Systemic immune dynamics in cancer

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CHAPTER 9

Discussion

Translational Research: Bridging the Gap Between the Lab and Clinic

In the modern landscape of biomedical science, a significant gap often exists between clinical practice and fundamental research. Clinical trials are pivotal for validating new interventions, ensuring their safety and efficacy. However, these trials often provide limited mechanistic insight, leaving many unanswered questions about how and why certain treatments work or do not work. Translational research—the crucial link between basic science and clinical application—plays a vital role in bridging this gap. It is particularly important in the context of immuno-oncology, where understanding the complex interplay between the immune system and cancer can lead to extremely durable remissions.

Clinical trials assess treatment efficacy through outcomes like safety, toxicity, tumor volume, and survival but often fail to reveal the biological mechanisms behind their success or failure. For example, immunotherapy has revolutionized cancer treatment, yet responses vary dramatically among patients. Some exhibit long-term remission, while others experience primary resistance or relapse. Without deeper mechanistic insight into immune cell behavior, tumor microenvironment dynamics, and genetic mutations, it remains challenging to predict which patients will benefit most. This limited understanding can impede the development of personalized treatment strategies. While a therapy may demonstrate clinical efficacy, understanding the specific immune pathways it targets is essential for refining its application, reducing side effects, and enhancing patient outcomes. Here, the importance of fundamental and translational research becomes clear.

Moreover, translational research takes discoveries from the lab—such as insights into immune cell signaling or tumor-immune interactions—and applies them to develop new treatment strategies. For instance, experiments using genetically engineered mouse models have revealed key details about the mechanisms of immune cell activation and suppression in the tumor microenvironment[1-3]. These findings can inspire new clinical trials. In immuno-oncology, fundamental and translational research has been instrumental in advancing therapies such as immune checkpoint inhibitors. By understanding the mechanisms of immune evasion employed by tumors, researchers have identified novel targets for intervention, leading to next-generation immunotherapies that can potentially overcome resistance. This coupling of mechanistic insights with clinical observations allows for a more personalized treatment approach, helping explain the varied responses seen in patients and identifying new avenues for therapeutic development. An example of this "from bench to bedside and back again" principle is described in this thesis in **Chapter 7**, where fundamental research both informs and is informed by clinical trial outcomes[4].

Mechanistic research clarifies how and why treatments work, enabling the development of targeted, effective therapies with fewer side effects. In the context of cancer immunotherapy, this might involve tailoring treatments based on an individual's unique immune landscape, tumor genetics, or environmental factors. By unraveling the mechanisms

and regulation of neutrophil migration in cancer for example, researchers can identify potential therapeutic targets. Modulating neutrophil migration may offer new strategies to manipulate the tumor microenvironment, enhance anti-tumor immune responses, and ultimately impact cancer progression and treatment outcomes.

To fully unlock the potential of translational research, strong collaborations between clinical and fundamental researchers are essential. Interdisciplinary teams combining clinical insight with deep mechanistic expertise can accelerate the development of novel treatment strategies. Furthermore, feedback loops between the clinic and the lab are crucial—clinical observations must inform fundamental research, and laboratory discoveries must be rapidly integrated into clinical trials. Fostering strong collaborations between clinical and fundamental scientists, has proven to be essential to keep advancing cancer immunotherapy. This thesis was shaped in such a collaborative work environment, serving as a testament to how strong partnerships can drive significant advancements in both scientific progress and the patient care of the future.

The influence of breast cancer subtype and disease stage on the circulating immune landscape

Cancer dysregulates intratumoral innate-adaptive immune cell crosstalk[5,6], but how the systemic immune landscape is altered during breast cancer progression remains largely unknown. Understanding these systemic immune modifications is crucial for uncovering mechanisms driving tumor growth, metastasis formation, and treatment resistance. A deeper insight into circulating immune alterations is essential for developing more precise and effective immunotherapeutic strategies. In **Chapters 3** and **4**, we investigated the circulating immune compartment in breast cancer patients compared to healthy donors (HDs) to determine how different stages of disease progression shape systemic immunity. We found that more advanced disease stages were associated with greater immune dysregulation, particularly characterized by immunosuppressive shifts. For example, we observed increases in neutrophils and (non-)classical monocytes, both described to be linked to poor prognosis and known to inhibit anti-tumor immune responses[7-13]. Transcriptional and proteomic analysis, alongside *ex vivo* functionality assays, of freshly isolated peripheral neutrophils revealed increased migratory capacity, higher abundance of granule proteins, and elevated ROS production in patients with mTNBC compared to HDs. The increased migratory capacity of neutrophils was already evident in patients with stage I-III TNBC. This means that neutrophils in patients with TNBC are not only more abundant, but are also phenotypically and functionally aberrant. Furthermore, we reported a reduction in CD8+ and CD4+ T cells and differentiated B cells. These changes could be partly driven by prior treatment, which we described for the triple negative subtype in **Chapter 4**. These findings align with previous reports that the immune system becomes progressively compromised

as the tumor grows and metastasizes, allowing the cancer to evade immune surveillance[5,14-17].

In addition, we explored the relationship between breast cancer subtypes and the circulating immune compartment. We hypothesized that distinct immune profiles would correlate with specific tumor subtypes, potentially providing insights into prognosis and therapeutic opportunities. Although we identified some subtype-specific immune changes, the influence of tumor subtype on the circulating immune compartment was less pronounced than expected. This may be partly due to the heterogeneity within subtypes, as some ER+ tumors exhibit basal-like transcriptional profiles and share characteristics with TNBC. In addition, also tumor-extrinsic factors may contribute to shaping the systemic immune landscape. This was well illustrated by the natural variation in immune profiles among HDs, which could be influenced by factors such as germline genetics, lifestyle, pathogenic infections, microbiome composition, and hormonal fluctuations. The relatively modest subtype-specific differences in circulating immune profiles suggest that disease stage may play a more pivotal role in systemic immune alterations than the tumor's molecular characteristics. While the systemic immune profiles provide valuable insights, it seems to be less related to breast cancer subtype than anticipated. This raises an important question for future research: should the focus shift toward tumor-intrinsic factors, such as mutations and gene expression profiles instead of focusing on the molecular tumor subtype?

The Role of Tumor Genetics in Shaping Immune Responses and Therapeutic Outcomes

Cancer cell intrinsic features, like the genetic makeup of the tumor, are increasingly recognized as a critical determinant of its behavior, immunogenicity, and therapeutic response[18-20]. Genomic and molecular studies have identified tumor-specific alterations, including mutations, copy number variations, and epigenetic modifications, that drive cancer progression and influence tumor-immune interactions. Mutations in TP53, KRAS, or PIK3CA can affect antigen presentation and immune cell recruitment to the tumor microenvironment (TME)[21-23]. Similarly, epigenetic changes, such as DNA methylation and histone modifications, modulate the expression of immune checkpoint molecules and cytokines, shaping immune evasion strategies[24-26]. Systemic immune changes, including T cell dysfunction and dysregulated cytokine levels, are integral to cancer progression, but understanding tumor-intrinsic factors enhances insights into the immune contexture of the TME. After all, the presence of tumor-specific neoantigens, arising from somatic mutations, can strongly dictate the tumor's immunogenic potential. Tumors with a high mutational burden or mismatch repair deficiencies often exhibit robust T cell infiltration and improved responses to checkpoint inhibitors[27-30]. Conversely, tumors with low immunogenicity, such as those with PTEN loss or Wnt/β-catenin pathway activation, are linked to immune exclusion and therapy resistance[31-34]. By focusing on the genetic landscape of tumors, researchers and clinicians may be able to identify actionable biomarkers and therapeutic targets. Genomic profiling

facilitates the development of precision medicine strategies, such as the use of PARP inhibitors in *BRCA*-mutant cancers or immune checkpoint inhibitors in tumors with high microsatellite instability. This tailored approach enables more precise treatment by exploiting tumor-intrinsic vulnerabilities.

In essence, integrating systemic immune modulation with tumor-specific genetic and molecular analyses offers a comprehensive understanding of cancer immunobiology. Systemic immune changes play essential roles in cancer progression, while tumor-intrinsic features, including mutations and gene expression profiles, shape the tumor microenvironment and guide therapeutic strategies. Our data show that in breast cancer, tumor molecular subtypes only have a modest impact on the circulating immune compartment compared to disease stage. However, the tumor's genetic landscape remains critical for advancing personalized therapies that integrate systemic and localized approaches to improve clinical outcomes.

Exploring Immune Cell States in mTNBC: Methodological Insights

Chapter 5 highlights the challenges and insights associated with analyzing systemic immune dysregulation in mTNBC using single-cell RNA-sequencing and matched TCR/BCR sequencing. Despite extensive analysis, no significant transcriptional differences or unique cell states were identified between patients with mTNBC and HDs, likely due to limited sample size and substantial inter-individual variability. Even though not disease-specific, the identification of eight distinct neutrophil states highlights the potential for further exploration of neutrophil diversity in cancer. This is particularly relevant given the clear differences observed in our bulk RNA sequencing data between neutrophils isolated from patients with mTNBC and HDs.

Key methodological insights derived from the experiments described in this chapter, include the benefits of leveraging barcoded antibodies to minimize batch effects, accurately retain low-RNA content cells like neutrophils, and enhance doublet removal. These approaches provide a framework for improving data quality and capturing underrepresented cell populations in future studies. To address the limitations encountered, future research should prioritize increased sample sizes and improved sequencing depth for neutrophils by targeted pre-processing of immune cell populations. Purifying neutrophils and integrating transcriptomic data with functional studies could yield critical insights into the role of neutrophil states in mTNBC. These refinements hold promise for uncovering immune dysregulation in cancer and advancing strategies for immune modulation in oncology.

Targeting Neutrophils: Harnessing Plasticity in Cancer Immunotherapy

Despite the growing attention directed toward neutrophils in recent years, their role in modulating cancer progression and immunotherapy responses remains a topic of considerable debate[35,36]. This ongoing controversy is largely attributable to the remarkable

heterogeneity and plasticity of neutrophils, which exhibit diverse phenotypes and functions depending on factors such as tumor subtype, disease stage, and the type of therapy employed[37-39]. The dynamic and context-dependent nature of neutrophils complicates efforts to define their exact role in either promoting or hindering therapeutic efficacy. Moreover, our understanding of their contribution to cancer progression and immunotherapy response is further hampered by the scarcity of robust tools for selectively and effectively targeting neutrophils in preclinical mouse models[40-42]. Although the studies are not always entirely unambiguous, accumulating data suggests that neutrophils in (breast) cancer predominantly exhibit a pro-tumorigenic functionality[43]. Previous research from our lab and others demonstrated that tumor-induced neutrophils promote mammary tumor progression and metastatic spread in mice[20,44-46]. In line with this, patients with TNBC with increased neutrophil-to-lymphocyte ratio (NLR) have a worse clinical prognosis[12]. Our data of **Chapter 4** demonstrate that neutrophils were the most profoundly and significantly increased immune cell type in the circulation of patients with mTNBC compared to HDs. This triggers the question whether it is possible to target neutrophils in cancer patients.

Reducing Neutrophil Migration

Since neutrophils are an indispensable part of the body's first line of defense against pathogenic infections, simply depleting a substantial proportion of the neutrophils in circulation is not a viable option. Therefore, one particular line of research is directed towards interfering with neutrophil recruitment to the TME: neutrophil migration. Data presented in **Chapter 4** further support that targeting neutrophil migration might be an interesting approach, because we showed that neutrophils from patients with mTNBC exhibit increased migratory capacity compared to those from healthy donors[47]. Given the central role of CXCR2 in guiding neutrophil migration to sites of inflammation and the TME, it has emerged as a promising therapeutic target. CXCR2 antagonists aim to reduce neutrophil migration, without compromising their systemic availability and functionality[48,49]. Preclinical studies in mouse models have shown that CXCR2 inhibition reduces neutrophil infiltration into tumors, decreasing their pro-tumorigenic activities, such as promoting angiogenesis and facilitating metastasis. For instance, Steele *et al.* demonstrated that pharmacological inhibition of CXCR2 in murine pancreatic cancer reduced tumor growth and metastasis by limiting neutrophil infiltration and enhancing T cell responses[50]. Similarly, in breast cancer models, inhibiting CXCR2 limited neutrophil recruitment and improved the efficacy of immune checkpoint inhibitors[51]. In human studies, CXCR1/2 antagonists, such as reparixin, have shown promise in early-phase trials. Reparixin, investigated in HER2-negative metastatic breast cancer, demonstrated safety, tolerability, and a 30% overall response rate with durable responses lasting over 12 months[52]. The CXCR2 antagonist navarixin, combined with pembrolizumab, failed to show efficacy in advanced cancers like prostate, colorectal, or lung cancer[53]. Another trial with the CXCR2 inhibitor AZD5069 and enzalutamide in metastatic castration-resistant prostate cancer (CRPC) was well tolerated and reduced neutrophil and myeloid cell infiltration, with some patients experiencing durable benefits[54]. These findings support targeting myeloid chemotaxis in metastatic CRPC and other cancers, but varied trial results highlight the need for further clinical evaluation to identify optimal patient populations.

Reducing Pro-Tumorigenic Inflammation

Beyond inhibition of neutrophil migration, targeting inflammatory mediators that mobilize neutrophils from the bone marrow has emerged as another promising approach to modulate neutrophil biology in cancer. IL-1β and other tumor-derived pro-inflammatory mediators (G-CSF, IL-6) signal to the bone marrow, altering hematopoiesis and increasing myeloid cells, especially neutrophils[5,20,44,55,56]. In the CANTOS trial (NCT01327846)[57], a monoclonal antibody targeting IL-1β called canakinumab was investigated in over 10,000 participants, primarily for cardiovascular outcomes. Unexpectedly, IL-1β inhibition reduced lung cancer incidence and mortality, prompting further trials[58]. However, initial adjuvant trials failed to meet efficacy goals[59-61]. Since systemic inflammation and neutrophilia are more pronounced in metastatic disease, IL-1β targeting may be more effective in advanced cancer. Another IL-1α/β inhibitor called anakinra has until now only been studied in clinical trials for patients with non-cancer inflammatory diseases like rheumatoid arthritis, hidradenitis suppurativa and COVID-19 with a favorable safety profile[62-65]. Its safety in cancer-related contexts requires additional clinical studies to fully evaluate and confirm potential long-term risks and benefits. In a mouse model of lung adenocarcinoma, blocking IL-1β slowed tumor progression, and targeting both IL-1α and IL-1β using anakinra prevented tumor initiation. The study suggests that targeting IL-1α and IL-1β with anakinra could help disrupt tumor growth and progression[66]. Further research is needed to confirm the benefits of targeting IL-1α and/or IL-1β in larger clinical cohorts and to determine the optimal timing for treatment, including its potential use as a preventative strategy in high-risk patient groups.

Harnessing Neutrophil Plasticity: From Tumor Promotion to Suppression

Another, potentially even more challenging yet promising line of research focusses on the phenotype conversion of neutrophils, turning them from a pro-tumorigenic into an anti-tumorigenic cell type[67,68]. Neutrophils have the potential to eliminate target cells through phagocytosis and directly kill cancer cells through the release of granules and via a process called antibody-dependent cellular cytotoxicity (ADCC)[69]. Neutrophil biology is influenced by a variety of factors such as cytokine signals like TGF-β and IFN-β. Studies suggest that TGF-β inhibition promotes anti-tumorigenic properties of neutrophils, enhancing anti-tumor

immunity and IFN-β supplementation can repolarize pro-tumoral neutrophils into a more anti-tumor state[68,70,71]. However, this classification of pro- and anti-tumorigenic neutrophils may oversimplify neutrophil diversity, as phenotypic plasticity exists within both mature and immature neutrophils. Recent studies also show that neutrophils can be influenced by immunotherapies, such as checkpoint inhibitors or tyrosine kinase inhibitors, which may alter their tumor-promoting or tumor-suppressing functions[72,73]. The exact origin of the phenotypic changes observed in neutrophils—whether occurring in fully matured cells or during the differentiation of progenitors—remains under investigation[69]. The growing understanding of neutrophil plasticity highlights their potential as therapeutic targets, with manipulation of their polarization offering promising strategies to enhance anti-tumor immunity.

Immune Profiling in Clinical Trials: Advancing Immunotherapy Treatment

Over the past decade, immunotherapy has revolutionized cancer treatment by targeting the immune system. While much research has focused on local immune responses within the TME, effective antitumor immunity requires ongoing coordination with the peripheral immune system[74]. In **Chapters 6**, the potential of short-term immune checkpoint blockade (ICB) was explored, to induce immune activation in patients with early-stage TNBC (BELLINI trial). The aim was to explore the potential of treating non-metastatic TNBC patients with neoadjuvant ICB in the absence of chemotherapy. The translational research project described in **chapter 7** aims to identify factors associated with the response to PD-1 blockade in patients with mTNBC (TONIC trial). I focused on the role of the circulating immune compartment in relation to ICB treatment in breast cancer. My primary objective was to identify baseline immune profiles that could serve as predictive biomarkers for treatment response. Specifically, we sought to determine whether particular (combinations of) immune cell populations in the blood could predict the efficacy of ICB therapy. However, after extensive analysis, we were unable to identify any reliable predictive biomarkers in blood that could be associated with treatment outcomes. This negative finding highlights the complexity of the immune response to ICB and suggests that predictive markers may reside in other compartments, such as the tumor microenvironment (TME) and/or lymph nodes, or be more dependent on dynamic changes during treatment.

Eosinophils in Cancer Immunotherapy: Enhancing ICB Response

In addition to baseline profiling, we examined the effects of ICB on the broader immune landscape during treatment. Notably, we observed a significant increase in circulating eosinophils upon ICB treatment in patients with TNBC who responded to ICB, a phenomenon absent in non-responders (**Chapter 7**). This discovery prompted further investigation into the role of eosinophils in the context of anti-tumor immunity. Mechanistic studies using

genetically engineered mouse models that develop spontaneous mammary tumors provided critical insights. We demonstrated that CD4+ T cell-derived IL-5 drives systemic eosinophil expansion, enabling their infiltration into the TME upon interleukin 33 (IL-33) induction. Within the TME, eosinophils actively contribute to an anti-tumor immune response by supporting CD8+ T cell-mediated tumor control (**Chapter 7**). This demonstrates that eosinophils are not merely passive bystanders but are actively involved in the anti-tumor immune response, contributing to the success of ICB in a subset of patients. Also in other cancer types like melanoma , eosinophil accumulation has been shown to positively correlate to ICB treatment outcomes[75].

The identification of eosinophils as key players in the immune response to tumors opens up new avenues for improving the efficacy of ICB. The fact that an increase in eosinophils upon ICB treatment is associated with response suggests that strategies aimed at modulating eosinophil activity in the TME could further enhance therapeutic outcomes. Future research efforts are therefore directed toward elucidating the precise mechanisms by which eosinophils contribute to the therapeutic benefits of ICB. A deeper understanding of how eosinophils interact with other immune cells, such as T helper cells, and how they influence the tumor milieu will be critical for developing new therapeutic approaches.

One of the key questions for future research is whether non-responders to ICB can be converted into responders by actively engaging eosinophils. This will require innovative strategies to recruit and activate eosinophils within the tumor. One potential approach is the use of intra-tumoral delivery of cytokines such as IL-33, which is known to attract and activate eosinophils. By enhancing eosinophil recruitment and activation in the TME, we may be able to convert immunologically “cold” tumors into “hot” tumors, thus improving the response to ICB. In summary, while circulating biomarkers for ICB response remain elusive, the role of eosinophils within the TME offers a promising new direction for improving cancer immunotherapy outcomes.

Exploring Chemotherapy-Free Immunotherapy in TNBC

To better understand how ICB can be leveraged in the treatment of early-stage TNBC, novel approaches are being explored. The aim was to explore the potential of treating early-stage TNBC patients with neoadjuvant ICB in the absence of chemotherapy. **Chapter 6** of this thesis discusses the BELLINI trial (trial registration number NCT03815890)[76], which explored the prospects of short-term ICB to induce immune activation in patients with early-stage TNBC. Three cohorts are described in this trial. In cohort A, patients received 4 weeks of anti-PD-1 therapy, while cohort B involved 4 weeks of anti-PD-1 combined with anti-CTLA4. In these two window-of-opportunity cohorts, patients could continue their treatment with standard of care neoadjuvant chemotherapy followed by surgery. High baseline levels of tumor-infiltrating lymphocytes (TILs) were found to correlate with treatment response. This

finding prompted the opening of cohort C, which enrolled patients with high TIL levels (≥50%). These patients received 6 weeks of neoadjuvant anti-PD-1 + anti-CTLA4, followed by surgery. The primary endpoint for cohorts A and B was immune activation, defined as a twofold increase of intra-tumoral CD8+ T cells or interferon-gamma gene expression. However, achieving this endpoint was more challenging in patients with an already high baseline TIL-score. After all, it is easier to go from 2% to 4% TIL than from 45% to 90%, and moreover, some patients already had a TIL score of 90% at the start of treatment, making further doubling impossible. Consequently, the primary endpoint for cohort C was adjusted to pathological complete response (pCR). Immune activation was observed in 53% (8/15) of patients in cohort A and 60% (9/15) in cohort B. In cohort C, 53% (8/15) of patients exhibited a major pathological response (<10% viable tumor at resection), with 33% (5/15) achieving a pCR. These results suggest that short-term ICB can induce immune activation and contribute to meaningful pathological responses even without chemotherapy in a substantial subset of patients with early-stage TNBC.

To gain a deeper understanding of immunotherapy response in early-stage TNBC, we performed spatial analyses and conducted in-depth comparisons between clinical responders and non-responders. These analyses included bulk RNA sequencing across all cohorts, as well as single-cell RNA-sequencing and TCR-sequencing in cohorts A and B, both pre- and post-treatment. Spatial analysis revealed that responders had shorter distances between tumor cells and the nearest CD8+ T cells, along with a higher density of double-positive CD8+PD-1+ cells and PD-1+ cells. Unsupervised sub-clustering of T cells from our single-cell RNA-sequencing dataset revealed multiple subpopulations, including a distinct CD8 T cell cluster with multiple previously described features of tumor-specific T cells. This cluster exhibited the highest clonality among all subclusters and showed the strongest enrichment of previously reported anti-tumor CD8 T cell signatures derived from functional tumor recognition experiments[77,78]. Single-cell RNA-sequencing revealed that higher pre-treatment levels of tumor-reactive CD8+ T cells and follicular helper T cells, correlated with treatment response, while elevated post-treatment regulatory T cells were linked to non-response. Flow cytometry of fresh blood samples showed an increase in Ki-67+ cells within the PD-1+ conventional CD4+ T cell population in responders compared to non-responders, with a similar trend observed for CD8+ T cells. This proliferative activity of PD-1+CD4+ T cells in the blood was also traced to the tumor, where responders had higher levels of Ki-67+ TFH cells—the CD4+ T cell cluster with the highest PDCD1 expression in tumor single-cell RNA-sequencing data. Notably, PD-1+ proliferating CD8+ T cells did not differ significantly between responders and non-responders, suggesting a special role for proliferating CD4+ T cells both systemically and within the tumor microenvironment. The observed proliferation of PD-1+ CD4+ T cells in responders, could suggest a potential role for the CD4-B cell axis in shaping the anti-tumor immune response. Given that Tfh cells are essential for B cell

activation and germinal center formation[79,80], they may contribute to the development of tertiary lymphoid structures (TLS), which have been strongly correlated with clinical benefit in multiple cancer types[80-82]. However, while this association is compelling, direct evidence linking the proliferation of PD-1+ CD4+ T cells to TLS formation and improved treatment outcomes remains to be established.

Our findings described in **Chapter 6** suggest that neoadjuvant immunotherapy, without chemotherapy, shows promising efficacy and could be a viable approach for patients with early-stage TNBC, particularly those with high levels of stromal TILs. Reducing the reliance on chemotherapy in a subset of patients is an important goal given the commonly experienced side-effects like gastrointestinal and neurological side effects, dermatologic and hair changes, fatigue, and the detrimental effect that chemotherapy has on the host immune system, but a careful evaluation of the potential benefits and risks of administering ICB in the neoadjuvant setting is essential. Long-term immune-related adverse events, such as adrenal gland insufficiencies or diabetes, could outweigh the anticipated benefits in early-stage disease. Therefore, it must be established whether the toxicities associated with ICB are indeed less severe than those induced by chemotherapy. If so, stromal TIL scores could serve as a promising biomarker to identify patients who may benefit from less aggressive treatment while maintaining excellent outcomes, paving the way for more personalized and less toxic therapeutic strategies.

Immunomonitoring HPV-specific T cell responses in blood

Breast cancer arises from genetic mutations that drive uncontrolled cell growth. However, some cancers are caused by viral infections, which in turn also introduce changes into the host DNA. One of the most well-known oncogenic viruses is human papillomavirus (HPV). HPV produces the oncoproteins E6 and E7, which inactivate the tumor-suppressor proteins p53 and RB, respectively. This disruption allows cells to evade apoptosis and bypass cell cycle regulation, leading to malignant transformation. HPV is linked to several cancers, including cervical and vulvar cancer. Vulvar intraepithelial neoplasia (VIN), the pre-malignant stage of vulvar cancer, is often associated with HPV. Despite being detectable due to viral epitopes, spontaneous regression of HPV-induced VIN is rare and progression to vulvar cancer is observed in 2-8% of cases[83-88]. Current treatments for VIN, including surgery and topical therapies, can have uncomfortable side effects and high recurrence rates, severely impacting women's quality of life. Therefore, immunotherapeutic strategies targeting the crucial oncogenic HPV proteins E6 and E7 are being explored as a potential approach to eliminate VIN lesions.

In **Chapter 8**, the clinical and translational results of the N16SIG trial were presented (trial registration number: NTR4607)[89], focusing on the efficacy of an HPV E6/E7 vaccine for patients with HPV-induced VIN lesions. Clinical responses were observed in 6 out of 14

patients (43%), with 2 complete responses and 4 partial responses. Interestingly, 5 of these 14 patients exhibited HPV-specific T-cell responses in the blood, as measured by *ex vivo* reactivity assays. Notably, all five patients with detectable HPV-specific T-cell responses had a corresponding clinical response, suggesting a strong correlation between immunological activity and clinical benefit.

To further investigate the reasons behind the lack of clinical benefit in the non-responders, tumor-infiltrating lymphocytes (TILs) were analyzed in both baseline and post-vaccination (day 56) tissue samples of the VIN lesions. The goal was to understand whether the presence or absence of TILs could explain the differential response to the vaccine. However, there was no statistically significant difference in TIL scores between responders and non-responders, either at baseline or after treatment (Figure 1a). This finding suggested that TIL levels alone might not be sufficient to predict clinical outcomes in patients receiving the HPV E6/E7 vaccine. Surprisingly, a statistically significant increase in TILs was observed between baseline and post-treatment samples in non-responders (Figure 1b). This increase was absent in the responding patients (Figure 1b). This observation raised several questions, particularly about the nature of the infiltrating immune cells. Since the increase in TILs did not correspond with clinical benefit, it is possible that the immune cells were not predominantly cytotoxic T cells, which would be expected to contribute to tumor control. Instead, the infiltrating cells might have included regulatory T cells or other immunosuppressive cell types that could dampen the anti-tumor immune response. In addition to quantifying stromal TILs, neutrophils and eosinophils were also assessed based on their morphology in the HE slide. No statistically significant differences were found in neutrophil and eosinophil counts between responders and non-responders, either at baseline or post-vaccination (Figure 1 c, e). Furthermore, we did not observe a statistically significant increase or decrease in neutrophils or eosinophils in VIN lesions during treatment (Figure 1 d, f).

Unfortunately, due to limitations in biopsy material, we were unable to conduct additional analyses, such as immunohistochemistry (IHC), high-dimensional imaging mass cytometry, or spatial transcriptomics. These techniques would have been valuable for identifying the specific immune cell populations present in the tumor microenvironment and determining whether immunosuppressive mechanisms were at play in the non-responding patients. Such insights could have provided guidance for future combination therapies aimed at increasing the response rate, potentially by adding immune checkpoint inhibitors or other agents that could counteract immunosuppression.

The results of the N16SIG trial highlight the complexity of immune responses in cancer patients and the need for deeper biological understanding to inform therapeutic strategies. While the HPV E6/E7 vaccine showed promising clinical activity in a subset of patients, it is clear that not all patients respond, and understanding the reasons behind this variability is

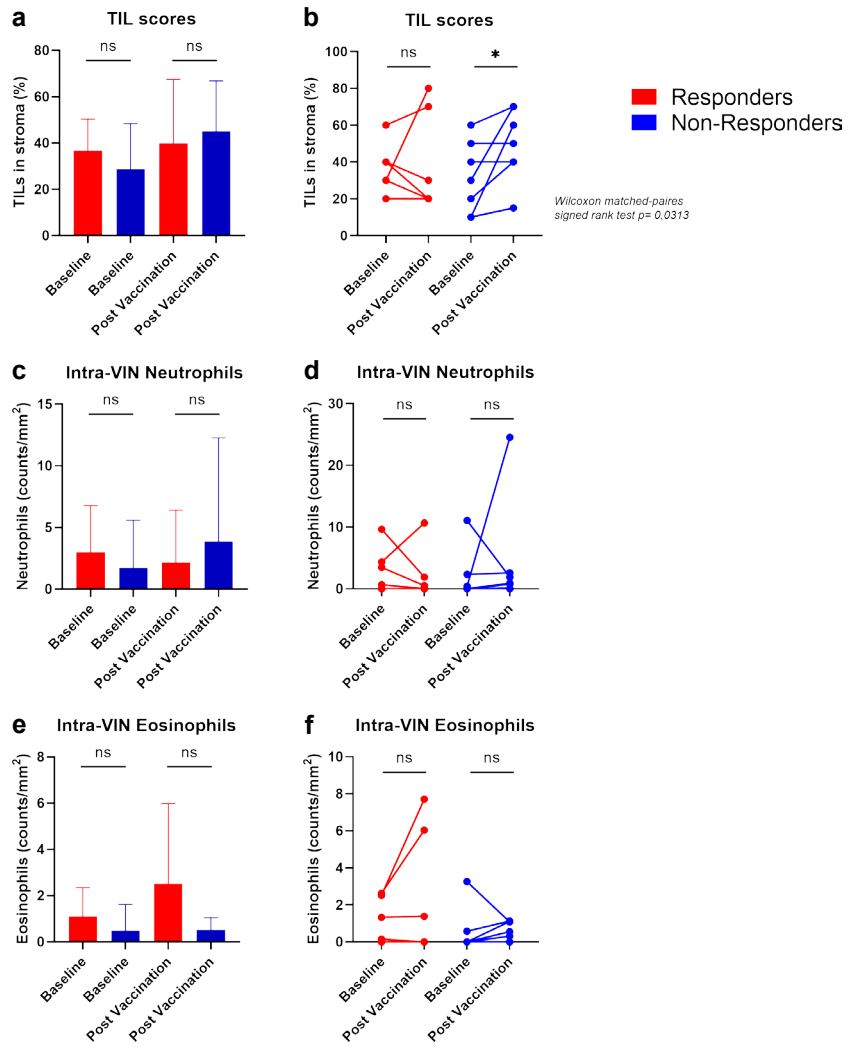

***Figure 1**: **(a-b)** Percentage of lymphocytes infiltrating the stroma of the VIN lesions, comparing **(a)** responders and non-responders at baseline and post treatment and **(b)** dynamic changes over time in responders and non-responders. **(c-d)** Neutrophil counts/mm² in VIN lesions, comparing **(c)** responders and non-responders at baseline and post treatment and **(d)** dynamic changes over time in responders and non-responders. **(e-f)** Eosinophil counts/mm² in VIN lesions, comparing **(e)** responders and non-responders at baseline and post treatment and **(f)** dynamic changes over time in responders and non-responders.*

crucial. In this context, the importance of obtaining and storing sufficient patient material for translational research cannot be overstated. By ensuring adequate tissue and blood samples are available, future studies can perform comprehensive analyses to uncover the mechanisms underlying both response and resistance.

Looking forward, therapeutic cancer vaccines targeting HPV epitopes hold significant potential. If these new treatment options prove to be long-term successful in HPV-related pre-malignancies such as vulvar intraepithelial neoplasia (VIN), they could potentially be extended to other HPV-induced conditions, including penile intraepithelial neoplasia (PIN) and anal intraepithelial neoplasia (AIN). Furthermore, their use might extend beyond pre-malignancies to encompass localized and advanced HPV-induced cancers, representing a significant breakthrough in the management of HPV-associated diseases. This progress could translate into improved outcomes and quality of life for patients globally. Additionally, combining these vaccines with other immune-modulating therapies, such as checkpoint inhibitors, may further enhance clinical outcomes by overcoming immunosuppressive mechanisms, particularly in non-responders.

Integrating vaccines with other immune-modulatory drugs may provide more durable responses for a broader range of patients[90]. This potential is becoming increasingly evident in various HPV-induced malignancies, where vaccines can counteract immune suppression and bolster anti-tumor immune responses. For instance, the phase II study NCT02426892 demonstrated encouraging results with the ISA101 synthetic long peptide vaccine combined with nivolumab, an anti-PD-1 checkpoint inhibitor. This combination achieved a 33% response rate (8 out of 24 patients) and a median survival of 17.5 months in individuals with incurable HPV-16-positive cancers, including oropharyngeal, cervical, and anal malignancies[91]. These findings highlight the promise of therapeutic vaccines to synergize with checkpoint blockade therapies, effectively targeting the immune-evasive mechanisms of HPV-driven tumors. Ongoing investigations continue to explore these synergies. Basket trials such as NCT04432597, NCT03439085, and NCT04287868 are currently evaluating therapeutic HPV vaccines in combination with diverse immunotherapy agents, targeting patients with locally advanced or metastatic HPV-positive cancers. These studies hold the potential to refine combination strategies and enhance the clinical efficacy of HPV vaccines. Moving forward, identifying optimal combinations, treatment sequences, and patient selection criteria will be crucial in translating these approaches into routine clinical practice. With continued research and innovation, the integration of therapeutic HPV vaccines into multimodal regimens may reshape the treatment landscape for HPV-related cancers.

Future Directions and Considerations for Improvement

There remain several promising avenues for advancing our research, particularly in enhancing our understanding of the interplay between the circulatory immune system and

the tumor microenvironment (TME). One critical next step would be to establish a direct link between systemic immune profiles and the TME, utilizing paired tumor and blood samples from the same patient. This would allow for a more comprehensive understanding of how systemic immune dynamics reflect or influence the local tumor environment or *vice versa*. While this approach was initially explored in my study on mTNBC, practical challenges impeded its full implementation. In The Netherlands, the majority of mTNBC patients undergo neoadjuvant chemotherapy, and as a result, the tumor samples I received post-treatment were not suitable for assessing intra-tumoral immune profiles. To effectively correlate the TME with systemic immune profiles, it is crucial to use pre-chemotherapy biopsies. Fortunately, the required approvals from the Medical Ethics Committee (METC) have recently been achieved, simplifying the process for subsequent researchers to carry out such studies. Analyzing matched blood and tumor samples can reveal important immunological shifts. For example, previous studies on a limited set of paired samples have demonstrated that a decline in memory B cells in the blood corresponds with an accumulation of class-switched memory B cells in the tumor[92]. This approach would provide valuable insights into how immune characteristics within the blood mirror those within the TME, uncovering a wealth of scientific knowledge and potentially offering novel biomarkers for treatment response.

Another important research focus is the long-term impact of various chemotherapy regimens on the peripheral immune composition in cancer patients. Recent findings show sustained lymphocyte depletion up to a year after chemotherapy for early breast cancer, similar to what we found in **Chapter 4**, highlighting potential long-term immune suppression[93]. While immediate immune effects post-treatment are well-documented, the prolonged influence of various chemotherapeutic regimens on both innate and adaptive immune compartments remains insufficiently explored. This line of research would involve the collection and analysis of fresh blood samples, which would enable the creation of comprehensive immune profiles that capture both adaptive and innate immune responses. By categorizing patients according to the types of chemotherapy received—such as taxanes, anthracyclines, and platinum-based agents—we could determine the specific immune alterations induced by each class of drugs. I recommend complementing these immune profiling efforts with functional assays to assess the key functional aspects of immune cells, such as cytotoxicity, antigen presentation, cytokine production, and several functional assays on neutrophils described in **Chapters 2** and **4**. Such a holistic approach would provide a deeper understanding of how different chemotherapy regimens influence immune competence, offering valuable insights into the interplay between chemotherapy and potential immune responses. This knowledge could be instrumental in guiding more tailored decision-making regarding chemotherapy and immunotherapy treatment strategies. Moreover, these insights would be crucial not only for refining current treatment protocols

to optimize patient outcomes but also for identifying specific patient populations that may derive the greatest benefit from combination therapies involving both chemotherapy and immunotherapy.

Concluding Remarks

Scientific progress is both purposeful and beautiful, driven by humanity's deep-seated curiosity to explore and understand the world. It reflects our innate desire to ask questions, seek answers, and expand the boundaries of knowledge. This pursuit is valuable in its own right, as each discovery adds to the intricate mosaic of human understanding and inspires future exploration. Beyond its intrinsic worth, scientific progress has a profound impact on our lives, especially in medicine. In breast cancer research, the quest to understand the disease's complexities has led to groundbreaking advancements that directly benefit patients. Insights into cancer subtypes, molecular mechanisms, and immune interactions have paved the way for innovative treatments such as immunotherapies and targeted therapies. These developments not only improve survival but also offer patients renewed hope and a better quality of life. It is my hope, that science may continue to serve a dual purpose: satisfying human curiosity while creating tools that can change lives. It is through this harmony of exploration and application that science achieves its fullest potential.

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