Pharmacology & Therapeutics 256 (2024) 108613

Contents lists available at ScienceDirect

Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/pharmthera

Immunotherapy in melanoma: Can we predict response to treatment with circulating biomarkers?

Elena Splendiani [a], Zein Mersini Besharat [a], Alessia Covre [b,c], Michele Maio [b,c], Anna Maria Di Giacomo [b,c], Elisabetta Ferretti [a,*]

[a] Department of Experimental Medicine, Sapienza University, Rome, Italy
[b] Center for Immuno-Oncology, Medical Oncology and Immunotherapy, Department of Oncology, University Hospital of Siena, 53100 Siena, Italy
[c] Medical Oncology, Department of Molecular and Developmental Medicine, University of Siena, 53100 Siena, Italy

ARTICLE INFO

Available online 16 February 2024

Associate editor: F Limana

Keywords:
Melanoma
Immune checkpoint inhibitors
Immunotherapy response
Liquid biopsy
Circulating biomarkers

ABSTRACT

Melanoma is the most aggressive form of skin cancer, representing approximately 4% of all cutaneous neoplasms and accounting for up to 80% of deaths. Advanced stages of melanoma involve metastatic processes and are associated with high mortality and morbidity, mainly due to the rapid dissemination and heterogeneous responses to current therapies, including immunotherapy. Immune checkpoint inhibitors (ICIs) are currently used in the treatment of metastatic melanoma (MM) and despite being linked to an increase in patient survival, a high percentage of them still do not benefit from it. Accordingly, the number of therapeutic regimens for MM patients using ICIs either alone or in combination with other therapies has increased, together with the need for reliable biomarkers that can both predict and monitor response to ICIs.

In this context, circulating biomarkers, such as DNA, RNA, proteins, and cells, have emerged due to their ability to reflect disease status. Moreover, blood tests are minimally invasive and provide an attractive option to detect biomarkers, avoiding stressful medical procedures.

This systematic review aims to evaluate the possibility of a non-invasive biomarker signature that can guide therapeutic decisions. The studies reported here offer valuable insight into how circulating biomarkers can have a role in personalized treatments for melanoma patients receiving ICIs therapy, emphasizing the need for rigorous clinical trials to confirm findings and establish standardized procedures.

Contents

Abbreviations: MM, Metastatic Melanoma; ICIs, Immuno-Checkpoint Inhibitors; FC, Flow Cytometry; Mono, Monotherapy; Combi, Combined therapy; PB, Peripheral Blood; UltraC, Ultracentrifugation.

* Corresponding author.
E-mail address: elisabetta.ferretti@uniroma1.it (E. Ferretti)
Social media:

1. Introduction

Since William B. Coley's first observation in the late 19th century regarding the capability of immune response to trigger tumors' spontaneous regression, several advancements have been made to

https://doi.org/10.1016/j.pharmthera.2024.108613

the point that immunotherapy is widely used in cancer therapy today.

Melanoma is a malignant skin cancer arising from melanocytes. Once it becomes metastatic, it undergoes rapid systemic dissemination, resulting in a very poor prognosis. The transformation of melanocytes to melanoma is primarily driven by oncogenic signaling pathways, triggered by a combination of environmental and genetic factors. Environmental risk factors include UV light exposure, the presence of atypical nevi, and a family history of melanoma. In clinical practice, melanoma is classified based on the evolution and progression of the lesion into 4 stages: stage 0 - a pre-invasive lesion confined in the epidermis, stages I and II - a series of initial phases of local invasion, stage III- invasion of the regional lymph nodes and stage IV - presence of metastases in distal sites.

Studies on the efficacy of immune checkpoint inhibition (ICI) against cancer led to the Nobel Prize in 2018 for James Allison and Tasuku Honjo. ICI stands out as one of the most active and promising approaches in the treatment of advanced melanoma, contributing to an increase in patient survival (Esfahani et al., 2020; Toor, Sasidharan Nair, Decock, & Elkord, 2019).

Based on these and many other studies, the Food and Drugs Administration (FDA) approved in 2011 the first immunotherapy agent, ipilimumab, for advanced melanoma treatment. In 2014 two other ICIs were approved, namely pembrolizumab and nivolumab, both PD-1 inhibitors. In 2016, atezolizumab, a PD-L1 inhibitor, received approval for the treatment of advanced cancer. More recently in 2022, Opdualag was approved by the FDA for metastatic melanoma treatment; this is a combination of nivolumab and relatlimab-rmbw, which is a LAG-3 inhibitor (FDA Approves Opdualag for Unresectable or Metastatic Melanoma, 2022).

Despite recent progress in treatments, advanced melanoma is still characterized by high mortality. Consequently, there is an urgent need to identify non-invasive and robust biomarkers useful for distinguishing patients who will benefit from adjuvant therapy from those who will not (Balch et al., 2009; Gershenwald & Scolyer, 2018).

In this context, blood-based liquid biopsy has garnered increasing interest due to its non-invasive nature and reliable performance (Heitzer, Haque, Roberts, & Speicher, 2019). The abundance of detectable and quantifiable molecules (DNA, RNA, and proteins) and immune cells released into the blood circulation, represents a valuable source of potential biomarkers. These biomarkers hold significant promise for the prognosis, diagnosis, and prediction of treatment response (Lim, Lee, Diefenbach, Kefford, & Rizos, 2018). Circulating biomarkers have been identified in both preclinical and clinical settings, but only a small number have received FDA approval for use in clinical applications, primarily to assist in cancer detection. Consequently, the investigation of blood-based biomarkers could have profound implications for melanoma patients undergoing ICIs therapy, with the potential to transform personalized medicine.

This review offers a systematic overview of blood-based biomarkers associated with melanoma patients' clinical outcomes when treated with immune checkpoint inhibitors (ICIs), either alone or in combination with other therapies, described in the last five years.

2. Study design (Prisma model)

A systematic literature research was conducted on Scopus and Web Of Science (WOS) database using the following keywords:

"Melanoma AND biomarkers AND immunotherapy AND circulating AND response",

"Melanoma AND biomarkers AND immunotherapy AND blood AND response".

The last search was performed on 9 September 2023 and the identified records were 647.

Study selection was performed following the PRISMA workflow, summarized in Fig. 1 (Page et al., 2021). Duplicate articles were removed through the Mendeley software and the timeframe taken into consideration included manuscripts published from 2019 to 2023.

In the screening phase, book, review, comment, communication, meta-analysis, non-English manuscripts or not congruent with the scope of this review were excluded by title or abstract.

Full-text articles, which have been assessed for eligibility, were excluded following the listed criteria:

- Not congruent: no blood biomarkers, immunotherapy response or melanoma patients enrolled;
- Only *in vivo* / *in vitro* studies: no human blood biomarker collection;
- Reduced number of subjects: <4 patients;
- Non-ICIs therapy or combined ICIs-immunotherapy.

In accordance with the inclusion criteria, described above, the bibliographic research in screening phase was evaluated by two researchers independently.

3. ICIs biomarkers of response

Immune checkpoints are key molecules involved in cell regulation of immune response and could be used by tumor cells to hijack immune surveillance (Fig. 2).

The response rate observed in advanced melanoma patients treated with anti-CTLA-4 (ipilimumab) or anti-PD-1 (nivolumab or pembrolizumab) monotherapy ranges between 20% and 40% but can reach up to 60% with combination therapy of anti-CTLA-4 plus anti-PD-1 (Esfahani et al., 2020; Toor et al., 2019).

Unfortunately, not every stage III and IV melanoma patient that receives ICIs treatment benefits and clinical response is often associated with immune-related adverse events (irAEs) (Larkin et al., 2015). In this context, the possibility of identifying the patients that will benefit the most with low invasive biomarkers able to guide the therapeutical choice and monitor the response to treatment is of utmost importance, and it could lie in blood-based liquid biopsy.

3.1. Routine blood parameters and white blood cell count

Routine blood parameters, such as lactate dehydrogenase (LDH), white cell blood count, hemoglobin, or cholesterol, could be useful biomarkers to indicate ICIs therapy response or patient survival improvement (Table 1).

Serum LDH levels are used in clinical practice as they can offer important insights into the prognosis of the disease. Monitoring serum LDH levels could help guide treatment decisions and assess intervention effectiveness due to their association with disease progression. Several studies have explored the link between LDH levels and response to ICIs therapy.

Overall, findings indicate that patients with high LDH levels before and during treatment are less likely to respond favorably to monotherapy or combined ICIs therapies, leading to a shorter survival rate (Ascierto et al., 2019; Bai et al., 2021; Burgermeister et al., 2022; Ma, Shang, Shanshan, Wang, & Cao, 2022; Pickering et al., 2023; Rogala et al., 2022).

Studies also investigated LDHB, an LDH isoform that was found to be elevated during treatment. LDHB was associated with disease progression and negatively correlated with progression-free survival (PFS) (Babačić, Lehtiö, De Coaña, Pernemalm, & Eriksson, 2020; Karlsson et al., 2021).

White cell blood count (WBC) and WBC ratios are important predictive factors of ICIs response and survival regardless of ICIs treatment. They were reported to be useful to individuate an infection status, inflammation, or autoimmune immunity response.

High baseline platelets-to-lymphocytes ratio (PLR), neutrophils-to-lymphocytes ratio (NLR or derivate NLR), monocyte-to-lymphocytes ratio (MLR), and neutrophil-to-eosinophil ratio (NER) were associated with poor prognosis and survival in patients treated with anti-PD-1,

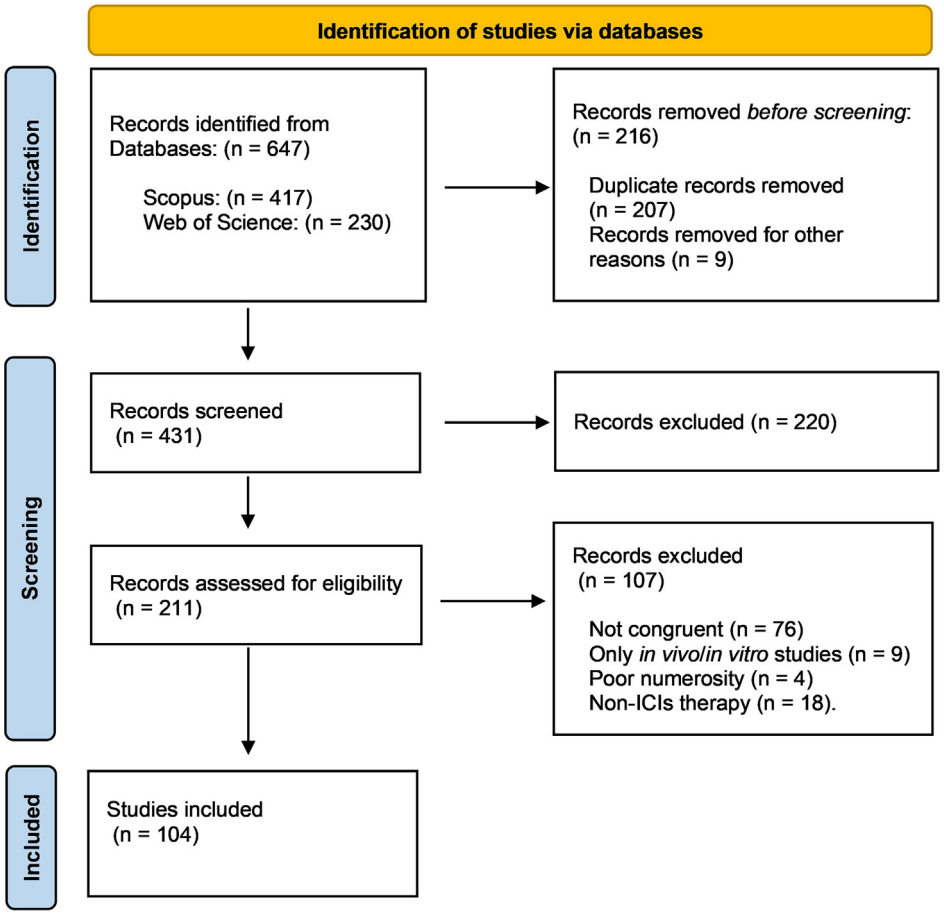

Fig. 1. Study selection process using PRISMA flow diagram. From the initially identified studies (n = 647) the duplicates were removed and then records were screened by title, abstract and full text leading to inclusion of 104 studies (Page et al., 2021).

anti-PD-L1 therapy and/or anti-CTLA-4 (Afzal, Sarwar, & Shirai, 2019; Bai et al., 2021; Bilen et al., 2019; Criscitiello et al., 2020; Guida et al., 2022; Ma et al., 2022; Varayathu et al., 2020).

Similarly, low levels of erythrocytes and lymphocytes were associated with shorter survival and disease progression (Indini et al., 2019; Kudura, Nussbaumer, Foerster, & Basler, 2022).

These parameters (LHD, NLR, PLR, or eosinophil counts) were also reported as prognostic biomarkers in more heterogeneous cohorts that included other advanced cancers as well as uveal melanoma patients.

These findings suggest a possibility of a common relapse mechanism underlying ICIs therapy (Criscitiello et al., 2020; Kartolo et al., 2020; Krishnan, Tomita, & Roberts-Thomson, 2020; Valero et al., 2021; Waninger et al., 2022).

Markers of cell activity detected in blood samples can also be informative for patient response to therapy. Baseline serum levels of eosinophil activity marker, eosinophil peroxidase (EPX), and high level of eosinophil cationic protein (ECP) were associated respectively with longer PFS and response to ICIs therapy (Ammann et al., 2022; Wendlinger et al., 2022).

Among routine blood parameters, hypercholesterolemia, a condition characterized by chronic inflammation, was associated with better outcomes in ICIs-treated cancer patients (Perrone et al., 2020).

These routinely tested laboratory markers could be used to build together with other variables (*i.e.* tumor mutational burden (TMB), number of metastases, and thyroid function) a significant predictive model for ICIs therapy response (Silva et al., 2022; Bai et al., 2021; Zhao et al., 2022).

A recent study used a machine learning model to predict ICIs response in a large cohort of advanced cancer patients, including melanoma.

Among the features that contributed most to the model, the following were reported: TMB, NLR, age, hemoglobin, platelets, and fraction of copy number alteration (Chowell et al., 2022).

3.2. Peripheral blood mononuclear cells (PBMC)

Emerging evidence suggests that blood cell composition can be evocative of a responsive environment and thus crucial for the identification of therapy response possibilities. Some cell subsets have been studied deeply for this purpose, such as T cells, B cells, myeloid-derived suppressive cells (MDSCs), and Natural Killer (NK) (Table 2).

3.2.1. $CD8^+$ T cells

$CD8^+$ T cells, cytotoxic T cells, are a type of white blood cells with an effector role in eliminating malignant but also damaged or infected cells. These cells activated by antigens, express on their surface a specific T-cell receptor (TCR) that is involved in antigen identification.

It has been shown that patients who benefit from anti-PD1 therapy are characterized by an increased $CD8^+$ T cell density and a higher TCR diversity (Andrews et al., 2021). $CD8^+$ T cell proliferation and increase of $CD8^+$ effector memory ($CD8^+CD45RA^-CD45RO^+CCR7^-$) in ICIs responders can be found before but also after treatment, while $CD28^+$ $CD8^+$ T cells increase only after treatment. In contrast, non-responder patients at the baseline are characterized by a decrease of CD28, an increase of inhibitory receptor-expressing cells (2B4 and

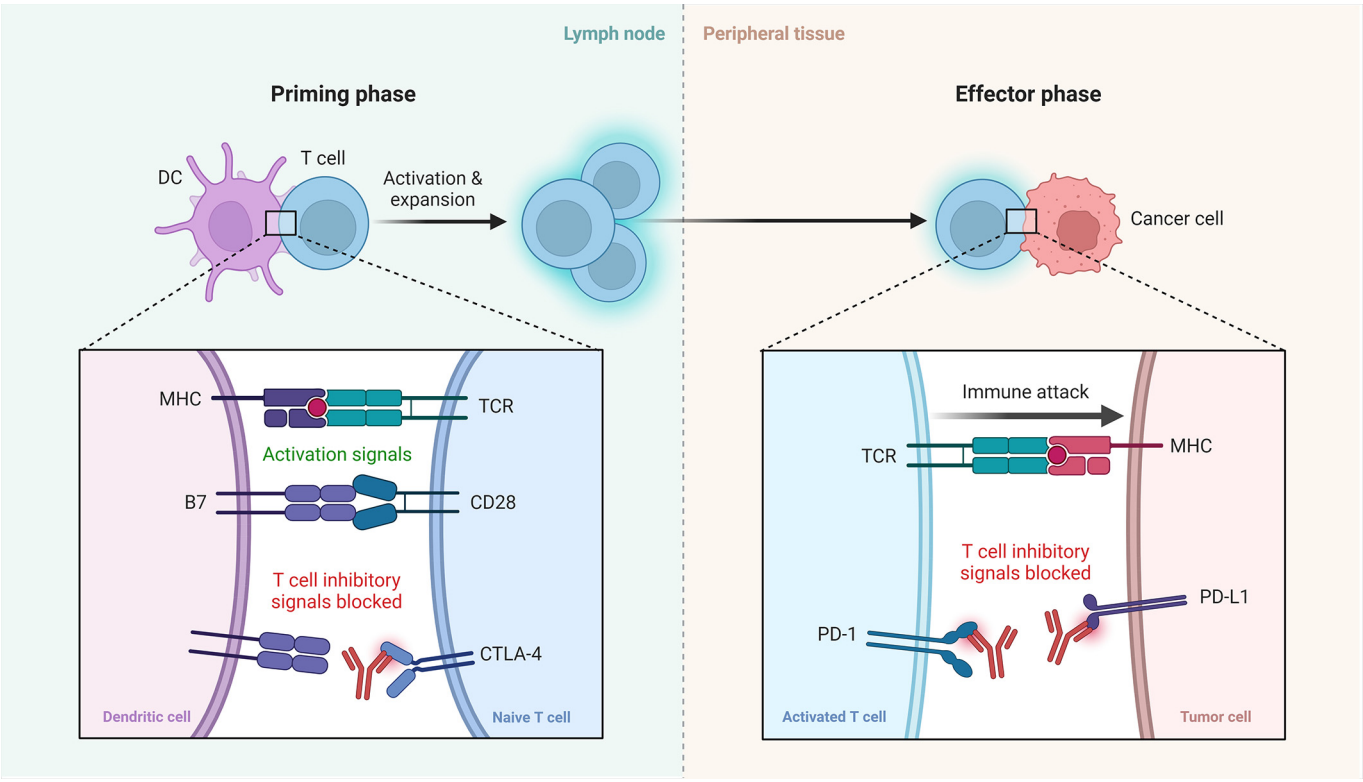

Fig. 2. Overview of the ICIs therapy mechanism. Reprinted from "Blockade of CTLA-4 or PD-1 Signaling in Tumor Immunotherapy", by BioRender.com (2023). Created with BioRender.com. Retrieved from https://app.biorender.com/biorender-templates

Table 1
List of routine blood parameters and WBC biomarkers of response to therapy.

Circulating biomarker	Source	Treatment	Method	Expression Level	Prognosis	Cohort	References
LDH	Plasma/Serum	ICIs (mono / combi)	Blood test	High	Poor	Melanoma and other cancers	(Bai et al., 2021), (Rogala et al., 2022), (Ma et al., 2022), (Burgermeister et al., 2022), (Pickering et al., 2023), (Ascierto et al., 2019)
LDHB	Plasma	ICIs (mono / combi)	Blood test	High	Poor	Advanced melanoma	(Babačić et al., 2020), (Karlsson et al., 2021)
NLR/dNRL	Plasma/Serum	ICIs (mono / combi)	Blood test	High	Poor	Melanoma and other cancers	(Babačić et al., 2020), (Ma et al., 2022), (Bai et al., 2021), (Varayathu et al., 2021), (Kudura et al., 2022), (Silva et al., 2022), (Kartolo et al., 2020), (Criscitiello et al., 2020), (Waninger et al., 2022), (Bilen et al., 2019), (Guida et al., 2022), (Valero et al., 2021)
PLR	Serum	ICIs (mono / combi)	Blood test	High	Poor	Melanoma and other cancers	(Ma et al., 2022), (Varayathu et al., 2021), (Kartolo et al., 2020), (Guida et al., 2022), (Bilen et al., 2019)
MLR	Blood	ICIs (mono / combi)	Blood test	High	Poor	Melanoma and other cancers	(Bilen et al., 2019)
NER	Serum	Anti-PD-1	Blood test	High	Poor	Melanoma and other cancers	(Varayathu et al., 2021)
Erythrocyte	PB	ICIs (mono / combi)	Blood test	Low	Poor	Advanced melanoma	(Kudura et al., 2022)
Lymphocyte count	PB	ICIs (mono / combi)	Blood test	High / Low	Good / Poor	Advanced melanoma	(Indini et al., 2019), (Kudura et al., 2022)
EPX, ECP	Serum	ICIs (mono / combi)	Elisa	High	Good	Advanced melanoma	(Ammann et al., 2022), (Wendlinger et al., 2022)
Cholesterol	Plasma	ICIs (mono / combi)	Blood test	High	Good	Melanoma and other cancers	(Perrone et al., 2020)

Lactate Dehydrogenase (LDH), Neutrophils-to-Lymphocytes Ratio (NLR), derivate NLR (dNLR), Platelets-to-Lymphocytes Ratio (PLR), Monocyte-to-Lymphocytes Ratio (MLR), Neutrophil-to-Eosinophil Ratio (NER), Eosinophil Peroxidase (EPX), Eosinophil Cationic Protein (ECP), Peripheral Blood (PB), Immuno-Checkpoint Inhibitors (ICIs), Monotherapy (Mono), Combined therapy (Combi).

Table 2
List of PBMC biomarkers of response to therapy.

Circulating biomarker	Source	Treatment	Method	Expression level	Prognosis	Cohort	References
$CD8^+$ T cells ($CD28^+$, effector memory)	PB	ICIs (mono / combi)	FC	High	Good	Advanced Melanoma	(Andrews et al., 2021), (Edner et al., 2023), (Reschke et al., 2021), (Salih et al., 2022)
$CD8^+$ T cells ($CD28^-$, $2B4^+$, $KLRG1^+$, OXPHOS,)	PB	ICIs (mono / combi)	FC and scRNA seq	High	Poor	Advanced Melanoma	(Andrews et al., 2021), (Edner et al., 2023), (Li et al., 2021)
$CD8^+$ naïve T cells	PB	ICIs (mono / combi)	FC and scRNA seq	Low	Good	Melanoma and other cancers	(Kasanen et al., 2020), (Khojandi et al., 2023),
TCR diversity	PB	Anti-PD-1	TCR seq and multiplex PCR assay	High	Good	Melanoma and other cancers	(Khojandi et al., 2023), (Salih et al., 2022), (Hogan et al., 2019)
TCR convergence	PB	ICIs (mono / combi)	TCR seq and scRNA seq	High	Good	Melanoma and other cancers	(Pan & Li, 2022)
$CD4^+$ T cells ($CD39^+$, HLA-DR^+, $CD26^{high}$)	PB	Anti-PD-1 and Anti-CTLA-4	FC, Mass Cytometry (CyTOF)	High	Good	Melanoma and other cancers	(Clouthier et al., 2019), (Friedman et al., 2022), (Pirozyan et al., 2020), (Strippoli et al., 2021), (Galati et al., 2023), (Edner et al., 2023)
$CD4^+$ T Treg ($CTLA4^+$, $ICOS^+$ $CXCR3^+$)	PB	ICIs (mono / combi)	FC	High	Poor	Advanced Melanoma	(Edner et al., 2023), (Krebs et al., 2021)
$CD14^+$ monocyte (S100A9 +)	PB	Anti-PD-1	FC, scRNA seq, Mass Cytometry (CyTOF)	High	Poor	Advanced Melanoma	(Pour et al., 2021), (Valpione et al., 2022), (Pirozyan et al., 2020)
$CXCR3^+$ T cells	PB	Anti-PD-1	FC, Mass Spectrometry	High	Poor	Melanoma and HNSCC tumors	(Han et al., 2019)
NKT cells	PB	ICIs (mono / combi)	FC, Mass Cytometry (CyTOF)	High	Good	Advanced Melanoma	(Strippoli et al., 2021), (Kasanen et al., 2020)
NK cells ($CD45RO^+$, $CD25^+$, $CD56^{dim}$ $CD16^+$DNAM-1^+)	PB	ICIs (mono / combi)	FC, Mass Cytometry (CyTOF)	High / Low	Good / Poor	Advanced Melanoma	(Pirozyan et al., 2020), (Kasanen et al., 2020)
$CD4^-$/$CD8^-$ (DNT) T cells	PB	ICIs (mono / combi)	FC	Low	Good	Advanced Melanoma	(Strippoli et al., 2021)
TNFα and IL-6 producing peripheral B cells	PB	ICIs (mono / combi)	FC	High	Good	Advanced Melanoma	(Jonge et al., 2021)
IgG	Serum	ICIs (mono / combi)	FC	High	Good	Advanced Melanoma	(Diem et al., 2019)
MDSCs cells (polymorphonuclear (PMN), arg 1^+ PMN and monocytic (M))	PB	ICIs (mono / combi)	FC	High / Low	Poor / Good	Advanced Melanoma	(Petrova et al., 2023), (Gondois-rey et al., 2021), (Krebs et al., 2021), (Gaißler et al., 2023)

T-cell receptor (TCR), Natural killer (NK) cells, Double Negative T cells (DNT), Myeloid-derived suppressive cells (MDSCs), Peripheral Blood (PB), Immuno-Checkpoint Inhibitors (ICIs), Monotherapy (Mono), Combined therapy (Combi), Single Cell RNA Sequencing (scRNA seq), Flow Cytometry (FC), Ultracentrifugation (UltraC).

KLRG1), and after therapy by an increase of CTLA-4^+ Treg (Edner et al., 2023; Reschke et al., 2021).

A specific population of $CD8^+$ T cells with high levels of oxidative phosphorylation (OXPHOS), CD38/CD39 ectonucleotidases, cytotoxic and exhaustion markers correlated with ICIs resistance and along with the transcriptomic information could predict therapy response (Li et al., 2021).

A deep analysis of $CD8^+$ T cells transcriptome and TCR revealed a loss of transcripts associated with a naïve phenotype in treatment responders and patients with better PFS, and the expansion of most rare and diverse cytotoxic $CD8^+$ T cell clones (Khojandi et al., 2023).

Immune activation induced by ICIs could be affected by age and this can have an impact on the response to treatment. It was suggested that older age may contribute to the presence of a clinical benefit. In elderly melanoma patients, before anti-PD-1 treatment, low levels of naïve $CD8^+$ T cells, an immune aging hallmark, and elevated serum levels of MCP-4 and OPG were associated with prolonged PFS (Kasanen et al., 2020). Additionally, old age responder patients were characterized by an increase in TCR clonality rearrangement, while younger responders (< 70 years) by an increase in TCR diversity (Salih et al., 2022). However, a previous work described differences in TCR repertoire related to different ICIs therapeutical approaches. Low clonality and high TCR diversity at the baseline have been reported to be associated with a favorable response and extended progression-free survival in anti-PD-1 therapy. Conversely, in the context of anti-CTLA-4 treatment, low clonality, and high TCR diversity have been linked to an unfavorable prognosis (Hogan et al., 2019). ICIs responders presented a higher level of TCR convergence with identical amino acids but different DNA sequences and this condition was significantly associated with OS and PFS. Convergent TCRs were more prevalent in $CD8^+$ T cells and enriched with antigen-experienced receptors, suggesting that convergence could be a mechanism to ensure the expansion of the antigen-specific T cells (Pan & Li, 2022).

3.2.2. $CD4^+$ T cell

Among the key players that finely regulate immune processes and thus could be involved in therapy response, $CD4^+$ T cells are well known for their ability to help B cells and $CD8^+$ T cells. An increase of $CD4^+$ T cells and $CD39^+$ $CD4^+$ T cells were associated with clinical benefit for patients treated with anti-CTLA-4 and anti-PD-1 monotherapy (Clouthier et al., 2019; Edner et al., 2023; Friedman et al., 2022; Pirozyan et al., 2020). A high percentage at the baseline of $CD4^+CD26^{-high}$ T cells were associated with anti-PD1 clinical benefit (Galati et al., 2023). In another study, $CD4^+$ T helper response to anti-TERT was most frequently detected before therapy in responders and was linked to improved PFS (Nardin et al., 2022). Anti-PD-1 treated melanoma patients with a high frequency of monocytes, in particular the S100A9 + subset, and low $CD4^+$ T cell/monocyte ratios were linked with poor response to therapy. Before treatment, a $CD14^+$ monocyte subpopulation expressing more HLA-I and HLA-II antigens, genes

involved in the adaptative immune response, inflammatory cytokine production, chemotaxis, and NF-kB regulation could be predictive of response (Pirozyan et al., 2020; Pour et al., 2021; Valpione et al., 2022). Following combined ICIs or anti-PD-1 therapy patients with poor response were characterized by an increase in proliferating CD4 $^{+}$ T Treg cells ($CD25^{+}CD127^{-}Foxp3^{+}CTLA\text{-}4^{+}$). Moreover, an increased CXCR3 $^{+}$, inducible T cell co-stimulator (ICOS), and CTLA-4 levels on Treg correlated with short OS (Carbone et al., 2022; Edner et al., 2023; Krebs et al., 2021). $CXCR3^{+}$ T cells ($CD4^{+}$ and CD8 $^{+}$ T cell) were found to be high in a percentage of patients with disease progression after anti-PD-1 treatment. Blockage of CXCR3 on mice *in vivo* experiments could facilitate tumor growth. It was proposed that $CXCR3^{+}$ cells can migrate into the tumor to activate IFN-γ signaling, which in turn increases CXCL9 and CXCL10 expressions, recruiting T cells into the tumor and inhibiting tumor growth (Han et al., 2019).

3.2.3. Natural killer cells

Natural killer (NK) cells are innate lymphoid cells involved in immune surveillance with a cytotoxic effect, characterized by the expression of CD56 and the lack of CD3 protein on their surface. Higher (bright) and lower CD56 (dim) density expression is associated with distinct immunoregulatory roles. In general, an increase of $CD3^{+}$-$CD56^{+}$NK T cells (NKT), $CD56^{+}$ NK, and $CD4^{+}$T along with a decrease in $CD4^{-}/CD8^{-}$ double-negative T cells (DNTs) were observed in ICIs responder patients (Pirozyan et al., 2020; Strippoli et al., 2021). During anti-PD-1 treatment, a significant increase of $CD3^{bright}CD56^{+}$ NKT cells together with increased expression of CD45RO and CD25 on $CD56^{dim}$ NK cells was observed only in responder patients (Kasanen et al., 2020). In addition, the presence of $CD56^{dim}CD16^{-}$DNAM-1^{-} NK cells was associated with disease progression, while $CD56^{dim}CD16^{+}$DNAM-1^{+} cells were associated with stable disease (Rethacker et al., 2021).

An ongoing clinical research study, involving approximately 200 participants, is investigating circulating lymphoid cells, particularly NK cells, before and during immunotherapy. The aim is to identify early and late indicators of both favorable response and resistance.

Given its substantial number of participants, the research has the potential to shed light on the role of lymphoid cells as biomarkers for responding to ICIs therapy (ClinicalTrials.gov ID NCT05062096).

3.2.4. B cells

The abundance of B cell clonotype as well as immunoglobulin expression could help identify responses to ICIs and B cells could contribute to anti-tumor immunity interacting with APC through MHC molecules. Jonge, K. De et al. investigated the role of B cells in promoting an inflammatory microenvironment in melanoma patients treated with ipilimumab and found a negative correlation between TNFα and IL-6-producing peripheral B cells with ICIs response (Jonge et al., 2021). High levels of serum immunoglobulin IgG (total and subclass) were associated with prolonged PFS (Diem et al., 2019).

3.2.5. Myeloid cells

Myeloid-derived suppressive cells (MDSCs) are also involved in ICIs therapy response and increased frequencies before and after therapy were associated with non-responder melanoma patients. Moreover, an absence of MDSCs was observed in melanoma patients without visible metastasis. In melanoma patients who did not benefit from therapy, there was a tendency at the baseline toward the accumulation of polymorphonuclear (PMN)- MDSCs, and monocytic (M)- MDSCs, both exerting a strong inhibition in T cell proliferation. Moreover, a correlation between increasing levels of IL-6, IL-8, TNFa, and PMN-MDSCs accumulation was found in metastatic patients (Petrova et al., 2023). Differences in the maturation status of PMN-MDSCs were also reported to be predictive of response to ICIs therapy. Patients who did not benefit from ICIs therapy presented at baseline higher frequencies of the mature subset PMN-MDCs while responders were characterized by immature PBMC-MDCs subset. Moreover, high PMN-MDC levels (arginase 1^{+}) and M-MDSCs were found to be associated with poor OS and PFS. Patient stratification based on M-MDSCs (high and low) levels revealed differences in all immune cell populations and importantly high M-MDSCs frequencies and LDH levels were independent prognostic biomarkers (Gaißler et al., 2023; Gondois-rey et al., 2021; Krebs et al., 2021).

3.3. ICI-expressing cells and circulating tumor cells

Recent studies have reported changes in the composition of inhibitory receptor-expressing cells during ICIs treatment, linked to response to treatment. However, the role of PD-1 on T cell surface as a predictive biomarker for ICIs therapy remains unclear due to conflicting results (Table 3).

Low baseline percentage of PD-1^{+} $CD73^{+}$ $CD8^{+}$ T cells and low PD-1^{+} CD3 $^{+}$ DNT cells were observed respectively in melanoma and heterogeneous cancer cohort that benefit from anti-PD-1 therapy (Bornschlegl et al., 2021; Capone et al., 2020).

Other researchers have reported a rise in PD-1 expression on CD8+ T cells among patients who responded to combined or ICIs monotherapy at the start of treatment, but not during. Additionally, these patients displayed heightened cell reactivity following *ex vivo* stimulation of the T cell receptor (Reschke et al., 2021). Moreover, a high score of "PD-1^{+} $CD8^{+}$ T cells x HLA-$DR^{+}CD4^{+}$ T cells x Treg" was shown to significantly correlate with ICIs response and long PFS in a cohort of different tumors, including melanoma patients (Mispelbaum, Hattenhauer, Held, Brossart, & Heine, 2022).

With decreased PD-1^{+} $CD3^{+}$ $CD8^{+}$ T cells in anti-PD-1 treated patients, high levels of LAG-3^{+} on $CD8^{+}$ or $CD4^{+}$ T cells were associated with poor PFS in both anti-PD-1 mono or combined therapy (Machiraju et al., 2021; Shen et al., 2021). In a large cohort of cancer patients treated with anti-PD-1, $CD8^{+}$ T cells, especially $K67^{low}$ cells, expressing intracellular but not surface LAG-3 were associated with poor prognosis and decreased cell function. Non-responder patients displayed activation of IL-6, STAT3, intracellular LAG-3 pathway, and T cell dysfunctions (Somasundaram et al., 2022). The expression of PD-1/PDL-1 was also detected in blood extracellular vesicles as an indicator of ICIs response. PDL-1^{+} Extracellular vesicles (EVs) from melanoma cells and $CD8^{+}$ T cells were lower in responder to combined ICIs therapy patients and described to present immunosuppressive properties. Furthermore, PD-1^{+} EVs were more abundant in non-responder patients and associated with poor prognosis (Cordonnier et al., 2020; Serrati et al., 2022). An ongoing clinical trial will investigate whether exosomal mRNA expression of PD-L1 and IFN-γ can predict the response to treatment with PD-1 and CTLA-4 inhibitors in a large cohort of approximately 150 patients (ClinicalTrials.gov ID NCT05878977). The total number of circulating tumor cells (CTCs) did not differ between responder and non-responder patients treated with ICIs, but it was reported that PDL-1^{+} CTCs were higher in responders at the baseline and decreased after treatment. Moreover, PDL-1^{+} CTCs were also associated with better PFS (Khattak et al., 2020). Immunotherapy-resistant melanoma patients expressed high levels of nuclear lysine-specific demethylase 1 phosphorylated at serine 111 (nLSD1p) in mesenchymal CTCs and in PD-1^{+} $CD8^{+}$ T cells. The role of LSD1 as an epigenetic enzyme can be crucial for CTCs dissemination and T cell dysfunctions. Indeed, LSD1 phosphorylation is associated with the co-expression of stem-like mesenchymal genes and regulates exhaustion transcription factor EOMES with a demethylation/acetylation switch (Tu et al., 2020).

3.4. Circulating molecules

3.4.1. Circulating free nucleic acids

A recent study introduced a novel Multicancer Early Detection (MCED) blood test, approved by the FDA in September 2023, that can detect various types of cancer at an early stage. By examining circulating tumor DNA (ctDNA) methylation patterns, this test can identify up to 50

Table 3
List of ICIs-expressing cells biomarkers of response to therapy.

Circulating biomarker	Source	Treatment	Method	Expression Level	Prognosis	Cohort	References
PD-1$^+$ CD73$^+$ CD8$^+$ T cells	PB	Anti-PD-1	FC	Low	Good	Advanced Melanoma	(Capone et al., 2020)
PD-1$^+$ CD3$^+$ DNT cell	PB	Anti-PD-1	FC	Low	Good	Melanoma and other cancers	(Bornschlegl et al., 2021)
PD-1$^+$ (CD3$^+$) CD8$^+$ T cells	PB	ICIs (mono / combi)	FC	High / Low	Good / Poor	Melanoma and other cancers	(Reschke et al., 2021), (Machiraju et al., 2021), (Mispelbaum et al., 2022)
LAG3$^+$ CD8$^+$/CD4$^+$ T cells	PB	ICIs (mono / combi)	FC	High	Poor	Advanced Melanoma	(Shen et al., 2021), (Machiraju et al., 2021)
CD8$^+$ T cells expressing IC-LAG-3	PB	Anti-PD-1	FC, scRNA seq	High	Poor	Melanoma and other cancers	(Somasundaram et al., 2022)
PDL-1 $^+$ EV	PBMC, Plasma	ICIs (mono / combi)	UltraC, FC, ELISA	Low / High	Good / Poor	Advanced Melanoma	(Serrati et al., 2022), (Cordonnier et al., 2020)
PD-1 EV	PBMC	ICIs (mono / combi)	UltraC, FC	High	Poor	Advanced Melanoma	(Serrati et al., 2022)
PDL-1$^+$ CTCs	PB	Anti-PD-1	FC	High at baseline and decrease at follow-up	Good	Advanced Melanoma	(Khattak, Reid, et al., 2020)
nLSD1p in CTCs and PD-1$^+$ CD8$^+$ T	PB	ICIs (mono / combi)	CD45 depletion	High	Poor	Advanced Melanoma	(Tu et al., 2020)

Double Negative T cells (DNT), Peripheral Blood Mononuclear Cells (PBMC), Peripheral Blood (PB), circulating tumor cells (CTCs), Immuno-Checkpoint Inhibitors (ICIs), Monotherapy (Mono), Combined therapy (Combi), Flow Cytometry (FC), Ultracentrifugation (UltraC).

types of cancer, including skin melanoma. Furthermore, it may facilitate the identification of the source of cancer and enhance both early detection and patient outcomes. Such methods detecting circulating nucleic acids show high potential in directing treatment and tracking disease advancement in individuals with cancer (Schrag et al., 2023). Recently a predictive model for PDL-1 inhibitor therapy was proposed based on the differences that occur in 3D genome architecture in T cell-related loci and their relationship to clinical response. The research group described 8 blood-based biomarkers using a 3D genomic approach to identify cancer patients, including melanoma, that are likely to benefit from anti-PDL-1 monotherapy (Hunter et al., 2023). Droplet digital PCR (ddPCR) is a useful technology that allows molecule quantification, including residual mutated BRAF and NRAS after ICIs treated patients. With this technology increased circulating tumor DNA (ctDNA) was observed in melanoma patients who presented disease progression (Keller et al., 2019).

Detectable ctDNA levels in patients who stopped anti-PD-1 therapy were reported to be highly predictive of recurrence risk, while undetectable ctDNA levels were associated with a low risk of relapse and response. The tumor mutational burden detected by liquid biopsy in patients treated with ICIs therapy was significantly decreased or undetectable in responder compared to non-responder patients and was also associated with longer PFS (Forschner et al., 2019; Warburton et al., 2020). Low copy number mutations were reported in the plasma of responder patients treated with anti-PD-1 and in particular BRAF V600 E/K and NRAS Q61/G12/G13.

The majority of patients with complete or partial response present undetectable ctDNA levels. Undetectable ctDNA or early decline of ctDNA levels were also associated with long PFS and OS in melanoma patients (Awada et al., 2021; Seremet et al., 2019).

Germline variants (single nucleotide polymorphisms - SNPs) have also been investigated for their usefulness as biomarkers. Variants in ILI1RL1 could have an impact on ICIs therapy response, the rs4988956 variant was found to be enriched in responder patients with specific genotype A/A and A/R compared to R/R (Montaudié et al., 2021).

SNPs in the PD-1 gene were related to response in melanoma patients treated with PD-1 inhibitors. Patients with the PD1.3 variant and the G/G genotype were more responsive than patients with the A/G genotype and the G/G genotype was associated with longer PFS compared to the AG allele (Parakh et al., 2021). In melanoma patients receiving either a combination of ICIs or monotherapy cell-free mRNA was evaluated for its prognostic value.

Several mRNAs related to immune activation were identified to be highly expressed in responders: BCL2L1, CXCL9, IDO1, IL13, MIF, CCL4, CCL5, CCR2, MYD88, TLR4, MYC, TGFB1, HLA—B, HLA—C, IRF1 compared to non-responders. Moreover increased BCL2L1, CCL4, CCL5, CXCL9, GZMB, and TNFSF10 gene levels at follow-up compared to baseline were reported (Ita et al., 2021).

Circulating non-coding RNAs, such as microRNAs are ideal candidate biomarkers due to their sensitivity and specificity. Recently, microRNAs were evaluated in a cohort of melanoma stage III-IV undergoing ICIs therapy and it was reported that their expression changed significantly during therapy and was related to response to treatment.

MiR-4649-3p, miR-1234-3p, and miR-615-3p expression levels significantly decreased after treatment in stage IV patients with complete response. Evaluating microRNA expression levels from stage III and stage IV melanoma patients, miR-1273e, miR-584-5p, and miR-1290 were increased in non-responders (Bustos et al., 2020). In a different study stable disease and responders treated with anti-PD-1 presented an increase in miR-16, miR-19b, and miR-25 expression levels after one month of treatment (Carbone et al., 2022). Findings on these circulating free nucleic acids reported in this paragraph are listed in Table 4.

3.4.2. Circulating proteins

Proteomic analysis revealed differences between responder and non-responder melanoma patients to ICIs therapy (Table 5). In particular, regarding metabolic proteins responders were characterized by a higher respiratory capacity reserve and an elevated basal glycolytic activity. Moreover, in responders was found the LDHC upregulation and the glucose transporter (Glut-14) was reported as the most significant upregulated gene on the T cell surface (Triozzi et al., 2022). Liquid Chromatography-Mass Spectrometry (LC-MS) combined with artificial intelligence delineated a glycoproteomic signature able to stratify ICIs treated patients about to the likelihood of benefit and fucosylation was a critical parameter (Pickering et al., 2023). Serum VEGF levels were significantly higher in non-responder patients before treatment with anti-CTLA-4 but they were not predictive of response in anti-PD-1 therapy or ICIs therapy combination (Khattak et al., 2020). In a larger cohort including non-small-cell lung cancers (NSCLC) high kinase activity of VEGF, STAT3, ERBB, and EGF signaling pathways were associated with response to anti-PD-1 therapy while PTEN activation was associated with resistance (Hurkmans et al., 2020). Low levels of hepatocyte growth factor (HGF) were associated with response, while high levels of leukemia inhibitory factor (LIF) with poor outcomes in anti-PD-1/PDL-1 treated patients and correlated negatively with the presence of tertiary lymphoid structures (Kubo et al., 2019; Loriot et al., 2021).

Table 4
List of circulating nucleic acid biomarkers of response to therapy.

Circulating biomarker	Source	Treatment	Method	Expression level	Prognosis	Cohort	References
Eight 3D genomic biomarkers	Whole Blood	Anti-PDL-1	EpiSwitch® protocols	Detected	Good	Melanoma and other cancers	(Hunter et al., 2023)
ctDNA	Plasma	ICIs (mono / combi)	ddPCR, NGS, Idylla	High / Low	Poor / Good	Advanced Melanoma	(Keller et al., 2019), (Warburton et al., 2020), (Forschner et al., 2019), (Awada et al., 2021), (Seremet et al., 2019)
SNPs IL1RL1 (rs4988956)	Whole Blood	ICIs (mono / combi)	WES, Sanger seq, qPCR	Detected	Good	Advanced Melanoma	(Montaudié et al., 2021)
SNPs PD1.3 (rs11568821, G > A)	Plasma	Anti-PD-1 and Anti-PDL-1	PCR	Detected	Good	Advanced Melanoma	(Parakh et al., 2021)
BCL2L1, CXCL9, IDO1, IL13, MIF, CCL4, CCL5, CCR2, MYD88, TLR4, MYC, TGFB1, HLA-B, HLA—C, IRF1	Plasma	ICIs (mono / combi)	qPCR	High	Good	Advanced Melanoma (Stage IV)	(Ita et al., 2021)
MiR-4649-3p, MiR-1234-3p, and MiR-615-3p	Plasma	ICIs (mono / combi)	NGS	Low	Good	Advanced Melanoma (Stage IV)	(Bustos et al., 2020)
MiR-1273e, MiR-584-5p, and MiR-1291	Plasma	ICIs (mono / combi)	NGS	High	Poor	Advanced Melanoma (Stage III-IV)	(Bustos et al., 2020)
MiR-19b, MiR-16 and MiR-25	Plasma	Anti-PD-1	qPCR	High	Good	Advanced Melanoma	(Carbone et al., 2022)

Circulating tumor DNA (ctDNA), Single nucleotide polymorphism (SNPs), Immuno-Checkpoint Inhibitors (ICIs), Monotherapy (Mono), Combined therapy (Combi), Polymerase chain reaction (PCR), Droplet digital PCR (ddPCR), Quantitative PCR (qPCR), Whole exome sequencing (WES), Next generation sequencing (NGS).

Collagen-rich peritumoral stroma characterizes immune excluded tumors and could interfere with CD8+ T cell activity. Stroma components and extracellular matrix (collagen III and vimentin turnover) were proposed as blood-based biomarkers for ICIs therapy response. High baseline levels of collagen III pro-peptide (PRO—C3) and crosslinked pro-peptide (PC3X) were associated with worse PFS while an increase of MMP- degraded type III (C3M), citrullinated, MMP- degraded vimentin (VICM) and decrease of PRO-C3 were related to better OS (Hurkmans et al., 2020). High baseline levels of granzyme B-mediated degradation of type IV collagen (C4G) and low PRO-C3 characterized patients responding to anti-CTLA-4 therapy (Jensen et al., 2020). High-resolution isoelectric focusing (HiRIEF) LC-MS/MS was used to evaluate plasma proteins in patients treated with anti-PD-1 and 31 plasma proteins were reported as differentially expressed between responder and non-responder patients. sPDL-1, sPD-1, IL-6, IL-10, acute phase inflammation, cell adhesion protein, neutrophil degranulation, and T cell response proteins were among the plasma proteins associated with response to therapy (Babačić et al., 2020). Low apolipoproteins and high inflammation markers (including LDHB) were related to ICIs therapy resistance and poor PFS (Karlsson et al., 2021). A proteomic test, BDX008, combining acute phase response, wound healing, and complement activation has been reported to stratify metastatic melanoma patients that will benefit from anti-PD-1 therapy. BDX008^{+} patients were reported to present better OS and a positive trend for PFS compared to BDX008^{-}. The efficacy of this biomarker was observed especially in BRAF wild type and low NLR subgroup (Ascierto et al., 2019). Coagulation parameters such as von Willebrand factor (vWF), D-dimers, and ADAMTS13 activity have been associated with clinical response to immunotherapy in melanoma-treated patients. Low baseline levels of vWF antigen were higher in responder patients and correlated with good OS, while vWF antigen increased levels were observed in patients with disease progression (Stadler et al., 2023). The early disappearance of tumor-associated antigen (TAA) reactive T cell either NY-ESO-1 or Melan-A was associated with better PFS and OS in anti-PD-1 treated patients (Bochem et al., 2021). Soluble urokinase plasminogen activator receptor (suPAR), a marker of immune activation, was associated with good response and better OS when detected before and after therapy at low levels in serum samples of ICIs treated patients (Loosen et al., 2021).

3.4.3. Cytokines and chemokines

Effector molecules may also provide insight into the mechanisms underlying resistance to immune checkpoint inhibitors, which are often associated with the development of immune-related adverse events (irAEs). Elevated levels of CXCL9, CXCL10, and CXCL11 along with high baseline expression levels of CXCL5 and CD25 were found in responder patients to anti-PD-1 treatment (Carbone et al., 2022; Fujimura et al., 2019; Kasanen et al., 2020). CXCL9/CXCL10/CXCL11 and interferon-g (IFN-γ) elevated levels after therapy were identified as markers of higher irAEs risk. A partial overlap was observed between response to therapy and irAE markers after ICIs therapy. Patients who were more likely to respond to therapy presented increasing levels of IL-17A, IFN-γ, and IFN-γ related proteins and a high frequency of proliferating T cells (Gérard et al., 2021; Nuñez et al., 2023). Increased INF-γ levels after treatment were also found in patients who benefit from anti-CTLA-4 monotherapy and combined with anti-PD-1, while non-responder patients had higher baseline levels of IL-10 (Friedman et al., 2022). Different treatment strategies result in distinct sets of cytokines and chemokines associated with patients' outcomes. Indeed, therapy response and better OS /PFS were associated with high levels of IFN-γ, TNFα, IL8, IP-10, CXCL10, IL-10 in combined ICIs therapy, while high TRAIL, MCP-1, IL-2, IP-10, MCP-4, TARC, ENA78, CXCL5 and low levels of IFN-γ and IL-6 in anti-PD-1 monotherapy (Su Y Lim et al., 2019; Pedersen et al., 2022). A group of cytokines (IFN-γ, INF-α, TNFα, sTIM-3, IL-1b, IL-1a, IL-12p70, MIP-1b, IL-13, sCD28, sGITR, sPDL-1 and IL-10) expressed in levels below the median were associated with longer OS in cancers including uveal melanomas treated with anti-PD-1 monotherapy (Botticelli et al., 2022).

3.4.4. Soluble ICIs

Soluble immune checkpoint molecules detection in blood samples, such as sPD-1, sPDL-1, sCTLA-4, sLAG-3, and sTIM, could be informative of response to ICIs therapy. Resistance to anti-PD-1 was associated with a high baseline level of sLAG-3 while resistance to combined therapy was associated with high sPD-1 detected in serum samples (Machiraju et al., 2021). Low plasma levels of sPD-1 and high levels of butyrophilins (BNT2A-1) were observed at the baseline of responders to anti-PD-1. In the same study, better OS was associated with low sPD-1 and high BMI (BMI ⩾ 25) (Incorvaia et al., 2023). In contrast, Pederson et al. found that

Table 5
List of proteins biomarkers of response to therapy.

Circulating biomarker	Source	Treatment	Method	Expression level	Prognosis	Cohort	References
LDHC and Glut-14 on T cell	Plasma	Anti-PD-1	UPLC/MS-MS	High	Good	Advanced Melanoma	(Triozzi et al., 2022)
Glycoprotein fucosylation	Plasma	ICIs (mono / combi)	LC–MS	Detection	Poor	Advanced Melanoma	(Pickering et al., 2023)
VEGF	Serum	Anti-CTLA-4	Milliplex panel	High	Poor	Advanced Melanoma	(Khattak, Abed, et al., 2020)
VEGF, STAT3, ERBB and EGF Kinase activity	PBMC	Anti-PD-1 and Anti-CTLA-4	PTK- PamChip-96 microarrays	High	Good	Melanoma and other cancers	(Hurkmans, Verdegaal, et al., 2020)
PTEN Kinase activity	PBMC	Anti-PD-1 and Anti-CTLA-4	PTK- PamChip-96 microarrays	High	Poor	Melanoma and other cancers	(Hurkmans, Verdegaal, et al., 2020)
HGF	Serum	Anti-PD-1	ELISA	Low	Good	Advanced Melanoma	(Kubo et al., 2019)
LIF	Plasma	Anti-PDL-1 (mono / combi)	Proteomics (Olink® Explore 1536 panel)	High	Poor	Melanoma and other cancers	(Loriot et al., 2021)
PRO-C3, PC3X	Serum	Anti- PD-1 and Anti-CTLA-4	ELISA, MS	High / Low	Poor / Good	Advanced Melanoma	(Hurkmans, Jensen, et al., 2020), (Jensen et al., 2020)
C4G	Serum	Anti-CTLA-4	MS, C4G ECLIA assay	High	Good	Advanced Melanoma	(Jensen et al., 2020)
MMP-C3M, MMP-VICM, citrullinated	Serum	Anti- PD-1	ELISA	High	Good	Advanced Melanoma	(Hurkmans, Jensen, et al., 2020)
31 proteins, including sPD-1, sPDL-1, IL-6, IL-10	Plasma	Anti- PD-1	HiRIEF-LC–MS/MS	High	Good	Advanced Melanoma	(Babačić et al., 2020)
Apolipoproteins	Plasma	ICIs (mono / combi)	LC-MS/MS	Low	Poor	Advanced Melanoma	(Karlsson et al., 2021)
Inflammation markers	Plasma	ICIs (mono / combi)	LC-MS/MS	High	Poor	Advanced Melanoma	(Karlsson et al., 2021)
BDX008	Serum	anti- PD-1	BDX008 test	Positive	Good	Advanced Melanoma	(Ascierto et al., 2019)
vWF	Plasma	anti- PD-1	ELISA	Low / High	Good / Poor	Advanced Melanoma	(Stadler et al., 2023)
NY-ESO-1, Melan-A	PB	anti-PD-1	FC	Low	Good	Advanced Melanoma	(Bochem et al., 2021)
suPAR	Serum	ICIs (mono / combi)	ELISA	Low	Good	Melanoma and other cancers	(Loosen et al., 2021)
CXCL9, CXCL10, CXCL11, CD25, MCP-4, OPG	Serum	Anti-PD-1	Proximity extension assay,	High	Good	Advanced Melanoma	(Kasanen et al., 2020)
CXCL5	Serum	Anti-PD-1	ELISA	High	Good	Advanced Melanoma	(Fujimura et al., 2019)
CXCL9, CD25	Serum	Anti-PD-1	ELISA	High	Good	Advanced Melanoma	(Carbone et al., 2022)
IFN-γ, IL-17 A	Serum, PB	ICIs (mono / combi)	Proteomics: (Olink 96 Inflammation panel), Single- cell 32- plex IsoCode chip	High / Low	Good / Poor	Advanced Melanoma	(Nuñez et al., 2023), (Friedman et al., 2022), (Gérard et al., 2021)
IL-10	PB	Anti-CTLA-4 (mono / combi)	Single- cell 32- plex IsoCode chip	High	Poor	Advanced Melanoma	(Friedman et al., 2022)
TNFa, IL-8, IP-10	Plasma	ICIs (combi)	Multiplex immuno- assay	High	Good	Advanced Melanoma	(Lim et al., 2019)
TRAIL, MCP-1, IL-2, IP-10, MCP-4, TARC, and ENA78	Plasma	Anti-PD-1	Multiplex immuno- assay	High	Good	Advanced Melanoma	(Lim et al., 2019)
sPD-1, sPD-L1, IFNγ, IL10, CXCL10, and TNFα	Plasma	ICIs (combi)	Meso Scale kits	High	Good	Advanced Melanoma	(Pedersen et al., 2022)
CXCL5	Plasma	Anti-PD-1	Meso Scale kits	High	Good	Advanced Melanoma	(Pedersen et al., 2022)
IFNγ and IL-6	Plasma	Anti-PD-1	Meso Scale kits	High	Poor	Advanced Melanoma	(Pedersen et al., 2022)
IFN-γ, INFa, TNFa, sTIM3, IL-1b, IL-1a, IL-12p70, MIP-1b, IL-13, sCD28, sGITR, sPDL-1 and IL-10	Serum	Anti-PD-1	Multiplex immuno- assay	Low	Good	Melanoma and other cancers	(Botticelli et al., 2022).
sLAG-3	Serum	Anti-PD-1	ELISA	High	Poor	Advanced Melanoma	(Machiraju et al., 2021).
BNT2A-1	Plasma	Anti-PD-1	ELISA	High	Good	Overweight melanoma patients	(Incorvaia et al., 2023)
sPD-1	Plasma	Anti-PD-1	ELISA	Low / High	Good / Poor	Overweight melanoma patients	(Incorvaia et al., 2023)
sCTLA-4/ipilimumab	Serum	Anti-CTLA-4	ELISA, nSMOL assay	High	Good	Advanced	(Koguchi et al., 2021),

(continued on next page)

Table 5 (*continued*)

Circulating biomarker	Source	Treatment	Method	Expression level	Prognosis	Cohort	References
						Melanoma	(Pistillo et al., 2019).
sCD73	Serum	Anti-PD-1	ELISA	High	Poor	Advanced Melanoma	(Turiello et al., 2020)
sBTLA	Serum	ICIs (mono / combi)	Multiplex immuno- assay	High / Low	Poor / Good	Melanoma and other cancers	(Gorgulho et al., 2021)

Peripheral Blood Mononuclear Cells (PBMC), Peripheral Blood (PB), Immuno-Checkpoint Inhibitors (ICIs), Monotherapy (Mono), Combined therapy (Combi), Liquid Chromatography-Mass Spectrometry (LC-MS), Ultra-high performance liquid chromatography (UPLC)-MS/MS, High-resolution isoelectric focusing (HiRIEF) LC–MS/MS, Flow cytometry (FC).

high plasma levels of sPD-1 as well as sPDL-1 were associated with favorable outcomes (longer PFS) in patients treated with combined therapy (Pedersen et al., 2022). Increasing plasma sPDL-1 and sPD-1 levels during treatment were associated with response to anti-PD-1 therapy in melanoma patients (Babačić et al., 2020). Increased serum sPD-1 levels were described in several studies of anti-PD-1 treatment; however, the results are contradictory. Of note, in some works, no correlation between sPD-1 and response to treatment was reported (Music et al., 2019). In melanoma patients treated with ipilimumab monotherapy, detection of serum sCTLA-4 or trough levels of ipilimumab (post-treatment) may predict longer or favorable clinical outcomes but are associated with higher irAEs risk (Koguchi et al., 2021; Pistillo et al., 2019). High soluble CD73 baseline levels before and during ICIs treatment were associated with a lack of response to treatment, low PFS and OS, while it was identified as an independent prognostic factor (Turiello et al., 2020). The soluble form of the B and T lymphocyte attenuator (sBTLA) was described as an independent prognostic factor with a negative association with OS. sBTLA was also significantly associated with the frequency of circulating $CD3^+$ $CD8^+$ $BTLA^+$ T cells, and it was proposed as a biomarker for poor ICIs therapy response (Gorgulho et al., 2021).

4. Emerging combined strategies and their biomarkers

Despite advancements in combined therapeutic strategies involving ICIs, a significant number of patients still experience disease progression or develop resistance to treatment. The potential of combining ICIs with other therapeutic approaches and immune modulators is currently under investigation as an encouraging prospect. However, there is a need for a systematic method to assess the effectiveness of different combination therapies, and blood-based biomarkers may offer a practical solution for implementing this approach. Below we report some blood biomarkers associated with combined therapy strategies.

4.1. Radiotherapy + ICIs

A study investigating *in vitro* stimulated serum of anti-PD-1 plus radiotherapy-treated patients reported that specific cytokines (Th1, Th2, and Th17) correlated with response to treatment and longer PFS. Moreover, non-responder patients had lower levels of IL-17 A at baseline and lower INF-γ levels during treatment, both correlating with poor PFS (Gérard et al., 2021).

In patients undergoing combined treatment of anti-PD-1 and radiotherapy, proliferation ($Ki67^+$) of $PD\text{-}1^+$ $CD8^+$ T cells and $PDL\text{-}1^+$ $CD8^+$ T cells before treatment characterized responder patients, while PD-1/PDL-1 expression on non-proliferating $Ki67^-$ $CD8^+$ and $CD4^+$ T cell correlated with worse outcomes (Meireson et al., 2021).

While radiotherapy combined with anti-CTLA-4 showed an increase in $CD4^+$ and $CD8^+$ $ICOS^+$ T cells, only the $CD8^+$ increase was associated with better PFS (Boutros et al., 2020).

Radiotherapy associated with both ICIs agents suggests a general activation of T cells and an increase in the expression of ICOS and PD-1 on $CD8^+$ T cells. Moreover, baseline low levels of $CD8^+$ naïve T-cells and higher $TIM\text{-}3^+$ $CD4^+$ T cells could predict response to therapy (Ratnayake et al., 2022). Melanoma patients with brain metastasis who benefited from a combination of ICIs and radiotherapy showed a high frequency of activated memory $CD8^+$ T cells and $ICOS^+$ CD8 T cells. Additionally, there was a significant increase in tumor-specific antigen reactivity in T cells secreting INF-γ. On the other hand, patients with progressive disease displayed an increase in $CD4^+$ T reg (Hassel et al., 2022). Both NLR and dNLR were significantly lower after the treatment in responders, and an increase in $CD8^+$ T cells before and during treatment was associated with better PFS (Boutros et al., 2020).

4.2. Immunotherapy combinations

The expected antitumor response from anti-CTLA-4 plus a high dose of IFN-α could be affected by gender differences. Indeed, it was reported that female patients were associated with longer PFS and OS and characterized with higher $CD3^+$ T cells, $CD3^+$ $CD4^+$ T helper cells and a trend of higher IL-1b levels compared to male patients (Saad et al., 2022).

Patients treated with the same therapeutic strategy that presented high levels of Th1 cell and high $CD8^+$ T cell were characterized by complete response, while patients presenting higher Th2 cell levels were associated with progressive disease (Khunger et al., 2021).

A clinical trial, where patients were treated with anti-CTLA4 and IFN-α, reported that early on treatment a decrease of T cell clonality in blood was associated with improved OS and PFS (Khunger, Rytlewski, Fields, Yusko, & Tarhini, 2019). Another study where patients were treated with a high dose of anti-CTLA4 and IL-2, described granzyme B and HLA-DM /HLA-DO ratio as suitable blood biomarkers of response to treatment and their association with tumor burden decrease (Silk et al., 2020).

An increase in tumor INF-γ signature was also detected in hTERT vaccination plus anti-CTLA4 treated responder patients. Of note, in these patients vaccine enriched TCR clones were detectable in blood biopsy (Ellingsen et al., 2022). Similarly, patients receiving anti-PD1 treatment combined with a personalized neoantigen vaccine (NEO-PV-01) that had increased clonal baseline TCR repertoires and longitudinal stability were characterized by a longer PFS whilst the frequencies of antigen-experienced T and B cells correlated with TCR repertoire characteristics (Poran et al., 2020).

In another study that evaluated anti-ICOS alone or in combination with anti-PD-1, $ICOS^{high}$ $CD4^+$ T cells characterized patients with greater clinical benefit (Yap et al., 2022).

Patients with prolonged PFS treated with combined anti-PD-1 therapy and anti-CD155 therapy presented a higher percentage of effector memory $CD8^+$ T cells and a lower number of $CD8^+$ $PD\text{-}1^+$ and $CD4^+$ $PD\text{-}1^-$ cells (Beasley et al., 2022).

5. Conclusions

Blood-based biomarkers have the potential to enhance the effectiveness of immunotherapy and provide valuable insights into the tumor microenvironment's responsiveness. This review emphasizes recent research on identifying circulating biomarkers associated with therapy

resistance or benefit. It also highlights the importance of utilizing these biomarkers in clinical practice to personalize cancer treatment and optimize patient outcomes.

Despite variations in immunotherapy agents and their administration, common results from the analyzed studies indicate encouraging possibilities for markers like LDH, whole blood cell counts, and their ratios to predict response to ICIs. Furthermore, patients who benefit from ICIs appear to exhibit an increase in cytotoxic activity within specific lymphocytes ($CD8^+$ or $CD4^+$), while non-responders show a more immune suppressive profile along with increased ctDNA levels (Fig. 3).

Effector molecules such as sPD-1, sPDL-1, IL-6, IL-10,TNFα,and IFN-γ demonstrate ambiguous roles; however, some evidence suggests predictive values vary based on whether patients are receiving combined or monotherapy.

While these findings suggest the possibility of identifying blood-based biomarkers of therapeutic response, better standardization is needed due to variability across studies.

Establishing a standard protocol for blood collection, processing, and the examination of immune cell subpopulations is crucial to minimize bias. Especially those caused by differences in flow cytometry protocols or single-cell analysis (Badalà, Nouri-mahdavi, & Raoof, 2008; Heumos et al., 2023; Kalina, 2020).

The analysis of CTCs, circulating RNA, and EVs may not be accurate or reproducible due to material scarcity or differences in isolation protocols from blood samples (Mondelo-Macía, Rodríguez-Ces, Suárez-Cunqueiro, & Romay, 2022; Xu et al., 2018).

Moreover, despite the high sensitivity and specificity of microRNAs as molecular biomarkers there are still gaps in understanding their involvement in antitumor immunity and cancer immune evasion mechanisms, rendering challenging their translation to a clinical test (Pogribny, 2018).

Indeed, molecular regulatory networks conferring resistance to immunotherapy in melanoma patients are currently under investigation and one such network was recently identified using single-cell spatial multi-omics (Pozniak et al., 2024).

However, in the rapidly advancing field of cancer research, accurate detection of biomarkers to develop personalized medicine regimens and guide the selection of appropriate therapies is becoming feasible goal with the development of new kits and biosensors able to detect molecules minimizing user-introduced bias from blood, plasma, or cell suspensions (Arifuzzman et al., 2023; Calvo-Lozano et al., 2022).

FDA-approved tests have been developed using circulating cells and tumor DNA as indicative markers of therapeutic response. These tests support the diagnostic process but are not diagnostic themselves. CELLSEARCH Circulating Tumor Cells kit is the only FDA-approved solution capable of detecting circulating tumor cells in prostate, breast, and colorectal cancer (Riethdorf et al., 2007).

Detecting circulating tumor cells can be challenging due to their low levels in certain types of tumors including melanoma. However, advancements have been made including tools development such as size filtration and fluorescent *in-situ* hybridization that suggest a possibility of overcoming these limitations (Yanagita, Luke, Hodi, Jänne, &

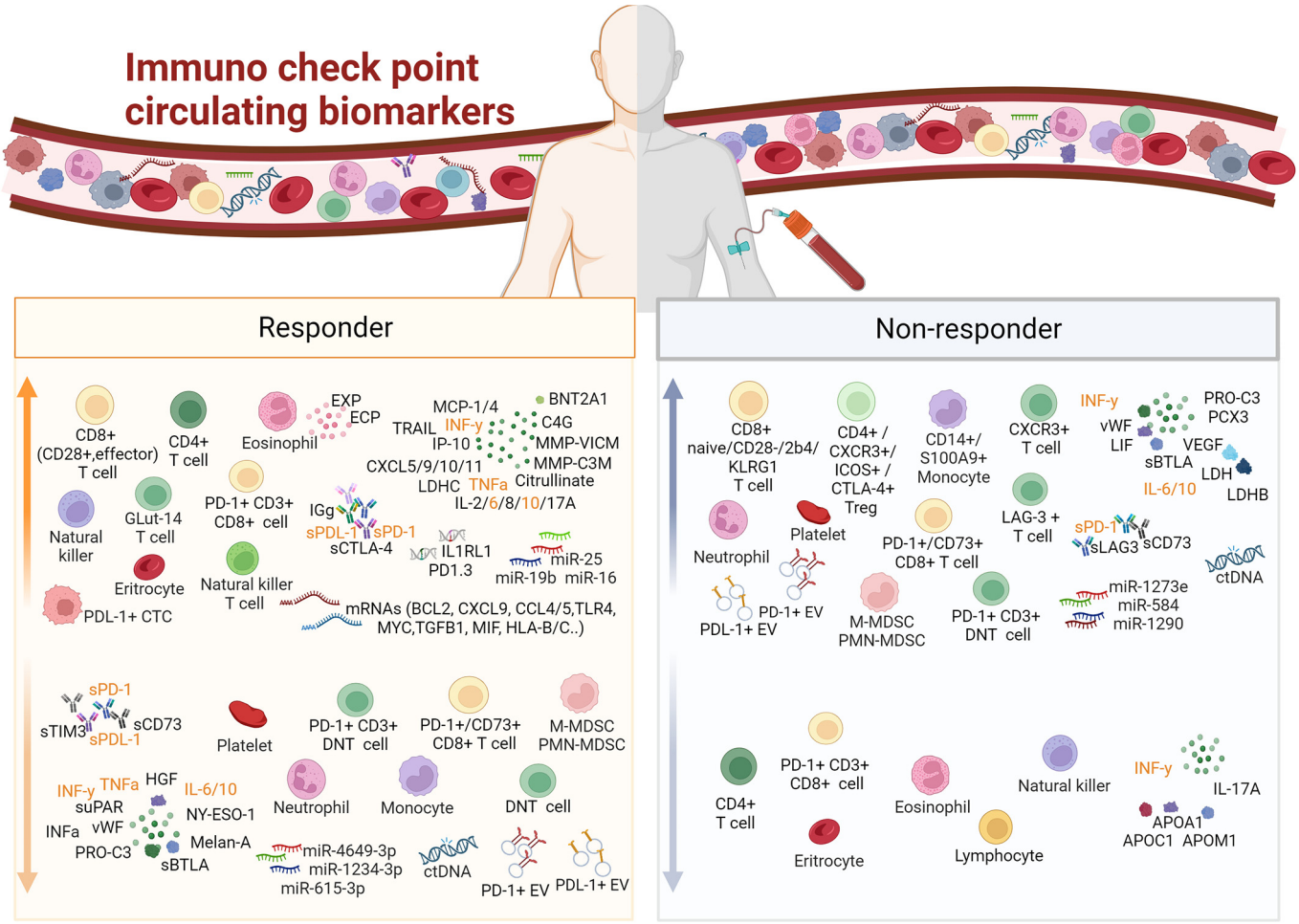

Fig. 3. Overview of the blood-based biomarkers identified in this review that could discriminate patients that benefit from immune checkpoint inhibitor (ICI) therapy. In orange the molecules with differences between mono or combined therapy. Created with BioRender.com.

Paweletz, 2018). Multicancer early detection (MCED) blood tests are the most promising blood-based biomarker tests to become the first that could be used in clinical practice. MCED leverages circulating DNA methylation patterns or SNPs to detect cancer or therapy response, including immunotherapy (Brunsgaard et al., 2023; Eroglu et al., 2023; Khaddour et al., 2022; Schrag et al., 2023). Current tools show great promise for future use in therapy follow-up, particularly with immunotherapy. However, this will require extensive research to incorporate it into clinical practice for therapy patient stratification. It is also essential to keep exploring new combination therapies and conducting trials while utilizing, when possible, kits or biosensors to draw more robust conclusions and accelerate their application. Furthermore, efforts must be made to collect and standardize data through platforms and consortia. That will ultimately allow researchers to reach a turning point by integrating predictive models and artificial intelligence to identify signatures of impactful biomarkers and guide treatment choices.

CRediT authorship contribution statement

Elena Splendiani: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing, Formal analysis, Methodology, Visualization. **Zein Mersini Besharat:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Alessia Covre:** Data curation, Investigation, Validation, Writing – review & editing. **Michele Maio:** Funding acquisition, Investigation, Supervision, Validation, Writing – review & editing. **Anna Maria Di Giacomo:** Formal analysis, Investigation, Validation, Writing – review & editing. **Elisabetta Ferretti:** Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was supported by co-funding of the European Union - The National Recovery Plan, Mission 4 Component 2 Investment 1.4 - Next Generation EU project CN3 - Spoke 2 Cancer - code CN_00000041 (CUP: B83C22002870006) and Associazione Italiana per la Ricerca sul Cancro (AIRC) Project "5 per Mille" EPICA [Grant Number 21073].

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