NIHR HTA Programme

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1. Project title

Can the intra-tumoral immune signature of primary colorectal tumours identify a subset of patients who do not require follow up after resection?
HTA project application number: 11/136/83

2. How the project has changed since the outline proposal was submitted

The key objectives of this project are the same as those described in the outline proposal. The primary focus remains to identify immunological markers in primary colorectal cancer that predict outcome following resection. The aim is to identify a subset of patients who may not require follow up after surgery for the primary tumour. This would not only benefit the individual patients, but also represent a significant cost saving to the NHS.

This project will utilise the tissue samples and clinical data collected in the context of the UK FACS (Follow up After Colorectal Cancer Surgery) Trial. This is a unique cohort of patients in which to both identify and validate immunological markers of prognosis since it is the only large, prospective cohort of patients who have undergone detailed staging that the applicants are aware of. The system proposed by Galon et al. (1) will be validated in this study. However other immune markers, including simpler methodologies, will also be evaluated to enhance the generalisability of immune scoring in the NHS.

A secondary objective not detailed in the outline proposal is to include analysis for microsatellite instability (MSI). MSI is a well documented marker of prognosis in primary colorectal cancer and the prognostic benefit is thought to relate to the increased immunogenicity of MSI-High tumours (2-6). The technique to assess MSI is in standard use in NHS laboratories and we predict will form part of the risk stratification algorithm we propose to develop.

The funding request has been increased in order to cover Trials Unit work to negotiate Tissue Transfer Agreements with Trusts involved in the study. The FACS Trial predated the European Clinical Trials Directive and the Human Tissue Act and agreements related to the transfer of tissue blocks were not included although consent has been obtained. Putting in place such agreements will require Trials Unit time and therefore represents an additional cost. The other key addition to the costings is for statistical support from the University of Oxford since full economic costings were not anticipated during the development of the outline proposal. Such support will be invaluable to the analysis stage of this project.

An extension of the project length to 24 months, from the 12 months stated in the outline proposal, has been requested for two reasons. Firstly following advice from the University of Southampton Clinical Trials Unit the time taken to formally obtain Tissue Transfer Agreements in order to acquire tissue blocks from other Trusts has been extended to make this process feasible in the lifetime of the grant. Secondly, the guidance notes state that time for write up should be included and this was not in the outline proposal. This extended project length has no funding consequences.

Finally this application permits the inclusion of much more detail than the outline proposal. As such both the world-class expertise in translational immunology in Southampton and the

statistical knowledge of the co-applicants at the University of Oxford is highlighted. This demonstrates how well placed the applicants are to undertake this research.

3. Planned investigation

i. Research Objectives

Primary objective

- To use the large prospective cohort of patients from the FACS (follow up after colorectal surgery ISRCTN 41458548) trial to identify immunological markers which may predict the need for follow up after resection of primary colorectal cancer

Secondary objectives

- To use the FACS cohort of patients to validate the immune score proposed by Galon et al (1)
- To develop a scoring system based on prognostic immunological markers that could be readily introduced in NHS pathology departments
- To determine the association between microsatellite instability (MSI) and the immunological response to the tumour and whether MSI has any application in a prognostic scoring system

The FACS Study has produced a patient cohort which is unique in colorectal cancer oncology. Although these patients were managed in a variety of hospitals, they were fastidiously staged at recruitment such that they had undergone a complete resection of the primary tumour, had a normal post operative (or post adjuvant chemotherapy if given) CT scan of the abdomen and chest and a normal blood carcinoembryonic antigen (CEA) level. If recurrence was detected they were managed by multidisciplinary teams which included access to liver and lung surgery. No other such cohort is known to exist and thus there is a unique opportunity to examine biomarkers with near-clinical application. The focus of this research is to identify immunophenotypic markers in the formalin fixed paraffin embedded (FFPE) tissue for the cancers removed in the trial patients. The study will be used to validate the immune score proposed by Galon et al (1) but in addition explore whether alternative/simpler scoring systems can predict the need for follow up with accuracy. Such a score could be used to both inform the need for follow up of these patients in addition to allowing selection for adjuvant treatments.

Genetic biomarkers such as k-Ras have purposefully not been included within the above research objectives. Whilst there is much interest in genetic biomarkers such as k-Ras these do not have similar prognostic power to immune markers in colorectal cancer. Thus although mutant k-Ras is predictive for non response to EGF receptor blockage in patients with metastatic disease, its prognostic value in primary disease can only be demonstrated with very large numbers of samples. It has no value in determining the prognosis in an individual patient. The same can be said of B-raf mutations, highly associated with poor outcomes in metastatic disease but much less so with primary disease and apparently not at all with microsatellite unstable tumours. However this is a unique patient cohort and the tissue/DNA bank which will be created allows a large number of potential biomarkers to be studied by us and other groups. We currently have a collaboration with Professor Sabine Tejpar, Leuven, Belgium, who is currently a world leader in this area.

ii. Existing Research

Surgery remains the primary curative treatment for patients with colorectal cancer. However, it is thought that following surgery approximately half of patients will go on to develop incurable recurrent disease. As such the majority of clinicians follow up patients in an attempt to detect recurrence at an early and treatable stage. The majority use a combination of routine blood CEA (carinoembryonic antigen) testing and interval CT scanning, however, there is currently a lack of evidence base for such follow up. Indeed the FACS trial was commissioned because there is no compelling evidence that any mode of colorectal cancer follow-up of asymptomatic patients after surgery is cost-effective in reducing morbidity or mortality.

In this trial patients were randomised in a 2×2 trial design to intensive versus minimal imaging and CEA (carcinoembryonic antigen) monitoring in primary care versus no CEA monitoring. Complete evaluation by CT chest, abdomen, pelvis and a normal CEA after surgery or adjuvant chemotherapy were prerequisites to trial entry. Follow up data including site of recurrence and further surgery has been collected prospectively.

An observational analysis has been performed on the entire cohort (7). At a median follow up of 60 months 85.6% of patients were alive without recurrence (stage I 90.9%, stage II 86.7%, stage III 80.4%). Of the 178 recurrences the majority were loco-regional or at multiple sites and were not amenable to curative treatment. Only 53 patients have gone on to have potentially curative revisional surgery, which represents 4.2% of the study cohort. These data demonstrate not only a relatively low level of recurrence but that few patients are eligible for curative resections at relapse. Whilst the by group analysis will not be permitted until Autumn 2012, the preliminary data suggest that it will be difficult to justify intensive follow up strategies for all patients. Furthermore, whilst TNM staging clearly has significant prognostic power, these data highlight the need for a more precise mechanism of identifying those patients who will relapse in order to inform follow up.

The balance of immune control versus immune escape of cancer is now thought to play a key role in the development of a number of cancers and has found its recognition in the recent revision of the 'hallmarks of cancer' by Weinberg et al (8). The anti-tumour activity of the immune system is initially mediated by innate immunity, mainly with effector cells such as Natural Killer (NK) cells and macrophages. Subsequently adaptive immune mechanisms are activated with tumour-specific responses and which generate memory immune cells, mainly B and T cells. A number of studies have now proposed immunological criteria as predictive for outcome of patients with colorectal cancer (9-12).

Whilst such data has only recently received such high profile recognition, studies of the immune infiltrates of primary colorectal cancer have been undertaken for a number of decades. In 1987 Jass et al. reported that lymphocytes at the invasive margin of rectal cancers may be an independent predictor of prognosis (13). Subsequently work has demonstrated that tumour infiltrating lymphocytes were a predictor of both disease-free and overall survival independent of stage (12). Perhaps the most striking and detailed research has been completed by Galon et al. This group performed a study of the immune infiltrate at both the centre of the tumour and the invasive margin of approximately 400 primary colorectal tumours using gene expression and immunostaining. Markers of the adaptive immune system, specifically for cytotoxic and memory T cells, performed well as prognostic indicators. The group conclude that the type, density and location of immune cells, has a prognostic value superior to and independent of those of the UICC-TNM

classification (for colorectal cancer stages I-III) (14). Based on this an "immune score" has been proposed, which provides a combined assessment of memory (CD45R0) and cytotoxic (CD8) T cell phenotypes at both the centre of the tumour (CT) and the invasive margin (IM) (1). Scores vary from 0 to 4 to reflect the level of infiltrate of these lymphocyte subtypes. Overall survival for patients with score 0 was just 27.5% at 5 years compared to 86.2% for patients with a score of 4. Whilst such data does appear compelling, the authors have had to combine cohorts of patients in order to achieve adequate numbers. Furthermore all analyses have been retrospective. To be fully accepted these findings need validation in a well-staged cohort of patients studied prospectively. The FACS study provides this opportunity.

A genetic marker of prognosis is the microsatellite status of the tumour. This has been shown to correlate both with survival and the immune infiltrate. Microsatellite instability (MSI) is the hallmark of a deficient mismatch repair system and results in a failure to correct replication-associated errors, particularly in regions of repetitive DNA known as microsatellites. It is observed in approximately 15% of all sporadic colorectal cancers (15). High graded microsatellite unstable (MSI-H) colorectal malignancies have an improved prognosis which is postulated to result from higher immunogenicity due to the large number of altered peptides generated (2-6). Indeed a number of studies have noted a pronounced lymphocytic infiltrate to these tumours (6, 16-18). New data suggests that the incidence of MSI differs by stage of colon cancer and that the prognostic impact of MSI is substantially stronger in stage II compared with stage III patients (19). Of note Galon et al have not evaluated the presence of MSI in their cohorts.

Despite such compelling data that both the immune infiltrate and microsatellite status of colorectal cancer are of prognostic importance these have yet to be incorporated into clinical practice. This may in part reflect the detailed nature and complexity of the scoring systems that have been proposed. This is mainly due to the perception that they are not as easily applicable in the clinical setting as the standard staging using the TNM (tumour, node, metastases) classification.

There are data to support more simplistic measures of the immune response to colorectal cancers. A study from some of our own group (20) confirmed that the number of lymph nodes harvested in a large dataset of over 5000 patients correlated with survival. This study also confirmed the correlation between the number of lymph nodes harvested and the degree of immune reaction to the tumour. Of key interest this research demonstrated that such data could be derived in NHS pathology laboratories where pathologists were not analysing the specimens for purposes of research.

In summary the preliminary data from the FACS trial supports the need for better methodologies to predict the outcome, and therefore need for follow up, of patients with colorectal cancer treated with curative intent, as the conventional UICC-TNM system appears inadequate. There is already an extensive research base to support the use of immunological scoring in this setting. The MSI status of the tumour may also form part of such a scoring system, however, clarification of the variability in its prognostic strength by stage of tumour is required, as is its contribution to the presence of an immune infiltrate. Such validation and derivation of a simple, clinically applicable method requires a large, prospective, accurately staged cohort of patients. The FACS cohort provides a unique and timely opportunity to perform such research.

iii. Research Methods

1. Tissue collection

The FACS study includes 1260 patients recruited from 38 centres around the UK for which clinicopathological data has been prospectively recorded. Ethical consent for the use of tissue is in place. Formalin-Fixed, Paraffin Embedded (FFPE) tissue blocks will be collected from these centres. Approximately 80% block recovery is anticipated based on experience in Southampton where block retrieval has been piloted. As such, there are 138 tissue blocks from the Southampton cohort upon which analysis can commence in November 2012 if funding is secured.

2. Tissue microarray (TMA) construction

The use of tissue microarrays will facilitate staining of multiple samples at once. This method is both time and cost efficient as compared with standard immunohistochemistical staining of multiple slides. In order to determine the areas of the blocks from which to select the cores, permanent section H&E slides of the blocks will be reviewed with Professor Gareth Thomas. Three cores, 1mm each, will then be taken from each of the centre of the tumour (CT) and the invasive margin (IM) containing the highest density of immune cells. Arrays will be constructed using the semi-automated Minicore arrayer (Mitogen, UK).

3. Immunohistochemistry

The H&E slides of the tumours will be categorised as having either a low, moderate or dense immune infiltrate. The TMA sections will be stained for markers which have been demonstrated in the literature to be of interest. This will include CD3 (a marker of T cells), CD8 (a marker of cytotoxic T cells) and CD45R0 (a marker of memory T cells). This will allow validation of the immune score proposed by Galon et al (1). Other key markers will include CD45 (a marker of lymphocytes in general), CD56 (a marker of NK cells), and CD4 (a marker of T helper cells). Further staining will depend upon preliminary data analyses and hypothesis generation. The researchers have a number of ideas to explore with the aim of identifying unique immunological markers which may predict the need for follow up after resection of primary colorectal cancer

Immunochemistry will be performed using automated systems (Dako) in the UHS Cellular Pathology Department. This clinical diagnostic laboratory is CPA-accredited and performs immunochemistry to national standards (NEQAS) which guarantees quality control. The immune markers described within the application are used routinely on the tissue types of interest as part of the diagnostic service. We have in addition, stained slides from ten patients as an additional “check” that there are no issues with using these slides from these stored samples. Any novel antibodies will be appropriately optimised in the usual manner. The slides will then be analysed using an image analyser workstation after manual counting has confirmed the accuracy and reproducibility of the methodology. Measurements will be recorded as number of positive cells per tissue surface unit.

4. DNA extraction and MSI testing

DNA will be extracted from the FFPE tissue using a QIAGEN kit. This has already been used successfully on 80 specimens. Using polymerase chain reaction (PCR), DNA from the tumours and adjacent normal mucosa will be used to test for microsatellite instability. A reference panel of 5–10 microsatellite loci is used to diagnose MSI cases (21), for which three categories have been established: MSI-High (MSI-H), unstable for 30% of markers used; MSI-Low (MSI-L), unstable for 10%–30% of markers used; and microsatellite stable (MSS), for cases that display no MSI. DNA will then be stored to permit other analyses in the

future.

5. Statistical analysis
Patient groups will be stratified on the basis of density of immune markers (low, moderate, dense) and MSI status, and Kaplan Meier Curves used to describe differences between groups on disease free, disease specific and overall survival. Statistical support will be sought from the University of Oxford, and indeed this in part explains the funding for statistician's time requested, in order to use the immunological data generated to develop a prognostic immune score if possible. It is hoped that it will be possible to generate this from the Southampton cohort of patients thereby utilising the remaining 1100 samples as a validation set. Briefly, cox proportional hazard models will be employed to determine if the presence of these immune markers is associated with survival (all three outcomes). We will check for non-informative censoring to assess if this is associated with survival. The proportional hazard assumption will be verified by visual inspection of graphs showing logarithms of the estimated cumulative hazard function. Should evidence of assumption violation exist, alternative regression strategies will be used.

iv. Planned Interventions

No interventions are planned in the patients. This is a solely laboratory based analysis. For reference we attach the trial protocol/summary in the appendix.

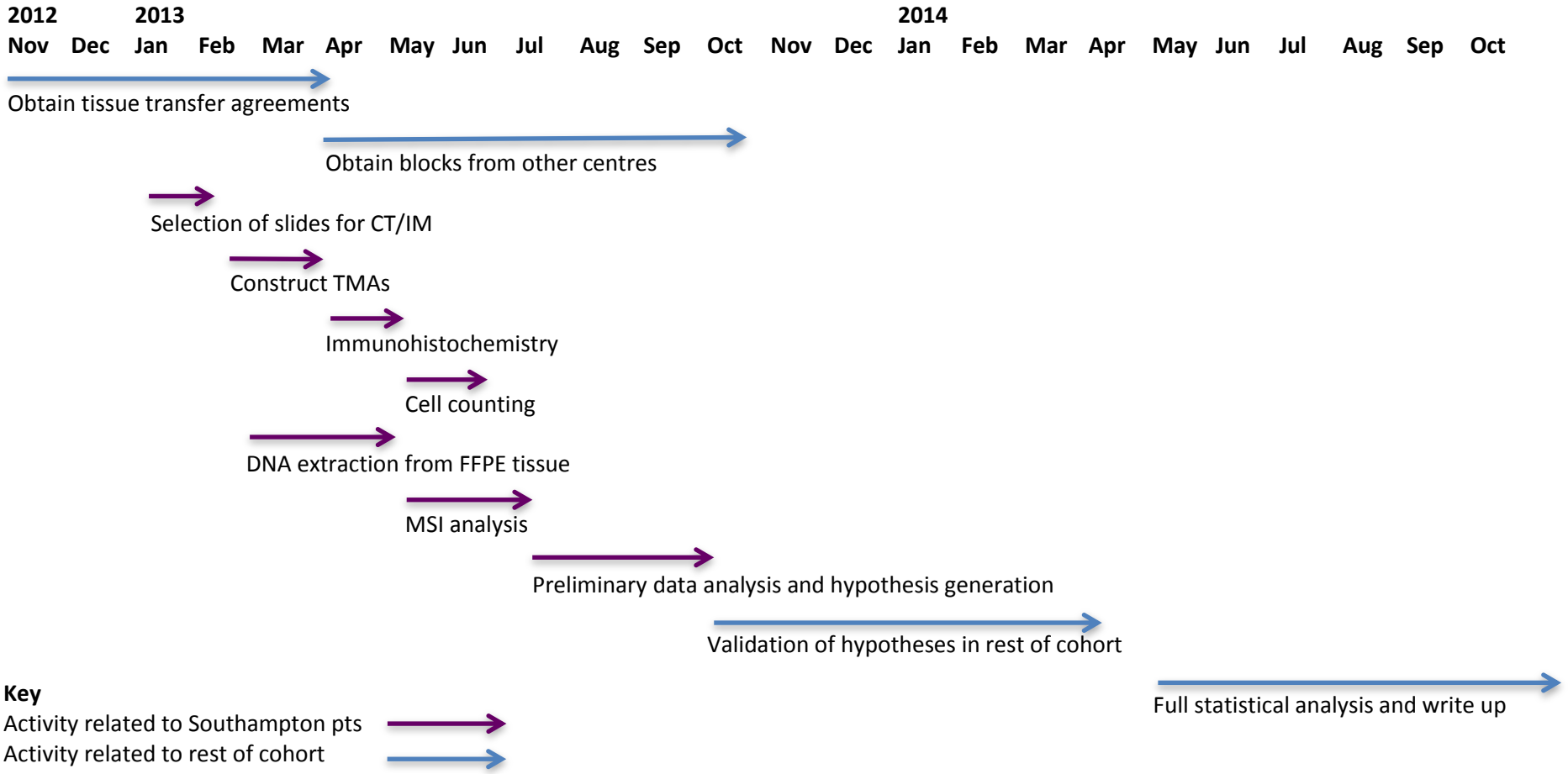
v. Planned inclusion/exclusion criteria
N/A

vi. Ethical arrangements
All patients recruited to the trial gave written ethical approval for their anonymised data and tissue samples to be used for the purpose of research into ways in which the follow up of patients with colorectal cancer could be improved.

vii. Research Governance
Research governance for the FACS trial is provided by both an existing Steering Committee and Data Monitoring Committee. The Steering Committee have approved the proposal to conduct this translational work on the FACS cohort of patients to further inform the need for follow up after resection of primary colorectal cancer. The Data Monitoring Committee has recommended breaking the randomisation code in autumn 2012. Whilst this project will commence before this date it will be conducted on the entire cohort and as such will not prejudice the integrity of the main trial.

4. Project timetable and milestones

The projected timescales are outlined in the diagram below. FFPE tissue and H&E slides have already been obtained from the Southampton cohort (138 patients) and it has been established that good quality DNA can be obtained from the FFPE tissue. The Southampton cohort will be used as a test set to optimise methods and for creation of hypotheses whilst tissue transfer agreements are set up and blocks are obtained from the other centres around the UK.



Key Milestones

Obtain tissue transfer agreements	March 2013
Obtain rest of blocks from other centres	October 2013
Preliminary analysis and hypothesis generation	October 2013
Completion of work on rest of cohort/validation of hypotheses	April 2014
Full statistical analysis and write up suitable for publication	December 2014

5. Expertise

The experimental work will be carried out within the Cancer Sciences Division of the Faculty of Medicine at the University of Southampton. This centre has international standing in translational immunology with programmatic funding from CRUK and other funding bodies. It has both the necessary facilities and expertise required to undertake this study. We will exploit the Southampton Experimental Cancer Medicine Centre and the strong links between the University and its NHS partner for translation of the findings into routine practice in the University Hospitals Southampton NHS Foundation Trust. The University of Southampton Clinical Trials Unit has appropriate experience in obtaining Tissue Transfer Agreements with the Trusts involved in the study and in obtaining tissue blocks. Once obtained the tissue will be stored prior to analysis in a HTA licensed tissue bank supported by the Southampton ECMC. The tissue microarrays will be created using a semi-automated Minicore Arrayer (Mitogen, UK) and there is core technician support for this facility. Tissue microarrays are routinely constructed in our unit. The immunophenotyping has been set up, validated and is being applied within the department on other tumour types.

The tissue immunophenotyping will be led by Professor Christian Ottensmeier, Professor of Experimental Cancer Medicine and Professor Gareth Thomas, Professor of Experimental Pathology at the University of Southampton. Professor Ottensmeier has developed extensive expertise in the evaluation of human immunological studies and holds programmatic funding in this field, supported by Cancer Research UK, the NIHR/EME and by the Department of Health (Southampton Experimental Cancer Medicine Centre, ECMC). His group has made an international contribution to the evaluation of T cell responses to cancer immunotherapy and the specific area of research to be undertaken in this project is a direct extension of his ongoing work. The intention is that beyond the clinical implementation as proposed in the application, it will feed directly into the evaluation of clinical trials of immunotherapy in colorectal cancer, which are under setup.

Professor Thomas has extensive experience in cancer biomarker research, including the morphological evaluation and immunophenotyping of human cancers. He sits on the NCRI Biomarkers and Imaging Clinical Studies Group, and is Chief Investigator for NIHR portfolio studies in head and neck and pancreatic cancers. His research group is supported by Cancer Research UK, the Medical Research Council and The Health Foundation. He will oversee the tissue microarray construction and pathological analysis for the study. Professor Thomas is the designated individual overseeing the HTA tissue bank facility in Southampton.

Professor Primrose is Professor of Surgery at the University of Southampton and Director of the Hampshire and Isle of Wight CLRN. As the FACS Study co-chief investigator he has extensive experience of trials and translational research in gastrointestinal cancer and on the clinical management of colorectal cancer, specifically liver metastases. He chairs the NCRI Upper GI Clinical Studies Group and holds several grants from CRUK and other funding bodies for clinical trials and translational studies in gastrointestinal and liver cancer. He will provide oversight to the potential application of the project read-outs in the clinical environment.

The statistical analysis will be undertaken in Oxford under the guidance of Dr's Rafael Perera (the FACS trial statistician) and Richard Stevens (and their team of statisticians) who have internationally acknowledged methodological skills and expertise in such analyses.

6. Service Users

The proposed project is in part a reflection of some of the initial comments from patients during the pilot phase of the trial. For a number of patients it was apparent that a major concern was the intensive nature of the follow up in group four which included the most intensive follow up regime. Given that the majority of patients are elderly, attending hospital on a six monthly basis for CT scanning and undergoing three monthly blood tests is a significant undertaking. If this further translational work can identify a subgroup at such low risk of relapse that follow up is deemed unnecessary this would be of significant benefit to those patients.

The FACS TSG has a PPI representative who will input as required to this investigation. In addition we are participating in the Citizen Scientist Programme which is being developed in relation to Breast Cancer by the University of Southampton CTU and CRUK. In summary this is a project whereby the public can assist in scoring the immunohistochemical staining of tissue microarrays. If the study is successful with the POSH trial (breast cancer) we plan to roll this study out subsequently.

7. Justification of support required

This translational project can be undertaken at relatively low cost. There is no requirement for funding of salary to undertake the laboratory work since this will be performed by a NIHR funded Academic Clinical Fellow in General Surgery. Senior staff are funded by the Universities of Southampton and Oxford, although there is a cost for statistical support from the University of Oxford. We are requesting funding for 37 days of statistician support (22 days of a junior statistician and 15 days of a senior statistician). In this time the statistician will undertake four tasks:

a) Conduct a Cox proportional hazard analysis to replicate Galon's results (i.e. to confirm that his proposed 0-4 score does indeed predict outcome better than traditional staging); in conducting this analysis it will be necessary to check that the data does meet the required proportional hazard assumption and if necessary to apply alternative regression strategies.

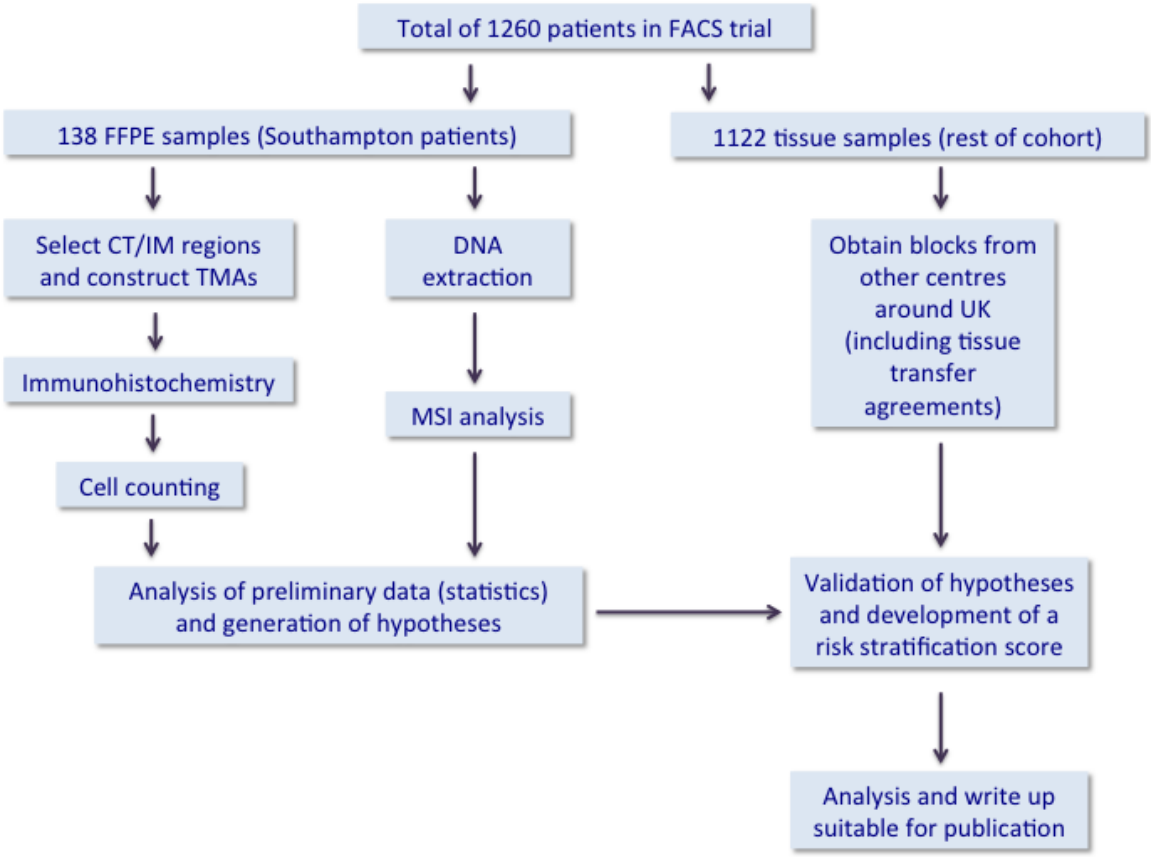
b) Construct a new regression model to explore which markers of the density and location of immune cells best predict outcome.

c) Develop and validate alternative scoring systems if this exploratory analysis suggests that the Galon score might be simplified or its performance otherwise improved (by the inclusion of other immune marker variables or different combination of variables).

d) Contribute to the drafting and writing-up of the scientific reports and papers resulting from this research.

The equipment to permit this high throughput validation is already in place in addition to an established tissue bank with a full time core funded technician. Clinicopathological data are being obtained as part of the FACS trial, the cost of which is already covered. Funding is required to support the clinical trials unit in arranging Tissue Transfer Agreements with involved Trust and there is a cost for tissue retrieval which is costed at the standard rate which would be reimbursed by CRUK in translational trials. Consumables, particularly antibodies, are required and the predicted costs of these have been outlined.

8. Flow diagram



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