Thesis

Predictive biomarkers in cancer patients receiving immune checkpoint inhibitors: Early C-reactive protein kinetics as a prognostic and predictive marker for response and survival in a multi-cancer collective

submitted by

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Graz, 12.10.2023

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Graz, 12.10.2023 Amelie Sandner m.p.

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Abstract in German

Hintergründe

Rezent durchgeführte Studien, welche den prädiktiven Wert der frühen longitudinalen Kinetik des C-reaktiven Proteins (CRP) hinsichtlich der Effektivität von Immuncheckpoint Inhibitoren (ICIs) untersuchten, schlugen diesen als Biomarker für die Vorhersage des klinischen Behandlungserfolgs von Patient*innen mit fortgeschrittener Krebserkrankung vor. Ziel dieser Studie war es, die Genauigkeit der frühen CRP-Kinetik für die Vorhersage der ICI-Wirksamkeit anhand einer großen Kohorte, welche verschiedene solide Krebsarten beinhaltet, zu validieren.

Methoden

562 Patienten mit soliden malignen Erkrankungen, die sich in zwei österreichischen Krankenhäusern einer palliativen Behandlung mit Immuncheckpoint Inhibitoren unterzogen, bildeten die Studienkohorte. In einem ersten Schritt wurden die Patient*innen anhand ihrer spezifischen longitudinalen Serum-CRP-Verläufe während der ersten drei Monate der ICI-Behandlung gemäß dem zuvor definierten Drei-Gruppen-Modell von Fukuda et al. (2021) klassifiziert und dessen Genauigkeit analysiert. In einem zweiten Schritt wurde das etablierte CRP-Kinetik-Modell um eine vierte Gruppe erweitert. Das neu definierte Vier-Gruppen-Modell (CRP-Flare-Responder, CRP-Responder, CRP-Non-Responder, All-Normal-CRP) wurde hinsichtlich seiner prädiktiven und prognostischen Präzision bewertet. Als Co-primäre Endpunkte wurden die Objektive Ansprechrate (ORR), das Progressionsfreie Überleben (PFS) und das Gesamtüberleben (OS) definiert. Für die statistische Analyse der Zusammenhänge kamen uni- und multivariable logistische Regressionsmodelle, Landmark-Analysen und Cox-Proportional-Hazard-Modelle, welche die CRP-Kinetik als zeitabhängige Variable behandelten, zum Einsatz.

Ergebnisse

Die ORR für Patient*innen mit All-Normal-CRP, CRP-Responder, CRP-Flare-Responder und CRP-Non-Responder betrug 41%, 38%, 31% bzw. 12%. Das mediane OS und das PFS betrugen 24,5 Monate (95%CI 18,5 - nicht erreicht) und 8,2 Monate (95%CI 5,9-12,0) für Patient*innen mit All-Normal-CRP, 16,1 Monate (95%CI 12,6-19,8) und 6,1 Monate (95%CI 4,9-7,2) für CRP-Responder, 14,0 Monate (95%CI 8,5-19,4) und 5,7 Monate (95%CI 4,1-8,5) für CRP-Flare-Responder und 8,1 Monate (95%CI 5,8-9,9) und 2,3 Monate (95%CI 2,2-2,8) für CRP-Non-Responder (log-rank p für PFS und OS <0,001). Diese Ergebnisse setzten sich in der multivariablen Analyse fort. Der prognostische und prädiktive

Wert des nach Fukuda et al. prädefinierten Drei-Gruppen-Modells der frühen CRP-Kinetik konnte bestätigt sowie mittels des erweiterten Vier-Gruppen-Modells präzisiert werden.

Conclusio

Die frühe CRP-Kinetik fungiert als präziser Biomarker im Hinblick auf eine frühzeitige Vorhersage des klinischen Behandlungserfolges bei Patient*innen, die unter diversen Krebserkrankungen soliden Typus leiden und sich einer Immuncheckpoint Inhibitor Therapie unterziehen.

Abstract in Englisch

Background

Recently, early C-reactive protein (CRP) kinetics have been suggested as biomarkers predicting the clinical outcome of patients with advanced cancer treated with immune checkpoint inhibitors (ICIs). The aim of this study was to validate the accuracy of early CRP kinetics for the prediction of ICI efficacy in a large multi-cancer cohort.

Methods

562 patients with solid malignancies undergoing palliative ICI treatment at two Austrian hospitals formed the studied cohort. In a first step, the patients were classified by their specific longitudinal serum CRP response patterns during the first three months after ICI treatment initiation according to the previously defined three-group model by Fukuda et al., 2021, and its accuracy was analysed. In a second step, the established CRP kinetics model was expanded by a fourth pattern of CRP kinetics. The refined four-group model (CRP-flare responders, CRP responders, CRP non-responders, all-normal CRP) was evaluated regarding its prognostic and predictive accuracy. Objective response rate (ORR), progression-free survival (PFS) and overall survival (OS) were defined as co-primary endpoints. Uni-and multivariable logistic regression models, Landmark analysis and Cox-proportional hazard models including CRP kinetics as time-dependent variable were implemented.

Results

The ORR in patients with all-normal CRP, CRP responders, CRP flare-responders and CRP non-responders was 41%, 38%, 31% and 12%, respectively. The median OS and PFS estimates were 24.5 months (95%CI 18.5 – not reached) and 8.2 months (95%CI 5.9-12.0) in patients with all-normal CRP, 16.1 months (95%CI 12.6-19.8) and 6.1 months (95%CI 4.9-7.2) in CRP-responders, 14.0 months (95%CI 8.5-19.4) and 5.7 months (95%CI 4.1-8.5) in CRP flare-responders and 8.1 months (95%CI 5.8-9.9) and 2.3 months (95%CI 2.2-2.8) in CRP non-responders (log-rank p for PFS and OS <0.001). These findings prevailed in multivariable analysis. The prognostic and predictive value of the established three-group model for early CRP kinetics by Fukuda et al. could be confirmed and refined by means of the extended four-group model.

Conclusion

Early on-treatment CRP kinetics represent an accurate biomarker for early prediction of treatment response, progression risk and clinical outcome in patients undergoing ICI therapy across various solid cancer entities.

Publications

Co-authorship of “Early kinetics of C-reactive protein for cancer-agnostic prediction of therapy response and mortality in patients treated with immune checkpoint inhibitors: a multi-center cohort study”, currently under revision for publication in the “Journal for ImmunoTherapy of Cancer” (manuscript ID jitc-2023-007765.R1)

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List of Abbreviations

APC	Antigen Presenting Cell
APP	Acute Phase Protein
ASCO	American Society of Clinical Oncology
BSA	Body Surface Area
CD	Cluster of Differentiation protein
CI	Confidence Interval
CR	Complete Remission
CRP	C-Reactive Protein
CTCAE	Common Terminology Criteria for Adverse Events
CTL	Cytotoxic T-Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen-4
DC	Dendritic Cell
dNLR	derived Neutrophil-to-Lymphocyte Ratio
DRESS	Drug Rash with Eosinophilia and Systemic Symptoms
ECOG PS	Eastern Cooperative Oncology Group Performance Score
ESMO	European Society for Medical Oncology
FDA	U.S. Food and Drug Administration
FT4	Free Tetraiodothyronine
GEJ	Gastroesophageal Junction
HCC	Hepatocellular Cancer
HNSCC	Head and Neck Squamous Cell Carcinoma
ICI	Immune Checkpoint Inhibitor
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IMDC	International Metastatic Renal-Cell Carcinoma Database Consortium Score
IQR	Interquartile Range
irRECIST	immune-related Response Evaluation Criteria In Solid Tumors
LDH	Lactatedehydrogenase
mAbs	monoclonal Antibodies
MCC	Merkel Cell Carcinoma
mCSCC	Metastatic Cutaneous Squamous Cell Carcinoma
MHC	Major Histocompatibility Complex
MMF	Mycophenolate-Mofetil
MMR	Mismatch Repair
MSI	Microsatellite Instability
MSI-H/dMMR	Microsatellite Instability-High/ Mismatch Repair-Deficient
mUC	metastatic Urothelial Carcinoma
mut/Mb	mutations per Megabase
NG	Neutrophil Granulocyte
NK	Natural Killer Cell
NSCLC	Non-Small Cell Lung Cancer
ORR	Overall Response Rate
PD-L2	Programmed Cell Death Ligand 2
PD-1	Programmed Cell Death-1
PD-1/L-1/ PD-L1	Programmed Cell Death-1/ Ligand-1
PI3K	Phosphoinositide-3-Kinase
PMBCL	Primary Mediastinal B-Cell Lymphoma
PR	Partial Remission

RCC	Renal Cell Carcinoma
SBRT	Stereotactic Body Radiation Therapy
SCLC	Squamous Cell Lung Cancer
SHP-1	Src homology 2 domain-containing protein tyrosine phosphatase 1
SHP-2	Src homology 2 domain-containing protein tyrosine phosphatase 2
TCR	T-Cell Receptor
TEN	Toxic Epidermal Necrolysis
TIL	Tumor Infiltrating Lymphocyte
TKI	Tyrosine Kinase Inhibitor
TMB	Tumor Mutation Burden
TMB-H	Tumor Mutational Burden-High
TME	Tumor Microenvironment
TNBC	Tripple Negative Breast Cancer
Tregs	regulatory T-cells
UC	Urothelial Carcinoma
ULN	Upper Limit of Normal

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1 Introduction

The use of immune checkpoint inhibitors (ICIs) has revolutionized treatment for cancer patients. An increasing understanding of the tumor-host interaction at molecular and cellular immunological levels revealed the therapeutic potential of procedures that support the body's natural antitumor defense (1). The growing knowledge in this field has paved the way for a range of new antitumor therapies based on immunologically mediated processes. The discovery that tumor cells express tumor-specific and tumor-selective antigens due to genetic alteration and epigenetic dysregulation provided the basis for the development of tailor-made therapies against molecular components of tumor cells (1–3).

The function of ICIs focuses on the disinhibition of the native immune response and therefore the activation of antitumor immunity which opened groundbreaking opportunities regarding therapeutic options in multiple cancer types (4). Some researchers even consider them potential "cancer terminators". As a result, immunotherapy is currently used as a supportive approach in the treatment of various cancer entities (5). To date, the most important targets used in immune checkpoint inhibitor therapy are CTLA-4 (cytotoxic T-lymphocyte antigen 4), PD-1 (programmed cell death 1), and its ligand PD-L1(programmed cell death ligand 1).

To identify patients who may be likely to respond to ICI therapy and to spare those who may not from unnecessary and potentially threatening side effects, the great need for predictive and prognostic biomarkers for clinical usage is currently driving scientific research in this area.

This work aims to contribute in parts to the investigation of a promising predictive and prognostic biomarker for the setting of immunotherapy: the C-reactive protein (CRP) and its early kinetics during ICI application.

Moreover, a theoretical overview of the immune system's role in carcinogenesis and the operating principle of ICIs with their potentially threatening effects will be provided.

2 General introductory section

2.1 The role of the immune system in carcinogenesis

2.1.1 Operating principle of the immune system

The immune system can be divided into two parts: the innate and the acquired immune system. In simplified terms, the first consists predominantly of its cellular components, the phagocytes which include macrophages, neutrophil granulocytes (NG), dendritic cells (DC), and natural killer cells (NK), and its soluble components, the complement system, different types of cytokines and acute phase proteins (APP). The innate immune system represents the rapidly reacting response to potentially host-threatening elements (6–8). The more sophisticated and specific acting adaptive immune system develops its full scope of action only a few days after the initial activation (7,8).

The innate immune system

The primary function of the innate immune system is the cytokine-modulated recruitment and activation of neutrophil granulocytes which are aimed to destroy embedded pathogens (9). The cells of this part of the immune system have tightly linked receptors that serve for recognizing components of potentially infectious microorganisms and dead cellular material (6). The NKs play a major role in differentiating between endogenous and foreign antigens by recognizing surface receptors for MHC class I molecules that are inevitably expressed by host cells. Endogenous cells are identified by the absence of these surface structures as well as by immunoglobulin receptors (10). Chemokines, also known as chemoattractant cytokines, adhesion molecules, and pro-inflammatory mediators are used by the NGs in a multi-step process to create an inflammatory environment that attracts other proinflammatory cells and ultimately phagocytes foreign, introduced components (9). While macrophages and NGs form the first line of defense in the invasion of microorganisms, DCs represent a key site in the interaction with the adaptive immune system (6).

The adaptive immune system

Being equipped with clonally expressed antigen-specific receptors, the T- and B-cells which represent the characteristic elements of the adaptive immune system can mediate targeted immune responses. Developing from precursor cells in the bone marrow and undergoing various gene rearrangements to form their antigen-specific receptors, the production of new T- and B-cell clones continues throughout life. For the initiation of a targeted immune

response following the detection of an antigen presented by several antigen-presenting cells (APC), a cell development cascade in the lymphatic tissue must be induced, starting from cell priming and subsequent cell activation toward cell proliferation and differentiation of the naive T- and B-cells.

B-cells have the task of antigen presentation and forming antibodies (10). T-cells, on the other hand, mature in the thymus where, through negative and positive selection, T-cell tolerance of self-antigens is established (11). Those in self-tolerance "trained" T-cells are now prepared to encounter APCs for priming. APC (including DCs and macrophages besides B-cells) can display both endogenously established antigens as they are produced within the cell in the context of viral infection or tumor disease as well as exogenously absorbed antigens via endocytosis. Being bound to MHC class I or II molecules, the antigens are detected by $CD8^+$ cytotoxic T-cells and $CD4^+$ helper T-cells, respectively. Unlike $CD8^+$ cells, $CD4^+$ cells are not directly cytotoxic. Their activation results in the production of cytokines and the subsequent activation of other immune components and an inflammatory response. For this initiated activation to happen at all, and overall important exclusively in the defense of antigens penetrating from outside the cell or inducing inflammation, stimulation from coreceptors is crucial. The main representatives of costimulatory receptors in T-cell activation are CD80 (B7-1), CD86 (B7-2), and CD40 and their upregulation is promoted by inflammatory mediators (10,12). These receptors are then either binding to the CD 40-ligand, to CTLA-4 on activated T-cells for signal damping or to CD28, the major costimulatory molecule for the initiation of the activation process of resting T-cells (6,10). Therefore, T-cell activation results from a complex antigen receptor signaling and co-stimulating process.

Due to the T-cells' ability of specific antigen recognition and their performance as effector cells combined with their property to form memory cells after the initial contact with an antigen and thus, to initialize prompt immune response upon further contact with the same antigen, T-cells combine relevant aspects that lead to significant participation in the maintenance of the balance between pro- and anticarcinogenic immune responses (13,14). Finally, the just mentioned properties of T-cells lead to the adaptability of the T-cell mediated immune response, including the advantageous possibility to withstand both tumor heterogeneity and the wide variability of tumor antigens, and therefore, to counteract the mutagenicity and adaptability of cancer (5). In this regard, a special role is assigned to memory cells which are crucial for long-term therapeutic response (14).

Immunological mechanisms required for tumor and immunity control: the Cancer-Immunity Cycle

Successful tumor control depends on the coordination of the T-cell-mediated immune response and might ideally consist of the following cyclic steps of a process called "cancer-immunity-cycle" after Chen et al. (15): cancer cell death may result in the release of cancer cell antigens. After the captivation and processing of these neoantigens by DCs, the next step of the cancer-immunity cycle is the presentation of the captured antigens on APCs' MHC molecules to T-cells. The priming and the activation of antigen-specific effector T-cells are succeeding. Consequently, the clonal replication of a subset of $CD8^+$ cytotoxic lymphocytes (CTL) which can migrate to and infiltrate tumor tissue and/or the $CD4^+$ T-cell conducted stimulation of an inflammatory response is induced (10,15). When encountering cancerous cells, the CTLs can then specifically interact with the cognate antigen bound to the MHC I molecule via their TCR. If cancer cells are then recognized as abnormal, they can get killed by these antigen-specific effector T-cells. Finally, this process of antigen-specific tumor immune response takes place iteratively as the previous killing of tumor cells releases new tumor antigens functioning as stimulator which bears the potential to extend the spectrum of T-cell responses until the tumor has ideally been completely destroyed (15).

Considering that the adaptive immune system sustains a latent destructive component through its potentially auto-reactive T-cell repertoire, the existence of multiple mechanisms against inappropriate reactions of the CTLs towards self-antigens is vital. They enable the immune system to regain homeostasis which Fife et al. characterized as a state of defensive readiness in the absence of active inflammation or immunity (12). One component of these dampening, protective mechanisms consists of regulatory T-cells (Tregs). A regulatory T-cell is defined as a subset of T cells that functionally limits immune responses by inhibiting other cell types' mode of action as well as by creating and maintaining peripheral tolerance (16). The second immunomodulating entity is represented by the immune checkpoints. They are to be understood as a small fraction of receptors and ligands of which the nowadays known function is to brake certain substeps of an immune response to avoid immune overreaction (17). Various checkpoints are necessary to run fail-safe processes in order to limit erroneous T-cell activation. The key immunological cascade of this immunomodulating entity is part of the antagonistic costimulatory-inhibitory pathway consisting of CD28, CTLA-4, and the B7-1/ B7-2 ligand pair. The dual specificity of B7-1 and B7-2 for the stimulatory receptor CD28 and the inhibitory receptor CTLA-4 induces the

equilibration of positive and negative signals guaranteeing the successful regulation of T-cell activity. On the one side, this regulatory process may promote T-cell activation and the expansion of T-cell lines as initial steps of a T-cell-mediated immune attack. On the downside, it may lead to T-cell control via the termination of T-cell response and the induction and maintenance of the overall important function of peripheral T-cell tolerance via the discrimination between self- and non-self-antigens in unaffected tissue (11,18). In a review published by a research team led by Noble Prize winner Tasuku Honjo, the authors characteristically sum up the effects of the antagonistic costimulatory-inhibitory pathway by stating: “The fate of lymphocytes after antigen encounter is determined by the integration of stimulatory and inhibitory signals from coreceptors” (19).

2.1.2 Mechanisms promoting tumor cell expansion

In cancer patients, the described cancer-immunity-cycle does not function as necessary to efficiently erase tumor cells (15). The following section provides an overview about processes and mechanisms performed by the interaction of the immune system and the cancer cells, eventually resulting in tumor cell expansion.

2.1.2.1 Cancer immunoediting

By defining the cancer immunoediting hypothesis, Schreiber et al. distinguish three different phases that tumor cells proceed independently or in sequence while gaining immunogenicity: the phase of elimination, the phase of equilibrium, and the phase of escape. The phase of elimination is understood as the stage of immunosurveillance in which, due to the interaction of the competencies of the innate and the adaptive immune system, the detection of malignly transformed cell material can occur and subsequently lead to the induction of a tumor-specific immune response. At this point, the expression of tumor-specific antigens already plays a crucial role: by establishing a tumor-hostile microenvironment through the release of pro-inflammatory and immunomodulatory cytokines after its initial activation, the innate immune system forms the basis for the development of an IFN-ϒ and T-cell-mediated tumor-specific immune response. According to non-in vivo and animal studies, whether successful elimination can take place depends on the specific characteristics of the tumor, its anatomical localization, and its growth rate. If successfully conducted, the elimination phase already represents the final phase of the immunoediting procedure without the development of a clinically manifest tumor (20–22).

Accordingly, the equilibrium phase is only entered by those tumor cell clones that were able to outlast the elimination phase. During this dynamic stage, the adaptive immune system prevents tumorous outgrowth but at the same time maintains tumorous residues in a functionally quiescent state. Thus, this phenomenon leads to an immunologically driven selection pressure that promotes the survival of tumor cell variants carrying different mutations, marking the starting point for increased resistance to immune attack and subsequent tumor cell differentiation (22). This mechanism also explains how therapy-refractory, latent tumor cells can become clinically manifest as (recurrent) primary tumors or peripheral metastases decades later (23–25).

The transition from the equilibrium to the escape phase emerges when the tumor cell population eventually is ideally adapted to the host's immune system due to the immunoediting processes and/or when the immune system succumbs to cancer-induced immunosuppression over time. Adaptation mechanisms of tumor cells favoring tumor escape are manifold. Of note is the mechanism of loss of tumor antigen expression, the origin of which can be traced to the clonal tumor cell selection by the host's immune system which is subject to the principles of evolutionary selection pressure. In addition, genetic instability in the corresponding tumor cells may have its share in the loss of tumor antigen expression. Tumor cells lacking tumor-specific antigens are invisible to the immune system, respectively the antigen receptors of $CD4^+$ and $CD8^+$ T-cells and can consequently expand into a progressively growing tumor (1,20–22). Other erroneous reactions within the cancer-immunity-cycle may consist of the incorrect classification of tumor antigens as self-antigens by DCs and T-cells, leading to regulatory T-cell responses instead of attacking the tumor cells, or the inhibition of TILs to actually infiltrate the tumor (15). Alternatively (and of high significance), the process of immunosurveillance escape seems to be based on the emergence of an immunosuppressive TME. Indeed, a manifest tumor does not only comprise tumor cells but forms a TME together with stromal and inflammatory cells as well as with vascular and extracellular matrix (26). Soluble factors such as immunosuppressive cytokines and surface ligands in the TME can affect T-cell function by preventing successful lymphocyte priming and impeding their migration into tumor tissue until, in the most extreme case, tumor-induced suppression of effector cells leads to a complete failure of tumor defense (12,13,26).

2.1.2.2 Cancer immunoresistance

To survive in the hostile microenvironment, the expression of PD-L1, a ligand of the immune checkpoint PD-1, is an essential "innate" requirement for the tumor. In conformity with Azuma et al, this constitutive mechanism may increase the resistance of PD-L1-expressing tumor cells to apoptosis by sending anti-apoptotic signals during the binding to PD-1 (27). Adaptive immune resistance is acquired by tumor cells as part of processes for adapting to tumor-specific immune responses, eventually leading to an increased survival chance (28). Tumor cells use the physiology of the induction of PD-1's ligands which is normally aimed at protecting unaffected tissue from infection- or tumor-induced immune responses. This ligand induction may happen because PD-L1 is expressed due to interferon - especially INF-ɣ- stimulation, and INF-ɣ acts in inflammatory environments as TMEs can create them. In sum, this mechanism accounts for the following process: INF-ɣ induces the expression of PD-L1, and PD-L1 in turn inhibits the activity of PD-1^+ T-cells within their proapoptotic function (29).

2.1.2.3 T-cell exhaustion

T-cell exhaustion is characterized by Chow et al. as "a state of reduced effector T-cell function at the time of the encounter with tumor cells associated with the manifestation of co-inhibitory markers and reduced production of inflammatory cytokines" (30). Continuous TCR stimulation during chronic antigen exposure leads to the transcriptional upregulation of the inhibitory immune checkpoint PD-1 and results in functional impairment and possibly a complete loss of function and replication capacity in $CD8^+$ T-cells (31,32). Characteristically, the condition of exhaustion affects T-cells that have already undergone priming by APCs and now face de novo antigens within the TME (30). Besides excessive TCR stimulation due to antigen persistence, other factors as hypoxia, an unfavorable TME and the resistance of target tumor cells to cytotoxicity may promote T-cell exhaustion (30). Various research work concluded that T-cell exhaustion is epigenetically encoded (33,34). The effect of the upregulated expression of the negative co-stimulatory molecule respectively inhibitory immune checkpoint PD-1 as one of the hallmarks of T-cell exhaustion is intended to be illustrated more precisely by the following example: tumor-infiltrating T cells (TILs) exhibit upregulated PD-1 levels (12,35,36). Tumor cells recognize those infiltrating T-cells by the detection of the IFN-ɣ which those T-cells release in high concentration. In anticipation of an imminent immune attack, IFN-ɣ is crucial for inducing the expression of PD-L1, another inhibitory checkpoint receptor, on the tumor cell surface

(37). If the TILs now bind to the PD-L1 receptor of the tumor cells via their PD-1 receptor, they may be able to recognize the tumor antigens as potentially threatening but they are entirely blocked by the binding process so that no further immune response can occur. This hypothesis has been supported by findings in melanomas where PD-1^+ TILs produced and/ or secreted fewer cytokines and consequently faced impaired effector function or functional exhaustion compared to PD-1^- TILs (15,32). Acquiring and making use of such an effector cell impairing mechanism prepares the path for the progression of tumor disease (12,35,36). In histochemical analyses, T-cell exhaustion can be determined by PD-1 expression in TILs (30). This fact might serve as a further explanation approach for the correlation of PD-1 levels of tumor-infiltrated tissue with tumor prognosis (38–40). PD-1 is, thus, understood as an exhaustion and prognosis marker. The abolition of this molecular T-cell braking mechanism and the re-enforcement of the $CD8^+$ T-cells in the form of clonal expansion can therefore in parts be achieved through an effective blockade of PD-1/ PD-L1 proteins, i.e., through the action of ICIs (30).

2.2 Immune checkpoint inhibitors

Unlike conventional tumor therapies that directly target cancer cells, immune checkpoint inhibitors try to overcome tumor-induced immunosuppression (20). Immune checkpoint inhibitors are monoclonal antibodies (mAbs) that block immunosuppressive receptors on immune effector cells or their ligands on tumor cells and APCs as previously described (11,20). Therefore, the blockade of "immune checkpoints" is accompanied by the potential to strengthen and maintain endogenous immunity to mutant and non-mutated antigens, ideally leading to lasting tumor control (2). The clinical exploitation of this potential was preceded by many decades of intense and partially controversial research.

2.2.1 A brief historical overview

2.2.1.1 Scientific milestones in the preclinical development of ICIs

In the late 1980s, researchers discovered the need for costimulatory signals for successful T-cell activation (18). Cytotoxic T-lymphocyte antigen 4 (CTLA-4) was identified in 1987, but its function in the process of regulating the immune system remained unclear until the 1990s (38,41). The discovery that T-cell priming induces the expression of CTLA-4 on activated T-cells also revealed the proliferation-dampening and secretion-inhibiting effects that CTLA-4 exerts on those activated T-cells following its expression (38,39). This

observation had a lasting effect on the development of immunotherapies leading to a paradigm shift from the strategy of therapeutically supported T-cell activation to the approach of therapeutically inactivating this immune checkpoint induced blockade to strengthen antitumor immunity (6). The finding that CTLA-4 and CD28 have opposite effects on the T-cell-mediated immune response was convincingly supported in a 1996 published paper by Leach et al. (40). Ten years later, Quezada et al. demonstrated that improved antitumor effectiveness was accompanied by an increased ratio of T-cell effector cells to regulatory T-cells (42). The first study results proofing that anti-CTLA-4 antibodies could induce cancer regression in metastatic melanomas were published in 2003 (43).

The identification of other intrinsic inhibitory T-cell pathways including those mediated by PD-1, program cell death 1, and its ligand PD-L1, followed. After the discovery of its ligand, PD-1 was assessed as another checkpoint molecule in 2000 (44,45). Much clarification about its function was obtained through murine experiments proofing that PD-1 deficiency provokes autoimmunity which formed the basis of the assumption that it may regulate peripheral tolerance (46,47). In the subsequent years, the identification of PD-L1 as a distal immune modulator led to the initiation of preclinical studies whose results formed the basis for the utilization of anti-PD-1 and anti-PD-L1 agents in tumor therapy (13). At the same time, Barber et al. succeeded in demonstrating that PD-1/PD-L1 antibody blockage can "save" "helpless" $CD8^+$ T-cells affected by exhaustion (48).

Thanks to these and many more preclinical results much of which can be attributed to the pioneering work of the winners of the Nobel Prize in Physiology or Medicine in 2018, Dr. James Allison and Dr. Tasuku Honjo, the research around CTLA-4 and PD-(L)1 inhibitors has been shifted from the preclinical to the clinical field (49).

2.2.1.2 From preclinical to clinical usage

Clinical trials conducted with the human CTLA-4 antibody ipilimumab initially showed significant success in phase I/II clinical trials: a decrease in tumor growth was observed for several tumor entities including malignant melanoma, renal cell carcinoma, prostate carcinoma, urothelial carcinoma, and ovarian carcinoma (50–53). These clinical trials were accompanied by phase III clinical trials with ipilimumab performed in patients with advanced melanoma disease (54,55). The results in the work of Robert et al. included, among other findings, that more than 20% of those treated with a combination of ipilimumab and dacarbazine had a survival expectancy of more than three years, leading to the conclusion on the possibility of a long-term response to anti-CTLA-4 therapy (54). The publication of

the work of Hodi et al. marked the first randomized protocol that was able to evidence a survival benefit resulting from ipilimumab in patients with metastatic melanoma. Moreover, based on the findings that 28.5% of the patients responding to ipilimumab experienced partial or complete response or the stabilization of their disease and among them, a proportion of 60% maintained the objective response for at least two years, the superiority of the ipilimumab regimen was exemplified (55). 2011 is finally to be understood as the birth year of immune checkpoint inhibitor therapy: ipilimumab obtained approval for the treatment of patients with melanoma by the FDA (6).

Analogously, response to PD-L1 antibody therapy was found in various tumor entities such as melanoma, renal cell carcinoma (RCC), non-small cell lung cancer (NSCLC), and bladder cancer in the subsequent years (56,57). In the course of the first therapy trials with anti-PD-1 antibodies, therapeutic response likewise was observed in melanoma, RCC, NSCLC Hodgkin's lymphoma, and head and neck cancer (6,58,59). After a large multicenter phase I clinical trial in a patient collective with advanced melanoma undergoing treatment with an anti-PD-1 antibody called pembrolizumab could demonstrate response rates of 37-38% even in patients previously treated with ipilimumab, pembrolizumab was also approved by the FDA in 2014 followed by nivolumab, another anti- PD-1 inhibitor, in 2015 (60). Fundamental knowledge with regard to the effectiveness of pembrolizumab was gained through the KEYNOTE-024 trial and its corresponding update: in a first-line setting, a significant prolonged median OS for patients suffering from advanced NSCLC with PD-L1 expression on at least 50% and no controllable mutations receiving pembrolizumab monotherapy in comparison to chemotherapy could be determined (61). At about the same time, trials investigating the clinical activity and safety profile of anti-PD-L1 therapy were conducted due to an increasing number of research work reporting a possible correlation between the prevalence of PD-L1 on tumor cells with the response rate. Blocking PD-L1 with a monoclonal antibody produced an ORR of 6-17% reflecting durable tumor regression and a prolonged disease stabilization for at least 24 weeks in patients with metastatic NSCLC, melanoma, renal-cell cancer, and ovarian cancer in a multicenter phase I clinical trial (57).

Table 1 provides an overview of the currently approved ICI agents and their FDA-approved indications (the state in 2021):

Drug	Target	Approval	FDA-Approved Indications
Ipilimumab	CTLA-4	August 2010	• Inoperable or metastatic melanoma *(1st line)* • Adjuvant treatment of stage IIIa cutaneous melanoma • Advanced, intermediate, or poor risk RCC *(1st line)* • Microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer
Nivolumab	PD-1	March 2015	• Adjuvant/ *1st line* inoperable or metastatic melanoma • Adjuvant treatment of melanoma • Metastatic NSCLC *(2nd line)* • Metastatic SCLC *(3rd line)* • Advanced, intermediate, or poor risk RCC *(1st line)* • Advanced RCC *(2nd line)* • Classical Hodgkin's lymphoma *(3rd/ 4th line)* • Recurrent or metastatic head and neck squamous cell carcinoma (HNSCC) *(1st line)* • Locally advanced or metastatic urothelial carcinoma (UC) *(1st/ 2nd line)* • MSI-H or dMMR metastatic colorectal cancer)
Pembrolizumab	PD-1	October 2016	• Adjuvant treatment of melanoma • Inoperable or metastatic melanoma *(1st line)*

			• Metastatic Merkel cell carcinoma (MCC) *(1st line)* • Metastatic NSCLC with PD-L1 expression *(2nd line)* • Metastatic (non)squamous NSCLC *(1st line)* • Metastatic NSCLC with high PD-L1 expression *(1st line)* • Recurrent or metastatic HNSCC *(1st/ 2nd line)* • Refractory classical Hodgkin's lymphoma *(4th line)* • Refractory primary mediastinal B-cell lymphoma (PMBCL) (*3rd line)* • Locally advanced or metastatic UC *(1st/ 2nd line)* • MSI-H or dMMR cancers • Recurrent locally advanced or metastatic gastric GEJ adenocarcinoma • Recurrent or metastatic cervical cancer • Hepatocellular carcinoma (HCC) *(2nd line)*
Atezolizumab	PD-L1	October 2016	• Locally advanced or metastatic UC *(1st/ 2nd line)* • Metastatic nonsquamous NSCLC *(1st line)* • Metastatic NSCLC *(2nd line)* • Unresectable locally advanced or metastatic triple-negative breast cancer (TNBC) *(1st line)*

Durvalumab	PD-L1	February 2016	• Unresectable stage III NSCLC • Metastatic bladder cancer • Locally advanced or metastatic UC *(1st/ 2nd line)*
Avelumab	PD-L1	March 2017	• Histologically confirmed metastatic MCC *(1st line)* • Locally advanced or metastatic UC *(1st 2nd line)*
Cemiplimab	PD-1	September 2018	• Metastatic cutaneous squamous cell carcinoma (mCSCC) *(1st line)*

Table 1:List of FDA-approved ICI agents and the corresponding cancer type indication, reviewed in (5,62), own depiction.

Since CTLA-4 and PD-1 regulate different pathways resulting in T-cell inhibition, the striking idea was also to design therapies that benefit both pathways and block different regulatory molecules. Showing an improved anti-tumor response in a pre-clinical murine model, the first phase I clinical trials investigating the combined application of anti-CTLA-4 and anti-PD-1 was found to lead to tumor regression in 50% of treated patients with advanced melanoma and on top of that, a tumor regression of 80% or higher was observed for most cases (62,63). Ever since then, the combined treatment with anti-CTLA-4 and anti-PD-1 or anti-PD-L1 antibodies was the subject of further research in other tumor types to establish an effective therapeutical strategy for oncological patients.

2.2.2 General functioning of immune checkpoint inhibitors

In comparison to the established tumor therapies, immunomodulators target to interact with the immune system or the tumor microenvironment (TME) instead of attacking tumor cells directly. The TME consists of tumor cells, stromal cells, inflammatory cells, vasculature, and extracellular matrices which can all serve as potential attack points in the context of targeted immunomodulators (26). Successful tumor control by immune checkpoint therapy is based on boosting the immune system via physiological pathways that were previously described in the context of the cancer-immunity-cycle by using the lynchpins that regulate those pathways: the T-cells and their corresponding immune checkpoints (15,64). To achieve this, immune checkpoint inhibitors perform as immunomodulatory antibodies by counteracting the via the immune checkpoints CTLA-4 and PD-(L)1 initialized suppressive and T-cell function dampening signals (5). James P. Allison described this key mechanism within the mode of action of ICIs as the “release of the brakes” of T-cell mediated immune

response (48). Immune checkpoint inhibitors are highly selective humanized monoclonal antibodies that aim to (re-)initiate self-sustaining immunity reactions to tumors by unleashing previously inhibited T-cells, therefore allowing the immune system to evade its regulatory mechanisms in favor of the promotion of cancer cell elimination (5,65).

Physiological operating principle of immune checkpoints

Dying tumor cells – which may have been destroyed due to previous therapies i.e., chemotherapy or in the scope of generating immunity to cancer within the cancer-immunity-cycle- release tumor antigens (15). APCs capture and present them to T-cells, employing costimulatory signals transmitted by the B7-molecules located on the surface of APCs besides the interaction between the TCR and the MHC introducing the antigen. Successful antigen recognition by the T-cell requires the delivery of both signals from the same APC. During the antigen detection process, the costimulatory engagement of CD28 via B7 enhances in a subsequent step the activation and clonally expansion of T-cells as CD28 delivers pro-survival signals through growth factors such as IL-2 or survival-promoting enzymes such as PI3K. Concurrently, the co-stimulation via CD28 leads to the preparation of the system for initiating regulation by the means of upregulating the inhibitory checkpoints CTLA-4 and PD-1 on the T-cells' surface to regain immune homeostasis after the fulfilment of its duties in anti-tumor control (6,12). The main task of CTLA-4 is to regulate early APC-mediated T-cell activation by suppressing the positive signals of CD28 via the competitive inhibition of CD28-B7 interaction as well as by mediating Treg suppressive function whereas PD-1 acts in limiting the T-cell effector function and in controlling autoimmunity via the maintenance of peripheral tolerance (13,64,66). While CTLA-4 is a ligand-independent regulator that still can exert its regulatory influence on resting T-cells if expressed as variation that is missing the B7 ligand-binding domain, T-cell suppression via PD-1 demands the binding to one of its ligands (11,13). On this occasion, an important role is played by PD-L1. Expressed on most types of tumor cells, it ligates PD-1 which is upregulated on tumor-infiltrating lymphocytes which in turn renders those TILs unresponsive and subsequently unfunctional by limiting their capability of producing IFN-ɣ and proliferating. Secondly, the ligation of PD-1 with PD-L1 also increases T-cell apoptosis rate (12,66).

Altogether, the actions conducted by immune checkpoints in the immunological synapsis are limiting the lifespan of the effector T-cells in order to regain T-cell homeostasis and immune quiescence (12).

Mechanisms of immune checkpoint inhibitors

As discussed earlier, cancer cells use the checkpoint molecule to escape T-cell mediated anti-tumor response. ICIs empower the immune system to recognize tumor antigens and at the same time, they prevent the weakening of the T-cell-mediated immune response by impeding cancer cell receptors from submitting dampening or immunosuppressive signals to the T-cells (5). Those actions require the activation of the T-cell-mediated part of the immune response in the first place, the expansion of the effector cells, the migration of the activated effector cells to the tumor tissue, and overall important their infiltration to the TME to eventually result in a systemic anti-tumor response and the destruction of tumor cells (26). Thus, to be effective, immunotherapies must increase the proportion of immune effector cells, reveal additional protective tumor antigens, and/or overcome immunosuppressive mechanisms which were either pathophysiologically induced by the tumor and its corresponding TME to inhibit its destruction or physiologically implemented by the regulatory instances of the immune system to limit the effector's activity (12).

Inhibiting the immune checkpoints CTLA-4 and PD-1 as well as the ligands PD-L1 and PD-L2 of the latter results in the following important interims on the way to release the brakes of the immune system for the establishment of tumor control:

if CTLA-4 gets antagonized by pharmacologically designed antibodies, the antibodies inhibit the negative signals delivered by this immune checkpoint which empowers CD28 to outcompete CTLA-4 for B7 engagement. The result of this newly empowered interaction is an increase in effector T-cell activation, expansion, and trafficking, therefore mainly affecting the $CD4^+$ compartment (12,64).

To counteract the functioning of the PD-1:PD-L1/-L2 pathway, anti-PD-L1 antibodies abolish the tumor cell protection caused by the immune checkpoint and thereby reestablish the tumor's visibility to the immune system (12). Restoring the tumor's visibility in a second step reinforces the killing capacity of T-cells by freeing CTLs within the TME from negative regulation (66). To fully restore CTLs function, antibodies targeting PD-1 ideally inhibit the interaction of PD-1 with both of its ligands whereas blocking one single ligand only releases those CTLs expressing the blocked ligand (67). Anti-PD-(L)1 antibodies, therefore, modulate the $CD8^+$ cell line and particularly the exhausted $CD8^+$ compartment (64).

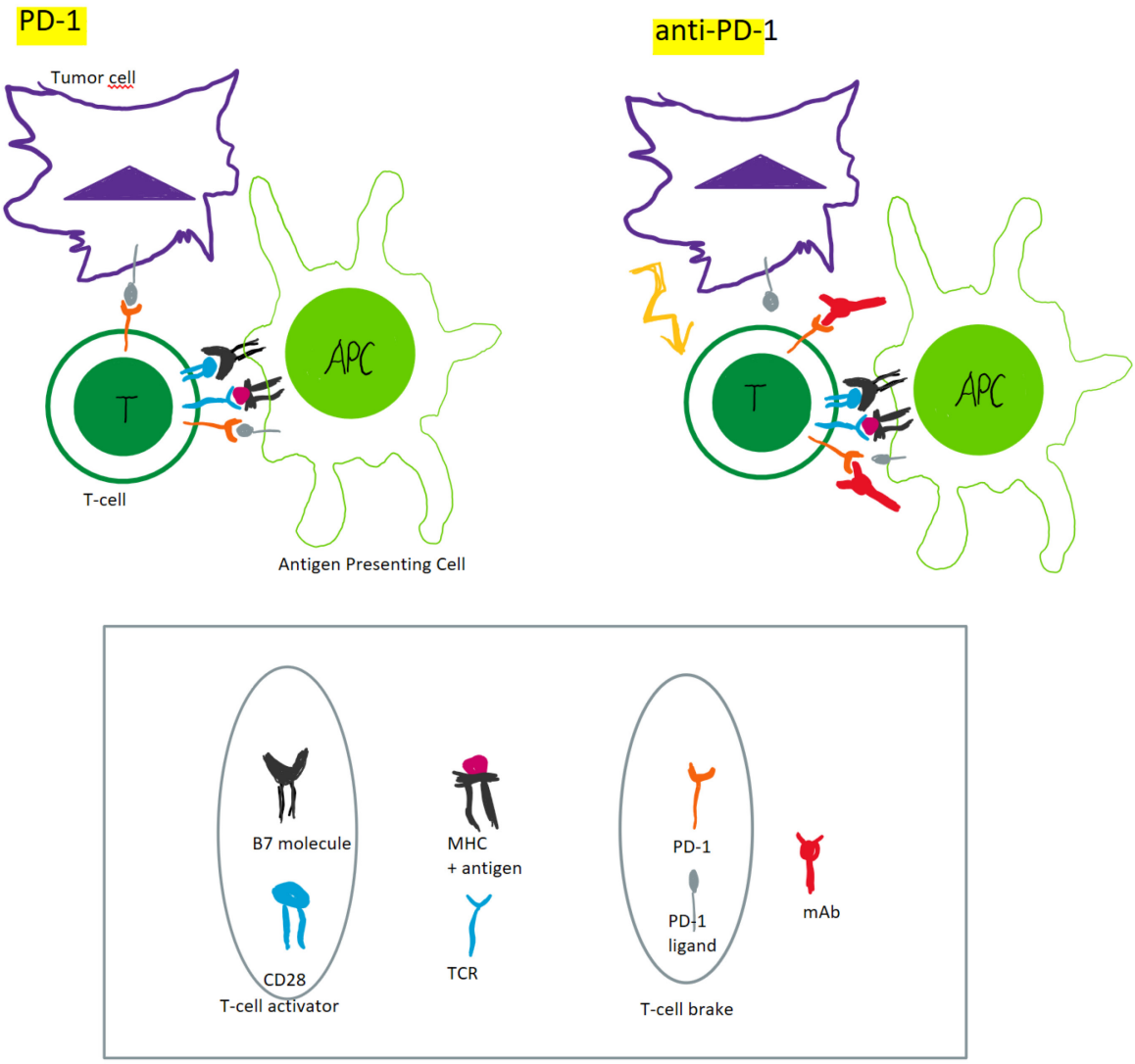

Figure 1: Mode of action of the mAb checkpoint inhibitor anti-PD-1

If successful, the binding of the ICI antibodies to the distinguished immune checkpoints obtains or restores T-cell function so that they can follow their mission of destroying tumor cells (6,12). Then, consecutively following the processes of the physiological immune response, some of these T-cells may differentiate into memory cells that can be reactivated in the future if the tumor reappears (6).

There are different categories of immune checkpoint inhibitors: the currently most commonly used and to date best studied active substances are targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death-1 (PD-1), or are binding to one of the ligands of PD-1, PD-L1 (PD-1/L-1) (4). As the expression of the distinct checkpoint molecules and their ligands differs in regard of their temporal and spatial patterns, taking a closer look at the time and site of action of the checkpoint molecules may help in

understanding the particularities and differences of the operating principles of these immune checkpoint inhibitors (12).

2.2.2.1 Cytotoxic T-lymphocyte antigen-4 (CTLA-4) and CTLA-4 inhibitors

CTLA-4: Mechanism of action

CTLA-4 (synonym CD152) is a protein that is specifically expressed on the surfaces of T-cells. As an inhibitory checkpoint molecule, it plays an important role in the self-regulatory processes of the immune system as it can limit the amplitude of T-cell activity at early stages of cell-mediated immune response (12). It is constitutively expressed on regulatory T-cells and predominantly works in lymph nodes (68,69). Also, CTLA-4 can be found in large proportions on the surface of activated T-cells where it usually is highly upregulated at the latest 2-4 days after both $CD4^+$ and $CD8^+$ T-cells have experienced initial activation. The enrolment of this process marks the launch of the inhibitory program restraining T-cell activity (38,39). This inhibitory program is believed to comprise two major effects: for one thing, CTLA-4 can inhibit T-cell activation by outcompeting its closely related homolog CD28 for binding B7 due to its much higher affinity for the shared ligands of the B7 family. Secondly, CTLA-4 can provoke reduced TCR and CD28 engagement either T-cell intrinsically through the induction of a negative feedback loop or by removing B7 molecules from APCs in a T-cell extrinsically manner (12,68,70). Limiting the signal exchange between these two components engenders the downmodulation of helper T-cell activity by impeding cytokine production, cell cycle progression, and biochemical signaling. In the meantime, the effector T-cell to regulatory T-cell ratio is shifting in favor of the Tregs, resulting in the enhancement of the immunosuppressive effects of regulatory T-cells that can suppress the proliferation capability of other T-cells (11,12,71). Despite these mechanisms which are in total opposing the effects of CD28, the ligation of CTLA-4 with B7-1 and B7-2 also forces the inhibition of IL-2 production and IL-2 receptor expression (38,39). IL-2 physiologically fulfills its function in maintaining T-cell growth and preventing T-cell anergy (72). Considering all its properties, CTLA-4 is to be understood as a signal dampener and negative regulator of CD28-dependent T-cell mediated immune responses (11).

CTLA-4 immune checkpoint inhibitors

The first CTLA-4 immune checkpoint inhibitor to be administered to patients was ipilimumab (73). As a monoclonal antibody blocking CTLA-4 and subsequently discontinuing its inhibitory signaling, ipilimumab develops its action through the

concomitant modulation of different T-cell compartments. On the one hand, it is targeting the compartment of intratumorally located effector T-cells via the enhancement of their activity. On the other hand, anti-CTLA-4 is selectively hindering the Tregs in downregulating T-cells via "switch-off signals" within tumor tissue (64,71,74). Consequently, this blockade results in the expansion of antigen specific CTLs within the tumor and tumor-draining lymph nodes, an increased $CD4^+$ and $CD8^+$ effector cells to Tregs ratio and prevents tolerance induction in tumor tissue (12,74,75). The observation of the effects of CTLA-4 inhibition led to the assumption that this blockade may act during the early stages of immune response and within the lymphatic system (12).

To date, ipilimumab is used for adjuvant treatment of stage IIIa cutaneous melanoma, for 1st line monotherapy in inoperable or metastatic melanoma and advanced, intermediate, or poor-risk renal cell carcinoma as well as for the treatment of MSI-H/dMMR metastatic colorectal cancer. Since 2020, ipilimumab is also administered in a combined regimen with nivolumab for malignant pleural mesothelioma, NSCLC (with tumor PD-L1 expression ≥1% and no EGFR/ALK aberrations), and hepatocellular carcinoma (76).

2.2.2.2 Programmed cell death protein 1 (PD-1)/ programmed cell death 1 ligand 1 (PD-L1) and PD-1/ PD-L1 inhibitors

PD-1 and its ligands PD-L1/ PD-L2

PD-1 (synonym CD279) is a type I transmembrane protein belonging to the superfamily of immune globulins and is also classified as a checkpoint molecule with negative immune regulatory functions. PD-1 has two ligands, PD-L1 (synonym CD274 and B7 homolog 1/ B7H1) and PD-L2 (synonym CD273 and B7-DC) (6).The physiological task of PD-1 is to limit primed T-cell activity in peripheral tissue during an immune response. Therefore, by controlling peripheral tolerance through inhibitory signals, it probably acts as the last instance that in various ways can prevent the self-destruction of body tissue by the T-cells as part of an autoimmune response (48). Hence, its ligands PD-L1 and PD-L2 are constitutively expressed in peripheral tissues (12). The interaction of PD-1 with its ligand PD-L1 regulates both the induction and maintenance of peripheral T-cell tolerance by inhibiting the initial activation of self-reactive effector $CD4^+$ and $CD8^+$ T-cells. Thus, it can be ensured that a peripheral immune response only occurs within the appropriate organs and if functionally necessary, and that usually, it does not degenerate into a systemic reaction (66). In addition, PD-1 and PD-L1 are also expressed by Tregs, thereby having a share in the suppression of effector T-cells (12). Besides functioning as a T-cell activity-controlling

entity, PD-1 is an overall important negative immune regulator that participates in complex immunologic processes such as T-cell exhaustion, the maintenance of T-cell tolerance, and the resolution of inflammation (19).

Analogously to CTLA-4, PD-1 is mostly expressed on activated T-cells, especially on Tregs, after the TCR engagement in the face of antigen presentation and CD28 co-stimulatory signals as well as on activated B- and myeloid cells (44,77). Its expression is enrolled within 24 hours after T-cell activation and diminishes after the disappearance of the triggering antigen (48,77). The binding of PD-L1/ PD- L2 and PD-1 recruits the Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) and 2 (SHP-2) (64). The recruitment of these phosphatases goes along with the dephosphorylation of TCR signaling molecules and the mitigation of TCR stimulation and consequently, T-cell activation and cytokine production are diminished as well (13,64). The latter impairs the killing capacity of cytotoxic T-cells (78). Furthermore, PD-1 promotes the differentiation of Tregs from naive T-cells, enhancing the growth of another immunity-controlling entity which in turn can interfere with the effector T-cells function and limit it (79). On top of that, it was found that PD-1 signals can change the motility of T-cells as well as the duration of their engagement with DCs and target cells (80).

PD-1 is also found in a large proportion on TILs of many different tumor types and on exhausted T-cells whereas PD-L1 and PD-L2 are expressed on various tumor cells (32,64,81). It was shown that the degree of the expression of PD-L1 and PD-L2 can increase under the influence of either oncogenic pathways as PI3K or inflammatory cytokines, among them predominantly interferon-gamma (IFN-ɣ), on many somatic cells (44,67,81). Of note is that IFN-ɣ is produced by $CD4^+$ and $CD8^+$ T-cells in the TME during a concurrently ongoing antitumor response (67,72). Succeeding such an inflammatory stimulation, PD-L2 is primarily up-regulated on APCs, PD-L1 on various cell populations including hematopoietic, epithelial, and endothelial cells (2,29). Under physiological conditions, this process of inhibitory checkpoint upregulation serves as negative feedback mechanism to limit effector T-cell activity and to prevent excessive, unnecessary tissue damage (13,28). Conversely in tumor tissue, this upregulation represents a mechanism of adaptive resistance, resulting in the favor of the tumor's protection from anti-tumor response. PD-L1/ PD-L2 signaling further seems to protect tumor cells from apoptosis and facilitate tumor growth, in contrast. Instead, PD-L1 is nowadays known to have an impact on the T-cells' proliferation rate and the apoptosis susceptibility of effector $CD8^+$ T-cells (82). According to this, the

overexpression of PD-L1 is analogously seen in most types of epithelial cancer whereas PD-L2 overexpression is more likely to occur in lymphoid malignancies (2). Despite these functions, PD-L1 has the ability to interact with the B7-1 molecule expressed on T-cells, exerting T-cell activation interfering effects and therefore, it serves as an interfacing structure between the CD28-CTLA-4 and the PD-1 pathway (83).

PD-1 and PD-L1/-L2 immune checkpoint inhibitors

Naturally, the compartment in which the blockade of PD-1:PD-L1/-L2 develops its effect is the tissue site where it acts in releasing exhausted and anergic T-cells as well as in causing autoimmunity (12). Consequently, this blockade leads to the restauration of effector T-cell function resulting in increased cytotoxicity, pro-inflammatory cytokine production, and proliferation of TILs which in total re-enables the preexisting anticancer immune cells to exert antitumor effects and to mediate tumor cell killing (66). The advantage of directly inhibiting PD-L1 lies in the reduced probability of provoking the development of severe adverse events when sparing PD-L2 that is barely expressed on human cancer cells (57,58). Initially, it was hypothesized that PD-1 and its ligands PD-L1 and PD-L2 may play an important role in preserving an immunosuppressive state within the TME and that the destination of PD-1:PD-L1/-L2 blockade may therefore lie within the microenvironment of the targeted tumor (2,66). More recent data suggests that the crucial target of anti-PD-(L)-1 therapy can be found in the discontinuance of the priming procedure carried out between DCs and precursor-exhausted $CD8^+$ T-cells in tumor-draining lymph nodes (30).

The data collected from various clinical trials since the authorization of anti-PD-1/ PD-L1 mAbs for the treatment of cancer suggest that blocking the PD-1 pathway in terms of clinical outcome is more frequent in some tumor entities when PD-L1 is expressed within the TME (66). In addition, various study results indicate that high expression levels of PD-L1 in tumor cells promote tumor progression and tumor invasion (12). The reason that PD-1 only acts as an inhibitor of lymphocyte function when engaged by one of its ligands might serve as a reference for this phenomenon. Using immunohistochemical techniques and flow cytometry-based analyses to detect PD-1 ligands' expression levels and patterns is therefore crucial for therapeutical decisions in finding the suitable ICI agent (17). Despite that, several trials have confirmed that pembrolizumab can induce complete and durable responses in a tumor-agnostic manner which indicates that it is rather targeting the immune system than the tumor cells (84).

To date, immune checkpoint inhibitors targeting the PD-1 pathway - nivolumab, pembrolizumab, and cemiplimab - have proven as effective agents in the treatment of different cancer types, among them Merkel cell carcinoma, melanoma, renal cell carcinoma, head and squamous cell carcinoma, SCLC and NSCLC. The PD-L1 inhibitors atezolizumab, durvalumab, and avelumab, have received FDA approval for some solid tumors such as NSCLC, NCSCC, melanoma, and MCC, and the latter one also for urothelial cancer that progressed during or after platinum-based chemotherapy (76). Following the "tissue agnostic" approval of pembrolizumab for adult and pediatric patients with unresectable or metastatic solid tumors with positive MSI-H or mismatch repair (MMR)-deficiency in 2017, in June 2020, the FDA moreover approved pembrolizumab for the treatment of tumor mutational burden-high (TMB-H) solid tumors [≥ 10 mutations/ megabase (mut/Mb) based on the FoundationOnce CDy assay] (57,85). This approval was based on the findings of the KEYNOTE-158 study which was able to identify an association between the TMB status and a robust tumor response for an ORR of 29% in patients with solid tumors (86).

2.3 Immune-related adverse events

ICI therapy-related adverse events can emerge in a range of symptoms, and they can affect almost any organ system. Generally, they occur within three months after ICI initiation whereby the time of onset possibly varies between the first days of treatment and a point in time after the termination of ICI therapy (68,87). The incidence of all grade ICI related adverse events established through large meta-analyses is <83% for anti-CTLA-4 agents, <72% for anti-PD-1 and <59% for anti-PD-L1 inhibitors with subgroup analyses for the incidence of all-grade adverse events varying from 44.53% for avelumab (anti-PD-L1) to 86.78% for tremelimumab (anti-CTLA-4) (88).

The pathophysiology of adverse events within the framework of ICIs is not yet fully understood, but it is assumed that their occurrence relates to the regulatory function of the distinct blocked immune checkpoints and the location of their expression. Existing on both immune and tumor cells, the blockade of these negative regulators of T-cell activity that are under physiological conditions responsible for balancing immunological processes and inhibiting overreaction of the T-cell mediated immune response against normal tissue now artificially imbalances the immunological homeostasis (89). By antagonizing these physiological processes in order to support the immune system in its capacity of destroying cancer cells, the application of ICIs can alter normal self-tolerance and evoke a variety of

local and systemic inflammatory side effects due to an unintentional excessive immunity against normal tissue in organs (90). At this point, cytokines may play a distinct role (91). This immunological explanatory approach is the reason why ICI therapy-related adverse events are in general called immune-related adverse events (irAEs). Among them, the most frequent all-grade irAEs are pulmonal (pneumonitis), gastrointestinal (diarrhea, colitis, nausea), musculoskeletal (arthralgia and myalgia), skin-related (most common: rash, pruritus, and vitiligo; rare: TEN, Steven-Johnson syndrome, DRESS) and endocrine (thyroid disorders such as hypothyroidism and hyperthyroidism followed by pituitary and adrenal dysfunction as well as diabetes). Constitutional symptoms of fatigue, pyrexia, and anorexia are also commonly detected (90).

As CTLA-4 and the PD-1 pathways are assigned to different positions in the regulatory framework of immunoregulation, their toxicity profiles manifest differently and vary with respect to their severity (91): in a murine model, mice with CTLA-4 gene depletion die from lymphoproliferation (92). On the other hand, those mice that lack PD-1 develop autoimmune arthritis or cardiomyopathy (46). Accordingly, the side-effect profile also varies between anti-PD-1, anti-PD-L1, and anti-CTLA-4 agents. Khoja et al. found in a 2017 published metanalysis that colitis, hypophysitis, and rash occurred more frequently as part of a CTLA-4 blockade, while pneumonitis, hypothyroidism, arthralgia, and vitiligo showed a higher incidence during anti-PD-1 therapy (90).

The severity of irAEs and the pronounced symptom complex are determined according to the classification system of the Common Terminology Criteria for Adverse Events. (CTCAE). Fife grades referring to the degree of manifestation of the different adverse events are distinguished: grade 1 (G1) implies irAEs of mild severity, grade 2 (G2) symptoms of moderate extent, grade three (G3) includes severe or medically significant adverse events and life-threatening symptoms are classified as grade 4 (G4) irAEs. Grade 5 (G5) signifies death related to any irAE (93). Each grade indicates stage-appropriate and compartment-related therapeutic management (68). **Table 2** exemplarily provides an overview of commonly detected irAEs in the setting of ICI therapy, the toxicity grade related symptoms and the recommended therapeutic management escalation pathway in accordance with the ESMO guidelines (87).

Affected compartment and recognised adverse events	*Toxicity grades and associated symptoms*	*Therapeutic management pathway*
Pulmonal - Pneumonitis	*G1*: radiographic changes (ground glass opacities, interstitial pneumonia pattern)	- **Delay of treatment if necessary** - Monitoring of symptoms
	G2: mild/ moderate new symptoms (dyspnoea, cough, chest pain)	- **Interruption of ICIs** + Antibiotics if signs for infection + Prednisolone if no infection and no improvement after 48h
	G3/4: severe new symptoms (new/ worsening hypoxia, difficulty in breathing, ARDS)	- **Permanent discontinuation of ICIs** - High dose IV (methyl)prednisolone +Empiric antibiotics +Ventilation if necessary +Escalation with infliximab, cyclophosphamide or MMF if no improvement after 48h
Gastrointestinal - Diarrhea - Colitis	*G1*: <4 liquid stools/ day over baseline +asymptomatic	- Continuation of ICI - Symptomatic: oral fluids, antidiarrheals, electrolyte supplementation, dietary change - Prednisolone if persistent (>14d)/ alarm symptoms

	G2: 4-6 liquid stools/ day over baseline +symptoms (abdominal pain, haematochezia, nausea)	- **Interruption of ICIs** - Symptomatic treatment as for G1 - Prednisolone if persistent (>3d)/ alarm symptoms
	G3/4: ≥7 stools/ day over baseline or episode within 1h of eating	- **ICI discontinuation** - IV (methyl)prednisolone + Sigmoido/colonoscopy (perforation!) + Infliximab, MMF or tacrolimus if no improvement after 72h
Musculoscelettal - Arthralgia	*G1:* mild pain +signs of inflammation, erythema, or joint swelling	- **Continuation of ICIs** - Analgetic (paracetamol, ibuprofen)
	G2: moderate pain +symptoms as above +limited instrumental activities of daily living	- **Interruption of ICI treatment to be considered, rechallenge upon symptom control** - Escalate analgesia (diclofenac, naproxen, etoricoxib**)** - For adequate control: prednisolone orally or intraarticular steroid injections

	G3: severe pain, irreversible joint damage, +disabled condition +limited self-care	- **Interruption of ICIs** - Prednisolone - Anti-TFNά if no improvement after 4 weeks

Table 2: Common irAEs - toxicity grading and associated management escalation (87)

In general terms and irrespective of the affected organ, the ESMO clinical practice guidelines (87) and the ASCO clinical guideline summary (94) recommend the following steps in case of the occurrence of irAEs: as soon as new symptoms manifest during ICI treatment, further investigation with a high level of suspicion that the symptoms are treatment related is required (87,94). Other causes as tumor progression, infections, virus reactivation, or toxicity related to other drugs must be excluded in the first place (95). Grade 1 symptoms usually -with the exception of some neurologic, hematologic, and cardiac toxicities- do not require any further intervention besides close clinical monitoring or diagnostic observation, ICI therapy should be continued. In case of most grade 2 irAEs, a temporarily discontinued treatment to achieve symptom regression and/or laboratory value reduction to ≤G1 may be sufficient. Besides, minimal intervention in the form of corticosteroids at an initial dose may be indicated. Grade 3 irAEs require the interruption of ICI therapy and the initiation of high-dose steroid therapy or immunosuppressants in case of no improvement after 48 to 72 hours as well as the hospitalization of the affected patient. ICI rechallenge may be discussed cautiously after a clinical and/ or laboratory-analytical return to toxicity grade ≤1. If grade 4 irAEs manifest, therapy must be permanently discontinued, and urgent intervention is indicated (87,93,94). Death (G5) in terms of irAEs on ICI treatment is a rare condition but severe irAEs such as myocarditis, pneumonitis, and neurologic events can cause death in some cases (91,93). More specific drug management may be necessary in case of skin-related toxicities and endocrinopathies (**Table 3**). In respect to the latter, hormone replacement may establish sufficient control over the distinct endocrine disorder, eventually allowing ICI rechallenges even in case of a G4 endocrinopathy (94):

Affected compartment and recognised adverse events	*Classification and associated symptoms*	*Therapeutic management pathway*
Skin *Most common* - Rash - Erythema	*Grade 1:* skin rash, <10% BSA, +/- symptoms	- Topical steroids of mild strength - +/- oral or topical antihistamines - **ICI treatment continuation**
	Grade 2: rash 10-30% BSA	- Topical steroids of moderate strength - +/- oral or topical antihistamines - **ICI treatment continuation**
	Grade 3: rash >30% BSA or grade 2 + symptoms (pruritus)	- Topical steroids of potent strength - Initiation of steroids (prednisolone if mild or moderate, IV (methyl)prednisolone if severe) - **Interruption of treatment, considering re-initiation at grade 1/ mild grade *2***
	Grade 4: skin sloughing >30% BSA with erythema, purpura, epidermal detachment	- IV (methyl)prednisolone - **Discontinuation of ICI treatment**
Endocrinopathies *Thyroid disorders*	*Elevated TSH:* **Normal FT4**	- **Next cycle**

- Hypothyroidism - Hyperthyroidism - Thyrotoxicosis *Hypophysitis* *Diabetes*	+symptoms **Low FT4** if asymptomatic +symptoms	- Consider thyroxine (TSH>10) - **Next cycle** - Thyroxine
	Normal TSH: **Elevated FT4** **Low FT4** if asymptomatic	 - Repeat - Discuss with endocrinologist if persistent - **Next cycle** - 9 am cortisol (hypopituitarism!)
	Low TSH: **Elevated FT4** if asymptomatic +symptoms hyperthyroidism **Low FT4**	 - **Next cycle** - Beta blocker, thyroid Abs, uptake scan - **Withhold ICIs until symptom control** - 9 am cortisol (hypopituitarism!)

Table 3: irAEs – classification and management escalation for skin related toxicity and thyroid disorders in accordance with the ESMO guidelines (87)

In general, most irAEs can be eliminated by delaying the ICI application or with the onset of a temporary immunosuppressive therapy (91). It is of utmost importance to point out that in accordance with the ESMO guidelines, the ICI related clinical outcome of those patients receiving immunosuppressive agents for the medication-based control of irAEs is not affected by these immunosuppressants (87).

It is still unclear why some patients suffer from severe irAEs and others do not. Eventually, analogous to the etiology of autoimmune diseases, a genetic influence may play a role (91). Closely connected to this is the hypothesis of the individual preexistence of shared antigens between the tumor and normal tissue provoking T-cell cross-reactivity which is causing the

broad symptomatic spectrum of irAEs (96). The question of whether the occurrence of irAEs is associated with a better therapeutic response remains controversial. It seems consistent that the manifestation of irAEs during ICI therapy speaks for the activation of the immune system by the ICI drug (91). For example, studies in melanoma patients showed a link between the occurrence of vitiligo lesions and positive clinical outcomes during ICI therapy (97). Also, a retrospective trial observing 559 NSCLC patients who received anti-PD-1 agents regarding the relationship between the development of irAEs and clinical benefit during immunotherapy came to pertinent results: higher ORR, longer PFS, and longer OS were each associated with the manifestation of any-grade irAEs (98). Nevertheless, neither the fact of their manifestation nor the extent of side effects seems to allow a generalized statement about the association of irAEs and OS in clinical outcomes (91). Only correlations of low strength and varying magnitude can be distinguished according to a recently published systematic review and meta-analysis of randomized studies investigating irAEs as potential surrogates of ICI efficacy (99). However, it should be noted that some patients need to interrupt or even discontinue immunotherapy due to irAEs. Therefore, the early detection of patients of high tisk for irAEs is of high interest for clinicians. This is where the search for suitable biomarkers and the question of their predictive and prognostic value comes into play.

2.4 Biomarkers

2.4.1 Definition of prognostic and predictive biomarkers

According to Clark et al. (100), a prognostic biomarker is to be understood as a measurement reflecting the natural enrolment of a disease while being associated with the patient's clinical outcome in the absence of therapy or as a consequence of the application of a standard therapy. A predictive biomarker is a measurement that is associated with response or non-responsiveness to a distinct therapy provided that response is defined by using any of the commonly applied clinical endpoints in clinical trials (100). Moreover, a predictive biomarker can also be thought as a measurement projecting the risk for treatment related adverse events and toxicity (101).

2.4.2 The need of prognostic and predictive biomarkers for ICI efficacy and examples of suggested biomarkes

Only a few biomarkers have been able to establish as clinically relevant for monitoring ICI therapy during the recent years of intensive research, including the aforementioned prevalence of PD-L1 in tumor tissue and on the surface of TILs (102). Directly assessing the PD-L1 expression rate on tumor cells to predict the clinical outcome of the use of anti-PD-1 or anti-PD-L1 therapy appears as a logical approach. Being supported by initial data from a phase I clinical trial investigating the effects of nivolumab in a study collective including patients with melanoma, NSCLS, RCC, prostate cancer, and colorectal cancer, the immunohistochemical analysis measuring tumor-cell PD-L1 expression did not make the transition into widespread clinical screening (58). However, PD-L1 can only be used for clinical evaluation considering its limiting factors. First, it can be obtained from tumor biopsy specimens only which is time-consuming and costly for both patients and medical staff (103). Second, its positive and negative predictive values are suboptimal, patients suffering from PD-L1 negative disease can still experience beneficial effects from anti-PD-(L)1 therapy, exemplified by Gibney et al. relying on the extraordinary proportions of patients with PD-L1 negative melanoma found in the CheckMate 067 trial (104) which are showing an ORR of 41% under nivolumab monotherapy and 54% under nivolumab and ipilimumab combination therapy. Based on the Checkmate 067 data (104), Gibney et al. determined the negative predictive value of anti-PD-(L)1 therapy at 58% for nivolumab and 45% for combined nivolumab and ipilimumab regimen (105). This is leading to the third point that its validity can only be transferred to patient collectives of certain tumor entities (103).

Associating the fact that it is of high interest to identify tumor-agnostic biomarkers, to date even fewer agents with predictive character in ICI therapy have been proven to function as reliable biomarkers for ICI treatment effect across different tumor entities. Among them are phenomena of genetic modification in tumors that can be revealed by molecular testing methods. The first one to name is the phenomenon of microsatellite instability (MSI) emerging from an impaired DNA mismatch repair. Recent study results indicate that the presence of MSI-high cancer may predict the clinical benefit of immune checkpoint blockade with pembrolizumab or other PD-1/ PD-L1 blockers as it was shown for subpopulations with colorectal or endometrial cancer (84,106). Yamashita et al. found that in a studied collective in which the participants' tumors were classified as MSI-high, the presence of $CD8^+$ TILs and PD-L1/ PD-1 expression was significantly higher compared to

the microsatellite-stable group which may be related to the observed higher odds of good response to immunotherapy in this group (106). As a rare condition in an extremely small subgroup of patients suffering from NSCLC, MSI-high was also found to be a predictive biomarker for the clinical outcome of lung adenocarcinoma (107). However, Bose (108) already pointed out the controversy about MSI-high as a potential tumor-agnostic biomarker: based on the results of Goodmann et al. (109) defining endometrial, colorectal, and small intestine cancer to be the tumor entities with the highest odds to contain a high percentage of MSI-H fields within their tissue samples, the research group argues that if the aforementioned carcinomas are excluded the spectrum of cancer entities which can be treated in a tissue-agnostic manner by pembrolizumab, the remaining spectrum of tumor entities that may profit from this drug becomes very narrow (109). The introduction of this observation questions the validity of the MSI as a tumor-agnostic biomarker if its presence will mainly be determined for some tumor types.

Another proposal in the context of molecular testing methods in the search for predictive biomarkers focused on the tumor mutation burden (TMB). The tumor mutation burden is defined as the frequency of certain mutations within a tumor's gene which bears the potential to generate immunogenic antigens. Furthermore, this explains the interest in investigating its predictiveness with regard to the host's response to ICI therapy. The promising results of former research considering the TMB as a biomarker with significantly positive predicting value for better PFS and OS were more recently ruled out by the discrepancy concerning the detection methods, the absence of a standardized definition of high TMB status, and the finding that its validity is only transferable to a subset of cancer types (85,110).

2.4.3 C-reactive protein

In the search for suitable biomarkers, the C-reactive protein (CRP) was eventually identified as one. Its objective measurability, high sensitivity, specificity and reproducibility, its non-invasiveness, and affordability in clinical routine, as it is extracted from peripheral blood samples, allow the CRP to meet many important criteria for a sufficient biomarker. Furthermore, the increasing data diversity around its predictive importance underlines its value in this regard (111). Various research findings concentrating on the identification of predictive biomarkers in the framework of ICI therapy have already brought the CRP into the focus of attention as an elevated CRP is associated with irAEs in several tumor entity subgroups e.g., melanoma, and its increased serum level can proceed with clinical symptoms (95).

The CRP is a member of the group of pentameric proteins (pentraxin) and is mainly secreted by hepatocytes in response to IL-6 and IL-1β stimulation (reviewed in (112), (113)). Belonging to the group of the APPs, non-specific markers of systemic inflammation, the serum CRP level is highly elevated during an acute inflammatory reaction as well as to a lesser degree in the course of chronic inflammation and therefore considered a reliable reflector of systemic inflammation (103,111,113). Within the first hours after tissue injury or infection, the rate of CRP synthesis is known to increase sharply, suggesting that it contributes to the innate immune response (114). As a pattern recognition molecule, the CRP binds at a molecular level to lysophosphatidylcholine which is released during the process of cell death or expressed on the surface of pathogens. This binding process will then lead to the activation of the complement system via C1q which has the effect that CRP can participate in host defense (113,114).

The link between inflammation, immunity, and cancer is widely assured and can be observed either as a response to cancer therapy or as part of carcinogenesis. During carcinogenesis, inflammation is considered a tumor growth-promoting characteristic and a pioneer for the activation of tumor hallmark-facilitating programs leading to cell proliferation, angiogenesis, and cancer cell migration (65,115). Inflammatory processes in tumor-affected tissue are not only found in the presence of underlying inflammatory diseases which have been shown to increase the risk of cancer. On intrinsic paths, signs of inflammation associated with cancer also appear to be due to the oncogenic activation of transcription factors supporting inflammation. It is, therefore, reasonable to assume that the phenomenon that of almost all tumors and regardless of their causal trigger, the TME consists of inflammatory cells and mediators, may originate from oncogenic-induced inflammation (116). Furthermore, preclinical models investigating the effects of T-cell exhaustion due to chronic virus infections at the cell receptor level point out the observed increase of inflammatory cytokines following T-cell activation after blocking the PD-1/ PD-L1 axis, a process that promotes the retrieval of exhausted T-cells (48). This result, evidence of the recovery of important cellular functions for immunity, may also be interpreted as evidence for the occurrence of an inflammatory reaction and thus a drug response (117). Given the functional mechanism of ICIs, the measurement of an elevated serum CRP may originate from systemic inflammation and the release of inflammatory mediators that reflects the induction of an antitumor response (118)

The prognostic value of systemic inflammation-based prognostic scores was acknowledged in a variety of randomized clinical trials and its effectiveness has been systematically reviewed by Dolan et al (119). In 2015, a large systematic literature analysis comprising both prospective and retrospective studies of which the study object was to analyse the relationship between CRP and life expectancy in patients with solid tumors found that elevated CRP levels are associated with higher mortality (120).

Detection of increased CRP level reflects the tumor's ability to produce significant amounts of pro-inflammatory cytokines, including IL-6, the main regulator of the CRP.

Recent findings on the background of the increase in CRP associated with a worse clinical outcome in tumor patients obtained in vitro from tissue samples from patients with metastatic melanoma indicate that the CRP also interferes with adaptive T-cell immunity. By binding T-cells, the CRP in vitro inhibits the proliferation and effector function of both $CD4^+$ and $CD8^+$cells and therefore exerts an influence on the ICI-mediated antitumor immune response which is based on the functioning of the effector T-cells (113,121). Besides binding T-cells, the CRP was also found to be internalized by a portion of T-cells 24 hours after exposure. At a certain serum level, the CRP affects the function of activated T-cells by increasing the protein expression of several checkpoints such as CTLA-4 and PD-1. Furthermore, the binding process of the CRP to the T-cells can inhibit TCR engagement at early stages as well as the expansion of antigen-specific T-cells and the downregulation of DC function by reducing the expression of co-stimulatory molecules. As another result of CRP stimulation on TILs obtained from melanoma specimens in vitro, an increase in IL-1β expression was noticed which further stimulates the de-novo production of CRP in the liver. In patients with NSCLC, high preoperative CRP serum levels were associated with PD-L1 positivity (122). Therefore, it seems plausible that high CRP levels at baseline may reflect a CRP-caused state of additional systemic immune suppression and that they are associated with worse PFS and/or OS during ICI therapy (113,123). Over the last few years, various research confirmed the reliability of CRP as a predictive biomarker for ICI treatment prognosis in several tumor types. Among them were studied groups with melanoma receiving anti-PD-(L)1 agents, with renal or bladder cancer receiving anti-PD-1 agents, with metastatic urothelial carcinoma (mUC) receiving pembrolizumab, with NSCLC receiving anti-PD-(L)1 agents and with advanced gastric cancer receiving nivolumab (103,124–126).

Beyond that, these works in parts also were able to prove the CRP's superiority in comparison to other prognostic risk models as demonstrated by Abuhelwa et al. for CRP and the IMDC risk model in patients with renal cell carcinoma receiving

atezolizumab/bevacizumab (127). More importantly, Hopkins et al. were able to define and validate a pretreatment prognostic tool within a large pooled post hoc analysis of trials dealing with patients receiving atezolizumab for advanced NSCLC. The pretreatment prognostic tool divides the patients into different risk groups concerning their mean OS based on an optimal OS multivariable Cox proportional hazard model consisting of CRP, LDH, dNLR, albumin, PD-L1 expression, ECOG PS, time since metastatic diagnosis and metastatic sites count. Among all these prognostic markers, CRP was the most predictive univariable for OS (128). In 2022, Minichsdorfer et al. came up with another striking retrospective analysis considering the value of serum CRP as a prognostic biomarker: in a real-world cancer population comprising 114 patients with solid malignancies of multiple tumor types treated with ICI at the Medical University of Vienna, pre-treatment CRP was found amongst other serum parameters as LDH and albumin to be strongly prognostic for a poor 6-month OS. The fact that these results were of prognostic value regardless of tumor type adds to the evidence of a significant prognostic role of serum parameters as the CRP in patients undergoing ICI treatment (129).
Other research work concentrating on identifying biomarkers that may help to predict the probability of experiencing irAEs during ICI therapy also concluded that the CRP can be considered as such. The following is an example for this: Husain et al. detected that the combined increase of IL-6 and CRP serum levels correlated with the early onset of irAEs for different organ systems in a patient collective with metastatic melanoma treated with ICI (130).

In summary, the CRP can therefore serve as a risk factor for the development of cancer, as an indicative or alarming serological parameter for the early diagnosis of cancer entities, and as a prognostic and disease course monitoring marker as it may represent the early stage within the activation of the immune cells (111,117). For the use of primary risk stratification in immune checkpoint inhibitor therapy, it is inevitable to understand the dynamics of the CRP level in serum samples for being able to draw substantial and reliable prognostic and predictive conclusions regarding the potential clinical outcomes as progression risk and treatment response and finally overcome resistance to immune checkpoint blockade.

2.4.4 CRP dynamics

In 2014, Simeone et al. succeeded in demonstrating that in patients with melanoma, both disease control and median OS can be monitored by CRP dynamics during the time of ipilimumab treatment. Disease control and median OS were both significantly associated with declining levels of CRP between the baseline and the end of ICI application (131).

Five years later, Ozawa et al. hypothesized that an early elevation of the inflammatory cytokines IL-6 and CRP following the initiation of PD-1/ PD-L1 checkpoint inhibitors may represent early activation of the immune cells. Investigating this hypothesis in NSCLC patients found that responses occurred invariably within patients who presented either IL-6 or CRP elevation within the first week after treatment commencement. Moreover, the patients of the cytokine elevation group showed a tendency to live longer than their counterparts. Of high interest in this case is the observation that the CRP levels reverted to the pretreatment levels within the second week after treatment commencement and did not increase again after the second ICI application (117). By analysing longitudinal repetitive CRP levels in the course of anti-PD-(L)1 inhibitor immunotherapy, Riedl et al. hypothesized that longitudinal trajectories of CRP might serve as a marker of treatment response and disease outcome. The results of a clinical trial conducted in patients with advanced NSCLC elucidated that an early CRP decline within the first 8 weeks after treatment initiation emerged as a strong predictor of favorable outcomes and that it was significantly associated with a risk reduction for experiencing a PFS event, whereas elevated CRP trajectories, as well as faster increases of CRP levels over the same time were independently found to be associated with higher progression risk. Assuming that the repetitive collection of the researched disease monitoring parameter may more accurately reflect disease activity than single pretreatment biomarkers, Riedl et al. suggested that longitudinal CRP trajectory may contain personalized dynamic information allowing the prediction of progression risk (125).

2.4.4.1 Model for early CRP kinetics

Inspired by the promising findings of earlier research work analysing the prognostic value of early CRP kinetics in mRCC during multimodal therapy including cytokine and TKI therapy, Fukuda et al. aimed to investigate if such early dynamic changes in CRP levels also occur in mRCC patients treated with nivolumab and whether they might be able to predict treatment efficacy in terms of tumor response to the ICI and patient survival, evaluating their potential serving as predictive biomarkers. A retrospective study including mRCC patients receiving nivolumab as second-line therapy following TKI therapy gave the occasion to

define three groups based on their different early CRP kinetics: the first group consisted of patients presenting at least a doubling of baseline CRP level within one month after initiation of ICI therapy with nivolumab which was characterized as “flare” and then followed by a decrease in values below the baseline within 3 months (group of CRP flare-responders); in the second group, CRP levels declined by ≥30% within 3 months without presenting a prior “flare” dynamic (group of CRP responders); and the remaining patients were subsumed under the term of non-CRP responders. The treatment outcome correlated with the different three kinetic groups. While CRP flare-responders and CRP-responders presented a maximum change in target lesion of -38% and -13%, respectively, an ORR of 73% and 27%, respectively, and a median PFS value that was not reached for both groups, the maximum target change, the ORR, and the median PFS for CRP non-responders were 16%, 6% and 12 months, respectively. The significant correlation of early CRP kinetics with better tumor response, improved PFS and higher rates of objective response within the CRP flare-response group impressively demonstrated the utility of evaluating early CRP kinetics. (132).

Since then, this model for on-treatment CRP kinetics has been validated in small observational cohorts for mUC and mRCC as well as in small retro- and prospective NSCLC cohorts (118,133–135). Further subgroup analysis of CRP flare responders among patients with mUC receiving anti-PD-(L)1 ICIs was able to show even better PFS and OS in completed flare-response kinetics during a period ≥ 6 weeks. Cautiously considering the small subcohort obtaining this favorable and yet statistically significant outcome, long-flare responses might correlate with more durable anti-tumoral responses (134). Another interesting finding in the NSCLC anti-PD-1 ICI cohort led to the suggestion to differentiate at an early stage of 4 weeks after treatment initiation between responders and non-responders as two-thirds of the CRP flare-responders and >90% of the CRP responders could already be correctly classified 4 weeks after the start of ICI therapy. This observation may increase the clinical value of monitoring CRP kinetics for early therapy adjustments by precociously identifying the vulnerable CRP non-responder subgroup for initiating a therapy shift or escalation (133). The established model of Fukuda et al. has remarkably redefined the CRP as a diagnostic-relevant biomarker in the setting of ICI therapy and facilitated its clinical utility (132).

Aim of the present Early CRP Kinetics Study

The aim of this study was to validate the prognostic and predictive accuracy of early CRP kinetics for the prediction of ICI efficacy in a large multi-cancer cohort to transfer the promising results of previously conducted studies on small cohorts of several tumor entities to a larger patient collective.

3 Materials and Methods of the Early CRP Kinetics Study

3.1 Study design and patient cohort

In this multicenter cohort study, 562 consecutive patients with various cancer types undergoing palliative ICI treatment at the Medical University of Graz, Austria (Department of Internal Medicine, Division of Oncology and Division of Pulmonology as well as Department of Dermatology) and the State Hospital of Feldkirch, Austria, were included in a registry called AUTRICHE (AUsTrian Registry for Immune Checkpoint Inhibitors) which was set up by the oncology department of the Medical University of Graz.

3.1.1 Inclusion criteria

Cancer patients undergoing ICI treatment in a palliative setting were included.

The inclusion period for the cohort was set from January 2015 to November 2021. Patients who started ICI treatment between January 2015 and august 2018 were included retrospectively. From September 2019, patients were followed prospectively.

3.1.2 Exclusion criteria

Missing CRP values at baseline led to the exclusion from the study.

3.2 Parameters

The clinicopathological characteristics which formed the basis for this cohort study included age, sex, gender, ECOG performance score, tumor type, ICI agent, Charlson comorbidity score, cancer stage, and of most interest for this study, all available CRP serum levels for up to three months from ICI treatment onset. CRP levels were reported in mg/L. All assessed parameters were gathered from both the electronic database systems of the participating hospitals and from paper chart documentation. Data collection and sorting was performed using RedCAP database. In case of death, dates of death were obtained from the Austrian social security database.

3.3 Ethics approval

The study was approved by the Institutional Review Board of the Medical University of Graz (No. 31-357 ex 18/19). Written informed consent was obtained from all prospectively enroled patients.

3.4 Refined CRP kinetics model

By defining the CRP kinetics model, Fukuda et al. (132) distinguished three CRP-response groups according to the different early CRP kinetics following ICI treatment initiation: the group of CRP-flare responders, the group of CRP responders, and the group of CRP-non responders. In the work of Fukuda et al., 2021, CRP-flare response was defined as at least a doubling of baseline CRP values and therefore an increase of ≥ 100% within one month after the start of ICI therapy, followed by a drop in CRP below baseline within the consecutive two months. CRP response was defined as a decrease in CRP levels of at least 30% from baseline within three months after treatment initiation. All other patients were classified as CRP-non responders (132).

During the sorting and analysis of the present data, the finding that a considerable number of patients presented only one longitudinal CRP readout (n=21) resulted in the reevaluation and redefinition of the existing model by adding a fourth CRP response group: the all-time normal CRP responders. This group was defined by CRP levels that consistently stayed below the ULN (≤5 mg/L) throughout the first three months following baseline.

3.5 Primary endpoints

The co-primary endpoints were the objective response rate, the progression-free survival and the overall survival.

The overall response rate was defined as the proportion of patients having complete remission (CR) or partial remission (PR) defined by the in-house radiologists in analogy to immune-related response evaluation criteria in solid tumors (irRECIST). In case of a patient's death prior response assessment via radiology, the best treatment response was defined as progressive disease.

Progression-free survival was defined as the time in months from the date of ICI treatment start until the date of radiological assessment of cancer progression or death from any cause.

Overall survival was defined as the time in months from the date of ICI treatment start to death of any cause.

3.6 Statistical analyses

The statistical analyses were performed using Stata for Windows version 16.1 (StataCorp LP, Collage Station, TX, USA).

Chi-square-tests, t-tests, and Kruskal-Wallis tests were employed to evaluate associations between the different CRP response groups and the baseline collected clinicopathological parameters.

Kaplan-Meier analysis compared with log-rank tests was used for calculating ORR, PFS and OS for all CRP response groups.

In addition, landmark analysis was conducted to minimize the inflation of survival times due to varying degrees of immortal time bias this study may be subject to. As the defined interval of observation during which the outcome event can occur varies among CRP response groups, artificial and differing impacts on survival times makes this an important potential cofounder. While CRP non-responders by definition need to live for at least 3 months to be classified in their associated group, CRP flare response and CRP response can occur and be assessed at any earlier date of follow-up. Therefore, the landmark date was empirically set at day 40 of follow-up and meets the median time to the definition of flare response. In the second step, PFS and OS curves from landmark analysis were compared with Mantel-Byar tests. Furthermore, the fitting of uni- and multivariable Cox proportional hazards models including CRP response groups as time-dependent variables was performed incorporating this immortal time bias in time-to-event regression.

For multivariable logistic and Cox regression modeling, only variables which were univariably associated with the outcome at the 5% significance level ($p<0.05$) were considered. In addition, a follow-up cut-off at 5 years after baseline was determined for all survival outcomes in the Cox regression models.

In the absence of validated cut-offs for the distinction of high vs low CRP at baseline in logistic regression and Cox models, an empirical cut-off at the 50^{th} percentile was used to implement CRP at baseline as dichotomized variable (high vs. low).

In the case of missing baseline parameters for the variables ECOG, tumor stage at ICI treatment start, and treatment line, multiple imputation models using chained equations with 100 (m=100) imputations for each missing variable were applied and the outcomes and longitudinal CRP values were not imputed.

4 Results of the Early CRP Kinetics Study

4.1 Cohort baseline characteristics

In total, the studied collective was composed of 562 patients with solid malignancies who were receiving palliative ICI treatment between January 2015 and November 2021. 432 patients underwent treatment at the Medical University of Graz, Austria, and 130 patients at the State Hospital of Feldkirch, Austria. The designated sex of 350 (62.3%) patients was male, and 212 (37.7%) patients were reported to be of female sex. The median age within the studied collective was 66.3 years [IQR 58.4-72.7]. The median calculated value for the Charlson Comorbidity Index was 10 [IQR 10-13]. For 455 patients, the ECGO score at baseline could be defined. Among them, 59 (10.5%) were classified as ECOG 0 and 396 (70.5%) as ECOG >0. The most frequent tumor entities were NSCLC (n=231, 41.1%), melanoma (n=95, 16.9%), RCC (n=73, 13%), and UC (n=46, 8.2%). Following their cancer diagnosis, 285 (50.7%) patients were treated in a palliative care setting. All patients were diagnosed with metastatic sites at the time of ICI start, the median number of metastatic sites was 1 [IQR 1-2]. 94% of patients had stage IV cancer at baseline assessment, the percentage of missing data for this characteristic was 1.25%. At the time of ICI treatment initiation, 280 (55.2%) patients showed a high baseline CRP. 250 (44.5%) patients received ICI as 1st line treatment whereas for 183 (55%) patients, the application of ICI marked the 2nd or a higher treatment line. For a proportion of 0.5%, the treatment line could not be assessed. Within the studied multi-cancer cohort, the most commonly applicated ICI agents were pembrolizumab (n=248, 44.1%) and nivolumab (n=234, 41.6%). 30 (5.3%) patients received atezolizumab, 10 (1.8%) ipilimumab, 4 (0.7%) durvalumab and none avelumab. A total of 36 (6.4%) patients received a combination of nivolumab and ipilimumab. Some patients underwent additional treatment during the enrolment of ICI therapy: 53 (9.4%) received chemotherapy, 12 (2.1%) radiotherapy, and 11 (2.0%) targeted therapy, respectively (**Table 4**).

	Number of cases with available data	**AUTRICHE cancer cohort (n=562)**
	n (%miss.)	Summary measure
Sex ---male ---female	562 (0%)	 350 (62.3%) 212 (37.7%)
Age (years)		66.3 [IQR 58.4-72.7]
ECOG	455 (19%)	

---ECOG 0		59 (10.5%)
---ECOG >0		396 (70.5%)
Charlson Comorbidity Index	562 (0%)	10 (IQR 10-13)
Palliative at diagnosis	562 (0%)	285 (50.7%)
Cancer types	562 (0%)	
---NSCLC		231 (41.1%)
---Melanoma		95 (16.9%)
---RCC		73 (13%)
---UC		46 (8.2%)
---other		117 (20.8%)
Tumor stage at ICI start	555 (1.25%)	
---II+III		25 (4.4%)
---IV		530 (94.3%)
Number of metastatic sites	562 (0%)	1 [IQR 1-2]
High baseline CRP	562 (0%)	280 (55.2%)
ICI		
Treatment line	559 (0.5%)	
---1st line		250 (44.5%)
---2nd line or higher		183 (55%)
ICI agent	562 (0%)	
---Nivolumab		234 (41.6%)
---Pembrolizumab		248 (44.1%)
---Atezolizumab		30 (5.3%)
---Durvalumab		4 (0.7%)
---Ipilimumab		10 (1.8%)
---Avelumab		0 (0%)
---Nivolumab/Ipilimumab		36 (6.4%)
Additional treatments	562 (0%)	
---Chemotherapy		53 (9.4%)
---Radiotherapy		12 (2.1%)
---Targeted therapy		11 (2.0%)
CRP response	562 (0%)	819 (89.6%)
---CRP non-responder		249 (44%)
---CRP responder		242 (43%)
---CRP flare-responder		71 (13%)

Table 4: Descriptive characteristics of the study population

With a median of 4 CRP values per patient, a total amount of n=3,652 CRP values assessed during the first three months of ICI therapy were included in the analysis. The minimum amount of obtained CRP values for one single patient was 1, the maximum was 21. The median CRP level at baseline was 14.4 mg/L.

According to the definition of Fukuda et al. (132), 249 (44%), 242 (43%), and 71 (13%) patients were classified as CRP non-responders, CRP-responders, and CRP-flare responders, respectively (**Table 4**). The ORR was 27%, the median PFS and OS estimates were 4.6 months (95% CI 4.0-5.2) and 10.0 months (95% CI 8.6-11.6).

4.2 Early CRP kinetics model as biomarker for ICI response and clinical outcome in the AUTRICHE cohort

The groups of both the CRP responders and CRP flare-responders had significantly higher odds of ICI response than CRP non-responders in univariable logistic regression models evaluating the impact of early CRP kinetics on treatment response. Adjusting these associations for age, baseline CRP, cancer type, and treatment line in the multivariable analysis could verify the prevalence of the former finding (**Table 5**).

Variable	**Univariable analysis**		**Multivariable analysis**	
	OR (95% CI)	***p*-value**	**OR (95% CI)**	***p*-value**
Age (continuously coded)	1.028 (1.003-1.034)	**0.022**	1.025 (1.006-1.045)	**0.011**
Sex				
Male	1 (reference)			
Female	0.771 (0.528-1.126)	0.178		
ECOG				
ECOG 1	1 (reference)			
ECOG >1	0.690 (0.395-1.204)	0.191		
Charlson Comorbidity Index	0.995 (0.889-1.026)	0.209		
Baseline CRP				
Low (<median)	1 (reference)		1 (reference)	
High (>=median)	0.676 (0.465-0.983)	**0.040**	0.584 (0.386-0.886)	**0.011**
Cancer type				
NSCLC	1 (reference)			
Melanoma	1.270 (0.761-2.120)	0.361		
RCC	1.078 (0.606-1.918)	0.797		
UC	0.526 (0.233-1.188)	0.122		
Other	0.714 (0.424-1.203)	0.206		
Metastatic sites	0.988 (0.831-1.175)	0.896		
Stage at ICI start				
II+III	1 (reference)		1 (reference)	
IV	0.284 (0.126-0.641)	**0.002**	0.319 (0.130-1.780)	**0.012**
Treatment line				
1st line	1 (reference)		1 (reference)	

2nd line or higher	0.448 (0.307-0.655)	**<0.001**	0.500 (0.333-0.751)	**0.001**
CRP response CRP non-responder CRP responder CRP flare-responder	 1 (reference) 4.230 (2.714-6.593) 3.030 (1.639-5.603)	 **<0.001** **<0.001**	 1 (reference) 4.803 (3.001-7.687) 3.026 (1.603-5.714)	 **<0.001** **0.001**

Table 5: Uni- and multivariable logistic regression models for the established early CRP kinetics model

In CRP non-responders, Kaplan-Meier analysis showed significantly shorter PFS (log-rank p<0.001) and OS (log-rank p<0.001) as compared to CRP responders and flare-responders (**Figure 2A-B**).

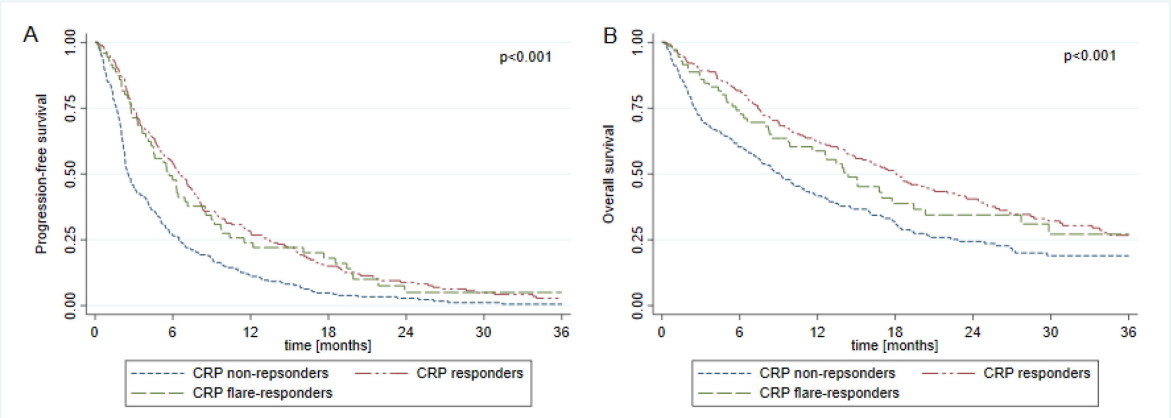

Figure 2: Kaplan-Meier curves showing progression-free survival (PFS) and overall survival (OS) stratified by the previously established three-group CRP response model by Fukuda et al. 2021

As the median time to definition for CRP flare-responders was 41.5 days, a period of 40 days was considered a reasonable cut-off for landmark analysis for all further analyses.

Uni- and multivariable Cox proportional hazard models which treated CRP kinetic groups as time-dependent variables provided further confirmation for the consistency of the association between early CRP kinetics and PFS and OS outcomes: the results of these analyses indicated longer PFS (**Table 6**) and OS (**Table 7**) in patients with CRP-flare response and CRP response.

Variable	**Univariable analysis**		**Multivariable analysis**	
	HR (95% CI)	***p*-value**	**HR (95% CI)**	***p*-value**
Age (continuously coded)	0.99 (0.99-1.00)	0.096		
Sex Male Female	 1 (reference) 1.13 (0.94-1.35)	 0.202	 	
ECOG ECOG 1 ECOG >1	 1 (reference) 1.50 (1.12-2.00)	 **0.007**	 1 (reference) 1.28 (0.95-1.73)	 0.103

Charlson Comorbidity Index	1.00 (0.97-1.03)	0.936		
Baseline CRP				
Low (<median)	1 (reference)		1 (reference)	
High (>=median)	1.46 (1.22-1.75)	**<0.001**	1.65 (1.36-2.01)	**<0.001**
Cancer type				
NSCLC	1 (reference)		1 (reference)	
Melanoma	0.90 (0.70-1.16)	0.426	1.03 (0.79-1.34)	0.838
RCC	0.73 (0.55-0.98)	**0.035**	0.63 (0.47-0.85)	**0.002**
UC	1.37 (0.99-1.89)	0.057	1.37 (0.99-1.90)	0.057
Other	1.13 (0.89-1.44)	0.315	1.19 (0.94-1.53)	0.152
Metastatic sites	1.08 (0.99-1.17)	0.076		
Stage at ICI start				
II+III	1 (reference)			
IV	1.57 (1.00-2.49)	0.053		
Treatment line				
1st line	1 (reference)			
2nd line or higher	1.20 (1.00-1.44)	0.050		
CRP response				
CRP non-responder	1 (reference)		1 (reference)	
CRP responder	0.63 (0.51-0.77)	**<0.001**	0.52 (0.42-0.65)	**<0.001**
CRP flare-responder	0.68 (0.51-0.92)	**0.013**	0.62 (0.46-0.84)	**0.002**

Table 6: Uni- and multivariable Cox regression models for PFS for the established early CRP kinetics model

In the univariable Cox regression model, ECOG performance, RCC as cancer, type, and CRP at baseline were found to be factors that are significantly associated with PFS after ICI treatment initiation besides the early CRP kinetics of CRP flare-response and CRP-response. In the multivariable Cox regression model, baseline CRP and RCC as cancer type remained significant besides the significantly associated impact of early CRP kinetics of CRP flare-response and CRP-response on PFS. In contrast, the ECOG score no longer was significantly associated with PFS in multivariable Cox regression analysis (**Table 6**).

Variable	**Univariable analysis**		**Multivariable analysis**	
	HR (95% CI)	***p*-value**	**HR (95% CI)**	***p*-value**
Age (continuously coded)	1.00 (0.99-1.01)	0.385		
Sex				
Male	1 (reference)			
Female	1.08 (0.87-1.34)	0.477		
ECOG				
ECOG 1	1 (reference)		1 (reference)	
ECOG >1	2.07 (1.39-3.10)	**<0.001**	1.70 (1.13-2.585)	**0.010**
Charlson Comorbidity Index	1.10 (1.06-1.14)	**<0.001**	1.07 (1.03-1.11)	**<0.001**
Baseline CRP				
Low (<median)	1 (reference)		1 (reference)	
High (>=median)	1.79 (1.45-2.20)	**<0.001**	1.87 (1.49-2.36)	**<0.001**
Cancer type				
NSCLC	1 (reference)		1 (reference)	
Melanoma	0.76 (0.57-1.02)	0.071	0.83 (0.61-1.12)	0.219
RCC	0.46 (0.31-0.69)	**<0.001**	0.41 (0.27-0.63)	**<0.001**
UC	1.66 (1.14-2.41)	**0.008**	1.77 (1.21-2.59)	**0.003**
Other	1.11 (0.84-1.47)	0.462	1.14 (0.86-1.53)	0.363

Metastatic sites	1.21 (1.10-1.33)	**<0.001**	1.18 (1.06-1.32)	**0.003**
Stage at ICI start II+III IV	 1 (reference) 2.03 (1.08-3.80)	 **0.028**	 1 (reference) 1.63 (0.84-3.16)	 0.151
Treatment line 1st line 2nd line or higher	 1 (reference) 1.14 (0.92-1.41)	 0.315		
CRP response CRP non-responder CRP responder CRP flare-responder	 1 (reference) 0.73 (0.58-0.92) 0.85 (0.60-1.19)	 **0.008** 0.339	 1 (reference) 0.57 (0.45-0.73) 0.68 (0.48-0.96)	 **<0.001** **0.029**

Table 7: Uni- and multivariable Cox regression models for OS for the established early CRP kinetics model

The analysis of univariable Cox regression models for OS revealed that only the group of CRP responders among the three predefined CRP response groups was significantly associated with OS. Besides, ECOG score, Charlson Comorbidity Index, baseline CRP, RCC or UC as cancer type, the presence of metastatic sites, and stage of cancer disease at ICI start were found to be significantly associated with OS. In multivariable analysis, all those factors remained significantly associated except for the stage of disease at ICI start. Moreover, CRP flare response was also found to have a significant impact on the OS in multivariable Cox regression (**Table 7**).

4.3 Refined CRP kinetics model accounting for patients with all-normal CRP

Throughout the first three months after ICI initiation, a total of n=58 (10.3%) patients consistently had CRP levels below the ULN (i.e., 5 mg/L). According to the previously defined model for early CRP kinetics by Fukuda et al. (132), n=23 (39.7%), n=33 (56.9%) and n=2 (3.4%) patients would have been grouped as CRP non-responders, CRP responders and CRP flare-responders, respectively. Accounting for this phenomenon, a four-group CRP kinetics model was subsequentially defined: n=58 (10.3%), n=209 (37.2%), n=69 (12.3%) and n=226 (40.2%) were classified as patients with all-normal CRP, CRP responders, CRP flare-responders and CRP non-responders, respectively (**Table 8**):

	Number of cases with available data	**AUTRICHE cohort (n=562)**
	n (%miss.)	Summary measure
CRP response --CRP non-responder --CRP responder --CRP flare-responder --All-normal CRP	562 (0%)	 226 (40.2%) 209 (37.2%) 69 (12.3%) 58 (10.3%)

Table 8: CRP group stratification according to the refined model for early CRP kinetics

4.3.1 Predictive and prognostic accuracy of refined early CRP kinetics model

Uni- and multivariable logistic regression analysis of the refined CRP response classification revealed that in addition to CRP responders and CRP flare-responders, patients with all-normal CRP showed significantly higher odds of treatment response compared to CRP non-responders (**Table 9**).

Variable	**Univariable analysis**		**Multivariable analysis** (Only univariably with p<0.05 associated variables)	
	OR (95% CI)	***p*-value**	**OR (95% CI)**	***p*-value**
Age (continuously coded)			1.023 (1.004-1.043)	**0.031**
Baseline CRP Low (<median) High (>=median)			 1 (reference) 0.631 (0.401-0.993)	 **0.046**
Stage at ICI start II+III IV			 1 (reference) 0.294 (0.121 -0.716)	 **0.007**
Treatment line 1st line 2nd line or higher			 1 (reference) 0.496 (0.330-0.744)	 **0.001**
CRP response CRP non-responder CRP responder CRP flare-responder All-normal CRP	 1 (reference) 4.385 (2.702-7.117) 3.310 (1.741-6.294) 4.992 (2.592-9.614)	 **<0.001** **<0.001** **<0.001**	 1 (reference) 5.024 (3.006-8.398) 3.246 (1.672-6.301) 3.937 (1.955-7.929)	 **<0.001** **0.001** **<0.001**

Table 9: Uni- and multivariable logistic regression models for the refined model for early CRP kinetics

In detail, the ORR was 41%, 38%, 31% and 12% in patients with all-normal CRP, CRP responders, CRP flare-responders and CRP non-responders, respectively. Alongside the early CRP kinetics, age, treatment line and baseline CRP were significantly associated with higher odds of treatment response (**Table 9**).

In addition, Kaplan-Meier analysis revealed significantly increased PFS and OS in patients with all-normal CRP, CRP responders and CRP flare responders (**Figure 3 A and B**).

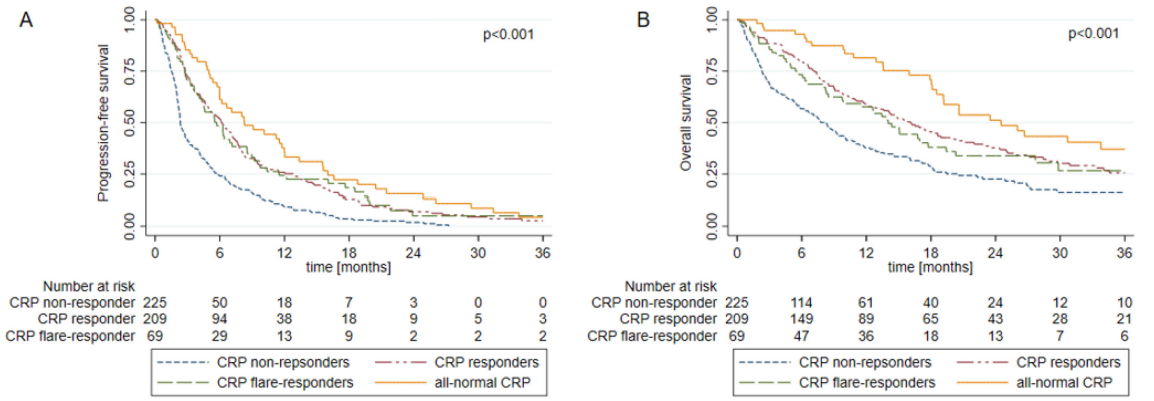

Figure 3: Kaplan-Meier curves showing progression-free survival and overall survival according to the refined four group early CRP kinetics model

This finding could be confirmed in Cox proportional hazards model (**Table 10**).

Variable	**Univariable analysis**		**Multivariable analysis** (Only univariably with p<0.05 associated variables)	
	HR (95% CI)	***p*-value**	**HR (95% CI)**	***p*-value**
ECOG ECOG 1 ECOG >1			 1 (reference) 1.32 (0.98-1.78)	 0.067
Baseline CRP Low (<median) High (>=median)			 1 (reference) 1.80 (1.46-2.22)	 **<0.001**
Cancer type NSCLC Melanoma RCC UC Other			 1 (reference) 1.04 (0.80-1.35) 0.66 (0.49-0.89) 1.29 (0.93-1.79) 1.23 (0.96-1.57)	 0.784 **0.006** 0.126 0.103
CRP response CRP non-responder CRP responder CRP flare-responder All-normal CRP	 1 (reference) 0.59 (0.48-0.73) 0.66 (0.49-0.89) 0.56 (0.40-0.78)	 **<0.001** **0.006** **0.001**	 1 (reference) 0.48 (0.38-0.59) 0.63 (0.46-0.85) 0.74 (0.52-1.05)	 **<0.001** **0.003** 0.096

Table 10: Uni- and multivariable Cox regression models for PFS for the refined model for early CRP kinetics

In detail, median PFS was 8.2 (5.9-12.0) months in patients with all-normal CRP, 6.1 months (95%CI 4.9-7.2) in CRP responders, 5.7 months (95%CI 4.1-8.5) in CRP flare-responders and 2.3 months (95%CI 2.2-2.8) in CRP non-responders. Median OS estimates of these four groups were 24.5 months (95%CI 18.5 – not reached), 16.1 months (95%CI 12.6-19-8), 14.0 months (95%CI 8.5-19.4) and 8.1 months (95%CI 5.8-9.9), respectively.

After adjusting for potential confounders including baseline CRP, the association between the all-normal CRP group and the survival endpoints slightly weakened, whereas the other CRP response groups fully prevailed as significant predictors. (**Table 10, Table 11**).

Variable	**Univariable analysis**		**Multivariable analysis** (Only univariably with p<0.05 associated variables)	
	HR (95% CI)	***p*-value**	**HR (95% CI)**	***p*-value**
ECOG ECOG 1 ECOG >1			 1 (reference) 1.74 (1.16-2.60)	 **0.008**
Charlson Comorbidity Index			1.08 (1.03-1.12)	**<0.001**
Baseline CRP Low (<median) High (>=median)			 1 (reference) 2.01 (1.58-2.57)	 **<0.001**
Cancer type NSCLC Melanoma RCC UC Other			 1 (reference) 0.84 (0.62-1.13) 0.42 (0.28-0.62) 1.66 (1.13-2.43) 1.14 (0.86-1.53)	 0.250 **<0.001** **0.009** 0.365
Metastatic sites			1.19 (1.07-1.33)	**0.001**
Stage at ICI start II+III IV			 1 (reference) 1.61 (0.83-3.13)	 0.159
CRP response CRP non-responder CRP responder CRP flare-responder All-normal CRP	 1 (reference) 0.64 (0.50-0.81) 0.75 (0.54-1.06) 0.46 (0.31-0.69)	 **<0.001** 0.104 **<0.001**	 1 (reference) 0.46 (0.35-0.59) 0.62 (0.44-0.88) 0.66 (0.43-1.01)	 **<0.001** **0.007** 0.053

Table 11: Uni- and multivariable Cox regression models for OS for the refined model for early CRP kinetics

5 Discussion

In the present study, the prognostic and predictive value of early CRP kinetics in the form of longitudinal CRP measurements to predict and monitor ICI efficacy could be demonstrated. By using a large multi-cancer cohort of patients receiving immune checkpoint inhibitors, this bi-centre study was able to confirm the prognostic and predictive validity of the model for early CRP kinetics established by Fukuda et al. (132) in 2021: based on the analysis of early CRP kinetics in 42 patients with mRCC undergoing treatment with nivolumab, Fukuda et al. had defined three groups of early CRP response patterns predicting different odds of responding to ICI therapy and experiencing more favorable clinical

outcome. According to the percentage change of their serum CRP level from baseline within the first 3 months after treatment initiation, patients were divided into the groups of CRP flare response, CRP response, and CRP non-response. CRP flare response or CRP response kinetics were significantly associated with a higher rate of treatment response and more desirable survival outcomes as compared to the CRP non-response group (132). Having since then only been applied to various smaller sub-cohorts of different cancer entities (118,133–135), the present study was able to externally validate the prognostic and predictive significance of this early CRP kinetics model for a considerable variety of ICI therapy regimen and tumor types. A significant association between the CRP response patterns and the study endpoints could be established, accurately reflecting clinically relevant CRP changes especially in patients with elevated baseline CRP.

For patients with normal baseline CRP, the present data generated from this large multi-cancer cohort led to the distinction of a fourth pattern of CRP kinetics: for a group of patients who consistently presented CRP values below the ULN (i.e. 5 mg/L) throughout the first three months of ICI therapy, superior response rates and survival outcomes alongside those of the groups of CRP responders and CRP flare-responders in comparison to CRP non-responders could be observed.

Therefore, a refined four-group CRP kinetics model serving as prognostic and predictive biomarker for ICI efficacy extended by the group of all-normal CRP was proposed in a second step. It was hypothesized that this distinct CRP response group consistently presenting measurements below the ULN throughout the first three months of ICI treatment also shows a favorable prognosis which can not sufficiently be taken into account by using the three-group CRP kinetics model. For instance, a patient with a baseline CRP of 2mg/L and consecutive CRP measurements of 3mg/L would be classified as a CRP non-responder. Accordingly, 10.3% of patients in the study cohort were reclassified as all-normal CRP of which a large proportion (39.7%) would have otherwise been grouped as CRP non-responders who previously have been found to show a poor prognosis. This four-CRP-group model showed improved discrimination of response rates, disease progression risk, and death. In detail, patients with all-normal CRP had 4-fold higher odds for treatment response than CRP non-responders and presented the numerically longest PFS and OS. Beside patients with all-normal CRP, CRP responders and CPR flare-responders also had significantly higher response rates and better prognosis than CRP non-responders. These findings are of high relevance regarding the ICI treatment management pathway as early on treatment CRP kinetics may facilitate the early identification of the vulnerable CRP non-

responders who may not profit from ICI therapy. Subsequently, earlier tumor staging, and the optimization of treatment can be performed and patients who are likely to not respond to ICI therapy can be spared from eventually life-threatening toxicity caused through the checkpoint blockade (87,94).

To date, the underlying pathophysiological mechanisms causing the described specific CRP dynamics are not yet fully understood as outlined in the introductory part. Early and rapid increase in CRP (CRP flare) was found to be associated with a favorable anti-tumor response and may therefore be interpreted as the reflection of the underlying inflammatory process as part of successful anti-tumor defense following earlier suggestions based on similar findings (117). Contrarily, elevated CRP level persisting for months after the initiation of ICI therapy should be analysed under the aspects of the established understanding that the CRP is a clinical marker for tumor-associated inflammation that may reflect an increased risk of tumor progression (120) as well as it is suggested to be indicative for insufficient tumor response to immune checkpoint blockade. This may be due to a variety of potentially underlying mechanisms: first, the CRP can directly suppress dendritic cell and T-cell activity, thereby interfering with essential components of the immune system that are required for the function of ICIs (113). Second, the CRP can enhance inflammatory response which in turn bears the potential to suppress immune function as well (113,136). Third, higher CRP levels have been reported to be associated with the formation of an immunosuppressive TME containing an increased amount of Tregs (137). On the other hand, the longitudinal decrease of the CRP level found after one month in the CRP flare responders and at least after three months in CRP responders may furthermore be understood as dynamic change of the cancer-immune system of the TME under the impact of ICI supported by earlier findings associating a CRP decrease after ICI initiation with a survival benefit (131). Elucidating the question in which matter high and low baseline CRP levels are related to the specific longitudinal CRP response patterns after the start of ICI therapy may also increase our understanding of the mechanisms responsible for early CRP kinetics. Klümper et al. suggested that baseline serum CRP may reflect the baseline immunogenicity of the tumor and the presence or absence of chronic tumor-induced inflammation (118). High baseline CRP levels may moreover signify worse health conditions and cancer circumstances of the affected patients which can have an impact on the effectiveness of ICIs as well (123). The in the framework of this study observed comparably high odds of response to ICI treatment (i.e. 41%) as well as the numerically longest PFS and OS (i.e. 8.2 months and 24.5 months,

respectively) of patients being assigned to the newly defined all-normal CRP group may support these hypotheses. Clarifying the association between the systemic inflammatory response and the tumor response to ICI therapy which may be reflected by the distinct CRP patterns could lead to further improvement of ICI treatment outcomes.

One of the noteworthy strengths of this study was the large cohort consisting of cancer patients suffering from different tumor entities who received ICI treatment in two different Austrian hospitals. The statistical power of this study was guaranteed by rigorously accounting for immortal time bias. Due to the time-dependent nature of the definitions of the CRP response groups, unaccounted immortal time bias as a special kind of selection bias affecting longitudinal data sets in observational studies leads to an overestimation of the favorable prognostic impact of being in any of the CRP response groups by falsely assuming the occurrence of CRP response at baseline (138).

Despite this, the limitations of the study must be acknowledged. First, as this study was mostly conducted in a retrospective design, selection bias cannot be entirely excluded. Therefore, the obtained results should be interpreted within the limitations of this design. Second, the limitations of the retrospective assessment of CRP values must be discussed. The collection of the CRP values was enrolled within routine clinical practice. Contrary to a collection process following a prospective, predefined protocol, the design of this study is more likely to be fraught with the risk that patients experiencing CRP flare response or CRP response between two measurements may not have been detected. Third, information on infections and antibiotic treatment within the first three months of ICI therapy was missing impeding the investigation of the potential influence of these two parameters on early CRP kinetics. Further research will be needed to determine if the predictive accuracy of the early CRP kinetics is independent of other factors that are usually associated with an increase of CRP levels, such as infection or other inflammatory conditions. Fourth, it might be worthwhile to investigate the influence other variables which are known to be of predictive potential for the clinical outcome in ICI therapy might exert on the early CRP kinetics' predictive accuracy in multivariable analysis. Variables to mention in this context are the TMB, the MSI and the PD-L1 status which were found to predict ICI effectiveness for a spectrum of tumor types (84,104,106,139). Recently, the negative predictive value of the PD-L1 tumor proportion score has been shown to increase in the form of a combined biomarker evaluation in NSCLC patients by adding plasma CRP levels to the analysed parameters (140). For some cancer types, the CRP's predictive value may depend on the

impact of the PD-L1 tumor proportion score so that this combined biomarker evaluation might also work vice versa. Fifth, it is necessary to discuss the heterogeneity of the studied cohort regarding the baseline characteristics and especially other accompanied treatments the patients were receiving during the period of inclusion. Stereotactic body radiation therapy (SBRT) for example was reported to enhance the efficacy of ICIs by variable immunomodulatory processes. A SBRT induced increase of the cytokine production, such as IL-1 and IL-6, has been observed in previous studies which, with the knowledge that these two cytokines boost CRP production, may also affect early CRP kinetics of those patients that were concurrently undergoing radiotherapy and ICI treatment (141). Studies investigating the interdependency of the different adjuvant therapies and the predictive accuracy of the CRP as biomarker for ICI treatment effectiveness are thus of great importance for the successful transfer of the within this trial gained knowledge to the clinic where practitioners are confronted with a heterogeneous patient collective in all regards.

In conclusion, this study emphasizes that early CRP kinetics represents an easily available and cost-effective predictive and prognostic biomarker for treatment response, disease progression, and survival outcomes in patients undergoing ICI therapy across various cancer types. Further validation and the establishment of an accurate and precise CRP value assessment and monitoring interval in a prospective design is warranted to clarify if patients that are found to be CRP non-responders should discontinue ICI treatment.

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