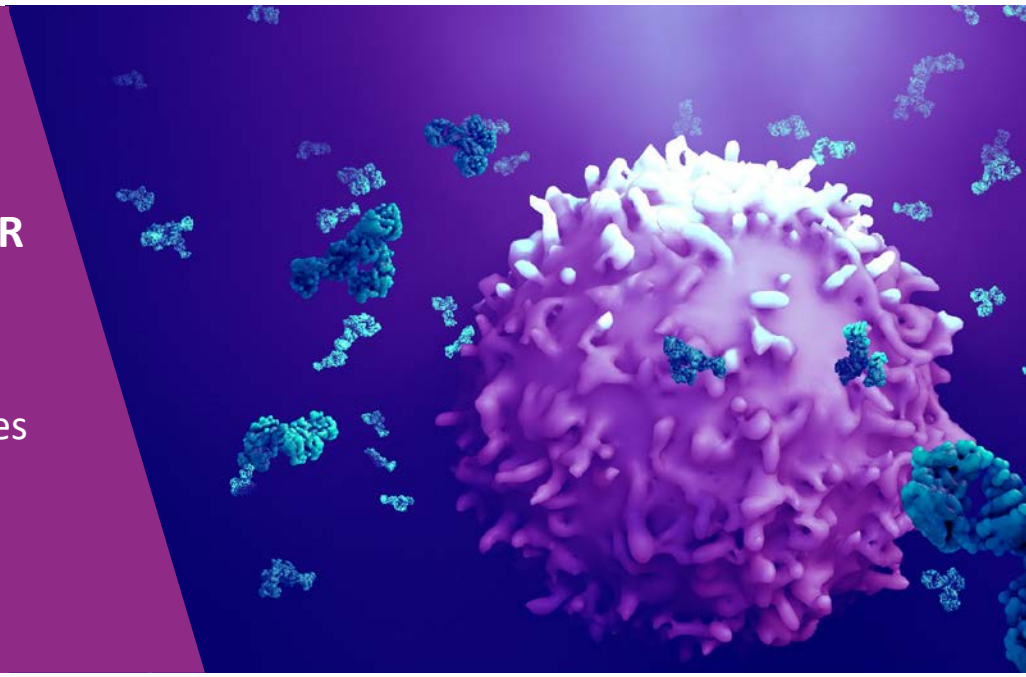
imSAVAR

**IMMUNE SAFETY AVATAR**

Nonclinical mimicking of the immune system effects of immunomodulatory therapies



# Deliverable 4.8

# Second round of study protocols and analyses plans for biomarker development including mechanistic insights from deep molecular profiling

**DELIVERABLE REPORT**

This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking (JU) under grant agreement No 853988.
The JU receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA and JDRF INTERNATIONAL.

imi innovative medicines initiative

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## Abstract

Work package 4 (WP4) focuses on the development of biomarkers for predicting the risk of observing harmful adverse outcomes in first-in-human (FIH) studies of immunomodulatory therapeutics. Current nonclinical models to assess safety of immunotherapies are often species-dependent and incomplete, since they reflect only limited areas of the human immune system, which often leads to wrong predictions of human immune-related adverse events (irAEs). Hence, WP4 aims at establishing biological characteristics (biomarkers) that are measurable and evaluable and can be integrated into safety models in order to (i) assess if the model mimics the underlying human biological processes leading to an immune-related adverse outcome as closely as possible, to (ii) assess if the biomarker is reliably predicting the risk of harmful adverse outcomes in FIH studies, and to (iii) support safe starting dose selection for FIH studies.

In imSAVAR four firstly defined mode of actions (MoAs) of immunomodulatory therapeutics will be addressed and require development and confirmation of biomarkers: (i) CAR (chimeric antigen receptor) T-cells, (ii) BiTEs (bispecific T-cell engagers), (iii) CPI (checkpoint inhibitors) and (iv) immuno-inflammatory disease therapeutics (e.g., IL-2). In imSAVAR, we align biomarker development with immune-related AOPs (irAOPs) to foster a common understanding of the processes triggered through a molecular initiating event and eventually leading to adverse outcomes.

With Deliverable D4.8, we sought to implement a second round of study protocols and analyses plans for biomarker development including mechanistic insights from deep molecular profiling data. Based on the literature research conducted in Deliverable D4.1 (First round of study protocols and analyses plans for biomarker development) and the iterative schemes we generated to develop biomarkers for nonclinical assessment of immunomodulatory therapeutics, we proceeded to further identify and fill knowledge gaps and gain mechanistic insight on molecular level for the different MoAs and baseline immune signatures and advanced irAOPs for the different MoAs. We summarized assays to model irAEs available inside and outside the consortium. Furthermore, we identified data sets already available in the consortium and subjected them to further analyses as well as conducted / are conducting biomarker studies, including the generation of molecular data. We are continuing to plan and carry out further studies to be able to advance our knowledge meeting the expected deliverables at the end of the project.

## Document Information

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# 1. Methods

**Organisation of WP4 to foster exchange between partners:**
To extend the knowledge gained in the first round of study protocols and analyses plans for biomarker development (Deliverable D4.1.), we established working groups in WP4 conducting biomarker-relevant studies:

1) Non-human primates (NHP) working group (MoA: baseline/cross-species)
2) Patient Study working group (MoA: CAR T cells)
3) T cell engaging bispecific working group (MoA: BiTEs)
4) CAR atlas working group (MoA: CAR T cells)

Furthermore, WP4 cooperates closely with WP2 and WP3 and the working groups established therein to support biomarker development for the MoAs CPI and IL-2. Specifically, we contribute to the irAOP working group.

As implemented before, we considered the MoAs individually where sensible. However, we as a consortium identified that a stringent separation of MoAs is not helpful for all steps. We thus combined MoAs wherever possible (e.g., combined/harmonized irAOP for Cytokine Release Syndrome (CRS)).

WP4 meets bi-weekly to fosters exchange within and between working groups.

Additionally, one in-person study-a-thon was held for focused analyses and discussions in a multidisciplinary team of experts.

**Extending irAOPs to cover biomarker development:**
After establishing a general conceptual view on how to integrate biomarker development into the concept of irAOPs, we developed several irAOPs for different MoAs. Using the MINERVA platform, we visualized some of the irAOPs developed. MINERVA enables a machine-readable representation for irAOPs allowing for interactive modelling of key events and key event relationships. Digital representations of irAOPs facilitate shared development, evaluation and refinement of mechanisms leading to immune-related adverse events. As a proof-of-concept study and a first iteration to characterize molecular mechanisms leading to irAEs, we selected the irAOP describing IL-2-mediated skin rash and a first version was implemented in MINERVA (see D4.5). This initial version was extended and MINERVA networks for additional irAOPs implemented (see D4.6). In D4.7, we report establishing a resource combining five irAOPs related to CRS into a single molecular interaction map.

**Systematic Review:** We conducted systematic reviews on CPI therapies (according to the PRISMA statement (1)) to summarize current knowledge of biomarkers related to adverse events (CRS, neurotoxicity and hepatotoxicity). With conducting systematic reviews our objective is to identify pre-existing datasets of human and non-human data that can be used to answer questions such as:

- Which molecules are involved in biological process (i.e., are the biological objects which are perturbed in a key event (KE) of an irAOP)?
- Which of those molecules are measurable as readouts of methods/models to evaluate the biological state of the KE?
- Which molecules define the structural and functional relationship between pairs of KEs?
- Which observed changes (time and amount) of molecule abundancy of an upstream KE are critical leading to measurable and evaluable changes in the downstream KE?

The outcome of the review were biomarkers used to guide clinical management of patients receiving CPI therapy. The datasets are included in a data catalogue comprising the current knowledge of biomarkers for predicting irEAs.

**Planning and conducting of studies relevant for biomarker development**

Based on the state-of-the-art assessed in the systematic reviews and the data catalogue we set up with publicly available datasets as well as datasets available in the consortium, we identified knowledge gaps. To fill these gaps, we plan(ned) and conduct(ed) studies to generate further insights into the MoAs of immunotherapies to eventually develop and advance biomarkers for safety assessment. To achieve this, we followed three different pathways:

1) Establishing and employing *in vitro* models to assess irAEs directly advancing assays and biomarkers.

2) Collecting and analyzing patient samples to characterize the patient's immune system on cellular and molecular level and relating the characteristics to irAEs. This identifies clinically relevant biomarkers and components essentially needed to model the irAEs in nonclinical assays.

3) Using computational strategies, we processed publicly available data and data generated in the consortium to set up *in silico* models for advanced data integration and generate biomarker readouts from complex and large datasets.

The results generated and knowledge acquired can be used for driving forward biomarker development by taking the output of one pathway and feeding it into another one. This flexible approach enables us to take into account the heterogenous knowledge base on the different MoAs and irAEs they evoke as well as the diverse levels of development of the models and assays.

# 2. Results

## 2.1 Concept – Biomarkers for early safety assessment of novel immunotherapies

AOPs describe the interconnection of a molecular initiating event with a series of key events eventually leading to an adverse outcome. The interconnection between two subsequent key events (KEs) is described by key event relationships (KERs). Central components of KERs are (i) a description of the structural and functional relationships between subsequent pairs of KEs (**biological plausibility**), (ii) a description of empirical evidences supporting the relationship between pairs of KEs (**empirical evidence**), and (iii) importantly a description which changes in the upstream $KE_{n-1}$ lead to a change in the downstream $KE_n$ (**quantitative understanding**). While the description of these components often cover different molecules, not all molecules or biological characteristics related to KEs and KERs are biomarkers meeting the definition of the Biomarkers Definition Working Group (3). Only biological indicators that are measurable (i.e. molecule status or biological characteristics can be quantified in a reproducible way using a defined measurement method) and are evaluable (i.e. changes of the quantified molecule status or biological characteristics can be (statistically) associated to an event) are biomarkers. Hence, WP4 assigned biomarkers to the concept of irAOPs in order to establish a common ground of definitions and understanding (Figure 1).

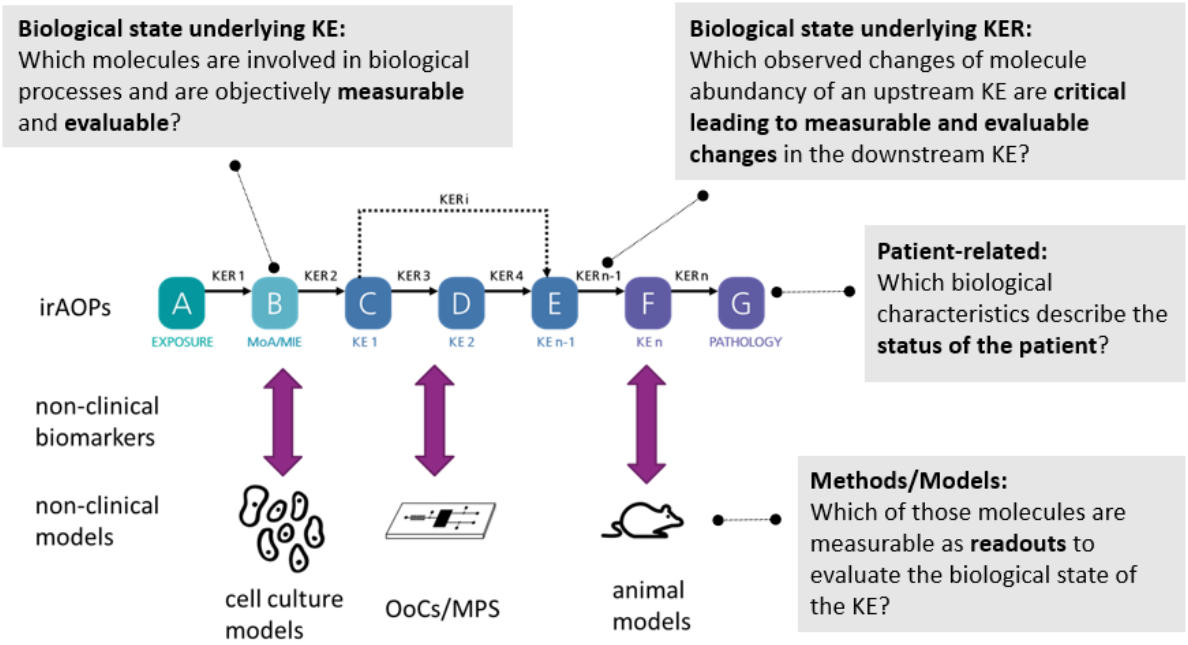


*Figure 1: Schematic overview of aligning biomarker development with irAOPs and assessment of preclinical safety models. Abbreviations: irAOP – immune-related adverse outcome pathway, KE – key event, KER – key event relationship, MIE – molecular initiation event, MoA – mode-of-action, MPS – microphsiological system, OoC – organ-on-chip*

Furthermore, to transfer biomarkers to nonclinical development of immunomodulatory therapies we added new biomarker classes required for the development of immune-related safety models to a list of known classes (4). We assigned each biomarker class a function with respect to irAOPs, safety models (nonclinical models mimicking KEs of irAOPs) and FIH studies (Figure 2). A systematization of biomarker types and a detailed understanding of their roles in nonclinical development of immunomodulatory therapeutics supports the definition of biomarker development strategies, and again fosters a common ground of understanding in the imSAVAR consortium.

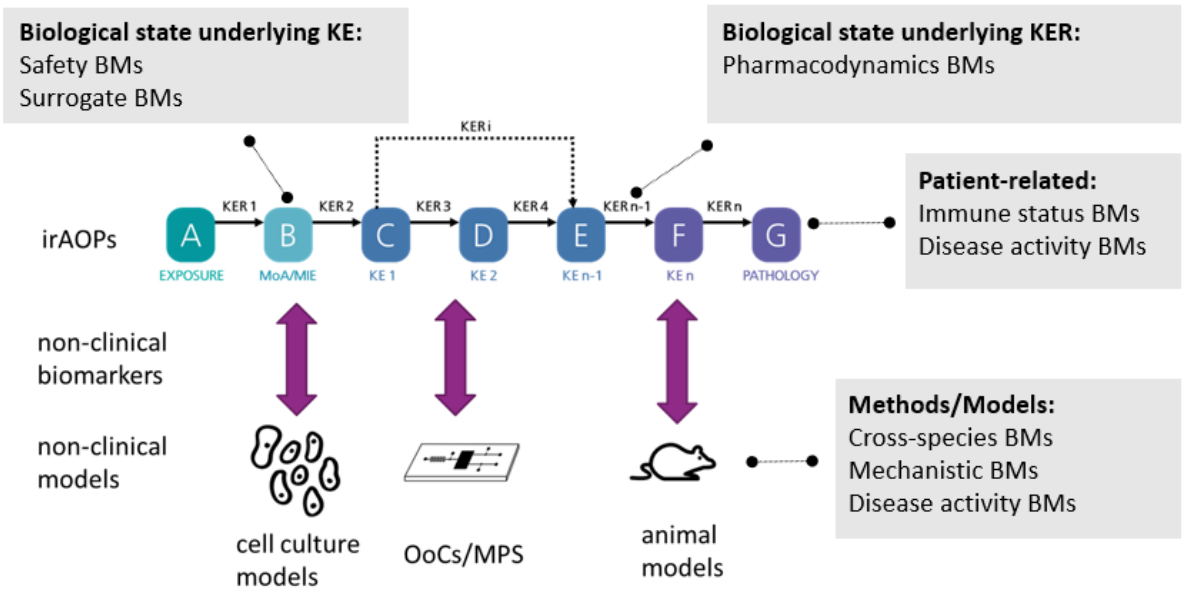


*Figure 2: Overview of types of biomarkers for preclinical assessment of immunomodulatory therapeutics and their association to KEs, KERs of irAOPs or to preclinical models. Abbreviations: BM – biomarker, irAOP – immune-related adverse outcome pathway, KE – key event, KER – key event relationship, MIE – molecular initiation event, MoA – mode-of-action, MPS – microphsiological system, OoC – organ-on-chip*

In summary, biomarkers related to safety models of immunomodulatory therapeutics are measurable biological characteristics that are an indicator of suitability of a model, e.g., mechanistic BMs, disease activity BMs or cross-species BMs. Biomarkers related to irAOPs are, on the other hand, measurable biological characteristics that are indicators of either key events (mechanistic BMs, safety BMs, pharmacodynamic BMs) or key event relationships (pharmacodynamic BMs) leading to adverse outcomes (surrogate BMs) in response to an immuno-therapeutic intervention.

With the concept outlined in Figures 1 and 2 we compiled a detailed list of tasks relating biomarkers to central components of irAOPs. This forms the basis to define MoA-specific iterative schemes to develop biomarkers for preclinical assessment of immunomodulatory therapeutics:

**Step 1 – Assess state-of-the-art for each selected MoA:** The first step towards biomarker development are systematic reviews for each MoA. We conducted systematic reviews following the PRISMA statement (1). The objective of the systematic reviews is to list state-of-the art (clinical) biomarkers for each of the selected irAOPs using a systematic, repeatable and documented approach. This enables us to organize obtained knowledge in machine readable formats and make it available to all consortium partners.

**Step 2 – Feedback loop to preclinical models:** With the outcome of the systematic reviews, we identify (clinical) biomarkers and corresponding statistical models that can be transferred to first experimental studies including novel preclinical models. Here, a direct interconnection with the imSAVAR research and development group for MPS supports the integration of biomarkers into novel models.

**Step 3 – Align biomarkers to irAOPs and identify current knowledge gaps:** A further outcome of systematic reviews are datasets suitable for re-analysis or meta-analyses. Here we aim at describing biological mechanisms triggering transition from an upstream $KE_{n-1}$ to downstream $KE_n$. This supports the identification of current knowledge gaps and detection of novel biomarkers. The datasets will be included in a data catalogue comprising the current knowledge of molecular mechanisms leading to KEs. Based on such a consolidated data catalogue we will identify those KEs and KERs with a high need for novel biomarkers.

**Step 4 – Detailed planning and conducting individual biomarker studies:** Based on steps 1-3 we iteratively define and refine experimental designs for biomarker studies. This will fill current knowledge gaps and improve current safety models. Here we aim at targeted use of multi-omics methods, integrative bioinformatics and artificial intelligence to support uncovering molecular and cellular processes affecting safety. Unbiased multi-omics strategies pave the way for novel biomarkers combined in multi-parametric models (artificial intelligence or machine learning) predicting adverse events.

## 2.2 Assess the state-of-the-art for each selected MoA

In Deliverable D4.1, we conducted systematic reviews for MoAs CAR T cells and BiTEs to identify immune cell datasets for predicting toxicities associated with these MoAs and developed first versions of roadmaps towards the development of novel biomarkers. Taken together, for CAR T cells we identified three studies with publicly available omics data, but none of them with metadata, and four studies describing biomarkers in statistical models predicting adverse events (CRS, neurotoxicity), which were added to a data catalogue. This was used for a combined analysis describing molecular mechanisms related to adverse events of CAR T cell therapies using self-organizing maps, which is available via our interactive results-sharing tool oposSOM-Browser: https://www.izbi.uni-leipzig.de/opossom-browser/. For BiTEs, we found ten studies with publicly available omics data and five studies describing biomarkers in statistical models predicting adverse events (CRS, neurotoxicity). We furthermore set up MoA-specific roadmaps for

CAR T cells, BiTEs and baseline/cross species towards novel biomarkers for the prediction of adverse events. The systematic review on CPIs was still ongoing.

In this deliverable, we report the results of the systematic review using specific search terms for CPIs aiming to identify studies covering irAEs (CRS, neurotoxicity, hepatotoxicity) observed in CPI therapy. We compiled a list of information resources between 11/2020 and 09/2021 (figure 3). We used the following search terms to search on PubMed:

Taken together, we identified 239 publications with this strategy and additional 8 publications from other sources that contained omics data. All publications were collected in a Citavi project (https:\\www.citavi.com) and evaluated according to pre-defined eligibility criteria. After removing duplicates, 210 publications remained for screening (Figure 3).

We used a rating system to evaluate all publications.

| **Rating** | **Criteria** |
|---|---|
| 5 Stars | Article reports on biomarkers for CRS or neurotoxicity or hepatotoxicity as side effects of CPI therapy |
| 4 Stars | Article reports on correlating factors of CRS or neurotoxicity or hepatotoxicity as side effects of CPI therapy or factors that prevent CRS or neurotoxicity |
| 3 Stars | Article reports on CRS or neurotoxicity or hepatotoxicity as side effects of CPI therapy or includes omics data in the context of CPI therapy |
| 2 Stars | Article reports on CPI therapy, but not on CRS or neurotoxicity or hepatotoxicity as side effects |
| 1 Star | Article does not report on CPI therapy or study is not written in English or is earlier than 2010 or study is a review |

We excluded publications with rating below three stars (n = 129) and conducted a full-text screening with the remaining 81 publications (Figure 3). We defined two types of studies we would include:

- Studies that identified biomarkes for irAEs of CPI therapy and report a clear cause effect relationship between the biomarker and the adverse event in a statistical model
- Studies that included omics data in the context of CPI therapy and its irAEs.

Fitting these criteria, we identified one study with meta-analyses and thirteen studies that included omics datasets for bioinformatics analysis.

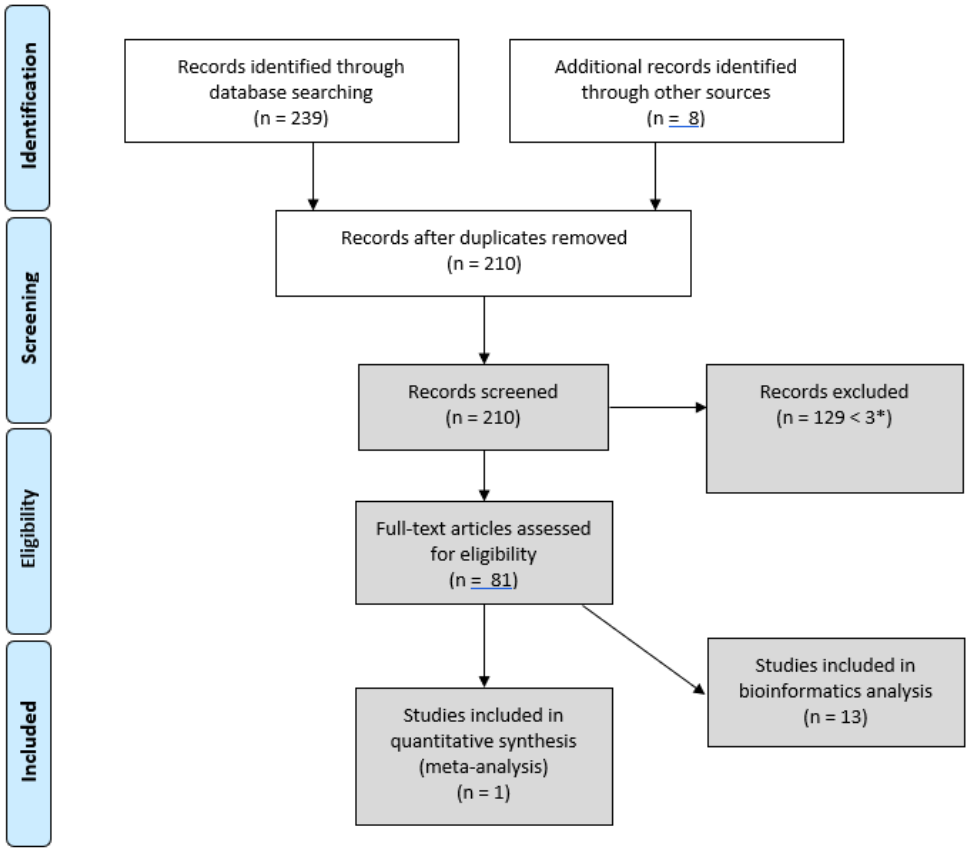


*Figure 3: Flow diagram documenting detailed steps of the systematic review including identification and evaluation of studies relating treatment with CPIs with adverse outcomes. Flow diagram includes all minimal categories to report systematic reviews according to the PRISMA statement (1).*

**MoA-spanning:**

Immune response, and thus also anticipated and unwanted immune-related responses after immunotherapy, is amongst other biological determinants crucially influenced by sex. Sex chromosome genes and sex hormones, including oestrogens, progesterone and androgens, contribute to the differential regulation of immune responses between the men and women (Klein and Flanagan, 2016).
Interaction with patient advocate groups (MPNE, CML Advocates network, LuCE, Patvocates) led us to explore potential differences in irAEs related to sex and gender. To this end, we conducted a literature research identifying publically available datasets that shed light on the sex-differences of irAEs after immunotherapy.

We identified 21 studies that addressed immunotherapy-mediated irAEs and distinguished between females and males. Nine of these studies observed that women develop significantly more irAEs following immunotherapy compared to men, while the others did not identify a sex-related difference. Closer looking into the MoA of CPIs, we found three studies showing that women overall develop significantly more irAEs compared to men (4–6).

The literature suggests, that reasons behind this gap in observed irAEs in females and males can be attributed to sex as well as to gender. Average body type differences between men and women lead to women possibly receiving greater relative doses in immunotherapy. There may also be differences in pharmacokinetics, -dynamics and -genetics that can be attributed to sex. Also, sex and gender may have influence on medication adherence for oral therapies, biases in interpretation and reporting of AEs and

differences in symptoms perception as well as differences in the gut microbiome, which regulates metabolic and immune inflammatory pathways.

## 2.3 Feedback loop to nonclinical models

**Data sharing agreement as cross-consortium effort**

Being able to sharing data from patient derived material is a crucial prerequisite for cooperation within the consortium. Driven by WP4, a data sharing agreement was drafted and approved by all imSAVAR partners.

**Models reflecting KEs in irAOPs**

In several studies, we explored in more detail, how different models can be utilized to assess irAEs after immunotherapy:

In one study, a breast-cancer-on-chip model was used to assess safety and efficacy of CAR T cells in treatment of solid tumors (figure 4). This assay permits recapitulation of the challenges of solid tumors and the tumor environment, integrating an endothelial barrier that enables transmigration of perfused immune cells and their infiltration of the tumor. The continuous perfusion of the chip allows for a time-resolved effluent analysis. Thus, cytokine release upon CAR T cell perfusion that could lead to CRS can concomitantly be monitored. Further, intervention strategies of cytokine release can be assessed. The breast-cancer-on-chip model has the power of modelling aspects of early KEs at the tumor site upon therapy administration in a physiologically relevant coculture model and applied clinically relevant readouts. The study was published in Cell Stem Cell (7).

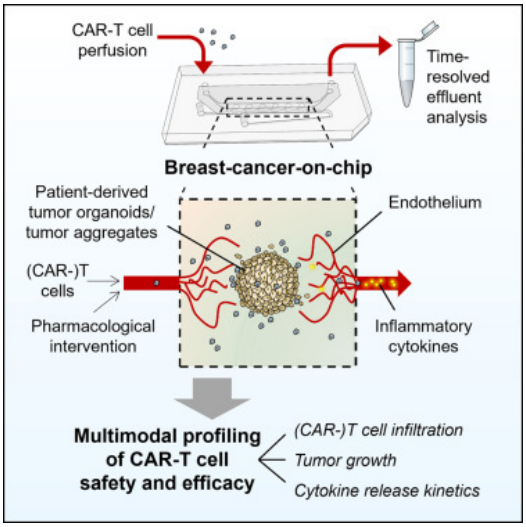


*Figure 4: Breast-Cancer-on-chip model allows for multimodal profiling of CAR T cell safety and efficacy. Abbreviation: CAR – chimeric antigen receptor*

Furthermore, the consortium recently published a joint collection of manuscripts to the Journal of Immunotoxicology for a special issue. In this thematically connected collection of manuscripts, the authors elaborate on the usage of the irAOP concept to identify and fill knowledge gaps on irAEs upon treatment with immunotherapies, also to guide the understanding of these irAEs, as well as employing certain assays for the assessment of irAEs. One study in the collection analyses anti-CD19 CAR T cell-induced cytokine release using Sanofi's MIMIC® assay (8). In this assay, a static microphysiological system harboring a co-culture of red blood cell-depleted whole blood and endothelial cells is successfully utilized to assess cytokine release upon CAR T cell treatment that could result in CRS (also see below for more details). Another manuscript in the collection is dedicated to summarize novel strategies to assess

cytokine release mediated by CAR T cells using the irAOP concept (9). Here, the authors describe simplistic and advanced cytokine release assays (CRAs), assign the KEs these assays are able to reflect, list possible readouts, name involved molecules / biomarkers and state limitations. In addition, they compile available mouse models that can be used to investigate cytokine release upon CAR T cell administration.

Currently, another manuscript is finalized, summarizing CRAs able to model CRS after immunotherapy with BiTEs (Perkins et al., finalizing manuscript for submission). The authors reference models available for cytokine release assessment, the assays' formats and readouts / endpoints. They also determine which KE in the irAOP of CRS mediated by BiTEs is reflected by the assay. Furthermore, the authors generated a decision trees - one to assess whether or not a CRA is necessary to include into nonclinical development of an immunotherapy and a second one to determine which type of CRA would be most appropriate to achieve a meaningful readout.

To identify, which assays modelling irAEs are available within the consortium, we compiled a detailed list of *in vitro* models for the different MoA and irAOPs and assigned the KE reflected in the assay as well as the type of biomarker that can determined with the readout. We also included information on the assay system, the assay's principle and what organs and tissues are represented.

## 2.4 Align Biomarkers to irAOPs and identify current knowledge gaps

We proceeded to further develop the irAOPs and aim for their visual representation in a machine-readable format in MINERVA. In this process, we include the alignment of biomarkers / bio-related molecules of each step in the irAOP. In a first study-a-thon, we developed an irAOP for CRS for five different MoAs (Figure 5, deliverable D4.7).

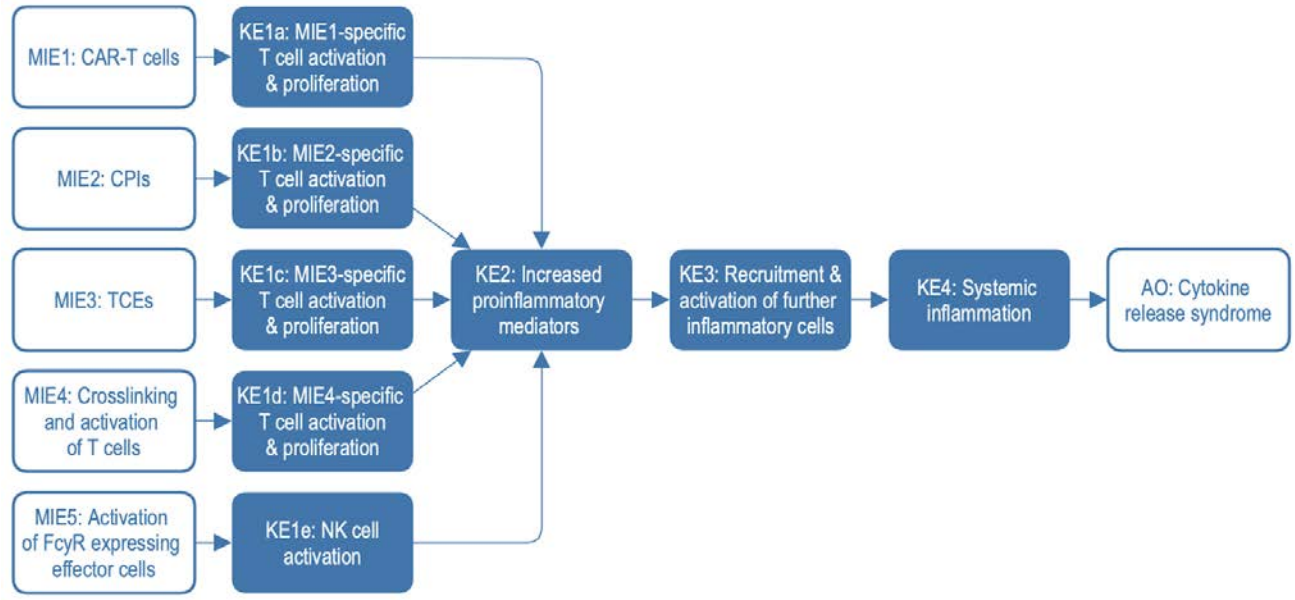


*Figure 5: Adverse outcome pathway (AOP) network with five different molecular initiating events. Abbreviations: MIE, molecular initiating event; KE, key event; AO, adverse outcome.*

Using MINERVA, we transformed the irAOP descriptions to a single interactive, machine-readable and expandable representation of molecular mechanisms describing the irAOP for CRS. This diagram, and its interconnected network version are continuously refined and are available as public demonstrator at:

| URLs of public demonstrators depicting a map and a network integrating CRS irAOPs |
| --- |
| A. CRS irAOP Map: https://imsavar.elixir-luxembourg.org/minerva/?id=CRSmap121<br><br>B. CRS irAOP network: https://imsavar.elixir-luxembourg.org/minerva/?id=crs115 |

This work has been described in a manuscript and is currently under peer review for publication and already available on Research Square (10).

Furthermore, we developed and matured three irAOPs describing the events in recombinant IL-2 treatment induced toxicities skin rash, vascular leakage and hepatotoxicity. Additionally, we developed an irAOP for CPI treatment-induced hepatotoxicity. These, too, are undergoing MINERVA representation. We started a process harmonizing these irAOPs.

To gain further insight and connections to the disease maps community, imSAVAR partners joined the 8th Disease Maps Community Meeting in Belval, Luxembourg and held the first imSAVAR symposium. Here, advanced methodologies in biomedical knowledge modeling, particularly through knowledge graphs were explored and integrated into the irAOP concept.

## 2.5 Detailed planning and conducting individual biomarker studies

**MoA CAR T**

BOEC CD19 CAR-T CRA: In an autologous co-culture assay using CD19-specific CAR-T or untransduced T cells with blood outgrowth endothelial cells from the same donor, we were able to optimize the sensitivity of relevant cytokine detection, incl. IL-6, compared to models that utilize heterogenous human umbilical cord blood endothelial cells. Furthermore, the dose-dependent killing of an antigen-expressing tumor cell line could be monitored over time. Thus, such a model can be used for efficacy and safety testing of cellular immunotherapies and could be further refined by incorporating primary samples from CAR-T patients.

MIMIC CD19 CAR-T: We were able to recapitulate essential features of CD19 CAR-T therapy in the MIMIC system of partner Sanofi. This includes B-cell aplasia as well as release of proinflammatory cytokines by CAR-T cells that was dependent on the endothelial component of the system. With such models, i.e. models incorporating endothelial cells, biomarkers that are associated with cell types beyond CAR-T can be monitored, allowing for an improved risk assessment. Results were recently published in our imSAVAR joint issue (8).

Breast-Cancer-on-Chip (ROR1 CAR-T): As outlined in section 2.3, we developed a breast-cancer-on chip-model that allows for perfusion with ROR1-specific CAR-T or untransduced T cells and subsequent transmigration of the lymphocytes into the tumor microenvironment. In this model, we were able to collect samples over 8 days from the endothelial channel for multiplex cytokine analysis. Here, we were able to detect relevant cytokines such as IL-6 only in chips that were perfused with CAR-T cells targeting our cell line-derived tumor aggregates or patient-derived tumor organoids. We further could show that the level of released cytokines correlates with the level of target expression by the tumor. Thus, such a model is able to monitor the efficacy as well as safety of cellular immunotherapies for solid tumors (bench to bedside and back translation).

Vessel-on-Chip (ROR1 CAR-T): Using supernatants from the breast cancer on chip model that was perfused with ROR1-specific CAR-T cells we were able to confirm that cytokines that were released from CAR-Ts

upon activation can trigger platelet aggregation in the vessel chip. Such a combination of models might be useful for back translation to patient real-world data sets, e.g. markers identified from our own imSAVAR patient study, and/or for mechanistic insights in e.g. KE3 within our CRS irAOP.

Based on the previously conducted literature research, that provided datasets (11,12) for characterizing adverse outcomes in CAR T cell (see analysis results provided on https://www.izbi.uni-leipzig.de/opossom-browser/), we expanded the search to datasets that provide publicly available single cell RNA sequencing data from patients that received anti CD19 CAR T cell therapy. With these data, we aimed to develop a specialized single-cell atlas for CAR T-cells, achieving a level of resolution that is required to distinguish the spectrum of transcriptional states which possibly relate to key events of the irAOPs. This atlas enables elucidating the functional fidelity and activity of CAR T-cells with the aim of defining therapeutically relevant CAR T-cells and to relate those to side effects. However, defining T-cell states poses an inherent challenge, due to technical and biological variables. Large sample sizes are needed to encompass divergence. Therefore, we collected publicly available single-cell sequencing data from approximately 250 samples of CAR T-cells, including infusion products as well as pre- and post-infusion samples, obtained from peripheral blood mononuclear cells (PBMCs) of 98 patients with Non-Hodgkin's lymphoma or acute lymphocytic leukemia. Together with other modalities such as single-cell TCR sequencing to characterize the fate of T-cells in response to therapy, we develop a unified and reliable CAR T cell reference atlas.

In addition, a CAR T cell reference atlas enables the annotation and interpretation of new datasets, for example on a cross-species level. The ability to embed query datasets in a stable reference landscape enables robust and reproducible interpretation of new samples related to curated and annotated cell identities. This also allows comparisons between different conditions such as pre- and post-treatment or mutant and wild type in a reference landscape. This methodology can improve preclinical models for the development of T-cell therapies by correlating preclinical datasets with clinical datasets. Finally, without a reliable CAR T -cell reference cell atlas, researchers have to manually annotate their datasets, which is a time-consuming process.

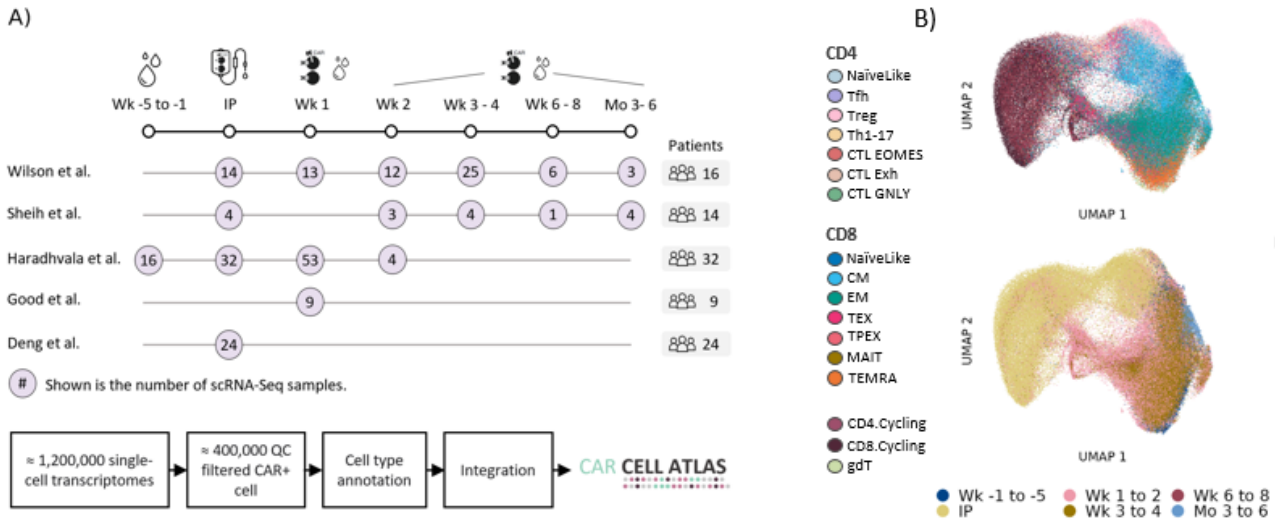


*Figure 6: (A) Overview of single-cell data sets that are integrated in the first version of the single cell CAR T cell atlas. (B) Integrated transcriptome profiles. Each dot represents a cell and is colored according to cell subtypes or states. Abbreviations: IP – infusion procedure, QC – quality control, Wk – week*

To gain a more detailed knowledge about CAR T cell therapy-mediated toxicities and identify involved cell types and molecules, we conduct studies characterizing individual patients treated with CAR T cells on cellular and molecular level and seek correlations with toxicities that emerge. With the deep knowledge gained of cellular and molecular factors involved in CAR T cell-mediated toxicities, we then can take the identified biomarkers and relevant factors from the clinical context back to appropriate *in vitro* models to achieve meaningful predictions of toxicities.

In a patient research study, we collected blood from up to now 213 patients at the UKW partner site and 60 patients at the Leipzig partner site with hematological malignancies (e.g. DLBCL, MM) that received approved CAR T cell products at several timepoints before and after treatment (see workflow figure 7). This enables us to correlate the occurrence of toxicities like CRS and cytopenias with patient characteristics present already before the treatment. We assess immune cell composition by flow cytometry, measure >30 serum cytokines and molecularly characterize the immune cell compartment by single cell RNA sequencing. In addition, the patients' clinical data are collected.

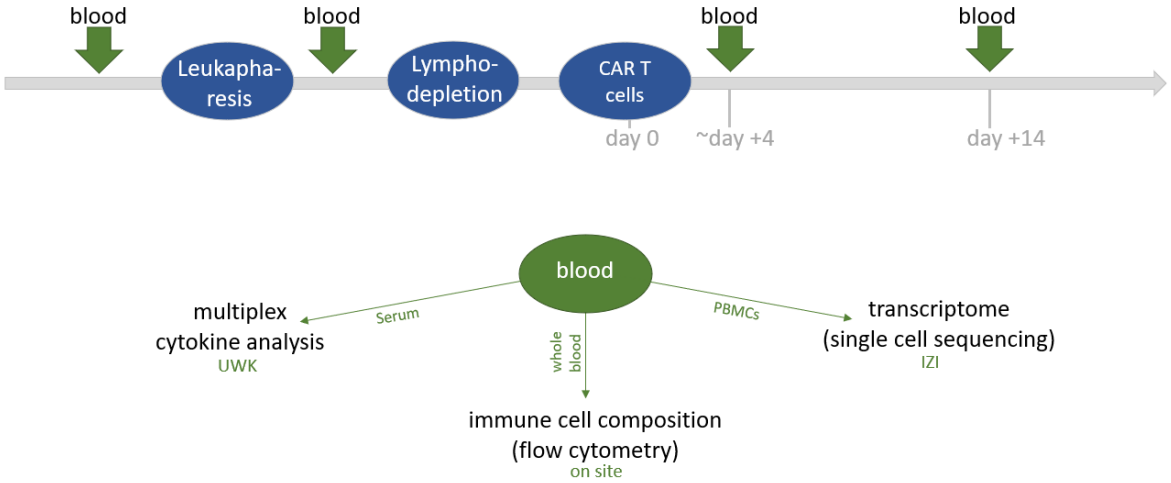


*Figure 7: Workflow of the patient study. Abbreviation: CAR – chimeric antigen receptor, PBMC – peripheral blood mononuclear cell*

Multiplex cytokine analysis was performed on 57 patients undergoing CAR-T therapy (MM/ide-cel n=33; DLBCL/axi-cel n=24) using samples collected before lymphodepletion and around day 4 after CAR-T cell infusion at the UKW partner site. Cytokine profiling revealed that both severe neutropenia, thrombopenia and anemia were highly associated with markers of endothelial dysfunction early after CAR-T infusion (d3-7). Of note, these markers also showed a strong correlation with high-grade ICANS and CRS. Further investigation revealed that patients with elevated markers of endothelial dysfunction and hyperinflammation prior to lymphodepletion were at high risk for prolonged severe neutropenias. A manuscript for publication is in preparation (Scheller et al.). Currently, we are validating these results in an independent patient cohort.

In autologous CAR T cell therapy, a bridging therapy bypasses the time from apheresis to CAR T cell administration. Using immunotherapies as bridging therapies may have implications on efficacy and safety of CAR T cell treatment, as they could target the same molecules and may lead to the same irAEs. In a patient research study we assessed the effects of BiTEs directed against B cell maturation antigen (BCMA)/CD3 (teclistamab) and G-protein coupled receptor family C group 5 member D (GPRC5D)/CD3 (talquetamab) as bridging therapy in heavily pretreated multiple myeloma patients that were to receive BCMA-directed CAR T cells. We assessed clinical outcome, the differentiation of the T cell compartment,

*in vivo* CAR-T cell expansion and *in vitro* cytotoxicity to elucidate the impact of BiTEs on CAR T cell proliferation and functionality.

Using BiTEs as bridging therapy, three out of four patients experienced low grade CRS (I and II) during bridging, but in general lower incidence of CRS after subsequent CAR T cell therapy than patients that didn't receive BiTEs as bridging therapy. Blood parameters to monitor cytokine-driven inflammation showed no differences between bridging regiments. BiTEs lead to an effective debulking of tumor mass, as shown by decreased soluble BCMA in the blood, and increased the progression free survival after CAR T cell therapy by allowing for effective T cell expansion, while not increasing T cell exhaustion markers as compared to other bridging therapies.

This study highlights the necessity to also consider sequential administration of immunotherapies in nonclinical assessment of safety, as it is already part of the clinical routine for relapsed/refractory patients. Further studies need to elucidate to which extend this needs to be represented in updated irAOPs and taken into consideration in *in vitro* models. This study has recently been published (13).

**MoA BiTEs**

The performance and reliability of in vitro models to assess irAEs after immunotherapy rely – amongst other factors – on the characteristics of the cellular donor material used. A frequent irAE after therapy with bispecific T cell engagers is Cytokine Release Syndrome (CRS). As our literature research showed that publicly available data on biomarkers predicting toxicities associated with BiTEs is sparse (D4.1), we proceeded to establish a working group to conduct a cross-consortium study assessing the variability of outcomes of cytokine release assays (CRA) depending on donor phenotypes. We aimed for identifying CRS-associated phenotypic factors that may impact susceptibility for this irAE (Figure 8). We employ different *in vitro* CRA formats in different labs to identify potentially distinct donor phenotypes that correlate with high or low responses in the *in vitro* assays. Our approach also allows for identifying CRS-dependant factors that appear across all assays and are independent of CRA format, rendering the biomarkers identified in this study highly transferable.

The models used in the study are non-standard/novel assays, provided by imSAVAR industry partners: i) the MIMIC-system, ii) blood outgrowth endothelial cell (BOEC) assay, iii) co-cultures of peripheral blood mononuclear cells (PBMC) and human umbilical vein endothelial cells (HUVEC), iv) whole blood assay. Three BiTE molecules were chosen to be tested in this study. Donors to assess were determined according to baseline phenotyping based on hematology, clinical chemistry and deep immunophenotyping of immune cells. Performing the CRAs with the selected donor material and BiTEs, cytokine and chemokine levels (and endothelial activation markers where applicable) were determined to reveal….

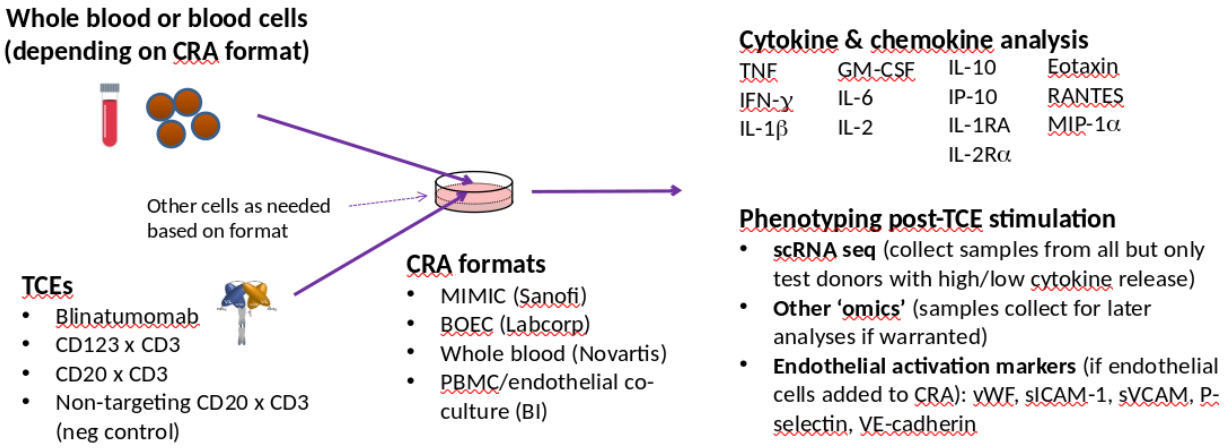


*Figure 8: Experimental design for cross-consortium systematic comparison of novel in vitro cytokine release assays. CRA – cytokine release assay, TCE – T cell engager, BOEC – blood-outgrowth endothelial cells*

In a next step, the donors identified as high and low responders will be further characterized by in-depth molecular phenotyping using single cell RNA sequencing. These data will then be correlated to the cytokine profiles acquired in the CRAs, allowing for detailed comparison of low vs. high responders. Further, the insights obtained in this study will fuel efforts to identify potential biomarkers for patient stratification translation and translating them to the clinic.

**MoA IL-2**

Vascular leakage is a severe irAE that can occur e.g., after treatment with IL-2. To gain closer understanding of this potentially lethal toxicity and to be able to reliably model this irAE *in vitro*, we established a trans-well based vascular leakage assay (figure 9, left). Using this assay, we conducted a study assessing the role of endothelial cell activation in vasculature permeability upon several stimuli (e.g., cytokines, endotoxins) in the presence or without immune cells. We were able to show that the presence of immune cells crucially determines the readout of the assay and identified stimuli leading to increased leakage (figure 9, right). In-depth analyses of the underlying mechanisms will enable biomarker development and identification of potential treatment targets.

A manuscript is submitted (Gogesch and Ortega Iannazzo et al.).

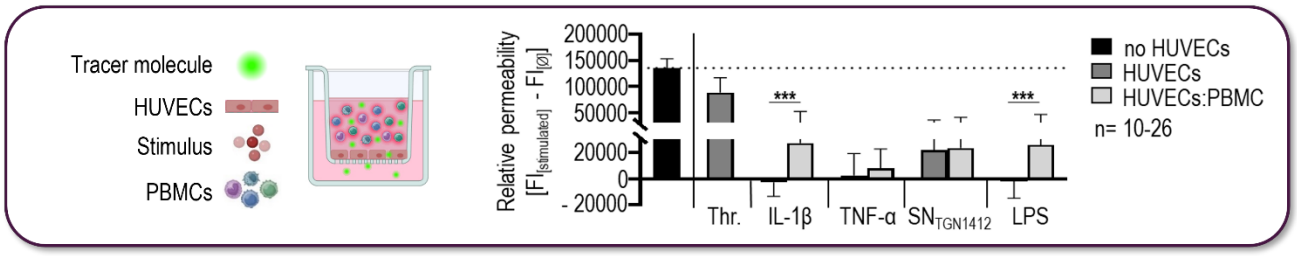


*Figure 9: Left: Assay design of HUVEC-based leakage assay. Right: Relative permeability upon different stimuli as determined in the assay. HUVEC - human umbilical vein endothelial cells, PBMC – peripheral blood mononuclear cells. Modified from Gogesch and Ortega Iannazzo et al. (submitted).*

**MoA-spanning**

The results of the literature research on sex differences in irAE manifestation after immunotherapy were presented at the annual consortium meeting in 2023. Awareness to the importance for considering the sex of patients and donors was raised throughout the consortium. The consortium agreed on documenting and providing information on the sex of the donor or patient whose biomaterial is used in an assay.

***In silico* Models**

Quantitative Systems Pharmacology/ Toxicology (QSP/T) models have been developed by imSAVAR partner Servier to model efficacy and safety of treatment with monoclonal antibodies of patients with autoimmune disease (systemic lupus erythematosus and primary Sjögren's syndrome). Also, the extensive molecular profiling of the patients required to set up the models allowed for identifying clinical subtypes further finetuning the prediction capabilities in heterogenous patient populations. Furthermore, these models provide useful information regarding dosing regimens, route and schedule of administration. QSP/T models can additionally be used to generate virtual patients supporting predictions on responses to drug candidates.

These models can be transferred to other immunotherapies and indications supporting preclinical predictions of efficacy and safety and can support the predictive capabilities of *in vitro* models developed in imSAVAR.

**Baseline/cross-species**

Within the consortium, we identified immunomics datasets for human and animals that allow characterizing immune cell baseline characteristics. These data were comprised in an immune cell atlas. To improve the utility of the data within and outside the consortium, we applied the FAIR prinicples to the dataset to render the data findable, accessable, interaperable and re-usable in collaboration with the IMI project FAIRplus. The data is provided as entry in the IMI data catalogue (https://datacatalog.elixir-luxembourg.org/develop/e/study/f85998b0-7479-11ed-a0d3-acde48001122).

We are furthermore setting up computational deconvolution methods for non-human primates (NHPs). This will enable resolving fractions of particular cell types from bulk RNA seq data for NHP samples and thus allow easier cell type identification from bulk data that are currently only available for human and mouse models. Based on the study design (figure 10), we used public and imSAVAR internal datasets to generate gene signatures for nine different immune cell types, which is a crucial part in developing the deconvolution methods. Further validation and testing of the proposed methods are ongoing.

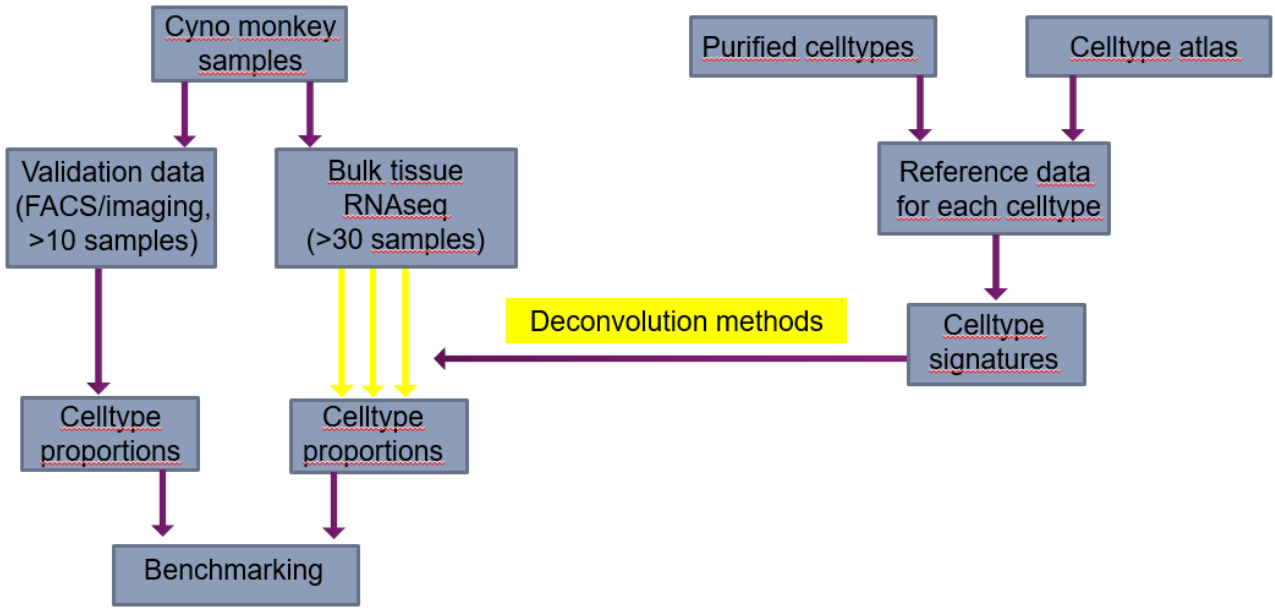


*Figure 10: Study design for development of deconvolution methods.*

In another task, we conducted a study deciphering T cell activation in a time-resolved manner. Here, human T cells were activated via CD3 and gene signatures were analyses longitudinally using next generation sequencing (NGS). This study provides general biomarker signatures robustly evaluating time-resolved T cell activation in humans. The results are published in Rade et al. (14).

**Deep learning-aided inter-species-comparison of immune response in drug development involving cynomolgus monkey:** Partner ULEI and IZI examine the efficacy and safety of therapeutic agents in the preclinical stage is integral to the early phase of drug development. Cynomolgus monkeys (Macaca fascicularis) are a commonly used animal model in preclinical studies investigating the immune system. Transferring findings from such studies to humans remains challenging. Here, we aim at identifying shared characteristics and divergences within T Cell-triggered immune response between cynomolgus monkeys and humans using deep learning and 'traditional' bioinformatics. We envision our computational workflow will support immunomodulatory therapies in drug development strategies involving animal models.

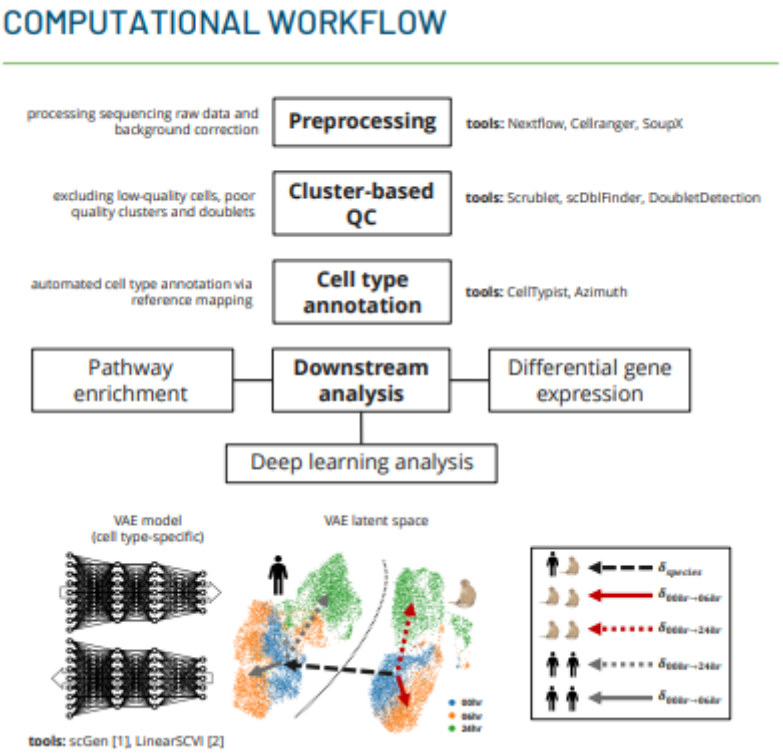


*Figure 11: Workflow of the analyses. VAE – Variational Autoencoder*

**Advancing safety models to meet upcoming deliverables D4.10, D4.11, D4.12, D4.13**

Until the end of the imSAVAR project, there are four deliverables due that build on the models we developed in imSAVAR and the results and insights we gained so far. Specifically, we will take the following actions to meet these deliverables:

***A) D4.10: BMs for determining TI and MRSD for new immunomodulatory therapeutic modalities*** *[month 67, 30/06/2025, lead: Fraunhofer, type: public report]*
*A set of biomarkers that includes imSAVAR generated confirmatory data and a defined pathway for their validation.*

***D4.11: Novel endpoints for determining mPAD and MABEL*** *[month 71, 31/10/2025, lead: Fraunhofer, type: public]*

*A set of model endpoints that includes imSAVAR generated confirmatory data and a defined pathway for their validation.*

The more complex models we developed in imSAVAR allow us to use newly determined biomarkers and endpoints, reflecting later KEs in the irAOPs of interest, to calculate dose-response curves. This enables an assessment of new biomarkers that can be used for determining TI and MRSD as well as the evaluation of novel endpoints that support the determination of mPAD and MABEL.

For these analyses, we aim to focus on the assessment of CRS induced by BiTEs, as this are the MoA and the irAOP we gained the most experience with over the course of the imSAVAR project. To assess the dose-response curves, we selected four models across the consortium: three OoCs (Tumor-on-

chip (Uni Tübingen), Neuroinflammation model (UT), Vascular Model (Dynamic42)) and one well-based model (Vascular Leakage Assay (PEI)), and will examining one or two different BiTE molecules. Samples will be provided by partner NVS.

**B)** ***D4.12: Standard for the development of immune safety assessments*** *[month 70, 30/09/2025, lead: Labcorp, type: public]*
*This standard will combine together the process and the insights and results to develop a standard for immune safety assessment. This will also include regulatory input.*

To specify and streamline the actions to be taken to meet this deliverable, the imSAVAR partners have agreed to understand "standard" as a set of recommendations that lay the path on how to go forward in developing of immune safety assessment. These recommendations can then fuel the Innovation Task Force (ITF) of the European Medicines Agency (EMA), translating them into regulation.

To compile these recommendations, we use three sources:

1. A white paper on irAOPs that is currently prepared by the consortium. Here, we elaborate on the nature and benefit of irAOPs. We see the irAOP concept to model immune-related AEs on a molecular level and align biomarkers as a beneficial concept at the basis of immune safety assessment.
2. The manuscript currently finalized for submission by Perkins et al. (see above), summarizing CRAs able to model CRS after immunotherapy with BiTEs. Here, the authors state recommendations on when to use a CRA to assess CRS in pre-clinical development of an immunotherapy and on which assay to use to achieve a meaningful readout. (lead: Labcorp)
3. A Manuscript currently prepared by imSAVAR partners with recommendations for model developers (lead: UT).

**C)** ***D4.13: Systematic comparison of non-standard/novel in vitro CRA formats incl. definition of transferable SOPs for models and biomarkers*** *[month 60, 30/11/2024, lead: NVS, type: confidential, only for members of the consortium (including Commission Services)]*
*Cross-partner study conducting systematic comparison of non-standard/novel in vitro cytokine release assay formats using tool immune cell engaging bispecific molecule*

We will conduct a cross-consortium study, generating dose-response curves for cytokine release upon stimulus with BiTEs to meet deliverables D4.10 and D4.11 (see above). Here different assays are conducted in different labs, achieving different endpoints. From this, we will derive transferable protocols.

## 3. Summary

We have advanced our knowledge on the different immunotherapies by following the MoA-specific roadmaps we defined in D4.1 using deep molecular profiling. We completed assessing the state-of-the-art by systematic reviews. We developed and advanced preclinical models and used them to generate further knowledge. All data obtained from the literature and our own work was comprised in irAOPs that we developed for the MoAs and several irAEs. We started creating harmonized irAOPs as several MoAs can lead to the same irAEs. Furthermore, we created machine-readable graphical representations to make our work accessable. On this basis, we identified knowledge gaps and conducted biomarker studies for

the different MoAs. We started a cross-consortium study comparing cytokine release assays available at the industry partner sites. Also, we created a large collecion of samples of patients that received immunotherapy (CAR T) and conducted deep molecular profiling. Using in silico models, we furthermore advanced our knowledge on baseline characteristics of the immune system in humans and non-human animals, also enabling deconvolution and cross-species comparison. Our efforts resulted in several peer-reviewed publications and mansucripts in preparation. We planned concrete actions for the remainder of the project time to meet the last, for imSAVAR very central, deliverables.

## 4. Discussion

We have advanced our knowledge on the different immunotherapies by following the MoA-specific roadmaps we defined in D4.1 using deep molecular profiling and have completed assessing the state-of-the-art by systematic reviews. On this basis we were able to developed and advance preclinical models and used them to generate further knowledge.

Comprising the data obtained from the literature and our own work allowed us to develop irAOPs for the MoAs and several irAEs, thereby structuring and contextualizing the knowledge we have gathered. This also revealed that different MoAs can lead to the same irAEs by the similar pathways. With starting to harmonize these irAOPs, we laid the foundation of a more generalized understanding of the molecular events leading to an irAE. The machine-readable graphical representations using the MINERVA platform we created makes the knowledge we obtained accessible to the scientific community.

This work also allowed us to identify knowledge gaps, which we started to fill by subsequently conducting biomarker studies for the different MoAs. The strong molecular data basis we created and continue to create with these efforts, e.g., a cross-consortium study comparing cytokine release assays available at the industry partner sites or creating a large collecion of samples of patients that received immunotherapy (CAR T) and conducting deep molecular profiling, further improves our insight in molecular mechanisms and allows us to better understand and evaluate the models we developed and utilized. All this information then feeds back into the irAOPs.

Using in silico models, we furthermore advanced our knowledge on baseline characteristics of the immune system in humans and non-human animals, also enabling deconvolution and cross-species comparison. This supports the nonclinical assessment in animal models in the development of new immunotherapies and allowes inferring the results on humans. With the CAR T cell reference atlas we created, we supply another building block for the improved understanding of molecular signatures.

## 5. Conclusion

With our substantial efforts we gained deeper molecular and mechanistic insights of the development of irAEs and not only produced new data but also established new in vitro and in silico models for the nonclinical assessment of immunotherapies, while also making the work of the individual partners available to the whole consortium. On this basis, we planned concrete actions for the remainder of the project time to meet the last, for imSAVAR very central, deliverables.

Our work resulted in several peer-reviewed publications and mansucripts in preparation.

## Abbreviations

AO – adverse outcome
BCMA – B cell maturation antigen
BiTE – bispecific T-cell engagers
BM – biomarker
BOEC – blood outgrowth endothelial cell
CAR – chimeric antigen receptor
CML – chronic myeloic leukemia
CPIs – checkpoint inhibitors
CRA – cytokine release assay
CRS – cytokine release syndrome
EMA – European Medicines Agency
GPRC5D – G-protein coupled receptor family C group 5 member D
FIH – first-in-human
HUVEC – human umbilical cord endothelial cell
irAEs – immune-related adverse events
irAOPs – immune-related adverse outcome pathways
ITF -- Innovation Task Force
KE – key event
KER – key event relationships
LION – Leipzig immune oncology
LuCE – Lung Cancer Europe
MABEL – minimum anticipated biological effect level
MIE – molecular initiating event
MoA – mode of action
mPAD – minimal pharmacological active dose
MPNE – Melanoma Patient Network Europe
MPS – microphysiological systems
MRSD – maximum recommended safe dose
NHP – non-human primates
OoC – organ-on-chip
PBMC – peripheral blood mononuclear cell
QSP/T – Quantitative Systems Pharmacology/ Toxicology
RNA-seq – RNA sequencing
SOP – standard operating procedure
TI – therapeutic index

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