

RESEARCH PROPOSAL

David N Church on behalf of the Oxford LynchVax Team

TITLE

LynchVax – a precision prevention vaccination strategy for Lynch Syndrome (LS)

PURPOSE

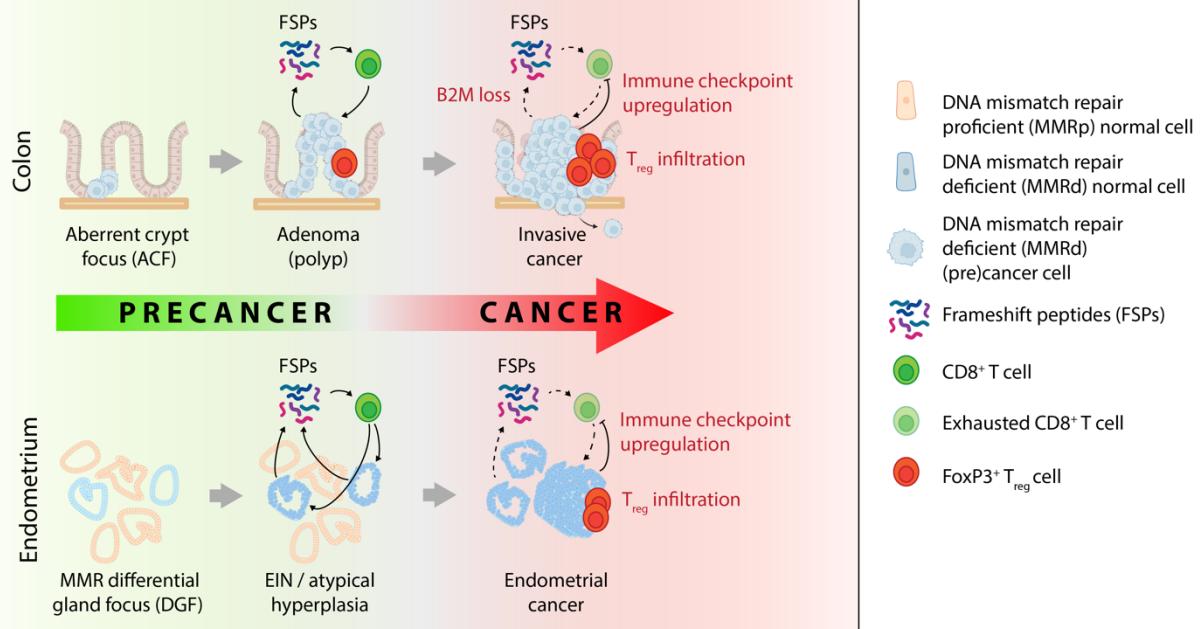
Lynch syndrome (LS) is the most common hereditary cancer syndrome, affecting 175,000-250,000 people in the UK (1). LS causes substantial morbidity and mortality owing to the inability to prevent cancers in carriers. Due to a genetic defect causing DNA mismatch repair deficiency (MMRd), LS-associated cancers are characterised by high mutational burden, including frameshift mutations which generate frameshift peptide (FSP) neoantigens. Two other groups have developed prophylactic vaccines against MMRd-associated FSPs, with both showing promising safety and immunogenicity in clinical trials (2, 3). This supports vaccination as a prevention strategy for LS. However current vaccines are based on low-coverage DNA sequencing of locally advanced invasive cancers and not the precancers they seek to prevent. **We hypothesise** that defining the immunogenic frameshift mutation landscape and epithelial-stromal-immune interactions in these early lesions, rather than in cancers, will enable the design of a rational, effective anti-cancer vaccine in this population ([Figure 1](#)). Our objectives include:

1. Defining the repertoire of potentially immunogenic frameshift mutations in LS-associated precancers;
2. Determining immune escape mechanisms in LS-associated precancers that may require targeting alongside vaccination;
3. Characterising expression and presentation of candidate FSPs by immunopeptidomic analysis of LS and MMRd precancers and cancers;
4. Assessing immunogenicity and population-scale immunoreactivity of candidate frameshift epitopes by functional and bioinformatic analysis for vaccine development;
5. Identifying enablers and barriers to prophylactic vaccine uptake in people with LS.

Our results will represent the most detailed study of LS precancers to date. Our outputs will:

- (i) advance mechanistic understanding of LS oncogenesis;
- (ii) support subsequent vaccine design, choice of adjuvant ± concomitant therapy and clinical testing
- (iii) guide future clinical trial design and messaging to the LS community around a preventative vaccine;
- (iv) catalyse and inform similar precision prevention efforts in other high-risk populations.

A. Steps in Lynch syndrome tumorigenesis



B. LynchVax precision prevention

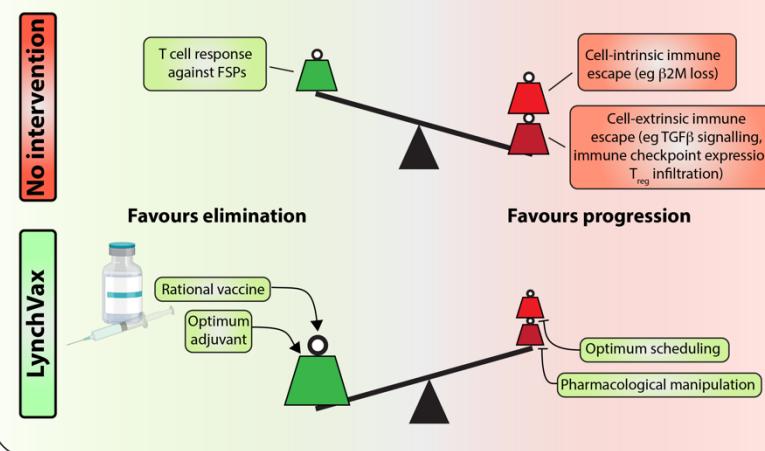


Figure 1. LynchVax project - graphical abstract showing relevant mechanisms and LynchVax aims

Upper panel (A) shows steps in Lynch syndrome (LS)-associated colorectal and endometrial tumorigenesis based on published literature and our preliminary data. The first detectable abnormality is a colonic/rectal aberrant crypt focus (ACF) or endometrial mismatch repair (MMR) differential gland focus (DGF) in which cells show DNA mismatch repair (MMR) deficiency (MMRd) (NB this is not always observed in the colon). It is believed that some lesions are eliminated at this stage by an effective immune response (not shown here). The next step is typically formation of a macroscopically detectable adenomatous polyp (adenoma) in the colon (though some lesions may develop submucosally) or endometrial atypical neoplasia (EIN)/atypical hyperplasia, the majority of which are MMRd. MMRd leads to generation of frameshift peptides (FSPs) which induce an antitumour CD8⁺ T-cell response. The T cell response is attenuated by acquisition of mechanisms of immune escape, including loss of antigen presentation (eg β2-microglobulin (B2M) mutation), immune checkpoint upregulation and infiltration by immunosuppressive regulatory T-cells (T_{reg}) which collectively enable progression to invasive cancer. Lower panel (B) shows overview of LynchVax project, which seeks to identify optimum FSPs for a prophylactic vaccine, and to delineate the dynamic microenvironment of LS precancers to define mechanisms of immune escape for possible targeting alongside vaccination. In the absence of intervention, cell-intrinsic and extrinsic immune escape mechanisms attenuate the anti-tumour T-cell response and enable disease progression. LynchVax aims to boost the antitumour with a rational vaccine while potentially also targeting mechanisms of immune escape, to skew the dynamic equilibrium against progression and towards elimination of precancerous MMRd clones.

BACKGROUND

Lynch syndrome (LS) is the most common hereditary cancer syndrome (1). Individuals with LS are born with a defective copy of one of several genes responsible for DNA mismatch repair (MMR) – a process which suppresses mutagenesis during DNA replication (4). Loss of the functional allele causes MMRd, accumulation of mutations, and malignancy – most commonly colorectal and endometrial cancer (CRC and EC) – with lifetime risk of >75% (5). Consequently, LS carriers are advised to have regular surveillance colonoscopy (6) and consider aspirin chemoprophylaxis (7). These interventions are costly, cause morbidity, and are only partially effective since LS still accounts for 3-4% of all newly diagnosed CRC and EC in unselected populations. Therefore, effective cancer prevention in LS would address a major unmet need.

LS-associated invasive cancers typically show MMRd (8, 9), and like cancers with sporadic MMRd caused by aberrant DNA methylation, display distinct characteristics which vary according to the MMR gene affected. They are hypermutated (≥ 10 mutations/Mb) and typically show microsatellite instability (MSI) owing to failure to repair insertion-deletion events at microsatellites. This results in recurrent frameshift mutations and potentially antigenic FSPs. LS-associated cancers typically harbour a prominent lymphocytic infiltrate, thought to represent an immune response against these non-self FSPs and other mutations. Further, they are exceptionally sensitive to immune checkpoint blockade (10, 11), with response greater in early, localized disease (12, 13).

These characteristics have prompted investigation of MMRd-associated FSPs as vaccine targets. Early support for this came from demonstration of patient T-cell reactivity against FSPs derived from mutant *TGFB2* (14) and other genes (15, 16), and the identification of FSP-reactive T-cells in cancer-free LS carriers (17). Three recurrent FSPs (from *AIM2*, *HT001*, and *TAF1B*) were subsequently targeted by a therapeutic vaccine that demonstrated cellular and humoral responses in patients (2). The Cancer Genome Atlas (TCGA), with thousands of sequenced cancers, has enabled the unbiased search for FSPs at greater scale. A recent study by Roudko of 338 TCGA MMRd cases revealed recurrent frameshift mutations across 83 CRC, 170 EC and 85 gastric cancers (GC) (18). After selection based on predicted immunogenicity, translation of polyepitopes, binding across multiple HLA alleles and prevalence of >20% across cancers, they identified 5 candidates: *SLC35F5*, *SEC31A*, *TTK*, *SETD1B* and *RNF43*. TCGA data also informed the design of the Nous-209 vaccine, which targets 209 FSPs detected in >5% of samples across MMRd cancers (3), and has demonstrated safety and immunogenicity in early trials (19).

While these studies support the feasibility of vaccination against FSPs for MMRd cancers, it is presently unclear whether the targets and technologies used are optimum for a prophylactic LS vaccine. This is because previous studies, based on sequencing of established MMRd cancers have ([Box 1](#)):

- (i) used targeted sequencing/low-coverage TCGA whole exome sequencing (WES) which failed to detect frameshift drivers such as *BAX* (20), and detected other known antigenic mutations such as *TAF1B* (17) at surprisingly low frequency
- (ii) typically not differentiated early clonal mutations from later subclonal mutations despite the likelihood that targeting the former is likely to be more effective (21)
- (iii) not examined whether the LS precursor tissue microenvironment is conducive to vaccine activity despite evidence that an immunosuppressive tissue microenvironment in invasive cancers is responsible for vaccine failure (22) and thus critical for choice of adjuvant ± concomitant therapy

Study/ method	Frameshifts identified by	Confirmation of targets in precancers	Analysis of target (precancer) microenvironment	Rational choice of adjuvant/ concomitant therapy	Public consultation
Kloor ²	Targeted sequencing of MMRd cancers	✗	✗	✗	✗
Roudko ¹⁸	30x WES on 338 MMRd cancers (TCGA)	✗	✗	NA	✗
Nous-209 ¹⁹	30x WES on 338 MMRd cancers (TCGA)	✗	✗	GAd and MVA viral vectors (non-empirical)	✗
CEA/MUC1 ⁴⁷	NA	✗	✗	✗	✗
LynchVax	100x WGS on 562 MMRd cancers	✓	✓	✓	✓

Box 1. How does LynchVax differ from existing approaches?

MMRd – DNA mismatch repair deficient; WES – whole exome sequencing; TCGA – The Cancer Genome Atlas; WGS – whole genome sequencing

Furthermore and importantly, for a preventative therapy there has been no systematic assessment of how acceptable a vaccination strategy would be to people with LS. Identifying enablers and barriers to vaccine uptake in LS carriers is critical to inform future clinical trials.

To address these shortcomings, we established the LynchVax group, including bioinformaticians, cancer geneticists, immunologists, gastroenterologists, oncologists, pathologists, triallists and individuals with LS. We first leveraged our detailed analysis of cancers with whole genome sequencing (WGS) to 100x by the Genomics England 100,000 Genomes Project (100KGP), including >2,023 CRC (lead: Tomlinson; manuscript under revision, *Nature* (23)) and 665 EC (lead: Church; manuscript in preparation). Among the 360 CRC and 202 EC with MMRd (considerably more cases than the Roudko study (18)), we identified 29 unique frameshift mutations occurring in ≥25% tumours of both types, including *TAF1B*, and multiple clonal drivers including variants in TGF-β signalling particularly common in MMRd CRC ([Figure 2A,B](#)). To confirm if these mutations occur early in tumorigenesis (important for a prophylactic vaccine), we analysed 19 colonic polyps and paired normal colon samples from 16 LS carriers (due to pathogenic variants in *MLH1*, *MSH6* or *PMS2*) by WGS (n=16) or WES (n=3) ([Figure 2C](#)). Results varied by polyp histology (reviewed in 16 polyps). 7 of 8 (88%) adenomatous polyps were hypermutated (defined as ≥100 nonsynonymous exonic mutations), with 5 (63%) showing MSI (≥1% instability across DNA microsatellites by MSI SensorPro (24)) ([Figure 2D](#)). In contrast, none of 8 serrated lesions showed hypermutation or MSI ($P=0.0014$ and $P=0.026$ respectively, Fisher exact test). The lower prevalence of MSI/MMRd in our small LS adenomatous polyps (adenomas) compared to what has been published for LS advanced adenomas (80%) (25) and CRCs (>90%) (8, 9, 26, 27), may reflect weaker phenotype of *PMS2* defects underlying hypermutated non-MSI adenomas (28, 29). The results from serrated lesions suggest they are not LS precancers (30). Importantly, MSI adenomatous polyps carried many of the 29 recurrent frameshift mutations identified in the MMRd CRCs/ECs (median 15, range 10-17), including two variants present in all 5 cases with likely clonal variant allele fraction (e.g. median=0.44, range 0.37-0.79) and 20 variants present in ≥40% cases ([Figure 2D](#)).

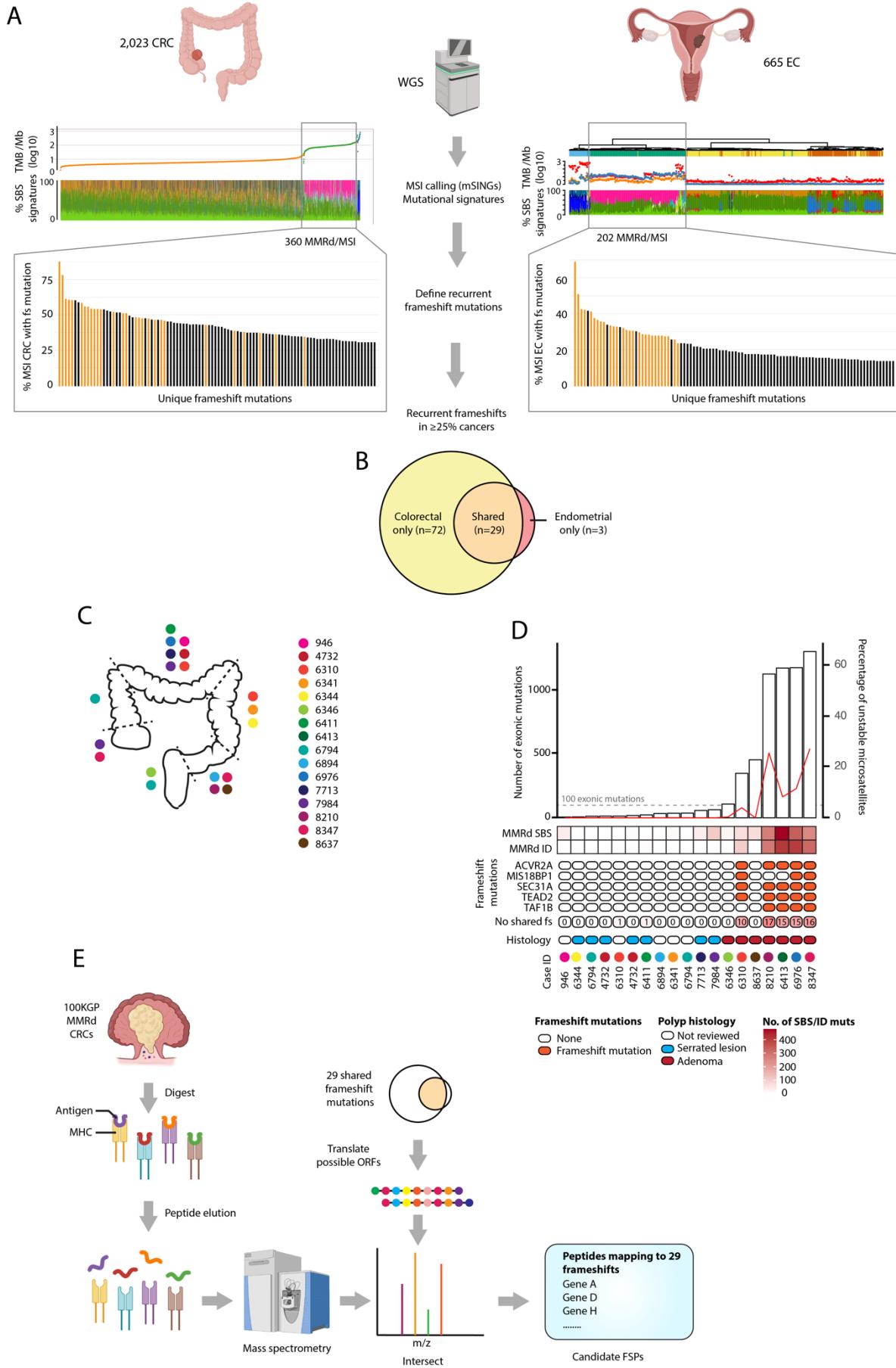


Figure 2 (previous page). Recurrent frameshift mutations in mismatch repair deficient (MMRd) colorectal and endometrial cancers and in Lynch syndrome-associated colonic precancers

(A) Workflow for detection of shared frameshift mutations in MMRd CRC and EC. Analysis of 2,203 CRC and 665 EC identified 360 and 202 MMRd/MSI cases respectively (note elevated tumour mutation burden (TMB) and single base substitution (SBS) signature 44 in pink). These cases were used to identify recurrent frameshift mutations in coding microsatellites, with variants that occurred in $\geq 25\%$ of MMRd/MSI cancers of both types prioritised for further analysis. Shared frameshift mutations present in both cancer types are highlighted in orange (B) Proportional Venn diagram showing overlapping unique frameshift mutations in MMRd/MSI CRC and EC. (C) Schematic showing location of colonic polyps analysed in Lynch syndrome (LS) carriers. (D) Nonsynonymous exonic mutation burden (white bars), percentage of unstable DNA microsatellites (red line), MMRd/MSI SBS and insertion-deletion (ID) mutational signatures and recurrent frameshifts shown in (A) determined by whole genome sequencing of colonic polyps from LS carriers. Polyp histology is indicated. (E) Workflow and results of detection of frameshift peptides eluted from tumour HLA class I molecules in MMRd/MSI invasive CRC from GEL 100KGP. 5 unique peptides matching shared frameshift mutations were detected including from most commonly frameshift-mutated gene. (NB the small polyps analysed so far have insufficient tissue for immunopeptidomic analysis, however analysis of larger polyps is planned in this proposal within WP2).

To directly identify FSPs, we designed a bespoke workflow to search for the 29 candidates in immunopeptidomic data from invasive MMRd/MSI CRCs from the GEL 100KGP. Five shared FSPs were identified, confirming their endogenous processing and HLA-mediated presentation ([Figure 2E](#)). Thus, our candidate neoepitopes are early clonal events in LS-associated tumorigenesis and are presented by HLA, making them rational vaccine targets.

The immunosuppressive tumour microenvironment of invasive cancers is a key reason for the failure of many vaccine studies (22), and MMRd cancers escape immunosurveillance by multiple mechanisms, including HLA class I downregulation/loss (31) and upregulation of immunosuppressive immune checkpoints (32). These mechanisms may also contribute to the lack of *in vivo* priming of T-cells against FSPs in patients with invasive MMRd cancers (18). While RNAseq of the 19 polyps confirmed downregulation of $\beta 2$ microglobulin (B2M) (the HLA-I light chain) in hypermutated adenomas compared to adjacent normal colon ([Appendix A](#)), immune checkpoint molecules were not upregulated, suggesting this occurs later in tumorigenesis, possibly mediating disease progression ([Figure 3A](#), [Appendix A](#)). Interestingly, RNAseq also revealed increased cytotoxic T-cells in a subset of hypermutated polyps, and significantly decreased polyp infiltration by monocytes and neutrophils, the relevance of which is presently unclear ([Figure 3A](#)). Multiplex immunofluorescence (mIF) confirmed dense CD8 $^{+}$ cytotoxic and CD4 $^{+}$ helper T-cell infiltrate in hypermutated polyps ([Figure 3B](#)), with evidence of tertiary lymphoid structures (data not shown) – both of which predict immunotherapy benefit (33, 34). Spatial localisation and quantification of mIF data by an AI-based method we recently developed to analyse >3,000 CRCs (35, 36) revealed similar density of intraepithelial and intrastromal CD8 $^{+}$ T-cell infiltrate in hypermutated polyps compared to adjacent normal colon ([Figure 3C](#)), in contrast to the depletion seen in LS CRCs (37). However, greater CD4 $^{+}$ and FoxP3 $^{+}$ cell density in these lesions suggested an early mechanism of immunosuppression. Preliminary immunohistochemistry (IHC) analysis indicated that despite reduced expression by RNAseq, HLA class I protein is detectable in all 7 hypermutated polyps with exception of subclonal loss in one lesion ([Figure 3D](#)). Collectively, our preliminary data ([Box 2](#)) demonstrate that recurrent frameshift mutations occur early in LS tumorigenesis, when pre-cancers lack some, but not all immune escape mechanisms acquired during disease progression. While this supports their targeting by a preventative vaccine, further work is required to define the optimum design and timing of such an intervention.

Box 2. Preliminary data – key messages

- DNA mismatch repair deficient (MMRd) cancers (CRC, EC) carry highly recurrent frameshift mutations
- These frameshift mutations are detectable in (MMRd) LS colonic precancers (polyps)
- FSPs are expressed and presented by HLA in MMRd cancers
- LS colonic precancers suppress immune cytolysis by potentially targetable mechanisms and lack important immune escape mechanisms acquired by invasive cancers

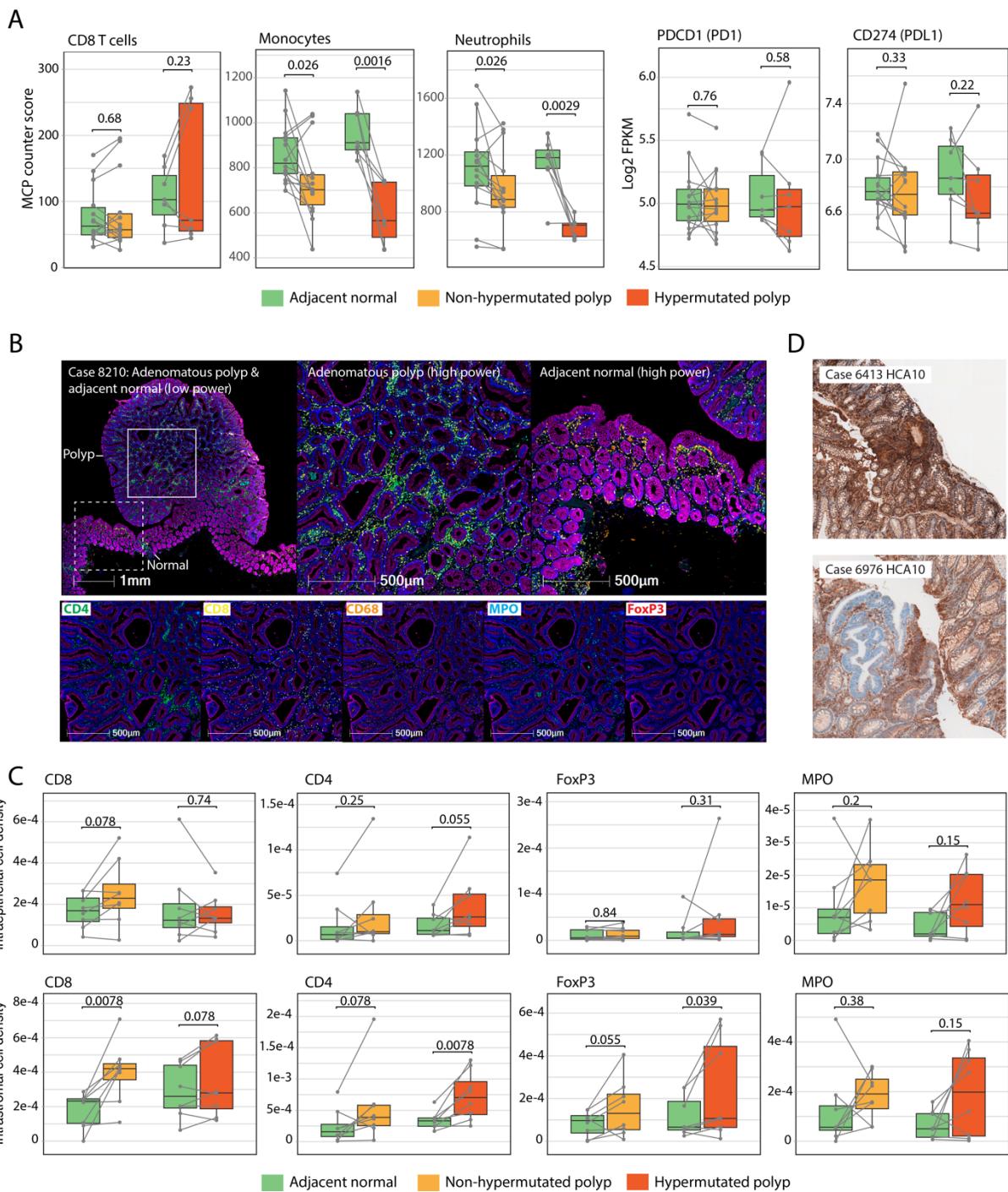


Figure 3. Immune microenvironment of Lynch syndrome-associated colonic polyps

(A) MCP counter estimates of infiltrating immune cell types and immune checkpoint expression derived from bulk RNAseq analysis of polyps and paired adjacent normal colonic mucosa. Polyps are stratified according to hypermutation status. (B) Multiplex immunofluorescence (mIF) staining of hypermutated polyps with microsatellite instability (MSI). Upper left panel shows overview with upper centre and upper right panels showing polyp and adjacent normal colon. Lower panels show high power views of individual marker channels CD4 (T helper cells), CD8 (cytotoxic T cells), CD68 (macrophages), MPO (neutrophils) and FoxP3 (regulatory T cells) in indicated colours. (C) Quantification of intraepithelial (upper panels) and intrastromal (lower panels) immune cell infiltrate by AI-based quantification (Refs 31, 32). (D) Immunohistochemistry for HLA class I (clone HCA10) in two hypermutated MSI polyps, showing retained expression (upper image) and subclonal loss (lower image). P values in (A) and (C) were determined by unadjusted Mann-Whitney test.

RESEARCH PLAN

To confirm the optimum vaccine target FSPs for delivery via the most effective platform and adjuvant ± concomitant therapy at a timepoint when intervention is most efficacious and acceptable to people with LS, we propose four work packages (WPs):

WP1. Frameshift mutation and immune landscape in Lynch syndrome-associated precancers and cancers (Objectives #1-2)

Hypotheses to test: (i) 95% of MSI LS precancers carry ≥ 10 shared frameshift mutations; (ii) an equilibrium of pro- and antitumorigenic immune cells in LS precancers can be skewed to the latter with vaccination; (iii) LS precancers lack immune escape mechanisms causing vaccine failure in invasive cancers and employ alternative targetable mechanisms (e.g inhibition of TGF- β signalling).

Plan: To give confidence a LS vaccine will prevent multiple cancer types regardless of the underlying MMR gene defect, we will increase our sample size and breadth to include: (i) 20-25 additional LS-associated colonic adenomatous polyps identified retrospectively and prospectively (from Oxford's Translational Gastroenterology Unit Biobank and St Mark's Hospital, collaboration Graham, Monahan; see letters); (ii) 20-25 LS-associated endometrial precancers (or early invasive cancers) (collaboration Crosbie, de Bruyn, Bosse; see letters); (iii) other LS/MMRd cancers (e.g. gastric) from the GEL 100KGP and TCGA cohorts. LS-associated lesions will be selected to ensure representation of all underlying MMR gene defects. We will perform WES, RNAseq, mIF, IHC and focused analyses ([Table 1](#)) to define the frameshift mutation and immune landscape in these lesions. We will use these data to:

- (i) confirm the most common shared frameshift mutations in LS precancers and cancers;
- (ii) define the type, density and localisation of infiltrating lymphoid and myeloid cells in neoplastic lesions and adjacent normal tissue;
- (iii) identify known cell-intrinsic (e.g. β 2-microglobulin loss) and cell extrinsic (e.g. inflammatory signalling, T_{reg} infiltration) mechanisms of immune escape;
- (iv) explore other immune-escape mechanisms such as TGF- β -driven exclusion of cytotoxic lymphocytes (38, 39);
- (v) generate bespoke markers of early, truncal mutations (ViewRNA for frameshifted drivers) to determine clonal expansion/competition in early lesions and surrounding normal tissue as a potential surrogate biomarker of immune editing pre-/post-vaccine delivery.

Key outputs:

- define top candidate FSPs for functional validation;
- identify immunosuppressive mechanisms for targeting alongside vaccination (e.g with adjuvant or therapy)

WP2. Direct identification of neoepitopes in LS-associated precursor lesions and cancers by immunopeptidomics (Objective #3)

Hypotheses to test: (i) recurrent FSPs are expressed and thus 'visible' to the immune system; (ii) malignant progression correlates with immunopeptidomes characterised by both reduced peptide levels and fewer tumour-specific peptides.

Table 1. Analyses planned in this project

WP	LS-associated samples*	Analysis	Key outputs
WP1	(i) Colonic adenomas (polyps); (ii) Endometrial precancers/early cancers	Whole exome sequencing (WES)	Single nucleotide variant (SNV) and frameshift mutational burden Shared frameshift mutations, including the 29 candidate genes identified in preliminary analysis (Figure 2) Driver mutations, particularly those reported to mediate immune evasion Genetic cell-intrinsic mechanisms of immune escape (e.g. <i>B2M</i> mutation)
		Bulk RNAseq	Expression of frameshift mutant reads Bulk estimation of infiltrating immune cell populations Mechanisms of immune escape (e.g. immune checkpoint upregulation)
		mIF with AI-based analysis	Type, density, and localization of immune cells in intraepithelial and intrastromal compartments, submucosa etc Detailed analysis of tertiary lymphoid structures
		IHC	Cell-intrinsic mechanisms of immune escape (e.g. MHC class I loss) Focused analysis of epithelial cell-extrinsic mechanisms of immune escape (e.g. phospho-Smad signalling for TGFβ signalling)
		ViewRNA	In situ identification of frameshift mutations (e.g. <i>ACVR2A</i> , <i>TGFB2</i>) to define the role of TGFβ signalling in cell-extrinsic immune escape in the colon (intersection with mIF and IHC data)
	Other MMRd cancer types (e.g. stomach)	WGS (GEL) WES (TCGA)	SNV and frameshift mutational burden Shared frameshift mutations among 29 candidates
		Bulk RNAseq (TCGA)	Infiltrating immune cell types Relevant mechanisms of immune escape
WP2	(i) Colonic adenomas (polyps) (ii) Endometrial precancers/early cancers	Immunopeptidomics	Confirmation of HLA-I-mediated presentation of FSPs Variation between immunopeptidome of LS-associated precursor lesions and invasive cancers
WP3	Candidate frameshift peptides (FSPs)	Predicted binding to HLA haplotypes	<i>In silico</i> prioritisation of candidate FSP targets based on predicted presentation by diverse HLA alleles in the UK population
	(i) Colonic adenomas (polyps) (ii) MMRd endometrial cancers	WES RNAseq	As for WP1 Confirmed presence of candidate frameshift mutations against which to screen lymphocyte reactivity
	(i) Peripheral blood mononuclear cells (PBMCs) (ii) Colonic adenoma (polyp) tissue	Dextramer assay IFNγ ELISPOT assay	Immunogenicity assessment of top candidate HLA-I neoepitopes samples Detection of pHLA-CD8+ restricted T-cells in subject-matched PBMCs and/or tumour infiltrating T lymphocytes (TILs)
	Colonic adenoma (polyp) and adjacent normal tissue	scRNAseq <i>In situ</i> T-cell receptor (TCR) profiling by Slide-TCR-seq	Define TCR of reactive clones Map neoepitope-specific TCRs in precancers and normal tissue at single cell level

*unless explicitly stated otherwise

Plan: We will identify frameshift mutation-derived neoantigens endogenously processed and presented by HLA class I/II in LS-associated advanced adenomatous polyps (n=3-5, prospective collection of lesions of sufficient size with WES and RNAseq) and MMRd ECs (n=10 stored samples with WGS available) (analysis of endometrial precancers is unfeasible owing to need for pathological review). Frameshift mutations and generated FSPs will be predicted using our established pipeline and patient-specific neoantigens predicted via binding affinity to HLA alleles using NetMHCpan 4.1 (40) – where feasible this will be confirmed experimentally by indirect fluorescence polarisation (routine in Elliott lab). As neoantigens can represent as few as 0.01% unique peptides identified in data-dependent discovery experiments (41), we will increase depth and sensitivity with a novel in-house timsTOF workflow ([Figure](#)

2E). Fresh-frozen tissue samples will be prepared using established workflows, HLA ligands will be isolated (42) and presented peptides profiled using the timsTOF SCP LC-MS system (Brucker) (43) (to be done in Oxford with support from Dr Ternette (Utrecht)).

Key output:

- confirm HLA-restricted presentation of FSPs

WP3. Functional prioritisation of FSPs as targets for vaccination (Objective #4)

Hypotheses to test: (i) shared FSPs that bind a broad range of HLA haplotypes can be identified; (ii) there is specific CD8⁺ and CD4⁺ immunoreactivity to novel FSPs in LS carriers with no cancer history; (iii) T-cell escape mechanisms are established in preneoplastic lesions; (iv) reactive TCRs are detectable both in polyps and in adjacent macroscopically normal colon (e.g. in MMRd glands).

Plan: Top candidate neoepitopes identified from WP1-2 will be further prioritised for vaccination by functional validation. Our strategy will focus on both epitope number and HLA coverage breadth (to optimise probability of generating at least one response in as many individuals as possible across multiple ancestries/ethnicities (40)). We will deploy in-house modelling to assist this and to minimise negative effects of intracellular epitope competition (44). The most promising neoepitopes from this screen will be tested for their immunogenicity. The frequency, and phenotype of neoepitope-specific CD8⁺ T-cells isolated directly from peripheral blood and matched tumour tissue disaggregates will be determined using peptide-HLA matched dextramers (45) (Immudex) for each candidate neoepitope by flow cytometry. For this, PBMCs ± paired colonic adenomas or cancer tissue (where applicable) will be obtained from 5-15 individuals from the following groups: (i) LS carriers with no cancer history; (ii) LS carriers with advanced adenomas/CRC; (iii) patients with sporadic MMRd CRC and (iv) control healthy volunteers. Pre-existing CD8⁺ T-cell immunity to candidate neoepitopes will also be determined by their *ex vivo* expansion upon antigen stimulation using overlapping peptide pools spanning top candidate FSPs. To characterize neoantigen-specific T-cell function, short-term expanded CD8⁺ T-cell lines will be assessed for IFN γ production upon antigen (unique peptide) stimulation. In a separately funded study, we will isolate paired TCR- α and TCR- β sequences from scRNAseq analysis of short-term cultures with individual unique peptides to derive reagents (RNA probes etc) to localise peptide-reactive T-cells in precancers and normal tissue using Slide-TCR-Seq (46) (collaboration Sud; see letters).

Key outputs:

- Define best FSPs for vaccine
- Inform choice of vaccine technology and adjuvant

WP4. Public consultation (Objective #5)

This proposal has been developed with and is strongly supported by our active and committed LS patient/carrier advisory group (see letters), who have also co-produced the plan for a wider consultation on the acceptability of a prophylactic LS vaccine as part of this work (see PPI plan).

Conclusions

Collectively the work proposed will identify and characterise the best FSPs for inclusion in a prophylactic LS vaccine and inform the choice of backbone, adjuvant and concomitant therapy, if any, for further development. Our unprecedented user consultation will help ensure subsequent clinical testing and implementation maximise uptake and benefit. Discussions with leading vaccine manufacturers are ongoing and we plan to either partner with these or leverage Oxford vaccine expertise and obtain further academic funding for the next steps in vaccine development. A future clinical trial of the vaccine would be run by Oxford's Oncology Clinical Trials Unit (see letters). Our project has a clear line of sight to clinical application for cancer prevention and is strongly endorsed by our PPI group: "very exciting", "huge potential benefits" who have highlighted the potential psychological benefits of a successful vaccine.

TEAM COMPOSITION

Our team and their interactions are shown ([Figure 4](#)).

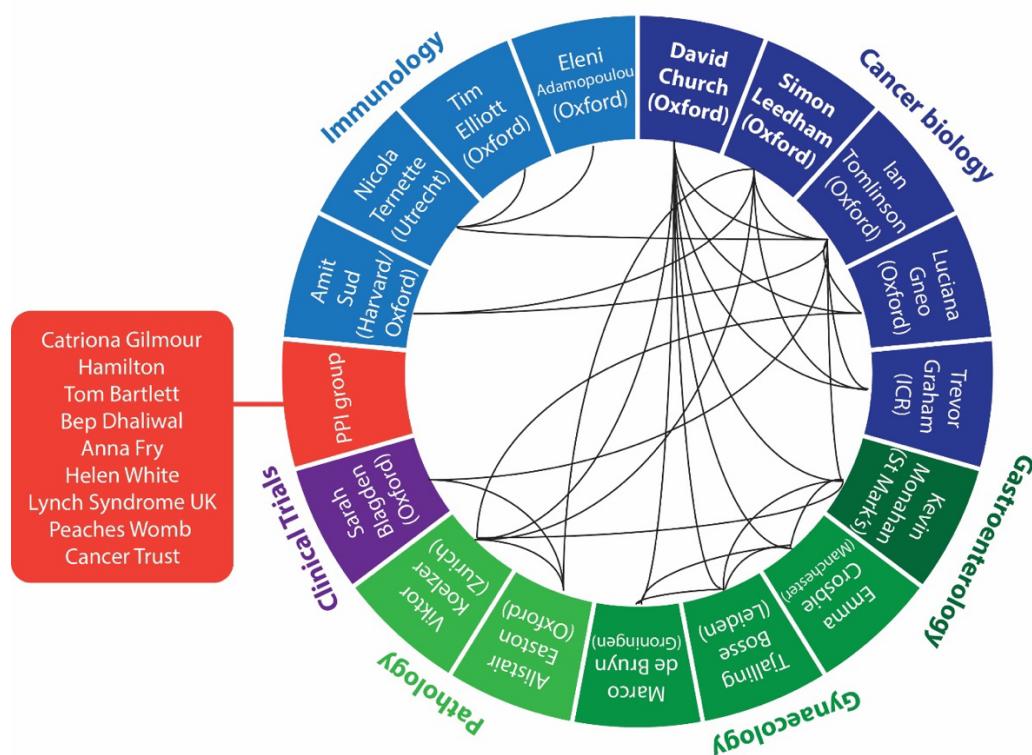


Figure 4. The LynchVax team composition

Black lines indicate previous shared publications and include: Church-Bosse (22 shared publications); Church-Tomlinson (32); Church-Koelzer (9); Church-De Bruyn (8); Church-Crosbie (6) and Leedham-Tomlinson (23); Leedham-Graham (13); Leedham-Koelzer (9).

TIMESCALE AND POTENTIAL PROBLEMS

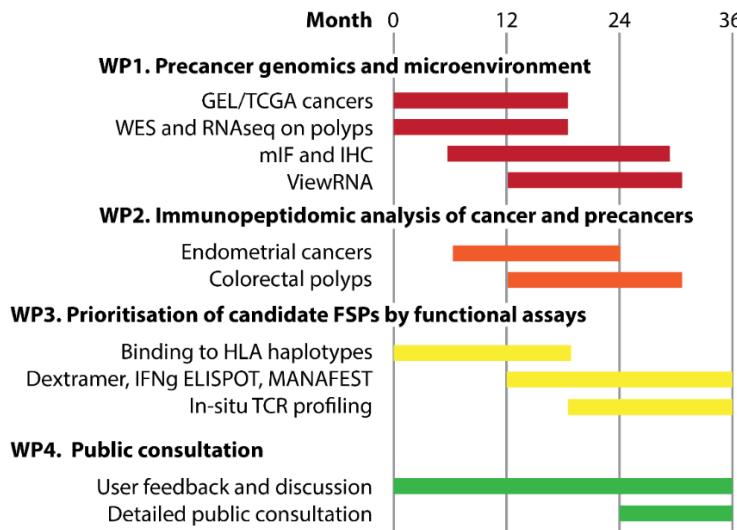


Figure 5. LynchVax project timelines

Potential problems and solutions

Problem: Slow prospective recruitment of cases in Oxford

Solution: Increase prospective recruitment of cases from St Mark's Hospital – the National Bowel Hospital with a very substantial population of LS carriers under colonoscopic surveillance

Problem: Difficulty in obtaining fresh frozen endometrial precancers owing to need for pathological review to differentiate endometrial epithelial neoplasia (EIN)/atypical hyperplasia from grade 1 endometrioid endometrial carcinoma (EEC)

Solution: Microdissect formalin fixed paraffin embedded (FFPE) samples for duplex whole exome sequencing (WES) optimised by the Graham lab. Complement with WES of grade I EEC of which samples are already available in Leiden and Groningen.

Problem: Poor immunogenicity of FSPs among the 29 candidates with prevalence $\geq 25\%$ in both MMRd colorectal and endometrial cancers.

Solution: Expand search space to multiple additional FSPs with prevalence e.g. $\geq 15\%$ informed by sequencing of cancers and precancers

Problem: Inability to find industry partner for vaccine development

Solution: Leverage world-leading academic vaccine expertise (Jenner Institute) and capability (Clinical BioManufacturing facility) available in Oxford for academic development

JUSTIFICATION FOR SUPPORT

Total project cost = £542,925

Staff costs (£397,275)

Support is requested for two post-doctoral research associates (**2 FTE, 3 years, £361,509**) essential for project delivery. One (**Dr Luciana Gneo**) will undertake the wet-lab assays proposed, including the sequencing experiments (whole exome and RNA), spatial analysis of the precancer and cancer

microenvironment (assisted by relevant core facilities) and the functional immunogenicity assays. We anticipate that from October 2024 Dr Gneo will supervise a graduate student (prospective candidates awaiting shortlisting) who will be able to help with experiments and sample collection. The second post-doctoral researcher will be responsible for data analysis, including the sequencing data workflows, immunopeptidomics and some elements of the spatial analysis in collaboration with the Koelzer group.

Differentiation of colonic and endometrial precancers requires expert pathologist review and so we include costs for pathologist support (**Dr Alistair Easton, 0.05 FTE, 3 years, £26,297**), who will annotate samples and images. Dr Easton will also assist with the analysis of the multiplex immunofluorescence and immunohistochemistry images.

Engagement with our target population of individuals with Lynch Syndrome is a central pillar of this proposal and so we include costs for leadership of the LynchVax patient and public involvement group (**Dr Catriona Gilmour Hamilton, 0.05 FTE, 3 years, £9,469**). Dr Gilmour Hamilton has very substantial experience in developing PPIE strategies and designing consultations to define patient priorities.

Non-staff costs (£145,650)

We have allocated the follow consumables/running costs:

- Sample collection (**£13,200**) to obtain colorectal tissue (polyp and adjacent normal) and blood samples (PBMCs) from people with Lynch syndrome from Oxford's Translational Gastroenterology Unit biobank. Similar samples will also be obtained from our collaborator Kevin Monahan at St Mark's. We will obtain endometrial tissue and blood samples from our collaborators Emma Crosbie, Marco de Bruyn and Tjalling Bosse.
- Sequencing costs (**£40,000**) of 80 samples (precancers with paired normal) each for whole exome sequencing and RNA sequencing.
- Spatial analysis (**£14,400**) of 30 samples to cover costs of multiplex immunofluorescence (£250 per sample), immunohistochemistry (£500 per antibody) and ViewRNA (£2,500 for 3 frameshift mutant and 3 wild-type probes).
- Immune assays (**£60,000**), to cover costs of immunopeptidomics (£600 per sample), dextramers (£1,000 each), peptide synthesis, reagents, and instrument running costs (eg £50/hour for flow cytometer, mass spectrometer)
- Data storage (**£12,000**) to cover the storage and processing of all the resulting data. This infrastructure leverages support from the CRUK Oxford Centre for Andrew Blake (Data Manager) and Guanke Bao (Data Analyst).
- PPI (**£6,050**) for PPI advisory panel reimbursement, facilitation for the online consultation, and travel to the Lynch Syndrome UK annual meetings.

Costs of additional complementary analyses such as those of scTCRseq and in-situ TCR profiling will be funded by other existing awards.

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