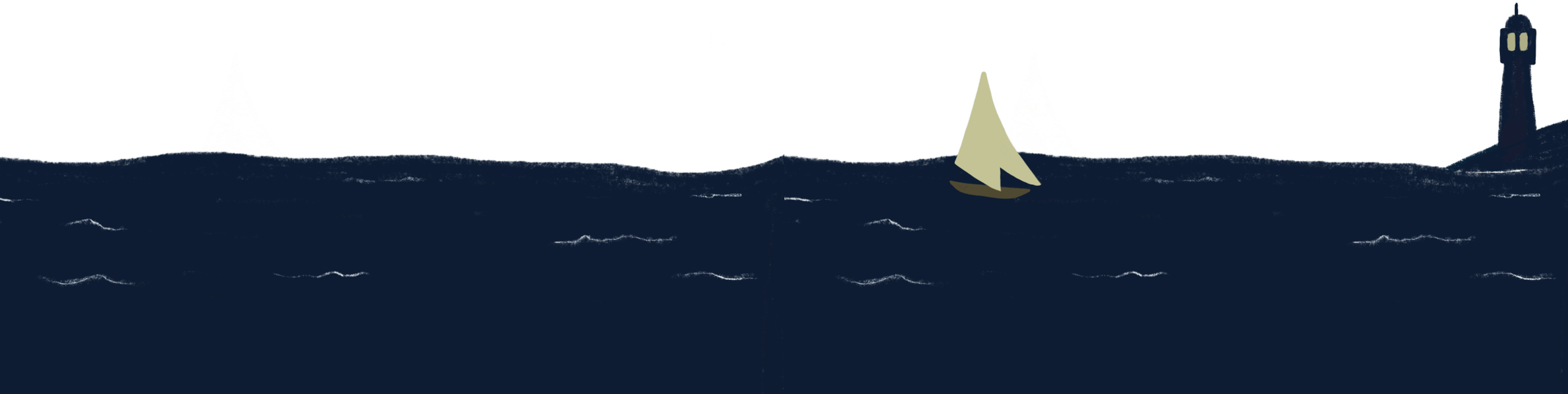
Enhancing visualization of gastrointestinal tumors: molecular targets and tracers for intraoperative optical imaging

Houvast, R.D.

CHAPTER 10

General discussion and future perspectives



This thesis evaluated biomarkers and novel tracers for molecular imaging in gastrointestinal cancers, with a focus on pancreatic and gastric cancer. This chapter reflects on the key findings and implications of this thesis as well as on future perspectives.

Exploration of novel imaging biomarkers: tumor-associated glycans and mucins

In the search for novel biomarkers suitable for molecular imaging, this thesis identified tumor-associated glycans and heavily glycosylated proteins, including mucins, as targets of particular interest (**Chapters 2 and 3**). Moreover, due to their superior tumor-specificity and expression in most patients, $Le^{a/c/x}$ and sdi-Le^{a} were identified as promising biomarkers for molecular imaging of PDAC (**Chapter 3**), as well as gastric and colorectal tumors (**Chapters 6 and 7**). While tumor expression of $Le^{a/c/x}$ and sdi-Le^{a} was mostly strong, their main strength lies in the differential expression between tumor and benign/healthy surrounding tissues, except for $Le^{a/c/x}$ in colorectal tumors. In PDAC, for instance, this differential expression was larger compared to EGFR, HER2, EpCAM and VEGF in PDAC, and, in gastric cancer, compared to $\alpha_V\beta_6$ and HER2 (**Chapter 3**).[1,2] This indicates that imaging tracers targeting tumor-associated glycans $Le^{a/c/x}$ and sdi-Le^{a} may offer superior imaging capabilities compared to several established molecular imaging tracers. Nevertheless, intratumoral heterogeneity of $Le^{a/c/x}$ and sdi-Le^{a} expression was still observed in **Chapters 3, 5, 6 and 7**, indicating that some limitations of current imaging targets have not yet been overcome. Although identifying a 'perfect' imaging biomarker may be an utopia, targeting tumor-associated glycans is currently in an early stage, partially relating to the intrinsic complexity of the glycome accompanied by a lack of robust glycoanalytical methods.[3] It is expected that advances in glycobiology research, such as the application of MALDI-TOF mass spectrometry imaging, will contribute to the discovery of novel tumor-associated glycans, potentially with superior suitability for molecular imaging.[3,4]

Targeting the tumor stroma

Apart from tumor-associated glycans and mucins, stromal targets represent another promising class of biomarkers for tumor imaging. As the stromal compartment comprises up to 90% of the mass of some pancreatic tumors, these targets are of particular interest in PDAC.[5] One example of such a target is fibroblast activating protein (FAP), which has been found promising not only for pancreatic cancer, but is also expressed on cancer-associated fibroblasts of more than 90% of carcinomas.[6,7] Regarding FAP-targeted positron emission tomography (PET) imaging, several clinical trials have been performed for many tumor types, with encouraging results.[8,9] For instance, the FAP-targeting PET tracer ^{18}F-FAPI-04 has shown to outperform conventional [^{18}F]Fluorodeoxyglucose (FDG)-PET imaging of primary and metastatic PDAC in the clinical setting.[10,11] A notable limitation of FAP, however, is its expression in tissue remodeling processes, such as wound healing and chronic inflammation, which can compromise the distinction between benign and malignant.[12] This of particular importance in patients who have received neoadjuvant therapy (NAT), a treatment known to induce increased fibrosis and necrosis.[13] The extent to which FAP expression is altered by NAT in PDAC remains insufficiently studied. Given the growing application of NAT in this tumor type, it is crucial to identify and target biomarkers that remain consistently expressed after NAT, such as the targets investigated in **Chapters 3 and 5**.

Underexplored differential expression of current imaging biomarkers

Chapter 4 identified CEACAM5, EGFR and EpCAM as suitable imaging biomarkers for gastric cancer. Several targeted PET and NIRF imaging tracers targeting these biomarkers have been evaluated in clinical trials. For instance, a phase 1 trial evaluating CEACAM5-targeted tracer [^{111}In]In-DOTA-labetuzumab-IRDye800CW for multimodal preoperative PET and intraoperative radioguidance and NIRF imaging in peritoneal metastases of colorectal cancer was recently conducted and allowed pre- and intraoperative identification of previously undetected lesions, altering clinical strategy in three patients.[14] Also, EGFR-targeting panitumumab-IRDye 800CW allowed detection of occult lymph node metastases using bimodal NIRF/PA imaging in proof-of-concept study in head and neck cancer.[15] These encouraging clinical results, supplemented with the immunohistochemical (IHC) data presented in **Chapter 4**, allow and necessitate rapid clinical evaluation of such tracers in gastric cancer.

As development and characterization of novel molecular imaging tracers can take years, it is crucial to evaluate the potential of current imaging tracers' targets across different tumor types to maximize their utility. IHC studies can provide sufficient evidence to apply (clinically evaluated) imaging tracers in other tumor types. Given the limitations of most biomarkers for molecular imaging, identifying novel biomarkers also remains essential. The optimal strategy combines both approaches, through which we can provide short-term solutions for

pre- and intraoperative tumor visualization, while simultaneously investing in the development of novel tracers which may offer superior specificity or sensitivity. This ensures that patients could benefit from the latest advances without unnecessary delays.

Selecting molecular imaging biomarkers: revised criteria and implications for practice

Chapter 3 and 4 show that it is crucial to investigate expression of biomarkers in the full anatomical context of the tumor to establish their potential as imaging biomarkers. Traditionally, however, most IHC studies investigating biomarker expression in human tissue specimens have solely investigated the 'tumor-versus-normal' expression of biomarkers. Instead, their expression should be evaluated in primary tumors after neoadjuvant therapy, in healthy surrounding tissues frequently invaded by the primary tumor (eg. duodenum in distal gastric cancer), in tumor-positive and -negative lymph nodes, distant metastases, as well as on benign surrounding tissue (eg. pancreatitis in PDAC). Lastly, but not evaluated in this thesis, biomarkers could be selected based on their expression in premalignant tissue, such as pancreatic intraepithelial neoplasia (PanIN) lesions or polyps with high-grade dysplasia in the case of PDAC and colorectal cancer, respectively. Future work should investigate these 'revised' criteria to guide tracer development.

Besides demonstrating (un)suitability of biomarkers for molecular imaging, these data also facilitate selective employment of tracers in certain conditions. For instance, application of a CEACAM5-targeted imaging tracer would not be recommended in proximal gastric cancer invading the gastro-esophageal junction (**Chapter 4**). Nevertheless, as limitations of IHC are present (discussed in detail below), it remains to be clarified to what extent quantified IHC staining truly reflects protein expression. Clinical imaging studies are therefore required to definitely establish the true impact of biomarker expression on surrounding tissue on a tracer's imaging potential.[16]

Addressing heterogenetic expression of current imaging biomarkers

This thesis additionally evaluated a workflow for preoperative biomarker screening using IHC in PDAC (**Chapter 5**). Although the predictive value of FNB expression for primary tumor expression was considerable for most biomarkers, concordance was not always present. This may have been caused by intratumoral heterogeneity of biomarker expression in PDAC, which was also described in **Chapter 3**. Assuming heterogeneity, biomarker expression in biopsy material may not invariably include positive biomarker expression in the tumor, and vice versa (**Chapter 5**). Diminishing intratumor heterogeneity as a source of bias could, for instance, be achieved by taking multiple core biopsies of the tumor, however this may not be feasible in clinical practice.[17]

Also, heterogeneity of biomarker expression between multiple tumor lesions in one patient may be present, which could complicate adequate tumor staging as not all lesions show positive signal during imaging.[18,19] Although the workflow described in **Chapter 5** cannot fully address this, concordance between biomarker expression in biopsies, primary tumors and (lymph node) metastases could be studied for the biomarkers evaluated in **Chapter 5**, as was performed for folate receptor-α by Boogerd et al. in breast and lung cancer.[19] Alternatively, targeted PET imaging could be performed to validate expression of an imaging biomarker, followed by administration of a NIRF imaging tracer. Another solution to the heterogeneity problem may be the employment of multiple tracers simultaneously or using a bispecific tracer. As demonstrated by the co-expression analyses of **Chapters 3 and 4**, a theoretical bispecific tracer could increase imaging capabilities, for some biomarker combinations, to virtually all patients within a single tumor type. Of note, targeting two biomarkers simultaneously has shown to increase tumor uptake *in vivo*.[20]

As more molecular imaging tracers become available for a single tumor type, biomarker screening will play a central role in personalizing imaging approaches, allowing suitable tracer selection to optimize tumor visualization. Upon clinical validation, the biomarker screening workflow could be conveniently integrated into clinical practice, as IHC is commonly performed on FNB tissue specimens for histological diagnosis of PDAC.[21] It is crucial that future studies investigate similar biomarker screening workflows in other gastrointestinal cancers. However, several drawbacks of IHC should be considered which will be discussed below.

IHC and scoring: critical evaluation

Technical biases of IHC, a technique extensively used in **Part I** of this thesis, include, but are not limited to tissue fixation and processing, antigen retrieval methods, endogenous peroxidase activity, and the secondary antibody used in the process, all of which can affect final staining results.[22] Moreover, primary antibodies used in IHC often bind different epitopes on the protein than the associated molecular imaging tracer. As different epitopes on the same protein may exhibit varying expression in tissues, the translational value of IHC studies may become

compromised.[23,24] IHC studies must therefore align the IHC antibody's epitope with the epitope targeted by the imaging tracer.

Another issue is the manual scoring of IHC staining, which suffers from intra- and interobserver variability, especially when staining is heterogenous, as was observed for some biomarkers evaluated in **Chapters 3**, **4 and 5**.[25-27] Semi-automated image analysis provides a solution, allowing highly reproducible and accurate cell detection and staining quantification using the H-score (range: 0-300), as was demonstrated in **Chapters 3 and 5** using QuPath.[28] However, training such software is labor intensive and still requires involvement of a dedicated pathologist for classification of tumor and non-malignant tissue, as well as validation of cell detection classifier algorithms. Despite these challenges, using QuPath offers significant advantages, especially for difficult-to-identify tumor types such as PDAC, and holds promise for clinical application. Although limitations of IHC warrant cautious interpretation of results, it offers a flexible, efficient and low-cost approach to evaluate biomarker expression in human tissue specimens, offering crucial information for suitability of molecular imaging biomarkers, as demonstrated in **Part I** of this thesis. Lastly, while selecting an appropriate biomarker is essential for successful tumor visualization, effective molecular imaging hinges on a complex interplay between biomarker properties, (photo) chemical properties and pharmacokinetics of the tracer, imaging system characteristics, to name just a few. This highlights the relevance of thorough *in vitro* and *in vivo* testing of imaging tracers, as was performed in **Part II**.

Targeting tumor-associated glycans for NIRF/PA imaging of gastrointestinal tumors

In **Part II** of this thesis, the preclinical potential of tumor-associated glycan-binding tracers CH88.2-800CW and CH129-800CW for bimodal NIRF and photoacoustic (PA) imaging of gastrointestinal cancers was demonstrated (**Chapters 6 and 7**). Thereby, combined with the findings from **Chapters 2, 3 and 5**, this thesis brought the concept of glycan targeting an important step forward. Despite the potential of tumor-associated glycans as biomarkers for imaging, a limited number of preclinical and clinical studies into glycan imaging has been conducted, as was discussed in **Chapter 2**. Clinically, sLe^a (also known as CA19-9), a glycan structurally related to Le$^{a/c/x}$ and sdi- Le^a, has been targeted for PET imaging in pancreatic cancer using the antibody-based tracer [^{89}Zr]-DFO-HuMab-5B1, and was able to detect primary tumors and distant metastases, as well as sub-centimeter lymph node metastases not visible with conventional imaging.[29,30] As demonstrated in **Chapter 3**, expression of Le$^{a/c/x}$ and sdi-Le^{a}on chronic pancreatitis was lower compared to sLe^a expression, thereby suggesting the superiority of both glycans as PDAC imaging biomarkers. Further clinical evaluation of CH88.2-800CW and CH129-800CW for bimodal NIRF/PA imaging of gastrointestinal cancers is warranted.

DARPins as targeting moieties for NIRF/PA imaging of gastrointestinal tumors

Chapter 8 provided the first preclinical evidence that bimodal NIRF/PA imaging of tumors using DARPins is feasible. DARPin-based molecular imaging has particularly focused on PET imaging.[31] The first in-human DARPin imaging study used HER2-targeted tracer ^{99m}Tc-(HE)$_3$-G3 for SPECT imaging of breast cancer and showed clear visualization of both primary and metastatic breast cancer, with a favorable safety and tolerability profile.[31] Preclinically, the EpCAM-targeting DARPin tracer [^{125}I]I-PIB-Ec1 was evaluated for PET imaging in an ovarian cancer xenograft model and showed a tumor-to-blood ratio of 31 at 24 hours post-injection, providing high-contrast tumor delineation.[32] Also, the tracer showed similarly promising results for SPECT/CT of triple-negative breast cancer.[33] A phase 1 trial evaluating this tracer for PET imaging of the tracer in ovarian and lung cancer is currently ongoing (NCT06386653) and the results are eagerly awaited. The promising clinical findings of DARPin-based imaging tracers combined with the (over)expression of EpCAM in virtually all gastrointestinal malignancies, including gastric cancer (**Chapter 4**), lung, breast, prostate, bladder, ovarian, thyroid and head and neck carcinomas, suggest broad application of EpCAM-binding DARPins as molecular imaging tracers.[34]

Selecting targeting moieties

Although mAbs have been the first targeting moiety of choice for molecular imaging, owing to their high specificity, affinity and stability, an increasing number of smaller-sized alternatives, such as DARPins, have become available.[35] Although this thesis did not directly compare mAbs and DARPins, as their targets were different, various factors should be considered when choosing a targeting moiety, which include molecular size, conjugation method, and several practical aspects.

As the hydrodynamic volume of a targeting moiety is inversely correlated with its extravasation and tumor penetration potential, tumor accumulation of mAbs take longer compared to DARPins, often requiring 3-5 days between injection and

imaging.[36] Conversely, DARPins extravasate quickly and allow a shorter interval between injection and imaging but require a higher (picomolar) affinity for similar tumor uptake to prevent backflow into the circulation and subsequent renal elimination.[36-38] Also, DARPins may provide better tumor penetration compared to mAbs due to their smaller size.[39]

The conjugation method, i.e. the attachment of a dye/radiolabel to a targeting moiety, is a second critical factor. The heterogeneous mixture after N-hydroxy succinimide-ester coupling of mAbs is susceptible to self-quenching and blocking of antigen-binding domains, thereby reducing brightness and binding potential of the construct, respectively.[40-42] Conversely, DARPins can be conjugated site-specifically through maleimide labelling using free cysteines that can be introduced site-specifically.[39] Simultaneously, a synthetic amino acids such as azidohomoalanine (Aha) can be introduced to allow coupling of effector moieties via click chemistry, allowing conjugation of two different dyes and allowing multimodal tumor imaging.[43]

Thirdly, practical aspects should be considered, such as production costs, which are substantially higher for mAbs compared to DARPins. However, retooling therapeutic mAbs for imaging purposes through conjugation with approved dyes or radiolabels, may reduce production costs and speed-up clinical translation.[44,45] Also, the interval time between injection and imaging longer than one day requires patient to make two hospitals visits, which is less attractive from a practical standpoint. Noteworthy, this timing has proven feasible in clinical practice, as indocyanine green (ICG) is also administrated 24 hours before colorectal liver metastasis resection.[46]

Eventually, it is to the end-user who decides if the benefits outweigh the drawbacks. In our experience, mAbs tend to show higher tumor signal than smaller targeting moieties, but tumor-to-background ratios may be similar.[47] High tumor signal remains a key condition for adequate tumor visualization, especially of smaller lesions that may not be expected to be present based on preoperative imaging. Conversely, DARPins offer a highly promising alternative to mAbs for molecular imaging considering their optimal pharmacokinetic profile, rapid production, and versatile engineerability.[38]

Tumor models in preclinical molecular imaging research

A final topic of discussion involves the tumor models used during *in vivo* experiments. While tumor models, by definition, aim to approximate clinical practice, several drawbacks of the *in vivo* models used in **Chapters 6, 7 and 8** can be identified. First, these models do not reflect the level tumor heterogeneity and histomorphology of human tumors, including their vascular, lymphatic and immune compartments.[48] Consequently, the enhanced permeability and retention (EPR) effect - which results in intratumoral uptake and trapping of tracers due to hyperpermeable tumor vasculature and inadequate lymphatic drainage - is not well modeled.[49] This can lead to aberrant tumor uptake of imaging tracers in preclinical models, potentially under- or overestimating their clinical utility.[36] This underlines the importance of a negative control tracer, ideally consisting of a similar targeting vehicle conjugated to the same fluorophore but directed at a target that is not expressed in the tumor, which would theoretically suffer similarly from the EPR effect.

Despite these limitations, our preclinical *in vivo* studies provide crucial information for the clinical translation of molecular imaging tracers. After all, the primary aim of *in vivo* studies in molecular imaging research is to determine whether an imaging tracer reaches its intended molecular target on the tumor in a living organism and can be visualized using a dedicated imaging system. A second application of *in vivo* studies is to study pharmacokinetic properties of the tracer, including hepatic or renal clearance. Lastly, while preclinical molecular imaging studies remain indispensable, one must realize that the true potential of a molecular imaging tracer can only be assessed in the clinical setting.

FUTURE PERSPECTIVES

The field of molecular imaging is rapidly evolving and increasingly adopted within surgical oncological care. The current arsenal of targeting imaging biomarkers needs to be expanded to overcome limitations of current biomarkers. To achieve this, future studies could employ innovative biomarker discovery strategies such as multi-omics analysis, which offers high-throughput biomarker screening across multiple molecular levels.[50,51] Research has shown the suitability this approach for molecular imaging biomarkers, but acknowledged the need of validating such targets on the protein level, for instance using IHC (De Muynck et al., manuscript in preparation).

This thesis highlighted the potential of glycan-targeted tumor imaging, however the field will advance further once novel targeting vehicles against promising tumor-associated glycan biomarkers have been developed. Glycan targeting has been hindered by the low immunogenicity and structural similarity of

glycans, requiring the employment of complex immunization strategies to develop suitable glycan-specific antibodies.[52] Therefore, an additional focus on non-mAb-derived vehicles is crucial. The development of glycan-targeting DARPins has not yet been successful despite substantial efforts.[53] It may be that the emerging class of glycan-targeting nanobodies could offer a promising, smaller-sized targeting alternative to mAbs.[54]

Several interesting developments regarding NIRF imaging are currently taking place. For instance, pH-activable tracers have become available for NIRF imaging, which owe their efficacy to the acidic environment of the tumor.[55,56] Such tracers may eventually circumvent the need for tumor targeting through tumor-specific biomarkers. Also, topical application of tracers is an interesting development as this can be done instantaneously and theoretically results in low or absent systemic exposure. In animal models, topically applied NIRF tracers have successfully visualized nerves, a development with potential applications in rectal or aortic surgery.[57] In addition to tumor imaging, future research should focus on intraoperative imaging of anatomical structures that should be avoided during surgery, such as ureters or nerves, which could enhance surgical safety by reducing iatrogenic damage.[57,58]

To overcome the limited penetration depth of NIRF imaging (NIR-I; 700-900 nm), NIR-II imaging has gained significant attention (1000-1400 nm). Due to reduced autofluorescence and scattering, NIR-II imaging may result in higher tumor-to-background ratios, allowing improved tumor detection with an increased depth of up to 20 mm.[59,60] As spectral characterization studies have shown long emission tails of ICG and IRDye 800CW in the NIR-II spectrum, applicability of current tracers for NIR-II without the immediate need for development of novel dyes is feasible.[61] However, as for PA imaging, suitable clinical camera systems capable of NIR-II imaging are required to bring this closer to the clinic. Close collaboration with developers of imaging systems is therefore essential. A last development of particular interest are molecular imaging tracers that allow bimodal NIRF/PET imaging.[62] Although currently in preclinical development, it is expected that such tracers will make their way to the clinic in the foreseeable future to allow pre- and intraoperative imaging using a single tracer injection.

As may be deducted from this thesis and developments outlined above, it appears that molecular imaging holds great promise for revolutionizing surgical oncological care. With the first NIRF imaging tracer being FDA-approved (OTL-38 in ovarian cancer), the results of the phase 3 trial evaluating CEACAM5-targeting SGM-101 in colorectal cancer are eagerly anticipated (NCT03659448). To arrive at a bright future, it is pivotal for researchers to adopt a truly multidisciplinary approach to pursue future endeavors. Such within the molecular imaging research field hinge on the reciprocal collaboration between preclinical and clinical researchers, as well as industrial partners and physicians from various clinical disciplines. Ultimately, problems must originate from the clinic and suggested solutions must be clinically feasible. On a personal note, the Green Light Leiden research group exemplified this collaborative approach, resulting in notable successes, with numerous studies currently ongoing.[63]

CONCLUSION

Molecular imaging may enhance pre- and intraoperative tumor identification. Preoperatively, targeted PET imaging is of particular interest. Intraoperatively, NIRF imaging, optionally combined with PA imaging, holds great promise. This thesis addressed several challenges regarding biomarkers and tracers for molecular imaging in gastrointestinal cancers, with a focus on pancreatic and gastric cancer. By evaluating biomarkers - such as tumor-associated glycans - and innovative tracers - such as glycan-binding and DARPin-based NIRF/PA tracers - this thesis contributed to the groundwork for improved pre- and intraoperative visualization of gastrointestinal cancers. Further development, optimization and clinical evaluation of molecular imaging tracers directed at the biomarkers identified in this work, and novel tracers evaluated in this thesis is warranted. Ultimately, such tracers may optimize surgical care and improve patient outcomes.

REFERENCES

1 de Geus SW, Boogerd LS, Swijnenburg RJ, Mieog JS, Tummers WS, Prevoo HA, et al. Selecting Tumor-Specific Molecular Targets in Pancreatic Adenocarcinoma: Paving the Way for Image-Guided Pancreatic Surgery. Mol Imaging Biol. 2016;18(6):807-19.

2 Tummers WS, Farina-Sarasqueta A, Boonstra MC, Prevoo HA, Sier CF, Mieog JS, et al. Selection of optimal molecular targets for tumor-specific imaging in pancreatic ductal adenocarcinoma. Oncotarget. 2017;8(34):56816-28.

3 Mereiter S, Balmaña M, Gomes J, Magalhães A, Reis CA. Glycomic Approaches for the Discovery of Targets in Gastrointestinal Cancer. Front Oncol. 2016;6:55.

4 Heijs B, Holst S, Briaire-de Bruijn IH, van Pelt GW, de Ru AH, van Veelen PA, et al. Multimodal Mass Spectrometry Imaging of N-Glycans and Proteins from the Same Tissue Section. Anal Chem. 2016;88(15):7745-53.

5 Tian C, Clauser KR, Öhlund D, Rickelt S, Huang Y, Gupta M, et al. Proteomic analyses of ECM during pancreatic ductal adenocarcinoma progression reveal different contributions by tumor and stromal cells. Proc Natl Acad Sci U S A. 2019;116(39):19609-18.

6 Zhao X, Zhang G, Chen J, Li Z, Shi Y, Li G, et al. A rationally designed nuclei-targeting FAPI 04-based molecular probe with enhanced tumor uptake for PET/CT and fluorescence imaging. Eur J Nucl Med Mol Imaging. 2024;51(6):1593-604.

7 Liu R, Li H, Liu L, Yu J, Ren X. Fibroblast activation protein: A potential therapeutic target in cancer. Cancer biology & therapy. 2012;13(3):123-9.

8 Gilardi L, Airò Farulla LS, Demirci E, Clerici I, Omodeo Salè E, Ceci F. Imaging Cancer-Associated Fibroblasts (CAFs) with FAPi PET. Biomedicines. 2022;10(3).

9 Arçay Öztürk A, Flamen P. FAP-targeted PET imaging in gastrointestinal malignancies: a comprehensive review. Cancer Imaging. 2023;23(1):79.

10 Li X, Lu N, Lin L, Chen Y, Yang S, Wang H, et al. (18) F-FAPI-04 Outperforms (18)F-FDG PET/CT in Clinical Assessments of Patients with Pancreatic Adenocarcinoma. J Nucl Med. 2024;65(2):206-12.

11 van Dam MA, Vuijk FA, Stibbe JA, Houvast RD, Luelmo SAC, Crobach S, et al. Overview and Future Perspectives on Tumor-Targeted Positron Emission Tomography and Fluorescence Imaging of Pancreatic Cancer in the Era of Neoadjuvant Therapy. Cancers. 2021;13(23):6088.

12 Kessler L, Ferdinandus J, Hirmas N, Zarrad F, Nader M, Kersting D, et al. Pitfalls and Common Findings in (68)Ga-FAPI PET: A Pictorial Analysis. J Nucl Med. 2022;63(6):890-6.

13 Vuijk FA, de Muynck L, Franken LC, Busch OR, Wilmink JW, Besselink MG, et al. Molecular targets for diagnostic and intraoperative imaging of pancreatic ductal adenocarcinoma after neoadjuvant FOLFIRINOX treatment. Sci Rep. 2020;10(1):16211.

14 de Gooyer JM, Elekonawo FMK, Bremers AJA, Boerman OC, Aarntzen E, de Reuver PR, et al. Multimodal CEA-targeted fluorescence and radioguided cytoreductive surgery for peritoneal metastases of colorectal origin. Nat Commun. 2022;13(1):2621.

15 Vonk J, Kukačka J, Steinkamp PJ, de Wit JG, Voskuil FJ, Hooghiemstra WTR, et al. Multispectral optoacoustic tomography for in vivo detection of lymph node metastases in oral cancer patients using an EGFR-targeted contrast agent and intrinsic tissue contrast: A proof-of-concept study. Photoacoustics. 2022;26:100362.

16 Muynck L, Gaarenstroom KN, Sier CFM, Duijvenvoorde MV, Bosse T, Mieog JSD, et al. Novel Molecular Targets for Tumor-Specific Imaging of Epithelial Ovarian Cancer Metastases. Cancers (Basel). 2020;12(6).

17 Goethals L, Perneel C, Debucquoy A, De Schutter H, Borghys D, Ectors N, et al. A new approach to the validation of tissue microarrays. J Pathol. 2006;208(5):607-14.

18 Lower EE, Glass E, Blau R, Harman S. HER-2/neu expression in primary and metastatic breast cancer. Breast Cancer Res Treat. 2009;113(2):301-6.

19 Boogerd LS, Boonstra MC, Beck AJ, Charehbili A, Hoogstins CE, Prevoo HA, et al. Concordance of folate receptor-α expression between biopsy, primary tumor and metastasis in breast cancer and lung cancer patients. Oncotarget. 2016;7(14):17442-54.

20 Kwon LY, Scollard DA, Reilly RM. (64)Cu-Labeled Trastuzumab Fab-PEG(24)-EGF Radioimmunoconjugates Bispecific for HER2 and EGFR: Pharmacokinetics, Biodistribution, and Tumor Imaging by PET in Comparison to Monospecific Agents. Mol Pharm. 2017;14(2):492-501.

21 Luu TT. Review of Immunohistochemistry Biomarkers in Pancreatic Cancer Diagnosis. Front Oncol. 2021;11:799025.

22 Matos LL, Trufelli DC, de Matos MG, da Silva Pinhal MA. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. Biomark Insights. 2010;5:9-20.

23 Gan HK, Burgess AW, Clayton AH, Scott AM. Targeting of a conformationally exposed, tumor-specific epitope of EGFR as a strategy for cancer therapy. Cancer Res. 2012;72(12):2924-30.

24 Baeuerle PA, Gires O. EpCAM (CD326) finding its role in cancer. Br J Cancer. 2007;96(3):417-23.

25 Loughrey MB, Bankhead P, Coleman HG, Hagan RS, Craig S, McCorry AMB, et al. Validation of the systematic scoring of immunohistochemically stained tumour tissue microarrays using QuPath digital image analysis. Histopathology. 2018;73(2):327-38.

26 Adams EJ, Green JA, Clark AH, Youngson JH. Comparison of different scoring systems for immunohistochemical staining. J Clin Pathol. 1999;52(1):75-7.

27 Feuchtinger A, Stiehler T, Jütting U, Marjanovic G, Luber B, Langer R, et al. Image analysis of immunohistochemistry is superior to visual scoring as shown for patient outcome of esophageal adenocarcinoma. Histochem Cell Biol. 2015;143(1):1-9.

28 Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open source software for digital pathology image analysis. Sci Rep. 2017;7(1):16878.

29 Lohrmann C, O'Reilly EM, O'Donoghue JA, Pandit-Taskar N, Carrasquillo JA, Lyashchenko SK, et al. Retooling a Blood-Based Biomarker: Phase I Assessment of the High-Affinity CA19-9 Antibody HuMab-5B1 for Immuno-PET Imaging of Pancreatic Cancer. Clin Cancer Res. 2019;25(23):7014-23.

30 Lwin TM, Hoffman RM, Bouvet M. The development of fluorescence guided surgery for pancreatic cancer: from bench to clinic. Expert Rev Anticancer Ther. 2018;18(7):651-62.

31 Bragina O, Chernov V, Schulga A, Konovalova E, Garbukov E, Vorobyeva A, et al. Phase I Trial of (99m)Tc-(HE)(3)-G3, a DARPin-Based Probe for Imaging of HER2 Expression in Breast Cancer. J Nucl Med. 2022;63(4):528-35.

32 Vorobyeva A, Konovalova E, Xu T, Schulga A, Altai M, Garousi J, et al. Feasibility of Imaging EpCAM Expression in Ovarian Cancer Using Radiolabeled DARPin Ec1. Int J Mol Sci. 2020;21(9).

33 Vorobyeva A, Bezverkhniaia E, Konovalova E, Schulga A, Garousi J, Vorontsova O, et al. Radionuclide Molecular Imaging of EpCAM Expression in Triple-Negative Breast Cancer Using the Scaffold Protein DARPin Ec1. Molecules. 2020;25(20).

34 Gires O, Pan M, Schinke H, Canis M, Baeuerle PA. Expression and function of epithelial cell adhesion molecule EpCAM: where are we after 40 years? Cancer Metastasis Rev. 2020;39(3):969-87.

35 Ryman JT, Meibohm B. Pharmacokinetics of Monoclonal Antibodies. PETT: Pharmacometrics & Systems Pharmacology. 2017;6(9):576-88.

36 Xenaki KT, Oliveira S, van Bergen En Henegouwen PMP. Antibody or Antibody Fragments: Implications for Molecular Imaging and Targeted Therapy of Solid Tumors. Front Immunol. 2017;8:1287.

37 Schmidt MM, Wittrup KD. A modeling analysis of the effects of molecular size and binding affinity on tumor targeting. Mol Cancer Ther. 2009;8(10):2861-71.

38 Plückthun A. Designed Ankyrin Repeat Proteins (DARPins): Binding Proteins for Research, Diagnostics, and Therapy. Annual Review of Pharmacology and Toxicology. 2015;55(1):489-511.

39 Stumpp MT, Binz HK, Amstutz P. DARPins: A new generation of protein therapeutics. Drug Discovery Today. 2008;13(15):695-701.

40 Mädler S, Bich C, Touboul D, Zenobi R. Chemical cross-linking with NHS esters: a systematic study on amino acid reactivities. J Mass Spectrom. 2009;44(5):694-706.

41 Agarwal P, Bertozzi CR. Site-specific antibody-drug conjugates: the nexus of bioorthogonal chemistry, protein engineering, and drug development. Bioconjug Chem. 2015;26(2):176-92.

42 Zhegalova NG, He S, Zhou H, Kim DM, Berezin MY. Minimization of self-quenching fluorescence on dyes conjugated to biomolecules with multiple labeling sites via asymmetrically charged NIR fluorophores. Contrast Media Mol Imaging. 2014;9(5):355-62.

43 Simon M, Zangemeister-Wittke U, Plückthun A. Facile double-functionalization of designed ankyrin repeat proteins using click and thiol chemistries. Bioconjug Chem. 2012;23(2):279-86.

44 Lyu X, Zhao Q, Hui J, Wang T, Lin M, Wang K, et al. The global landscape of approved antibody therapies. Antib Ther. 2022;5(4):233-57.

45 Hernot S, van Manen L, Debie P, Mieog JSD, Vahrmeijer AL. Latest developments in molecular tracers for fluorescence image-guided cancer surgery. The Lancet Oncology. 2019;20(7):e354-e67.

46 van Manen L, Handgraaf HJM, Diana M, Dijkstra J, Ishizawa T, Vahrmeijer AL, et al. A practical guide for the use of indocyanine green and methylene blue in fluorescence-guided abdominal surgery. J Surg Oncol. 2018;118(2):283-300.

47 Baart VM, van Manen L, Bhairosingh SS, Vuijk FA, Iamele L, de Jonge H, et al. Side-by-Side Comparison of uPAR-Targeting Optical Imaging Antibodies and Antibody Fragments for Fluorescence-Guided Surgery of Solid Tumors. Mol Imaging Biol. 2021.

48 Gengenbacher N, Singhal M, Augustin HG. Preclinical mouse solid tumour models: status quo, challenges and perspectives. Nat Rev Cancer. 2017;17(12):751-65.

49 Danhier F. To exploit the tumor microenvironment: Since the EPR effect fails in the clinic, what is the future of nanomedicine? J Control Release. 2016;244(Pt A):108-21.

50 Das T, Andrieux G, Ahmed M, Chakraborty S. Integration of Online Omics-Data Resources for Cancer Research. Front Genet. 2020;11:578345.

51 Biswas N, Chakrabarti S. Artificial Intelligence (AI)-Based Systems Biology Approaches in Multi-Omics Data Analysis of Cancer. Front Oncol. 2020;10:588221.

52 Prendergast JM, Galvao da Silva AP, Eavarone DA, Ghaderi D, Zhang M, Brady D, et al. Novel anti-Sialyl-Tn monoclonal antibodies and antibody-drug conjugates demonstrate tumor specificity and anti-tumor activity. mAbs: Taylor & Francis; 2017. p. 615-27.

53 Warkentin R. Toward the use of Ankyrins and Affibodies as Scaffolds for Glycan Binding Proteins: A Directed Evolution and Computational Approach. Concordia University; 2022.

54 Khilji SK, Goerdeler F, Frensemeier K, Warschkau D, Lühle J, Fandi Z, et al. Generation of glycan-specific nanobodies. Cell Chem Biol. 2022;29(8):1353-61.e6.

55 Voskuil FJ, Steinkamp PJ, Zhao T, van der Vegt B, Koller M, Doff JJ, et al. Exploiting metabolic acidosis in solid cancers using a tumor-agnostic pH-activatable nanoprobe for fluorescence-guided surgery. Nat Commun. 2020;11(1):3257.

56 Golijanin J, Amin A, Moshnikova A, Brito JM, Tran TY, Adochite RC, et al. Targeted imaging of urothelium carcinoma in human bladders by an ICG pHLIP peptide *ex vivo*. Proc Natl Acad Sci U S A. 2016;113(42):11829-34.

57 Barth CW, Rizvi SZH, Masillati AM, Chakraborty S, Wang LG, Montaño AR, et al. Nerve-Sparing Gynecologic Surgery Enabled by A Near-Infrared Nerve-Specific Fluorophore Using Existing Clinical Fluorescence Imaging Systems. Small. 2023:e2300011.

58 de Valk KS, Handgraaf HJ, Deken MM, Sibinga Mulder BG, Valentijn AR, Terwisscha van Scheltinga AG, et al. A zwitterionic near-infrared fluorophore for real-time ureter identification during laparoscopic abdominopelvic surgery. Nature Communications. 2019;10(1):3118.

59 Hu Z, Fang C, Li B, Zhang Z, Cao C, Cai M, et al. First-in-human liver-tumour surgery guided by multispectral fluorescence imaging in the visible and near-infrared-I/II windows. Nat Biomed Eng. 2020;4(3):259-71.

60 He S, Song J, Qu J, Cheng Z. Crucial breakthrough of second near-infrared biological window fluorophores: design and synthesis toward multimodal imaging and theranostics. Chem Soc Rev. 2018;47(12):4258-78.
61 Guo X, Li C, Jia X, Qu Y, Li M, Cao C, et al. NIR-II fluorescence imaging-guided colorectal cancer surgery targeting CEACAM5 by a nanobody. EBioMedicine. 2023;89:104476.
62 Schouw H, Huisman L, Janssen Y, Slart R, Borra R, Willemsen A, et al. Targeted optical fluorescence imaging: a meta-narrative review and future perspectives. European journal of nuclear medicine and molecular imaging. 2021:1-21.
63 Mieog JSD, Achterberg FB, Zlitni A, Hutteman M, Burggraaf J, Swijnenburg R-J, et al. Fundamentals and developments in fluorescence-guided cancer surgery. Nature Reviews Clinical Oncology. 2021.