Improving diagnostic, prognostic and predictive biomarkers in colorectal cancer: the role of proteomics and stromatogenesis

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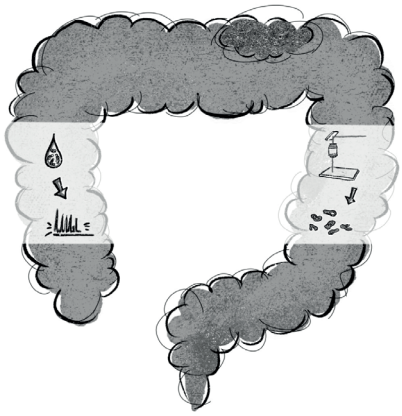

General Discussion and Future Perspectives

Colorectal cancer forms a major health burden. It is one of the most frequently occurring cancers worldwide next to lung and breast cancer [1]. In 2018, 14.200 newly diagnosed patients were reported in The Netherlands. Although the incidence rate of colorectal cancer (CRC) in the Dutch population has increased, the mortality rate has decreased due to continuous improvements in the diagnostic process and treatment options [2]. Nevertheless, CRC is still the second leading cause of cancer related deaths. Tumor stage is one of the most important prognosticators for colon cancer. Therefore, early diagnosis is of great importance to reduce disease-related mortality [3, 4].

This doctoral thesis analysed the pathologic and molecular characterizations of colorectal cancer, with a focus on the role of (diagnostic, prognostic and predictive) biomarkers and an aim to improve disease specific survival. The thesis was divided into two parts.

In the first part, the research analysed the role of proteomics as a *diagnostic* biomarker for early colorectal cancer detection. This part of the research is important because through its use as a diagnostic biomarker, proteomics may improve screening applications.

The second part of this research examined the role of stromatogenesis as a *prognostic* and *predictive* biomarker, and as such the role of stromatogenesis on risk stratification of colorectal cancer patients. This part is of great clinical relevance, because stromatogenesis in our research provides a robust, reliable biomarker. In addition, it gives future leads to develop new biomarkers that will contribute to risk stratification of colorectal cancer beyond clinical staging.

PART 1 PROTEOMICS AS A DIAGNOSTIC BIOMARKER

As mentioned before, early diagnosis of colon cancer is important to reduce disease-related mortality. Therefore, non-invasive screening methods can offer a vital improvement for survival. However, current screening protocols have a limited sensitivity and specificity [5-8]. We therefore chose to study whether the use of serum biomarkers to distinguish cancer patients from healthy persons could be a tool to improve screening programs. Serum is an ideal sample type for early detection markers since samples can be obtained in a straightforward, standardised manner at minimal cost, minimal risk and, most importantly, in a less-invasive manner compared to existing detection methods, such as colonoscopy [9].

Chapters 2 and 3 therefore focused on proteomic serum biomarkers. This could provide a non-invasive *diagnostic* biomarker. Mass spectrometry based proteomics (MS) is a technology used for mapping and identifying peptides and proteins in body fluids [10-14]. In **chapter 2**, an overview of protein profiling methods for CRC and breast cancer (BC) proteomic serum biomarkers is provided with translation to implementation in clinical setting and potential screening programs. Several case-control studies described favourable reports on serum protein profiling of BC and CRC. Comparing the reported sensitivities and specificities with current screening techniques, MS would appear to be a very promising tool. However, these results are likely to be overoptimistic when compared to a screening population. The described series analysed selected groups of patients with a priori a higher chance of having CRC compared to a screening population. As the control groups of those studies only consisted of healthy people, it is impossible to determine whether the discriminatory peaks are actually (colon) cancer specific or more general disease-specific. In addition, these studies used different sample processing methods. In order to apply MS in a routine clinical setting, collecting, measuring and processing of data must have strict protocols and guidelines to make it a robust and reproducible method [15, 16]. The current robotic platforms facilitate standardized methods and high throughput. It sometimes seems almost elusive to reproduce MS outcome into clinical practice, but focusing on analysing specific sets of identified proteins (targeted proteomics) instead of different protein spectra might give further direction into clinical translation. More comparative and prospective studies are needed to determine the value of MS in clinical practice and the possible superiority to other screening methods.

Therefore, we designed our study in the manner described in **chapter 3**: a case-controlled study that identifies proteomic profiles and their potential for colorectal cancer screening. For this study, a mass spectrometry based serum peptide and protein biomarker signature was found with a high discriminative power to distinguish CRC patients from healthy controls. The area under the curve (AUC) was 0.95 with a high specificity of 94-95%. Full automation of the preparation and analysis process in our robotic platform ensures standardization and robustness. The current screening with immunochemical faecal blood test (iFOBT) requires many additional colonoscopies. Almost 90% of those colonoscopies following a positive iFOBT are negative [17]. Colonoscopies are invasive procedures that are not without risks and often require sedation. It is time consuming and requires bowel preparation. Instead, the serum proteomics test is based on the analysis of one tube of peripheral blood. It is easy to apply, cheap and patient friendly with good sensitivity and specificity. Comparing this test performance in a population cohort and to the current screening methods may result in additional possibilities for less invasive screening programs. However, despite

the high discriminative power and automated handling, larger studies are essential to evaluate the 'tumor-specificity' of the obtained discriminative signatures.

Since conducting our research, a large number of additional CRC biomarkers were identified by proteomics using diverse approaches. Alnabulsi et al and Binetti et al, give a detailed review of recent achievements of clinical implementation of those biomarkers [18,19]. They again conclude that the clinical potential of proteomic biomarkers will not be fully determined without improvements in the validation process. Continued advancements in sample processing, detection technologies and computational analysis will gradually address the challenges in proteomics and hopefully enable the safe implementation in clinical setting.

PART 2 STROMATOGENESIS AS A PROGNOSTIC AND PREDICTIVE BIOMARKER

Chapters 4-6 focused on stromatogenesis and its possible role as a new *prognostic* and *predictive* biomarker. Stromatogenesis is the formation of new specific types of tumor stroma. Apart from the importance of early detection, the stage-independent outcome variability is a topic of great interest. Some patients with early stage CRC may show relapse, cancer progression and worse survival compared to other early stage CRC patients. To evaluate this risk stratification of colon cancer beyond current clinical staging, understanding the molecular heterogeneity enhances the ability to select patients in need of additional or adjusted treatment protocols [20]. Tumor stroma facilitates tumor cell invasion and migration. Therefore, tumor stroma and cancer cell interactions may be key elements in the puzzle of tumor survival, growth, invasion and metastasis [43=21]. The tumor stroma percentage in colon cancer patients has previously been reported by our research group as a strong independent prognostic parameter [22, 23]. Patients with a high stroma percentage within the primary tumor have a poor prognosis. In **chapter 4**, validation of the tumor-stroma ratio (TSR) as a prognostic biomarker in a large study population of the VICTOR trial is described, confirming the TSR to be an independent strong prognostic factor. Our study confirms that an increased amount of stromal involvement, even if it is detected in only a small part of the total tumor mass, can be linked to an unfavourable prognosis, independent of other prognostic parameters. Next to histopathological staging, the microsatellite instability (MSI) status is advised as an indicator for therapy choice and possible predictor for prognosis [24-27]. In this study the MSI status showed no significant difference in survival, but TSR and MSI were found to be associated. Furthermore, our high inter-observer agreement in this and previous studies indicates that the

TSR is a highly reproducible measurement. It is remarkable that a simple tissue-based parameter can possess such a high discriminative power without any additional costs. It would therefore be of great importance to implement TSR into daily routine diagnostics next to the TNM classification, to better predict prognostic outcome of CRC patients.

In our study, the worse prognosis of TSR high patients was again confirmed. However, there is no suitable therapy or even a lead for new therapy developments for this high-risk group. We therefore investigated the stromatogenesis process to discover perspectives for new treatment options. One of our hypotheses was that because one of the factors of tumor progression facilitated by the tumor stroma is angiogenesis, anti-angiogenetic therapy could help increase survival of this high-risk patient group. In **chapter 5**, we therefore evaluated the TSR in the QUASAR 2 trial. We investigated whether anti-angiogenic therapy might improve survival of patients with a stroma-high profile. The QUASAR 2 trial is a large phase III randomized trial of adjuvant capecitabine (CAP) ± bevacizumab after complete surgical resection of high-risk stage II and stage III colorectal cancer [28]. Bevacizumab is a monoclonal antibody against vascular endothelial growth factor, which therefore might interfere with the stromatogenesis. Importantly, although the study population only consisted of high-risk patients, the study confirmed again that TSR is an independent prognostic factor for colon cancer patients by showing that this parameter is strong enough to differentiate patients even in an already selected group. In addition, a worse survival for patients with vascular invasion was confirmed. Nonetheless, our hypothesis failed because no effect in disease free survival was seen with respect to additional bevacizumab treatment. Furthermore, a significant difference in survival was seen comparing groups with or without vascular invasion. And above that, a correlation between vascular invasion and stroma-high was seen, supporting the negative prognostic value of both high-risk factors. The relation between patients with a stroma-high tumor and vascular invasion has not been described earlier. This correlation could confirm the important role angiogenesis plays in the stromal environment.

But besides bevacizumab, different treatment regimens should be evaluated. Further knowledge of the stromal composition might lead to new targeted treatment regimens. In **chapter 6** we therefore evaluated this stromal composition to identify its activated pathways and the possible interactions for therapy targets. We described a pilot study where stromal tissue was analysed using laser capture microdissection coupled to broad-scale protein pathway activation mapping using reverse phase protein microarrays. We performed this pilot to try to better understand the way stromatogenesis originates and evolves and why patients with a stroma-high tumor

have a poor prognosis, what causes the aggressiveness of tumors with high stromal formation and what pathways are involved in this process. Patients with histologically proven stage II and stage III colon cancer were selected from the LUMC database. Reverse phase protein microarray was performed using microdissected tissue material to generate multiplexed pathway profiling.

Statistical comparison showed the potential presence of biochemical derangements in the tumor stroma from patients with stroma-high colon cancer with increased activation of VEGFR-2 and decreased activation of ZAP70, eNOS and ICAM-1 compared to stroma-low tumors. VEGFR2 is one of the most prominent ligand-receptor complexes in the VEGF system. It can lead to endothelial cell proliferation, migration, survival and new vessel formation involved in angiogenesis [29]. High levels of VEGF expression are related to poorer survival and an increased rate of distant metastases in colorectal cancer patients [30]. ZAP70 encodes an enzyme belonging to the protein tyrosine kinase family and plays a role in T-cell development and lymphocyte activation. It is used as a prognostic marker in identifying different forms of chronic lymphocytic leukaemia (CLL). The expression of ZAP70 is associated with a significantly lower overall survival [31]. Its role in CRC is not described yet. Our study showed a lower expression of ZAP70 in the stroma-high group. This correlates with our visual finding of stroma-high tumors having microscopically less lymphocytic infiltration compared to the stroma-high tumors. Further research is necessary to unravel the mechanism and the possible clinical implications behind this. eNOS is known to be involved in the production of nitrogen oxide (NO) through L-arginine. Literature suggests that NO plays a key role in physiological regulations, including defence mechanisms against infectious disease and tumors [32]. ICAM-1 is a surface glycoprotein and is known to be a member of the immunoglobulin gene superfamily of adhesion molecules. It is expressed on vascular endothelium and plays a key role in the trans endothelial migration of neutrophils and T-cell activation [33]. It has been suggested that ICAM-1 can inhibit cancer progression by activation of the host immune surveillance system by adherence to the extracellular matrix and thereby alleviating or eliminating metastasis of CRC [33, 34].

Correlation analysis also showed more interconnections in the stroma-low group compared to the stroma-high group. The stroma-low group showed two major interconnection nodes: eNOS and ARPC2. Furthermore, within the stroma-low group, there is a significantly higher expression of eNOS with many interconnections including ARPC2. With the characteristics of eNOS as described above, it may be an important player contributing to the better prognosis of patients with a stroma-low tumor compared to the stroma-high group.

The other lead in the correlation map within the stroma-low group is ARPC2. In literature ARPC2 in gastric cancers showed significant associations with large tumor size, lymph node invasion, and high tumor stage. In addition, in the same study ARPC2-positive patients had lower recurrent free and overall free survival rates compared to ARPC2-negative patients [35]. In breast cancer, ARPC2 is described to promote cancer proliferation and metastasis [36]. In colon cancer, so far only an under expression of ARPC2 in early colorectal cancer is described [37]. In our study, ARPC2 is equally expressed in the stroma-high and stroma-low group. But ARPC2 shows many correlations and might be an important part of the stroma-low micro-environment network.

The aforementioned interconnections might play an important contribution to the favourable prognosis of the stroma-low group. These findings in our study could give a new lead for additional research to better understand the different tumor phenotypes of these two prognostically different groups based on their stroma amount.

FUTURE PERSPECTIVES

Proteomics future prospects

The field of proteomics is constantly changing. In earlier days biomarker discovery was performed using protein profiling or (untargeted) proteomics. Nowadays targeted quantitative proteomics, with predefined set of biomarkers is performed. Quantitative proteomics using mass spectrometry allows for system-wide identification and quantification of proteins and targeted proteomics applications. Quantitative mass spectrometry analyses can detect and quantify thousands of proteins in a single experiment. Furthermore, combining laser capture microdissection and proteomics techniques is a promising way to find significant differentially expressed proteins in target tissues [38, 39]. Furthermore, like mentioned earlier, the challenge of clinical implementation depends largely on the possibility of a reproducible and well validated biomarker. Continued advancements in knowledge, technologies and computational analysis will hopefully enable the safe implementation of proteomic biomarkers in clinical setting.

TSR Prospective multicentre study

To further refine the prognostic prediction strategies of CRC patients, it would be of great importance to implement TSR into daily routine diagnostics next to the TNM classification. It is a low-cost test, performed on standard HE slides and requiring only a small amount of time. The TNM Evaluation Committee (UICC) and the College of

American Pathologists (CAP) stated the TSR has the potential to be included in the TNM staging algorithm but needs validation in a prospective cohort. Therefore, the UNITED study has been designed [40]. This international multicentre study investigates the reproducibility of scoring the TSR amongst pathologists, using an E-learning module. Stage II and III colon cancer patients are simultaneously included to validate the prognostic value of the TSR in a European prospective observational cohort. The inclusion of patients is still ongoing. After the results of this prospective study are published, which confirm that the TSR is an independent strong diagnostic biomarker, we expect the TSR to be implemented next to the routinely used TNM classification.

Stromatogenesis

The mechanism by which tumor stroma facilitates tumor progression has not yet been fully unravelled. However, a key hypothesis is that stroma producing factors influence local and systemic inflammation, tumor pH and tumor metabolism [41]. An improved understanding of tumor and stroma metabolism could give insights and possible leads for new therapy strategies. Normal differentiated cells primarily rely on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes. In contrast, most cancer cells rely on aerobic glycolysis, a phenomenon called "the Warburg effect" [42]. Aerobic glycolysis is an inefficient way to generate energy. The advantage it might confer to cancer cells has been unclear, but this process might be facilitated by the tumor-supporting stroma. Giatromanolaki et al. reported that increased tumor cell expression of enzyme pathways associated with anaerobic metabolism and lactate extrusion, including lactate dehydrogenase isoenzyme 5 (LDH-5) and monocarboxylate transporter 1 (MCT-1), increased the ability of cancer-associated fibroblasts to uptake and oxidate lactate, supporting tumor cell metabolism [21].

As Roseweir et al. described in their study that the combination of TSR and tumor cell expression of cytoplasmic MCT-2 or nuclear LDH-5 is associated with poor prognosis for stage I-III CRC. Moreover, the combination of TSR and nuclear LDH-5 was significantly associated with increased tumor budding and decreased stromal CD3+ T-lymphocytes. Tumor budding is associated with poor prognosis in CRC and is thought to be the histological representation of epithelial-mesenchymal transition. Decreased T-lymphocytes might suggest that highly metabolically active tumor cells utilize metabolites that are needed by T-lymphocytes to survive and function [43,44]. This supports the hypothesis that one mechanism by which increased stromal invasion promotes tumor progression is through modulation of tumor metabolism. Blocking this metabolic support could be of great therapeutic relevance. Inhibitors of lactate dehydrogenase or blockers of monocarboxylate transporters would severely compro-

mise the metabolic activity and may provide promising therapeutic targets for patients with stroma-high CRC [21,44].

Biopsy TSR

The TSR is assessed on resection specimen of CRC, but it could be interesting to evaluate the value of TSR pre-operatively on biopsy tissue. To that end, it seems feasible to examine the tumor microenvironment on endoscopic biopsy specimen. Park et al. analysed biopsies and resection specimens and found stroma-high in biopsies predicted stroma-high in resected specimens associated with cancer specific survival [45]. However, due to intratumor heterogeneity this also has its limitations. A single biopsy might not adequately represent the stromal makeup of the tumor. In addition to tissue biopsies, liquid biopsies are described as a new method for early detection and tracking of biomarkers during treatment, especially in blood [46]. Zheng et al. suggested that some of the essential interactions between proliferating cells and tumor stroma can in part be monitored through stromal liquid biopsies where the extracellular proteins are found as a proteomic pattern in the general blood circulation (serum) of patients with different types of cancer [47,48]. More research in this area therefore looks promising for early prediction of prognosis or even prediction and monitoring therapeutic benefit in both stroma-high and -low CRC patients.

Digitalizing, Artificial Intelligence and Deep Learning

Recent years, pathology has moved towards a more digitalized workflow. Pathology sections are more and more scanned for digital viewing on a computer instead of examined by the pathologist using conventional microscopy. In this shift towards a digital workflow, automation of tissue parameters and even deep learning to evaluate specimen is of growing interest. Current research is exploring possibilities of developing new algorithms to support the pathologists in daily practice and to reduce their workload. Zhao et al. confirm again the prognostic effect of the TSR for overall survival of colon cancer patients, showing the robustness of the TSR method. But above all they show the possibility to quantify the tumor-stroma percentage by artificial intelligence. Although there are still challenges to overcome, this is a huge step forward. One of those challenges is, for example, the importance of stain normalization before running the algorithm, because of its sensitivity to variation in colours [49]. Skrede et al. recently published an article in the Lancet describing the use of a prognostic marker algorithm based on TSR, which was developed by using deep learning methods [50]. While artificial intelligence may play a role in future clinical decision making, caution has to be taken. For example, Specogna et al. stated important limitations within the training set of Skrede et al. and also an automation bias. A system that is automated is usually entirely data driven and not trained to understand why. Using a causal perspec-

tive, an outcome occurs. Attention should be given to how learned biases might relate to errors eventually translated into clinical decisions with the potential to harm patients. Artificial intelligence, automation and deep learning can bring research to a next level. However, they are unlikely to eliminate the need for expert human intervention, even though they could allow for greater efficiency. Prospective validation studies are needed to assure the safeness of implementation for routine clinical use [51].

Introduction of new biomarkers in the clinic

Implementation of new biomarkers in clinical guidelines and daily practice is time consuming and may take more than a decade. Clinical guidelines should be based on the highest quality of evidence leading to the best available treatment and a standardized approach to patient care [52]. New robust biomarker application is challenging sometimes because of methodological aspects, such as robustness and reproducibility, related to the quality of the technology, the sample, or sometimes just because of the complexity of the tumor biology. More efficient sampling and the use of high-sensitive methodologies within clinical multidisciplinary trials that meet the highest quality standards may overcome the influence of tumor heterogeneity and result in reproducible highly reliable biomarkers. But even when biomarkers fulfil all the criteria, implementation might eventually not be achieved [53].

CONCLUSIONS

This thesis highlights, firstly, the importance of early CRC detection by presenting results of a CRC *diagnostic* proteomic biomarker signature with high discriminative power. Secondly, the strong robust, independent *prognostic* TSR biomarker confirms to be of important clinical value. The TSR has the ability to stratify colon cancer patients according to their prognostic outcome in a highly reproducible and low-cost manner. It has shown to link patients with a high intra tumor stromal content and a worse prognosis. Literature shows a wealth of evidence that supports this prognostic value in CRC as well as in other cancers. This PhD research therefore concludes that it should be implemented in the official guidelines of the TNM classification to improve stratification for CRC patients in daily routine pathological evaluation. The prospective, international, multicentre UNITED study will hopefully overcome the last hurdle for this clinical implementation. Lastly, this thesis offers more insight in the elusiveness of the tumor microenvironment and stromatogenesis that contributes to the aggressiveness of some CRC tumors. The biological differences, interconnections and changes in the microenvironment presented give multiple leads for further research and new personalized treatment possibilities.

REFERENCE LIST

1. Bray, F., et al., Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin, 2018. 68(6): p. 394-424.
2. Brouwer, N.P.M., et al., An overview of 25 years of incidence, treatment and outcome of colorectal cancer patients. Int J Cancer, 2018. 143(11): p. 2758-2766.
3. Labianca, R., et al., Early colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol, 2013. 24 Suppl 6: p. vi64-72.
4. Bresalier, R.S., Early detection of and screening for colorectal neoplasia. Gut Liver, 2009. 3(2): p. 69-80.
5. Imperiale, T.F., et al., Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population. N Engl J Med, 2004. 351(26): p. 2704-14.
6. Yang, H., et al., Effectiveness of the immunofecal occult blood test for colorectal cancer screening in a large population. Dig Dis Sci, 2011. 56(1): p. 203-7.
7. Rozen, P., et al., Cumulative evaluation of a quantitative immunochemical fecal occult blood test to determine its optimal clinical use. Cancer, 2010. 116(9): p. 2115-25.
8. Zavoral, M., et al., Colorectal cancer screening in Europe. World J Gastroenterol, 2009. 15(47): p. 5907-15.
9. Ganepola GA, et al., Use of blood-based biomarkers for early diagnosis and surveillance of colorectal cancer. World J Gastrointest Oncol, 2014.6: p. 83-97.
10. Aebersold, R. and M. Mann, Mass spectrometry-based proteomics. Nature, 2003. 422(6928): p. 198-207.
11. Wulfkuhle, J.D., L.A. Liotta, and E.F. Petricoin, Proteomic applications for the early detection of cancer. Nat Rev Cancer, 2003. 3(4): p. 267-75.
12. Ikonomou, G., M. Samiotaki, and G. Panayotou, Proteomic methodologies and their application in colorectal cancer research. Crit Rev Clin Lab Sci, 2009. 46(5-6): p. 319-42.
13. Silvestri, A., et al., Individualized therapy for metastatic colorectal cancer. J Intern Med, 2013. 274(1): p. 1-24.
14. Pierobon, M., et al., Application of molecular technologies for phosphoproteomic analysis of clinical samples. Oncogene, 2015. 34(7): p. 805-14.
15. West-Norager, M., et al., Unravelling in vitro variables of major importance for the outcome of mass spectrometry-based serum proteomics. J Chromatogr B Analyt Technol Biomed Life Sci, 2007. 847(1): p. 30-7.
16. Baumann, S., et al., Standardized approach to proteome profiling of human serum based on magnetic bead separation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Chem, 2005. 51(6): p. 973-80.
17. Zorzi M, et al. High sensitivity of five colorectal screening programmes with faecal immunochemical test in the Veneto Region, Italy. Gut 2011 Jul;60(7):944-9.
18. Alnabulsi A, Murray GI. Proteomics for early detection of colorectal cancer: recent updates. Expert Rev Proteomics. 2018 Jan;15(1):55-63.
19. Binetti M, et al., Proteogenomic biomarkers in colorectal cancers: clinical applications. Expert Rev Proteomics. 2020 May;17(5):355-363.
20. Dotan, E. and S.J. Cohen, Challenges in the management of stage II colon cancer. Semin Oncol, 2011. 38(4): p. 511-20.
21. Giatromanolaki, A., E. Sivridis, and M.I. Koukourakis, The pathology of tumor stromatogenesis. Cancer Biol Ther, 2007. 6(5): p. 639-45.

22. Mesker, W.E., et al., The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Cell Oncol, 2007. 29(5): p. 387-98.
23. Mesker, W.E., et al., Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I-II colon cancer patients. Cell Oncol, 2009. 31(3): p. 169-78.
24. Ribic, C.M., et al., Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med, 2003. 349(3): p. 247-57.
25. Kim, S.T., et al., Clinical impact of microsatellite instability in colon cancer following adjuvant FOLFOX therapy. Cancer Chemother Pharmacol, 2010. 66(4): p. 659-67.
26. Sargent, D.J., et al., Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. J Clin Oncol, 2010. 28(20): p. 3219-26.
27. Bertagnolli, M.M., et al., Microsatellite instability predicts improved response to adjuvant therapy with irinotecan, fluorouracil, and leucovorin in stage III colon cancer: Cancer and Leukemia Group B Protocol 89803. J Clin Oncol, 2009. 27(11): p. 1814-21.
28. Kerr, R.S., et al., Adjuvant capecitabine plus bevacizumab versus capecitabine alone in patients with colorectal cancer (QUASAR 2): an open-label, randomised phase 3 trial. Lancet Oncol, 2016. 17(11): p. 1543-1557.
29. Abhinand, C.S., et al., VEGF-A/VEGFR2 signaling network in endothelial cells relevant to angiogenesis. J Cell Commun Signal, 2016. 10(4): p. 347-354.
30. Cho, T., et al., The role of microvessel density, lymph node metastasis, and tumor size as prognostic factors of distant metastasis in colorectal cancer. Oncol Lett, 2017. 13(6): p. 4327-4333.
31. Cramer, P. and M. Hallek, Prognostic factors in chronic lymphocytic leukemia-what do we need to know? Nat Rev Clin Oncol, 2011. 8(1): p. 38-47.
32. Zhao, Y., P.M. Vanhoutte, and S.W. Leung, Vascular nitric oxide: Beyond eNOS. J Pharmacol Sci, 2015. 129(2): p. 83-94.
33. Hubbard, A.K. and R. Rothlein, Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. Free Radic Biol Med, 2000. 28(9): p. 1379-86.
34. Wang, Q.L., et al., Polymorphisms of the ICAM-1 exon 6 (E469K) are associated with differentiation of colorectal cancer. J Exp Clin Cancer Res, 2009. 28: p. 139.
35. Zhang, J., et al., Role of ARPC2 in Human Gastric Cancer. Mediators Inflamm, 2017. 2017: p. 5432818.
36. Cheng, Z., et al., ARPC2 promotes breast cancer proliferation and metastasis. Oncol Rep, 2019. 41(6): p. 3189-3200.
37. Lau, T.P., et al., Pair-wise comparison analysis of differential expression of mRNAs in early and advanced stage primary colorectal adenocarcinomas. BMJ Open, 2014. 4(8): p. e004930.
38. Mustafa, D., J.M. Kros, and T. Luider, Combining laser capture microdissection and proteomics techniques. Methods Mol Biol, 2008. 428: p. 159-78.
39. Liu, N.Q., et al., Quantitative proteomic analysis of microdissected breast cancer tissues: comparison of label-free and SILAC-based quantification with shotgun, directed, and targeted MS approaches. J Proteome Res, 2013. 12(10): p. 4627-41.
40. Smit, M., et al., Uniform Noting for International Application of the Tumor-Stroma Ratio as an Easy Diagnostic Tool: Protocol for a Multicenter Prospective Cohort Study. JMIR Res Protoc, 2019. 8(6): p. e13464.
41. Park, J.H., et al., Evaluation of a tumor microenvironment-based prognostic score in primary operable colorectal cancer. Clin Cancer Res, 2015. 21(4): p. 882-8.
42. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science, 2009. 324(5930): p. 1029-33.

43. van Wyk, H.C., et al., The role of tumour budding in predicting survival in patients with primary operable colorectal cancer: a systematic review. Cancer Treat Rev, 2015. 41(2): p. 151-9.
44. Roseweir, A.K., et al., The association between markers of tumour cell metabolism, the tumour microenvironment and outcomes in patients with colorectal cancer. Int J Cancer, 2019. 144(9): p. 2320-2329.
45. Park, J.H., et al., Preoperative, biopsy-based assessment of the tumour microenvironment in patients with primary operable colorectal cancer. J Pathol Clin Res, 2019.
46. Herrera, M., et al., A Snapshot of The Tumor Microenvironment in Colorectal Cancer: The Liquid Biopsy. Int J Mol Sci, 2019. 20(23).
47. Wang, X. and M. Kuruc, Functional Proteomics: Methods and protocols, Methods in Molecular Biology. 2019. Vol. 1871.
48. Zheng, H., et al., Stroma Liquid Biopsy - Proteomic Profiles for Cancer Biomarkers. Cell, 2011. 141: p. 646-674.
49. Ciompi, F., et al. The importance of stain normalization in colorectal tissue classification with convolutional networks. ArXiv 170205931 Cs. 2017.
50. Skrede OJ, et al. Deep learning for prediction of colorectal cancer outcome: a discovery and validation study. Lancet. 2020 Feb 1;395(10221).
51. Specogna AV, Sinicrope FA. Defining colon cancer biomarkers by using deep learning. Lancet. 2020 Feb 1;395(10221):314-316.
52. Rauh, S., et al., Challenge of implementing clinical practice guidelines. Getting ESMO's guidelines even closer to the bedside: introducing the ESMO Practising Oncologists' checklists and knowledge and practice questions. ESMO Open, 2018. 3(5): p. e000385.
53. Prudkin, L. and P. Nuciforo, Obstacles to precision oncology: confronting current factors affecting the successful introduction of biomarkers to the clinic. Cell Oncol (Dordr), 2015. 38(1): p. 39-48.