**Justus-Liebig-Universität Gießen**

**Fachbereich Medizin**

Zentrum für Frauenheilkunde und Geburtsmedizin

# Entwicklung Antikörper basierter Ansätze zur spezifischen Erkennung und Behandlung von Tripel-negativen Brust- und Eierstockkrebs

„Development of Antibody-Based Approaches for Specific Detection and Treatment of Triple Negative Breast and Ovarian Cancers“

Habilitationsschrift in kumulativer Form
zur Erlangung der Lehrbefähigung für das Fach Experimentelle Molekulare Gynäkologie im Fachbereich Medizin der Justus-Liebig-Universität Gießen
vorgelegt von

Dr. rer. nat. Ahmad Fawzi Hussain

Gießen (2024)

**Bibliographische Beschreibung und Referat**

Hussain, Ahmad Fawzi
Entwicklung Antikörper basierter Ansätze zur spezifischen Erkennung und Behandlung von Tripel-negativen Brust- und Eierstockkrebs
Habilitation am Fachbereich Medizin, Zentrum für Frauenheilkunde und Geburtsmedizin der Justus-Liebig-Universität Gießen
Justus-Liebig-Universität Gießen
Habilitationsschrift in kumulativer Form, 2024, 178 Seiten, 8 Abbildungen, 1 Tabelle.

Die vorliegende Arbeit zielt darauf ab, die Methoden zur Entwicklung einer zielgerichteten Krebstherapie zu verfeinern und die Mängel der verfügbaren Therapiemodalitäten von Tripel-negativem Brustkrebs „Triple-negative breast cancer“ (TNBC) und Eierstockkrebs wie geringe Effizienz und Arzneimittelresistenz zu überwinden. Im Rahmen dieser Arbeit wurde die SNAP-Tag-Technologie verwendet, die eine schnelle und effiziente ortsspezifische Konjugation einer Vielzahl von Effektormolekülen unter physiologischen Bedingungen ermöglicht.
Die therapeutischen Strategien der Nahinfrarot-Photoimmunotherapie (NIR-PIT) und der Antikörper-Wirkstoff-Konjugat ADC-Reagenzien (in Kombination und/oder einzeln) zeigten in-vitro gegen verschiedene TNBC- und Eierstockkrebszellen starke bildgebende Eigenschaften und/oder starke therapeutische Aktivität. Trotz der vielversprechenden Ergebnisse sind weitere Experimente erforderlich, um die pharmakokinetischen Eigenschaften besser zu charakterisieren und die Bildgebung, sowie die therapeutische Aktivität in vivo zu verifizieren.
In einem weiteren Therapieansatz wurden zwei humanbasierte Immuntoxine generiert und untersucht. Sowohl in-vitro- als auch in-vivo-Ergebnisse zeigten, dass die Immuntoxine scFv-CSPG4-MAP und GbR201K-scFvEpCam (vollständiges humanes Immuntoxin) ein hohes Translationspotenzial für die gezielte Eliminierung von TNBC aufweisen.

**Stichworte:** Eierstockkrebs, Tripel-negativer Brustkrebs, Antikörper, Nahinfrarot-Photoimmunotherapie, Antikörper-Wirkstoff-Konjugat, Immuntoxin, ortsspezifische Konjugation (site-specific conjugation), SNAP-tag.

## Table of Contents

# 1. Introduction

## 1.1. Cancer

Every day, approximately 60 billion cells die in the human body, and new cells are produced to replace them. For each generated cell, the whole genetic material needs to be correctly duplicated and transferred to the new cell [1]. The cells have a highly controlled system for DNA replication, including proofreading and repair processes, which guarantee that less than one mistake occurs for each billion nucleotides during DNA replication [2]. However, mistakes occurred and lead to changes in the properties or the number of proteins involved in cell-cycle control system, this could lead to uncontrolled cell division which is a characteristic of cancer [3].

Cancer arises as a result of dynamic alteration in the genetic material of normal cells leading to transforming them into a malignant phenotype. These genetic alterations allow the cancer cells to gain function in the case of growth promoting genes, while experiencing loss of function in case of tumor suppressor genes and stability genes [4].

In general, there are more than 100 different cancer types, however six characteristic changes (hallmarks) are shared by most cancer types. These include sustaining proliferative signals, evading growth suppressors, resistance to cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis [3]. Furthermore, enabling factors such as genome instability and mutation, tumor promoting inflammation and the emerging hallmarks such as deregulating cellular energetics and avoiding immune destruction are a distinguish properties for malignant cells [4].

As a disease, cancer is the main cause of death in economically developed countries and it ranks as the second leading cause of death in the developing countries, making it the most threatened and prevalent disease worldwide. Around 19.3 million new cases and approximately 10 million death-associated with cancer were registered in 2020 worldwide. The most diagnosed cancer is the breast cancer, which is responsible for about 11.7% of total new cancer cases, followed by lung and colorectal cancers, which count 11.4% and 10% of overall cancers, respectively. Lung cancer is responsible of 18% of total death, while around 7% of mortality is caused by breast cancer [5].

In women, breast cancer comprises round quarter of all new cases, followed by colorectal (9.4%) and lung (8.4%) cancers, while ovarian cancer represents 3.4% of these cases. Although, ovarian cancer incidence is low, it is responsible of around 5% of the death in women, while 15.5% of the females' death are associated with breast cancer [6].

Despite the great and rapid developments in cancer early diagnosis and treatment, still around one in six deaths are caused by cancer worldwide, and it is one of the major obstacles for the improvment of life expectancy [7].

### 1.1.1. Triple negative breast Cancer

Female breast cancer is the leading cause of cancer worldwide, contributing to 2.3 million new cases and responsible for 1 in every 6 cancer-related deaths among women, resulting in 685,000 deaths in 2020 worldwide [8]. Based on the expression of estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2), breast cancer is classified into five molecular subtypes: luminal subtype A; luminal subtype B; basal-like subtype; HER2 positive subtype; normal breast-like subtype (Figure 1) [9, 10]. Triple negative breast cancer (TNBC) is identified by the clinical absence of ER, PgR and HER2, through immunohistochemistry and accounts for 10-20% of all breast cancer cases [11]. Even though defined by different methods, TNBC is often, but not always, equal to basal-like subtype, as most of basal-like subtypes are TNBC and about 80% of TNBC are also basal-like breast cancer [12]. TNBC shows a more aggressive biology compared to other subtypes characterized by increased likelihood of distant recurrence and death ratio [13].

Due to the heterogenicity of TNBC and the lack of ER, PgR and HER2 [14], classical receptor-targeting therapies such as tamoxifen or trastuzumab are therefore unsuitable. Consequently TNBC patients faces challenges due to limited therapeutic options including surgery and systematic chemotherapy [15].

The treatment options for TNBC patients have changed with the recent approving of the antibody–drug conjugate (ADC) Sacituzumab govitecan for treating metastatic breast cancer. This ADC is targeting the highly expressed cell surface receptor trophoblast cell surface antigen-2 (Trop-2) in TNBC [16].

Due to TNBC inter- and intratumor heterogeneity of TNBC, it is unlikely that any single targeting molecule will be efficacious for detecting or treating all TNBC patients [17].

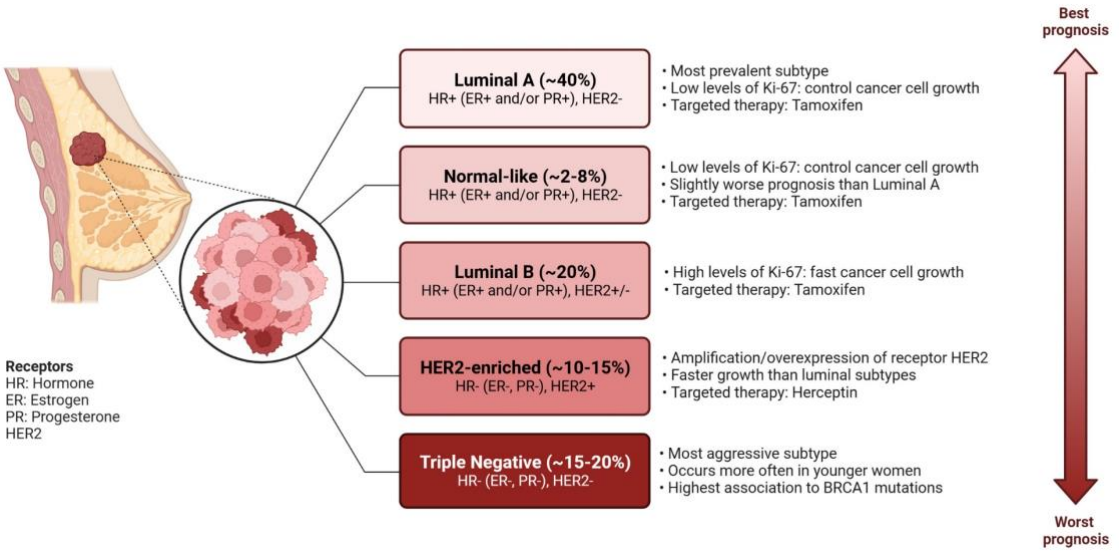


***Figure 1:*** *Five main intrinsic or molecular subtypes of breast cancer. Created with BioRender.com*

### 1.1.2. Ovarian cancer

Ovarian cancer is one of the most common gynecological tumors, with more than 200,000 new cases diagnosed each year and more than 100,000 deaths per year worldwide [18]. The gold standard therapy for ovarian cancer involves an intensive surgical cytoreduction, followed by a platinum and taxane-based cytostatic treatment [19]. Till now, no significant improvements have been achieved by adding further cytotoxic agents to this regimen [20]. However, the addition of the antiangiogenic drug bevacizumab has been shown to increase the disease-free interval [21, 22].

The fatality rate of ovarian cancer is high because these therapeutic modalities fail to cure ovarian cancer patients with advanced disease, classified in the international federation of gynecology and obstetrics (FIGO) stages II – IV (Figure 2) [23], which are representing around 75% of patients with ovarian cancer patients. For these patients, the five-year survival rates are range from 20% to 30% compared to 70% to 90% cure rates for those diagnosed when the disease is confined to the ovary (Stage I) [24].

The spreading of ovarian tumors into the peritoneum, retroperitoneal space and the serosa layer of the gut is one of the major limitations preventing the removal of residual tissues during cytoreductive surgery [25]. Patients with no residual disease have a median survival rate of ~99 months, but this survival rate decreases to ~36 months if residual tumors are persent. However, the detection of non-resectable macro-metastasis and micro-metastasis poses a significant challenge during regular surgery [26]. This emphasizing the need to develop new molecular targeting approaches for robust elimination of cancer cells and inducing long-term antitumor responses to improve the ovarian cancer patients' survival rate.

## 1.2. Cancer therapy

Although cancer is defined as a modern disease, its roots and treatment attempts can be traced back to ancient Egyptian and Greek civilizations [27]. The first documented case was around 3000 B.C, which describe a breast cancer and the surgical procedure used to remove the tumor mass [28]. Cancer has been considered as a curse and therefore treated with radical surgery and cautery. Although Hippocrates recognized cancer as a disease with biological origins around 400 B.C., its treatment methods remained limited until the end of the nineteenth century focusing on food diet, radical surgery and cautery [28].

The real changes in treating cancer started when Wilhelm Röntgen in 1895 discovered the X-Ray. This was followed by a discovery of Radium in 1898 by Marie and Pierre Curie, which was able to destroy the treated cancerous. Independently, Emil H. Grubbé has used the X-Ray for treating breast cancer in 1896, while in 1899, Anton Ultimus Sjögren used it for treating epithelioma of mouth. These experiments have paved the way for the modern radiotherapy,

which has been established by Claudius Regaud, who could minimize the adverse effects of radiation and use it for treating different types of tumors [29].

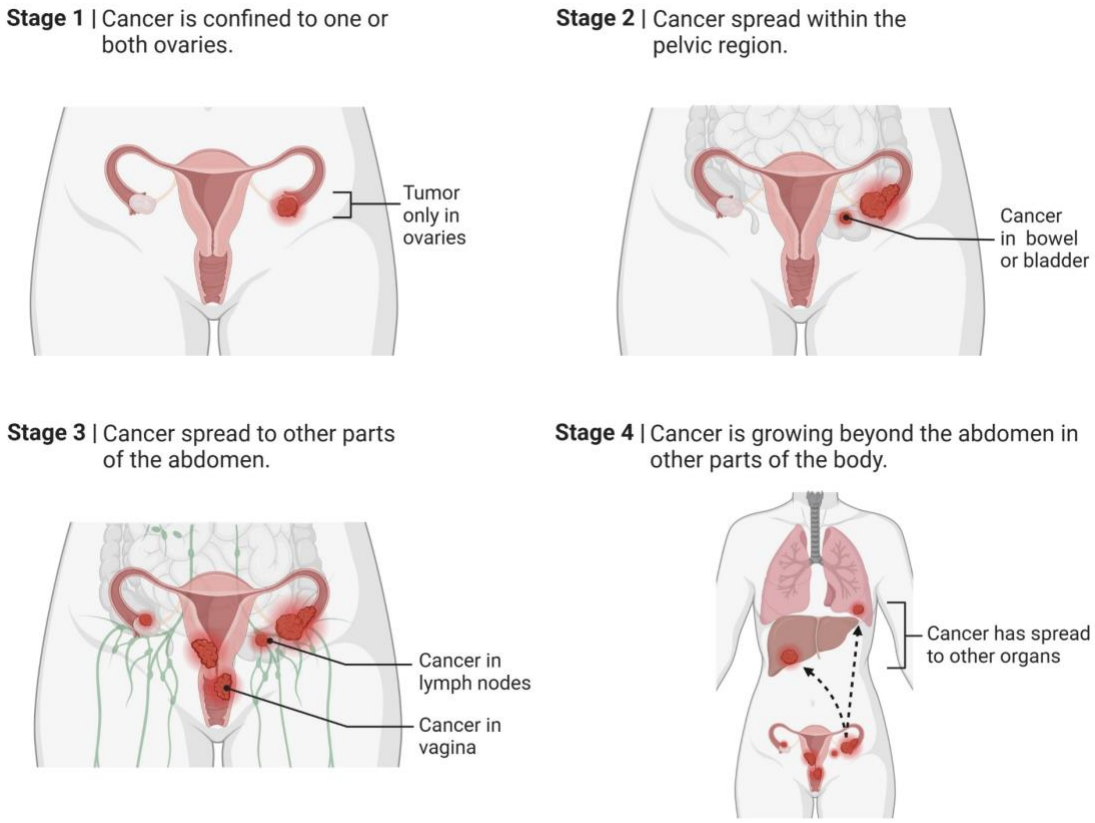


***Figure 2:*** *FIGO staging of ovarian cancer. Created with BioRender.com.*

Mid of the twentieth century, a new era of cancer therapy has started with the discovery and the use of nitrogen mustards and antifolate cytostatic agents. The cytotoxic effect of the nitrogen mustards, which has been used as a chemical weapon, was discovered accidently during the second war by observing its toxicity in the bone marrow and depletion of immune cells [30]. Nitrogen mustards are DNA alkylating agents, which bind to the guanine bases through their aziridinium group and replace chlorine to create interstrand cross-links. This prevents cell proliferation through blocking the DNA replication [31]. Mechlorethamine, first generation nitrogen mustard, was clinically used later to treat patients with lymphoid and prostate cancers. However, severe side effects were associated with this agent and therefore it is not used anymore [28].

In the same period, a different therapeutic option, called antimetabolites was investigated. This is mainly based on blocking vital cell proliferation processes or pathways such as DNA, RNA or protein synthesis using molecules that are similar to the metabolites in their structure but not functional. The first antimetabolites agent used to treat cancer patients was aminopterin,

which belongs to the antifolates family [32]. In 1947, Sidney Farber was able to inhibit tumor cell growth in patient with acute lymphoblastic leukemia after treating him with aminopterin [33].

These and other attempts have encouraged the development of different cytostatic agents, which have the ability to inhibit cell proliferation by blocking or preventing the synthesis of DNA, RNA and their metabolism. These agents can be classified into four groups: alkylating agents, antimetabolites, antibiotics, and alkaloids [34].

A new boost to the cancer treatment has occurred when Bernhard Fisher reported in 1981 that using systemic adjuvant therapy in combination with local surgery led to comparable results to radical surgery and could improve the treatment of cancer dissemination and metastasis [35].

Despite the huge improvement in surgical procedures, as well as in radiation, and chemotherapy these approaches are still associated with serious side effects during and after treatment. Therefore, significant efforts have been made to develop therapeutic modalities with no or limited impact on normal cells while exhibiting high potency and toxicity against cancer cells.

### 1.2.1. Tumor targeted therapy

Although the principle of the targeted therapy has been suggested in 1907 by Paul Ehrlich through his theory of using what he called a “magic bullet” to treat cancer cells specifically [36], the first breakthrough in this field was achieved in early ‘80s by attempting to use small molecules or macromolecules, such as monoclonal antibodies, nucleic acids and polypeptides to block different molecular targets [36].

Cancer targeted therapy can be divided into two major classes: small molecules and macromolecules. Small molecules cancer targeted therapy class is directed to kinases, DNA damage repair enzymes, epigenetic regulatory proteins and proteasomes. Although small molecules have advantages over macromolecules in some respect such as high tumor tissue penetration, pharmacokinetic (PK) properties and costs, they are associated with several limitations such as low therapeutic activity and resistance [37]. To date, there are 68 anti-cancer small molecules approved by Food and Drug Administration (FDA). They fall into seven broad subclasses: receptor tyrosine kinase inhibitors, serine / theonine kinase inhibitors, epigenetic inhibitors, B-cell leukemia/lymphoma-2 (BCL-2) inhibitors, hedgehog pathway inhibitors, proteasome inhibitors, poly-ADP ribose polymerase (PARP) inhibitors [38].

The macromolecule cancer targeted therapy class is relying on using monoclonal antibodies, polypeptides, antibody-drug conjugates, and nucleic acids to block specific pathways or indue cell death [37]. The first clinical use of targeted macromolecule was based on treating the lymphoma-associated antigen in non-Hodgkin’s (NHL) lymphoma patients using the monoclonal antibody (AB89) [39]. Although, the treated patients have not benefit significantly from this therapeutic approach, this attempt together with new discoveries and developments

in the fields of molecular biology and immunology spurred major leaps in the field of cancer targeted therapy [28].

These great efforts had led to the approval of the first monoclonal antibody (mAb) (Rituximab) targeting CD22 for treating NHL in 1997 by the FDA [40]. Subsequently, one year later, the mAb Trastuzumab targeting HER2 had been approved by FDA to treat breast cancer [41]. The mechanisms of action of these antibody-based tumor therapies, along with other 17 agents (marked in blue in Table 1), are based on the antibody natural properties. These include, blocking the pathways for tumor growth and progression as well as evoking the proteins and cells of the immune system through triggering complement dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP) mechanism (Figure 3) [42].

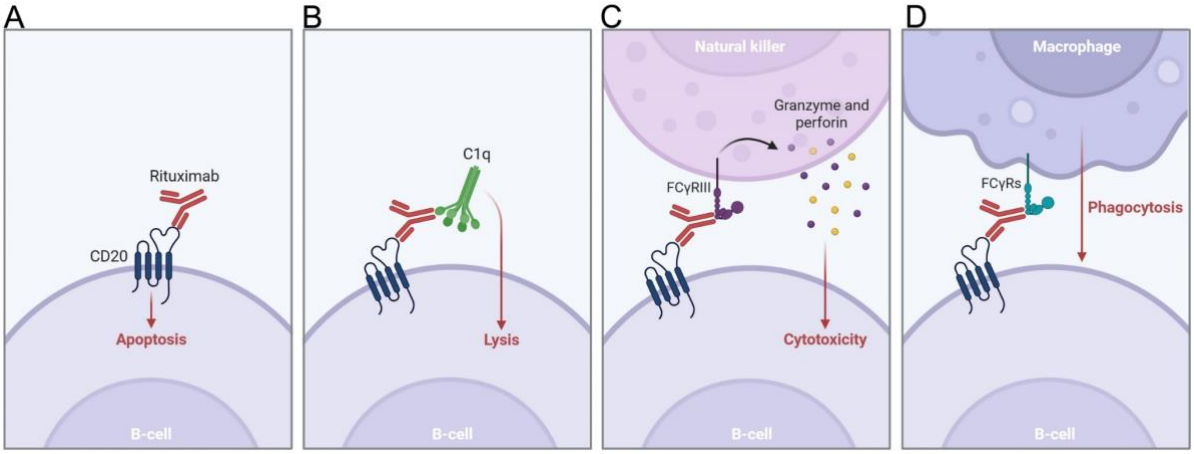


***Figure 3:*** *Therapeutic mechanisms of Rituximab against B cell lymphoma. The binding of Rituximab to the CD20 expressing B cell lymphoma induces (A) direct killing of the cells activating downstream signaling pathways, (B) CDC through the binding of C1q the Fc region of Rituximab-CD20, (C) ADCC through the binding to the FCγRIII of natural killer cells which lead to the release of granzyme and perforin, (D) ADCP by activating B cell phagocytosis due to the binding of Macrophage FCγ receptors to the Rituximab FC region. Created with BioRender.com.*

Beyond the antibody natural properties, antibodies are exploited to engage the adaptive immune system to attack cancer cells. Here, thirteen different antibodies, which have the ability to evoke the cytotoxic activity of T cells, have been approved by FDA [43]. These include modifying the transmembrane segment in T cells, which is fused to cytoplasmic signaling domain of a T cell costimulatory receptor (CD28 or 4-1BB) or the cytoplasmic signaling domain of CD3ζ of the T cell receptor (TCR) complex with extracellular antibody fragment (single-chain variable fragment; scFv), creating chimeric antigen receptor T cells (CAR-Ts) (Figure 4). At the time of writing this thesis, four CAR-Ts (Tisagenlecleucel, Axicabtagene Ciloleucel, Brexucabtagene Autoleucel, and lisocabtagene Maraleucel), which are directed to CD19, are applied to treat relapsed or refractory (r/r) B-cell acute lymphoblastic leukemia (B-ALL) or B-cell non-Hodgkin lymphoma B-NHL. Additionally, CAR-T-based therapy (idecabtagene vicleucel) targeting B-cell maturation antigen (BCMA), is available for treating r/r multiple myeloma (marked in red in Table 1) [44].

***Table 1:*** *Antibody-based therapy approved by FDA and in clinical use.*

| Name | Target | mAb format | Payload | Linker | DAR | Cancer | Approval |
|---|---|---|---|---|---|---|---|
| Rituximab (Rituxan®) | CD20 | $IgG1_K$, chimeric mouse/human | - | - | - | B-NHL, CLL | 1997 |
| Trastuzumab (Herceptin®) | HER2 | $IgG1_k$, humanized | - | - | - | Breast Stomach | 1998 |
| Ibritumomab tiuxetan (Zevalin®) | CD20 | $IgG1_k$, mouse | $^{90}Y$ | Non-cleavable | Unknown | B-NHL | 2002 |
| Cetuximab (Erbitux®) | EGFR | $IgG1_K$, chimeric mouse/human | - | - | - | Colorectal, h & n | 2004 |
| Bevacizumab (Avastin®)®) | VEGF | $IgG1_k$, humanized | - | - | - | Colorectal, lung, brain, kidney, cervical, ovarian, fallopian, peritoneal, liver | 2004 |
| Panitumumab (Vectibix®) | EGFR | $IgG2_K$, human | - | - | - | colorectal | 2006 |
| Ofatumumab (Arzerra®) | CD20 | $IgG1_K$, human | - | - | - | CLL | 2009 |
| Ipilimumab (Yervoy®) | CTLA4 | $IgG1_K$, human | - | - | - | Melanoma, kidney, MSI-H/dMMR colorectal, liver, lung, mesothelioma | 2011 |
| Brentuximab vedotin (Adcetris®) | CD30 | $IgG1_K$, chimeric mouse/human | MMAE | Cleavable | 4 (mean) | HL, T-NHL | 2011 |
| Pertuzumab (Perjeta®) | HER2 | $IgG1_k$, humanized | - | - | - | Breast | 2012 |
| Ado-trastuzumab emtansine (Kadcyla®) | HER2 | $IgG1_k$, humanized | DM1 | Non-cleavable | 3.5 (mean) | Breast | 2013 |
| Obinutuzumab (Gazyva®) | CD20 | $IgG1_k$, humanized | - | - | - | CLL, B-NHL | 2013 |
| Ramucirumab (Cyramza®) | VEGFR2 | $IgG1_k$, humanized | - | - | - | Stomach, colorectal, liver, lung | 2014 |
| Pembrolizumab (Keytruda®) | PD1 | $IgG4_k$, humanized | - | - | - | Melanoma, lung, h & n, HL, bladder, MSI-H/dMMR, stomach, cervical, B-NHL, liver, kidney, esophageal, endometrial, TMB-H, skin, breast (TNBC) | 2014 |
| Blinatumomab (Blincyto®) | CD19, CD3 | (scFv)2 (BiTE), mouse | - | - | - | B-ALL | 2014 |
| Nivolumab (Opdivo®) | PD1 | $IgG4_k$, humanized | - | - | - | Melanoma, lung, kidney, HL, h & n, bladder, MSI-H/dMMR colorectal, liver, esophageal, mesothelioma | 2014 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Dinutuximab (Unituxin®) | GD2 | IgG1κ, chimeric mouse/human | - | - | - | Neuroblastoma | 2015 |
| Daratumumab (Darzalex®) | CD38 | IgG1κ, human | - | - | - | Multiple myeloma | 2015 |
| Necitumumab (Portrazza®) | EGFR | IgG1κ, human | - | - | - | Lung | 2015 |
| Elotuzumab (Empliciti®) | SLAMF7 | IgG1κ, humanized | - | - | - | Multiple myeloma | 2015 |
| Atezolizumab (Tecentriq®) | PDL1 | IgG1κ, humanized | - | - | - | Bladder, lung, breast (TNBC), liver, melanoma | 2016 |
| Olaratumab (Lartruvo®) | PDGRFA | IgG1κ, human | - | - | - | sarcoma | 2016 |
| Avelumab (Bavencio®) | PDL1 | IgG1 λ, human | - | - | - | Merkel cell carcinoma, bladder, kidney | 2017 |
| Durvalumab (Imfinzi®) | PDL1 | IgG1κ, human | - | - | - | lung | 2017 |
| Inotuzumab ozogamicin (Besponsa®) | CD22 | IgG4κ, humanized | Calichea-micin | Cleavable | 2-6 | B-ALL | 2017 |
| Tisagenlecleucel (Kymriah®) | CD19 | scFv-based CAR-T cells, mouse T | T cell | - | - | B-ALL, B-NHL (DLBCL, FL) | 2017 |
| Gemtuzumab ozogamicin (Mylotarg®) | CD33 | IgG4κ, humanized | Calichea-micin | Cleavable | 0-6 | AML | 2017 |
| Axicabtagene ciloleucel (Yescarta®) | CD19 | scFv-based CAR-T cells, mouse T | T cell | - | - | B-NHL (DLBCL, FL) | 2017 |
| Mogamulizumab-kpkc (Poteligeo®) | CCR4 | IgG1κ, humanized | - | - | - | T-NHL | 2018 |
| Moxetumomab pasudotox-tdfk (Lumoxiti®) | CD22 | dsFv, mouse | PE | - | - | B-NHL (hairy cell leukemia) | 2018 |
| Cemiplimab-rwlc (Libtayo®) | PD1 | IgG4κ, human | - | - | - | Cutaneous squamous cell carcinoma, basal cell carcinoma, lung | 2018 |
| Polatuzumab vedotin-piiq (Polivy®) | CD79B | IgG1κ, humanized | MMAE | Cleavable | 3.5 (mean) | B-NHL (DLBCL) | 2019 |
| Enfortumab vedotin-ejfv (Padcev®) | NECTIN4 | IgG1κ, human | MMAE | Cleavable | 3.8 (mean) | Bladder | 2019 |
| Fam-trastuzumab deruxtecan-nxki (Enhertu®) | HER2 | IgG1κ, humanized | DXd | Cleavable | | Breast, stomach | 2019 |
| Isatuximab-irfc (Sarclisa®) | CD38 | IgG1κ, chimeric mouse/human | - | - | - | Multiple myeloma | 2020 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Sacituzumab govitecan-hziy (Trodelvy®) | Trop2 | IgG1$_k$, humanized | SN38 | Cleavable | 7-8 | Breast (TNBC), bladder | 2020 |
| Brexucabtagene autoleucel (Tecartus®) | CD19 | scFv-based CAR-T cells, mouse T | T cells | - | - | B-NHL (mantle cell lymphoma) | 2020 |
| Tafasitamab-cxix (Monjuvi®) | CD19 | IgG1$_k$, humanized | - | - | - | B-NHL (DLBCL) | 2020 |
| Belantamab mafodotin-blmf (Blenrep®) | BCMA | IgG1$_k$, humanized | DM1 | Non-Cleavable | 4 (mean) | Multiple myeloma | 2020 |
| Naxitamab-gqgk (Danyelza®) | GD2 | IgG1$_k$, humanized | - | - | - | Neuroblastoma | 2020 |
| Margetuximab-cmkb (Margenza®) | HER2 | IgG1$_K$, chimeric mouse/human | - | - | - | Breast 2020 | 2020 |
| Lisocabtagene maraleucel (Breyanzi®) | CD19 | scFv-based CAR-T cells, mouse T | T cells | - | - | B-NHL (DLBCL, FL) | 2021 |
| Idecabtagene vicleucel (Abecma®) | BCMA | scFv-based CAR-T cells, mouse T | T cells | - | - | Multiple myeloma | 2021 |
| Mirvetuximab Soravtansine-Gynx | FOLR1 | IgG1, humanized | DM4 | cleavable linker | 3.5 (mean) | Platinum-resistant epithelial ovarian, fallopian tube or primary peritoneal cancer | 2022 |

*The different colors represent the of mechanism of action of antibody based therapeutic agents; natural therapeutic properties of mAbs (blue), specific delivering therapeutic or imaging agents (orange), immune checkpoint blockade (green), engaging T-cell using bispecific antibodies (black), CAR-t cells (red)*

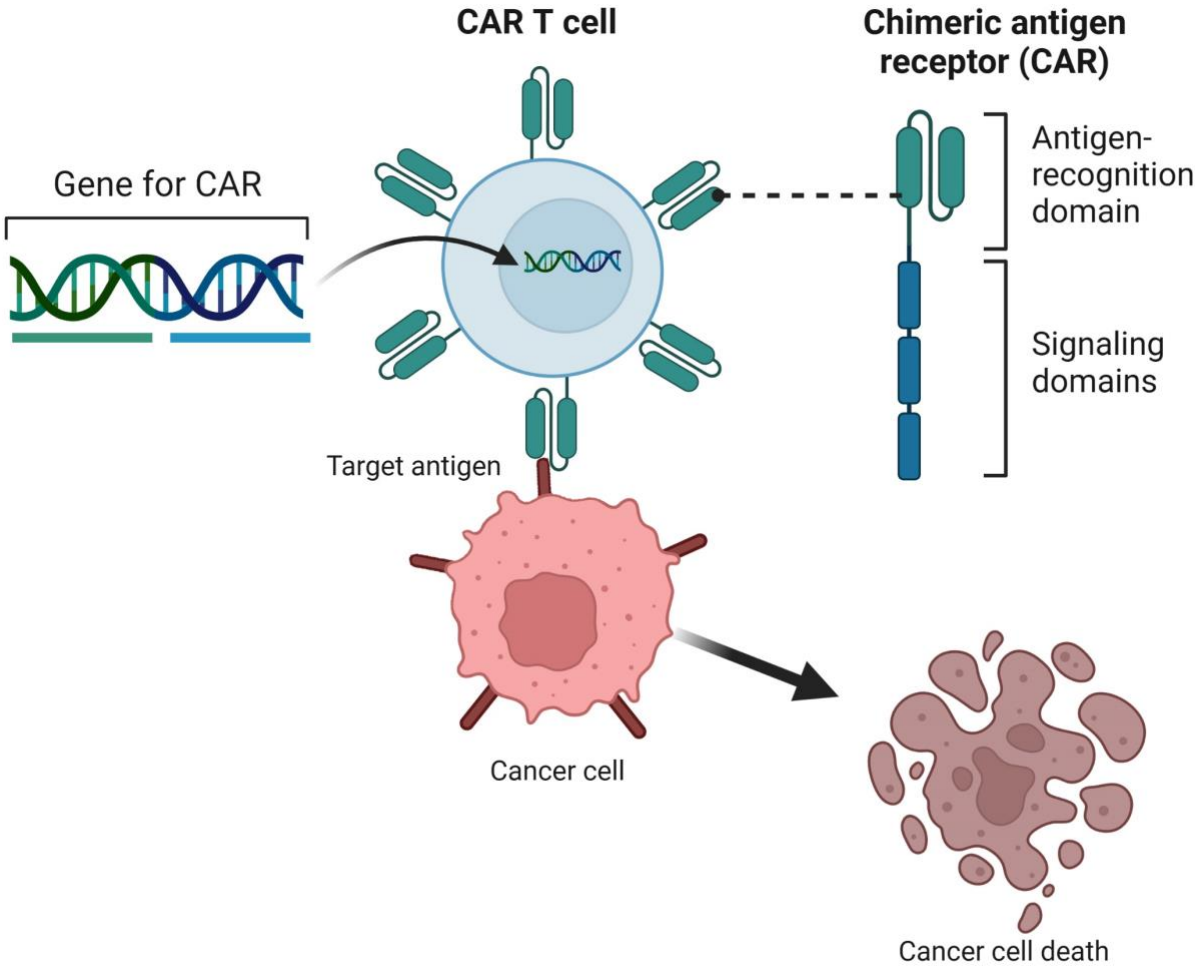


***Figure 4:*** *Schematic illustration of a CAR-T generation and therapy. Genetically modifying T cells with CAR gene composed of antigen recognition domain and signaling domains. The generated CAR-T cells can recognize targeted antigen on tumor cells leading to inducing cancer cell death.* ***Created with BioRender.com.***

Immune checkpoint inhibitors (ICIs), are part of the antibody-based therapeutic approach, and currently, seven anti-immune checkpoint antibodies are available for clinical use [45]. These antibodies have the ability to activate the T cells through targeting them directly via blocking programmed cell death protein 1 (PD1) with Pembrolizumab, Nivolumab and Cemiplimab-rwlc or cytotoxic T-lymphocyte-associated protein 4 (CTLA4) with Ipilimumab. Additionally, they target cancer cells, dendritic cells and macrophages in the tumor microenvironment by targeting programmed cell death-ligand1 (PD-L1) with Atezolizumab, Avelumab and Durvalumab (marked in green in Table 1) (Figure 5) [46].

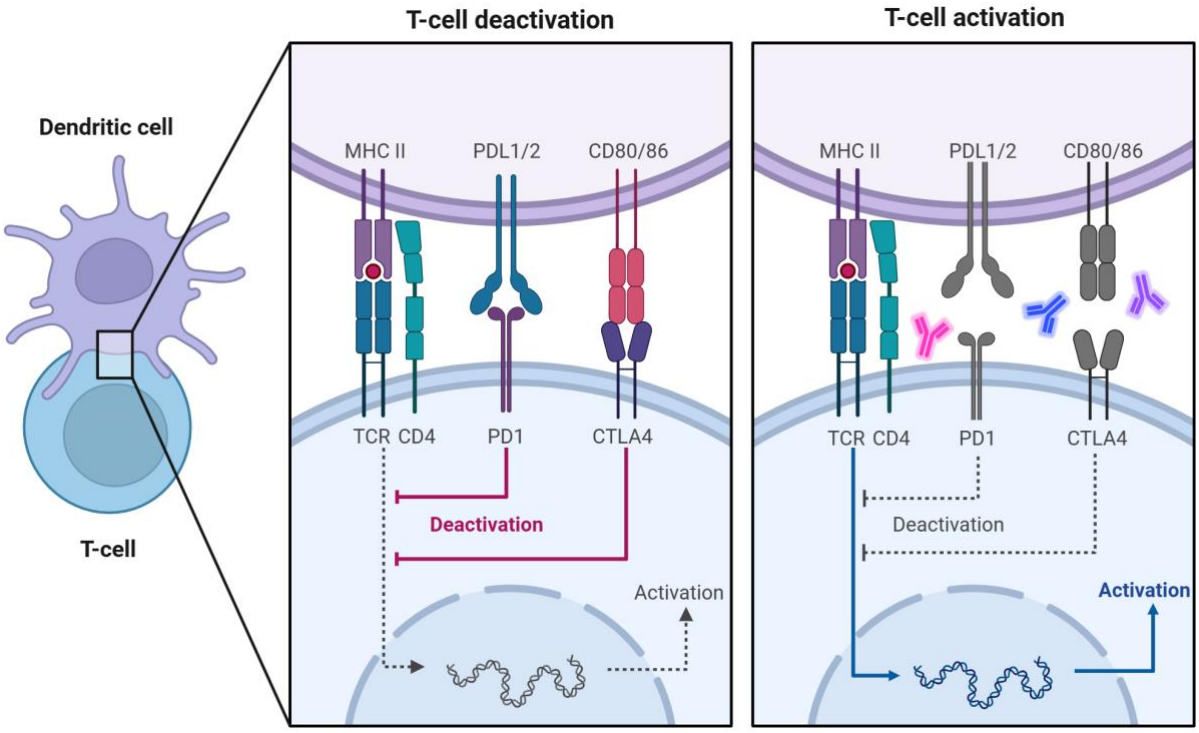


***Figure 5:*** *Schematic illustration of mechanisms of immune checkpoint inhibitors. PDL1 and CD80/86 on dendritic or tumor cells bind to PD1 and CTLA4, respectively and deactivate T cells. Blocking PDL1, PD1 and CTLA4 with Anti-PDL1 antibody (red), anti-PD1 antibody (blue) and anti-CLTA4 antibody (burble), respectively lead to activate the T cells and allows them to kill tumor cells. Created with BioRender.com.*

Another member of this antibody-based therapeutic group is the T-cell engaging bispecific antibodies (T-biAbs), which are designed to induce major histocompatibility complex (MHC)-independent recognition and destruction by bridging T cells to cancer cells [47]. Blinatumomab is the only T-biAbs approved for clinical use by FDA and it is applied for treating acute lymphocytic leukemia that bind to CD19 on malignant B cells and T-cell receptor CD3ε chain (marked in black in Table 1) (Figure 6) [48].

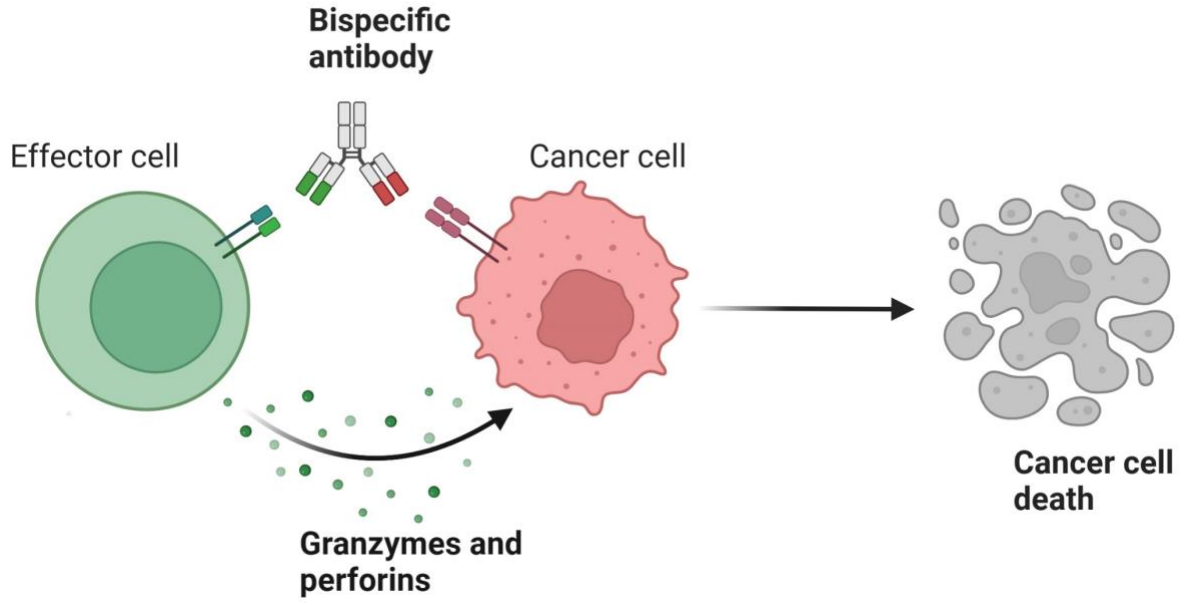

***Figure 6:*** *Schematic illustration of mechanism of action of bispecific antibody. As an example, the T-biAbs triggering T cells activation by increasing their proximity to tumor cells through cross-linking them. Created with BioRender.com.*

#### 1.2.1.1. Antibody drug conjugate (ADCs)

The combining of high antibody specificity with the therapeutic activity of powerful cytotoxic agents, constitutes a highly precise and effective class of antibody-based targeted therapy. In this class, the antibody is exploit as a transport vehicle for the highly cytotoxic agents, specifically to cancer cells expressing corresponding antigen and thereby spare healthy cells [49]. According to the molecules attached to the antibody, this class can be divided into antibody drug conjugates (ADCs), immunotoxins and radioimmunoconjugates subclasses [50].

Currently, only one radioimmunoconjugate (Ibritumomab Tiuxetan) is applied for the treatment of B-NHL. Ibritumomab Tiuxetan is generated by conjugation of the CD20-specific monoclonal antibody Ibritumomab with the chelator Tiuxetan, which allows the coupling of the radioactive isotope yttrium 90 (90Y) to the antibody (marked in orange in Table 1) [51].

Another subclass within antibody-based cytotoxic payloads delivery class is the immunotoxin, which involves fusing antibodies or antibody fragments with cytotoxic proteins. Moxetumomab Pasudotox-tdfk is the only immunotoxin approved for the treatment of hairy cell leukemia. It is a Fv fragment of anti-CD22 monoclonal antibody genetically fused to Pseudomonas Exotoxin A (PE) fragment (marked in orange in Table 1) [52].

The third class of the antibody-based cytotoxic payload delivery is the ADCs, which is the most growing class in tumor targeted therapy field. In general, ADCs are generated by bringing three component, mAb, linker and a cytotoxic payload, together in a rational design (Figure 7) [49].

The developments in the fields of chemistry and biochemistry, including mAbs modification and production, linker chemistry and design, conjugation methods as well as cytotoxic payload discovery, spurring major leaps in ADCs development [53]. At the time of writing this work, twelve ADCs have been approved by the FDA for treating patients with different types of cancers (marked in orange in Table 1) and seven ADCs are in advanced stages of clinical trials [54, 55].

Currently, there are five ADCs approved for solid tumors, these are; Ado-Trastuzumab Emtansine (Kadcyla®) approved in 2013 for treating patients with metastatic HER2-positive breast cancer that have received trastuzumab or taxane previously, Sacituzumab Govitecan-hziy (Trodelvy®) approved in 2020 as a second-line treatment for advanced or metastatic trophoblastic cell-surface antigen-2 (Trop-2)-positive breast cancer (TNBC), Tisotumab Vedotin approved in 2021 for the treatment of recurrent or metastatic tissue factor (TF)-positive cervical cancer patients, Mirvetuximab Soravtansine-gynx approved in 2022 for treatment of patients with folate receptor-α (FRα)-positive and platinum-resistant ovarian cancer, in 2022 Fam-Trastuzumab Deruxtecan-nxki (Enhertu) approved for adult patients with unresectable or metastatic HER2-low breast cancer, Enfortumab Vedotin-ejfv (Padcev) approved in 2023 for

treating patients with locally advanced or metastatic urothelial cancer in combination with pembrolizumab [56, 57].

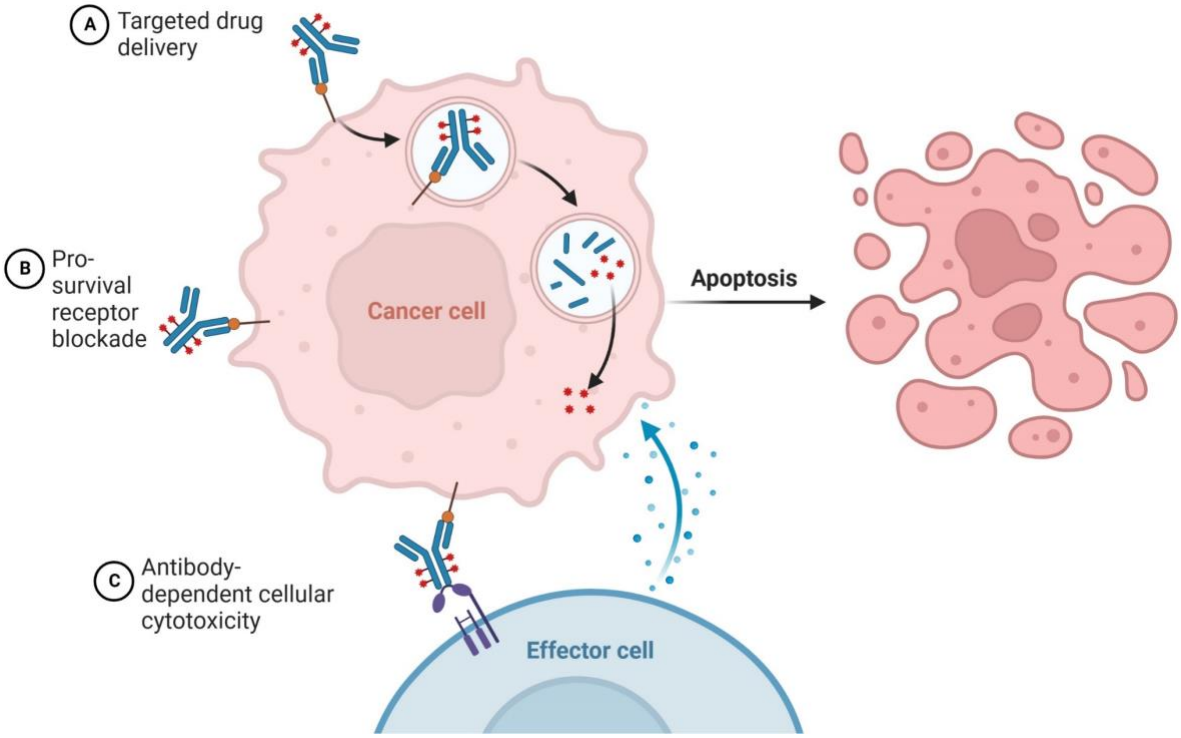


***Figure 7:*** *Schematic illustration of mechanism of action of ADC. (A) After binding to the targeted antigen, the ADC will be internalized into the cells and the payload will be released from the lysosomes into the cytoplasm and finally induce tumor cell death. Due to the natural properties of antibody, ADCs could also induce tumor cell death through blocking tumor growth and progression pathways (B) or triggering ADCC (C). Created with BioRender.com.*

The most frequently used toxic agents in approved ADCs are Auristatins including monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF) and they account for six out of thirteen of the ADCs in the clinical use [43].

Recently, we have conjugated the monomethyl auristatin E (MMAE) to recombinant antibody fragments targeting the tumor associated antigens epidermal growth factor receptor 1 (EGFR) and epithelial cell adhesion molecule (EpCam). These ADCs were able to kill TNBC cells expressing the corresponding antigen through inducing cell apoptosis at a nanomolar concentration [58].

Furthermore, near-infrared photoimmunotherapy (NIR-PIT) approach has been conditionally approved by the Pharmaceuticals and Medical Devices Agency in Japan for the treatment of recurrent head and neck squamous cell carcinoma (HNSCC). This approach based on arming EGFR specific mAbs (cetuximab) with the highly potent near-infrared (NIR) photosensitizer IRDye700 agent, which stay nontoxic till it is activated with NIR light at 690 nm. This causes the release of hydrophilic side chains of IRDye700 and generating an extremely hydrophobic side chain. Finally, the nearby cell membrane become damaged, causes water stream into the cell and lead to cell death [59].

In the last years, we have conjugated the IRDye700 with different cell-surface receptors specific recombinant antibody fragments, including EGFR, EpCam and chondroitin sulfate proteoglycan 4 (CSPG4). The specific imaging and therapeutic properties of these NIR-PIT agents were determined individually and in combination against different tumor entities expressing different levels of targeted cell-surface receptors [60, 61].

As an example, the viability of five different TNBC cell lines, expressing different levels of EGFR, EpCAM and CSPG4 were reduced with 50% elimination of the cells ($IC_{50}$) range from 62 to 165 nM of individual NIR-PIT agents, while treating the same cells with a combination of all three reagents improve their therapeutic activity by up to 40%. Furthermore, our studies show that treating cells with each of NIR-PIT approach elements (recombinant antibody, IRDye700 or the NIR-light) alone has generated minimal toxicity and the maximal treatment response can reached only when all the elements directed together to the appropriate cells simultaneously [60].

#### 1.2.1.2. Methods for conjugating therapeutic payload

To arm mAb with the therapeutic payloads, high potent drug molecules need to be conjugated to the mAb in most cases using an appropriate linker. To this background, different conjugation methods have been developed to create the ADCs and PIT reagents. These methods can be divided generally to random and site-specific conjugation methods.

##### 1.2.1.2.1. Random conjugation methods

Random conjugation methods are the most used methods to generate ADCs and PIT reagents and the first generation of clinically approved ADCs and PIT agents have been created using these methods. These methods are well established and easy to use, however, they lead to generate heterogeneous product with different pharmacological properties. This is mainly because they are relying on attaching the therapeutic molecules to the mAbs by directly functionalization of the highly abundance lysine or cysteine residues. Using both conjugation methods make it difficult to determine the conjugation site in the mAb and number of attached molecules to each mAb [62].

Recently approved ADCs famtrastuzumab deruxtecan-nxki (Enhertu® ) and sacituzumab govitecan-hziy (Trodelvy® ) were generated by conjugating the therapeutic payloads to all hinge cysteine residues involved in interchain disulfide bridges in the IgG molecule to generate a uniform drug-to-antibody ratios of 8 [63, 64]. However, the optimal DAR for the ADCs is 2 and therefore, several site-specific conjugation methods are developed and used to generate the Next-generation ADCs [65].

#### 1.2.1.2.2. Site-specific conjugation methods

Site-specific conjugation methods represent ideal approaches to overcome the limitations of random conjugation techniques and produce nearly uniform products with a defined DAR through conjugating a certain number of therapeutic molecules to specific sites in the antibody molecule. Currently, several methods have been established, these include cysteine-conjugation, glycol-conjugation, integrating unnatural or non-canonical amino acids, and enzyme-based conjugation methods [66].

The best studied methods here are the cysteine-based conjugation strategies THIOMAB and SELENOMAB. In THIOMAB method, cysteine residues are added at specific positions in the heavy or light chains of antibody. These unnaturally occurring cysteine residues are used later to conjugate the therapeutic molecules and keeping the structural disulfide bonds unaffected. The SELENOMAB conjugation strategy is based on introducing selenocysteine residues during protein translation, this allows the specific coupling of methysulfone-modified therapeutic molecules with near-uniform stoichiometry. Also, the controlled disulfide rebridging conjugation method, which rely on conjugating the therapeutic agent to two cysteine residues, retain the antibody structure stability [67].

Furthermore, different enzyme-based conjugation methods relying on coupling the therapeutic molecules enzymatically to a certain amino acid sequence with a define DAR have been recently established.

One of these enzymes is the *Staphylococcus aureus* Sortase A, which has the ability to recognize the LPXTG peptide sequence motif, specifically cleave the amine bond between the threonine and forming a new amide bond with the N-terminal of oligoglycine-modified molecule through an acyl-enzyme intermediate. Different fusion proteins including recombinant antibodies have been site-specifically modified with therapeutic or imaging agents using this method [68].

Another controlled enzymatic conjugation method is the O6-Alkylguanine-DNA alkyltransferase (AGT), also known as SNAP-tag, method. This method relies on genetically fusing the SNAP-tag to the recombinant antibody to allow the specific conjugation of O(6)-benzylguanine (BG)-modified molecules by forming a covalent thioether bond between the reactive cysteine in SNAP-tag and the alkyl group in the BG-modified molecules [69].

The SNAP-tag technology represents a simple, effective, specific and rapid conjugation method that results in more than 80% conjugation efficiency with the BG-modified molecule after 1 h incubation at room temperature in common physiological solution without any activation or reduction steps [70]. Due to the intrinsically monovalent and high specific conjugation properties of the SNAP-tag, this method generates a high homogeneous product. Furthermore, it is expected that SNAP-tag protein associated with low immunogenicity as the SNAP-tag is a modified human protein. These properties and the availability of broad range of

BG modified molecules and building blocks make SNAP-tag conjugation method one of the most promising site-specific conjugation strategies [71].

In the last years, we have exploited the highly efficient, specific, and monovalent conjugation properties of the SNAP-tag to conjugate BG-modified imaging agents, nanocarriers and therapeutic molecules to a set of recombinant antibody fragments targeting cancer cell-surface receptors. This method allows us to generate a homogenized tumor specific therapeutic and imaging agents with uniform pharmaceutical properties. One class of these targeted reagents is the NIR-PIT agents, which involve the conjugation of on demand and locally activatable IRDye700 agent to different cell-surface receptors specific recombinant antibody fragments using SNAP-tag conjugation method. The developed NIR-PIT reagents targeting EGFR, EpCAM and CSPG4 in different solid tumor types showed specific imaging properties and highly phototherapeutic toxicity *in vitro*. This approach could be used to select best fit therapy for each patient, to facilitate optical-image guided surgery, and in situ or on-demand drug activation as well as real-time treatment monitoring [61, 71-76].

Another class of targeted reagents that we have developed is ADCs for specific treating of TNBC cells by targeting EGFR and EpCam [58]. Here, BG-modified MMAE was conjugated to the above-mentioned SNAP-tag recombinant antibodies [58]. The specific binding and internalization activities of scFv-EGFR-SNAP and scFv-EpCam-SNAP based ADCs were confirmed using flow cytometry analysis and fluorescence microscopy. These ADCs were able to reduce the cell viability and induce cell apoptosis in the cells expressing the targeted cell-surface receptors at a nanomolar concentration in a dose-dependent manner. This study revealed that scFv-EGFR-SNAP and scFv-EpCam-SNAP based DACs represent promising targeting reagents for treating TNBC [58].

#### 1.2.1.2.3. Dual labeling of therapeutic antibodies

Equipping antibodies with two or more different effector molecules has been emerged as a promising approach for detecting and treating cancer cells simultaneously, or for overcoming cancer cells resistance to ADC therapies. One of the advantages of dual labeled antibodies is their ability to precisely map the ADC biodistribution and determine the side effects of off targeting effect in preclinical and clinical studies [77].

Here for example, indocyanine green (ICG)-sulfo-OSu and [111In] using the chelator diethylene triamine penta-acetic acid (DTPA) were conjugated to panitumumab (targeting EGFR) and trastuzumab (targeting HER2) and used to investigate molecular targeting by optical imaging (detecting ICG signal) determining the antibodies biodistribution and tumor accumulation by determining the radioactivity of 111In [78]. In another approach, trastuzumab, HER2 specific antibody, was labeled with IRDye 800 and [111In]-DTPA to study its imaging properties for detecting metastatic tissues in breast cancer patients [79]. The dual labeled

antibodies in these studies were generated using random conjugation methods, which lacks the specificity of site-specific labeling and is not associated with uniform DAR [78, 79].

Rijpkema, et al have investigate the intraoperative imaging potential of three antibodies (anti-carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5); MN-14; anti-carbonic anhydrase IX (CAIX); girentuximab; and anti-EGFR; cetuximab) labeled with both 111In through the chelator DTPA and IRDYe 800CW at different DARs (of 0, 1, 1.5, 2, and 3). The results of *in vivo* biodistribution and imaging experiments showed that antibody with DARs of 1−1.5 have the optimal imaging properties. Antibodies with lower or higher DARs tended to accumulate to unfavorably levels in the liver or inadequately in the tumor [80]. However, the optimal design of dual labeled conjugation is antibody dependent and need to be investigated for each dual-labeled antibody individually.

Moreover, pharmacokinetic properties, safety and therapeutic activity of antibody conjugates are relying on the conjugation sites of the effector molecules [81]. Therefore, several attempts have been made to generate dual labeled antibody with uniform pharmacokinetic, therapeutic and safety properties using site-specific conjugation methods.

One of these attempts is relying on the THIOMAB and SELENOMAB technology, which allows the attachment of iodoacetamide and methylsulfonemodified effector molecules to engineeringed of cysteine and selenocysteine residues at specific sites in the antibody, respectively, with near uniform stoichiometry [82, 83]. Another attempt exploits orthogonal conjugation methods based on transglutaminase and engineered cysteine, respectively. Here, Puthenveetil et al., used transglutaminase-mediated conjugation to modify the glutamine 295 with strained alkyne (BCN) in trastuzumab heavy chain, which is finally conjugated with Cy5.5 azide. The cysteine engineered residue at position 183 in trastuzumab light chain was conjugated with BODIPY TMR C5-maleimide [84].

Using controlled disulfide rebridging method, Wang et al., were able to site-specifically dual label proteins with water-soluble allyl sulfones. This method has several advantages over techniques relying on reduced cysteine residues. It maintains the stability of the antibody structure through the rebridging of disulfide bonds and its water solubility and reactivity enhance its application [85]. Moreover, non-amino acid–based conjugation methods such as glycoengineering allow the coupling of effector molecules to for example N297 glycans located in the CH2 domain [66].

Although the mentioned methods can generate nearly homogeneous antibody dual labeled products with high conjugation efficiency, they are associated with complicated production process and high costs. This is mainly due to the requirement of multiplex reaction and purification steps. The SNAP-tag and CLIP-tag simultaneous and one-step dual site-specific conjugation method overcome the limitation of other methods such as the need for separate conjugation steps, reduction steps or buffer exchange. This is mainly because the intrinsic

monovalent conjugation properties of both SNAP-tag and CLIP-tag, which allow a rapid one-step reaction, site-directed and autocatalytic labeling under physiological conditions with a uniform DAR [76].

#### 1.2.1.3. Human cytolytic fusion protein-based immunotoxin for treating breast cancer

Immunotoxins are chimeric proteins composed of a targeting moiety, generally full antibodies, antibody fragments, peptides, or growth factors, linked to toxin moiety, bacterial or plant toxic proteins or human cytolytic proteins [86]. Ideally, immunotoxin need to bind the targeted cell surface antigen, which allows the internalization of the immunotoxin via receptor mediated endocytosis, and finally induce cell death by inhibiting protein synthesis or triggering specific cell apoptosis pathways enzymatically [87].

Although, immunotoxins show high therapeutic activities in preclinical and early clinical experiments, they are associated with several limitations such as nonspecific toxicity, limited tissue penetration, heterogeneous composition and high immunogenicity [88].

Recombinant DNA techniques have been used to design and refine the immunotoxins. Most of the developed immunotoxins are relying on using bacterial toxins, such as diphtheria toxin (DT), PE or plant toxins, such as ricin and gelonin. These toxins have a binding domain that allow them to bind to the cells nonspecifically [89]. To overcome this shortcoming, the toxin binding domain was deleted to prevent its binding to normal cells. To increase tissue penetration of mAb-based immunotoxins, antibody fragments such as scFv (MW ≈ 25 kDa) or single domain antibody (MW ≈ 12 kDa) were used to generate the immunotoxins. This reduction in immunotoxin size not only increases its penetration rate, but also decreases the immunogenicity of the targeting domain, especially in case of murine antibodies, as scFv lack the antibody Fc region. Furthermore, scFv fragments from humanized or human antibodies were used to further reduce the targeting domain immunogenicity [63]. Another reason for high immunogenicity is the plant or bacterial-based toxin domain. Here, human cytolytic proteins have been used, due to their low immunogenicity and higher tumor selectivity. Different types of human cytolytic proteins, such as granzyme B (Gb), ribonucleases (RNases) or microtubule-associated protein were used for developing human cytolytic fusion proteins [90].

Our work aimed at refining immunotoxins for treating TNBC. We have used scFv fragments targeting two highly expressed TNBC cell receptors EpCam and CSPG4 and two human cytolytic proteins: Gb and microtubule-associated protein tau (MAP). Both the Gb and MAP proteins were genetically modified to improve their therapeutic activity. Naturally, Gb produced by cytotoxic T cells and natural killer cells as an immune response to combat viral infected and cancer cells through activating multiple apoptotic pathways directly processing caspases −3, −4, −8, and −10. However, several cancer cells have the ability to irreversibly inhibit Gb by upregulating the expression of its natural inhibitor serpin B9 (PI-9) [91]. Therefore, we have used

the mutant version of granzyme B (R201K) with strongly reduced sensitivity toward PI-9 to generate the full human immunotoxin GbR201K-scFvEpCam.

MAP protein has several phosphorylation sites that lead to release of tau from tubulin, therefore we have used the genetically modified MAP, with two depletion of phosphorylation sites (S156 and S204), with increased activity of binding and stabilizing of spindle microtubules [92]. This mutated MAP protein was used to generate scFv-CSPG4-MAP cytolytic fusion protein. The therapeutic activities of both cytolytic fusion proteins were investigated both *in vitro* and *in vivo*. The data represented in this work revealed that both GbR201K-scFvEpCam and scFv-CSPG4-MAP recombinant immunotoxins efficiently and specifically target counterpart cell surface receptors and induce cell death in different TNBC cell lines *in vitro*. Furthermore, the specific accumulation of these recombinant immunotoxins was confirmed in human TNBC xenograft mice model resulting in significant tumor regression [93, 94].

## 2. Discussion

The recent global cancer statistical studies show that breast and ovarian cancers represent a serious threat for females´ live and huge challenges to healthcare systems. Yearly, around 2.3 million new cases of breast cancer are diagnosed worldwide, accounting for 24.5% of all new cancer cases in females. Furthermore, it is the responsible for 15.5% of female cancer deaths. Although the incidence of ovarian cancer is not high (313,959 cases; 3,4%), its mortality-to-incidence ratio exceeds 0.6, contributing to 207,252 (4.7%) female deaths annually [5]. Furthermore, 1 from 6 patients with ovarian cancer die within the first 90 days of diagnosis [95].

In the last three decades, cancer detection and treatments methods have evolved significantly. This led to a 20% increase in the 5-year relative survival rate of cancer patients. The breast cancer collectively has a 5-year survival rate of 85% [96]. However, breast cancer is a very heterogeneous disease, which is classified based on genomic studies into five major subtypes: luminal A, luminal B, HER2-positive, normal-like and triple-negative [9, 10]. The best therapeutic outcomes are observed in most frequent breast cancer subtype luminal A-like with 95.1% overall survival rate, while the lowest overall survival rate (78.5 %) was observed in patients with the aggressive subtype TNBC [97].

Despite improvements in cancer diagnostics and therapy, the benefits for ovarian cancer patients is remain limited. The 5-years survival rates do not exceed 47% even in highly developed healthcare systems such as in the United States and Canada [95]. The limited improvements in 5-years survival rates is attributed to lack of specific symptoms and effective screening methods. Therefore, the majority (75%) of ovarian cancer cases are diagnosed with in stage III/IV with only 33% reaching a complete resection status [98].

Beside the established treatment approaches of breast and ovarian cancers, such as surgery and systemic chemotherapy, extensive research has shown that targeted therapy can improve patient overall survival rates and quality of life. This is because targeted therapies have a large therapeutic window with optimal therapeutic index [99].

One of the most promising targeted therapies is based on using antibody to specifically deliver therapeutic agents to cancer cells, particularly the ADC-based therapeutic approaches [49]. Currently, two ADCs, ado-trastuzumab emtansine (Kadcyla®), trastuzumab deruxtecan (Enhertu®), are used to treat patients with HER2 positive breast cancer and sacituzumab govitecan (Trodelvy®) has been approved by FDA in February 2023 for treating Trop-2 positive TNBC patients. For ovarian cancer, only one ADC (Mirvetuximab Soravtansine-gynx) has been approved in November 2022 for treating FRα positive ovarian cancer patients [56]. However, TNBC and ovarian cancer are a highly heterogeneous diseases including various subtypes with different histopathological features and clinical behaviors leading to different therapeutic outcome of surgery and/or chemotherapy and associated with inherent and acquired drug

resistance [17, 95]. This emphasizing the need to develop new targeted agents and targeting approaches to overcome the inter- and intra-tumor heterogeneity of TNBC and ovarian cancer. The aims of this work are to generate different targeting agents directed to different breast and ovarian cancer-associated antigens with different mechanisms of action to overcome breast and ovarian cancer heterogeneity and drug resistance. Here, three targeted therapeutic approaches; NIR-PIT [60, 61, 71, 72, 74-76], ADC [58], and immunotoxin [93, 94] were used to eliminate TNBC and ovarian cancer cells by targeting three highly expressed cell surface receptor (EGFR, EpCam and CSPG4) on TNBC and ovarian cancer cells .

The different mechanism of action can overcome the limitation of cancer cell-acquired and inherited drug resistance of TNBC and ovarian cancer, while targeting a set of three cell surface receptor can overcome the inter- and intratumor heterogeneity of TNBC and ovarian cancer.

The impact of developing antibody-based recombinant proteins, the use of SNAP-Tag technology for conjugating effector molecules, targeting three cell surface receptors (EGFR, EpCam and CSPG4) individually or in combination and the exploiting of different therapeutic strategies (NIR-PIT, ADC, and immunotoxin) for targeting TNBC and ovarian cancer were applied in the present work and the outcomes are discussed below.

## 2.1. Designing, Expression and Purification of Recombinant antibody-SNAP-tag fusion proteins

- **Hussain, A. F.**; Kampmeier, F.; von Felbert, V.; Merk ,H. F.; Tur, M. K.; Barth, S. SNAP-tag technology mediates site specific conjugation of antibody fragments with a photosensitizer and improves target specific phototoxicity in tumor cells. *Bioconjug Chem* **2011**, *22* (12), 2487-2495.
- von Felbert, V.; Bauerschlag, D.; Maass, N.; Bräutigam, K.; Meinhold-Heerlein, I.; Woitok, M.; Barth, S.; **Hussain, A. F.** A specific photoimmunotheranostics agent to detect and eliminate skin cancer cells expressing EGFR. J Cancer Res Clin Oncol **2016**, 142 (5), 1003-1011.
- Chouman, K.; Woitok, M.; Mladenov, R.; Kessler, C.; Weinhold, E.; Hanz, G.; Fischer, R.; Meinhold-Heerlein, I.; Bleilevens, A.; Gresch, G.; Haugg, A. M.; Zeppernick, F.; Bauerschlag, D.; Maass, N.; Stickeler, E.; Kolberg, K.; **Hussain, A. F.** Fine Tuning Antibody Conjugation Methods using SNAP-tag Technology. *Anticancer Agents Med Chem* **2017**, *17.*
- **Hussain, A. F.**; Heppenstall, P. A.; Kampmeier, F.; Meinhold-Heerlein, I.; Barth, S. One-step site-specific antibody fragment auto-conjugation using SNAP-tag technology. *Nat Protoc* **2019**, *14*, 3101-3125.

In this work, different recombinant antibodies to be used for cancer detection or therapy were designed, produced, and validated. These minimized recombinant antibodies composed of

two main domains: the binding domain (scFv fragments) and the site-specific conjugation domain (SNAP-tag).

The scFv composed of only the variable domains of the heavy chain (VH) and light chain (VL) of an antibody connected via a peptide linker, and therefore it is the smallest antibody format with full antigen-binding properties. Its small size (typically 27 kDa) and availability of different genetic engineering modifications make it favored in different diagnostic and therapeutic approaches [100]. However, the use of scFv-based fusion proteins as replacements for full-length mAbs in cancer treatment remains a subject of ongoing debate. The key advantages of using scFv-based recombinant proteins include higher tumor tissue penetration due to their small molecular weight, rapid blood clearance rate because proteins up to 45 kDa are rapidly filtered in the glomeruli [101], reduced immunogenicity due to the lack of the Fc region that leads to activate the host's immune system through triggering immune cells and antibody-dependent pathways such as ADCC, ADCP, CDC [102]. Furthermore, in comparison to the parental antibody, scFv fragment can be easily engineered, modified, produced quickly at lower costs in a higher yields using different expressing systems such as bacterial, plant, insect or mammalian expression systems [103].

Although different kind of peptide linker were established for generating scFvs, the 15-amino acid $(GGGGS)_3$ linker, who provide a 35-Å distance variable domains without effecting the conformation of the antigen binding site, was used in our work for constructing all scFvs due to its flexible nature and *in vitro* and *in vivo* properties [104]. To generate the scFvs the DNA sequence of the VH and VL of antibodies targeting EGFR, EpCam and CSPG4 were connected via the DNA sequence of $(GGGGS)_3$ peptide linker (Figure 8).

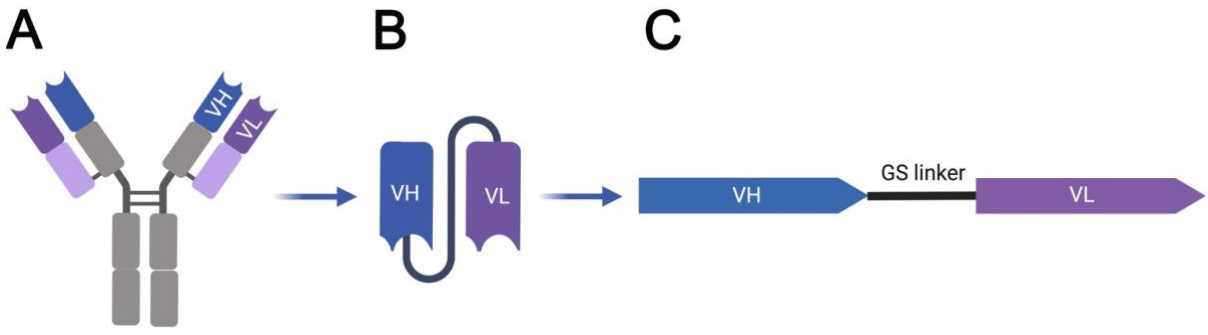


***Figure 8:*** *Schematic diagram of designing and construction single-chain variable fragment (scFc). (A) full length antibody, (B) protein structure of scFv, (C) DNA construct of scFv.*

The site-specific conjugation domain relays on SNAP-tag self-labeling enzyme. This method is based on the enzymatic activity of O6-alkylguanine-DNA alkyltransferase (AGT) in the DNA repair pathway, in which the alkyl group from the DNA transferred irreversibly to a cysteine

residue in the AGT protein, generating a thioether (S-alkylcysteine) and thus deactivate the AGT protein. The AGT protein has been engineered and modified to end up with 20 kDa engineered protein (SNAP-tag) with 50 times higher reactivity to BG modified molecules comparing to AGT [105-107].

Fusing SNAP-tag protein to scFv proteins allows the enzymatic conjugation of any BG-modified molecules to the scFv rapidly, efficiently and under physiological conditions. Beside these properties, SNAP-tag allows the site-specific conjugation of effector molecules with 1:1 stoichiometry, resulting in a high homogeneous product. Furthermore, SNAP-tag serve as a physical spacer, virtually separating the binding domain from the conjugated effector molecule and thus maintaining the binding activity of the antibody comparing to direct antibody conjugation methods, which may alter the physiochemical properties of the binding domain [108]. Also, SNAP-tag can improve the therapeutic properties of scFv fragments by increasing the protein size to around 50 kDa, to reduce the rapid clearance by the kidneys, It has been shown that the 50 kDa is optimal for achieving a maximum tumor-to plasma exposure ratio [109].

Therefore, SNAP-tag method has favorable conjugation properties in comparison to other enzyme-based conjugation methods developed to allow site specific modification of recombinant proteins with effector molecules.

One of these approaches is the sortase A (SrtA) enzyme-based conjugation method. SrtA is an extracellular membrane enzyme discovered in *Staphylococcus aureus.* It recognizes the amino acid sequence LPXTG and has the ability to cleavage the amide bond between the threonine (T) and the glycine (G) and anchore to surface proteins by couple the amino group in pentaglycine of peptidoglycan to the carboxyl group of threonine [110]. This enzymatic activity was used to generated ADCs, as an example C-terminal LPETG peptide was coupled to the heavy chain of anti-CD20 ofatumumab, which is finally conjugated to pentaglycine-modified MMAE using SrtA enzymatic reaction [111]. Another ADC was generated using two steps chemo-enzymatic. In the first step, anti-CD20 ofatumumab with LPETG peptide was conjugated to pentaglycine-modified azide and in the second step, DBCO-PEG-vc-PAB-MMAE was conjugated to the antibody by strain-promoted azide-alkyne cycloaddition [112]. Due to the low catalytic and reversible reactivity of SrtA, significant surplus of pentaglycine-modified molecules and large amount of SrtA are required to achieve high yields of the ADC. Therefore, this mothed is associated with high cost, synthesis challenging and limited production scaling up possibilities [113].

Another enzyme conjugation method relies on enzymatic activity of trypsiligase, an engineered trypsin protease, that recognizes the amino acid sequence YRH, cleave the Y-R peptide bond and ligating molecules harboring RH amino acids in the presence of $Zn^{2+}$ (for C-terminal labeling). N-terminal labeling, molecules modified with the arginine mimic 4-guanidinophenyl ester (OGp) can be ligated to the YRH tag. This method was used in combination with click

chemistry to arm the fragment antigen-binding region (FAB) of trastuzumab with DM1 cytotoxic reagent with around 70% coupling efficiency [114]. The requirement of short tag (only 3 amino acids) tag, is an advantage over other enzyme conjugation method, however concomitant proteolytic activity and the reversibility of enzymatic reaction produce unwanted side products [115].

Transglutaminase is also used for enzymatically conjugating effector molecules to antibodies due to its ability to catalyze the formation of amide bonds between a γ-carboxyamide group of a glutamine and a primary amine. Although, transglutaminase is also found in humans, the Streptomyces mobaraensis transglutaminase (mTG) is widely used because of its broader substrate specificity, lower deamidation activity, wider temperature and pH range of the reaction [116].

This approach was used to conjugate the cytotoxic reagent monomethyl auristatin D (MMAD) to three antibodies targeting EGFR, Her2 and M1S1. The amin modified MMAD was conjugated to the Q-tags that genetically inserted into 90 different sites of the antibody constant regions. From the 90 conjugation sites, only 12 sites show promising biophysical properties. The generated antibodies showed comparable effective property to ADCs generated with maleimide chemistry, however a rapid clearance and lower stability was determined when the MMAD was conjugated to the C-terminal of antibody heavy chain [81]. Furthermore, the functionalizing of lysine residue need larger peptide substrates and the requirement of significant surplus of substrate are still limit the use of mTG for antibody conjugation [7].

In our work, all scFv-SNAP recombinant proteins were expressed successfully in mammalian expression system to ensure proper protein folding and post-translational modifications [117]. The recombinant proteins were enriched from the culture media by immobilized metal ion affinity chromatography (IMAC) using the C-terminal polyhistidine-tag (6xHis-tag) with a high protein purity and high protein expression levels (10–15 mg/l culture supernatant) [71, 72, 74].

The targeting activity of the recombinant antibodies was determined by observing a strong and homogeneous membrane staining of cell lines expressing the corresponding surface antigen using confocal microscopy and flow cytometry, while no fluorescence signal was observed in cell lines lacking the corresponding surface antigen [71, 72, 74].

## 2.2. Generation of near infrared photo-immunotheranostics reagents (NIR-PIT) for treating TNBC and ovarian cancer cells

- Amoury, M.; Bauerschlag, D.; Zeppernick, F.; von Felbert, V.; Berges, N.; Di Fiore, S.; Mintert, I.; Bleilevens, A.; Maass, N.; Bräutigam, K.; Meinhold-Heerlein, I.; Stickeler, E.; Barth, S.; Fischer, R.; **Hussain, A. F.** Photoimmunotheranostic agents for triple-negative breast cancer diagnosis and therapy that can be activated on demand. *Oncotarget* **2016**, *7* (34).

- Bauerschlag, D.; Meinhold-Heerlein, I.; Maass, N.; Bleilevens ,A.; Bräutigam, K.; Al Rawashdeh, W.; Di Fiore, S.; Haugg, A. M.; Gremse, F.; Steitz, J.; Fischer, R.; Stickeler, E.; Barth, S.; **Hussain, A. F.** Detection and Specific Elimination of EGFR(+) Ovarian Cancer Cells Using a Near Infrared Photoimmunotheranostic Approach. Pharm Res **2017**, 34 (4), 696-703.

In this work, the theranostics agent IRDye700DX was successfully modified with BG-$PEG_{24}$-$NH_2$ building block, using the NHS ester–amino group reaction to generate BG-$PEG_{24}$-IRDye700. This ultimately allows the conjugation of IRDye700 to scFv-SNAP recombinant proteins targeting EGFR, EpCAM, and CSPG4 with a high labeling efficiency (~ 90%) [60, 61]. Furthermore, both scFv-SNAP recombinant proteins and IRDye700 showed a high stability in mouse serum for up to 6 h at 37°C [60].

The generated NIR-PIT reagents showed specific and clear binding towards corresponding target antigens in different TNBC and ovarian cancer cell lines. The fluorescently labeled fusion proteins were able to recognize the different expression levels of the targeted antigens in different cell lines expressing EGFR, EpCam, and CSPG4 after incubating them with the fluorescent labeled scFv-425-SNAP, scFv-EpCam-SNAP or scFv-CSPG4-SNAP, respectively. Under the same experimental conditions, no or low signal was detected in the cell lines lacking the target antigen [60, 61].

Furthermore, a homogeneous cell membrane staining was visualized using immunofluorescence microscopy in all $EGFR^+$, $EpCam^+$, and $CSPG4^+$ TNBC and ovarian cancer cell lines after 30-min incubation at 4 °C with the fluorescent labeled fusion proteins. The fusion proteins were internalized via receptor-mediated endocytosis as they were exclusively taken up into all cells expressing the corresponding cell receptor after max. 3 h of incubation at 37 °C. In contrast, no signal was detected when the cells lacking the targeted receptors incubated with fusion proteins under the same conditions [60, 61].

The ability of the NIR-PIT agents to specifically and on demand inducing cell death were investigated in this work in different TNBC and ovarian cancer cell lines using formazan dye-based cell proliferation XTT kit II. Significant, antigen expression level-dependent and dose-dependent reduction in cell viability were determined after incubating the cells with individual NIR-PIT reagents for 24 h at 37°C followed by light treatment at wavelengths ranging from 580–1400 nm. The $IC_{50}$ values for NIR-PIT reagents targeting $EGFR^+$, $EpCam^+$ and $CSPG4^+$ in TNBC and MCF-7 cells were of 62–165 nM [60]. Comparable results were determined in ovarian cancer cell lines [61]. Furthermore, a synergic effect by up to 40% compared to the individual reagents was observed, when equimolar mixture of EGFR, EpCam and CSPG4 targeting NIR-PIT reagents was used to treat TNBC and MCF-7 cells [60].

The high specificity of the NIR-PIT reagents was not only relied on the targeted antigens, but also dependent on the light treatments as no toxic effects were observed when the cells were treated with NIR-PIT reagents without NIR light treatment. Also, no reduction in cell viability

was detected when the cells treated with the unconjugated BG-PEG24-IRDye700 even after illumination [60, 61].

Apoptosis is highly regulated and controlled process that allows multicellular organisms to get rid of irreversibly damaged cells through entering a programmed cell death. The p53 dependent cell death pathways occur when caspase proteinases cleave their substrates [118]. Therefore, measuring the levels of caspase 3 and caspase 7 reveals the ability of NIR-PIT reagents to induce cell apoptosis. The results presented in this work showed that the levels of activated caspase-3 and caspase-7 increased in $EGFR^+$, $EpCam^+$ and $CSPG4^+$ treated cells and a direct correlation between the targeted antigens' level and the activated caspase-3 and -7 was observed. Importantly, the level of caspase-3 or caspase-7 was not signivicantly increased in cells treated with the unconjugated IR700, even upon NIR-light treatment [60].

Theranostics is a fast-growing field, and the main actor of this field is nanoparticle-based reagents due to their advantages properties such as high cellular uptake, high drug or drug encapsulation efficiency as well as precisely controlled drug release. However, Nanoparticles-based theranostics reagents are generally associated with several limitations such as complexity and high production costs, systemic and cellular toxicity and off-target accumulation [119].

In comparison to nanotheranotics approaches, the NIR-PIT approaches is a safe and minimally invasive approach that is composed of three components with minimal toxicity (on demand activatable thereanostics reagent, high specific antibody and harmless NIR-light). Toxicity is only achieved only when all these elements are applied together at the appropriate place and time [60, 61]. Furthermore, The production process of NIR-PIT reagents is similar to the well-established ADC production process with high probability of less side effects than ADC reagents [120].

One of the key differences between the NIR-PIT reagents generated in this work and other NIR-PIT reagents using IR700 [121-125] is the use of site-specific conjugation method to generate uniform products with a defined DAR. While a heterogeneous NIR-PIT reagents were generated using NHS ester–amino group reaction in the studies mentioned above, our BG-modified IRDye700 was conjugated site specifically, rapidly with high efficiency (~ 90%) and 1:1 stoichiometry to different SNAP-tag recombinant proteins. Another difference is the use of scFv antibodies instead of full-length antibodies, which could improve NIR-PIT reagents penetration into solid tumor and their rapid clearance by renal filtration to improve their therapeutic activities and safety profile [109].

Furthermore, other studies were focusing on targeting individual antigen to determine the therapeutic activity of NIR-PIT reagents. In this study, we investigated a cocktail of three NIR-PIT reagents targeting some of the most abundant receptors (EGFR, EpCAM and CSPG4) in TNBC cells. The favorable detection and therapeutic effect of these reagents were confirmed

individually and in combination in four different TNBC cell lines that express different levels of EGFR, EpCAM and CSPG4, with $IC_{50}$ values in the nanomolar range [60]. This approach could overcome the therapeutic limitations of targeting single antigen in tumors with high inter- and intra-tumor heterogeneity such as TNBC and ovarian cancer [126].

Despite the promising *in vitro* results, further experiments are clearly required to further confirm the therapeutic activities and safety profile of the generated NIR-PIT reagents *in vivo.*

## 2.3. Development of antibody drug conjugates for treating TNBC cells

- Zhang, C.; Sheng, W.; Al-Rawe, M.; Mohiuddin, T. M.; Niebert, M.; Zeppernick, F.; Meihold-Heerlein, I.; **Hussain, A. F**. EpCAM- and EGFR-Specific Antibody Drug Conjugates for Triple-Negative Breast Cancer Treatment. *Int J Mol Sci* **2022**, *23* (11).

In this work, two cell membrane receptors, namly EGFR and EpCAM, which are highly expressed in TNBC, were chosen as a potential target for the development of ADCs. Several studies have reported the EGFR overexpression in breast cancer, especially in TNBC, where it is associated with higher proliferation, recurrence and poor survival. The high expression of EGFR in TNBC (up to 65%) and its functional activation makes it an attractive target for diagnosis and therapy [127]. Several studies have reported elevated therapeutic activity in patients with TNBC when monoclonal antibodies against EGFR (cetuximab and panitumumab) combined with chemotherapeutic agents such as carboplatin, carboplatin/irinotecan, and paclitaxel are used [128]. EpCam expression was found in up to 35% of breast cancers and this is almost twice as much as the reported HER-2 over expression [129]. EpCam was used to detect circulation tumor cells (CTC) in the whole blood of metastatic breast cancer patients. The highest numbers of prognostic relevant EpCam positive CTC were found in TNBC [130].

The other important component of ADC is the linker used for conjugating the cytotoxic agent to the antibody. Generally, there are two classes of linkers, cleavable and non-cleavable linkers. Comparing to non-cleavable linkers, cleavable linkers allow the release of cytotoxic agents in targeted cells or tumor microenvironments. This can increase the bystander effect, through the diffusion of the released cytotoxic agent to extracellular environment and finally killing the adjacent cells [131].

Different types of cleavable linker were developed such as chemically cleavable linker (e.g., acid-cleavable linkers and reducible disulfides) and enzyme cleavable linker (e.g., dipeptide-containing linkers and glycosidase-cleavable linkers) [132]. The well-established protease-sensitive valine-citrulline (Val-Cit) dipeptide cleavable linker was used in this work. In contrast to acid-cleavable linker, Val-Cit dipeptide cleavable linker shows better human or mouse plasma stability and can be released in tumor extracellular environment due to the overexpressed lysosomal enzymes [133].

In order to facilitate the conjugation of MMAE to SNAP-tag fusion proteins, BG-GLA-PEG4-Val-Cit-PAB-MMAE (hereinafter called BG-MMAE) was successfully generated by coupling the BG-GLA-NHS to the amino-PEG4-Val-Cit-PAB-MMAE. By conjugating the generated BG-MMAE to the scFv-425-SNAP and scFv-EpCAM-SNAP fusion proteins, two ADCs targeting EGFR and EpCam were developed, respectively. Furthermore, the same SNAP-tag fusion proteins were conjugated with SNAP-Surface® Alexa Fluor® 647 to generate antibody-based imaging agents [58].

Specific and clear binding of generated ADCs toward corresponding target antigens in different TNBC cell lines (MDA-MB-468, MDA-MB-231, MDA-MB-453 and Hs578T) and one ER positive cell line (MCF7) were confirmed using flow cytometry analysis. Not only the detection ability of fluoresce-labeled fusion proteins, but also their ability to recognize different expression levels of the targeted antigens in different cell lines were determined. Meanwhile, no or low signal was detected in the $EGFR^-$ and $EpCam^-$ cell lines, under the same experimental conditions. Furthermore, a strong fluorescence staining was observed solely in the cells expressing the targeted antigen at 37 °C using fluorescence microscopy. This result confirms the cell uptake of the fusion proteins via receptor mediate internalization pathway [58].

The cytotoxicity of scFv-SNAP-MMAE was relying on the cell susceptibility for MMAE and the targeted cell surface receptor density. The highest cell proliferation inhibition was observed in MDA-MB-468 cells treated with scFv-425-SNAP-MMAE ($IC_{50}$: 114.7 nM) due to their high sensitivity to MMAE ($IC_{50}$: 61.16 nM) and high EGFR expression level [134, 135]. Lower cytotoxic activity of was observed, when MDA-MB-231and Hs578T cells, which have moderate sensitivity to MMAE and expressed moderate level EGFR [136, 137], treated with scFv-425-SNAP-MMAE, although not to the point where the fusion protein loss its therapeutic activity. No significant toxicity was observed when MDA-MB-453 and MCF-7 cell lines that expressing low level of EGFR [137] were treated with scFv-425-SNAP-MMAE [58].

The same cytotoxicity pattern was observed when TNBC cells treated with scFv-EpCAM-SNAP-MMAE. Here, the highest response was observed in MDA-MB-468 ($IC_{50}$: 135.2 nM), while moderate cytotoxicity was determined in MDA-MB-453 ($IC_{50}$: 551 nM). Comparing to other cell lines, MCF7 sensitivity to MMAE ($IC_{50}$: 660.6 nM) was low, however it still manifested specific cytotoxicity to scFv-EpCAM-SNAP-MMAE ($IC_{50}$: 981.7 nM). Furthermore, MDA-MB-231 and Hs578T cells that lack EpCam [138] were not significantly affected when treated with scFv-EpCAM-SNAP-MMAE. The cytotoxic response of the scFv-425-SNAP-MMAE and scFv-EpCAM-SNAP-MMAE was directly proportional to the expression level of the corresponding antigen, which confirmed the high specificity and safety of these agents [58].

The ability of the MMAE, scFv-425-SNAP-MMAE and scFv-EpCAM-SNAP-MMAE to induce cell apoptosis was determined using Annexin V/PI assay. Our results showed that MMAE induced apoptosis in all cell lines compared to negative control. After treating the cells with

scFv-425-SNAP-MMAE or scFv-EpCAM-SNAP-MMAE, the proportion of apoptotic cells were significantly increased in all $EGFR^+$ (MDA-MB-468, MDA-MB-231 and Hs578T) and $EpCam^+$ (MDA-MB-453, MDA-MB-468 and MCF7) cell lines, but not in the cell lines MDA-MB-453 and MCF7 expressing low level of EGFR or MDA-MB-231 and Hs578T cell lines that have a low EpCAM density. Unconjugated scFv-SNAP fusion proteins showed no cytotoxic effect in tested cell line [58].

Although, both 425-scFv-SNAP and EpCam-scFv-SNAP fusion proteins have demonstrated previously their promising targeting properties *in vivo* against several types of cancer [92, 93, 139, 140], further experiments are required to further confirm our results *in vivo*.

## 2.4. Simultaneous and Independent Dual Site-Specific Self-Labeling of Recombinant Antibodies for targeting ovarian cancer cells

- Wollschlaeger, C.; Meinhold-Heerlein, I.; Cong, X.; Bräutigam, K.; Di Fiore, S.; Zeppernick, F.; Klockenbring, T.; Stickeler, E.; Barth, S.; Hussain, A. F. Simultaneous and Independent Dual Site-Specific Self-Labeling of Recombinant Antibodies. Bioconjug Chem 2018, 29 (11), 3586-3594.

Arming antibody with two different molecules allows simultaneous use of individual antibody for different diagnosis and treatment strategies. For example, dual labeling the antibody with both radionuclide and NIR-dye allows nuclear imaging of whole-body to detect the tumor and facilitate surgery planning, while NIR-dye allows image-guided surgery [141]. Other approach for dual labeling is overcoming the high tumor heterogenicity and resistance to cytotoxic reagents. Here, two different cytotoxic reagents coupled to individual antibody can induce different cell death pathways and improve therapeutic and safety properties [142].

In this work, we have shown that self-labeling proteins (SNAP-tag and CLIP-tag) enable efficient and site-specific conjugation of two different effector molecules simultaneously and independently. This dual-labeled antibody allows both fluorescence imaging and photoimmunotherapy [76].

In addition to experimental techniques and rational design strategies, computational methods have been employed to aid in the design and engineering of proteins. One of these computational methods is the molecular dynamics (MD), which allows simulation the motions of proteins according to classical dynamics. Therefore, MD simulations were performed for SNAP-tag, CLIP-Tag and scFv proteins before cloning them together. The MD simulation results show that the CLIP-tag and SNAP-tag were well retained compared to the crystal structure of alkyl transferase (Cα RMSD < 2 Å). The folding pattern of the templet antibody is maintained in the tested scFv structure (Cα RMSD ranging from 2.9 ± 0.2 Å to 3.2 ± 0.4 Å) with slightly larger variations. In the simulations, we observed secondary-structure changes of

the first two β-strands of the light chain into β-bend structures. However, the Debye−Waller factor of this region was also high in the template crystal structure, indicating high flexibility. The simulation results confirm that all native folding propensity and structure are preserved in all tested structures (scFv, SNAP-tag and CLIP-tag) as one fusion protein. Furthermore, no interfering was observed between the functional sites of the scFv and the SNAP-tag and CLIP-tag self-conjugation sites. Both SNAP-tag and CLIP-tag in the fusion protein serve as a spacer between the scFv and the effector molecules and therefore reducing the chemical and physical effect of the direct interaction with scFv amino acids [76].

To confirm the simulation results, two fusion proteins (CLIP-scFv-425-SNAP and SNAP-scFv-425-CLIP) were designed and cloned. Furthermore, the cathepsin B-cleavable peptide linker GGGGSALAL (Cat-linker) [143] was inserted between the scFv-425 and SNAP-tag sequence to generate CLIP-scFv-425-Cat-linker-SNAP cassette. All three fusion proteins (CLIP-scFv-425-SNAP, SNAP-scFv-425-CLIP, and CLIP-scFv-425- Cat-linker-SNAP) were successfully expressed in HEK-293T cells and enriched using fast protein liquid chromatography (FPLC) system [76].

The BC-647 fluorescence dye and theranostics agents BG-$PEG_2$4-IRDye700 were conjugated specifically and simultaneously to the CLIP-tag and SNAP-tag moieties, respectively, of the fusion proteins CLIP-scFv-425-SNAP and SNAP-scFv-425-CLIP. A high conjugation efficiency was determined when CLIP-tag (86%) and SNAP-tag (84%) conjugated individually with BC-647 and BG-$PEG_{24}$-IRDye700 molecules, respectively and comparable conjugation efficiency was observed when CLIP-tag (89%) and the SNAP-tag (85%) conjugated with both dyes in combination. The high conjugation activity was associated with high site-specific conjugation. Pre-blocking of SNAP-tag or CLIP-tag with SNAP-tag blocking reagent bromothenylpteridine (BTP) or CLIP-tag blocking reagent, respectively, reveal that BG-$PEG_{24}$-IRDye700 and BC-647 dyes conjugate site-specifically and lonely to SNAP-tag and CLIP-tag, respectively [76].

The binding properties of the dual labeled CLIP-scFv-425-SNAP and SNAP-scFv-425-CLIP fusion proteins were determined in three $EGFR^+$ ovarian cancer cell lines (SKOV-3, OVCAR-3, and IGROV-1) and the $EGFR^-$ cell line (A2780). Both dual labeled fusion proteins bound specifically to the EGFR and were able to differentiate between $EGFR^+$ and $EGFR^-$ cells [76].

A clear fluorescent signal was observed in all $EGFR^+$ cell lines after 30 min incubation with the dual-labeled fusion proteins, while no signal was observed in the $EGFR^-$ cell line under the same experimental conditions. These results confirm that the binding activity of the scFv-425 does not significantly affected by fusing it to SNAP-tag and CLIP-tag proteins. As no differences were observed in the binding activities between both fusion proteins, the further experiments were performed using the CLIP-scFv-425-SNAP fusion protein [76].

The ability of dual-labeled CLIP-scFv-425-SNAP fusion protein to internalize the $EGFR^+$ cell lines via receptor-mediated endocytosis was determined by incubating the cells with CLIP-

scFv-425-SNAP labeled with both BC-647 and BG-Vista Green (VG) at 37 °C for 2 h. Both the Alexa 647 and VG fluorescence signals were detected intracellularly in $EGFR^+$ ovarian cell lines treated with 647-CLIP-scFv-425-SNAP-VG, while only VG fluorescence was observed in $EGFR^+$ cells after incubating them with scFv-425-SNAP-VG. No fluorescence signal was observed from any of the labeled fusion proteins in the $EGFR^-$ cell line A2780 after incubation under the same conditions [76].

As the BG-$PEG_{24}$-IRDye700 has a phototoxic activity and can induce cell death after NIR light treatment, we have determined the phototoxic effects of the NIR-PIT reagents 647-CLIP-scFv-425-SNAP-IRDye700 and scFv-425-SNAP-IRDye700 in four ovarian cancer cell lines expressing different level of EGFR receptor. After incubating the ovarian cancer cell lines (SKOV-3, OVCAR-3, IGROV-1 and A2780) with increasing concentrations of 647-CLIP-scFv-425-SNAP-IRDye700 and scFv-425-SNAP-IRDye700 and treating them with NIR light, the cell viability of the treated cells was determined using the formazan dye-based cell proliferation. Our results reveal that the cell viability of treated cells was reduced in a dose-dependent manner following light treatment after cell incubation with each of the 647-CLIP-scFv-425-SNAP-IRDye700 and scFv-425-SNAP-IRDye700 reagents for 24 h at 37°C. The $IC_{50}$ values for 647-CLIP-scFv-425-SNAP-IRDye700 were 45 nM (SKOV3), 50 nM (OVCAR-3), and 60 nM (IGROV-1), whereas those for scFv-425-SNAP-IRDye700 were 52 nM (SKOV-3), 66 nM (OVCAR-3), and 70 nM (IGROV-1). No significant reduction in cell viability was determined in A2780 cells treated with the same conditions. All the cells treated with 647-CLIP-scFv-425-SNAP-IRDye700 and scFv-425-SNAP-IRDye700 reagents without light treatment remain viable [76].

Overall, our research established site-specific dual labeling method, which enables generation of a homogeneous product with a DAR of 1 + 1 in one-pot reaction using SNAP-tag and CLIP-tag proteins. The *in vitro and ex vivo* results presented in this work provide sound evidence that this method can be used to equipping individual recombinant antibody with a wide range of effector molecules.

## 2.5. Application of immunotoxins for treating TNBC

- Amoury, M.; Kolberg, K.; Pham, A. T.; Hristodorov, D.; Mladenov, R.; Di Fiore, S.; Helfrich, W.; Kiessling, F.; Fischer, R.; Pardo, A.; Thepen, T.; **Hussain, A. F.**; Nachreiner, T.; Barth, S. Granzyme B-based cytolytic fusion protein targeting EpCAM specifically kills triple negative breast cancer cells in vitro and inhibits tumor growth in a subcutaneous mouse tumor model. Cancer Lett 2016, 372 (2), 201-209.
- Amoury, M.; Mladenov, R.; Nachreiner, T.; Pham, A. T.; Hristodorov, D.; Di Fiore, S.; Helfrich, W.; Pardo, A.; Fey, G.; Schwenkert, M.; Thepen, T.; Kiessling, F.; **Hussain, A. F.**; Fischer, R.; Kolberg, K.; Barth, S. A novel approach for targeted elimination of CSPG4-positive triple-negative breast cancer cells using a MAP tau-based fusion protein. Int J Cancer 2016, 139 (4), 916-927.

In this work, EpCam targeting Granzyme B and CSPG4 targeting MAP based human immunotoxins were generated and investigated in *in vitro* and *in vivo* studies. The scFv format chose to generate the immunotoxins due to its advantages over mAb such as, small size, higher penetration and clearance rates, lack of an Fc region which could be associated with unfavorable immune response as well as simplicity of the design and modification [144].

The DNA sequence for an enterokinase cleavage site followed by DNA sequence of the granzyme B mutant with reduced sensitivity to its natural inhibitor serpin B9 (GbR201K), was inserted successfully downstream of an immunoglobulin κ leader sequence. The enterokinase cleavage site allows the expression of inactive fusion protein EGbR201K-EpCam-scFv in mammalian cells. After protein purification, the enterokinase digestion releases the N-terminal peptide, which leads to activate the GbR201K moiety. The DNA sequence of EpCam-scFv was inserted downstream to the GbR201K and upstream to polyhistidine-tag (6xHis-tag) [93].

The CSPG4-scFv-ETA (Truncated version of *Pseudomonas aeruginosa* exotoxin A) bacterial expression plasmid was kindly provided by Prof. Dr. Georg Fey (University Nurnberg-Erlangen, Germany) [145]. The DNA sequence for functionally enhanced form of the human microtubule-associated protein (MAP) tau was inserted instead of the ETA DNA sequence to generate scFv-EpCam-MAP fusion protein [94]. As a positive control, we used the immunotoxin scFv-EpCam-ETA and scFv-CSPG4-ETA. Both fusion proteins were evaluated for their specific cell binding and internalization activity and their cytotoxicity *in vitro* and *in vivo* [93, 94].

The EGbR201K-scFv-EpCam was expressed successfully in the mammalian cells HEK293T as inactive immunotoxin due to the presence of N-terminal pre-peptide and released into the cell culture media. IMAC was used to enrich the EGbR201K-scFv-EpCam with final yield of 8 mg/l [93]. The immunotoxin scFv-CSPG4-MAP was expressed in E. coli BL21(DE3) under osmotic stress conditions in the presence of compatible solutes. The immunotoxin was purified using IMAC followed by Strep-Tactin affinity chromatography and size exclusion chromatography with >90% purity and a final yield of 4-5 mg /l of culture media [94].

An effective and safe immunotoxin need to specifically bind and internalized to the target cells; therefore, the binding and internalization specificity and activity were investigated for both EGbR201K-scFv-EpCam and scFv-CSPG4-MAP immunotoxins in TNBC expressing EpCam and CSPG4 cell receptors. Consistent with the expression of EpCam determined using commercial antibodies, flow cytometry analysis revealed that EGbR201K-scFv-EpCam binds specifically to EpCam-expressing MDA-MB-453 and MDA-MB-468 cells [93]. Similar to the results presented by Wang et al., [146], CSPG4-based immunotoxin binds to TNBC cell lines MDA-MB231 and Hs578T that expressing CSPG4 on their surface. In contrast, no significant binding was detected in CSPG4 negative cell line MDAMB-468 [94].

In order to be able to perform its function of killing targeted cells, immunotoxins need to be internalized specifically and efficiently into the cells. Therefore, we have determined the

binding and the internalization of EpCam and CSPG4-based immunotoxins using confocal microscopy. After 90 min incubation of fluorescence labeled immunotoxins with MDA-MB-468 and MDA-MB-231 TNBC cells, specific and strong fluorescence signal were detected in the MDA-MB-468 cells that expressing EpCam on their surface, while no signal was observed in EpCam negative cells (MDA-MB-231). Consistent with the flow cytometry results, fluorescence labeled scFv-CSPG4-MAP was solely uptake by CSPG4 positive cell line MDA-MB-231 [93, 94].

The cell viability assay (XTT) was used to investigate the cytotoxic activity *in vitro* of the generated immunotoxins in TNBC cell lines expressing different levels of EpCam and CSPG4. After treating the cells with the increased concentrations of immunotoxins, dose-dependent cell viability reduction was determined in the cells expressing the targeted receptor. The $IC_{50}$ range for ETA-based immunotoxin was 32.9 nM to 66.4 nM, for GbR201K-scFv-EpCam was 307 nM for MDA-MB-453 and 221.8 nM for MDA-MB-468, while the scFv-CSPG4-MAP $IC_{50}$ was 219.5 nM for MDA-MB-231 and 480 nM for Hs578T. Under the same experiment conditions, no significant cell growth inhibition was detected in EpCam negative cell line (MDA-MB-231) and CSPG4 negative cell line (MDA-MB-468) [93, 94].

The accumulation of immunotoxins in different organs (e. g. liver, spleen, kidneys, bladder) as well as subcutaneous tumor has be investigated at different time-points after i. v. injection (0, 4, 8 and 24 hr) into BALB/c nude mice bearing MDA-MB-231 (for GbR201K-scFv-EpCam and scFv-EpCam- ETA) or MDA-MB-468 (scFv-CSPG4-MAP and scFv-CSPG4-ETA) expressing red fluorescent Katushka2 protein using CRi Maestro Imaging System [93, 94].

After i.v. administration of DyLight-747-B3-labeled immunotoxins, a strong fluorescent signal was observed at the injection site and rapid distribution to highly perfused organs (e.g., liver, lung, kidney, spleen, and gut). The results reveal that immunotoxins were cleared from blood stream and most of the central compartment organs within few hours, apart from the kidneys where the signal remained for up to 24 hr. One hour post injection, the immunotoxins were detected in the tumor and their signals were maintain into the tumor for at least 24 hr. as demonstrated by the co-localization of the signal with the primary tumor signal. Furthermore, this observation were confirmed using immunofluorescence and immunohistochemistry [93, 94].

The specific toxic activity of GbR201K-scFv-EpCAM and scFv-CSPG4-MAP immunotoxins were investigated in subcutaneous Katushka2-transfected TNBC cells xenograft model. Tumor inhibition of EpCam-based immunotoxin was determined after i. v. injection of GbR201K-EpCam-scFv (2.5 mg/kg), EpCam-scFv-Eta (1.25 mg/kg), scFv-EpCam-SNAP (2.5 mg/kg) or PBS at days 13, 14, 17, 19 into BALB/c nude mice bearing Katushka2-transfected MDA-MB-231. The activity of CSPG4-based immunotoxin was investigating by treating BALB/c nude mice bearing Katushka2-transfected MDA-MB-468 with either scFv-CSPG4-ETA (1.2 mg/kg dose), scFv-CSPG4-MAP (2.5 mg/kg), scFv-CSPG4-SNAP (2.5 mg/kg), or PBS on days 22, 24, 27 and 29. Tumor inhibition was determined by monitoring the size of tumors through

determining the Katushka2 fluorescence signal intensity and using caliper measurements. Growth inhibition of 40% (GbR201K-scFv-EpCAM) and 60% (scFv-EpCam-ETA), respectively, were determined into BALB/c nude mice bearing Katushka2-transfected MDA-MB-231 comparing to the control group [93, 94].

Treatment with scFv-CSPG4-MAP or scFv-CSPG4-ETA resulted in comparable inhibition of tumor growth. Furthermore, no detectable tumor growth inhibition was observed after treatment with a non-cytolytic control proteins (scFv-EpCam-SNAP or scFv-CSPG4-SNAP), this confirms that the antitumor activity of immunotoxins is mediated by their effector domains. The results presented here show that the fusion scFv-EpCam or scFv-CSPG4 with GbR201K or MAP, respectively, did not affecting the tumor-selective binding activity of the scFv or the cytotoxic activity of GbR201K or MAP [93, 94].

Although both GbR201K and MAP based human immunotoxins could reduce immunogenicity and potential side effects associated with bacterial-based immunotoxins such as ETA, they show lower toxicity comparing to ETA-based immunotoxins, but not to the point where they loss their therapeutic activity. Around 40–50% overall inhibition of tumor growth with absent off-target toxicity were observed *in vivo* after a short treatment regimen consisting of a single course of 4 doses. However, the impact of longer or repeated treatment approaches needs to be further studied.

# 3. Publications

## 3.1. Designing, Expression and Purification of Recombinant antibody-SNAPtag fusion proteins

**Pages 39-66**

- **Hussain, A. F.**; Heppenstall, P. A.; Kampmeier, F.; Meinhold-Heerlein, I.; Barth, S. One-step site-specific antibody fragment auto-conjugation using SNAP-tag technology. Nat Protoc 2019, 14, 3101-3125.

**Pages 67-73**

- Chouman, K.; Woitok, M.; Mladenov, R.; Kessler, C.; Weinhold, E.; Hanz, G.; Fischer, R.; Meinhold -Heerlein, I.; Bleilevens, A.; Gresch, G.; Haugg, A. M.; Zeppernick, F.; Bauerschlag, D.; Maass, N.; Stickeler, E.; Kolberg, K.; **Hussain, A. F**. Fine Tuning Antibody Conjugation Methods using SNAP-tag Technology. Anticancer Agents Med Chem 2017, 17.

**Pages 74-82**

- von Felbert, V.**;** Bauerschlag, D.; Maass, N.; Bräutigam, K.; Meinhold-Heerlein, I.; Woitok, M.; Barth, S.; **Hussain, A. F.** A specific photoimmunotheranostics agent to detect and eliminate skin cancer cells expressing EGFR. J Cancer Res Clin Oncol 2016, 142 (5), 1003-1011.

**Pages 83-91**

- **Hussain, A. F.**; Kampmeier, F.; von Felbert, V.; Merk, H. F.; Tur, M. K.; Barth, S. SNAPtag technology mediates site specific conjugation of antibody fragments with a photosensitizer and improves target specific phototoxicity in tumor cells. Bioconjug Chem 2011, 22 (12), 2487-2495.

## 3.2. Generation of near infrared photo-immunotheranostics reagents (NIR-PIT) for treating TNBC and ovarian cancer cells

**Pages 93-100**

- Bauerschlag, D.; Meinhold-Heerlein, I.; Maass, N.; Bleilevens ,A.; Bräutigam, K.; Al Rawashdeh, W.; Di Fiore, S.; Haugg, A. M.; Gremse, F.; Steitz, J.; Fischer, R.; Stickeler, E.; Barth, S.; **Hussain, A. F.** Detection and Specific Elimination of EGFR(+) Ovarian Cancer Cells Using a Near Infrared Photoimmunotheranostic Approach. Pharm Res 2017, 34 (4), 696-703.

**Pages 101-113**

- Amoury, M.; Bauerschlag, D.; Zeppernick, F.; von Felbert, V.; Berges, N.; Di Fiore, S.; Mintert, I.; Bleilevens, A.; Maass, N.; Bräutigam, K.; Meinhold-Heerlein, I.; Stickeler, E.; Barth, S.; Fischer, R.; **Hussain, A. F.** Photoimmunotheranostic agents for triple-negative breast cancer diagnosis and therapy that can be activated on demand. Oncotarget 2016, 7 (34).

## 3.3. Development of antibody drug conjugates for treating TNBC cells

**Pages 114-126**

- Zhang, C.; Sheng, W.; Al-Rawe, M.; Mohiuddin, T. M.; Niebert, M.; Zeppernick, F.; Meihold-Heerlein, I.; **Hussain, A. F.** EpCAM- and EGFR-Specific Antibody Drug Conjugates for Triple-Negative Breast Cancer Treatment. Int J Mol Sci 2022, 23 (11).

## 3.4. Simultaneous and independent dual site-specific self-labeling of recombinant antibodies for targeting ovarian cancer cells

**Pages 128-136**

- Wollschlaeger, C.; Meinhold-Heerlein, I.; Cong, X.; Bräutigam, K.; Di Fiore, S.; Zeppernick, F.; Klockenbring, T.; Stickeler, E.; Barth, S.; **Hussain, A. F.** Simultaneous and Independent Dual Site Specific Self-Labeling of Recombinant Antibodies. Bioconjug Chem 2018, 29 (11), 3586-3594.

## 3.5. Application of immunotoxins for treating TNBC

**Pages 138-146**

- Amoury, M.; Kolberg, K.; Pham, A. T.; Hristodorov, D.; Mladenov, R.; Di Fiore, S.; Helfrich, W.; Kiessling, F.; Fischer, R.; Pardo, A.; Thepen, T.; **Hussain, A. F.**; Nachreiner, T.; Barth, S. Granzyme B-based cytolytic fusion protein targeting EpCAM specifically kills triple negative breast cancer cells in vitro and inhibits tumor growth in a subcutaneous mouse tumor model. Cancer Lett 2016, 372 (2), 201-209.

**Pages 147-158**

- Amoury, M.; Mladenov, R.; Nachreiner, T.; Pham, A. T.; Hristodorov, D.; Di Fiore, S.; Helfrich, W.; Pardo, A.; Fey, G.; Schwenkert, M.; Thepen, T.; Kiessling, F.; **Hussain, A. F.**; Fischer, R.; Kolberg, K.; Barth, S. A novel approach for targeted elimination of CSPG4-positive triple-negative breast cancer cells using a MAP tau-based fusion protein. Int J Cancer 2016, 139 (4), 916-927.

# 4. Summary

The hypothesis of a ‘‘one pill fits all’’ limits the success of cancer treatments and it is now acknowledged that no single therapeutic agent has the same effect on all patients with the same diagnosis. Precision medicine is a promising paradigm, mainly in cancer treatment, and it is widely believed that precision medicine will have a revolutionary impact on healthcare before, during and after disease by specifically targeting disease cells to ensure maximal efficacy and safety. However, the heterogeneity and complexity of tumors, along the development of different metastases set a countless challenge for cancer targeting therapies. The considerable increase in understanding molecular processes led to identification of numerous biomarkers for cancer detection and treatment. These biomarkers have paved the way for developing several tumor targeting therapies in recent years. It is now becoming increasingly clear that corresponding therapies have a great chance in improving cancer care by selectively targeting tumor cells and concomitantly reducing adverse side effects.

Despite recent reduction in breast cancer mortality, breast cancer remains the leading cause of cancer-related death among women worldwide. Breast cancer targeted therapies that rely on tumor cell expression of estrogen (ER), progesterone (PgR) and HER2 receptors can be effective in the treatment of luminal and HER2-positive breast cancers. However, TNBC presents a challenge as it is characterized by the absence of ER, PgR and HER2 receptors. TNBC patients are generally young premenopausal women accounting for up to 20% of breast cancer cases. Moreover, TNBC is associated with unfavorable outcome with decreased disease free and overall survival rates.

Ovarian cancer is one of the most common gynecological tumors worldwide. The gold standard therapy for ovarian cancer involves an intensive surgical cytoreduction, followed by a platinum and taxane-based cytostatic treatment. The antiangiogenetic bevacizumab and / or the novel drug class of PARP inhibitors – both effective in some high-grade serous cancers – have improved the progression-free survival as well as the overall survival. Nevertheless, the prognosis of ovarian cancer is poor and new therapeutic approaches warranted.

The fatality rate of ovarian cancer is high because the available therapeutic modalities have failed to treat ovarian cancer patients with advanced disease, who are representing more than 70% of patents with ovarian cancer. For these patients, the five-year survival rates are ranging from 20% to 30%, in some settings up to 50%, compared to cure rates of 70% to 90 cure rates for those diagnosed with the disease is confined to the ovary. This is mainly due to the spreading of ovarian tumors into the peritoneum, retroperitoneal space and the serosa layer of the gut, which is one of the major limitations preventing the removal and / or treatment of poor-vascularized macro-metastasis, local and/or long distant micro-metastases as well as non-resectable macro-metastasis during cytoreductive surgery. These untreated tumor tissues

usually cause tumor relapse, reducing the median survival rate from ~99 months for the patient with no residual disease to ~36 months if residual tumors are present.

The high inter- and intra-tumor heterogeneity of TNBC and ovarian cancer associated with different histopathological features and clinical behaviors that influence differentially the therapeutic outcome of surgery and/or chemotherapy and associated with inherent and acquired drug resistance. Therefore, there is there is an urgent, and till now unmet, medical need for new treatment options for TNBC and ovarian cancer.

This work represents the attempts to refine the methods for generating cancer targeting therapy and to overcome the shortcomings of the available therapeutic modalities of TNBC and ovarian cancer, such as tumor heterogeneity, low efficiency, and drug resistance. This is mainly achieved by using SNAP-tag technology as a site-specific conjugation method for arming antibody with therapeutic and / or imaging agents. Moreover, applying different therapeutic strategies (near infrared photo-immunotheranostics (NIR-PIT), antibody drug conjugate (ADC) , and immunotoxin) as well as targeting different highly expressed cell surface receptor (EGFR, EpCam and CSPG4) on TNBC and ovarian cancer cells.

SNAP-tag technology allows to site-specifically conjugate a wide range of effector molecules rapidly and efficiently under physiological conditions with 1:1 conjugation stoichiometry. The different mechanism of actions can overcome the limitation of cancer cell acquired and inherited drug resistance of TNBC and ovarian cancer, while targeting a set of different cell surface receptor can overcome the inter- and intra-tumor heterogeneity of TNBC and ovarian cancer.

The developed NIR-PIT and ADC reagents demonstrated strong imaging properties and / or potent therapeutic activity in combination and / or individually against different TNBC and ovarian cancer cells *in vitro*. Despite the impressive results, additional experiments are undoubtedly required to determine the pharmacokinetic properties and to verify the imaging and / or the therapeutic activity *in vivo*.

In another therapeutic approach, two human-based immunotoxins, scFv-CSPG4-MAP and GbR201K-scFvEpCam (full human immunotoxin), were generated and evaluated. Both *in vitro* and *in vivo* results showed that the immunotoxins have a high translational potential for targeted elimination of TNBC.

The impact of developing antibody-based recombinant proteins, the use of SNAP-Tag technology for conjugating effector molecules, targeting three cell surface receptors (EGFR, EpCam and CSPG4) in combination and / or individually and the exploiting of different therapeutic strategies (NIR-PIT, ADC, and immunotoxin) for targeting TNBC and ovarian cancer were investigated and discussed in this work.

# 5. Zusammenfassung

Genetische Veränderungen, die zur Entstehung von Krebs führen, variieren zwischen Patienten. Daher gewinnt die maßgeschneiderte Krebsbehandlung zunehmend an Bedeutung und die Präzisionsmedizin könnte in Zukunft eine personalisierte und zielgerichtete Therapie von Tumorzellen ermöglichen.

Es wird angenommen, dass die Präzisionsmedizin einen revolutionären Einfluss auf die Gesundheitsversorgung vor, während und nach einer Krankheit haben wird, indem sie gezielt auf erkrankte Zellen abzielt und damit maximale Wirksamkeit und Sicherheit gewährleistet. Allerdings stellt die Heterogenität und Komplexität des Tumors, sowie die Entwicklung von Metastasen eine große Herausforderung für zielgerichtete Krebstherapien dar.

Die beträchtliche Verbesserung des Verständnisses der molekularen Prozesse der Krebsentstehung führte zur Identifizierung zahlreicher Biomarker für die Diagnose und Behandlung. Diese Biomarker haben in den letzten Jahren den Weg geebnet für die Entwicklung mehrerer Tumor-spezifischer Therapien. Mittlerweile wird immer deutlicher, dass entsprechende gezielte Therapien ein großes Potential für die Krebsbehandlung haben. Solche Therapieansätze ermöglichen eine zielgerichtete Bekämpfung von Tumorzellen und gleichzeitig eine Verringerung von unerwünschten Nebenwirkungen.

Trotz des jüngsten Rückgangs der spezifischen Sterblichkeit bleibt Brustkrebs weltweit die häufigste Krebstodesursache bei Frauen. Auf Brustkrebs ausgerichtete Therapien, die auf der Tumorzellexpression von Östrogen (ER), Progesteron (PgR) und HER2-Rezeptoren beruhen, können bei der Behandlung von luminalem und HER2-positivem Brustkrebs wirksam sein. Dreifach negativer Brustkrebs (TNBC) ist klassifiziert durch das Fahlen der Expression von ER-, PgR- und HER2-Rezeptoren. Bei TNBC-Patienten handelt es sich im Allgemeinen um junge Frauen vor der Menopause, die bis zu 20% der Brustkrebsfälle ausmachen. Darüber hinaus sind TNBC mit einem ungünstigen Verlauf verbunden wie einem verringerten krankheitsfreien und Gesamtüberleben.

Eierstockkrebs gehört weltweit zu den häufigsten gynäkologischen Tumoren. Die Goldstandardtherapie ist hier eine intensive chirurgische Zytoreduktion, gefolgt von einer Platin- und Taxan-basierten Zytostatikabehandlung. Das antiangiogenetische Bevacizumab und/oder die neuartige Medikamentenklasse der poly-ADP-Ribose-Polymerase (PARP)-Inhibitoren, die bei einigen hochgradigen serösen Krebsarten wirksam sind, haben das progressionsfreie Überleben, sowie das Gesamtüberleben der betroffenen Patientinnen verbessert. Trotz dieser neuen Behandlungsmöglichkeiten ist die Sterblichkeitsrate bei Eierstockkrebs dennoch hoch, da die verfügbaren Therapiemodalitäten keine Behandlung für Patientinnen mit fortgeschrittenem Eierstockkrebs ermöglichen. Diese machen mehr als 70 % der Patientinnen mit Eierstockkrebs aus und die 5-Jahres-Überlebensraten liegt zwischen 20 und 30 %, in manchen Fällen sogar bis zu 50 %, im Vergleich zu 70 bis 90 % bei Patientinnen,

bei denen die Erkrankung auf den Eierstock beschränkt ist. Dies ist hauptsächlich auf die Ausbreitung von Ovarialtumoren in das Peritoneum, den retroperitonealen Raum und die Serosaschicht des Darms zurückzuführen, was eine der größten Einschränkungen darstellt, die die Entfernung und/oder Behandlung schlecht vaskularisierter lokaler und/oder lokaler Makrometastasen verhindern weit entfernte Mikrometastasen sowie nicht resezierbare Makrometastasen während zytoreduktiver Chirurgie. Diese unbehandelten Tumorgewebe verursachen in der Regel einen Tumorrückfall und reduzieren die mittlere Überlebensrate von etwa 99 Monaten für die Patientinnen ohne Resterkrankung auf etwa 36 Monate, wenn Resttumoren vorhanden sind.

Die hohe inter- und intratumorale Heterogenität von TNBC und Eierstockkrebs ist mit unterschiedlichen histopathologischen Merkmalen und klinischen Verläufen verbunden. Diese wirken sich unterschiedlich auf das therapeutische Ergebnis einer Operation und/oder Chemotherapie aus und sind mit inhärenter und erworbener Arzneimittelresistenz assoziiert. Daher besteht ein dringender und bislang ungedeckter medizinischer Bedarf an neuen Behandlungsmöglichkeiten für TNBC und Eierstockkrebs.

Die vorliegende Arbeit zielt darauf ab, die Methoden zur Entwicklung einer zielgerichteten Krebstherapie zu verfeinern und die Mängel der verfügbaren Therapiemodalitäten von TNBC und Eierstockkrebs wie geringe Effizienz und Arzneimittelresistenz zu überwinden.

Im Rahmen dieser Arbeit wurde die SNAP-Tag-Technologie verwendet, die eine schnelle und effiziente ortsspezifische Konjugation einer Vielzahl von Effektormolekülen unter physiologischen Bedingungen und einer 1:1-Konjugationsstöchiometrie ermöglicht. Die unterschiedlichen Wirkungsmechanismen der Effektormoleküle können die Einschränkungen der durch Krebszellen erworbenen und vererbten Arzneimittelresistenz von TNBC und Eierstockkrebs überwinden, während die gezielte Behandlung verschiedener Zelloberflächenrezeptoren die inter- und intra-tumorale Heterogenität von TNBC und Eierstockkrebs überwinden kann.

Die entwickelten therapeutischen Strategien Nahinfrarot-Photoimmunotherapie (NIR-PIT) und Antikörper-Wirkstoff-Konjugat ADC-Reagenzien zeigten in Kombination und/oder einzeln in-vitro starke bildgebende Eigenschaften und/oder starke therapeutische Aktivität gegen verschiedene TNBC- und Eierstockkrebszellen. Trotz der vielversprechenden Ergebnisse sind zweifellos weitere Experimente erforderlich, um die pharmakokinetischen Eigenschaften zu bestimmen und die Bildgebung und/oder die therapeutische Aktivität in vivo zu verifizieren.

In einem weiteren Therapieansatz wurden zwei humanbasierte Immuntoxine generiert und untersucht. Sowohl in-vitro- als auch in-vivo-Ergebnisse zeigten, dass die Immuntoxine scFv-CSPG4-MAP und GbR201K-scFvEpCam (vollständiges humanes Immuntoxin) ein hohes Translationspotenzial für die gezielte Eliminierung von TNBC vorweisen.

Die Auswirkungen der Entwicklung antikörperbasierter rekombinanter Proteine, der Einsatz der SNAP-Tag-Technologie zur Konjugation von Effektormolekülen, die gezielte Bekämpfung von drei Zelloberflächenrezeptoren (EGFR, EpCam und CSPG4) in Kombination und/oder einzeln sowie die Nutzung verschiedener therapeutischer Strategien (NIR- PIT, ADC und Immuntoxin) zur Bekämpfung von TNBC und Eierstockkrebs wurden in dieser Arbeit untersucht und diskutiert.

## 6. References


(1) Cotter, T. G. Apoptosis and cancer: the genesis of a research field. *Nature Reviews Cancer* **2009**, *9* (7), 501-507. DOI: 10.1038/nrc2663.

(2) Pray, L. A. DNA Replication and Causes of Mutation. *Nature Education* **2008**, *1* (1), 214. DOI: 10.1038/nrc2663.

(3)Hanahan, D.; Weinberg, R. A. The hallmarks of cancer. *Cell* **2000**, *100* (1), 57-70. DOI: 10.1016/s0092-8674(00)81683-9 From NLM.

(4) Hanahan, D.; Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **2011**, *144* (5), 646-674. DOI: 10.1016/j.cell.201 1.02.013From NLM.

(5) Sung, H.; Ferlay, J.; Siegel, R. L.; Laversanne, M.; Soerjomataram, I.; Jemal, A.; Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* **20** .249-209 ,(3) 71 ,21DOI: 10.3322/caac.21660 From NLM.

(6) Siegel, R. L.; Miller, K. D.; Fuchs, H. E.; Jemal, A. Cancer Statistics, 2021. *CA Cancer J Clin* **2021**, *71* (1), 7-33. DOI: 10.3322/caac.21654 From NLM.

(7) Debela, D. T.; Muzazu, S. G.; Heraro, K .D.; Ndalama, M. T.; Mesele, B. W.; Haile, D. C.; Kitui, S. K.; Manyazewal, T. New approaches and procedures for cancer treatment: Current perspectives. *SAGE Open Med* **2021**, *9*, 20503121211034366. DOI: 10.1177/20503121211034366 From NLM.

(8) Arnold, M.; Morgan, E.; Rumgay, H.; Mafra, A.; Singh, D.; Laversanne, M.; Vignat, J.; Gralow, J. R.; Cardoso, F.; Siesling, S.; et al. Current and future burden of breast cancer: Global statistics for 2020 and 2040. *Breast* **2022**, *66*, 15-23. DOI: 10.1016/j.breast.2022.08 010.From NLM.

(9) Perou, C. M.; Sørlie, T.; Eisen, M. B.; van de Rijn, M.; Jeffrey, S. S.; Rees, C. A.; Pollack, J. R.; Ross, D. T.; Johnsen, H.; Akslen, L. A.; et al. Molecular portraits of human breast tumours. *Nature* **2000**, *406* (6797), 747-752. DOI: 1.35021093/0.1038

(10) Sørlie, T.; Perou, C. M.; Tibshirani, R.; Aas, T.; Geisler, S.; Johnsen, H.; Hastie, T.; Eisen, M. B.; van de Rijn, M.; Jeffrey, S. S.; et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* **2001**, *98* (19), 10869-10874. DOI: 10.1073/pnas.191367098 From NLM.

(11) Elsawaf, Z.; Sinn, H. P. Triple-Negative Breast Cancer: Clinical and Histological Correlations. *Breast Care (Basel)* **2011**, *6* (4), 273-278. DOI: 10 000331643/1159.From NLM.

(12) Foulkes, W. D.; Smith, I. E.; Reis-Filho, J. S. Triple-negative breast cancer. *N Engl J Med* **2010**, *363* (20), 1938-1948. DOI: 10.1056/NEJMra1001389 From NLM.

(13) Nunnery, S. E.; Mayer, I. A.; Balko, J. M. Triple-Negative Breast Cancer: Breast Tumors With an Identity Crisis. *Cancer J* **2021**, *27* (1), 2-7. DOI: 10.1097/ppo.0000000000000494 From NLM.

(14) Yin, L.; Duan, J. J.; Bian, X. W.; Yu, S. C. Triple-negative breast cancer molecular subtyping and treatment progress. *Breast Cancer Res* **2020**, *22* (1), 61. DOI: 10.1186/s13058-020-01296-5 From NLM.

(15) Yao, H.; He, G.; Yan, S.; Chen, C.; Song, L.; Rosol, T. J.; Deng, X. Triple-negative breast cancer: is there a treatment on the horizon? *Oncotarget* **2017**, *8* (1), 1913-1924. DOI : /10.18632oncotarget.12284 From NLM.

(16) Sakach, E.; Sacks, R.; Kalinsky, K. Trop-2 as a Therapeutic Target in Breast Cancer. *Cancers (Basel)* **2022**, *14* (23). DOI: 10.3390/cancers14235936 From NLM.

(17) Asleh, K.; Riaz, N.; Nielsen, T. O. Heterogeneity of triple negative breast cancer: Current advances in subtyping and treatment implications. *J Exp Clin Cancer Res* **2022**, *41* (1), 265. DOI: 10.1186/s13046-022-02476-1 From NLM.

(18) Vargas, A. N. Natural history of ovarian cancer. *Ecancermedicalscience* **2014** .465 ,8 , DOI: 10.3332/ecancer.2014.465 From NLM.

(19) Coleman, R. L.; Monk, B. J.; Sood, A. K.; Herzog, T. J. Latest research and treatment of advanced-stage epithelial ovarian cancer. *Nature Reviews Clinical Oncology* **2013**, *10* (4), 211-224. DOI: 10.1038/nrclinonc.2013.5.
(20) Oliver, K. E.; McGuire, W. P. Ovarian cancer and antiangiogenic therapy: caveat emptor. *J Clin Oncol* **2014**, *32* (30), 3353-3356. DOI: 10.1200/jco.2014.57.4574 From NLM.
(21) Burger, R. A.; Brady, M. F.; Bookman, M. A.; Fleming, G. F ;.Monk, B. J.; Huang, H.; Mannel, R. S.; Homesley, H. D.; Fowler, J.; Greer, B. E.; et al. Incorporation of bevacizumab in the primary treatment of ovarian cancer. *N Engl J Med* **2011**, *365* (26), 2473-2483. DOI: 10.1056/NEJMoa1104390 From NLM.
(22) Perren ,T. J.; Swart, A. M.; Pfisterer, J.; Ledermann, J. A.; Pujade-Lauraine, E.; Kristensen, G.; Carey, M. S.; Beale, P.; Cervantes, A.; Kurzeder, C.; et al. A phase 3 trial of bevacizumab in ovarian cancer. *N Engl J Med* **2011**, *365* (26), 2484-2496. DOI: 10.1056/NEJMoa1103799 From NLM.
(23) Prat, J. FIGO's staging classification for cancer of the ovary, fallopian tube, and peritoneum: abridged republication. *J Gynecol Oncol* **2015**, *26* (2), 87-89.
(24) Rauh-Hain, J. A.; Krivak, T. C.; Del Carmen, M. G.; Olawaiye, A .B. Ovarian cancer screening and early detection in the general population. *Rev Obstet Gynecol* **2011**, *4* (1), 15-21. From NLM.
(25) Lengyel, E. Ovarian cancer development and metastasis. *Am J Pathol* **2010**, *177* (3), 1053-1064. DOI: 10.2353/ajpath.2010.10010 5From NLM.
(26) du Bois, A.; Reuss, A.; Pujade-Lauraine, E.; Harter, P.; Ray-Coquard, I.; Pfisterer, J. Role of surgical outcome as prognostic factor in advanced epithelial ovarian cancer: a combined exploratory analysis of 3 prospectively randomized phase 3 multicenter trials: by the Arbeitsgemeinschaft Gynaekologische Onkologie Studiengruppe Ovarialkarzinom (AGO-OVAR) and the Groupe d'Investigateurs Nationaux Pour les Etudes des Cancers de l'Ovaire (GINECO). *Cancer* **2009**, *115* (6), 1234-1244. DOI: 10.10/02cncr.24149 From NLM.
(27) Binder, M.; Roberts, C.; Spencer, N.; Antoine, D.; Cartwright, C. On the antiquity of cancer: evidence for metastatic carcinoma in a young man from ancient Nubia (c. 1200 BC). *PLoS One* **2014**, *9* (3), e90924. DOI: 10.1371/journal.pone.0090924 From NLM.
(28) Falzone, L.; Salomone, S.; Libra, M. Evolution of Cancer Pharmacological Treatments at the Turn of the Third Millennium. *Front Pharmacol* **2018**, *9*, 1300. DOI: 10.3389/fphar.2018.01300 From NLM.
(29) Connell, P. P.; Hellman, S .Advances in radiotherapy and implications for the next century: a historical perspective. *Cancer Res* **2009**, *69* (2), 383-392. DOI: 10.1158/0008-5472.Can-07-6871 From NLM.
(30) Smith, S. L. War! What is it good for? Mustard gas medicine. *Cmaj* **2017**, *189* (8 ,( E321-e322. DOI: 10.1503/cmaj.161032 From NLM.
(31)Weber, G. F. DNA Damaging Drugs. *Molecular Therapies of Cancer* **2014**, 9 - 112.
(32) Tiwari, M. Antimetabolites: established cancer therapy. *J Cancer Res Ther* **2012**, *8* (4), 510-519. DOI: 10.4103/0973-148 2.106526From NLM.
(33) Luengo, A.; Gui, D. Y.; Vander Heiden, M. G. Targeting Metabolism for Cancer Therapy. *Cell Chem Biol* **2017**, *24* (9), 1161-1180. DOI: 10.1016/j.chembiol.2017.08.028 From NLM.
(34) Brookes, P. The early history of the biological alkylating agents, 1918-1968. *Mutat Res* **1990**, *233* (1-2), 3-14. DOI: 10.1016/0027-5107(90)90145-t From NLM.
(35) Schirrmacher, V. From chemotherapy to biological therapy: A review of novel concepts to reduce the side effects of systemic cancer treatment (Review). *Int J Oncol* **2019**, *54* (2), 407-419. DOI: 10.3892/ijo.2018.4661 From NLM.
(36) Strebhardt, K.; Ullrich, A. Paul Ehrlich's magic bullet concept: 100 years of progress. *Nat Rev Cancer* **2008**, *8* (6), 473-480. DOI: 10.1038/nrc2394 From NLM.
(37) Zhong, L ;.Li, Y.; Xiong, L.; Wang, W.; Wu, M.; Yuan, T.; Yang, W.; Tian, C.; Miao, Z.; Wang, T.; et al. Small molecules in targeted cancer therapy: advances, challenges, and

future perspectives. *Signal Transduction and Targeted Therapy* **2021**, *6* (1), 201. DOI: 10.10/38s41392-021-00572-w.
(38) Roskoski, R., Jr. Properties of FDA-approved small molecule protein kinase inhibitors: A 2022 update. *Pharmacol Res* **2022**, *175*, 106037. DOI: 10.1016/j.phrs.2021.106037 From NLM.
(39) Nadler, L. M.; Stashenko, P.; Hardy, R.; Kaplan, W. D.; Button, L. N.; Kufe, D. W.; Antman, K. H.; Schlossman, S. F. Serotherapy of a patient with a monoclonal antibody directed against a human lymphoma-associated antigen. *Cancer Res* **1980**, *40* (9), 3147-3154. From NLM.
(40) Pierpont, T. M.; Limper ,C. B.; Richards, K. L. Past, Present, and Future of Rituximab-The World's First Oncology Monoclonal Antibody Therapy. *Front Oncol* **2018**, *8*, 163. DOI: 10.3389/fonc.2018.00163 From NLM.
(41) Swain, S. M.; Shastry, M.; Hamilton, E. Targeting HER2-positive breast cancer: advances and future directions. *Nature Reviews Drug Discovery* **2023**, *22* (2), 101-126. DOI: 10.1038/s41573-022-00579-0.
(42) Shuptrine, C. W.; Surana, R.; Weiner, L. M. Monoclonal antibodies for the treatment of cancer. *Semin Cancer Biol* **2012** .13-3 ,(1) 22 ,DOI: 10.1016/j.semcancer.2011.12.009 From NLM.
(43) Goydel, R. S.; Rader, C. Antibody-based cancer therapy. *Oncogene* **2021**, *40* (21), 3655-3664. DOI: 10.1038/s41388-021-01811-8 From NLM.
(44) Sengsayadeth, S.; Savani, B. N.; Oluwole, O.; Dholaria, B. Overview of approved CAR-T therapies, ongoing clinical trials, and its impact on clinical practice. *EJHaem* **2022**, *3* (Suppl 1), 6-10. DOI: 10.1002/jha2.338 From NLM.
(45) Marei, H. E.; Hasan, A.; Pozzoli, G.; Cenciarelli, C. Cancer immunotherapy with immune checkpoint inhibitors (ICIs): potential, mechanisms of resistance, and strategies for reinvigorating T cell responsiveness when resistance is acquired. *Cancer Cell Int* **2023**, *23* (1), 64. DOI: 10.1186/s12935-023-02902-0 From NLM.
(46) Shiravand, Y.; Khodadadi, F.; Kashani, S. M. A.; Hosseini-Fard, S. R.; Hosseini, S.; Sadeghirad, H.; Ladwa, R.; O'Byrne, K.; Kulasinghe, A. Immune Checkpoint Inhibitors in Cancer Therapy. *Curr Oncol* **2022**, *29* (5), 3044-3060. DOI: 10.3390/curroncol29050247 From NLM.
(47) Kamakura, D.; Asano, R.; Yasunaga, M. T Cell Bispecific Antibodies: An Antibody-Based Delivery System for Inducing Antitumor Immunity. *Pharmaceuticals (Basel)* **2021**, *14* (11). DOI: 10.3390/ph14111172 From NLM.
(48) Burt, R.; Warcel, D.; Fielding, A .K. Blinatumomab, a bispecific B-cell and T-cell engaging antibody, in the treatment of B-cell malignancies. *Hum Vaccin Immunother* **2019**, *15* (3), 594-602. DOI: 10.1080/21645515.2018.1540828 From NLM.
(49) Fu, Z.; Li, S.; Han, S.; Shi, C.; Zhang, Y. Antibody drug conjugate: the “biological missile” for targeted cancer therapy. *Signal Transduction and Targeted Therapy* **2022**, *7* (1), 93. DOI: 10.1038/s41392-022-00947-7.
(50) Bruins, W. S. C.; Zweegman, S.; Mutis, T.; van de Donk, N. Targeted Therapy With Immunoconjugates for Multiple Myeloma. *Front Immunol* **2020**, *11*, 1155. DOI: 10.3389/fimmu.2020.01155 From NLM.
(51) Caers, J.; Duray, E.; Vrancken, L.; Marcion, G.; Bocuzzi, V.; De Veirman, K.; Krasniqi, A.; Lejeune, M.; Withofs, N.; Devoogdt, N.; et al. Radiotheranostic Agents in Hematological Malignancies. *Front Immunol* **2022**, *13*, 911080. DOI: 10.3389/fimmu.2022.911080 From NLM.
(52) Lin, A. Y.; Dinner, S. N. Moxetumomab pasudotox for hairy cell leukemia: preclinical development to FDA approval. *Blood Adv* **20** .2910-2905 ,(19) 3 ,19DOI: 10.1182/bloodadvances.2019000507 From NLM.
(53) Aggarwal, D.; Yang, J.; Salam, M. A.; Sengupta, S.; Al-Amin, M. Y.; Mustafa, S.; Khan, M. A.; Huang, X.; Pawar, J. S. Antibody-drug conjugates: the paradigm shifts in the targeted

cancer therapy. *Front Immunol* **2023**, *14*, 1203073. DOI: 10.3389/fimmu.2023.1203073 From NLM.
(54) Maecker, H.; Jonnalagadda, V.; Bhakta, S.; Jammalamadaka, V.; Junutula, J. R. Exploration of the antibody-drug conjugate clinical landscape. *MAbs* **2023**, *15* ,(1) .2229101DOI: 10.1080/19420862.2023.2229101 From NLM.
(55) Najminejad, Z.; Dehghani, F.; Mirzaei, Y.; Mer, A. H.; Saghi, S. A.; Abdolvahab, M. H.; Bagheri, N.; Meyfour, A.; Jafari, A.; Jahandideh, S.; et al. Clinical perspective: Antibody-drug conjugates for the treatment of HER2-positive breast cancer. *Mol Ther* **2023**, *31* (7), 1874-1903. DOI: 10.1016/j.ymthe.2023.03.019 From NLM.
(56) Kondrashov, A.; Sapkota, S.; Sharma, A.; Riano, I.; Kurzrock, R.; Adashek, J. J. Antibody-Drug Conjugates in Solid Tumor Oncology: An Effectiveness Payday with a Targeted Payload. *Pharmaceutics* **2023**, *15* (8). DOI: 10.3390/pharmaceutics15082160 From NLM.
(57) Tong, J. T. W.; Harris, P. W. R.; Brimble, M. A.; Kavianinia, I. An Insight into FDA Approved Antibody-Drug Conjugates for Cancer Therapy. *Molecules* **2021**, *26* (19). DOI: 10.3390/molecules26195847 From NLM.
(58) Zhang, C.; Sheng, W.; Al-Rawe, M.; Mohiuddin, T. M.; Niebert, M.; Zeppernick, F.; Meihold-Heerlein, I.; Hussain, A. F. EpCAM- and EGFR-Specific Antibody Drug Conjugates for Triple-Negative Breast Cancer Treatment. *Int J Mol Sci* **2022**, *23* (11). DOI: 10.3390/ijms23116122 From NLM.
(59) Mohiuddin, T. M.; Zhang, C.; Sheng, W.; Al-Rawe, M.; Zeppernick, F.; Meinhold-Heerlein, I.; Hussain, A. F. Near Infrared Photoimmunotherapy: A Review of Recent Progress and Their Target Molecules for Cancer Therapy. *Int J Mol Sci* **2023**, *24* (3). DOI: 10.3390/ijms24032655 From NLM.
(60) Amoury, M.; Bauerschlag, D.; Zeppernick, F.; von Felbert, V.; Berges, N.; Di Fiore, S.; Mintert ,I.; Bleilevens, A.; Maass, N.; Bräutigam, K.; et al. Photoimmunotheranostic agents for triple-negative breast cancer diagnosis and therapy that can be activated on demand. *Oncotarget* **2016**, *7* (34), 54925-54936. DOI: 10.18632/oncotarget.10705 From NLM.
(61) Bauerschlag, D.; Meinhold-Heerlein, I.; Maass, N.; Bleilevens, A.; Bräutigam, K.; Al Rawashdeh, W.; Di Fiore, S.; Haugg, A. M.; Gremse, F.; Steitz, J.; et al. Detection and Specific Elimination of EGFR(+) Ovarian Cancer Cells Using a Near Infrared Photoimmunotheranostic Approach. *Pharm Res* **2017**, *34* (4), 696-703. DOI: 10.1007/s11095-017-2096-4 From NLM.
(62) Wagh, A.; Song, H.; Zeng, M.; Tao, L.; Das, T. K. Challenges and new frontiers in analytical characterization of antibody-drug conjugates. *MAbs* **20** .243-222 ,(2) 10 ,18DOI: 10.1080/19420862.2017.1412025 From NLM.
(63) Allahyari, H.; Heidari, S.; Ghamgosha, M.; Saffarian, P.; Amani, J. Immunotoxin: A new tool for cancer therapy. *Tumor Biology* **2017**, *39* (2), 1010428317692226. DOI: 10.1177/101042831769) 2226acccessed 2024/01/18.(
(64) Sasso, J. M.; Tenchov, R.; Bird, R.; Iyer, K. A.; Ralhan, K.; Rodriguez, Y.; Zhou, Q. A. The Evolving Landscape of Antibody-Drug Conjugates: In Depth Analysis of Recent Research Progress. *Bioconjug Chem* **2023**, *34* (11), 195 .2000-1DOI: 10.1021/acs.bioconjchem.3c00374 From NLM.
(65) Hamblett, K. J.; Senter, P. D.; Chace, D. F.; Sun, M. M.; Lenox, J.; Cerveny, C. G.; Kissler, K. M.; Bernhardt, S. X.; Kopcha, A. K.; Zabinski, R. F.; et al. Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin Cancer Res* **2004**, *10* (20), 7063-7070. DOI: 10.1158/1078-0432.Ccr-04-0789 From NLM.
(66) Agarwal, P.; Bertozzi, C. R. Site-specific antibody-drug conjugates: the nexus of bioorthogonal chemistry ,protein engineering, and drug development. *Bioconjug Chem* **2015**, *26* (2), 176-192. DOI: 10.1021/bc5004982 From NLM.
(67) Kang, M. S.; Kong, T. W. S.; Khoo, J. Y. X.; Loh, T. P. Recent developments in chemical conjugation strategies targeting native amino acids in proteins and their

applications in antibody-drug conjugates. *Chem Sci* **2021**, *12* (41), 13613-13647. DOI: 10.1039/d1sc02973h From NLM.
(68) Ritzefeld, M. Sortagging: a robust and efficient chemoenzymatic ligation strategy. *Chemistry* **2014**, *20* (28), 8 .8529-516DOI: 10.1002/chem.201402072 From NLM.
(69) Hussain, A. F.; Grimm, A.; Sheng, W.; Zhang, C.; Al-Rawe, M.; Bräutigam, K.; Abu Mraheil, M.; Zeppernick, F.; Meinhold-Heerlein, I. Toward Homogenous Antibody Drug Conjugates Using Enzyme-Based Conjugation Approaches. *Pharmaceuticals (Basel)* **2021**, *14* (4). DOI: 10.3390/ph14040343 From NLM.
(70) Kolberg, K.; Puettmann, C.; Pardo, A.; Fitting, J.; Barth, S. SNAP-tag technology: a general introduction. *Curr Pharm Des* **2013**, *19* (30), 5406-5413. DOI: 10.2174 138161281131999905 14/From NLM.
(71) Hussain, A. F.; Heppenstall, P. A.; Kampmeier, F.; Meinhold-Heerlein, I.; Barth, S. One-step site-specific antibody fragment auto-conjugation using SNAP-tag technology. *Nat Protoc* **2019**, *14* (11), 3101-3125. DOI: 10.103/8s41596-019-0214-y From NLM.
(72) Chouman, K.; Woitok, M.; Mladenov, R.; Kessler, C.; Weinhold, E.; Hanz, G.; Fischer, R.; Meinhold-Heerlein, I.; Bleilevens, A.; Gresch, G.; et al. Fine Tuning Antibody Conjugation Methods using SNAP-tag Technology. *Anticancer Agents Med Chem* **2017**, *17* (10), 1434-1440. DOI: 10.2174/1871520617666170213123737 From NLM.
(73) Hussain, A. F.; Amoury, M.; Barth, S. SNAP-tag technology: a powerful tool for site specific conjugation of therapeutic and imaging agents. *Curr Pharm Des* **2013**, *19* (30), 5437-5442. DOI: 10.2174/1381612811319300014 From NLM.
(74) Hussain, A. F.; Kampmeier, F.; von Felbert, V.; Merk, H. F.; Tur, M. K.; Barth, S. SNAP-tag technology mediates site specific conjugation of antibody fragments with a photosensitizer and improves target specific phototoxicity in tumor cells. *Bioconjug Chem* **2011**, *22* (12), 2487-2495. DOI: 10.1021/bc200304k From NLM.
(75) von Felbert, V.; Bauerschlag, D.; Maass, N.; Bräutigam, K.; Meinhold-Heerlein, I.; Woitok, M.; Barth, S.; Hussain, A. F. A specific photoimmunotheranostics agent to detect and eliminate skin cancer cells expressing EGFR. *J Cancer Res Clin Oncol* **2016**, *142* (5), 1003-1011. DOI: 10.1007/s00432-016-2122-7 From NLM.
(76) Wollschlaeger, C.; Meinhold-Heerlein, I.; Cong ,X.; Bräutigam, K.; Di Fiore, S.; Zeppernick, F.; Klockenbring, T.; Stickeler, E.; Barth, S.; Hussain, A. F. Simultaneous and Independent Dual Site-Specific Self-Labeling of Recombinant Antibodies. *Bioconjug Chem* **2018**, *29* (11), 3586-3594. DOI: 10.1021/acs.bioconjchem.8b00545 From NLM.
(77) Xu, L.; Kuan, S. L.; Weil, T. Contemporary Approaches for Site-Selective Dual Functionalization of Proteins. *Angew Chem Int Ed Engl* **2021**, *60* (25), 13757-13777. DOI: 10.1002/anie.202012034 From NLM.
(78) Ogawa, M.; Regino, C. A.; Seidel, J.; Green, M. V.; Xi, W.; Williams, M.; Kosaka, N.; Choyke, P. L.; Kobayashi, H. Dual-modality molecular imaging using antibodies labeled with activatable fluorescence and a radionuclide for specific and quantitative targeted cancer detection. *Bioconjug Chem* **2009**, *20* (11), 2177-2184. DOI: 10.1021/bc900362k From NLM.
(79) Wang, X.; Aldrich, M. B.; Marshall, M. V.; Sevick-Muraca, E. M. Preclinical characterization and validation of a dual-labeled trastuzumab-based imaging agent for diagnosing breast cancer. *Chin J Cancer Res* **2015**, *27* (1), 74-82. DOI: 10.3978/j.issn.1000-9604.2015.01.02 From NLM.
(80) Rijpkema, M.; Bos, D. L.; Cornelissen, A. S.; Franssen, G. M.; Goldenberg, D. M.; Oyen, W. J.; Boerman, O. C. Optimization of Dual-Labeled Antibodies for Targeted Intraoperative Imaging of Tumors. *Mol Imaging* **2015**, *14*, 348-355. From NLM.
(81) Strop, P.; Liu, S. H.; Dorywalska, M.; Delaria, K.; Dushin, R. G.; Tran, T. T.; Ho, W. H.; Farias, S.; Casas, M. G.; Abdiche, Y.; et al. Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. *Chem Biol* **2013**, *20* (2), 161-167. DOI: 10.1016/j.chembiol.2013.01.010 From NLM.

(82) Bhakta, S.; Raab, H.; Junutula, J. R. Engineering THIOMABs for site-specific conjugation of thiol-reactive linkers. *Methods Mol Biol* **2013**, *1045*, 189-203. DOI: 10.1007/978-1-62703-541-5_11 From NLM.
(83) Li, X.; Patterson, J. T.; Sarkar, M.; Pedzisa, L.; Kodadek, T.; Roush, W. R.; Rader, C. Site-Specific Dual Antibody Conjugation via Engineered Cysteine and Selenocysteine Residues. *Bioconjug Chem* **2015**, *26* (11), 2243-2248. DOI: 10.1021/acs.bioconjchem.5b00244 From NLM.
(84) Puthenveetil, S.; Musto, S.; Loganzo, F.; Tumey, L. N.; O'Donnell, C. J.; Graziani, E. Development of Solid-Phase Site-Specific Conjugation and Its Application toward Generation of Dual Labeled Antibody and Fab Drug Conjugates. *Bioconjug Chem* **2016**, *27* (4), 1030-1039. DOI: 10.1021/acs.bioconjchem.6b00054 From NLM.
(85) Wang, T.; Riegger, A.; Lamla, M.; Wiese, S.; Oeckl, P.; Otto, M.; Wu, Y.; Fischer, S.; Barth, H.; Kuan, S. L.; et al. Water-soluble allyl sulfones for dual site-specific labelling of proteins and cyclic peptides. *Chem Sci* **2016**, *7* (5), 3234-3239. DOI: 10.1039/c6sc00005c From NLM.
(86) Kim, J .S.; Jun, S. Y.; Kim, Y. S. Critical Issues in the Development of Immunotoxins for Anticancer Therapy. *J Pharm Sci* **2020**, *109* (1), 104-115. DOI: 10.1016/j.xphs.2019.10.037 From NLM.
(87) Li, M.; Liu, Z. S.; Liu, X. L.; Hui, Q.; Lu, S. Y.; Qu, L. L.; Li ,Y. S.; Zhou, Y.; Ren, H. L.; Hu, P. Clinical targeting recombinant immunotoxins for cancer therapy. *Onco Targets Ther* **2017**, *10*, 3645-3665. DOI: 10.2147/ott.S134584 From NLM.
(88) Li, M.; Mei, S.; Yang, Y.; Shen, Y.; Chen, L. Strategies to mitigate the on -and off-target toxicities of recombinant immunotoxins: an antibody engineering perspective. *Antib Ther* **2022**, *5* (3), 164-176. DOI: 10.1093/abt/tbac014 From NLM.
(89) Akbari, B.; Farajnia, S.; Ahdi Khosroshahi, S.; Safari, F.; Yousefi, M.; Dariushnejad ,H.; Rahbarnia, L. Immunotoxins in cancer therapy: Review and update. *Int Rev Immunol* **2017**, *36* (4), 207-219. DOI: 10.1080/08830185.2017.1284211 From NLM.
(90) Mungra, N.; Jordaan, S.; Hlongwane, P.; Naran, K.; Chetty, S.; Barth, S. Targeted human cytolytic fusion proteins at the cutting edge: harnessing the apoptosis-inducing properties of human enzymes for the selective elimination of tumor cells. *Oncotarget* **2019**, *10* (8), 897-915. DOI: 10.18632/oncotarget.26618 From NLM.
(91) Schiffer, S.; Hansen, H. P ;.Hehmann-Titt, G.; Huhn, M.; Fischer, R.; Barth, S.; Thepen, T. Efficacy of an adapted granzyme B-based anti-CD30 cytolytic fusion protein against PI-9-positive classical Hodgkin lymphoma cells in a murine model. *Blood Cancer Journal* **2013**, *3* (3), e106-e1 .06DOI: 10.1038/bcj.2013.4.
(92) Hristodorov, D.; Mladenov, R.; Pardo, A.; Pham, A. T.; Huhn, M.; Fischer, R.; Thepen, T.; Barth, S. Microtubule-associated protein tau facilitates the targeted killing of proliferating cancer cells in vitro and in a xenograft mouse tumour model in vivo. *Br J Cancer* **2013**, *109* (6), 1570-1578. DOI: 10.1038/bjc.2013.457 From NLM.
(93) Amoury, M.; Kolberg, K.; Pham, A. T.; Hristodorov, D.; Mladenov, R.; Di Fiore, S.; Helfrich, W.; Kiessling, F.; Fischer, R.; Pardo, A.; et al .Granzyme B-based cytolytic fusion protein targeting EpCAM specifically kills triple negative breast cancer cells in vitro and inhibits tumor growth in a subcutaneous mouse tumor model. *Cancer Lett* **2016**, *372* (2), 201-209. DOI: 10.1016/j.canlet.2016.01.027 From NLM.
(94) Amoury, M.; Mladenov, R.; Nachreiner, T.; Pham, A. T.; Hristodorov, D.; Di Fiore, S.; Helfrich, W.; Pardo, A.; Fey, G.; Schwenkert, M.; et al. A novel approach for targeted elimination of CSPG4-positive triple-negative breast cancer cells using a MAP tau-based fusion protein. *Int J Cancer* **2016**, *139* (4), 916-927. DOI: 10.1002/ijc.30119 From NLM.
(95) Lheureux, S.; Braunstein, M.; Oza, A. M. Epithelial ovarian cancer: Evolution of management in the era of precision medicine. *CA Cancer J Clin* **2019**, *69* (4), 280-304. DOI: 10.3322/caac.21559 From NLM.

(96) Miller, K. D.; Nogueira, L.; Devasia, T.; Mariotto, A. B.; Yabroff, K. R.; Jemal, A.; Kramer, J.; Siegel, R. L. Cancer treatment and survivorship statistics, 2022. *CA Cancer J Clin* **2022**, *72* .436-409 ,(5) DOI: 10.3322/caac.21731 From NLM.
(97) Hennigs, A.; Riedel, F.; Gondos, A.; Sinn, P.; Schirmacher, P.; Marmé, F.; Jäger, D.; Kauczor, H. U.; Stieber, A.; Lindel, K.; et al. Prognosis of breast cancer molecular subtypes in routine clinical care: A large prospective cohort study. *BMC Cancer* **2016**, *16* (1), 734. DOI: 10.1186/s12885-016-2766-3 From NLM.
(98) Matulonis, U. A.; Sood, A. K.; Fallowfield, L.; Howitt, B. E.; Sehouli, J.; Karlan, B. Y. Ovarian cancer. *Nat Rev Dis Primers* **2016**, *2*, 160 .61DOI: 10.1038/nrdp.2016.61 From NLM.
(99) Anand, U.; Dey, A.; Chandel, A. K. S.; Sanyal, R.; Mishra, A.; Pandey, D. K.; De Falco, V.; Upadhyay, A.; Kandimalla, R.; Chaudhary, A.; et al. Cancer chemotherapy and beyond: Current status, drug candidates ,associated risks and progress in targeted therapeutics. *Genes Dis* **2023**, *10* (4), 1367-1401. DOI: 10.1016/j.gendis.2022.02.007 From NLM.
(100) Chiu, M. L.; Goulet, D. R.; Teplyakov, A.; Gilliland, G. L. Antibody Structure and Function: The Basis for Engineering Therapeutics. *Antibodies* **2019**, *8* (4), 55.
(101) Schenk, S.; Schoenhals, G. J.; de Souza, G.; Mann, M. A high confidence, manually validated human blood plasma protein reference set. *BMC Med Genomics* **2008**, *1*, 41. DOI: 10.1186/1755-8794-1-41 From NLM.
(102) Kholodenko, R. V.; Kalinovsky, D. V.; Doronin, II; Ponomarev, E. D.; Kholodenko, I. V. Antibody Fragments as Potential Biopharmaceuticals for Cancer Therapy: Success and Limitations. *Curr Med Chem* **2019**, *26* (3), 396-426. DOI: 10.2174/09298673246661 70817152554From NLM.
(103) Ahmad, Z. A.; Yeap, S. K.; Ali, A. M.; Ho, W. Y.; Alitheen, N. B.; Hamid, M. scFv antibody: principles and clinical application. *Clin Dev Immunol* **2012**, *2012*, 980250. DOI: 10.1155/2012/980250 From NLM.
(104) Hoedemaeker, F. J ;.Signorelli, T.; Johns, K.; Kuntz, D. A.; Rose, D. R. A single chain Fv fragment of P-glycoprotein-specific monoclonal antibody C219. Design, expression, and crystal structure at 2.4 A resolution. *J Biol Chem* **1997**, *272* (47), 29784-29789. DOI: 10.1074/jbc 272.47.29784.From NLM.
(105) Gronemeyer, T.; Chidley, C.; Juillerat, A.; Heinis, C.; Johnsson, K. Directed evolution of O6-alkylguanine-DNA alkyltransferase for applications in protein labeling. *Protein Eng Des Sel* **2006**, *19* (7), 309-316. DOI: 10.1093/protein/gzl014 From NLM.
(106) Gronemeyer, T.; Godin, G.; Johnsson, K. Adding value to fusion proteins through covalent labelling. *Curr Opin Biotechnol* **2005**, *16* (4), 453-458. DOI: 10.1016/j.copbio.2005.06.001 From NLM.
(107) Juillerat, A.; Gronemeyer, T ;.Keppler, A.; Gendreizig, S.; Pick, H.; Vogel, H.; Johnsson, K. Directed evolution of O6-alkylguanine-DNA alkyltransferase for efficient labeling of fusion proteins with small molecules in vivo. *Chem Biol* **2003**, *10* (4), 313-317. DOI: 10.1016/s1074-5521(03 1-00068(From NLM.
(108) Wakankar, A.; Chen, Y.; Gokarn, Y.; Jacobson, F. S. Analytical methods for physicochemical characterization of antibody drug conjugates. *MAbs* **2011**, *3* (2), 161-172. DOI: 10.4161/mabs.3.2.14960 From NLM.
(109) Li, Z.; Li, Y.; Chang ,H. P.; Chang, H. Y.; Guo, L.; Shah, D. K. Effect of Size on Solid Tumor Disposition of Protein Therapeutics. *Drug Metab Dispos* **2019**, *47* (10), 1136-1145. DOI: 10.1124/dmd.119.087809 From NLM.
(110) Mazmanian, S. K.; Liu, G.; Ton-That, H.; Schneewind, O .Staphylococcus aureus sortase, an enzyme that anchors surface proteins to the cell wall. *Science* **1999**, *285* (5428), 760-763. DOI: 10.1126/science.285.5428.760 From NLM.
(111) Pan, L.; Zhao, W.; Lai, J.; Ding, D.; Zhang, Q.; Yang, X.; Huang, M.; Jin, S ;.Xu, Y.; Zeng, S.; et al. Sortase A-Generated Highly Potent Anti-CD20-MMAE Conjugates for Efficient Elimination of B-Lineage Lymphomas. *Small* **2017**, *13* (6). DOI: 10.1002/smll.201602267 From NLM.

(112) Xu, Y.; Jin, S.; Zhao, W.; Liu, W.; Ding, D.; Zhou, J ;.Chen, S. A Versatile Chemo-Enzymatic Conjugation Approach Yields Homogeneous and Highly Potent Antibody-Drug Conjugates. *Int J Mol Sci* **2017**, *18* (11). DOI: 10.3390/ijms18112284 From NLM.
(113) Antos, J. M.; Truttmann, M. C.; Ploegh, H. L. Recent advances in sortase-catalyzed ligation methodology. *Curr Opin Struct Biol* **2016**, *38*, 111-118. DOI: 10.1016/j.sbi.2016.05.021 From NLM.
(114) Liebscher, S.; Schöpfel, M.; Aumüller, T.; Sharkhuukhen, A.; Pech, A.; Höss, E.; Parthier, C.; Jahreis, G.; Stubbs, M. T ;.Bordusa, F. N-terminal protein modification by substrate-activated reverse proteolysis. *Angew Chem Int Ed Engl* **2014**, *53* (11), 3024-3028. DOI: 10.1002/anie.201307736 From NLM.
(115) Debon, A.; Siirola, E.; Snajdrova, R. Enzymatic Bioconjugation: A Perspective from the Pharmaceutical Industry. *JACS Au* **2023**, *3* (5), 1267-1283. DOI: 10.1021/jacsau.2c00617 From NLM.
(116) Strop, P. Versatility of microbial transglutaminase. *Bioconjug Chem* **2014**, *25* (5), 855-862. DOI: 10.1021/bc500099v From NLM.
(117) Khan, K .H. Gene expression in Mammalian cells and its applications. *Adv Pharm Bull* **2013**, *3* (2), 257-263. DOI: 10.5681/apb.2013.042 From NLM.
(118) Elmore, S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* **2007**, *35* (4), 495-516. DOI: 10.1080/01926 230701320337From NLM.
(119) Kashyap, B. K.; Singh, V. V.; Solanki, M. K.; Kumar, A.; Ruokolainen, J.; Kesari, K. K. Smart Nanomaterials in Cancer Theranostics: Challenges and Opportunities. *ACS Omega* **2023**, *8* (16), 14290-14320. DOI: 10.1021/acsomega.2c07 840From NLM.
(120) Kobayashi, H.; Choyke, P. L. Near-Infrared Photoimmunotherapy of Cancer. *Accounts of Chemical Research* **2019**, *52* (8), 2332-2339. DOI: 10.1021/acs.accounts.9b00273.
(121) Maruoka, Y.; Furusawa, A.; Okada, R.; Inagaki, F.; Fujimura, D ;.Wakiyama, H.; Kato, T.; Nagaya, T.; Choyke, P. L.; Kobayashi, H. Combined CD44- and CD25-Targeted Near-Infrared Photoimmunotherapy Selectively Kills Cancer and Regulatory T Cells in Syngeneic Mouse Cancer Models. *Cancer Immunol Res* **2020**, *8* (3), 345-355. DOI: 10.1158/2326-6066.Cir-19-0517 From NLM.
(122) Mitsunaga, M.; Ogawa, M.; Kosaka, N.; Rosenblum, L. T.; Choyke, P. L.; Kobayashi, H. Cancer cell-selective in vivo near infrared photoimmunotherapy targeting specific membrane molecules. *Nat Med* **2011**, *17* .1691-1685 ,(12) DOI: 10.1038/nm.2554 From NLM.
(123) Nagaya, T.; Nakamura, Y.; Okuyama, S.; Ogata, F.; Maruoka, Y.; Choyke, P. L.; Kobayashi, H. Near-Infrared Photoimmunotherapy Targeting Prostate Cancer with Prostate-Specific Membrane Antigen (PSMA) Antibody. *Mol Cancer Res* **2017**, *15* (9), 1153-1162. DOI: 10.1158/1541-7786.Mcr-17-0164 From NLM.
(124) Nakajima, T.; Sano, K.; Choyke, P. L.; Kobayashi, H. Improving the efficacy of Photoimmunotherapy (PIT) using a cocktail of antibody conjugates in a multiple antigen tumor model. *Theranostics* **2013**, *3* (6), 357-365. DOI: 10.7150/thno.5908 From NLM.
(125) Nishimura, T.; Mitsunaga, M.; Ito, K.; Kobayashi, H.; Saruta, M. Cancer neovasculature-targeted near-infrared photoimmunotherapy (NIR-PIT) for gastric cancer: different mechanisms of phototoxicity compared to cell membrane-targeted NIR-PIT. *Gastric Cancer* **2020**, *23* (1), 82-94. DOI: 10.1007/s10120-019-00988-y From NLM.
(126) Sun, X.-x.; Yu, Q. Intra-tumor heterogeneity of cancer cells and its implications for cancer treatment. *Acta Pharmacologica Sinica* **2015**, *36* (10), 1219-1227. DOI: 10.1038/aps.2015.92.
(127) Reddy, K. B. Triple-negative breast cancers: an updated review on treatment options. *Current oncology (Toronto, Ont.)* **2011**, *18* (4), e173-179. From NLM.
(128) Carey, L. A.; Rugo, H. S.; Marcom, P. K.; Mayer, E. L.; Esteva, F. J.; Ma, C. X.; Liu, M. C.; Storniolo, A. M.; Rimawi, M. F.; Forero-Torres, A.; et al. TBCRC 001: randomized

phase II study of cetuximab in combination with carboplatin in stage IV triple-negative breast cancer. *J Clin Oncol* **2012**, *30* (21), 2615-2623. DOI: 10.1200/jco.2010.34.5579 From NLM.
(129) Spizzo, G.; Obrist, P.; Ensinger, C.; Theurl, I.; Dunser, M.; Ramoni, A.; Gunsilius, E.; Eibl, G.; Mikuz, G.; Gastl, G. Prognostic significance of Ep-CAM AND Her-2/neu overexpression in invasive breast cancer. *Int J Cancer* **2002**, *98* (6), 883-888. From NLM.
(130) Peeters, D. J.; van Dam, P. J.; Van den Eynden, G. G.; Rutten, A.; Wuyts, H.; Pouillon, L.; Peeters, M.; Pauwels, P.; Van Laere, S .J.; van Dam, P. A.; et al. Detection and prognostic significance of circulating tumour cells in patients with metastatic breast cancer according to immunohistochemical subtypes. *Br J Cancer* **2014**, *110* (2), 375-383. DOI: 10.1038/bjc.2013.743 From NLM.
(131) Staudacher, A. H.; Brown, M. P. Antibody drug conjugates and bystander killing: is antigen-dependent internalisation required? *British Journal of Cancer* **2017**, *117* (12), 1736-1742. DOI: 10.1038/bjc.2017.367.
(132) Su, Z.; Xiao, D.; Xie, F.; Liu, L.; Wang, Y.; Fan, S.; Zhou, X.; Li, S. Antibody-drug conjugates: Recent advances in linker chemistry. *Acta Pharm Sin B* **2021**, *11* (12), 3889-3907. DOI: 10.1016/j.apsb.2021.03.042 From NLM.
(133) Balamkundu, S.; Liu, C. F. Lysosomal-Cleavable Peptide Linkers in Antibody-Drug Conjugates. *Biomedicines* **2023**, *11* (11). DOI: 10.3390/biomedicines11113080 From NLM.
(134) Abdollahpour-alitappeh, M.; Amanzadeh, A.; Heidarnejad, F.; Habibi-Anbouhi, M.; Lotfinia, M.; Razavi-vakhshourpour, S.; Jahandideh, S.; Najminejad, H ;.Balalaie, S.; Abolhassani, M. Monomethyl Auristatin E, a Potent Cytotoxic Payload for Development of Antibody-Drug Conjugates against Breast Cancer. *Novelty in Biomedicine* **2017**, *5* (3), 98-103. DOI: 10.22037/nbm.v5i3.15523 (acccessed 2024/02/28.(
(135) Hossein-Nejad-Ariani, H.; Althagafi, E.; Kaur, K. Small Peptide Ligands for Targeting EGFR in Triple Negative Breast Cancer Cells. *Scientific Reports* **2019**, *9* (1), 2723. DOI: 10.1038/s41598-019-38574-y.
(136) Burns, K. E.; Robinson, M. K.; Thévenin, D. Inhibition of cancer cell proliferation and breast tumor targeting of pHLIP-monomethyl auristatin E conjugates. *Mol Pharm* **2015**, *12* (4), 1250-1258. DOI: 10.1021/mp500779k From NLM.
(137) Subik, K.; Lee, J. F.; Baxter, L.; Strzepek, T.; Costello, D.; Crowley ,P.; Xing, L.; Hung, M. C.; Bonfiglio, T.; Hicks, D. G.; et al. The Expression Patterns of ER, PR, HER2, CK5/6, EGFR, Ki-67 and AR by Immunohistochemical Analysis in Breast Cancer Cell Lines. *Breast Cancer (Auckl)* **2010**, *4*, 35-41. From NLM.
(138) Martowicz ,A.; Spizzo, G.; Gastl, G.; Untergasser, G. Phenotype-dependent effects of EpCAM expression on growth and invasion of human breast cancer cell lines. *BMC Cancer* **2012**, *12*, 501. DOI: 10.1186/1471-2407-12-501 From NLM.
(139) Hristodorov, D.; Amoury, M.; Mladenov, R.; Niesen, J.; Arens, K.; Berges, N.; Hein, L.; Di Fiore, S.; Pham, A. T.; Huhn, M.; et al. EpCAM-selective elimination of carcinoma cells by a novel MAP-based cytolytic fusion protein. *Mol Cancer Ther* **2014**, *13* (9), 2194-2202. DOI: 10.1158/1535-7.163Mct-13-0781 From NLM.
(140) Kampmeier, F.; Niesen, J.; Koers, A.; Ribbert, M.; Brecht, A.; Fischer, R.; Kiessling, F.; Barth, S.; Thepen, T. Rapid optical imaging of EGF receptor expression with a single-chain antibody SNAP-tag fusion protein. *Eur J Nucl Med Mol Imaging* **2010**, *37* (10), 1926-1934. DOI: 10.1007/s00259-010-1482-5 From NLM.
(141) Azhdarinia, A.; Ghosh, P.; Ghosh, S.; Wilganowski, N.; Sevick-Muraca, E. M. Dual-labeling strategies for nuclear and fluorescence molecular imaging: a review and analysis. *Mol Imaging Biol* **2012**, *14* (3), 261-276. DOI: 10.1007/s11307-011-0528-9 From NLM.
(142) Yamazaki, C. M.; Yamaguchi, A.; Anami, Y.; Xiong, W.; Otani, Y.; Lee, J.; Ueno, N. T.; Zhang, N.; An, Z.; Tsuchikama, K. Antibody-drug conjugates with dual payloads for combating breast tumor heterogeneity and drug resistance. *Nature Communications* **2021**, *12* (1), 3528. DOI: 10.1038/s41467-021-23793-7.
(143) Schmid, B.; Chung, D. E.; Warnecke, A.; Fichtner, I.; Kratz, F. Albumin-binding prodrugs of camptothecin and doxorubicin with an Ala-Leu-Ala-Leu-linker that are cleaved by

cathepsin B: synthesis and antitumor efficacy. *Bioconjug Chem* **2007**, *18* (3), 702-716. DOI: 10.1021/bc0602735 From NLM.
(144) Lou, H.; Cao, X. Antibody variable region engineering for improving cancer immunotherapy. *Cancer Commun (Lond)* **2022**, *42* (9), 804-827. DOI: 10.1002/cac2.12330 From NLM.
(145) Schwenkert, M.; Birkholz, K.; Schwemmlein, M.; Kellner, C.; Kügler, M.; Peipp, M.; Nettelbeck, D. M.; Schuler-Thurner, B.; Schaft, N.; Dörrie, J.; et al. A single chain immunotoxin, targeting the melanoma-associated chondroitin sulfate proteoglycan, is a potent inducer of apoptosis in cultured human melanoma cells. *Melanoma Res* **2008**, *18* (2), 73-84. DOI: 10.1097/CMR.0b013e3282f7c8f9 From NLM.
(146) Wang, X.; Osada, T.; Wang, Y.; Yu, L.; Sakakura, K.; Katayama, A.; McCarthy, J. B.; Brufsky, A.; Chivukula, M.; Khoury, T.; et al. CSPG4 protein as a new target for the antibody-based immunotherapy of triple-negative breast cancer. *J Natl Cancer Inst* **2010**, *102* (19), 1496-1512. DOI: 10.1093/jnci/djq343 From NLM.

# 7. ERKLÄERUNG ZUR HABILITATION

Hiermit erkläre ich, dass ich die vorliegende Arbeit bzw. die mir zuzuordnenden Teile im Rahmen einer kumulativen Habilitationsschrift, selbstständig und ohne zulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nichtveröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Ich versichere, dass ich für die nach §2 (3) der Habilitationsordnung angeführten bereits veröffentlichten Originalarbeiten als Erst- oder Seniorautor fungiere, da ich den größten Teil der Daten selbst erhoben habe, für das Design der Arbeiten verantwortlich bin und die Manuskripte maßgeblich gestaltet habe. Für alle von mir erwähnten Untersuchungen habe ich die in der „Satzung der Justus-Liebig-Universität zur Sicherung guter wissenschaftlicher Praxis“ niedergelegten Grundsätze befolgt. Ich versichere, dass alle an der Finanzierung der Arbeiten beteiligten Geldgeber in den jeweiligen Publikationen genannt worden sind. Ich versichere außerdem, dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Weise einer anderen Prüfungsbehörde vorgelegt wurde oder Gegenstand eines anderen Prüfungsverfahrens war. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.

Gießen,

Dr. rar. nat. Ahmad Fawzi Hussain

# 8. Abbreviations list

| Abbreviation | Term |
|---|---|
| [%] | Percent |
| °C | Degree Celsius |
| µg | Microgram |
| µl | Microgram |
| nM | Nanomolar |
| 90Y | Radioactive isotope yttrium 90 |
| ADC | Antibody drug conjugate |
| ADCC | Antibody-dependent cellular cytotoxicity |
| ADCP | Antibody-dependent cellular phagocytosis |
| AGT | O6-Alkylguanine-DNA alkyltransferase |
| B-ALL | B-cell acute lymphoblastic leukemia |
| B-NHL | B-cell non-Hodgkin lymphoma |
| BCL-2 | B-cell leukemia/lymphoma-2 |
| BCMA | B-cell maturation antigen |
| BG | O(6)-benzylguanine |
| CAIX | Carbonic anhydrase IX |
| CAR-T | Chimeric antigen receptor T cell |
| CDC | Complement dependent cytotoxicity |
| CEACAM5 | Carcinoembryonic antigen-related cell adhesion molecule 5 |
| CTLA4 | Cytotoxic T-lymphocyte-associated protein 4 |
| CSPG4 | Chondroitin sulfate proteoglycan 4 |
| DAR | Drug-to-antibody ratios |
| DNA | Deoxyribonucleic acid |
| DT | Diphtheria toxin |
| DTPA | Diethylene triamine penta-acetic acid |
| EGFR | Epidermal growth factor receptor 1 |
| EpCam | Epithelial cell adhesion molecule |
| ER | Estrogen receptor |
| FDA | Food and Drug Administration |
| FIGO | International federation of gynecology and obstetrics |
| Gb | Granzyme B |
| HER2 | Human epidermal growth factor receptor 2 |
| $IC_{50}$ | 50% elimination of the cells |
| ICG | Indocyanine green |
| ICI | Immune checkpoint inhibitor |
| mAb | Monoclonal antibody |
| MAP | Microtubule-associated protein tau |
| MHC | Major histocompatibility complex |
| MMAE | Monomethyl auristatin E |
| MMAF | Monomethyl auristatin F |
| NIR | Near-infrared |
| NHL | Non-Hodgkin lymphoma |
| NIR-PIT | Near-infrared photoimmunotherapy |
| PgR | Estrogen receptor |
| PARP | Poly-ADP ribose polymerase |
| PD1 | Programmed cell death protein 1 |
| PD-L1 | Programmed cell death-ligand1 |
| PE | Pseudomonas exotoxin A |
| PK | Pharmacokinetic |
| PI-9 | Serpin B9 |
| R201K | Mutant version of granzyme B with strongly reduced sensitivity toward PI-9 |
| RNA | Ribonucleic acid |

| | |
|---|---|
| RNases | Ribonucleases |
| scFv | Single-chain variable fragment |
| T-biAbs | T-cell engaging bispecific antibodies |
| TCR | T cell receptor |
| TNBC | Triple negative breast cancer |
| Trop-2 | Trophoblast cell surface antigen-2 |

# 9. Acknowledgments

It would not have been possible to complete this work without the support and the assistance of the nice people around me, to only some of whom it is possible to give particular mention here.

First of all, I would like to gratefully and sincerely thank my wonderful family, specially, my parents for their faith in me and their encouragement from the very beginning. I cannot even begin to express how thankful I am for my wife Marwah for her encouragement, support, and great patience at all times.

I am sincerely grateful and appreciative to Prof. Dr. Ivo Meinhold-Heerlein for his supervision, understanding, and his friendship during my work at the RWTH-Aachen University Hospital and Medical Faculty, Justus-Liebig-University Giessen. Also, I would like to thank Prof. Dr. Stefan Barth for his kind supervision. I am most grateful for all collaboration partners for their kind collaboration and support.

I am so grateful to my colleagues in of the RWTH-Aachen University Hospital and Medical Faculty, Justus-Liebig-University Giessen for the amazing time both inside and outside the lab.