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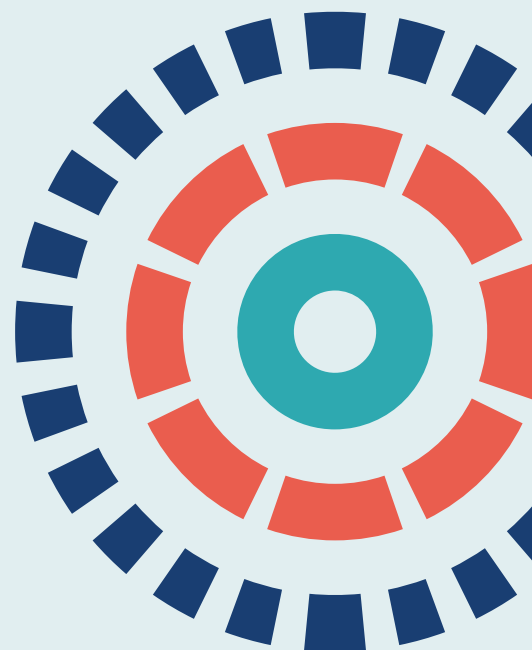
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This report

Abstract

Intratumoural immune signature to identify patients with primary colorectal cancer who do not require follow-up after resection: an observational study

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Background: Following surgical and adjuvant treatment of primary colorectal cancer, many patients are routinely followed up with axial imaging (most commonly computerised tomography imaging) and blood carcinoembryonic antigen (a tumour marker) testing. Because fewer than one-fifth of patients will relapse, a large number of patients are followed up unnecessarily.

Objectives: To determine whether or not the intratumoural immune signature could identify a cohort of patients with a relapse rate so low that follow-up is unnecessary.

Design: An observational study based on a secondary tissue collection of the tumours from participants in the FACS (Follow-up After Colorectal Cancer Surgery) trial.

Setting and participants: Formalin-fixed paraffin-embedded tumour tissue was obtained from 550 out of 1202 participants in the FACS trial. Tissue microarrays were constructed and stained for cluster of differentiation (CD)3+ and CD45RO+ T lymphocytes as well as standard haematoxylin and eosin staining, with a view to manual and, subsequently, automated cell counting.

Results: The tissue microarrays were satisfactorily stained for the two immune markers. Manual cell counting proved possible on the arrays, but manually counting the number of cores for the entire study was found to not be feasible; therefore, an attempt was made to use automatic cell counting. Although it is clear that this approach is workable, there were both hardware and software problems; therefore, reliable data could not be obtained within the time frame of the study.

Limitations: The main limitations were the inability to use machine counting because of problems with both hardware and software, and the loss of critical scientific staff. Findings from this research indicate that this approach will be able to count intratumoural immune cells in the long term, but whether or not the original aim of the project proved possible is not known.

Conclusions: The project was not successful in its aim because of the failure to achieve a reliable counting system.

Future work: Further work is needed to perfect immune cell machine counting and then complete the objectives of this study that are still relevant.

Trial registration: Current Controlled Trials ISRCTN41458548.

Contents

List of figures

List of abbreviations

CD	cluster of differentiation	H&E	haematoxylin and eosin
CEA	carcinoembryonic antigen	HTA	Health Technology Assessment
CRC	colorectal cancer	IDA	industrial denatured alcohol
CT	computerised tomography	NIHR	National Institute for Health Research
DFS	disease-free survival		
FACS	Follow-up After Colorectal Surgery	TMA	tissue microarray

Plain English summary

Bowel cancer (also known as colorectal cancer) is the fourth commonest cancer in the UK. When the cancer is confined to the bowel and/or the surrounding lymph nodes (early bowel cancer), it is typically treated with an operation to remove the cancer with or without the addition of chemotherapy. Following this treatment, many patients will be cured, but in approximately one in five patients the cancer may come back (recur) either in the bowel or in another organ (e.g. the liver). Consequently, after treatment of early bowel cancer, clinicians often follow up patients in the hope of detecting any recurrent cancer at an early and treatable stage. For the four out of five patients whose cancer will never recur, this follow-up is unnecessary and burdensome on both the NHS and the patients. Better markers are needed to inform which patients do and do not need to undergo this surveillance.

Over the last decade, evidence has accumulated to show that the way that a patient's immune system responds to a cancer influences the likelihood of the cancer recurring. It is plausible that those with the most immune cells in their cancer have such a small chance of recurrence that follow-up is not necessary. To validate this in an accurately followed-up population of patients with bowel cancer, we collected cancer tissue specimens from 701 patients in the Follow-up After Colorectal Surgery (FACS) trial and developed methods to count the number of immune cells in their cancers. At present, methods are still under development to automate the process. Indeed, if this were ever to become part of routine practice in NHS laboratories, then automation would be essential.

Scientific summary

Background

Following primary surgical and adjuvant treatment for colorectal cancer, patients are routinely followed up for ≥ 5 years. This involves blood testing for carcinoembryonic antigens and a variable number of computerised tomography scans. The Follow-up After Colorectal Surgery (FACS) trial showed that this follow-up is effective in detecting recurrences that are treatable with curative intent. However, as only 20% of patients relapse, many patients are followed up (resulting in the spend of attendant costs and inducing patient anxiety) but never develop recurrence. It is known that the immunological response to cancer manifested by the presence of intratumoural lymphocytes, especially T cells [cluster of differentiation (CD)3+] and memory T cells (CD45RO+), correlates strongly with outcome. We surmised that it may be possible to use the data from immunophenotyping of the primary tumours to identify a cohort of patients in whom relapse is unlikely and follow-up is not needed. The FACS trial is an ideal opportunity to investigate this possibility as patients have a defined follow-up strategy after standard of care treatment and good follow-up data.

Aim and objectives

The aim was to determine whether or not the density of CD45RO+ and CD3+ lymphocytes in the primary tumour from patients in the FACS trial can be used to predict the possibility of relapse. The data from the analysis of these initial markers were intended to provide guidance on what other markers may be of value in supporting the primary hypothesis.

Methods

Pathology blocks
Tissue was obtained from the primary tumours and from the FACS trial, a 2 × 2 pragmatic, randomised, factorial controlled trial comparing minimum post-surgery follow-up of colorectal cancer patients for 5 years with 3- to 6-monthly blood tests for carcinoembryonic antigen and 6- to 12-monthly computerised tomography imaging. As the overall survival was similar in all arms of the trial, we included all of the patients from the trial in the tissue collection. Tissue was stored in the Southampton Tissue Bank until it was used.

Tissue analysis
A full-face section was taken from each block and stained with standard haematoxylin and eosin. The blocks were marked to allow sampling of cores to create tissue microarrays. Three cores were taken from the centre of the tumour and three were taken from the invasive margin. The tissue microarrays were sectioned and stained for CD45RO+ and CD3+ T lymphocytes as well as being stained with standard haematoxylin and eosin in the first instance.

Cell counting
Initially, the intention was to manually count lymphocytes in the tissue microarrays. This was attempted but was abandoned because the time needed to undertake the work was not feasible for the individuals concerned. Counting was then attempted using an automated cell counter. A Zeiss Axio Scan.Z1 (ZEISS, Oberkochen, Germany) whole-slide scanner was utilised at a resolution equivalent to ×20 magnification. The initial image analysis was carried out using QuPath (developed at the University of Edinburgh; originally created at the Centre for Cancer Research & Cell Biology at Queen's University Belfast as

part of research projects funded by Invest Northern Ireland and Cancer Research UK). Various technical issues were encountered, including misregistration of the tissue microarray cores by the software (requiring manual relocation, which was a prolonged exercise). Some preliminary data were obtained on 287 patients prior to the scientist running the scanner relocating. These results represent only part of the cohort and, as such, are unsuitable for publication. However, they give an indication that a signal is present which warrants pursuing the project further.

Relevance and implications

This study was not feasible as originally conceived because manual counting of this sample size would be possible within a 'citizen science'-type programme only, because of the time-consuming nature of the task. It has been demonstrated that machine counting may be possible if there is suitably functioning hardware and software combined with available technical expertise. It seems likely that the use of machine counting of tissue microarrays of colorectal tumours will give prognostic information, and preliminary data suggest that the survival benefit associated with a high infiltrate of CD3- and CD45RO-positive immune cells is limited to those with left-sided tumours. Further work is required to verify the results and to include a much larger cohort to analyse additional immune markers to confirm these observations. It is not clear whether or not the immune signature as assessed by these methods will be able to identify a subset of patients in whom follow-up is not required.

Trial registration

This trial is registered as ISRCTN41458548.

Funding

Chapter 1 Introduction

International guidelines recommend that after completion of treatment for primary colorectal cancer with curative intent, some form of patient follow-up is instated with the aim of detecting treatable recurrence.[1-4] Indeed, there is abundant evidence that circumscribed recurrence is amenable to further potentially curative treatment.[5,6] However, the optimal methodology of follow-up is not known. A Cochrane review[7] was performed to evaluate this but included studies in which follow-up was not linked to any treatment that could improve survival. Therefore, the results are difficult to interpret. Of the trials published in the past decade, GILDA,[8] Follow-up After Colorectal Surgery (FACS) trial[9] and COLOFOL[10] are important to consider.

The GILDA[8] trial mainly utilised ultrasound-based imaging, which is insufficiently sensitive to detect recurrence and is not applicable to the current management of colorectal cancer. Furthermore, all of the patients had tumour marker [carcinoembryonic antigen (CEA)] analysis performed and, therefore, the GILDA trial did not evaluate follow-up strategies that did not involve regular blood tests. It was a negative trial. By contrast, COLOFOL[10] examined two intensive schedules of follow-up, both including computerised tomography (CT) imaging and CEA blood tests but with different frequencies. Crucially, it did not include stage I cancers. This was also a negative trial.

The FACS trial[9] evaluated the use of CEA and CT imaging in a 2 × 2 trial design. This included a minimum surveillance arm in which patients had purely symptomatic follow-up with the possible addition of a single CT scan at 12–18 months. This trial had an end point of identification of recurrent disease that was treatable with curative intent. The trial was positive by this primary end point. All three intensive arms (i.e. CEA only, CT only, and CEA and CT combined) identified more treatable recurrences than the minimum follow-up arm. Importantly, it showed that there was no benefit in having both regular CEA measurements and CT imaging.

The FACS trial included patients with stage I–III colorectal cancer. Although predictably, the incidence of relapse was higher among those with higher-stage cancer, patients with all stages of cancer benefited equally from follow-up. This is because a greater proportion of the patients with recurrence from early-stage cancers were able to undergo further treatment with curative intent.[5,9] Therefore, the benefit of follow-up is not stage dependent.

In recent years, the influence of the immune system in cancer has come to the fore and immunotherapy has become mainstream in the treatment of certain cancers. In colorectal cancer, the observation that a lymphocytic infiltrate in the tumour and enlargement of draining lymph nodes correlates with a better outcome was made a long time ago.[11,12] A seminal study in 2006[13-18] refined this further, reporting that density of infiltration of colorectal cancer with T lymphocytes [cluster of differentiation (CD)3+] and memory T cells (CD45RO+) correlates better with outcome than conventional tumour node metastasis (TNM) staging. Although subsequent studies have not shown the same strength of correlation with outcome as the initial paper,[13] there is a consistent relationship between immune cell infiltration and cancer recurrence.

It is important to consider why the initial reports demonstrated the density T-cell infiltrate to be such a strikingly strong prognostic marker. Careful evaluation of the survival curves reveals frequency of recurrence to be far higher than one would expect by stage. By contrast, the recurrence rate in the FACS trial was lower than many anticipated for a cohort of stage I–III colorectal cancer, but it is in fact in keeping with other modern studies. This is most likely explained by the robust staging that patients underwent prior to being eligible for recruitment to FACS (CT imaging of the chest/abdomen and a CEA level of ≤ 10 μg/l on completion of treatment) meaning that a large number with residual disease were excluded from the study. It is therefore surmised that the cohorts of patients utilised for some studies of immune cell infiltrates may have used understaged patients.

It is therefore crucial that the prognostic relevance of T-cell infiltrates are assessed in a well-staged cohort of patients managed in the modern era of imaging, surgery and adjuvant treatments. The FACS cohort provides such a population and allows us to determine whether or not there is a group of patients in whom the risk of relapse is so low that follow-up is not required.

The work by Galon et al.[13] generally uses full-face tissue block sections. This is not suitable for routine use because of the level of difficulty and the cost involved. If widespread application is to be introduced then any process has to be suitable for routine use in histopathology laboratories. Therefore, it was decided to use tissue microarrays (TMAs). This is a system whereby multiple cores of tissue can be examined in a single section, greatly decreasing the time and cost of processing because material from multiple patients can be managed together. The disadvantage is that only six cores, in general, will be sampled from each tumour; therefore, less of the tumour is assessed when using TMAs than when using full-face tissue block sections.

Aim and objectives

The aim is to determine if immunophenotypic analysis of T lymphocytes within the primary tumour can identify:

1. the risk of relapse
2. a subset of patients in whom follow-up is not necessary.

Chapter 2 Methods

Design

This is a translational observational study utilising tissue and clinicopathological data from the completed FACS trial.[9] The intention is to correlate the incidence of recurrence and the recurrence that is treatable with curative intent with the immune cell milieu in the primary tumour.

Participants

Participants were recruited from the centre at which they received their primary treatment for colorectal cancer, spanning 39 NHS hospitals across all regions of England. To be eligible for the study, participants had to have undergone curative surgery for primary colorectal cancer and, after extensive testing (histology and imaging, and a CEA level of ≤ 10 μg/l), were confirmed to have no residual disease. Further detail regarding the study setting, participant inclusion and exclusion criteria, and randomisation procedures can be found in the original publication.[9]

Patient and public involvement

The original FACS study had significant patient and public involvement. This substudy did not.

Tissue collection

The original FACS proposal did not include funding to obtain tissue from the primary tumours of the patients in the study but the ethics permissions and patient consent allowed for this. The present grant funding enabled the collection of tissue blocks from the centres. This work was undertaken by the Southampton Clinical Trials Unit and tissue stored in the Human Tissue Authority-registered Southampton Tissue Bank until used.

Tissue processing

The tissue blocks were sectioned and stained with routine haematoxylin and eosin (H&E) on an automated Dako CoverStainer (Agilents Diagnostics, Santa Clara, CA, USA) (see *Appendices 2* and *3*). The sections were examined by a pathologist and the sites for the six cores to be taken were marked. Three cores were taken from the centre of the tumour and three were taken from the invasive margin. TMAs were produced on a MiniCore® machine (ALPHELYS, Plaisir, France) (see *Appendix 4*) and recorded on the map shown (*Figure 1*). Sections from the TMA blocks were stained with H&E (see *Appendix 2*) and then for the T-cell markers CD3+ (all T cells) and CD45RO+ (memory T cells). CD3+ and CD45RO+ immunohistochemistry staining was performed on an automated Dako Autostainer 48 Link (Agilents Diagnostics) (see *Appendix 3*). The CD3+ antibody (Dako, Agilents Diagnostics) is ready to use and the CD45RO+ antibody (Dako, Agilents Diagnostics) is concentrated and was used at a dilution of 1 : 2500. The methodology is detailed in *Appendices 5* and *6*.
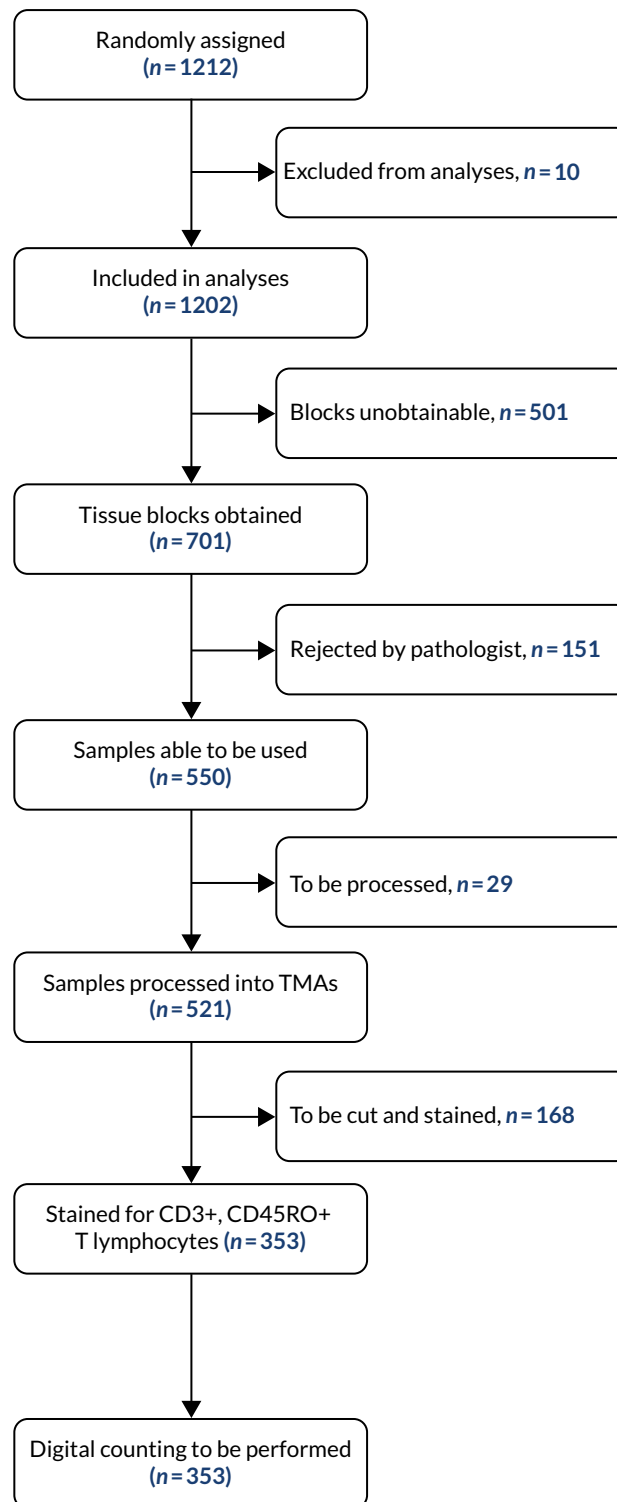
FIGURE 1 Tissue acquisition and processing.

Chapter 3 Results

*F*igure 1 illustrates the tumour tissue available for analysis of patients in the FACS trial. Of the 1202 randomised patients, tissue blocks were obtained for 701 patients. In cases where the initial block sent contained no cancer, another block was requested. Samples from a total of 151 patients were judged to be inadequate by the study pathologists (i.e. they contained no cancer). As a result, tumour tissue blocks were processed for 550 of the 1202 randomised patients. This is an acceptable proportion for a retrospective tissue collection when the collection was not built into the original protocol.

Cell counting

The initial intention was to manually count cells using a standard microscope. Although this proved possible, rapidly became clear that the time required for the project was not feasible. Our calculation was that counting 17 TMAs for two antibodies only would take around 480 hours or 80 6-hour days. Consideration was given to using a 'citizen science'-type programme,[19] but there were no resources to organise such a complex system. In 2015, it became clear that automated analysis was coming to the fore, and in 2016 a Digital Pathology Accelerator award to Belfast and Southampton enabled appropriate hardware and software to be installed. Attempts were made to scan the TMAs on a Zeiss Axio Scan.Z1[20] (ZEISS, Oberkochen, Germany) whole-slide scanner at resolution equivalent to ×20 magnification. Initial image analysis was carried out using QuPath (developed at the University of Edinburgh; originally created at the Centre for Cancer Research & Cell Biology at Queen's University Belfast as part of research projects funded by Invest Northern Ireland and Cancer Research UK).[21] Unfortunately, neither the hardware nor the software functioned correctly.

Numerous problems occurred, including the misregistration of the cores (the number assigned by the machine being at variance with the actual core number) in the software (*Figure 2*) requiring laborious manual reassignment. These problems were compounded by the loss of key science staff following the 2016 referendum, a situation only remedied in 2019. Some preliminary data were obtained (for the abstract, see *Appendix 1*). For each tumour region, high (Hi) and low (Lo) CD3+ and CD45RO+ densities were determined according to the median of the cohort. To investigate the combined effect of CD3+ and CD45RO+ densities in both tumour regions, patients were divided into three groups: Lo (low density of both markers in both tumour regions), Hi (high density of both markers in both tumour regions) and Het (heterogeneous, i.e. high density in some regions and low in others). Examples of the stained TMAs are shown in *Figures 2–5*. The data indicated a potential survival benefit associated with tumours exhibiting a high infiltrate of CD3- and CD45RO-positive immune cells, but these results are preliminary and the reproducibility has not yet been established.

This benefit appeared to be limited to those with left-sided tumours. At present, an attempt is being made to analyse the entire cohort with different software (Definiens Developer XD 2.7; Definiens AG, Munich, Germany).
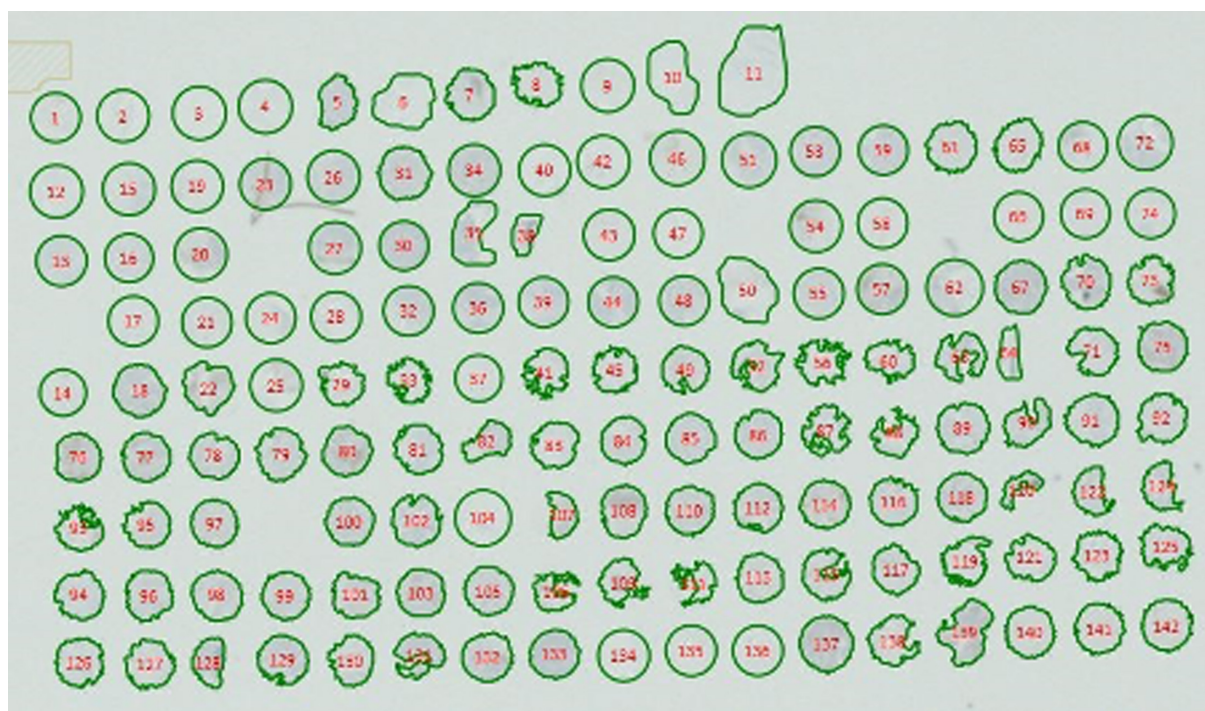
FIGURE 2 Example of a TMA map showing misregistration by the analysis software.
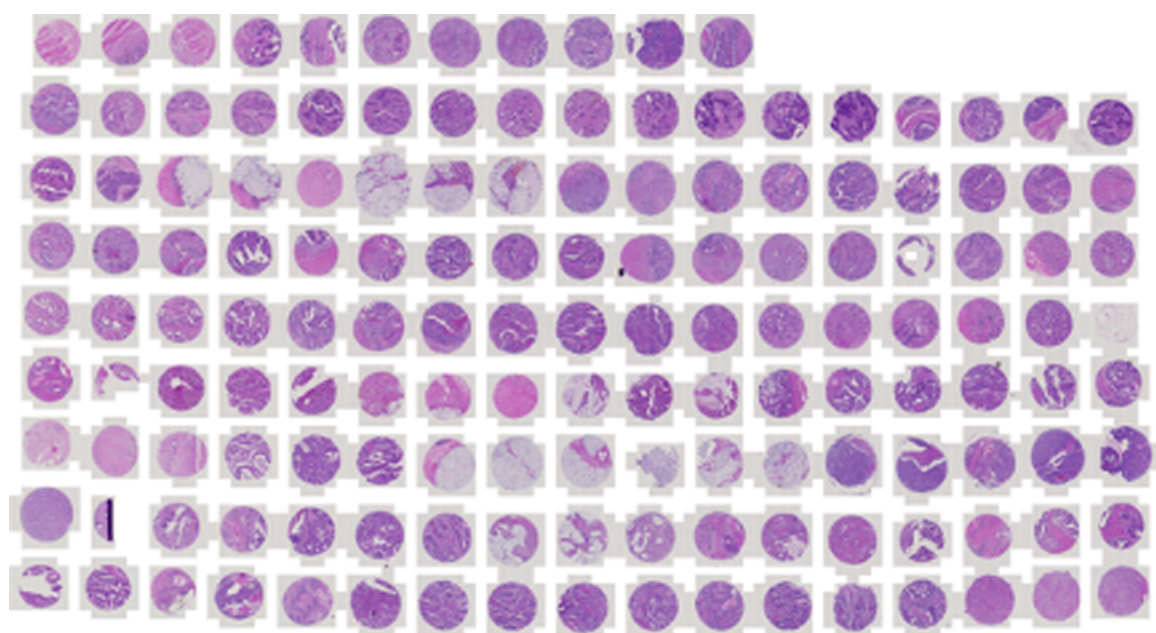

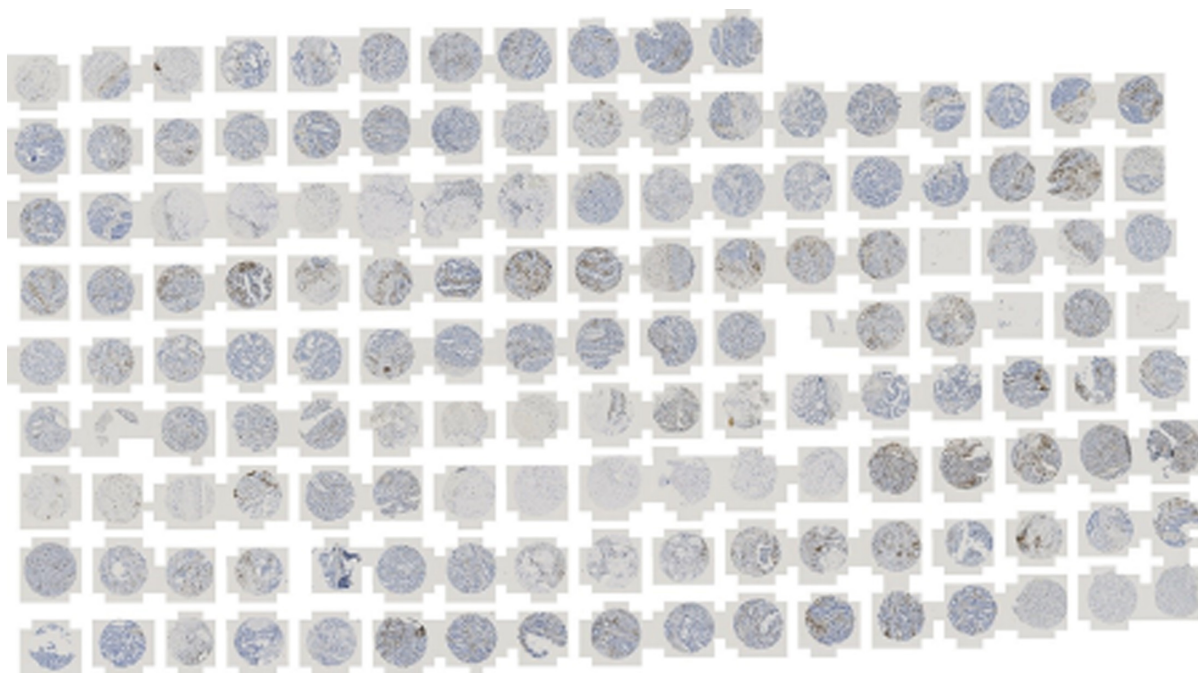FIGURE 3 Example of a TMA map stained with H&E.

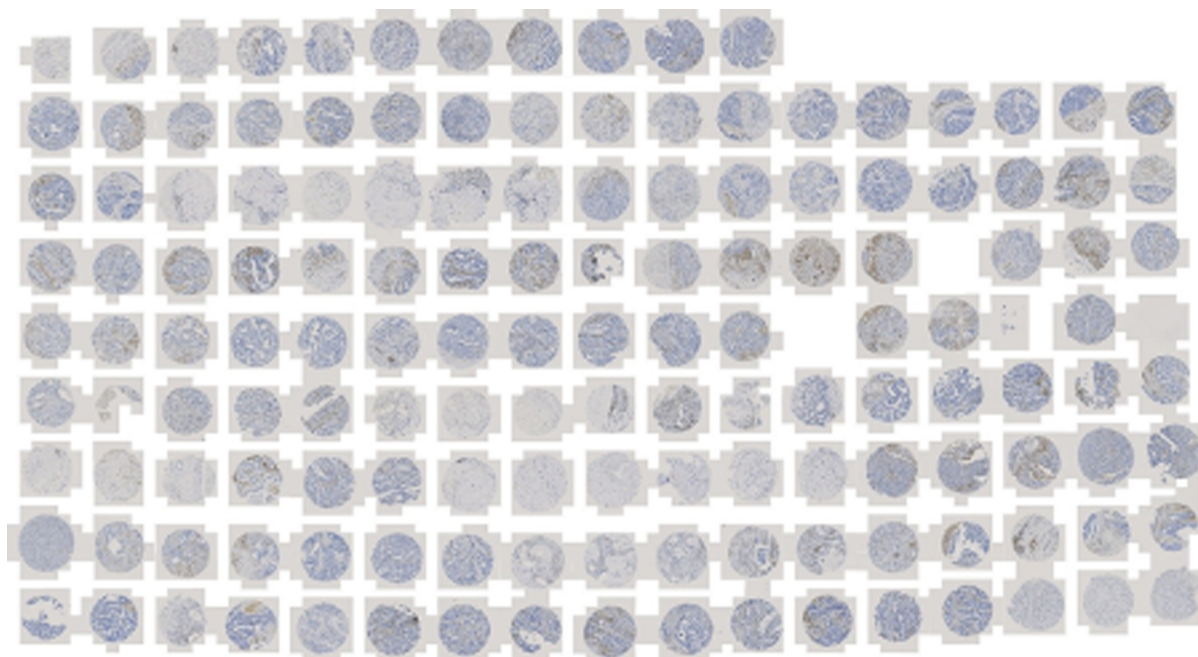FIGURE 4 A TMA map showing staining for CD3+ lymphocytes (staining brown).

FIGURE 5 A TMA map showing staining for CD45RO+ lymphocytes (staining brown).

Chapter 4 Discussion

This study aimed to build on the clinicopathological data acquired during the FACS trial of colorectal cancer follow-up, which is a unique resource. It provides a population of accurately staged patients and, unlike trials of adjuvant therapies, includes patients with early-stage cancers. Although the original grant application was written many years ago, it, fortunately, had the foresight to include consent to collect tumour tissue from these patients. Utilising the resource obtained in a subsequent grant has meant that is has been possible to assess the tumour tissue for approximately half of the patients in the trial.

Traditional prognostic tumour features, such as stage at presentation and differentiation, are predictive of relapse. However, the overall relapse rate of completely resected cancers is relatively low. These features are insufficient to identify a cohort that would not require follow-up.[9]

It is clear that the immune system is critical to the outcome in cancer cases and it is clear that the measurement of immune cells in the primary tumour has prognostic significance. In the original paper by Galon *et al.*,[13] the predictive value was dramatic. However, these patients were almost certainly not staged with the rigour that would be normal now, as carried out in the FACS trial, and it is likely that many of the supposedly early-stage patients would actually have had occult metastatic disease. More recent studies give a more reliable assessment in all probability, but the predictive value remains.[14–18]

The tissue blocks obtained from the patients in the FACS trial provide a resource for future research. We were able to use TMA technology to examine immune cells in multiple patients in one block, hence producing a method that is economical and could be adapted for routine clinical use. Unfortunately, we were not able to perfect machine counting of immune cells within the time frame of this study. We were able to make the system work on a small subset of the specimens, but these results are unvalidated and not suitable for publication at the present time.

The failure to achieve manual counting was not predictable as pathology experts advised that it was reasonable. We had anticipated using undergraduates during their special study module to perform this counting with the expectation of a publication. However, this proved unfeasible.

We have demonstrated that machine counting is possible. This possibility first became available in 2016 when the technology first became widespread. The Zeiss Axio Scan.Z1[20] was introduced to Belfast and Southampton as part of an Accelerator Award in Digital Pathology. There were significant problems with integration of both the hardware and the software (QuPath was used initially).[21] The scanner in Belfast was replaced as it was considered unfit for purpose, but the same scanner remains in Southampton. Scans were originally obtained in Belfast, but problems with the software made the results unable to be used. In 2017, images were acquired in Southampton from a subset of the cohort. As detailed, the misregistration of the cores by the software made any analysis very challenging. Enough data were acquired for an abstract but there has not been the opportunity to confirm the reproducibility of these results or complete the analysis on the whole cohort.

Scientists with the capability to use the hardware and the software are rare. After the 2016 referendum, the scientist involved in this work relocated to outside the UK. It proved impossible to replace this skill set until 2019. There is the intention to continue and develop this work further. The initial approach was to use a limited but validated range of markers (i.e. CD3+, CD45RO+) as proof of principle. However, any publishable work would at least include CD8+ (cytotoxic T cells) and FOXP3+ as a minimum.[14–18] It is also clear that the lymphocytic infiltrate is correlated with the more immunogenic microsatellite unstable [microsatellite instable (MSI) high] tumours, and this can also be assessed on the TMAs.[18]

In summary, despite the technical challenges, machine counting of tumour-infiltrating lymphocytes in TMAs that are formed from the tumours of patients with colorectal cancer is potentially feasible and this might be undertaken as a routine service in a regional pathology laboratory with an appropriate digital scanner and easily available software. However, high-level operator expertise is needed at present. It remains to be seen whether or not these biomarkers are sufficiently discriminating to have an impact on patient management.

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Data Monitoring and Ethics Committee

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Data-sharing statement

The data reported here are a subset extracted from the main FACS trial data set. Requests to access anonymised data from the FACS trial for the purposes of non-commercial research for patient benefit should be addressed to Professor Primrose (j.n.primrose@soton.ac.uk). At present, there are no data from this substudy that can be shared. It is likely that data will become available in due course and after a full publication will be made available as above.

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Appendix 1 Abstract from American Society of Clinical Oncology meeting 2018

Background

Accumulating evidence demonstrates an association between density of tumor infiltrating lymphocytes and outcome in colorectal cancer (CRC). This study sought to assess the prognostic utility using an accurately staged cohort of patients followed up in a clinical trial.

Methods

Observational analysis of data from the FACS (follow-up after CRC surgery) trial after 5 years of follow-up. All patients had undergone treatment with curative intent for stage I–III primary CRC, with microscopically clear margins, no evidence of metastases on axial imaging and CEA < 10 µg/l following completion of treatment. Immune cell densities were quantified in the centre (CT) and invasive margin (IM) of all tumors for both CD3 and CD45RO. For each tumor region high (Hi) and low (Lo) CD3 and CD45RO densities were determined according to the median of the cohort to investigate association with disease-free survival (DFS).

Results

Tumor samples have been analysed from 297 patients to date for which the combined 5-year DFS is 83% (left sided CRC 81%, right sided CRC 85%). High densities of CD3 and CD45RO positive cells in both tumor regions were associated with a superior outcome: $CD3_{CTIM}^{Hi}CD45RO_{CTIM}^{Hi}$ 94% 5-year DFS vs $CD3_{CTIM}^{Lo}CD45RO_{CTIM}^{Lo}$ 81%, HR 0.36 95% CI 0.15–0.89 $p = 0.04$. This difference was most notable in left sided CRC: $CD3_{CTIM}^{Hi}CD45RO_{CTIM}^{Hi}$ 96% 5yr DFS vs $CD3_{CTIM}^{Lo}CD45RO_{CTIM}^{Lo}$ 78%, HR 0.13 95% CI 0.04–0.41 $p = 0.02$. In right sided CRC the difference was not significant: $CD3_{CTIM}^{Hi}CD45RO_{CTIM}^{Hi}$ 91% 5-year DFS vs $CD3_{CTIM}^{Lo}CD45RO_{CTIM}^{Lo}$ 83%, HR 0.70 95% CI 0.17–2.89 $p = 0.62$.

Conclusions

In a well characterised and followed up cohort of CRC patients within a clinical trial we have demonstrated that $CD3_{CTIM}^{Hi}CD45RO_{CTIM}^{Hi}$ left sided tumors have a significantly better outlook. The potential for these data to impact on the need for clinical follow up in patients with left sided CRC should be examined in a prospective study. Clinical trial information: 61091474.

Appendix 2 The H&E protocol on Dako CoverStainer

Station	Reagent	Time (minutes)
1	Xylene	3.00
2	Xylene	3.00
3	95% IDA	0.10
4	95% IDA	2.00
5	70% IDA	2.00
6	Tap water	1.00
7	Haematoxylin	0.45
8	Distilled water	1.00
9	Blueing buffer	1.00
10	Tap water	1.00
11	70% IDA	1.00
12	Eosin Phlox B	1.30
13	95% IPA	1.00
14	100% IPA	1.00
15	100% IPA	1.00
16	100% IPA	1.00
17	Xylene	1.00
18	Xylene	Exit

IDA, industrial denatured alcohol; IPA, isopropyl alcohol.

Appendix 3 Immunohistochemistry procedures using the Dako PT Link and Dako Autostainer 48S Link platform

IMMUNOHISTOCHEMISTRY PROCEDURES USING DAKO PT LINK AND DAKO AUTOSTAINER 48S LINK PLATFORM

PURPOSE AND SCOPE

The purpose of this protocol is to define the procedure for using the automated platform, Dako Autostainer 48S Link for immunohistochemistry, including HIER with the Dako PT Link.

2.   RESPONSIBILITIES

2.1   SECTION HEAD BIOMEDICAL SCIENTIST

The BMS in charge of the Immunohistochemistry (IHC) area is responsible for ensuring that the IHC service is maintained to the appropriate standard.

2.2   BIOMEDICAL SCIENTISTS

May carry out the procedure once trained to do so. They are responsible for reporting any faults or staining issues to the section head or advanced biomedical scientist.

2.3 TRAINEE BIOMEDICAL SCIENTISTS AND BIOMEDICAL SUPPORT WORKERS

Trainee staff and BSWs may be called upon to work in the section under the supervision of qualified and trained staff.

3.   REFERENCES
EXHIST0020 - PT Link User Guide
EXHIST0021 - PT Link Quick Start Guide
EXHIST0024 – Autostainer Link 48 Quick Reference Guide
EXHIST0022 – Autostainer Link 48 User Guide

4. DEFINITIONS

IHC   Immunohistochemistry
HIER   Heat Induced Epitope Retrieval

5. DOCUMENTATION

5.1 RISK ASSESSMENTS

CPRA 002   Specimen Identification
CPRA 073   Handling Chemicals
CPRA 075   Preparing Solutions
CPRA 081   Preparing Slides for IHC

5.2 COSHH ASSESSMENTS

COSHH 015   Alcohol (IDA99%)
COSHH 385   Xylene
COSHH 240   Pertex
COSHH 266   Proteinase K

5.3 ASSOCIATED FORMS

LF 130 007a   New antibody titration form
LF 130 007b   Existing antibody titration form
LF 130 009   Antibody acceptance record

6 ACTIONS AND METHODS

6.1 PRINCIPLE OF THE METHOD

The Dako staining machines will dewax the slides, perform antigen retrieval where required and then carry out an immunohistochemical staining technique. In order to ensure optimal staining results, the appropriate antigen retrieval and antibody dilution need to be worked out for each new antibody. In some cases, variation between antibody batches/lot numbers may mean that existing antibodies sometimes need re-titrating.

6.2   SPECIMEN REQUIREMENTS

- Correctly labelled 4µ paraffin sections mounted on 'Superfrost Plus' slides.
- Appropriate positive and negative control material.

6.3   DATA ACQUISITION

- Antibody data sheets.
- Antibody titration forms (LF 130 007a & b)

6.4   REAGENTS, CONTROLS AND EQUIPMENT REQUIRED

Relevant control slides for each antibody to be stained

User fillable reagent pots

The relevant antibody

Antibody diluents

Pipettes/ Pipette tips

6.5   CALIBRATION

All calibration is carried out by service engineers.

6.6   STEPWISE DESCRIPTION OF THE PROCEDURE

6.6.1 Cutting sections for Immunohistochemistry.

- Sections should be cut at 4 µm onto superfrost slides with appropriate control section on the same slide, following the SOP LP130 007 Cutting Sections for Immunohistochemistry.
- Pre heat the slides in the 60°C oven for a minimum of 30 mins.
- Once the slides are ready generate slide labels as instructed in the Dako Autostainer user guide EXHIST0022.

6.6.1 Preparing slide labels

- Information to be added to slide labels should include:
    - The lab number and surname of the Patient.
    - Select the pathologist that has requested the slides
    - Select the antibody that has been requested

- Once all the antibodies for that request have been selected, press print labels and then case complete.

- The name of the antibody will automatically appear on the slide due to the way the labels are generated by the Dako system.

- The labels must be stuck centrally in the label area of the slide and kept as straight as possible.

- Rack the slides in the slide racks for the PT Link, separating the slides into the high and low pH retrieval solution.
- Slides that require Proteinase K pre-treatment must be dewaxed and taken to water prior to staining, do not put these slides into the PT Link. Once dewaxed the slides can put directly on the Autostainer. Make sure that the Proteinase K container is added to the reagents to be loaded onto the Autostainer.

6.6.2 Antigen Retrieval using the PT LInk

- Refer to:
    - EXHIST0020 and EXHIST0021 for how to use the PT Link

Chemical-protective gloves should be worn when handling parts immersed in any reagent used in PT Link.

- Fill tanks with desired Dako Target Retrieval Solutions (Codes S1699/S2367).
- Place slide racks with slides into tanks.
- Close and lock lid with external latch.
- Press **RUN** button for each tank to start run.

    a. If Preheat is enabled, CYCLE will show **PREHEAT 65°C**.

    b. Press **RUN** again to start the run, CYCLE will show **WARMUP** and the
    lid lock will engage.
- Unit will warm up to set temperature and then start countdown clock for retrieval cycle.
- When retrieval cycle is finished, CYCLE will show **COOL**.
- When COOL cycle is finished, CYCLE will show **DONE** and lid will unlock.
- Press **DONE** on the Dako Link software to acknowledge that you have removed the slides.
- Take one slide rack at a time out of PT Link and rinse all slides with warm (65°C) Dako Wash Buffer (Code S3006).
- Rinse slides with room temperature Dako Wash Buffer.
- Place the rack on a Dako Autostainer Link instrument.

6.6.3 Performing Immunohistochemistry with Dako Autostainer Link

- Prepare reagents required and load them onto the Dako Autostainer Link.
- Remove the lids and place them on the place holder under the instrument so that they match the position of the reagents. This will make sure that the lids are not mixed up when the run is complete and avoids contaminating the reagents.
- Make sure that the bulk reagents containers have been filled up with wash buffer and water, and that the hazardous waste container has sufficient capacity to hold the hazardous waste during the run.
- Prime the bulk solutions with the first run of the day following the on-screen instructions.
- Press **START** to start the run. The machine will scan all the slides and reagents and inform you if all the reagents that are required for the run are there. If not, it will tell you which reagents are required and then ask you to press **OK** and rescan the reagents.
- Once all the reagents are present, press start to start the run. The machine will ask you if you have enough bulk fluids and ask you to press **YES**

- At the end of the run press **DONE** and remove the slides and reagents.
- Remove the slides from the racks and transfer them to the racks for the coverslipper.
- Place slides in water to wash for 5 minutes, then dehydrate with 70% Alcohol, two changes of Absolute Alcohol and two changes of Xylene.
- Coverslip the slides on the Leica coverslipper CV5030.

6.7   QUALITY CONTROL / ASSESSMENT

The requesting Pathologist / Consultant Pathologist along with the Section Lead must evaluate the slides produced before a new antibody is added to the repertoire.

6.8   LIMITATIONS OF METHOD

The effects of antigen retrieval may vary with the length of time of fixation or the type of fixative used. These things are not always known, particularly with referred material.

All staff performing these tasks should be fully trained for the tasks set out above and have competency records to prove this, before they can perform the tasks unassisted.

6.9   REPORTING OF RESULTS AND INTERPRETATION

Reporting of results can only be carried out by the clinical pathologists. Biomedical Scientists and Biomedical Support workers cannot issue reports on Immunohistochemistry results. The Lead Biomedical Scientist must check the quality of the immunostaining on the control tissue before sending the slides to the requesting pathologist.

6.10   PROCEDURE NOTES AND OTHER PERTINENT INFORMATION

It is essential that control material used for antibody titration is selected carefully. Ideally, known positive AND known negative tissues should be used. Appropriate control material is usually suggested on the antibody data sheet and is crucial to identify genuine positive staining. If an antibody titre is too concentrated, non-specific staining may occur in known negative tissues while still staining appropriately on the positive control material. If possible a range of positive tissues should be used to include high, moderate and low expressors in order to correctly determine the optimum titration.

7.   TRACEABILITY AND UNCERTAINTY

7.1 TRACEABILITY

All antibodies have product inserts uploaded to Q-Pulse which provide information on dilutions, pre-treatments and control material and which can be used as reference material if required. Guidance on how to use the machines is traceable back to the manufacturer user manuals.

7.2 UNCERTAINTY

Please refer to QP 000 036 Measurement of Uncertainty and Criticality in Cellular Pathology

Appendix 4 Tissue microarray procedure

1. Gather all of the blocks based on the marked H&E-stained slides for TMA.
2. Go to TMA designer options to design your TMA.
2a. Choose your recipient block (the size of the block you are using).
2b. Make a new tissue array template design (this will include the punch size that you are going to use for making your TMA and the spacing of the spots; it will give you exact numbers of the spots you are going to create). The punch size we usually use is 1000 μm and a spacing between 800 μm and 1000 μm. This will also give you the total number of spots by row and by column, and you can also mark your orientation or even edit the spots you want by deleting the spots you will not be going to use. In addition, you can save this for your TMA project.
2c. List of tissue types options (you can save here the tissue types that you are going to use, i.e. orientation, colorectal central and colorectal invasive, and assign a colour code for this) and initial of the one making the TMA project.
2d. Making of Tissue Array Project design. Name of the project is the first step followed by downloading the list of your donor blocks that you created in an Excel file (Microsoft Excel®, Microsoft Corporation, Redmond, WA, USA) (this includes the histology number of the block and name of the patient; you may include the sex, hospital number, date of birth, date of diagnosis and site). It will also ask you to download the tissue types that you wanted and apply how many replicate numbers of cores you wanted for each block. Afterwards, the computer will fill the tissue array and will ask you to save the TMA project you created.
3. Click on the MiniCore icon and select the name of the project you saved, and the machine will tell you to load the blocks that you require and unload the blocks afterwards. The TMA removes a wax core from the recipient TMA block to allow space to insert a tissue core. The marked slide will be put on top of the donor block. The inbuilt camera can take a picture of this and the marked points on the slide are selected by clicking on the appropriate areas. This puts a marker where the donor block is going to get the specimen that it will transfer into a recipient block. It will do this repeatedly on each of the blocks depending on how many replicates (usually three per block) you created until it finishes the whole project.
4. Put the TMA block created in a 40-degree oven overnight to assist in the wax adhering to the tissue cores. This helps to reduce the number of cores that you lose when cutting sections. Sections are cut and stained with H&E and/or any other immunohistochemical stain that you want for the project.

Appendix 5 The CD3+ protocol on the Dako Autostainer 48 Link

Station	Reagent	Time (minutes)
1	FLEX buffer	0.00
2	Endogenous peroxidase block	5.00
3	FLEX buffer	0.00
4	CD3+ RTU	15.00
5	FLEX buffer	0.00
6	FLEX-labelled polymer/HRP	20.00
7	FLEX buffer	0.00
8	FLEX buffer	5.00
9	FLEX DAB + chromogen	5.00
10	FLEX DAB + chromogen	5.00
11	FLEX buffer	0.00
12	Haematoxylin	5.00
13	Distilled water	0.00
14	FLEX buffer	5.00
15	Distilled water	0.00

DAB, 3,3'-diaminobenzidine; HRP, horseradish peroxidase; RTU, ready to use.

Appendix 6 The CD45RO+ protocol on the Dako Autostainer 48 Link

Station	Reagent	Time (minutes)
1	FLEX buffer	0.00
2	Endogenous peroxidase block	5.00
3	FLEX buffer	0.00
4	CD45RO+ 1 : 2500	20.00
5	FLEX buffer	0.00
6	FLEX mouse linker	15.00
7	FLEX buffer	0.00
8	FLEX-labelled polymer/HRP	20.00
9	FLEX buffer	0.00
10	FLEX buffer	5.00
11	FLEX DAB + chromogen	5.00
12	FLEX DAB + chromogen	5.00
13	FLEX buffer	0.00
14	Haematoxylin	5.00
15	Distilled water	0.00
16	FLEX buffer	5.00
17	Distilled water	0.00

DAB, 3,3'-diaminobenzidine; HRP, horseradish peroxidase.