to eliminate the tumour, but at the same time exerts a selective pressure possibly leading to selection of more aggressive neoplastic clones [9, 10]. In fact, clinical and pathological observations have enriched the complexity of the picture, leading to the discovery that the patient immune cells in the tumor stroma, and tumor stroma itself, are crucial determinants of cancer biology and key factors for the success or failure of cancer therapy[11]. Nowadays, the capability to escape from immune destruction is recognized as one of the hallmarks of cancer[12] .

1.4 Immunological synapsis and immunotherapy

The recognition of cancer cells by the immune system takes place in the so called immunological synapsis, where the interaction with innate (natural killer cells, macrophages,

and dendritic cells) and adaptive (T lymphocytes) immunity cells occurs and an antigen presenting cell belonging to the innate compartment (macrophages, and dendritic cells) processes and presents, after phagocytosis, tumour antigens to a competent T lymphocyte. Any impairment in antigen expression or presentation by the malignant cells may render immune recognition and consequent lysis improbable. Moreover, a series of factors linked to the cell death process concur to influence the immunogenicity of a cancer cell; in fact, recent works highlight how the same antigen and histocompatibility repertoire leads to immune stimulation or not accordingly to different cancer cell death patterns: this is the concept of immunogenic cell death [13-16]. In addition, tumor cells may exert active immunosuppression, either by means of soluble factors, which may act directly or throughout immunosuppressive leucocyte populations (i.e. Treg cells, myeloid derived suppressor cells, immature dendritic cells), or by expression of negative immune checkpoint molecules [17].

The immunogenicity of melanoma has been known for long time [18], with MELAN-A and MART-1 among the first investigated tumor antigens [19-22]. Moreover, melanoma was recently classified as the top neoantigen expressing cancer, as a consequence of the high rate of somatic mutations, likely consequent to UV exposure adducts [2, 3]. This should grant, at least hypothetically, the antigens for the immunological synapsis. However, antigen presentation constitutes only the first step towards immune response against tumours.

Immunohistochemical (IHC) studies aimed to analyze tumor-infiltrating T lymphocyte cell (TIL) in melanoma and other cancers have shown a positive prognostic association between high density of effector cells (CD8 positive T lymphocytes) and patient overall survival [23]. Moreover, the importance of TIL activation against cancer cells is confirmed by gene expression analysis of tumour biopsies, revealing that activation of interferon (IFN) signal transduction pathway (i.e. IFNγ, phosphorylated STAT1, CCR5 and CXCR3) positively correlated with better response to treatment. In addition, studies performed on melanoma and

other cancers (among which colon, ovary and breast carcinomas) have shown a positive correlation between up regulation of genes involved in the CD4 positive lymphocytes, Th1 adaptive immune response and a more favorable prognosis [24-26]. As a conclusion, both adaptive immunity arms (CD8 positive driven, Th2, and CD4 positive driven, Th1) appear to be mandatory for an efficient antitumour response. A further confirmation of the importance of immune infiltrate in melanoma microenvironment comes from a recent large cooperative melanoma clinic-pathological and multi-dimensional genomic and proteomic analysis study, that found that there was no significant outcome correlation with genomic classification (BRAF mutated, RAS mutated, NF1 mutated or triple wild type), nevertheless, samples that were assigned an enriched for immune gene expression transcriptomic subclass, related to lymphocyte infiltrate on pathology review and high LCK protein expression (a T lymphocyte marker), were associated with better patient survival [27]. Nowadays, the complexity of the interactions between tumour, microenvironment and immune response are beginning to be unraveled, also thanks to the considerable technical improvements of the tools available for scientists. An attempt to update Schreiber immunoediting model was recently proposed as a network of interactions and balances called the Immunogram, which summarizes with seven parameters (general immune status, neo-antigen load, immune cell infiltration, absence of checkpoints, absence of soluble inhibitors, absence of inhibitory tumour metabolism, tumour sensitivity to immune effectors) cancer and immune system determinants that concur to influence the outcome of anti-tumour immunity [28].

The capability of T cells to selectively recognize and destroy tumour antigen expressing cells[19], intrigued the researchers since the first onco-immunology advances, and suggested that T cells were one of the most important immune effectors against tumor cells. Of consequence, notwithstanding studies aimed to investigate the antigen specificity, functional characteristics and to confirm the impact on prognosis[29] of spontaneous TILs are still ongoing, the focus of active immunotherapy has predominantly been set on T lymphocyte,

both Th1 and Th2 driven, response enhancement. Unfortunately, the first attempts of active specific immunization (i.e., vaccines [30, 31]) did not appear to considerably improve patients survival and immunostimulating cytokines (e.g., interferon alpha and interleukin-2) had a very limited effect [32, 33] in the metastatic setting and as consequence up to the first decade of XXI century immunology of tumours suffered from the skepticism derived by the lack of clinical proof of concept.

1.4.1 Cellular therapy

With the advances in cellular engineering, several cellular therapy approaches are under preclinical and early phase clinical study to elicit immune response against tumours using artificially powered T cells. For example, T lymphocytes may be stimulated *in vitro*, or genetically manipulated to express modified antigen receptors, to increase immune response against melanoma antigens [34].

Using the former method, lymphocytes with high affinity for tumour antigens could be isolated from the patient, stimulated and expanded by means of cytokine exposure, and, usually after systemic lymphodepleting therapy (there are evidences that low dose regimens may also be effective[35]), finally infused back into the patient. However, lymphocyte *ex vivo* isolation may present some difficulties due to the low number of circulating high affinity T cells.

Another source of tumor-reactive T cells is represented by TILs or by isolation from draining nodes[36]. Three early phase clinical trials studied the cellular therapy in stage IV melanoma patients using autologous TILs, boosted by administration of IL-2 after lymphodepleting therapy (chemotherapy alone or with 2 or 12 Gy total body irradiation) and observed objective-response rates of 49, 52, and 72%, respectively [37]. Most of patients who had a complete tumor regression (22%) had a disease-free survival longer than 8 years[38].

Significant overall response rate (40% out of 57 patients) were also shown in another phase II study by treating metastatic melanoma patients with unsorted TILs and high-dose IL-2 after lymphodepletion [39]. However, the difficulties in isolating high affinity T cells remain a major obstacle to effective large-scale exploitation of this adaptive cellular therapy.

With the advent of new generation of cell sorting and genetic engineering techniques, the latter approach to adaptive cellular therapy become available [40, 41]. High affinity human T-cell receptors (TCR) are obtained either from *in vitro* co-cultures of naïve human T lymphocytes and allogeneic tumour peptide-pulsed antigen-presenting cells [42], or from mice, transgenic for both human leukocyte antigen (HLA) alleles and human TCR, after tumour vaccination [43]. With these methods, TCRs with strongest avidity are identified and a lentiviral vector is then used to carry the genetic clone that will transduce autologous T lymphocytes from patients with matching HLA[44].

A further implementation, aimed to overtake the HLA allele restriction of antigen recognition, comes by the use of chimerical antigen receptors (CARs), in which an artificial single-chain antibody specific for the antigen is coupled to both the transmembrane and cytoplasmic signaling domains of the TCR complex and co-stimulatory molecules (CD137 or CD28), thus providing the necessary activation upon antigen encounter independently from the HLA match and co-stimulatory complex provided by the antigen presenting cells [45-47]. First CAR based therapeutic regimen is expected to be approved in 2017.

An additional improvement in T cell engineering is represented by hybrid CARs, with potentially no limit to the choice of dual target, co-stimuli or inducible activation systems that can be added to the molecule [48-51]. Moreover, cellular therapies may be combined with immunostimulating or anti-negative checkpoint molecules, because unfortunately also potentiated T cells may be hampered by immunosuppressive microenvironment[52, 53], but only preliminary data are available on safety and activity of this approach [54, 55].

NK cells may also be suitable effectors of immune response against melanoma, even if the lack of antigen specificity have been considered an obstacle in the past. However, NK cells may be more easily available than high affinity T cells, more cheap than engineered lymphocytes and a single donor may be used for many patients, with or without donor selection according to HLA or CD16 genotypes[56-58]. In fact, cytokine-induced killer cells (CIK) can be derived from NKs isolated in peripheral blood mononuclear cell fraction and easily expanded by means of cytokine-enriched *in vitro* culture. Moreover, the absence of genetic manipulation could be advantageous, given the stringent regulatory requirements for TCRs and CARs

(http://www.fda.gov/biologicsbloodvaccines/cellulargenetherapyproducts/default.htm).

However, despite the encouraging results of T adoptive therapy with TILs, CIKs, transduced TCRs or CARs, a clinical response is still not guaranteed for all patients. Moreover, several possible issue related to this therapies may rise, for example the toxicity profile (e.g., severe toxicities such as autoimmunity syndromes or cytokine storm [59-61]. In addition, the limited availability of authorized Centers for cellular therapy as well as the high costs of the procedures will possibly limit the availability of that approach..

1.4.2 Anti-inhibitory checkpoint monoclonal antibodies

1.4.2.1 Ipilimumab

The pivotal innovation in immunotherapy of melanoma, and cancer immunotherapy as well, came with the synthesis of monoclonal antibodies against the inhibitory checkpoints in the immune response cascade[4, 5].

The new era of immunotherapy of melanoma, and, maybe of immunotherapy for cancer, started with the fully humanized monoclonal anti-Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4, a competitor of the TCR early co-stimulus molecule family B7) antibody ipilimumab. Ipilimumab, inhibiting the binding between the TCR and CTLA4 that leads to a down modulation of T cell activation after antigen recognition by TCR, produced significant sustained durable responses and, and this translated in an improvement of metastatic melanoma patient survival [62]. A high mutational load had a good prognostic impact in patients with metastatic melanoma treated with ipilimumab, thus confirming the hypothesis of better immune response in presence of abundant neoantigens. Furthermore, very interestingly a set of neoepitopes expressed by melanoma cells could also predict response in the same case series [63]; however, the lack of validation makes this topic controversial and the identification of a predictive epitope fingerprint has yet to be confirmed and is currently object of study and discussion [64]. In addition to the inhibition of CTLA4 constrain signaling on effector T cells, another effect of ipilimumab is supposed to be the depletion of regulatory T cells (Tregs), via a mechanism of action that is yet not fully understood but seems to involve non classical macrophage antibody mediated cytotoxicity clearance of Tregs [65, 66].

In phase II clinical trials, treatment with ipilimumab (3mg/kg q3w) was associated with 5-year survival rates up to 16.5% and 17.0% in pre-treated and treatment-naïve patients, who received ipilimumab 3mg/kg q3w in phase II clinical trials, respectively[67, 68]. Food and Drug Administration and European Medicines Agency authorized Ipilimumab for metastatic melanoma in 2011 after its efficacy in prolonging overall survival in metastatic melanoma patients was proven in a phase III study[62].

However, treatment with ipilimumab may be associated with severe immunological toxicity, usually according to a time pattern that presents consistent risk of delayed adverse events (AEs) [69] [69]. The current toxicity scoring system is derived from the National Cancer

Institute's Common Terminology Criteria for Adverse Events (CTCAE). In clinical trials, patients treated with ipilimumab at the registered dose of 3mg/kg q21 days for 4 cycles experienced grade (G) 3 (not life-threatening) or G4 (life-threatening) immune related adverse events (irAEs) in up to 19.1% and 3.8% of patients, respectively, due to the development of autoimmunity effects. Of note, the reports from "real life" settings (i.e., outside clinical trials) describe even higher toxicity rates, G3-4 AEs being observed in up to up to 30% of patients[70]. The risk of ipilimumab toxicity is not limited to the treatment course but subsists after therapy completion (late or delayed AE). Apparently, the occurrence of a severe AE does not compromise the activity and efficacy of ipilimumab treatment, but can potentially be fatal and prolong exposure to immunosuppressant therapies (mainly corticosteroids) used to counterbalance the excessive immune upregulation by negative checkpoint inhibitors [71]. Up to date, no predictive factors for toxicity have been found. Given the possibility of durable responses, of late responses even after an initial progression and risk of immunological toxicity, several research groups are active in analyzing potential biomarkers able to identify patients who are likely to benefit from treatment with ipilimumab, thus sparing toxicity and resources. A number of potential biomarkers have been investigated so far, including serum lactic dehydrogenase (LDH) [72], the absolute number of lymphocytes and C-reactive protein (CRP), circulating tumour DNA (ctDNA) [73]. On the other hand, other biomarkers have not yet been extensively studied, for instance the count of other blood cell components, melanoma markers, circulating extracellular vesicles (for example exosomes, microvesicles) and serum inflammatory cytokines.

1.4.2.2 Second-generation anti-checkpoint antibodies

After ipilimumab, a new generation of anti-inhibitory checkpoint monoclonal antibodies has been proposed. One of the key processes of effector lymphocyte anergization used by tumour

cells to escape from immune destruction takes place through the Programmed Death-1 (PD1) and PD1-ligand 1 (PD-L1) and 2 (PD-L2) interaction, an inflammatory negative switch that prevents damages from sustained excessive inflammation under physiological or pathological conditions (for example, after infections)[74]. In addition, recent reports revealed that the PD1-PD1L axis inhibition in hosts without active immune system led to a suppression of tumour growth, without interactions with immunity, thus, the PD1-PD1L pathway might play a direct role in tumor genesis, too[75]. Several monoclonal antibodies against PD1 have been synthesized; two of them, nivolumab and pembrolizumab (formerly known as MK3475 or lambrolizumab), have been successfully tested in clinical trials for melanoma. They both are G4 immunoglobulins directed against the surface PD1 and prevent the interaction between lymphocytic PD1 and its ligands that would impair the T cell function. In particular, a phase III randomized study (CheckMate-066) that compared dacarbazine and nivolumab as first-line treatment for BRAF wild type metastatic melanoma patients (N=418), showed an objective response rate favoring nivolumab (40% vs. 14%), with 1-year overall survival (OS) rate 73% vs. 42% for dacarbazine. Median OS was not reached for nivolumab and was 10.8 months for dacarbazine, but median progression-free survival (PFS) was 5.1 months for nivolumab and 2.2 months for dacarbazine, with a hazard ratio (HR) of 0.42 in favor of nivolumab[76]. A phase III study (CheckMate-037, N=631) showed that nivolumab was advantageous compared with dacarbazine or carboplatin plus taxol in patients with metastatic melanoma progressed after anti-CTLA4 therapy (or after anti-CTLA4 therapy and anti-BRAF therapy if BRAF mutated melanoma) [77].

Pembrolizumab, administered in two arms with different drug dosage (2 and 3mg/kg, q3w), was investigated in a randomized pivotal phase II study and showed improved 6 month PFS rate compared with investigator's choice chemotherapy (34-38% vs. 16%) in pretreated and heavily pretreated patients with metastatic melanoma progressed after anti-CTLA4 therapy

(KEYNOTE-002, N=540), with HR for progression of 0.5-0.57 in favor of pembrolizumab[78].

Moreover, a phase III study (KEYNOTE-006, N=834) for metastatic melanoma patients who had received no more than one previous systemic therapy for advanced disease, irrespective of BRAF mutational status, compared two schedules (10mg/kg q2w and q3w) of pemblolizumab vs. ipilimumab and showed a HR for progression of 0.58 in favor of the two pembrolizumab arms, with improved response rates (approximately 33% for both pembrolizumab arms vs. 11.9% for ipilimumab) and 1 year OS (68-74 vs. 58%), with prolonged PFS (4.1-5.5 vs. 2.8 months) and better toxicity profile[79]. For those reasons, both nivolumab and pembrolizumab were granted a fast track to registration in metastatic melanoma.

1.4.2.3 New molecules

Following the wave of new immunotherapeutical approaches, other anti-inhibitory checkpoint molecules have been investigated; intuitively, the ligands of PD1 (PD-L1 and PD-L2) were among the first candidate targets and clinical studies are actually ongoing (NCT01772004, NCT01846416, NCT00658892, NCT01455103, NCT01656642, NCT01375842) or will start in the near future. Other possible candidate targets for immunotherapy in melanoma could be other members of the important regulatory family B7 (NCT02475213[80]), indoleamine 2,3-dioxigenase (NCT02327078) or the Lymphocyte Activation Gene 3 (LAG3) that, interacting with HLA molecules, stimulates Treg cells (NCT02061761, NCT01968109, NCT02460224[81]), and other immunological checkpoints like anti-OX40[82], anti-CD137[83-85], anti-CD27 (NCT02335918, NCT02413827 [86-88] and anti-glucocorticoid-induced TNFR family related gene [89]. Table 1 resumes a list of possible candidates for immunotherapy of melanoma (and, more in general, of tumours).

Tab. 1 Potential targets for future immunotherapy of tumors

Potential target	Expressing cells	Function	Reference
CD40	Antigen presenting cells	activation	[90]
TL1A	Antigen presenting cells	activation	[91]
GITR ligand	Antigen presenting cells	activation	[92]
4-1BB ligand	Antigen presenting cells	activation	[93]
OX40 ligand	Antigen presenting cells	activation	[94]
CD70	Antigen presenting cells	activation	[95]
HHLA2	Antigen presenting cells	inhibition	[96]
ICOS ligand	Antigen presenting cells and tumour cells	activation	[97]
CD80	Antigen presenting cells, tumour cells and T cells	Activating or inhibiting according to expression intensity	[98]
PDL1	Antigen presenting cells and tumour cells	inhibition	[99]
PDL2	Antigen presenting cells and tumour cells	inhibition	[100]
BTNL2	Antigen presenting cells	inhibition	[101]
B7-H3	Antigen presenting cells and tumour cells	inhibition	[102]
B7-H4	Antigen presenting cells and tumour cells	inhibition	[103]
BTNL1	Antigen presenting cells	inhibition	[104]
CD48	Antigen presenting cells	inhibition	[105]
HVEM	Tumour cells	Inhibition	[106]
Siglec family	Antigen presenting cells	activation	[107]
CD40L	T cells	activation	[108]
TNFRSF25	T cells	activation	[109]
GITR	T cells	activation	[110]
4-1BB	T cells	activation	[111]
OX40	T cells	activation	[112]
CD27	T cells	activation	[112, 113]
TMIGD2	T cells	activation	[114]
ICOS	T cells	activation	[115]
CD28	T cells	activation	[116]
LIGHT	T cells	activation	[117]
LAG3	T cells	inhibition	[118] [81]

CD244	NK cells	inhibition	[119]
TIM3	T cells	inhibition	[120]
BTLA	T cells	inhibition	[121]
CD160	T cells	inhibition	[122]
Butyrophilin family	Tumour cells	inhibition	[123]
CD155	Tumour cells	inhibition	[124]
VISTA	Myeloid cells	inhibition	[125]

1.4.2.4 Combination treatments

Combinations between new generation immunotherapeutic drugs are already under study. For example, the CheckMate-067 and 069 trials compared a "combo" arm with ipilimumab and nivolumab vs. ipilimumab or nivolumab: the response rate and progression-free survival resulted better with the combination of anti-PD-1 and anti-CTLA4 blockade; however, overall survival data are not available yet and the combination treatment increases grade 3 and 4 irAEs to more than 50%. As consequence, at the moment it is clear that anti PD-1 are better than ipilimumb in terms of ORR, TTR, MS and OS, but it is not possible to draw conclusions about the superiority of double checkpoint blockade[126-128] respect to anti PD-1 alone.

Considering that multiple processes are involved in immunogenicity of cancer cells and immune response stimulation, some possible future strategies may be represented by the following: (a) association of cancer vaccines (to boost specific anti-melanoma T lymphocytes) and immune checkpoint blockers to prevent lymphocyte anergization by tumor [129]; (b) combination between different immunostimulating agents and anti-inhibitory checkpoints or (c) association of anti-melanoma drugs (i.e. anti BRAF or anti MEK therapy or chemotherapy) with anti-inhibitory checkpoints (NCT02460224, NCT02475213, NCT02335918, NCT02413827, NCT01656642, NCT02357732, NCT02027961, NCT01656642, NCT02130466). This latter approach could possibly led to a significant improvement in patients with targetable mutations in the MAP Kinase pathway, as a recent

report highlighted the role of immune adaptation in disease progression under targeted drugs and the detrimental consequences of acquired resistance on CD8 intra-tumoral T cells [130]. Moreover, also the combination of checkpoint targeted drugs with molecules addressing suppressive cells like Tregs or myeloid derived suppressor cells (for example, all-trans retinoic acid should reduce myeloid derived suppressor cell detrimental effect on immune system[131, 132]) could be possible. However, all these approaches, even though very promising in theory or preclinical settings, should be tackled very carefully, because of possible toxicity issues that preclinical studies may be insufficient to enlighten adequately. For example, an early phase study that was considered very promising at the time of ideation, aimed to assess the safety profile of the association of vemurafenib and ipilimumab, reported unexpected severe toxicities, in particular hepatic adverse events that forced an early termination of the trial for safety concerns [133].

1.5 Adjuvant and neoadjuvant therapy

The revolutionary results with the new immunological drugs obtained in metastatic patients have encouraged researchers to design studies for the adjuvant setting as well. Ipilimumab has also been tested in high risk stage III radically operated melanoma patients, and both PFS and OS of this phase III study are indicating a clear benefit for ipilimumab compared with placebo[134-136]: Again the toxicity was not negligible with more than 50% Grade 3 and 4 irAEs and 5 toxic deaths, although the dose used was 10 mg/kg every 3 weeks for 4 cycles followed by maintenance every 3 months for 3 years (the actual standard dose for metastatic melanoma is 3mg/kg every 3 weeks for 4 cycles). A phase III randomized study comparing nivolumab vs. ipilimumab and a phase III randomized trial comparing pembrolizumab vs. placebo are ongoing (CheckMate-238 and KEYNOTE-054, respectively), patient accrual was completed and data are expected. Obviously, the safety profile in the adjuvant setting, with a

portion of patients potentially already cured by surgery, will be of even more importance than in the metastatic setting, and the possibility to observe late or delayed irAEs superior than with a population with consistent risk of dying before irAE occurrence. Moreover a less impaired immunosystem in the adjuvant setting could increase by itself the irARs.

A phase Ib study assessing the toxicity and activity of immunotherapy in the neoadjuvant and adjuvant setting for patients with locally advanced melanoma is currently ongoing: 20 patients were treated with nivolumab plus ipilimumab before (neoadjuvant) or after (adjuvant) surgery for stage III (with nodal metastases) palpable melanoma with no in-transit metastases. Ipilimumab was administered at 3 mg/kg and nivolumab was given at 1 mg/kg every 3 weeks. The preliminary results were presented at the European Society of Medical Oncology 2016 congress[137]. In the neoadjuvant arm, patients received 2 cycles of therapy prior to surgery followed by 2 cycles of the combination after resection. In the adjuvant arm, 4 cycles of nivolumab and ipilimumab were administered following resection. At the time of the most recent data cutoff (September 2016), 10 patients in the neoadjuvant arm were assessable. The overall response rate in the neoadjuvant setting was 80%, which included 3 patients (30%) who experienced a pathologic complete response. Six patients (60%) had a significant response, with only micrometastatic disease following resection, of which 4 were labeled as nearly pathologic complete response. A difference in surgery-related AEs was not observed between the two arms, suggesting that neoadjuvant ipilimumab plus nivolumab did not complicate resection. Overall, however, the rate of AEs seen in the study was much above expectations. In fact, only 2 of the 18 evaluable patients completed 4 courses of therapy as a result of severe adverse events.

1.6 Biomarkers of prognosis and toxicity

Latest, but not last, the advent of new drugs, effective but very expensive and potentially causing severe toxicity, will require a careful patient evaluation and selection to plan resource allocation and treatment planning[138]. For example, several research groups are active in analyzing potential biomarkers to identify patients who are likely to benefit from immunotherapy, and a number of candidates have been investigated so far, including serum lactic dehydrogenase (LDH) [72, 139], circulating lymphocytes[140] and neutrophils [139, 141], serum C-reactive protein (CRP) [73, 140], circulating tumour DNA [142] or morphemic parameters like muscular infiltration by fat [143]. T-cell Receptor (TCR) clonality and diversity analysis figures among the most promising candidates[144, 145]; however, the available data are based on small series. Moreover, the analysis of TCR DNA is unfortunately still too expensive to be recommended in daily practice. In addition, tumor burden, even if with inevitable obstacle in standardization of the measurement, seems to be one of the most important[78]. Of note, despite preliminary encouraging results, PD1-L expression has yet to be confirmed as a valid prognostic or predictive biomarker in melanoma patients treated with immunotherapy[146]. Notwithstanding the benefit of the combined blockage of CTLA4 and PD1 vs. monotherapy in patients with negative or low tumour PD1-L expression was shown, PD1-L testing is not yet standardized in melanoma and is not recommended by regulatory drug agencies for treatment decision [127].

The research field of toxicity of immunotherapy, even though fascinating and potentially informative about the immunotherapy mechanisms of action and caveats, seems to be a bit neglected in comparison to the resources dedicated to the study of activity and efficacy. Indeed studies dedicated to prognostic or predictive biomarkers of immunotherapy toxicity in patients with metastatic melanoma are numbered and no biomarkers have been identified so far [147, 148].

2 Patients and Methods

2.1 Patients and treatment

An observational prospective study was performed at the Veneto Institute of Oncology (IOV), the main inclusion criteria being the administration of ipilimumab 3mg/kg every 3 weeks for metastatic melanoma.

In the present work, we report the data of 113 patients for the prognostic analysis and of all 140 patients accrued for the toxicity; last data cut-off for the analysis was July 2015. The Istituto Europeo di Oncologia (IEO) and the University of Torino (UT) databases were queried for the values of the significant prognostic factors identified in the prognostic model as well as for survival data of patients, to provide the validation cohort (N=97).

2.2 Biomarkers and patient variables

We collected and analyzed the anthropometric features with potential influence on inflammatory or immunological status (age, gender), the treatment history and tumor burden surrogate markers (S-100 and lactic dehydrogenase [149]). Then, to investigate the inflammatory status of patients, we performed a study of biological blood markers of inflammation, such as acute or chronic phase proteins (C-reactive protein [CRP][65] and beta-2 microglobulin)[150-152]) and cytokines associated with inflammation and immune reaction (vascular endothelial growth factor-A [VEGF][153], interleukin 2 [IL2][154], interleukin 6 [IL6][155]). In addition, to assess the possible influence of different leukocyte subpopulations on treatment efficacy and toxicity, peripheral blood granulocytes and lymphocyte subpopulations were counted. Patient characteristics were recorded from clinical records; age and sex were included in the analysis. Baseline blood samples (before first ipilimumab

administration) were analyzed for the following levels of melanoma and inflammatory biomarkers: LDH (kinetic method optimized according to the German Society of Clinical Chemistry), CRP (nephelometric method), beta2-microglobulin (immunonephelometric method), VEGF (immunoenzymatic method), IL2 (immunoenzymatic method), IL6 (chemoluminescent immunoenzymatic method), S-100 (chemoluminescent immunodosing), peripheral blood leucocyte (cytometric method) and lymphocytes subpopulations (which were analyzed with cytofluorometry to identify membrane positivity for CD3, CD4, CD8, CD16, CD19, CD56). The following antibodies were also searched in the plasma of patients: anti-thyroperoxydase, anti-thyroglobuline, anti-neutrophil cytoplasmic and nucleus antigens, anti-Glutamic Acid Decarboxylase (anti-GAD), and anti-adrenal glands (indirect immunofluorescence, chemoluminescent immunodosage and immunoenzymatic method). Blood tests and clinical examination were performed before every ipilimumab cycle (time window from one week to the same day before administration) and then accordingly to scheduled follow-up surveillance (first visit 2 weeks after treatment completion and then approximately every 12 weeks). New regimen guidelines were also considered. Only one was lost after first follow up visit. In case of toxicity, blood tests and examination took place during an urgent unscheduled visit. Occurrence and outcome of AEs (according to CTCAE v.4.0), date of last follow up and cause of death (melanoma or other) were collected from clinical records. All patients gave informed consent to the treatments and to the use of their clinical records for scientific purposes.

2.3 Statistical analysis

Disease-free interval (DFI) was defined as the time from initial MM diagnosis to first inoperable disease recurrence onset. Overall survival (OS) was defined as the time from first ipilimumab administration to the date of death or last follow-up, and was estimated with the Kaplan-Meier estimator, the log-rank test being used to compare survival estimates of different groups. We used Cox proportional hazards regression analysis on the IOV dataset to examine the association between potential prognostic variables and survival. Schoenfeld residual methodology was used to check the proportional hazard assumption of the Cox model. The Wald test with Bonferroni correction for multiple testing was used to assess the significance of each variable included in the full model, fast-backward method (with Akaike Information Criterion [AIC] as a stopping rule instead of P-values, in order to weight the probability of both significance and prediction strength) was used to select the covariates in the final model. Model performance was measured with the Receiver Operating Curve simulation of the hazard prediction estimates at 6, 12 and 24 months; shrinkage slope (after 100 bootstrap replications) was used to calibrate the overfitting of the model, and discrimination (a measure of the correlation with the hazard of death) was determined with Somer's Dxy (that is also equal to 2*(Harrell's C-index - 0.5)). The prognostic model was then externally validated using the IEO and UT combined datasets. A nomogram was tailored on the final regression model, the total number of points derived by specifying values was used to calculate the expected survival probabilities at 6, 12 and 24 months. Missing values were estimated with multiple imputation using additive regression, bootstrapping, and predictive matching; a correction on the estimation procedure, based on 20 multiple imputations, was performed. Patients lost to follow-up, or whose death was unrelated to metastatic melanoma progression were censored at last follow-up. When analyzing the impact on survival of therapies after ipilimumab, the model was adapted with landmark analysis (i.e.

in which survival time was defined as the time from 12 weeks after first ipilimumab administration, at the time of first response assessment).

We used logistic regression analysis, corrected for the bias in prediction error estimates [156], to examine the association between toxicity and above mentioned biomarkers. The algorithm was constructed including stratification for time of observation. The model was fitted to data using Wald test with Bonferroni correction for multiple testing to assess the statistical significance of each covariate included in the model. Fast-backward method with AIC as a stopping rule was applied to test the covariates in the final model. Stratification for the time of observation was included in the model. Performance of this model was measured with the Receiver Operating Curve, Harrell's C-Index and standard error derived by the estimation were reported; smooth calibration was evaluated with shrinkage slope (after 200 bootstrap replications). The predictive effect of the model was then validated using bootstrap methodology (200 replications), as advised for small datasets [157]. Visual tree method was used to report cluster analysis for covariates. Overall survival (OS) was calculated from first ipilimumab administration to date of death or last follow-up. OS was estimated with the Kaplan-Meier survival method. Two-sided P-values were reported. Statistical analysis was performed with R 3.0.2 (survival and rms libraries, R Foundation for Statistical Computing, Vienna, Austria).

3 Results:

3.1 Prognostic study

3.1.1 Patients characteristics

Putative prognostic factors were collected from the IOV dataset. Table 2 summarizes the characteristics of patients included in the prognostic study. Table 3 shows the biomarker levels at the time of first ipilimumab administration.

Tab.2 Patients characteristics

Variable	N (%)	Median (range)
Sex (M)	47 (42)	
Weight (Kg)		69 (45-135)
Site of primary melanoma		
Trunk	51 (45)	
Limb	38 (34)	
Extremities (Acral and Head and Neck)	4 (4)	
Mucosal	5 (4)	
Uveal	5 (4)	
Unknown origin	10 (9)	
Ulceration		
Present	21 (18)	
Absent	12 (11)	
NA	80 (71)	
Molecular alterations		
BRAF and NRAS wt	29 (47)	
BRAF V600	26 (43)	
NRAS	6 (10)	
Not tested	*52*	
Disease-free interval (months)		27.8 (1.1-192.0)
N of therapies before ipilimumab		1 (0-4)
PS		
0	76 (67)	
1	27 (24)	
≥2	10 (9)	
Number of metastatic organs		3 (1-6)
Localization of metastases		
M1a or inoperable IIIC	16 (14)	
M1b	20 (18)	
M1c	77 (68)	

Characteristics of patients and melanoma. Older cases did not report ulceration in the diagnosis of primary melanoma, and were not tested for BRAF of NRAS mutation. Most patients had M1c disease, and the median number of metastatic organs was 3, only a minority of patients had oligometastatic disease.

Tab. 3 Biomarkers

Biomarker (normal range)	Median (range)
White blood cells (4.40-11.00 x 10^6/L)	6.2 (2.3-17.5)
Eosinophils (0-0.50 x10^6/L)	0.08 (0.01-0.89)
Neutrophils (1.80-7.8 x 10^6/L)	4.0 (1.1-16.2)
Lymphocytes (1.10-4.80 x 10^6/L)	1.3 (0.7-2.5)
CD3+ (7.0-27.0 %)	71.0 (42.0-92.0)
CD4+ (32-52 %)	39.0 (17.0-73.0)
CD8+ (16-33 %)	23.0 (5.3-79.0)
NK (7.0-27.0 %)	18.0 (5.6-35.6)
CD3/CD16/CD56+ (1-11 %)	3.0 (1.0-13.0)
LDH (<1, x UNL)	0.9 (0.4-11.56)
CRP (0-6 mg/L)	6.7 (2.9-214.0)
β2-microglobulin (1.09-2.53 ng/L)	2.3 (1.2-7.2)
IL6 (0-5.9 ng/L)	3.5 (2.0-658.0)
IL2 (0-2 ng/L)	7 (2-28.3)
S-100 (0.00-0.15 ug/L)	0.6 (0.03-97.0)
VEGF (62-707 ng/L)	431.5 (3.4-2100.0)

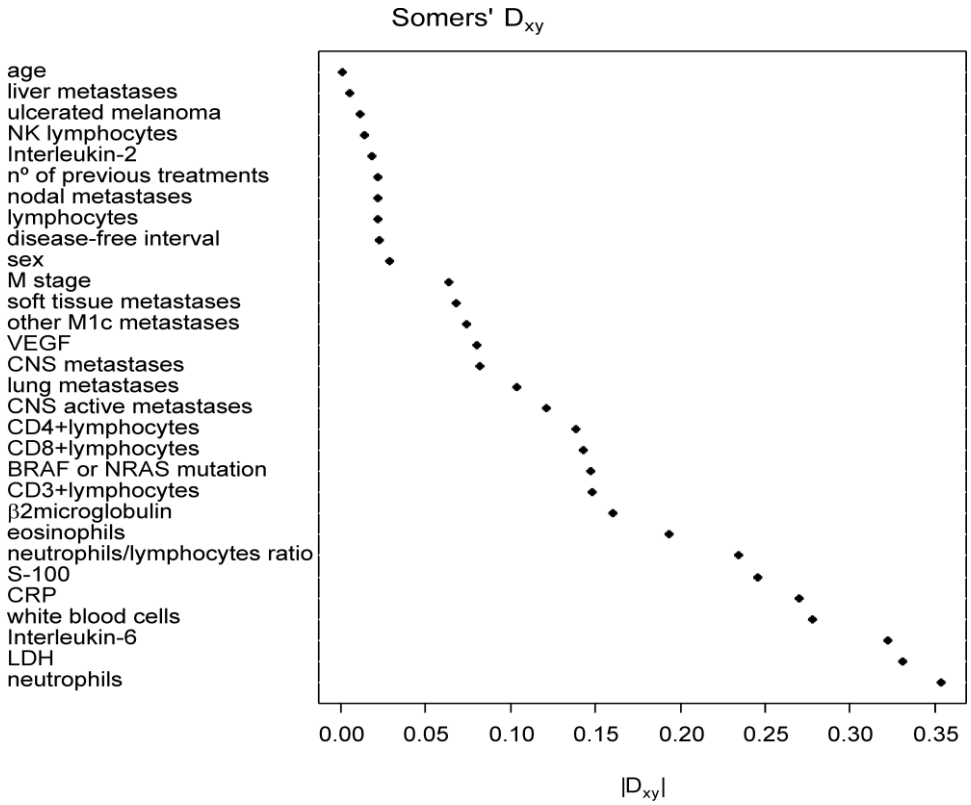
Median values of biomarkers were within the normal ranges, with the exception of CD3 lymphocytes, IL2 and S-100.

3.1.2 Survival and prognostic model

In the IOV cohort for the prognostic, 35 patients were alive after a median follow up of 8.2 months (95% CI=6.31-11.15). Median OS was 9.7 months, 1- and 2-year survival rates being 38.4% and 21.9%, respectively. After ipilimumab, 23 (20%) patients received at least one line of systemic treatment: among these patients, 7 (6%) received BRAF inhibitors, 6 (5%) anti-PD1 drugs, and one (1%) had surgical resection of residual disease.

In the validation cohorts, median OS was 4.9 months at IEO (95% CI 3.4-7.3; 15 patients alive after a median follow up of 14 months), the 1- and 2-year survival rates being 23.9% and 17.4%, respectively; at UT, median survival was 7.1 months (95% CI=2.9-na; 10 patients alive after a median follow-up of 15 months), the 1- and 2-year survival rates being 41% and 26%, respectively. Covariates collected in the IOV cohort were tested for their relationship with survival, and Figure 1 shows the discrimination (Somer's Dxy) performance of the variables tested the full model (i.e., the model including all available covariates): the highest the Dxy, the strongest the relation with survival.

Fig. 1

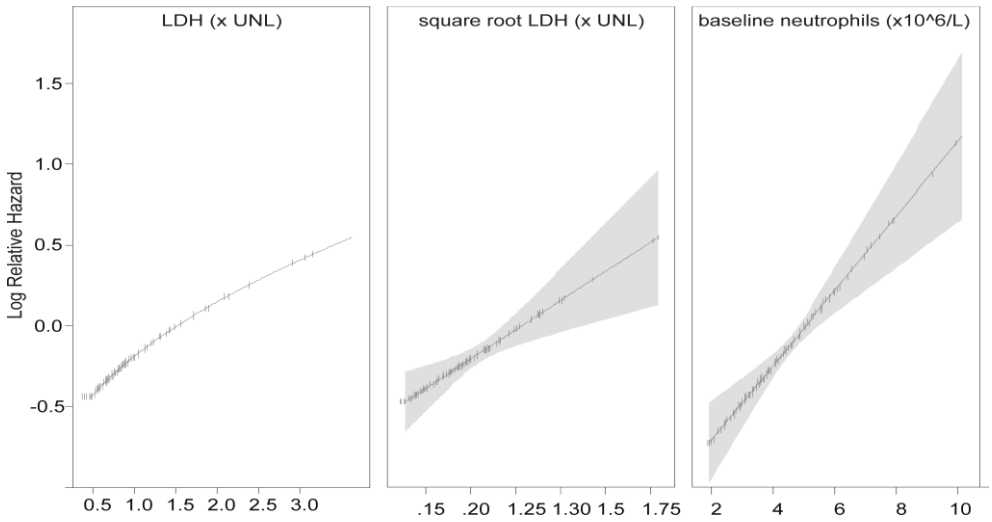


The Somer's Dxy was calculated for all the variables in the full model: the highest the Dxy, the strongest the relation with the hazard of death. In this representation, the result does not render if the correlation is direct or inverse, and if it is statistically significant. Baseline levels of IL6, LDH and neutrophils had the strongest relationship with the hazard, with Dxy>0.30. Metastatic sites were analyzed both according to AJCC stage (i.e. inoperable III plus M1a vs. M1b vs. M1c) and according to the specific location of metastases, focusing on the most common sites.

Baseline levels of IL6, LDH and neutrophils had the strongest relationship with the hazard of death. These 3 covariates were associated with prognosis in the full model after Wald test corrected for multiple testing (respectively, P=.046, P=.010, P=.001), while the presence of CNS active metastases (P=.081) and CD8 lymphocyte count (P=.052) showed a trend. After fast backward variable selection, only LDH and baseline neutrophils satisfied the AIC rule and were retained in the final model. In particular, higher baseline levels of LDH (HR=1.36, 95% CI 1.16-1.58, P<.001) and neutrophils (HR=1.76, 95% CI 1.41-2.10, P<.001) were associated with a worse prognosis. Figure 2 shows the predictive effect on the death relative

hazard of LDH (both as linear predictor and after square root transformation) and neutrophil level.

Fig. 2 Predictive effect on the death Relative Hazard of LDH and neutrophil count



The figure shows the predictive effect on the death relative hazard of serum LDH and neutrophil count. On the y-axis the Log of relative hazard is represented; this means that a Hazard Ratio (HR) of 1 corresponds to 0, upper values corresponds to HR>1 and lower values corresponds to HR<1. Point wise .95 confidence bands (shadowed area) are also shown. "Rug plots" on curves show the density of the predictor. LDH is represented both before (left) and after (centre) square root transformation, to show the relaxation of the relation: the curve is, in fact, straight in the center graph, as for neutrophil count (right), indicating a linear relation with the hazard.

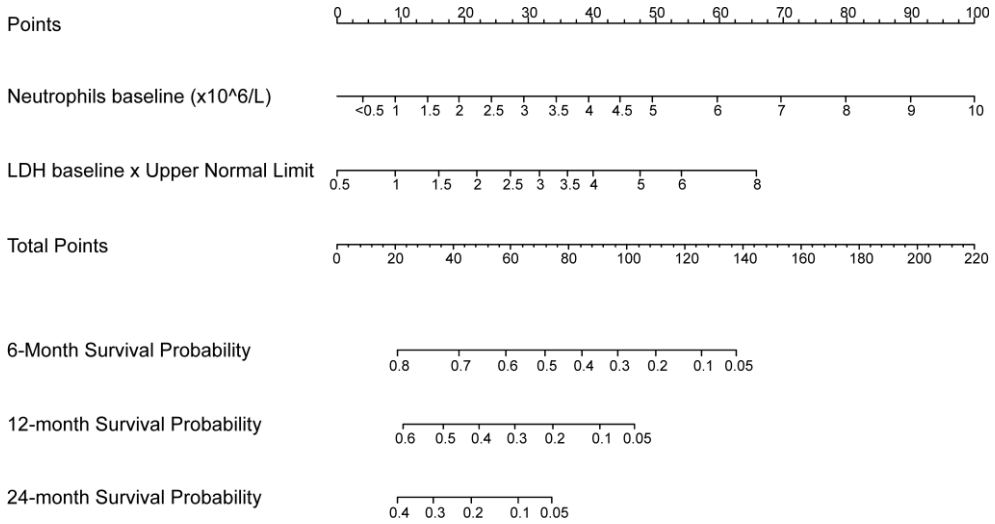
The analysis were adapted to include the therapy regimens after ipilimumab for patients who prosecuted active treatments at disease progression, and showed a non significant trend for survival benefit for PD1 inhibitors and, in the subgroup of patients BRAF mutated, for BRAF inhibitors (not shown).

The shrinkage factor (slope) of the prognostic model was 0.95 (range of the parameter 0-1, where 1 would be the ideally fitted model). Supplemental (??) Figure 1 shows the Receiver Operating Characteristic curves of the performance of survival prediction at 6, 12 and 24 months (the closeness of the lines refers to the reliability of the predictions).

The Proportional Hazard Hypothesis was confirmed both in the full and the final model. The prognostic model was validated internally with bootstrap methodology (200 bootstrap

replication) and the Dxy (the possible range of the parameter being 0-0.5, where 0.5 is the ideal model) resulted 0.42 (standard error [SE] .006). The Dxy of the external validation, performed using the conjoined IEO and UT datasets, was 0.40 (SE .007). A prognostic nomogram was tailored on the final prognostic model (Fig. 3); it could be used to calculate the hazard of death given the value of LDH and neutrophil count at baseline.

Fig. 3 Nomogram



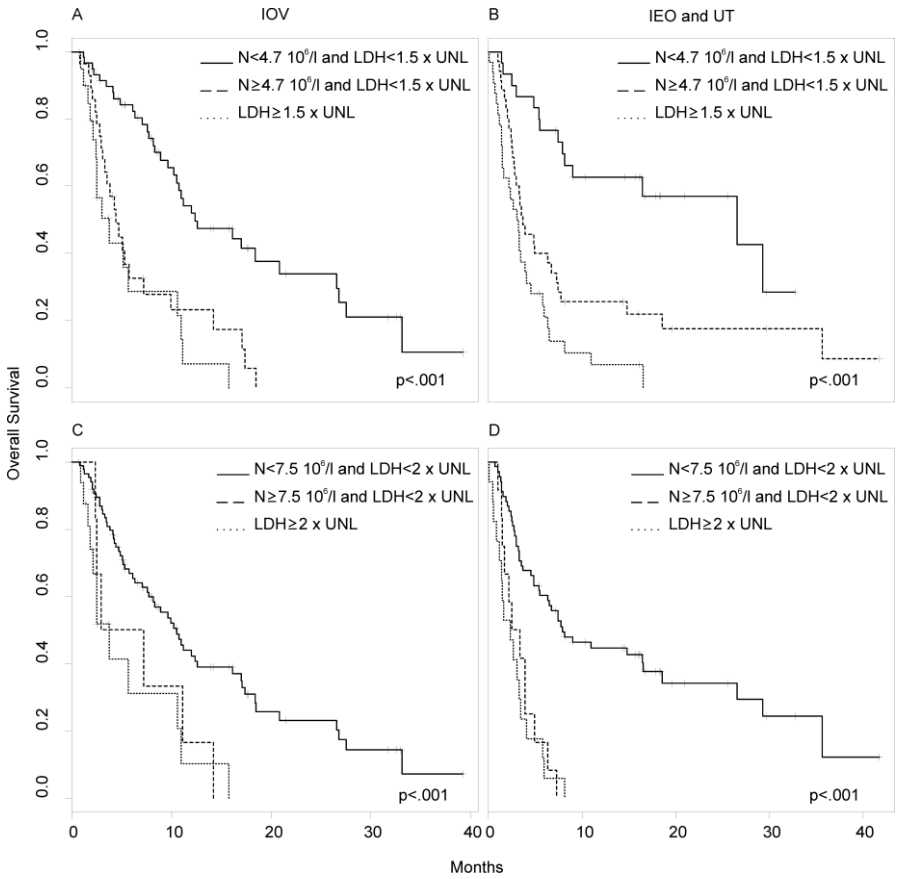
The sum of the prognostic factor points corresponds to the survival probability at 6, 12 and 24 months. For example, we sum the total points for a patient with LDH= 2.5 xUNL (upper normal limit) (for example, a value of 625U/L with a laboratory UNL=250U/L) and neutrophil count=$7x10^6$/L. Points for LDH=27, points for neutrophils=70 The sum of the prognostic factor points is 97, corresponding to a survival probability of 30% at 6 months, less than 10% at 12 months and tending to zero at 24 months.

3.1.3 Predictive value of the prognostic factors

We grouped patients from the two cohorts (IOV and the validation cohort consisting of IEO plus UT), separating the patients according to LDH and neutrophil baseline levels, to assess the ability of the prognostic factors to discriminate the patients who lived longer than 24 months. In the Cox regression final model, the value of neutrophils and LDH for which the HR was 1 were, approximately, $4.7x10^6$/L and 1.5 x UNL, respectively; therefore, we used these cut-offs to group patients. Both cut-offs rendered 3 groups of patients: (a) high LDH; (b)

low LDH and high neutrophils; (c) low LDH and low neutrophils. Survival of patients with high LDH did not diverge according to neutrophil value (data not shown). The 3 groups were significantly different at the survival analysis (Figure 4), being the median OS of patients with low LDH and low neutrophils (c) notably superior to that of the other 2 groups (Figure 4 A and B, and Table 3).

Fig. 4 Survival curves according to prognostic groups



Survival curves in patients treated with ipilimumab at IOV (left) and in the validation cohort (right, IEO and UT patients), according to cut off of LDH and neutrophils of 1.5x UNL and 4.7 x 10^6/L (upper) and of 2x UNL and 7.5 x 10^6/L (down).

Additionally, we also used the previously proposed prognostic cut-off for neutrophils[158] and LDH[72] of $7.5x10^6$/L and 2 x UNL, respectively, to gather the patients of IOV and validation cohort according to (a) high LDH; (b) low LDH and high neutrophils; (c) low LDH and low neutrophils. Again, at the survival analysis the 3 groups were significantly different

both for IOV and the validation cohort patients (Figure 4 C and D, and Table 4); moreover, these cut offs were more specific in discriminating long survivors. In particular, median OS of patients with baseline LDH superior to 2 xUNL (a) or more than 7.5 x10^6/L neutrophils (b) was far below 6 months, with zero patients alive at 24 months.

Tab. 4 Prognostic groups in IOV and validation cohorts

Cohort/prognostic group		N (deaths)	Median survival (months)	95% CI	6 m survival (%)	12 m survival (%)	24 m survival (%)	*P*
cut-of: LDH 1.5xUNL and neutrophils 4.7x10^6/L								
IOV	**Low LDH low neutrophils**	61 (34)	12.3	10.6-26.8	82.3	52.0	29.7	**<.001**
	Low LDH high neutrophils	28 (24)	4.4	3.3-9.9	27.9	17.5	0	
	High LDH	21 (16)	3.7	2.4-11.1	28.8	6.7	0	
IEO and UT	**Low LDH low neutrophils**	30 (14)	26.6	8.9-na	73.2	57.0	8	**<.001**
	Low LDH high neutrophils	35 (29)	3.7	2.8-7.5	37.1	22.0	8	
	High LDH	32 (30)	3.3	2.3-5.8	25.8	8.6	0	
cut-off: LDH 2xUNL and neutrophils 7.5x10^6/L								
IOV	**Low LDH low neutrophils**	87 (56)	11.6	7.7-17.0	66.7	43.9	20.2	**<.001**
	Low LDH high neutrophils	6 (6)	5.1	2.5-na	33.3	16.7	0	
	High LDH	17 (12)	3.7	2.1-na	31.1	10.4	0	
IEO and UT	**Low LDH low neutrophils**	68 (44)	7.9	5.5-26.6	58.7	42.7	29.4	**<.001**
	Low LDH high neutrophils	12 (12)	2.9	1.7-na	8.3	0	0	
	High LDH	17 (17)	2.9	1.6-5.8	0	0	0	

Survival cohorts in patients treated with ipilimumab at IOV and in the validation cohort (IEO and UT patients), according to cut off of LDH and neutrophils of 1.5x UNL and 4.7 x 10^6/L (upper) and of 2x UNL and 7.5 x 10^6/L (down). Both cut-off resulted in 3 groups (low LDH and low neutrophils vs. low LDH and high neutrophils vs. high LDH) that significantly differed for survival. Using the second cut-off set (down) the 2-year survival for patients with high neutrophils or high LDH is 0%.

3.2 Toxicity study

The toxicity study was performed on the full cohort of 140 patients, enriched with 27 patients enrolled after the completion of the prognostic study. Details about the patients are represented in Table 5.

Tab. 5 Patients characteristics for the toxicity study.

Patients characteristics	**Median or *N* (range or %)**
Sex male	86 (61.4)
female	54 (38.6)
Age	63.0 (27.0-85.0)
Number of previous treatments	1 (0-4)
Observation time (months)	6.2 (0.7-53.6)
Biomarker (normal range)	**Median (range)**
White blood cells (4.40-11.00 x 10^6/L)	6.2 (2.3-17.5)
Eosinophils (0-0.50 x10^6/L)	0.08 (0.01-0.89)
Neutrophils (1.80-7.8 x 10^6/L)	4.0 (1.1-16.2)
Lymphocytes (1.10-4.80 x 10^6/L)	1.3 (0.7-2.5)
CD3+ (7.0-27.0 %)	71.0 (42.0-92.0)
CD4+ (32-52 %)	39.0 (17.0-73.0)
CD8+ (16-33 %)	23.0 (5.3-79.0)
NK (7.0-27.0 %)	18.0 (5.6-35.6)
CD3/CD16/CD56+ (1-11 %)	3.0 (1.0-13.0)
LDH (<1, x UNL)	0.9 (0.4-11.56)
CRP (0-6 mg/L)	6.7 (2.9-214.0)
β2-microglobulin (1.09-2.53 ng/L)	2.3 (1.2-7.2)
IL6 (0-5.9 ng/L)	3.5 (2.0-658.0)
IL2 (0-2 ng/L)	7 (2-28.3)
S-100 (0.00-0.15 ug/L)	0.6 (0.03-97.0)
VEGF (62-707 ng/L)	431.5 (3.4-2100.0)

The table describes the features of the patients included in the study. Of note, most of the biomarkers lied within the normal ranges with the exception of CD3 positive lymphocytes, IL2 and S-100 levels, that were superior to the value of the average healthy population.

AEs are reported in Table 6 and reflect the typical toxicity pattern for ipilimumab in a real world setting. Sixty-five of 140 patients (46%) experienced some AEs (any grade, with 124 recorded AEs); of them, 49 had more than one AE, the commonest association being skin toxicity and constitutional symptoms (19 patients). Thirty-six patients (26%) experienced a severe adverse event (2 patients had 2 concomitant G3-4 AE, with a total of 38 recorded G3-4

AEs). Of note, two of them had late events (one G4 diarrhea 3 months after treatment completion and one G3 diarrhea plus hypophysitis 5 months after treatment completion).

Tab. 6 Adverse Events

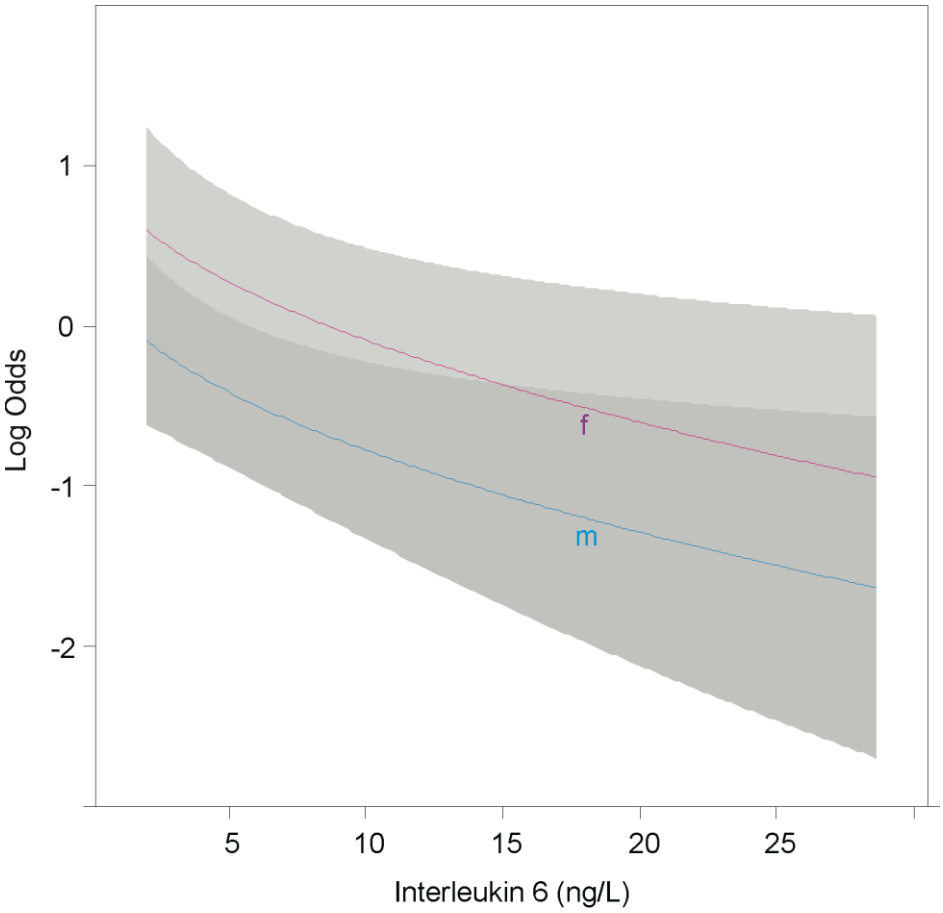
Adverse Event	***N* (%)** **tot=140 patients**	**G3-4 *N* (%),** **tot=140 patients**
Cutaneous	52 (37)	5 (4)
Pruritus	22 (16)	3 (2)
Rash	24 (17)	2 (1)
Vitiligo	6 (4)	0
Gastrointestinal	30 (21)	21 (15)
Diarrhea	21 (15)	19 (14)
Pancreatitis or lipase/amylase increase	5 (4)	2 (1)
Nausea/vomit	3 (2)	0
Constipation	1 (1)	0
Constitutional symptoms	21 (15)	0
Fatigue	13 (9)	0
Fever	7 (5)	0
Headache	1 (1)	0
Endocrine disorders	12 (9)	11 (8)
Hypophysitis	10 (7)	10 (7)
Thyroiditis	1 (1)	0
Hyperglycemia	1 (1)	1 (1)
Other	9 (6)	1 (1)
Arthralgia	5 (4)	1 (1)
Hepatotoxicity	2 (1)	0
Anemia	1 (1)	0
Posterior uveitis	1 (1)	0

The most frequent adverse events by all grades were cutaneous toxicity. On the other hand, gastrointestinal events accounted for the majority of severe (G3-4 according to the Common Terminology Criteria for Adverse Events) toxicities. Patients may have more than one toxicity event, in particular, out of 140 patients, 65 (46%) experienced some AEs and of them, 49 had more than one AE, for a total of 124 total recorded adverse events.

Grade 3-4 diarrhea, which occurred in 19 patients (14%), was the most frequent cause of treatment discontinuation due to toxicity, followed by hypophysitis, which occurred in 9 patients (6%). Patients experiencing G3-4 AEs remained on corticosteroid therapy for a minimum of 4 weeks, to a maximum of 8 months of mineral-corticoid replacement in a case of hypophysitis (treatment ongoing). Investigated serum antibody titers did not correlate with occurrence of AEs. Of note, we did not observe any correlation between baseline anti-thyroperoxydase titer and the occurrence of thyroiditis. One patient developed anti-GAD antibodies after treatment completion, without evidence of any AEs. One death was suspected caused by refractory hypophysitis because of clinical presentation with asthenia and declining PS associated with low Adrenocorticotropic Hormone and ionic imbalance, which worsened despite corticosteroids; however, the autopsy did not find evidence of immune aggression neither in the hypophysis nor in other organs.

The association between collected clinical figures, biomarkers and G3-4 AEs was investigated accounting for patient survival. Female patients and those with lower IL6 baseline serum levels had higher risk of developing G3-4 toxicity (OR=1.5, 95% CI 1.06-2.16 and OR=2.84 for 1ng/L variation, 95% CI 1.34-6.03, respectively). These two variables were also the only significant after backward selection (AIC rule satisfied, Chi-square 5.24, *P*=.022 and Chi-square 7.37, *P*=.007, respectively, Figure 5).

Fig. 5



The risk of adverse events decreases for increasing baseline levels of IL6. At parity of IL6 serum concentrations before ipilimumab treatment, the risk of toxicity is higher for women.

No significant correlation with the subtype of AE emerged from the cytokine analysis, as well as from the analysis of all considered biomarkers. Correlations and significance level for the full marker panel are reported in Table 7.

These findings were validated using bootstrap analysis; C-index was 0.65, standard error was 0.038.

Nine patients experiencing G2-4 AEs were treated with an anti-PD1 antibody, pembrolizumab or nivolumab, at melanoma progression; none of them had severe AEs with anti-PD1 therapy (one patient who had previously suffered from G3 arthritis experienced G1 arthritis after three pembrolizumab courses, resolved with short term low-dose corticosteroid therapy; one patient

who previously had G2 pruritus developed transient and self-limiting G1 pruritus after the first course of pembrolizumab).

Tab. 7 Biomarkers and associated risk of toxicity

Clinical or biological marker	Odds Ratio	*P*	95% CI
Interleukin 6	2.84	.007	1.34-6.03
Sex: Female	1.5	.022	1.06-2.16
LDH	1.18	.645	0.58-2.41
Age	2.82	.283	0.42-18.81
Interleukin 2	0.74	.934	0.00-1025.23
Beta2-microglobulin	0.16	.164	0.01-1.6
Natural Killer cells	0.63	.593	0.12-3.67
Total lymphocytes	0.28	.314	0.02-3.36
CD3 lymphocytes	0.41	.841	0-2500.35
CD4 lymphocytes	2.93	.722	0.01-1096.90
CD8 lymphocytes	14.04	.461	0.01-15879.76
Eosinophils	3.28	.151	0.65-16.63
S-100 protein	1.05	.489	0.91-1.21
C Reactive Protein	2.08	.308	0.51-8.52
White blood cells	15.02	.303	0.09-2621.67
Neutrophils	0.59	.704	0.04-8.95
Vascular Endotelial Growth Factor-A	0.65	.748	0.04-9.30

The table resumes the Odds Ratios (OR) for ipilimumab toxicity and significance levels for the markers analyzed in the study. Only interleukin 6 and sex had a significant association with the risk of immune-related adverse events (independently of toxicity subgroup). OR for continuous variables refers to the cumulative OR for one unit increase. LDH=lactic dehydrogenase.

Of note, two patients who had interrupted ipilimumab treatment because of G3 diarrhea and one patient who had interrupted ipilimumab because of G3 hyperglycemia were treated with anti-PD1s and did not experience any AEs, after a treatment time span between two and eight months.

4 Discussion, future perspectives and conclusions

With the advent of targeted therapies, oncologists need a reliable tool to personalize treatments in order to reduce the economic Healthcare System burden [159], as well as exposition to toxicity for patients who are unlikely to benefit from therapies. With the advent of second generation anti-inhibitory checkpoint monoclonal antibodies, ipilimumab can no more be considered the standard first line immunotherapy for metastatic melanoma patients; however, it is still a second line option for patients progressing after anti-PD1 inhibitors. In addition, it was approved even if only in US. So far, in the adjuvant setting: as consequence, this study could have a clinical relevance in a large patient population. Moreover, our findings should be tested in a cohort of patients treated with anti-PD1s or combination of anti-PD1 and anti-CTLA4 drugs, to verify if they are specific for ipilimumab or not.

4.1 Discussion

The aim of our study was to identify, in a real-world setting, prognostic factors that could be easily introduced in routine clinical practice, to screen the patients who are candidates to ipilimumab. LDH levels and neutrophil count were independent prognostic factors, regardless of melanoma origin and other clinical characteristics and biomarkers: the higher their values, the worse the patients' prognosis; LDH and neutrophils also resulted predictive factors of significant benefit from treatment with ipilimumab, i.e. a survival longer than two years. The nomogram we developed based on the findings of the prognostic model was externally validated, with satisfactory calibration and discrimination comparable to previously published

models for rare tumors [160] and we believe this nomogram could be very useful if implemented in the routine clinical practice. Objective response was not considered an end-point, because of the peculiar pattern of responses of ipilimumab: in fact, tumour response rates (TRR) are relatively low and it may take months for tumour shrinkage to occur, even after an initial progression [161]. This is why the most important results obtained with ipilimumab are usually measured in terms of OS and not in terms of TTR [62]. Our findings are consistent with previous works, concluding that neutrophils and LDH are independent prognostic factors for melanoma, in particular when patients are treated with immunotherapy [72, 158, 162], and the results of this part of the project have already been published by Valpione et al.[139] and confirmed on a larger cohort from the Italian Expanded Access Program data [141]. Moreover, high neutrophils were found to be an independent poor prognostic factor in metastatic renal cell carcinoma as well [163, 164]. The relationship between neutrophils and prognosis of melanoma is not fully known. One possible explanation is that the tumour microenvironment, which is believed to play an essential role in determining the response to immunotherapy [165, 166], could be influenced by neutrophils themselves, for example by producing tumor-stimulating or immunosuppressive cytokines [167-169], or coadiuvating tumor invasion with the release of metalloproteinases [170, 171]. An alternative hypothesis is that the circulating neutrophil count is the consequence of a cytokine stimulus conditioned by melanoma [172]: this way the neutrophils could be the expression of an immunosuppressive environment induced by the tumour itself. The absence of a significant correlation between neutrophil levels and toxicity looks like in favor of the latter hypothesis. The confirmation of the independent prognostic value of LDH is consistent with the well-established importance of this marker for melanoma [72, 173], at the point that it is included in the AJCC TNM classification for stage IV melanoma[174]. Although the importance of both neutrophils and LDH in melanoma patient prognosis was previously known, this is the first time that these parameters have been evaluated together with a large

series of clinical and biochemical markers; moreover, they were compared both for their prognostic and predictive implication, and resulted equally significant to be considered and helpful to stratify patient expected outcome. In our study, only LDH and neutrophils were retained in the final model, but interleukin-6 showed a trend of association with survival and it is likely that implementing the number of observations this factor reaches statistical significance; anyway, a planned extended analysis should give more insight into the prognostic value of an inflammatory marker as interleukin-6. The present study includes data from patients mainly treated before the approval of BRAF inhibitors and clinical implementation of PD1 inhibitors. In fact, patients treated with these drugs at disease progression after ipilimumab are a minority. Patients who progressed after ipilimumab had limited options at the time of the study, or their conditions allowed no active therapy. This reflects the oncologic treatment repertoire before the era of BRAF and PD1 inhibitors and enabled us to assess the value of prognostic factors without the confounding effect represented by post-ipilimumab therapies, which are expected to impact on survival[76, 127, 175-179]. BRAF status was unknown in a number of cases, because at the time no targeted therapies were available and as consequence the costs of the test were not justified; anyway, BRAF mutations have no influence on response to ipilimumab [68]. In the near future, due to the ever-growing diffusion of other target therapies, the conduction of a study on prognostic factors in patients treated with ipilimumab could be unfeasible because of the interference of subsequent treatments. In our opinion, the relevance of neutrophils and LDH as prognostic factors is worthy to be studied with the future therapeutic options, in particular for patients treated with the new generation immunotherapy (for example anti-PD1s or combined anti-PD1s plus ipilimumab). Interested in the possible predictive value of neutrophils and LDH, we looked for a cut-off that could discriminate the patients who did not survive more than 24 months, but additional studies are needed to clarify the best therapeutic approach for patients with high LDH or high neutrophils. The inefficacy of ipilimumab for patients with LDH

superior to 2 x UNL and neutrophils superior to 7.5 x 10^6/L undoubtedly, notwithstanding the difficulty to effectively quantify it in absence of a control group, raises the question whether the patients with high LDH and neutrophils are refractory to ipilimumab, or are affected by a more aggressive variant of the disease. In these subgroups of patients with worse prognosis, one possibility would be to test if newer therapies such as anti-PD1, combined anti-PD1 and anti-CTLA4, or anti-BRAF/anti-MEK inhibitors, could be more effective than ipilimumab alone. Moreover, although our results may give some indications regarding a subset of patients with a very poor prognosis, confounding factors are numerous and we encourage the research of efficient, reliable and possibly easy biomarkers that could be applied in the clinical practice to tailor the treatment of metastatic melanoma patients in the aim of avoiding wasting of resources and unnecessary toxicity, when possible.

To our knowledge, this is one of the first studies planned to look for predictors of toxicity with ipilimumab in patients with metastatic melanoma[147, 148].

Among a wide range of serum markers investigated, baseline levels of IL6, a well-known pro-inflammatory cytokine, can stratify the risk of developing AEs: in particular, the lower the level of IL6, the higher is the risk of AEs. Moreover, female patients have an increased risk than males. This may have implications for establishing personalized follow-up strategies for these patients.

CTLA4 blockade by ipilimumab provides suppression of the inhibitory signal to T-cells and increases the chances for activation against tumor cells. Activation of effector T-cells by CTLA4 is not antigen-specific, and the details of the process of tumor clearance and aggression of bystander cells are not completely understood. In fact, the pattern of immune deregulation occurring in individuals or animals with CTLA4 constitutive impairment does

not completely match with the most frequent AEs described for anti-CTLA4 antibodies, thus suggesting a toxicity mechanism for these drugs that is not limited to CTLA4 inhibition [180, 181]. For example, it is hypothesized that a prolonged depletion of Tregs following ipilimumab, disrupting the necessary immunomodulation at the gut interface, might be a cause for severe immune-mediated colitis[65]. Moreover, a direct action of ipilimumab on organs affected by toxicity may be hypothesized: for example, a potential explanation of immune-related hypophysitis comes from preclinical models that demonstrated CTLA4 expression in the pituitary gland [182]. However, this issue is still debated and the current hypothesis is that AEs follow a reduction in tolerance to antigens previously recognized as "self", which eventually leads to autoimmune events [183]. In this scenario, the inflammatory environment could play a pivotal role in regulating the development of an autoimmune disease.

Toxicity management in patients receiving immunostimulatory agents, such as ipilimumab, may be challenging, requiring a careful patient monitoring by experienced multidisciplinary teams. Despite appropriate patient education and guidelines for AE management, fatal events have been recorded in most studies and case series. Strikingly, AEs may occur after completion of treatment with ipilimumab, thus making often patients monitoring a difficult task. Moreover, AE management represents a meaningful economic burden [184] which adds to the already high drug costs. Taking these considerations together, identification of patients at risk of developing severe AEs is of paramount importance to plan personalized surveillance.

Researching for a possible association between treatment response and toxicity is challenging for at least two reasons. Firstly, although immunosuppressive therapy administered to manage AEs is considered not detrimental for anti-tumor response, its real impact on anti-tumor immune activation is unknown. Indeed, we could argue that patients with AEs might have a

better clinical effect than patients without AEs, but this is offset by the steroids necessary to resolve the toxicity, with the final result of making the patients no more responsive than others. Secondly, the probability of experiencing delayed AEs depends on patients' survival. Intuitively, a patient who dies because of rapid melanoma progression will not have any possibility to develop late AEs, despite an environment potentially favoring autoimmunity. However, the study performed on all the patients of the Ipilimumab Italian Expanded Access Program found no association between effectiveness and occurrence of any AEs [185]. In contrast, there appears to be a correlation between severe AEs and outcome for melanoma patients treated with anti-PD1s, according to a recent pooled analysis on nivolumab or nivolumab plus peptide vaccine treatment [186], which calls for further investigation in this field.

Inflammatory environment and microenvironment may play a role in immune-tolerance and tumour response, and inflammation could impact on AEs. A tumor-induced disruption of inflammatory cytokine network and balance between acute and chronic phase cytokines may have detrimental effects both on tumor response and, on the other hand, autoimmunity occurrence.

IL6 is an acute phase cytokine usually secreted during infections or tissue damage and its production is rapidly switched off after healing [187], but an aberrant production has been associated with several aspects of cancer biology [188].

Patients with higher levels of IL6 have lower risk of AEs; conversely, lower baseline levels of IL6 are associated with higher risk of AEs. Remarkably, metastatic melanoma patients with low IL6 serum levels had a better prognosis [155] and showed a trend for longer survival after ipilimumab treatment (considering the stringency of the statistical tests used in the present study, it is possible that a larger sample size would prove IL6 significance) [189]. These results are consistent with the findings of immune-suppression and tumour invasiveness

occurring after IL6 induction by colon rectal cancer cells [190]. In addition, a recent study demonstrated that melanoma cells, mainly via prostanoid signaling, induce negative immunomodulatory effects by means of a series of pro-inflammatory mediators, including the stimulus of IL6 secretion by myeloid cells, ending with the polarization of inflammation towards immune-suppression [191]. Moreover, IL6 might also directly contribute to cancer aggressiveness, since it has been linked to metastasis modulation and stemness in a number of cancers [192-195] and IL-6 production appears involved in the acquisition of an aggressive phenotype in mouse melanoma models [196]. In this context, metastatic melanoma may induce chronic high level of IL6, which can both confer aggressiveness and compromise the immune-inflammatory regulation, affecting the immune response elicited by CTLA4 blockade. Conversely, patients with low, normal physiological levels of IL6 (the cut-off we found is within the normal range) have more probability to respond to ipilimumab, but their immune system will also be at risk of significant AEs.

Given that females are at higher risk of several autoimmune diseases, it is not surprising that women have greater risk of AEs than men with the same levels of IL6 and time of observation. Interestingly, no gender effect was observed in the prognostic study; nonetheless, the prognosis of primary melanoma is different for the two genders, in favor of female patients. This difference was, in the past, justified with the dissimilar skin sun exposure habits and consequent differential UV damage sites and intensity, with relevant biological subgroups[197], but the influence of hormonal factors could be related to apoptotic pathways hormonal regulation [198]. The impact of the endocrine system on immune regulation in patients with melanoma is yet to be explored [199]. The results of this thesis corroborate the hypothesis of a significant role for endocrine elements in immunology of tumours, in a complex and not completely understood interaction of hormones with immune system.

Another finding of this study is the absence of correlation between auto-antibody tiers and the occurrence of AEs, confirming the hypothesis of cytotoxic lymphocyte mediated toxicity [200].

This study was designed to investigate markers commonly available at clinical laboratories in order to offer easy-to-obtain and reproducible biomarkers of toxicity and did not analyze immunosuppressive blood cells. The association between these cells, i.e. Tregs and myeloid derived suppressor cells, and adverse events is still controversial and more research is needed before they can be considered of clinical practice. However, a recent paper from Martens *et al.* [148] showed no association between immunosuppressive blood cells and adverse event occurrence in metastatic melanoma patients treated with ipilimumab.

A subgroup of patients who underwent treatment with anti-PD1 antibodies after severe toxicity from ipilimumab did not experience significant reactivation of AEs. This result should be viewed as hypothesis generating, as anti-PD1 antibodies could appear safe in patients who had severe AEs after ipilimumab. Further confirmation in larger cohorts is warranted, for no evidence of cross-linking toxicity emerged, as previously suggested by small series[201]. Of note, the diffusion of checkpoint inhibitors based immunotherapy for a growing number of cancers [202], the use of combination checkpoint inhibitors like ipilimumab plus nivolumab (with a significant risk of immune toxicity) [126, 127, 203], coupled with the implementation of ipilimumab for the adjuvant therapy of melanoma [136, 204], should increase the interest to extend the use of sex and IL6 for the prediction of AEs in patients affected with tumors other than melanoma and treatment setting other than metastatic.

In some measure, the calibration and the validation of the statistical model suffered from the small sample size, thus encouraging further validation of our results in larger series that could have a more significant predictive value.

4.2 Future perspectives

This study was specifically designed for biomarkers of response and toxicity easily available for daily clinical practice, but a number of other candidates could become accessible for extensive study in the near future and eventually proposed to clinicians for treatment decisions.

Possible nominees include TILs analysis in pre-treatment tumour biopsies, TCR clonality and diversity in TILs and circulating T cells[205] and functional assays of cancer-specific immune reactivity in patient lymphocytes[206]. Unfortunately, with the exception of TILs distribution and phenotype analysis in pre-treatment tumour biopsies for a subgroup of cancers (with the limitations concerned the availability of tissue) [207, 208], these tests are far from being validated or available in clinics.

Recently, JAK 1/2 loss has been highlighted as a biomarker of immunotherapy failure in patients with primary or acquired resistance to anti-PD1s. However, JAK 1/2 mutations or, more extended, IFN pathway impairments have only been demonstrated in a minority of cancers. As consequence, the majority of patients are left without an explanation for the disappointing response[209, 210]: such a prognostic biomarker would have an unacceptable false negative rate. Furthermore, specific gene sequence analysis offers only a narrow assessment and requires a biopsy that is not always easily available. In addition, DNA sequencing is expensive and whole exome sequencing of tumour biopsies for all patients would be unaffordable outside a clinical trial with dedicated resources. Unbiased analyses for mRNA or miRNA expression and proteomics suffer from the same limitations.

Liquid biopsy technologies (circulating tumour DNA [ctDNA] and its methylation, circulating RNA, exosomes and microvesicles) allow an in depth analysis of tumour

metastases without the burden and possible complications of surgical biopsy. For example, ctDNA has demonstrated to be useful for the study of resistance to targeted therapy and evolution of the tumour during treatments [211, 212]. Studying ctDNA fragment length and methylation status, useful information about actively transcribed or silenced genes could be available[213, 214], while circulating vesicles could provide an insight on the cross-talk between cancer cells and healthy cells, included immune cells, with potentialities that have only begun to be explored[215]. It's becoming clear that, with the progress of technical tools and the increasing sensitivity of the assays, liquid biopsy is looked upon at as the next frontier to study cancer metastases and microenvironment and these technologies could not only provide non-invasive tools to investigate the interactions between the tumour and the immune system, but might contribute to provide targets for personalized immunotherapy. For example, ctDNA sequencing could give information about tumour specific neo-antigens burden and neo-epitopes to be used as targets for CARs or bi-specific monoclonal antibodies. However, in addition to the costs, all of these innovative approaches are still to be tested or validated and are currently confined to research projects.

Finally, another implementation in the field of the present thesis research would be to study a mechanistic model to explain the biological reasons underlying neutrophil level prognostic value, and to study the interactions between immune cells, hormonal environment and IL6 and melanoma. This could not only provide a better understanding of melanoma biology, but also be useful for future therapeutic approaches, for example by means of anti-IL6 drugs or IL6 modulators that could have the dual benefit of inhibiting cancer cells and reprogramming immunosuppressive environments[216].

4.3 Conclusions

In conclusion, we propose that levels of neutrophils and LDH could help clinicians to select patients before treatment initiation, and ipilimumab may not be the best treatment in patients with higher neutrophil count and LDH, in particular when superior to $7.5x10^6$/L and x2 UNL, respectively. A comparative study would answer the question whether the patients with high LDH and neutrophils are refractory to ipilimumab, or are affected by a more aggressive variant of the disease. Serum baseline IL6 evaluated before ipilimumab treatment could be useful to identify patients at risk of toxicity and to plan a more specific monitoring during and after the treatment, aiming at increasing its safety. In particular, females with low IL6 serum levels should be carefully monitored for late AEs. This finding has implications for patients counseling and for planning appropriate toxicity surveillance even after treatment conclusion.

The results of this thesis could be proposed for prospective studies with new generation immunotherapy like anti-PD1s and combination immunotherapy (for example, combination of ipilimumab and nivolumab), or combination of immunotherapy and targeted agents, both in the metastatic and in the neoadjuvant or adjuvant settings.

In addition, there is space of hypothesis generating speculations about the biological mechanisms underlying the interactions between melanoma, neutrophils, IL6 and endocrine factors.

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