Towards implementation of the tumour-stroma ratio in colorectal cancer

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Chapter 7

Summary, general discussion and future perspectives

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The tumour microenvironment, with all its facets, is no longer deemed an innocuous compartment of a cancerous growth. As much research these past decades has proven, this thesis subsequently showcases the undeniable influence of the tumour stroma specifically on tumour behaviour and patient-related outcomes in colorectal cancer. The tumour-stroma ratio (TSR) is herein par excellence a biomarker capturing that effect, and is the subject of this current work. Commencing with the results of the by-international-instances-recommended, prospective study validating the TSR as prognosticator in colon carcinoma patients, subsequently proving the value of the TSR in predicting response to neoadjuvant therapy in rectal carcinoma patients, and researching this tumour stroma more in depth, the overview given here is concluded by a self-supervised and unbiased artificial intelligence-algorithm confirming the importance of tumour stroma. This thesis forms a theoretical framework, a step-by-step plan and concrete checklist ultimately aiming implementation of the TSR in national and international guidelines.

Previously, the TSR has iteratively shown to be a reproducible, solid and accurate biomarker [1-4]. However, upon the initial presentation of this evidence to the Union of International Cancer Control (UICC) and College of American Pathologists (CAP), advocating implementation of the TSR in routine pathology diagnostics, first, a prospective study was recommended [5]. Finalising the work of predecessors, this current work therefore starts off with the long-awaited results of that recommendation: the international multicentred UNITED study (**Chapter 2**). Over 1,500 stage II and III colon carcinoma patients were included in total by 27 centres from 12 countries worldwide. The TSR was scored by certified pathologists, participating after successfully finishing the official TSR E-learning [6], on the primary resected tumour material through standardized microscopic assessment [7]. Categorised with a stroma-high (>50%) or stroma-low (≤50%) colon carcinoma, included patients were in follow-up for a median of three years to evaluate the influence of the TSR on disease-free survival, and secondarily, overall survival and assessment of benefit from adjuvant chemotherapy.

Indeed, disease-free survival was significantly shorter in the patients with a stroma-high colon carcinoma in comparison to their stroma-low counterparts, illustrated by the respective 3-year disease-free survival rates of 70% and 83%. Correcting for potential bias of other risk factors, the TSR stayed an independent prognosticator for disease-free survival in multivariate analysis as well, with a hazard ratio of 1.49. Although the study was not specifically powered for the secondary outcomes, a strong trend towards worse overall survival was visible in stroma-high patients. Moreover, in the stage II and stage III stroma-high patients, a worse disease-free survival was observed despite their received adjuvant treatment, indicating a potential therapy resistance by the tumour stroma.

Hence, the UNITED study hereby unequivocally validates the TSR as an independent prognostic biomarker in predicting worse disease-free survival in colon cancer patients [8]. These results have been once again presented to the UICC, as well as to the Dutch national guideline committee [9], strongly urging implementation of the TSR in the tumour-node-metastasis (TNM) classification [10] as additional prognosticator. In response, first, the UICC congratulated us on the UNITED study, asking to share our work with their collaborators the American Joint Committee on Cancer (AJCC), and promised to add the TSR to the list of important prognostic factors in the upcoming next version of the TNM classification for colon and rectal cancer. Moreover, as the UICC suggested to work with the International Collaboration on Cancer Reporting (ICCR) committee to incorporate the TSR in the diagnostic pathology synoptic reports, we reached out to the ICCR, and in response, they agreed that it needs consideration for the next ICCR review [11]. Although the Dutch national guidelines committee also acknowledged the prognostic value and promised to submit the implementation request in their next meeting, the committee emphasized that a clinical need for ultimate validation of the predictive value of the TSR remained. An initial exploration of that value, predicting therapeutic effect or possible benefit from this therapy, is already endeavoured in this thesis in **Chapter 3**.

Here, the influence of the TSR score on biopsy material and response to neoadjuvant therapy in patients with rectal carcinoma is studied. As neoadjuvant therapy alters the tumour microenvironment, resulting in e.g. fibrosis [12], and a total neoadjuvant therapy regime, including chemoradiation variations, is currently standard protocol in rectal carcinomas [13], a biopsy-based and upfront selection of potential responders is imperative. Aiming to confirm the findings of the UNITED study as well as other existing, though insufficient and scare, literature [14, 15], we hypothesised that a biopsy-scored stroma-high rectal carcinoma patient would less often reach a major response to their neoadjuvant therapy. Ultimately, these potential non-responders could be for instance counselled in a shared decision-making setting to opt for a surgery-based approach, sparing them the burdensome and inefficient neoadjuvant therapy.

Harnessing data from two clinical trials, i.e. RAPIDO[16] and PROCTOR-SCRIPT[17], and integrating our LUMC patient database, a large multicentre cohort was therefore established. After first validating the TSR scoring method in biopsy material in our study as per literature [14, 15], we could indeed observe significantly less major response rates in a total neoadjuvant therapy approach in the biopsy-scored stroma-high rectal carcinoma patients compared to stroma-low patients (hazard ratio 0.63). Although the TSR had no singular prognostic effect on disease-free nor overall survival, this was however the case for response, to which the TSR was a major contributor. Lastly, emphasizing their inherent aggressiveness, locally advanced rectal carcinomas were mere often stroma-high. Hence, where the TSR was proven to be a prognostic biomarker in chapter 2, here in chapter 3, the TSR is also a proven predictive biomarker, albeit in a retrospective analysis and in rectal carcinoma. Currently,

novel established collaborations are utilised to accurately determine the predictive potential of the TSR in colon carcinomas as well, aiming ultimately unavoidable implementation in (inter)national guidelines: the UNITED-II. The clinical consequences of implementing this important parameter are far-reaching, identifying those stroma-high patients with predicted worse prognosis and less expected benefit from therapy. In frail elderly, this could potentially lead to a more supportive approach with a focus in shared-decision making on maintaining quality of life, while for the younger and fitter patients, more research is necessary for experimental and personalised therapies.

One of those potential experimental personalised therapies comprises the use of theragnostics [18]. Combining imaging and therapy by a single radioactive biologically-targeted substance, theragnostics can also pertain to pathways or cells in the tumour stroma compartment [19]. Specifically, the initially quiescent fibroblast, activated by an epithelial-to-mesenchymal transition and giving rise to the so-called cancer-associated fibroblast (CAF), is subject to an exponential amount of research [20]. CAFs, actively remodelling the tumour microenvironment and being important modulators of tumour aggressiveness, form therefore an attractive target [21]. Despite their large heterogeneity, a general CAF marker expressed and used in a majority of research is the fibroblast activation protein (FAP) [22]. Radioactively labelled and chemically modified, the FAP inhibitor (FAPI) is currently increasingly studied in improving tumour diagnostics and staging, specifically in identifying tumour-positive lymph nodes, laying groundwork for future theragnostics [23]. Hence, this thesis subsequently dives more into the tumour stroma in lymph node metastases, followed by an assessment of FAP-expressing CAFs in colorectal carcinomas, aiming to elucidate this phenomenon and forming a theoretical framework.

In **Chapter 4**, we first analyse tumour-positive lymph nodes and specifically, the stromal metastases therein. Since detailed literature on this was scarce, we aimed to determine whether stromal-based detection methods like the FAPI tracer would indeed theoretically improve nodal staging [24]. Protocols in imaging state that lymph nodes with a diameter in size >5-10 millimetres, along with other aspects like roundness of shape, are suspect for malignancy, but this is still prone to erroneous interpretations [25, 26]. An overview is provided in this work containing all features, sizes and tumour-stromal content of lymph node metastases in colon carcinoma material. We observed that lymph node size had positive correlations to the presence and the size of metastases, however, also small lymph nodes <5 millimetres in diameter could contain stromal metastases [27]. A theoretical basis was thus formed for the indeed often clinically observed improvement of nodal staging with the upcoming targeted FAPI tracer used in nuclear imaging compared to the common fluordeoxyglucose tracer, but also the potential limitations thereof [28].

Subsequently, in **Chapter 5**, we look even more in depth at the FAP marker by using immunohistochemical staining in colorectal carcinoma tissue. Aiming to fill the current gap in

knowledge and to form an extensive histological reference for future correlation to FAPI imaging studies [29], we described patterns of FAP expression in primary resected tumours, lymph nodes and biopsy material. Moreover, we assessed the correlation between the TSR and FAP expression, which later could constitute FAPI uptake: if positively correlated, on future imaging high FAPI uptake would thus identify stroma-high colorectal carcinomas, potentially enabling an even more improved upfront therapy selection. While the majority of colorectal carcinoma tissue did stain positively for FAP, this was mostly heterogeneous, and not only between tumours, but also within tumours. Also healthy colorectal and lymphoid tissue could stain for FAP. Furthermore, the TSR, mostly scored in the tumour centre, did not correlate to FAP, which was generally expressed at the invasive front. Therefore, care has to be taken directly translating FAP expression and inherently future FAPI uptake to disease activity and extent. This chapter emphasizes the importance of a multidisciplinary approach in studies, and the need for further correlation studies, some of which are currently ongoing in collaborative aspects, before patient treatment can be endeavoured, i.e. the FAPI-CRC1 (NCT05209750) and FoCus (NCT06191120) trials.

Historically, from the sixteenth century onward, the 'anatomists' increasingly gained knowledge of pathology. With the invention of the microscope, improved organisation and the distribution of that knowledge of the many pathological observations and drawings through publishing, this is often coined as the start of modern pathology [30]. However, after centuries, a new era has been slowly dawning: that of the digital pathology [31]. The traditional glass slides are currently generally scanned, giving rise to digitalized whole slide images. Using exponential computing power, artificial intelligence has emerged as an important tool to aid pathologists [32]. Initially a supportive feature, creating annotations or detailed assessments, saving and sharing analyses, nowadays the new era pertains to the development of more advanced algorithms gaining more and novel information from slides than with mere microscopy, and performing increasingly complicated tasks, for instance with deep learning algorithms. Deep convolutional networks, a large network of connected 'neurons' creating a computerized brain-like model, can for instance be trained on pathologists' annotations in order to predict tissue types on unannotated whole slide images [33]. This supervised deep learning is increasingly becoming more accurate, and enables prediction of patient-related outcomes as well as implementation of other biomarkers, such as the TSR, which our research group analysed previously [34].

However, to adequately train supervised learning models, time-consuming and extensive annotations by pathologists are required. Moreover, the results are limited to the prespecified outcomes, such as tissue types. Contrastingly, self-supervised learning merely necessitates unannotated whole slide images as input [35]. In the last scientific chapter of this thesis, **Chapter 6**, a self-supervised learning algorithm, specifically the Barlow Twins encoder, is trained to automatically extract histological patterns from group small image patches (tiles) of the whole slide images from colon carcinoma patients [36]. These

tiles are grouped based on the similarity of the extracted features in so-called histomorphological phenotype clusters (HPCs). An independent clinical trial cohort was then used to test their reproducibility. Ultimately, a total of 47 unique HPCs were identified. Each HPC was examined in detail, histopathologically as well as with an immune landscape and gene set enrichment analysis, and associated to overall survival and response to treatment therein. Of note, traits pertaining to tissue type, tissue quantity and tissue architecture were independently highlighted by the model as clinically significant. Moreover, survival predictions with this HPC-based risk grouping were state-of-art. The resulting atlas even uncovered some HPCs identifying stage II/III colon carcinoma patients who could potentially have benefited from the experimentally tested and controversially detrimental deemed bevacizumab [37]. Lastly, this unbiased self-supervised learning method specifically emphasised the importance of the tumour stromal compartment. Future HPC-assisted analyses could aid decision-making in multidisciplinary team meetings for an improved personalized colon carcinoma patient treatment.

Summarizing, this thesis confirms, from bench to bedside, the clinical importance of the TSR as parameter. Throughout these chapters, unequivocal evidence is collected, in line with previous literature. While this evidence in our current, modern, evidence-based health care is crucial, implementation of a biomarker in guidelines is not as straightforward as merely presenting this evidence. Part of an evidence-based approach is analysing this evidence and ranking or scoring this in a predefined hierarchical order [38]. However, this order is based on interventional medicine studies, mostly promoting randomised controlled trials, and pathology parameters are often inapplicable and poorly representable therein [39]. Hence, aiming to find gaps in the current World Health Organisation Classification of Tumours (WCT) [40], which is deemed the absolute fundament of all evidence and containing all carcinoma classifications, recently, a large Delphi study was performed amongst pathologists and other associated experts worldwide [41]. Collecting expertise, opinions, and discussions, this resulted in a consensus for a modified hierarchy of these levels of evidence for pathological parameters pertaining tumours, ranking evidence in level P1 (highest) to P5 (lowest) and specified for the various WCT topics. For the TSR, two are of relevance: those with a research focus on prognostics, and those pertaining to predictive biomarkers.

First, the prognostic biomarker assessment. Following the example research question, ‘What features influence the five-year survival?’, a list of study types is given in the sequence of weighed robustness, e.g. ‘greatest confidence’, to answer this question. Level P1 merely states that a systematic review with predominantly prospective cohort studies or studies derived from randomised-controlled trials, i.e. level P2, would suffice as evidence. For predictive biomarkers, answering a generic ‘can this marker predict treatment?’ research question, also randomised-controlled trials themselves, as an additional level P2 evidence, could be included in the similarly only deemed sufficient P1 systematic review.

Simultaneously, the work group admits that still pathological research is not always includable in these levels of evidence and that establishing this hierarchy was challenging and requires some nuances, also due to some unique considerations per topic. Although the large, international, multicentre UNITED study, would technically be deemed as level P2 evidence, combining all results with many (systematic) reviews and meta-analyses, previously performed by our research group [2] as well as worldwide [42-44], the TSR as prognostic biomarker can sufficiently with great confidence answer the research question, as the stroma-high feature indeed significantly negatively influences the five-year disease-free survival. Thus, implementation of the TSR as prognosticator additional to the TNM classification, as currently is being implored to the UICC, is undeniably pivotal. The TSR as predictive biomarker is still subject to ongoing, high-level research, of which the results will subsequently be presented in an implementation proposal as well.

Interestingly, no mention of a potential existence of expert consensus on the specific prognostic or predictive biomarker, the cost-effectiveness, educational nor distributive aspect of said biomarker is given in the novel modified hierarchy. However, in comparison to the relatively recently implemented tumour budding biomarker [45] or the Immunoscore [46], the TSR surpasses these parameters. Not only is the TSR endorsed by the European Society of Pathology with a consensus on how to score this parameter, with their support assuring the qualitative aspects, an official E-learning was developed to distribute knowledge on this consensus. Previously, our research group had shown that this E-learning could relatively easily instruct pathologists worldwide, experienced and those in training, the TSR scoring method, also on a long-term basis with consistently high interobserver agreement Cohen's kappa's >0.70 [6]. Moreover, the TSR requires no additional, time-consuming nor expensive analyses or staining, but is scored on the same haematoxylin-and-eosin stained routine diagnostic pathology slide used in staging of the local extent of the tumour, i.e. T-stage [7]. From 2007 onward, our research group was the first to describe this parameter, but nowadays, overwhelming evidence from exponential amounts of studies published worldwide has been collected [1].

In conclusion, the TSR is relatively easy, cost-effective, reproducible and valid pathological prognosticator. Stroma-high tumours are more aggressive and less likely to respond to treatments, based on biopsy data, the primary tumour itself, and lymph nodes. The TSR can support patient selection for specific treatment strategies. For example, frail and/or older patients with stroma-high tumours may be offered less or no (neo-)adjuvant therapy, while fitter and/or younger patients may be offered more intensive or experimental treatments. New techniques, such as FAPI PET-CT scans and artificial intelligence, may help map the TSR and other tumour characteristics more quickly and accurately in the future. Moreover, the TSR qualifies for the as abovementioned stated 'robustness' with highest levels of evidence, and although the TSR as predictive biomarker will be proven in the foreseeable nigh future, the TSR as prognostic biomarker should urgently be implemented in (inter)national guidelines.

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