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Applicant Details

Title	Dr
Forename	Jessica
Surname	Hale
Email Address	jessica.hale@liverpool.ac.uk
Telephone	07725365690
Institution	University of Liverpool
Address	Department of clinical cancer medicine, floor 2 Sherrington building, Ashton street Liverpool United Kingdom L69 3BX

Other Applicants

Title	Professor
Forename	William
Surname	Greenhalf
Email Address	00
Telephone	Reader Molecular & Clinical Cancer Medicine

Title	Professor
Forename	Eithne
Surname	Costello-Goldring
Email Address	00
Telephone	Professor of Clinical Cancer Medicine

Title	Professor
Forename	Daniel
Surname	Palmer
Email Address	00
Telephone	Professor and Honorary Medical Oncology Consultant

Title	Dr
Forename	Kenneth
Surname	Bruce
Email Address	00

Telephone	Senior Lecturer in Molecular Microbiology

Title	Dr
Forename	David
Surname	Fine
Email Address	00
Telephone	Visiting Senior Lecturer in Clinical Medicine

Title	Dr
Forename	Richard
Surname	Jackson
Email Address	00
Telephone	Lead Statistician, LCTC Liverpool

Title	Ms
Forename	Charlotte
Surname	Rawcliffe
Email Address	00
Telephone	ECMC operational director

Title	Ms
Forename	Sara
Surname	Martin

Email Address	00
Telephone	ECMC manager

Title	Professor
Forename	Christopher
Surname	Halloran
Email Address	00
Telephone	Clinical Lead for Europac

Institution Details

Name	University of Liverpool
Address	Department of Cellular and Molecular Physiology Crown Street, University of Liverpool Liverpool, United Kingdom L69 3BX

Abstract

Title of Study	Stratification of pancreatic cancer according to microbiome directed tumorigenesis
Abstract and methodological description	Abstract: The progression model of cancer assumes that benign lesions develop into malignant cancer through a series of mutations. Intraductal papillary mucinous neoplasm (IPMN) are assumed to be benign precursors of pancreatic cancer, partly because IPMN are often found alongside cancers. However, if an IPMN is still present it cannot have developed into the cancer and our hypothesis is that the IPMN has caused rather than developed into the malignancy. A possible mechanism for this is that oncogenic bacteria that have colonised

biofilms in IPMN spread out down the duct. In this collaboration, bacterial populations in IPMN associated with cancer will be compared to bacteria within the cancer and in benign cystic lesions. A microbial signature associated with IPMN related cancer will be identified and clinical outcomes for patients with this signature will be contrasted with outcomes in patients where cancer development may be independent of the IPMN-bacteria-cancer pathway.

Background:
Intraductal papillary mucinous neoplasm (IPMN) are benign lesions that have a close association with Pancreatic Ductal Adenocarcinoma (PDAC). This is usually assumed to be because they represent precursors of cancer. IPMN found in the main duct of the pancreas are linked to the greatest risk of malignancy and NICE guidelines state that patients with such lesions should be offered surgery to remove the IPMN if that is feasible. IPMN in the branch ducts are more frequently identified and are less closely associated with development of PDAC. Patients with these lesions are offered surveillance to monitor possible progression rather than surgery. In Liverpool we host Europe’s largest registry of families with genetic predisposition for pancreatic cancer. Individuals on this registry are offered secondary screening which involves imaging of the pancreas and IPMN are frequently identified. We have recently published on these findings, concluding that IPMN should be treated in the same way in high risk individuals as they are when encountered incidentally1. IPMN are found concomitant to PDAC in up to 10% of cancer cases, while confirming the association between IPMN and cancer this also shows that at least some IPMN lesions in cancer patients do not turn into pancreatic cancer. Suggesting that there is either a link via a common susceptibility for the separate lesions (pancreatic cancer and IPMN), that IPMN somehow causes pancreatic cancer or pancreatic cancer causes IPMN. In Familial Adenomatous Polyposis (FAP) individuals have a very high frequency of polyp formation and hence an increased risk of colorectal cancer. Significantly, polyps found in FAP patients with colorectal cancer are colonized with carcinogenic bacteria found in their cancers and in cancers from patients with sporadic colorectal cancer. These bacterial populations are found in biofilms, which emanate within the polyp but extend out beyond them into the gut, suggesting that the polyps may be causing cancer to develop outside of

the polyp and the link between the APC mutation and colorectal cancer may be due to polyps promoting cancer rather than cancer developing from the polyps2 . By analogy bacteria within biofilms may be the link between IPMN and pancreatic cancer. IPMN certainly can have bacterial populations that are associated with cancer development3, 4 . If the link between IPMN and pancreatic cancer is via bacteria in biofilms this would not of course mean that all cancers develop as a result of bacterial infiltration, but it could mean that this form of tumorigenesis is more likely where an IPMN is identified in a patient alongside a pancreatic cancer. Such cancers have been reported to be less aggressive than cancers where no separate IPMN is observed5 . If pancreatic cancer concomitant with IPMN on average behaves differently to other pancreatic cancers and if this is because more of these cancers are the result of biofilm microbiota, then it is probable that cancers with IPMN biofilm associated bacterial populations will behave differently to treatment than cancers with other forms of microbiome. The microbiome is known to contribute to immunosuppression and hence resistance to immunotherapy6 . It can also directly contribute to chemo resistance through bacterial inactivation of chemotherapeutic agents7 . Therefore, the proposed project may provide insight into therapeutic response because of differential tumorigenesis (IPMN related compared to other) or because of the nature of the cancer microbiome itself (association of bacterial species and/or diversity and response).

Methodological description:

Aims and Objectives:
Hypotheses:
1. The microbial population in IPMN associated with cancer will be similar to the microbial populations in cancers associated with IPMN (IPMN-Cancer pattern) and distinct from the microbial population in IPMN where no cancer develops and in cancers not associated with IPMN
2. Cancers with an IPMN-Cancer microbial pattern will have a different response to specific treatments than other cancers

Aims:

(i) To identify an IPMN-Cancer microbial signature
(ii) To establish associations between the IPMN-Cancer microbial signature with clinical outcomes and biomarkers (survival, progression, CA19-9 level, diabetes)
(iii) To establish associations between the IPMN-Cancer microbial signature in tissue and oral/intestinal microbial signatures

Objectives
1. Obtain aspirates of IPMN cyst fluid from patients under surveillance and from patients being screened for pancreatic cancer. Obtain saliva and faecal samples from these patients.
2. Obtain paraffin embedded fixed samples and frozen samples of resected main duct IPMN. Obtain saliva and faecal samples from these patients prior to surgery.
3. Obtain paraffin embedded fixed samples and frozen samples of pancreatic ductal adenocarcinoma from patients where no IPMN are identified. Obtain saliva and faecal samples from these patients prior to surgery.
4. Obtain paraffin embedded fixed samples and frozen samples of matched cancer and IPMN from patients with concomitant IPMN and pancreatic cancer. Obtain saliva and faecal samples from these patients prior to surgery.
5. Identification and quantification of bacterial genera in samples obtained as in objectives 1 to 4 by gene panel Next Generation Sequencing of bacterial DNA.
6. Carry out in-situ hybridisation of fixed samples to identify and localise particular microbial species.
7. Use of principal component analysis to identify discriminators amongst the bacterial populations between different sample types from the tumours (e.g. IPMN associated with cancer and their matched cancers against the rest, cancer associated with IPMN against cancer not associated with IPMN, IPMN from screened individuals against incidental IPMN from patients under surveillance etc.).
8. Identification of discriminating bacterial species (from objective 7) in fixed samples.
9. Use of data from objectives 7 and 8 to identify a possible IPMN-Cancer microbial signature.
10. Classification of patients according to presence or absence of the IPMN-

Cancer microbial signature in cancers or IPMN.
11. Use of principal component analysis to identify discriminators amongst the bacterial populations in stool and saliva samples between patients defined as in objective 10.
12. Association analysis of patient classification (according to microbial signature) with clinical outcomes and biomarkers.

Work Plan:
Methods for microbiota analysis have been developed and refined by the Bruce group for two decades. From each individual enrolled in this study, samples (saliva, faeces and where relevant IPMN cyst fluid) will be biobanked. SOPs for the analysis of the microbiota in many sample types e.g. faeces have been developed and are in constant use. Funding though is requested to derive a robust SOP for IPMN cyst fluid analysis. By applying these protocols, we will gain information of immediate clinical relevance e.g. in terms of the variation in microbiota composition in IPMN as sampled at different locations within the same individual or wider. Clinical samples will also be stored in the Study Biobank as paraffin embedded fixed samples and frozen samples. During this sample collection and process validation phase, it is likely the rate of accumulation of clinical material will vary with certain tissue/ specimen types more common. For these more common tissues/ specimen types, we will look to obtain material extending the initial remit from an exclusively cross-sectional to include longitudinal sets of data. Microbiota data – primarily bacterial genera identification (high through put sequencing) and bacterial load estimation (quantitative PCR) – will be combined with clinical metadata. By applying statistical tools e.g. principal component analysis, discriminators amongst the bacterial communities present on tumours (e.g. IPMN associated with cancer and their matched cancers against the rest, cancer associated with IPMN against cancer not associated with IPMN, IPMN from screened individuals against incidental IPMN from patients under surveillance etc.) will emerge. This may identify a possible IPMN-Cancer microbial signature which has in itself alone exciting applications e.g. as biomarkers. This application represents an opportunity for the Clatterbridge Cancer Centre, the University of Liverpool and King’s College London to forge a new collaboration. The samples will be collected and stored using the established expertise of the Liverpool GCPLab

	facility. Microbiome analysis will be carried out together with King's College London who are the established leaders in this field. Clinical data will be recorded and linked to laboratory data at the CCC and statistical analysis of associations will be carried out by the lead cancer statistician of the LCTC. References 1. Sheel ARG, Harrison S, Sarantitis I, et al. Identification of Cystic Lesions by Secondary Screening of Familial Pancreatic Cancer (FPC) Kindreds Is Not Associated with the Stratified Risk of Cancer. Am J Gastroenterol 2018. 2. Dejea CM, Fathi P, Craig JM, et al. Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria. Science 2018;359:592-597. 3. Gaiser RA, Halimi A, Alkharaan H, et al. Enrichment of oral microbiota in early cystic precursors to invasive pancreatic cancer. Gut 2019. 4. Li S, Fuhler GM, Bn N, et al. Pancreatic cyst fluid harbors a unique microbiome. Microbiome 2017;5:147. 5. Yamaguchi K, Kanemitsu S, Hatori T, et al. Pancreatic ductal adenocarcinoma derived from IPMN and pancreatic ductal adenocarcinoma concomitant with IPMN. Pancreas 2011;40:571-80. 6. Gopalakrishnan V, Helmink BA, Spencer CN, et al. The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy. Cancer Cell 2018;33:570-580. 7. Geller LT, Barzily-Rokni M, Danino T, et al. Potential role of intratumor bacteria in mediating tumor resistance to the chemotherapeutic drug gemcitabine. Science 2017;357:1156-1160. 8. Rogers GB, Bruce KD, Martin ML, et al. Corrections. The effect of long-term macrolide treatment on respiratory microbiota composition in non-cystic fibrosis bronchiectasis: an analysis from the randomised, double-blind, placebo-controlled BLESS trial. Lancet Respir Med 2015;3:e15.

Timetable

Name	Ethical approval from local committee at Clatterbridge Cancer Centre

Date	July 2021

Name	Biobanking of samples to begin in July 2021 and continue throughout the year as samples become available. Aim to collect 60 samples within a 12 month period.
Date	July 2021- August 2022

Name	Obtain microbiota data (bacterial genera identification using high throughput sequencing) and bacterial load estimation (quantitative PCR)
Date	August 2021- August 2022

Name	Statistical analysis (combine microbiota data with clinical metadata)
Date	September 2022

Name	preparation of manuscript for publication
Date	October 2022

Funding

Name	16S Metagemomic NGS
Amount	9073.5

Name	Immunohistochemistry
Amount	175.0

Name	Sample storage

Amount	60.0

Details of ethical approval

Biases caused by sample collection and processing means that this study is unsuitable for retrospectively collected samples and all samples will therefore be collected prospectively with full informed consent under the ethical approval auspices of the Biomarker Discovery Programme, ECMC.
Ethical approval for this study will be obtained from the relevant committee at Clatterbridge Cancer Centre.

Institutional approval information

The principal applicant (Prof Daniel Palmer) is Director of the Liverpool ECMC and, with Prof Bill Greenhalf, established the collaboration with Prof Ken Bruce and Prof David Fine specifically in order to address the clinical need described in this application. The study hypotheses were developed during discussions between Professors Palmer, Greenhalf, Bruce and Fine. Dr Jessica Hale carried out the necessary literature search for the application and will lead the laboratory work in Liverpool as part of her CCC-funded PhD clinical fellowship. Professor Halloran is a consultant surgeon and clinical lead for Europe's largest screening programme for pancreatic cancer (EUROPAC), he is therefore able to help in identification of patients and in obtaining samples. Dr Richard Jackson is the lead cancer statistician in the LCTC and as such has the expertise necessary to properly control for clinical variants in the complex analysis necessary for this study. Ms Charlotte Rawcliffe and Ms. Sara Martin are key members of the ECMC operational team who have co-ordinated the collaboration as it has developed to date and will continue to support the project during its progression.

Declaration

Confirm Declaration: Yes

Head of Department

Title	Professor
Forename	Andrew
Surname	Pettitt
Email Address	arp@liverpool.ac.uk
Telephone	0151 706 4297
Institution	University of Liverpool

Address	Department of Cellular and Molecular Physiology Crown Street, University of Liverpool Liverpool, United Kingdom L69 3BX

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